Retinal Cell Biology

A Combination Therapy Targeting Endoglin and VEGF-A Prevents Subretinal Fibro-Neovascularization Caused by Induced Müller Cell Disruption

Weiyong Shen,1 So-Ra Lee,1 Michelle Yam,1 Ling Zhu,1 Ting Zhang,1 Victoria Pye,1 Ashish Easow Mathai,1 Keichi Shibagaki,2 Jin-Zhong Zhang,2 Takeshi Matsugi,2 and Mark C. Gillies1

1The University of Sydney, Save Sight Institute Discipline of Ophthalmology, Sydney Medical School, Sydney, New South Wales, Australia
2Department of Non-Clinical Research, Global R&D, Santen Pharmaceutical Co. Ltd., Nara, Japan

Correspondence: Weiyong Shen, Save Sight Institute, The University of Sydney, 8 Macquarie Street, Sydney 2000, Australia; weiyong.shen@sydney.edu.au.
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PURPOSE. Subretinal fibro-vascularization is one of the most common causes of vision loss in neovascular AMD (nAMD). Anti-VEGF therapy effectively inhibits vascular leak and neovascularization but has little effect on fibrosis. This study aimed to identify a combination therapy to concurrently inhibit subretinal neovascularization and prevent fibrosis.

METHODS. We generated transgenic mice in which induced disruption of Müller cells leads to subretinal neovascularization, which is reliably accompanied by subretinal fibrosis. We conducted Western blots and immunohistochemistry to study changes in transforming growth factor-β (TGFβ) signaling including endoglin, a coreceptor essential for TGFβ signaling, and then tested the effects of monthly intravitreal injection of anti–VEGF-A and anti-endoglin, either alone or in combination, on the development of subretinal fibro-neovascularization in our transgenic mice.

RESULTS. Müller cell disruption increased expression of TGFβ1, TGFβ type 1 receptor, and phosphorylated-Smad3. Endoglin was strongly expressed in subretinal fibrovascular tissue. Fluorescein angiography and measurements of retinal vascular permeability indicated that intravitreal anti–VEGF-A in combination with anti-endoglin treatment more efficiently inhibited vascular leak compared with either monotherapy. Immunostaining of retinal wholemounts with antibodies against glial fibrillary acidic protein and ionized calcium binding adaptor molecule 1 indicated that the combination therapy also effectively prevented subretinal fibrosis and inhibited microglial activation. Luminex cytokine assays indicated that intravitreal anti–VEGF-A and anti-endoglin treatment, either alone or in combination, reduced the production of IL53 and macrophage inflammatory protein-3α.

CONCLUSIONS. Our findings offer a potentially novel combination approach to concurrently managing subretinal neovascularization and fibrosis in nAMD.

Keywords: neovascular age-related macular degeneration, fibrosis, transforming growth factor β, endoglin, vascular endothelial growth factor, combination therapy

Subretinal fibrosis that accompanies the growth of abnormal blood vessels is one of the most common causes of irreversible vision loss in neovascular AMD (nAMD). Drugs that target VEGF effectively treat blood vessel leak and neovascularization but demonstrate limited effect on retinal fibrosis.1,2 A major challenge in treating nAMD is to concurrently inhibit subretinal neovascularization and prevent fibrosis.

Retinal fibrosis is characterized by overexpression of extracellular matrix proteins by activated Müller cells and microglia, transformed RPE cells, fibroblast-like cells, vascular endothelial cells, and pericytes.3–4 Müller cells, the radial glial cells that span the full thickness of the retina, are important contributors to subretinal fibrosis and neovascularization. Müller cells become activated (or gliotic) under pathologic conditions and contribute to fibrosis by producing extracellular matrix proteins such as fibronectin, connective tissue growth factor, and α-smooth muscle actin.5,6 Activated Müller cells also produce angiogenic factors including VEGF matrix metalloproteinases, cytokines, and chemokines, thus contributing to blood vessel leak and neovascularization.7,9 Müller cell gliosis can form subretinal scars that disrupt the transport of nutrients from the choroid to the retina.

Microglia are the primary immune cells in the retina. “Resting” microglia play a housekeeping role in the normal retina and are activated under diseased conditions secrete cytokines and chemokines that contribute to tissue injury. Activated microglia have been reported to express extracellular matrix proteins and release angiogenic factors in diseases characterized by retinal neovascularization.6,7,11 suggesting that they, like Müller cells, have an important role in the development of fibrosis and neovascularization in retinal diseases.

The transforming growth factor-β (TGFβ) signaling pathway is an important intracellular mechanism that promotes fibrosis.12–15 Interaction of TGFβ receptors with their ligands leads to phosphorylation of Smad proteins, resulting in their...
translocation to the nucleus where they regulate expression of profibrotic genes. TGFβ signaling regulates the production of extracellular matrix proteins and is involved in cell migration, chemotaxis, and proliferation of fibroblasts and vascular endothelial cells in the retina, as well as epithelial–mesenchymal transition of RPE cells.14 TGFβ superfamily members mediate their effects through a receptor complex that contains endoglin, a type III coreceptor for TGFβ receptor type II and activin-like kinases 1 and 5.15,16 Endoglin has been found to be expressed in vascular endothelial cells, activated monocytes, tissue macrophages, stromal cells, pre-B cells, erythroid precursors, syncytiotrophoblasts, cytотrophoblasts, and tumor cells.14 Endoglin binds to TGFβ as part of a multireceptor complex that mediates signal transduction and plays an important role in tumor-associated fibrosis and angiogenesis.15–21

TGFβ signaling contributes to fibrosis in multiple diseases,12,13,15–22 but there is limited information about how it contributes to subretinal fibrosis and neovascularization. We generated transgenic mice in which induced disruption of Müller cells causes subretinal neovascularization that is reliably accompanied by subretinal fibrosis25–27 and can serve as a model system to test the effect of novel therapies on subretinal fibro-neovascularization. Here, we report changes in TGFβ signaling and endoglin expression, and further, the effects of intravitreal injections of antibodies against mouse endoglin and VEGF-A, either alone or in combination, on the development of subretinal neovascularization and fibrosis caused by induced Müller cell disruption.

METHODS

Induced Müller Cell Disruption in Transgenic Mice

This study was approved by The University of Sydney Animal Ethics Committee and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rlbp-CreERT2-DTA176 transgenic mice were generated by crossing Rlbp1-CreER mice with Rosa-DTA176 mice as described previously.25–27 Müller cell disruption was induced by cell-specific expression of an attenuated form of diphtheria toxin fragment A (DTA176) following intraperitoneal injections of tamoxifen in Rlbp-CreERT2-DTA176 mice at 8 to 10 weeks of age. Normal mice with the same genetic background were used as controls.

Intravitreal Injections

Intravitreal injections of test agents were performed using a 32-gauge needle attached to a Hamilton syringe as described previously.25–27 In brief, mice were anesthetized with ketamine (48 mg/kg) and medetomidine (0.6 mg/kg) and their pupils were dilated with one to two drops of 1% tropicamide and 0.5% phenylephrine. Intravitreal injections were performed 7 days before induced Müller cell disruption followed by two repeated injections 4 and 8 weeks after induced Müller cell disruption. One eye of each mouse received 2 μL test solutions, including a rat anti-mouse endoglin monoclonal antibody (M104317, 2.7 μg/μL; TRACON Pharmaceuticals, Inc., San Diego, CA, USA), a goat anti-mouse VEGF164 (2.5 μg/μL, #AF-493-NA; R&D Systems, Minneapolis, MN, USA), and a combination of both antibodies. PBS was injected in the same way in normal and transgenic eyes as controls.

Fluorescein Angiography

Retinal fluorescein angiography was performed to monitor changes in the retinal vasculature 6 and 10 weeks after induced Müller cell disruption as described previously.25,26 Images were taken between 2 and 5 minutes after an intraperitoneal injection of 0.05 mL of 10% sodium fluorescein (MW = 376) in each mouse.

Immunostaining Using Frozen Sections and Retinal Wholemounts

Eyes were fixed in 4% paraformaldehyde and embedded in optimal cutting temperature compound as described previously.25–27 Frozen sections were incubated with antibodies against glial fibrillary acidic protein (GFAP; rabbit polyclonal, #Z0334, 1:250; DAKO, Santa Clara, CA, USA) to study Müller cell gliosis, collagen type IV (rabbit polyclonal, #sc-2150-1470, 1:250; ABDSerotec, Kidlington, UK), or fluorescence-labeled isoelectric B4 (I54, L2895, 20 μg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) to study blood vessels. We used antibodies against endoglin (rat anti-mouse monoclonal, 1:100, Cat# MAB1320; R&D Systems), collagen I (rabbit polyclonal, #34710, 1:100; Abcam, Cambridge, UK), and fibronectin (rabbit polyclonal, #AB2035, 1:100; Chemicon, Temecula, CA, USA) to study their localization in normal and diseased retinas. After incubating with primary antibodies at +4°C overnight, the bound antibodies were detected with corresponding secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:1000; Invitrogen, Carlsbad, CA, USA) using a confocal laser scanning microscope.

For immunostaining on retinal wholemounts, dissected eye cups were fixed in 4% paraformaldehyde for 1 hour and placed in PBS at +4°C. The next day, retinas were isolated, permeabilized, and incubated in a solution containing peanut-agglutinin (PNA) conjugated with Alexa Fluor 594 (10 μg/ mL, L-32459; Invitrogen) for cone photoreceptor apical processes and an antibody against GFAP for Müller cell gliosis or ionized calcium binding adaptor molecule 1 (Iba1, 1:400, #019-19741; Wako, Osaka, Japan) for microglial infiltration in the subretinal space. Retinal wholemounts were counterstained with Hoechst stain, and confocal images were processed and quantitatively analysed as previously described.25–27

Western Blots

Proteins were extracted from retinas, and their concentrations were determined by detergent compatible protein assay. Equal amounts of protein were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were probed with primary Abs (Table) and then incubated with secondary Abs conjugated with horseradish peroxidase. Protein bands were visualized using the G:Box Biolmaging system (Syngene, Frederick, MD, USA) and quantified using the G:Box software package (Syngene). Protein expression was normalized to z/tubulin (rabbit polyclonal, 1:2000, #2148; Cell Signaling, Danvers, MA, USA), which served as a loading control.

Quantitative Measurement of Retinal Vascular Permeability

Retinal vascular leak was quantitatively assessed by measuring retinal vascular permeability to FITC-conjugated dextran as described previously with slight modifications.28,29 Briefly, FITC-dextran (MW = 70 kDa, 25 mg/kg; Sigma) was injected intravenously after deep anesthesia of mice. Twenty to 25 minutes after the injection, the chest cavity was opened, and a perfusion cannula was introduced into the aorta. A blood sample was collected from each mouse immediately before perfusion. After achieving drainage from the right atrium, each
mouse was perfused with saline (500 mL/kg) to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. After perfusion, the retinas were carefully removed, weighed, and homogenized to extract the FITC-dextran in 0.25 mL water. The extract was processed for fluorescence reading using a spectrofluorometer with excitation 485 nm and emission 538 nm. Corrections were made by subtracting the reading of water blank. For normalization, the amount of FITC-dextran within the retina was divided by the retinal weight and by the concentration of FITC-dextran in the plasma. Retinal vascular leak was calculated using an equation described previously,28,29 with the results being expressed as percentage of the permeability as measured in normal mice receiving intraocular injections of PBS.

Luminex Cytokine Assays

Proteins were extracted from retinas, and their concentrations were determined as described above. We performed Murine Luminex Screening Assays (R&D Systems) to profile 26 cytokines and chemokines associated with retinal glial activation, fibrosis, and neovascularization (Supplementary Table S1). The results were normalized to pg/mg proteins.

Statistical Analysis

Results are expressed as mean ± SEM. Data were analyzed using 1-way ANOVA to identify significant differences among groups. SPSS 17.0 for Windows version software (SPSS Inc., Chicago, IL, USA) with a post hoc Bonferroni's correction was used for the statistical analysis. P < 0.05 was regarded as statistically significant.

RESULTS

TGFβ Signaling Pathway Is Activated by Induced Müller Cell Disruption

We performed Western blotting to study changes in the TGFβ signaling pathway 2 weeks after induced Müller cell disruption.25 We found that induced Müller cell disruption resulted in significant overexpression of TGFβ1, which was accompanied by increased expression of TGFβ type 1 receptor and phosphorylated Smad3 (p-Smad3), whereas total Smad3 levels remained unchanged (Fig. 1). These results suggest that the TGFβ signaling pathway was activated by induced Müller cell disruption.

Endoglin Is Strongly Expressed in Subretinal Fibro-Neovascular Tissues

We performed double label immunostaining for endoglin and collagen IV, a marker that stains blood vessels, to study changes in endoglin during the development of retinal vascular abnormalities (Fig. 2). Endoglin was predominantly expressed in the normal retina in retinal and choroidal blood vessels but not in the RPE (Figs. 2A–C). In the transgenic retina, immunostaining for collagen IV confirmed that Müller cell disruption led to intraretinal vascular abnormalities (Figs. 2D–F, small arrows) and subretinal fibro-neovascularization (Figs. 2D–F, large arrows). Double immunostaining for collagen IV and endoglin indicated that the subretinal fibro-neovascular tissue and hyperplastic RPE cells were strongly positive for endoglin (Figs. 2D–F, 2G–I).

We also conducted immunostaining to study the localization of extracellular matrix proteins including collagen I and fibronectin in normal and transgenic retinas after induced Müller cell disruption (Fig. 3). Collagen I (Figs. 3A–C) and fibronectin (Figs. 3G–I) were weakly expressed in the superficial retina and choroidal vessels in the normal retina. In transgenic retinas where induced Müller cell disruption led to subretinal neovascularization, strong immunoreactivity was observed in the superficial retina (small arrows in Figs. 3F, 3L) and subretinal neovascular tissues (larger arrows in Figs. 3F, 3L).

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FIGURE 2. Double label immunostaining for collagen type IV and endoglin. (A–C) In the normal retina, endoglin was mainly expressed in retinal blood vessels (arrows) and choroidal capillaries (cc). (D–I) Endoglin was expressed in abnormal vessels within the retina (small arrows) and strongly expression in subretinal fibro-neovascular tissue (large arrows) and hyperplastic RPE cells (asterisks in I) 3 months after induced Müller cell disruption. G–I are higher-power images of the area with subretinal fibro-neovascularization in D–F. (J–L) Negative controls using rat and rabbit IgG to replace the primary antibodies along with the corresponding secondary antibodies used in A–I. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
the development of vascular lesions, we performed intravitreal injection 7 days before induced Müller cell disruption followed by two repeated monthly injections. As expected, transgenic mice receiving PBS developed vascular lesions showing focal intense leak during angiography, which remained unchanged 6 and 10 weeks after induced Müller cell disruption (Figs. 4A, 4B). Reduced vascular lesions were observed in eyes receiving anti-endoglin or anti–VEGF-A antibody as a monotherapy (Figs. 4G, 4H). The most robust inhibition observed in eyes receiving a combination of the two antibodies as evidenced by complete inhibition of the development of focal intense vascular leak in the majority of eyes receiving the combination therapy (Figs. 4I, 4J). The therapeutic effects of intravitreal injections of anti-endoglin and anti–VEGF-A antibody therapy were further confirmed by grading vascular lesions during periodical fluorescein angiography (Fig. 4I) and quantitative measurement of retinal vascular permeability to fluorescein labeled dextran when we terminated the study 3 months after induced Müller cell disruption (Fig. 4J). Our results also indicated that angiography using sodium fluorescein (MW = 376) appears to be more sensitive in detecting retinal vascular leak than the permeability assay using FITC-dextran (MW = 70,000; Fig. 4J).

**Intravitreal Injection of Anti-Endoglin in Combination With Anti–VEGF-A Antibodies Significantly Inhibits Phosphorylation of Smad3**

We performed Western blotting to study the impact of intravitreal injections of anti-endoglin and anti–VEGF-A antibodies on expression of TGFβ1, VEGF-A, total Smad3, and p-Smad3. As expected, levels of TGFβ1 and VEGF-A were significantly increased in transgenic eyes compared with normal eyes injected with PBS. Intravitreal injections of anti-endoglin and anti–VEGF-A antibody, either alone or in combination, did not alter the expression profiles of TGFβ1 or VEGF-A (Figs. 5A, 5B). The level of total Smad3 tended to increase in transgenic eyes, but the difference was not statistically significant compared with normal eyes receiving PBS, whereas p-Smad3 was significantly upregulated in transgenic eyes compared with normal eyes receiving PBS (Figs. 5C, 5D). Intravitreal injections of anti-endoglin or anti–VEGF-A antibody alone tended to inhibit the phosphorylation of Smad3, with a significant reduction of p-Smad3 observed in eyes receiving a combination of the two antibodies (Fig. 5D).

**Anti-Endoglin in Combination With Anti–VEGF-A Antibody Treatment More Effectively Prevents the Development of Subretinal Fibrosis Than Either Monotherapy**

We conducted immunostaining of GFAP in retinal whole-mounts to study the formation of scar tissue in the subretinal space (Figs. 6A–F). As GFAP expression is confined to the inner retina in normal mice, we did not observe any GFAP staining in the outer retina of normal mice receiving PBS (Figs. 6A, 6B). However, by 3 months after induced Müller cell disruption, transgenic mice receiving PBS had developed extensive scar tissue in the subretinal space (Fig. 6C). Less scarring was observed in eyes receiving anti-endoglin and VEGF-A antibodies, either alone or in combination (Figs. 6D–F). Quantitative image analysis indicated that the areas of GFAP-stained scar tissue were significantly reduced in eyes receiving anti-endoglin or anti–VEGF-A antibody, with the most robust

**Combination of Anti-Endoglin With Anti–VEGF-A Antibody Therapy More Effectively Inhibits the Development of Retinal Vascular Lesions Than Either Monotherapy**

We reported previously that vascular lesions caused by induced Müller cell disruption are associated with upregulation of VEGF-A. Because endoglin acts as a coreceptor for TGFβ signaling and contributes to fibrosis and neovascularization, we performed intravitreal injections of antibodies against mouse endoglin (M1043) and VEGF-A, either alone or in combination, to assess their effects on inhibiting the development of subretinal fibro-neovascularization. To study whether early interventions would prevent

**Figure 3.** Immunostaining staining for collagen type I (A–F) and fibronectin (FN, G–L) in combination with FITC-isolectin B4 (IB4). (A–C, G–I) Expression of collagen I (A–C) and fibronectin (G–I) in the normal retina. Arrows in C point to the superficial retinal vessels that were positive for both collagen I and IB4. (D–F, J–L) Strong immunoreactivity for collagen I and fibronectin in the superficial retina (small arrows) and subretinal neovascular tissue (large arrows) after induced Müller cell disruption. (M–O) Negative controls using rabbit IgG to replace the primary antibodies along with the corresponding secondary antibody used in A–L. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, photoreceptor inner segments.
inhibition observed in eyes receiving the combination therapy (Fig. 6G).

Anti-Endoglin and Anti–VEGF-A Antibody Treatments Have Little Effect on the Global Overexpression of GFAP and Extracellular Matrix Proteins

We performed immunostaining for GFAP using frozen sections to examine the status of Müller glia across the whole retina (Figs. 7A–F). In the normal retina, GFAP was expressed in the superficial retina and in the outer plexiform layer (Fig. 7A). Repeated intravitreal injections of PBS slightly activated Müller glia (Fig. 7B, arrows). Consistent with our findings from retinal wholemount immunostaining, transgenic retinas receiving PBS developed extensive scar tissue in the subretinal space (Fig. 7C, arrows) and anti-endoglin and anti–VEGF-A treatment, either alone or in combination, limited the formation of scar tissue in the subretinal space (Figs. 7D–F, arrows). However, Müller cell processes in the inner retina remained strongly positive for GFAP regardless of the types of treatments administered.

Western blotting of GFAP and extracellular matrix proteins, including connective tissue growth factor (CTGF), fibronectin, intergrin-α5, matrix metalloproteinases (MMP2), and MMP9, indicated that induced Müller cell disruption resulted in overexpression of these fibrotic markers. However, anti-endoglin and anti–VEGF-A antibody treatment, either alone or in combination, had little effect on the global expression of these markers (Figs. 7G, 7H, 8A–D).

Combination of Anti-Endoglin With Anti–VEGF-A Antibody Therapy More Efficiently Inhibits Subretinal Microglial Infiltration Than Either Treatment

As microglia are important contributors to subretinal fibrovascularization, we performed double labeling for Iba1 and PNA on retinal wholemounts to study their infiltration into the subretinal space (Figs. 9A–F). Microglia were absent from the outer retina of normal eyes receiving PBS injections (Figs. 9A, 9B) but were markedly activated by induced Müller cell disruption in transgenic mice receiving injections of PBS (Fig. 9D). Intravitreal injections of anti-endoglin and anti–VEGF-A antibodies, either alone or in combination, reduced microglial
infiltration into the subretinal space (Figs. 9D–F). Quantitative image analysis indicated that Iba1-stained areas were significantly reduced in eyes receiving anti-endoglin or anti–VEGF-A antibody, with the most robust inhibition observed in eyes receiving a combination of the two antibodies (Fig. 9G).

We next studied whether inhibiting microglial infiltration into the subretinal space would reduce photoreceptor degeneration. Quantitative measurement of PNA-stained photoreceptor apical processes indicated that there were no statistically significant differences between transgenic mice receiving PBS and those receiving anti-endoglin or anti–VEGF-A antibody, with the most robust inhibition observed in eyes receiving a combination of the two antibodies (Fig. 9G).

We next studied whether inhibiting microglial infiltration into the subretinal space would reduce photoreceptor degeneration. Quantitative measurement of PNA-stained photoreceptor apical processes indicated that there were no statistically significant differences between transgenic mice receiving PBS and those receiving anti-endoglin or anti–VEGF-A antibody, with the most robust inhibition observed in eyes receiving a combination of the two antibodies (Fig. 9G).

Anti-Endoglin and Anti–VEGF-A Therapies Reduced the Levels of IL33 and MIP3a

We performed Luminex ELISA assays using single mouse retinas as individual samples to examine changes in inflammatory cytokines and chemokines induced by Müller cell disruption. Among the 26 cytokines and chemokines we studied (Supplementary Table S1), only IL33 and macrophage...
inflammatory protein 3α (MIP3α) were reliably detected, and both were significantly increased by induced Müller cell disruption (Fig. 10). Anti-endoglin and anti–VEGF-A antibody therapies, either alone or in combination, significantly reduced the production of IL33 (Fig. 10A) and MIP3α (Fig. 10B) compared with transgenic mice injected with PBS.

**DISCUSSION**

We demonstrated that the TGFβ signaling pathway was activated by induced Müller cell disruption, and endoglin, a coreceptor essential for TGFβ signaling, was strongly expressed in subretinal fibro-neovascular tissue and hyperplastic RPE cells. We particularly tested the effects of monthly intravitreal injections of antibodies against endoglin and VEGF-A, either alone or in combination, on the retinal pathology caused by Müller cell disruption. We found that a combination of anti-endoglin and anti-VEGF treatment was more effective in inhibiting vascular leak and preventing subretinal fibrosis compared to either monotherapy. This study has particular clinical significance for the development of effective treatments for nAMD, which is characterized by subretinal fibro-neovascularization.

VEGF-A is a well-known mediator of angiogenesis and vascular permeability and anti-VEGF therapy has significantly improved the care of patients with nAMD. However, a significant proportion of patients with nAMD do not respond to anti-VEGF monotherapy despite maximizing dose and dosing frequency. Increasing the dose of VEGF inhibitors or changing regimens may not benefit some of these patients, indicating that the peak efficacy of anti-VEGF monotherapy may have been reached with available agents. Suboptimal response to intravitreal anti-VEGF agents may be due to the chronicity of nAMD, the involvement of cytokines and chemokines resulting from chronic inflammation, and the contribution of angiogenic factors other than VEGF including members of the TGFβ superfamily such as endoglin, angiopoietin 2,
endothelin 1, follistatin, heparin-binding epidermal growth factor (HB-EGF), hepatocyte growth factor (HGF), and IL8.35,36

TGF\(\beta\) signaling plays an important role in fibrosis and neovascularization in multiple organs, but there is limited information about how it contributes to subretinal fibro-neovascularization. TGF\(\beta\) signaling begins with the binding of TGF\(\beta\) superfamily ligands to the TGF\(\beta\) type II receptor, leading to activation of the TGF\(\beta\) type I receptor and phosphorylation of Smad proteins.13 We found that Müller cell disruption significantly increased the expression of TGF\(\beta\)1, TGF\(\beta\) type I receptor, and subsequent phosphorylation of Smad3, indicating that the TGF\(\beta\) signaling pathway was activated by induced Müller cell disruption in our transgenic mice (Fig. 1). Endoglin is a coreceptor for TGF\(\beta\) superfamily ligands and has been reported to promote VEGF-induced tip cell formation and fibro-neovascularization through sustaining VEGF receptor 2.16,21 We found that endoglin was strongly expressed on fibro-neovascular tissue and hyperplastic RPE cells (Fig. 2), and

![Figure 7. Effects of anti-endoglin and anti-VEGF-A therapies on global Müller cell gliosis and overexpression of GFAP and CTGF (A–F). Immunostaining for GFAP. (A, B) In the normal retina, GFAP expression was observed in the superficial retina and the outer plexiform layer (A), and repeated intravitreal injections of PBS slightly activated Müller cells (B, arrows). (C–F) Induced Müller cell disruption led to extensive invasion of Müller cell processes into the subretinal space (C, arrows), and anti-endoglin and anti-VEGF treatment, either alone or in combination, did not inhibit Müller cell gliosis in the inner retina, although they appeared to limit the invasion of Müller cell processes into the subretinal space (D–F, arrows). (G, H) Western blot analysis indicated that anti-endoglin and anti-VEGF treatment did not affect the global overexpression of GFAP and CTGF \(\ast P < 0.05\) and \(\ast\ast P < 0.01\) versus normal mice receiving PBS; \(N = 6–10\)/group.](image-url)
intravitreal injections of anti-endoglin in combination with anti–VEGF-A antibody therapy significantly inhibited the expression of p-Smad3 (Fig. 5D). These findings formed the basis for our hypothesis that intravitreal injections of antibodies against endoglin and VEGF inhibit subretinal fibro-neovascularization.

Indeed, our study demonstrated that a combination of anti-endoglin with anti–VEGF-A therapy was more effective in inhibiting retinal vascular lesions than either treatment alone (Fig. 4). We used two methods to assess the effectiveness of intravitreal injections of test antibodies on the development of retinal vascular lesions: grading vascular leak during angiography and quantitative measurement of retinal vascular permeability to fluorescently labeled dextran. Consistent with our previous studies, transgenic mice receiving PBS developed focal vascular lesions with intense leak of fluorescein, which remained relatively unchanged during the period of study. Intravitreal injections of anti-endoglin or anti–VEGF-A antibody reduced the number of vascular lesions, but incomplete inhibition of the development of vascular lesions was frequently observed in eyes receiving a monotherapy, indicating that multiple angiogenic factors are likely involved in subretinal neovascularization. By contrast, most eyes receiving anti-endoglin in combination with anti–VEGF-A antibody therapy did not develop focal intense vascular leak during angiography. Collectively, our data indicate that both VEGF-A and TGFβ signaling plays an important role in the

**Figure 8.** Effects of anti-endoglin and anti–VEGF-A therapies on global retinal overexpression of extracellular matrix proteins. Western blot analysis indicated that anti-endoglin and anti-VEGF treatment did not affect the global overexpression of fibronectin (A), integrin-α5 (B), MMP2 (C), and MMP9 (D). *P < 0.05 and **P < 0.01 versus normal mice receiving PBS; N = 4–10/group.
development of subretinal neovascularization caused by induced Müller cell disruption.

A combination of anti-endoglin and anti-VEGF-A therapy also effectively prevented the development of subretinal fibrosis (Fig. 6). Previous studies have identified endoglin as a critical component of TGFβ1 signaling in cardiac and renal fibrosis. Our study indicates that endoglin is an important mediator of subretinal fibro-neovascularization, which is consistent with the data from fibrotic models in other organs. A limitation of this study is the lack of efficacy in inhibiting global Müller cell gliosis (Fig. 7) and that even the combination therapy did not suppress the overall upregulation of extracellular matrix proteins in the whole retina (Fig. 8).

In addition to their definite effects on stimulating vascular leakage and growth, TGFβ and VEGF-A are also known to induce microglial migration and proliferation. We found that anti-endoglin and anti-VEGF-A therapy, either alone or in combination, also inhibited microglial infiltration into the subretinal space (Fig. 9). Activated microglia have been reported to express extracellular matrix proteins and release angiogenic factors in retinal vascular diseases. Our data from Luminex cytokine and chemokine assays indicated that anti-endoglin and anti-VEGF-A antibody treatment also significantly inhibited the production of IL-33 and MIP-3α (Fig. 10), both of which have been reported to contribute to angiogenesis and fibrosis. Inhibition of microglial infiltration may partially account for the reduced subretinal fibro-neovascular-
transgenic mice, subretinal new vessels actually originate from the retinal vasculature, which is different from the choroidal origin of new vessels in nAMD. In addition, the subretinal scar tissue in our transgenic model mainly consists of Müller cell processes protruding into the subretinal space, with less involvement of transdifferentiated RPE cells that are the major player in human nAMD. However, our study clearly indicates that aberrant VEGF and TGFβ signaling plays an important role in the development of subretinal fibro-neovascularization. Further studies are warranted to test whether the combination therapy is also effective in other animal models, which recapitulate other key features of the human disease.

In summary, we found that the TGFβ signaling pathway was activated, and endoglin, a coreceptor essential for TGFβ signaling, was strongly expressed in subretinal fibro-neovascular tissue caused by induced Müller cell disruption. Our central observation is that a combination of anti-endoglin and anti-VEGF-A antibody therapy is more effective in inhibiting subretinal fibro-neovascularization compared with either monotherapy. As the humanized anti-endoglin (DE122) is currently in a phase 2a clinical trial for the treatment of nAMD, our findings offer a potentially novel combination approach to concurrently managing subretinal neovascularization and fibrosis in nAMD.

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