

Involvement of Müller Glial Autoinduction of TGF- β in Diabetic Fibrovascular Proliferation Via Glial–Mesenchymal Transition

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PURPOSE. Müller glial–mesenchymal transition (GMT) is reported as the fibrogenic mechanism promoted by TGF- β –SNAIL axis in Müller cells transdifferentiated into myofibroblasts. Here we show the multifaceted involvement of TGF- β in diabetic fibrovascular proliferation via Müller GMT and VEGF-A production.

METHODS. Surgically excised fibrovascular tissues from the eyes of patients with proliferative diabetic retinopathy were processed for immunofluorescence analyses of TGF- β downstream molecules. Human Müller glial cells were used to evaluate changes in gene and protein expression with real-time quantitative PCR and ELISA, respectively. Immunoblot analyses were performed to detect TGF- β signal activation.

RESULTS. Müller glial cells in patient fibrovascular tissues were immunopositive for GMT-related molecular markers, including SNAIL and smooth muscle protein 22, together with colocalization of VEGF-A and TGF- β receptors. In vitro administration of TGF- β 1/2 upregulated *TGFB1* and *TGFB2*, both of which were suppressed by inhibitors for nuclear factor- κ B, glycogen synthase kinase-3, and p38 mitogen-activated protein kinase. Of the various profibrotic cytokines, TGF- β 1/2 application exclusively induced Müller glial *VEGFA* mRNA expression, which was decreased by pretreatment with small interfering RNA for SMAD2 and inhibitors for p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Supporting these findings, TGF- β 1/2 stimulation to Müller cells increased the phosphorylation of these intracellular signaling molecules, all of which were also activated in Müller glial cells in patient fibrovascular tissues.

CONCLUSIONS. This study underscored the significance of Müller glial autoinduction of TGF- β as a pathogenic cue to facilitate diabetic fibrovascular proliferation via TGF- β -driven GMT and VEGF-A–driven angiogenesis.

Keywords: diabetic retinopathy, fibrovascular proliferation, Müller glial–mesenchymal transition, transforming growth factor- β vascular endothelial growth factor-A

Pathologic retinal angiogenesis seen in proliferative diabetic retinopathy (PDR) takes place in the form of epiretinal tissue growth called fibrovascular proliferation, a vision-threatening complication characterized by tractional retinal detachment and vitreous hemorrhage resulting from fibrocellular and neovascular components, respectively. Fibrocellular contraction of the fibrovascular tissue depends on transdifferentiated myofibroblasts harboring both fibrogenic and contractile features.¹ To promote the pathogenesis of fibrovascular proliferation, profibrotic and proangiogenic molecules are theorized to function cooperatively with a biologically purposive link. Indeed, TGF- β and VEGF-A, the representative fibrogenic and angiogenic cytokines, were reported to correlate with high intravitreal levels in patients with PDR.² Consistently, both of these cytokines were shown to be expressed in surgically excised fibrovascular tissues from patients with PDR.^{3,4}

In contrast with the epiretinal fibrovascular proliferation occurring in PDR, another vision-threatening disease,

namely, neovascular AMD (nAMD), is complicated by subretinal fibrovascular proliferation, also known simply as choroidal neovascularization. Using patient surgical specimens, we recently demonstrated RPE cells as the cellular source of myofibroblasts through the epithelial–mesenchymal transition (EMT) in the pathogenesis of subretinal fibrovascular proliferation occurring in nAMD.^{5,6} TGF- β signaling via TGF- β receptor (T β R) was shown to be required for the upregulation of SNAIL, a key transcription factor triggering EMT in RPE cells and subsequent myofibroblastic differentiation.^{5–8} In parallel with TGF- β -initiated EMT, TGF- β induced the expression of TGF- β per se in RPE cells, suggesting the autoinduction of TGF- β as the amplifier of subretinal fibrosis in nAMD.^{6,7}

As concerns the cellular source of myofibroblasts in epiretinal fibrocellular proliferation seen in idiopathic epiretinal membrane, we recently revealed that Müller glial cells are equipped with fibrogenic EMT program for the expression of T β R, SNAIL, and smooth muscle protein

(SM)22, which would thus be termed the Müller glial-mesenchymal transition (GMT) as an alternative to EMT.⁹ In fibrovascular proliferation associated with PDR, however, little is yet known about the involvement of Müller GMT promoted by the TGF- β -SNAIL axis, although Müller glial cells in patient tissues were suggested to bear a contractile phenotype expressing α -smooth muscle actin (SMA).¹⁰ It is also yet to be elucidated whether Müller glial cells have any capacity for the profibrotic TGF- β autoinduction as seen in RPE cells during subretinal fibrovascular proliferation.⁶ In the present study, we investigated the multifaceted involvement of TGF- β signaling in diabetic epiretinal fibrovascular proliferation through Müller glial bioactions including GMT, TGF- β autoinduction, and VEGF-A production, using patient-excised tissues and human cultured cells.

METHODS

Human Surgical Samples

Four fibrovascular tissues were excised from PDR eyes during vitrectomy and used for immunohistochemistry. This study was conducted in accordance with the tenets of the Declaration of Helsinki and after receiving approval from the Institutional Review Board of the Hokkaido University Hospital. Written informed consent was obtained from all patients after an explanation of the purpose and procedures of this study.

Immunofluorescence Microscopy

Immunofluorescence analyses were performed as described previously.^{6,9} Serial paraffin sections at 4 μ m thickness were cut and mounted onto microscope slides. Sections were incubated with primary antibodies followed by secondary antibodies. All antibodies are detailed in the Supplementary Information. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and sections were examined using a Bioevo BZ-9000 epifluorescence microscope (Keyence, Osaka, Japan).

Cell Culture and Reagents

The human Müller glial cell line Moorfields/Institute of Ophthalmology-Müller 1 was provided from Dr. G. Astrid Limb (UCL Institute of Ophthalmology, London, UK).¹¹ The Moorfields/Institute of Ophthalmology-Müller 1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C. Human retinal microvascular endothelial cells (Cell Systems Corporation, Kirkland, WA) were cultured on type I collagen-coated plates with CS-C complete medium (Cell Systems Corporation). All reagents are detailed in the Supplementary Information.

Real-Time Quantitative PCR Analyses

Total RNA was isolated by using TRI reagent (Molecular Research Center, Cincinnati, OH), and reverse transcription to cDNA was then performed using GoScript reverse transcriptase (Promega, Madison, WI), according to the manufacturers' instructions. All primers are listed in Supplementary Table S1. Real-time quantitative PCR was performed with the GoTaq qPCR Master Mix (Promega) and StepOne Plus Systems (Thermo Fisher Scientific, Waltham, MA). Gene

expression levels were calculated by the 2^{-ddCt} method with glyceraldehyde-3-phosphate dehydrogenase levels as an internal control.

Immunoblot Analyses

Collected cells were lysed in sodium dodecyl sulfate buffer. After quantifying protein concentrations using BCA reagent (Thermo Fisher Scientific), proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked in 5% skim milk and incubated with primary antibodies followed by secondary antibodies. All antibodies are detailed in the Supplementary Information. Signals were visualized using a SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific).

ELISA

Protein levels of VEGF-A in the supernatant were determined with Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The optical density was measured using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland).

Cell Viability Assay

Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to quantify viable cells, according to the manufacturer's instructions. The optical density was measured using a microplate reader (Sunrise, TECAN).

Statistical Analyses

All results are expressed as the mean \pm SEM. The Student *t*-test was used for statistical comparison between groups, and one-way ANOVA followed by the Tukey-Kramer method as a post-hoc test was used for multiple comparison procedures. Differences between means were considered statistically significant when *P* values were less than 0.05.

RESULTS

Expression of GMT-Related Molecular Markers in Müller Glial Cells Migrated Into Diabetic Fibrovascular Tissues

Prerequisite factors for fibrogenic Müller GMT recently proved to be the essential combination of profibrotic signal transduction, mesenchymal transcriptional activation, and resultant myofibroblastic differentiation, corresponding respectively to the expression of T β R, SNAIL, and SM22.⁹ To investigate the involvement of GMT in diabetic fibrovascular proliferation, we carried out immunofluorescence analyses of these GMT-related molecular markers in fibrovascular tissues collected from PDR patients. Double-staining experiments for serial sections exhibited colocalization of an activated glial marker glial fibrillary acidic protein (GFAP) with a specific Müller glial marker glutamine synthetase (GS) (Figs. 1A–1C), T β RI (Figs. 1D–1F), T β RII (Supplementary Figs. S1A–S1C), TGF- β 1 (Supplementary Figs. S1D–S1F), TGF- β 2 (Supplementary Figs. S1G–S1I), SNAIL (Fig. 1G–1I), and a mesenchymal marker α -SMA

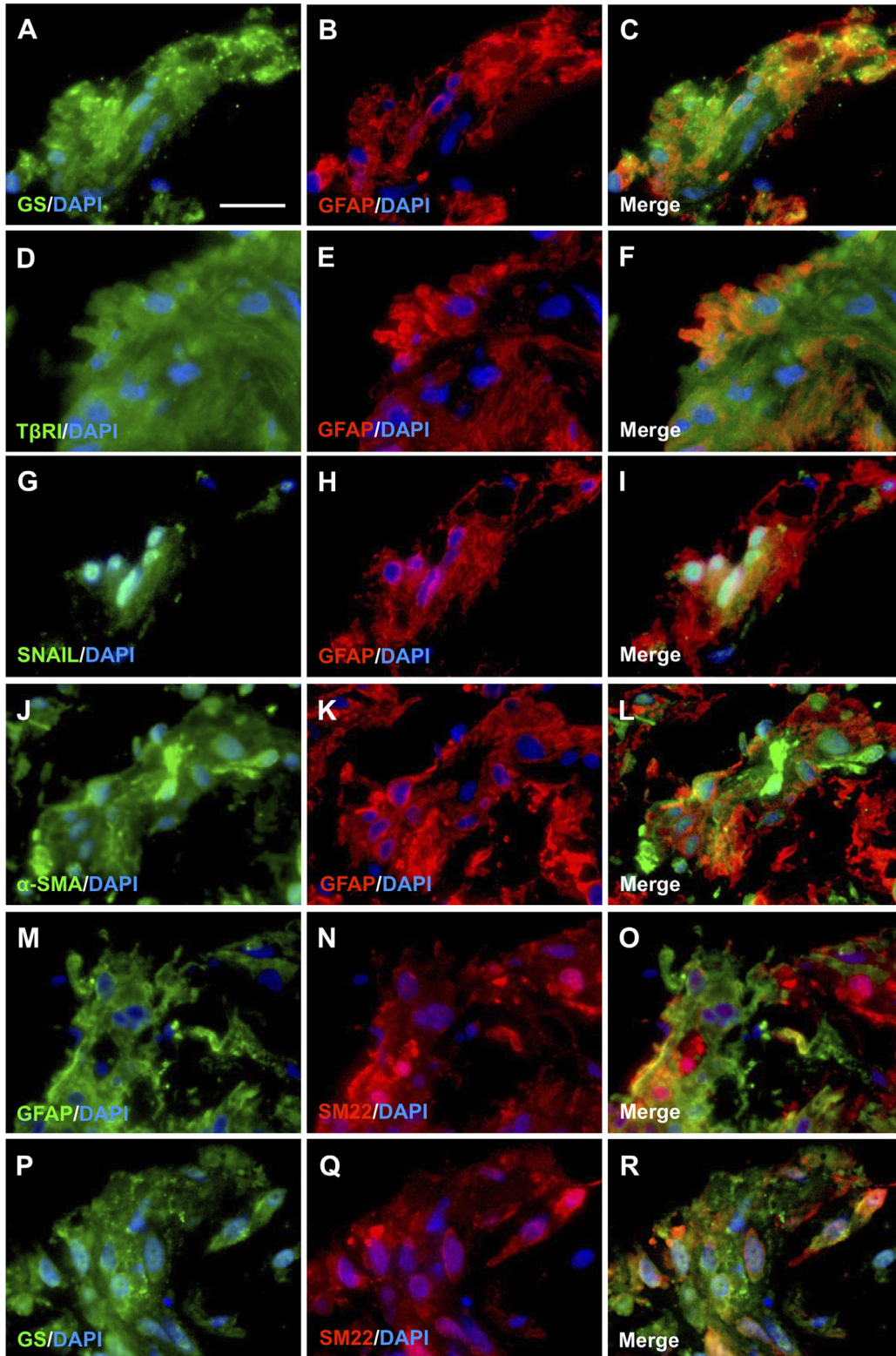


FIGURE 1. Expression of GMT-related molecular markers in Müller glial cells migrated into diabetic fibrovascular tissues. (A–R) Double labeling of GS (green) and GFAP (red) (A–C), T β RI (green) and GFAP (red) (D–F), SNAIL (green) and GFAP (red) (G–I), α -SMA (green) and GFAP (red) (J–L), GFAP (green) and SM22 (red) (M–O), and GS (green) and SM22 (red) (P–R) in diabetic fibrovascular tissue specimens with DAPI (blue) counterstain to nuclei. Scale bar = 20 μ m.

(Figs. 1J–1L), suggesting the establishment of Müller GMT in diabetic fibrovascular proliferation. Furthermore, SM22, an actin-binding cytoskeletal protein used as an EMT or GMT marker, colocalized with glial markers GFAP (Figs. 1M–1O) and GS (Figs. 1P–1R), indicating the contractile phenotype of activated Müller glial cells in the fibrovascular tissue.

Autoinduction of *TGFB1/2* Expression via Its Noncanonical Signaling Pathways in Müller Glial Cells

TGF- β autoinduction in RPE cells was recently reported as the amplifier of subretinal fibrosis owing to TGF- β -initiated EMT in the pathogenesis of nAMD.⁹ In general, TGF- β /T β R-induced EMT requires signaling via SMAD-dependent (canonical) and -independent (noncanonical) pathways, the latter of which includes extracellular signal-regulated kinase (ERK)1/2 and phosphatidylinositol 3-kinase (PI3K)/AKT.^{12–14} In particular, the activity of SMAD2, the key downstream molecule of TGF- β signaling, is critical for cancer EMT.¹⁵ SMAD signaling was also shown to be involved in proximal tubular epithelial TGF- β autoinduction together with ERK and p38 mitogen-activated protein kinase (MAPK) pathways.¹⁶ In the context of GMT, however, little is known about TGF- β signal transduction and TGF- β autoinduction in Müller glial cells. To explore the possible involvement of TGF- β autoinduction in Müller GMT, we next investigated the impact of TGF- β 1/2 application on *TGFB1/2* expression in human Müller glial cells. Stimulation with TGF- β 1 and TGF- β 2 to Müller cells significantly elevated mRNA levels of both *TGFB1* (Fig. 2A) and *TGFB2* (Supplementary Fig. S2A). To determine its downstream intracellular signaling pathways, we pretreated Müller cells with specific inhibitors for ERK1/2, nuclear factor (NF)- κ B, PI3K, c-Jun N-terminal kinase (JNK), p38 MAPK, and glycogen synthase kinase (GSK)-3. TGF- β 1/2-induced *TGFB1* expression levels were significantly decrease by NF- κ B, p38 MAPK, and GSK-3 inhibitors, but enhanced by PI3K inhibitor (Fig. 2B), which was also true of *TGFB2* expression changes (Supplementary Fig. S2B). Transfection with small interfering RNA (siRNA)-1 and siRNA-2 against *SMAD2* into Müller cells significantly suppressed *SMAD2* mRNA expression (Fig. 2C). Silencing of *SMAD2* did not cancel TGF- β 1/2-induced expression of *TGFB1* (Fig. 2D) or *TGFB2* (Supplementary Fig. S2C), indicating the absence of canonical signaling in Müller glial autoinduction of TGF- β 1/2. Consistently, the phosphorylation of NF- κ B, p38, and GSK-3 α/β increased substantially up to 24 hours after stimulation with TGF- β 1 (Fig. 2E) and TGF- β 2 (Supplementary Fig. S2D). In addition, the inhibition of PI3K alone did not increase *TGFB1* or *TGFB2* expression, but synergistically increased TGF- β 1/2-induced *TGFB1* and *TGFB2* expression (Supplementary Figs. S2E, S2F). These results indicated that Müller glial autoinduction of TGF- β 1/2 was positively regulated via noncanonical pathways by NF- κ B, p38 MAPK, and GSK-3, and negatively by PI3K/AKT.

We next investigated the role of T β RI/II in the mechanism of TGF- β 1/2 autoinduction in Müller glial cells. T β RI blockade by pretreatment with anti-T β RI-neutralizing antibody and T β RI kinase inhibitor significantly attenuated TGF- β 1/2-induced upregulation of *TGFB1* (Fig. 2F) and *TGFB2* (Supplementary Fig. S2G). Similarly, T β RII blockade by pretreatment with anti-T β RII neutralizing antibody signifi-

cantly attenuated TGF- β 1/2-induced upregulation of *TGFB1* (Fig. 2G) and *TGFB2* (Supplementary Fig. S2H). Moreover, *TGFB1* and *TGFB2* gene expression levels increased after TGF- β 1/2 stimulation (Supplementary Figs. S2I, S2J). These data suggested the facilitatory role of T β RI/II in Müller glial autoinduction of TGF- β 1/2.

Screening of PDR-Associated Profibrotic Cytokines and Müller Glial VEGF-A Induction Exclusively by TGF- β 1/2

During the pathogenesis of diabetic epiretinal fibrovascular proliferation, both profibrotic and proangiogenic cytokines such as TGF- β and VEGF-A were reported to increase often with mutual link in the vitreous of PDR patient eyes.^{2,17–20} On top of its profibrotic nature, TGF- β is also a classically known proangiogenic factor that induces VEGF-A in human RPE cells^{21,22} and rat Müller glial cells,²³ potentially promoting subretinal and epiretinal fibrovascular proliferations, respectively. Of various profibrotic stimuli with TGF- β 1/2, connective tissue growth factor (CTGF), fibroblast growth factor (FGF) 2, nerve growth factor (NGF), and platelet-derived growth factor (PDGF)-BB, all of which were elevated in PDR eyes^{2,17–20}; TGF- β 1/2 alone exclusively increased *VEGFA* mRNA levels in human Müller cells (Fig. 3A). The administration of TGF- β 1/2 to Müller cells upregulated *VEGFA* expression in dose- and time-dependent manners (Supplementary Figs. S3A, S3B). Consistent with the mRNA expression results, VEGF-A protein levels in the culture medium were elevated by TGF- β 1/2 stimulation in a dose-dependent manner (Fig. 3B). We next investigated the role of T β RI/II in the mechanism of VEGF-A induction in Müller glial cells. T β RI blockade by pretreatment with anti-T β RI-neutralizing antibody and T β RI kinase inhibitor significantly attenuated TGF- β 1/2-induced upregulation of *VEGFA* (Fig. 3C). Similarly, T β RII blockade by pretreatment with anti-T β RII neutralizing antibody significantly attenuated TGF- β 1/2-induced upregulation of *VEGFA* (Fig. 3D). These data suggested the crucial role of TGF- β 1/2–T β RI/II signaling in Müller glial expression of VEGF-A.

Next, we further confirmed whether TGF- β -stimulated Müller glial cells can indeed affect vascular endothelial cells through VEGF-A bioactivity. Human retinal microvascular endothelial cells were incubated with TGF- β 1/2-stimulated Müller cell culture medium. TGF- β 1/2 stimulation to Müller cells significantly increased endothelial cell viability, which was potently inhibited by pretreatment with anti-VEGF-A drugs bevacizumab and aflibercept (Fig. 3E). These results indicated the potentially high dependency of endothelial activation on Müller glial VEGF-A production by stimulation with TGF- β 1/2.

Colocalization of VEGF-A With T β RI/II in Müller Glial Cells Migrated Into Diabetic Fibrovascular Tissues

GFAP-positive cells present in diabetic fibrovascular tissues were previously shown to be the source of VEGF-A production with *in situ* hybridization.⁴ To examine the association of T β RI/II-bearing cells with the expression of VEGF-A in diabetic fibrovascular proliferation, we performed immunofluorescence analyses of VEGF-A and T β RI/II in Müller glial cells migrated into fibrovascular tissues excised

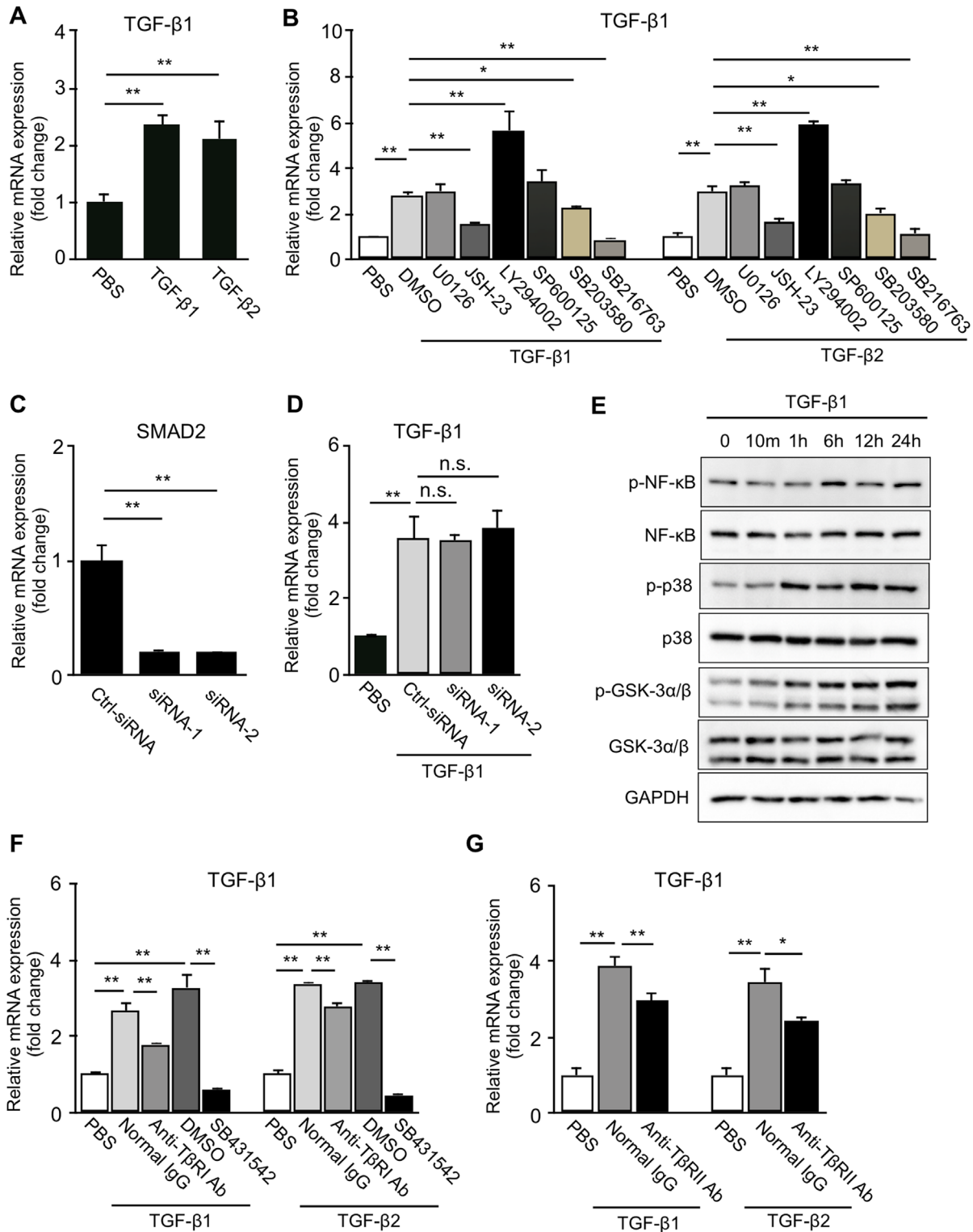


FIGURE 2. Autoinduction of *TGFβ1* expression via its noncanonical signaling pathways in Müller glial cells. (A) Müller glial cells were incubated with TGF-β1 or TGF-β2 (30 ng/mL) for 24 hours, and *TGFβ1* mRNA levels were analyzed. (B) Müller glial cells were pretreated with each inhibitor (U0126, ERK1/2; JSH-23, NF-κB; LY294002, PI3K; SP600125, JNK; SB203580, p38 MAPK; SB216763, GSK-3) at 10 μM for 30 minutes followed by stimulation with TGF-β1 or TGF-β2 (30 ng/mL) for 24 hours, and *TGFβ1* mRNA levels were analyzed. (C) *SMAD2*-siRNAs (siRNA-1 and siRNA-2) were transfected in Müller glial cells, and *SMAD2* mRNA levels were analyzed. (D) Control siRNA-treated (Ctrl-siRNA) and *SMAD2*-knockdown (siRNA-1 and siRNA-2) Müller glial cells were stimulated with TGF-β1, and *TGFβ1* mRNA levels were analyzed. (E) Müller glial cells were incubated with TGF-β1, and protein levels of phosphorylated and total forms of NF-κB, p38, and GSK-3α/β were analyzed at various time points. (F, G) Müller glial cells were pretreated with normal IgG (300 ng/mL), anti-TβRI antibody (300 ng/mL), TβRI kinase inhibitor SB431542 (10 μg/mL), or anti-TβRII antibody (250 ng/mL) for 30 minutes followed by stimulation with TGF-β1 or TGF-β2 (30 ng/mL) for 24 hours, and *TGFβ1* mRNA levels were analyzed. n = 3, *P < 0.05, **P < 0.01. n.s., not significant.

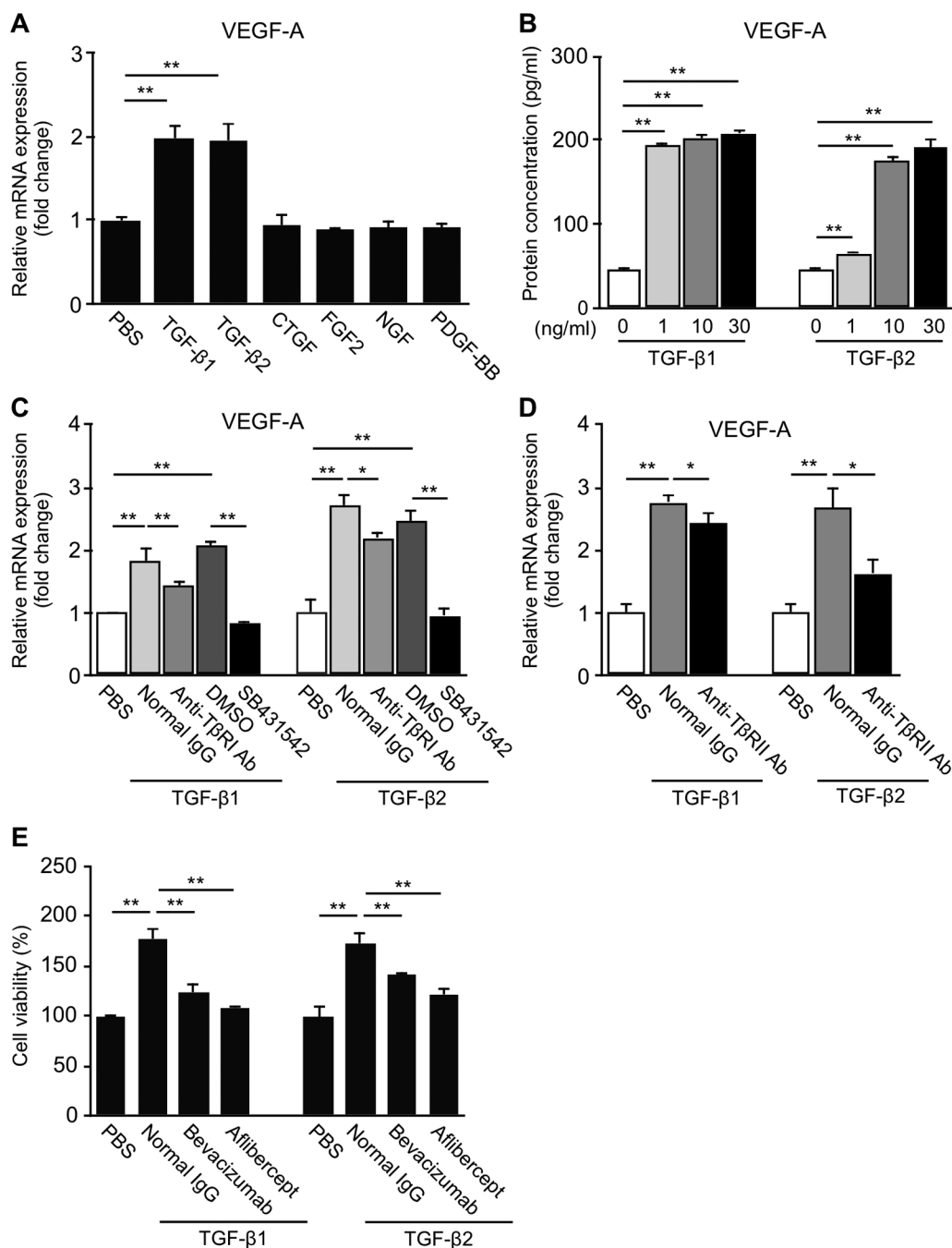


FIGURE 3. Screening of PDR-associated profibrotic cytokines and Müller glial VEGF-A induction exclusively by TGF- β 1/2. **(A)** Müller glial cells were applied with TGF- β 1, TGF- β 2, CTGF, FGF2, NGF, and PDGF-BB at the dose of 10 ng/mL for 24 hours, and *VEGFA* mRNA levels were analyzed. **(B)** Müller glial cells were incubated with TGF- β 1 or TGF- β 2 at different doses, and protein levels of VEGF-A were analyzed. **(C, D)** Müller glial cells were pretreated with normal IgG (300 ng/mL), anti-T β RI antibody (300 ng/mL), T β RI kinase inhibitor SB431542 (10 μ g/mL), or anti-T β RII antibody (250 ng/mL) for 30 minutes followed by stimulation with TGF- β 1 or TGF- β 2 (30 ng/mL) for 24 hours, and *VEGFA* mRNA levels were analyzed. **(E)** Müller glial cells were stimulated with TGF- β 1 or TGF- β 2 (30 ng/mL) for 24 hours, and supernatants were collected. Human retinal microvascular endothelial cells were preincubated with anti-VEGF-A drugs bevacizumab (0.3125 mg/mL), aflibercept (0.5 mg/mL), and normal IgG (0.5 mg/mL) for 15 minutes, followed by treatment with TGF- β 1/2-stimulated Müller cell culture medium for 48 hours, and processed for cell viability assay. $n = 3$, * $P < 0.05$, ** $P < 0.01$.

from eyes of PDR patients. Double-staining experiments for serial sections demonstrated colocalization of GFAP with GS (Figs. 4A–4C), T β RI (Figs. 4D–4F), and VEGF-A (Figs. 4G–4I). Importantly, VEGF-A colocalized with T β RI (Figs. 4J–4L) and T β RII (Figs. 4M–4O). These data suggested that Müller glial production of VEGF-A in the downstream of T β RI/II during

diabetic fibrovascular proliferation, in consistence with the in vitro results showing the requirement of T β RI/II in TGF- β 1/2-induced *VEGFA* expression (Figs. 3C, 3D). In contrast, VEGF-A application to Müller glial cells did not induce the expression of *TGFB1* or *TGFB2* (Supplementary Figs. S4A, S4B).

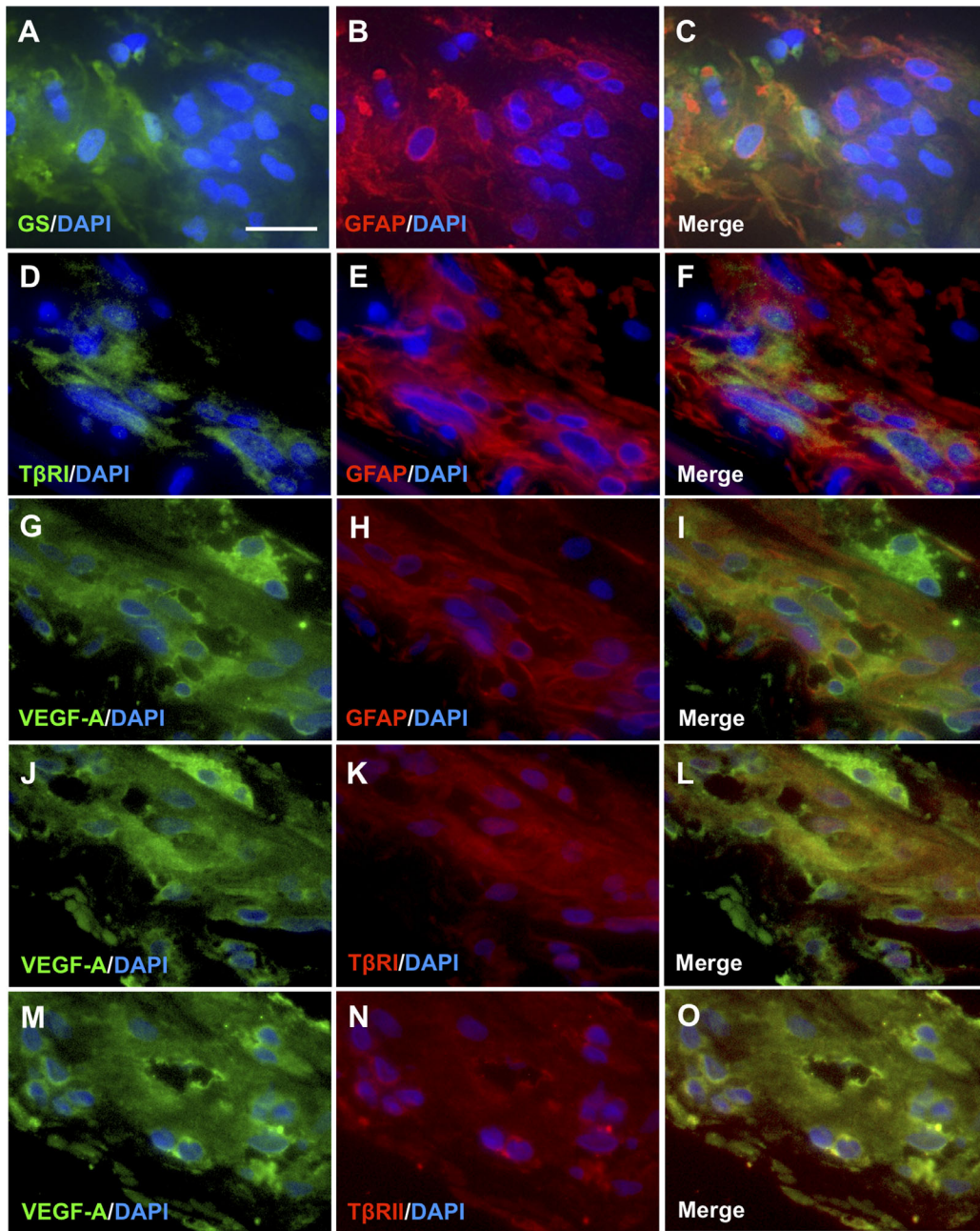


FIGURE 4. Colocalization of VEGF-A with T β RI/II in Müller glial cells migrated into diabetic fibrovascular tissues. (A–O) Double labeling of GS (green) and GFAP (red) (A–C), T β RI (green) and GFAP (red) (D–F), VEGF-A (green) and GFAP (red) (G–I), VEGF-A (green) and T β RI (red) (J–L), and VEGF-A (green) and T β RII (red) (M–O) in diabetic fibrovascular tissue specimens with DAPI (blue) counterstain to nuclei. Scale bar = 20 μ m.

TGF- β 1/2–Induced VEGF-A Expression via Its Canonical and Noncanonical Signaling Pathways in Müller Glial Cells

Although TGF- β was reported to induce VEGF-A expression in rat Müller glial cells,²³ its intracellular signal transduction remains to be clarified. We, therefore, used the same set of inhibitors with TGF- β autoinduction (Fig. 2) to detect signaling pathways to induce *VEGFA* expression in TGF- β 1/2–stimulated human Müller cells. TGF- β 1–induced *VEGFA* expression levels were significantly reduced

by PI3K and p38 MAPK inhibitors, but enhanced by JNK and GSK-3 inhibitors (Fig. 5A). Silencing of *SMAD2* significantly suppressed TGF- β 1–induced expression of *VEGFA* (Fig. 5B), indicating involvement of canonical signaling. Moreover, we verified the impact of pharmacologic inhibition of PI3K and p38 MAPK (Fig. 5C) and siRNA-based knockdown of *SMAD2* (Fig. 5D) on TGF- β 1–induced production of VEGF-A protein as well. Consistently, the phosphorylation of AKT and SMAD2 increased substantially up to 24 hours after stimulation with TGF- β 1 (Fig. 5E). TGF- β 1–induced phosphorylation of SMAD2 was mitigated by inhibition of p38 MAPK

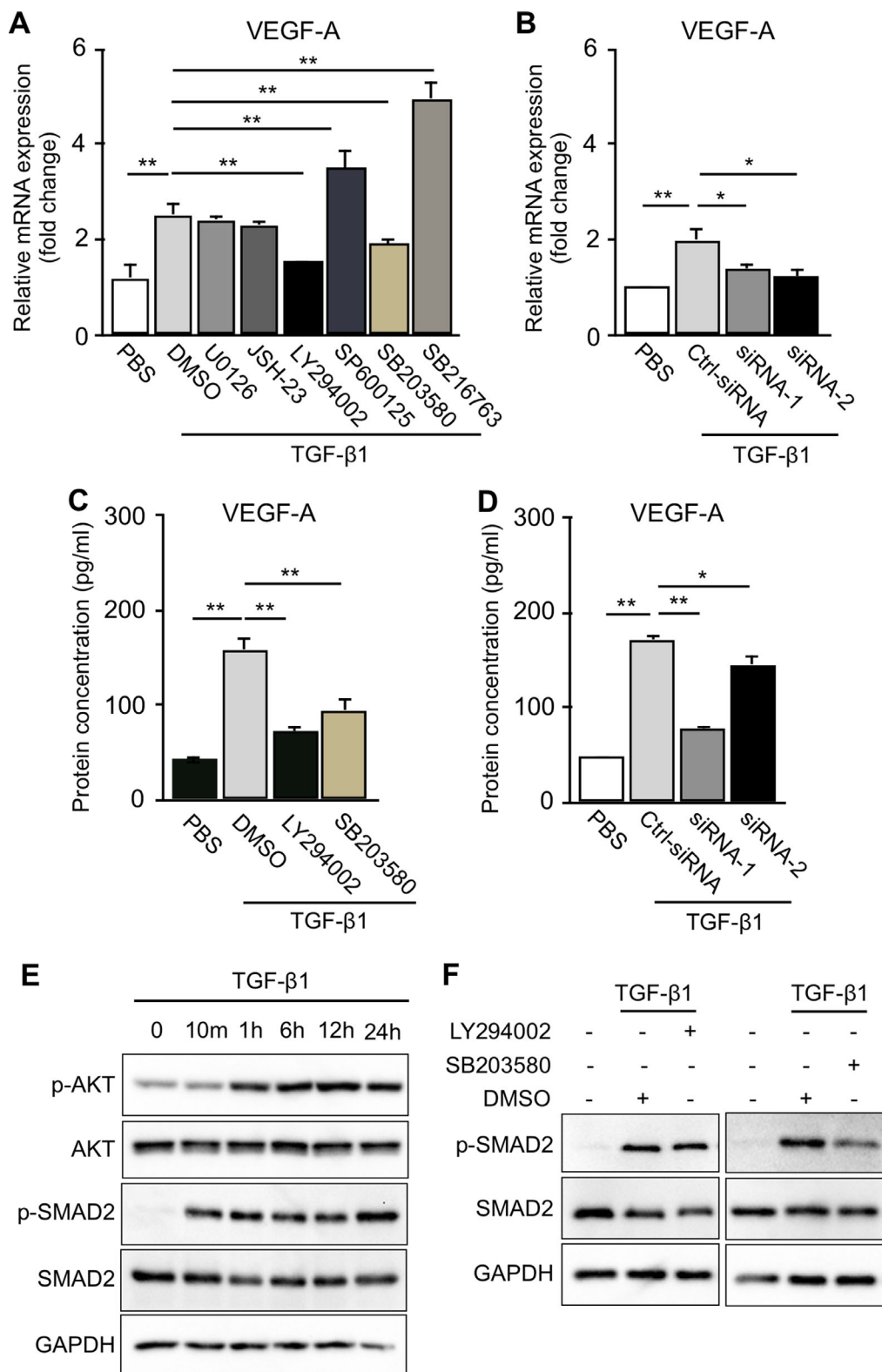


FIGURE 5. TGF- β 1-induced VEGF-A expression via its canonical and noncanonical signaling pathways in Müller glial cells. **(A)** Müller glial cells were pretreated with each inhibitor (U0126, ERK1/2; JSH-23, NF- κ B; LY294002, PI3K; SP600125, JNK; SB203580, p38 MAPK; SB216763, GSK-3) at 10 μ M for 30 minutes followed by stimulation with TGF- β 1 (30 ng/mL) for 24 hours, and *VEGFA* mRNA levels were analyzed. **(B)** Control siRNA-treated (Ctrl-siRNA) and *SMAD2*-knockdown (siRNA-1 and siRNA-2) Müller glial cells were stimulated with TGF- β 1 (30 ng/mL) for 24 hours, and *VEGFA* mRNA levels were analyzed. **(C, D)** Müller glial cells were pretreated with each inhibitor **(C)** and siRNAs against *SMAD2* **(D)** followed by stimulation with TGF- β 1 (30 ng/mL) for 48 hours, and protein levels of VEGF-A were analyzed. $n = 3$, * $P < 0.05$, ** $P < 0.01$. **(E)** Müller glial cells were incubated with TGF- β 1 (30 ng/mL), and protein levels of phosphorylated and total forms of AKT and SMAD2 were analyzed at various time points. **(F)** Müller glial cells were pretreated with each inhibitor followed by stimulation with TGF- β 1 (30 ng/mL) for 30 minutes, and protein levels of phosphorylated and total forms of SMAD2 were analyzed.

but not PI3K (Fig. 5F), suggesting the activation of SMAD2 in the downstream of p38 MAPK. Similar results were obtained in the above series of experiments using TGF- β 2 (Supplementary Figs. S5A–S5F).

In addition, the inhibition of JNK and GSK-3 alone per se increased *VEGFA* expression, and synergistically increased TGF- β 1–induced *VEGFA* expression (Supplementary Figs. S5G, S5H). These results indicated that TGF- β –induced VEGF-A expression was positively regulated by PI3K/AKT and p38 MAPK with its downstream SMAD2 canonical signaling, and negatively by JNK and GSK-3 in Müller glial cells.

Activation of TGF- β 1/2–Induced Signaling Molecules in Müller Glial Cells Migrated Into Diabetic Fibrovascular Tissues

To confirm the activation of TGF- β 1/2–induced signaling molecules (Figs. 2 and 5) in diabetic fibrovascular proliferation, we performed immunofluorescence analyses of their phosphorylated forms in Müller glial cells migrated into fibrovascular tissues excised from eyes of PDR patients. Double-staining experiments for serial sections demonstrated colocalization of GFAP with GS (Figs. 6A–6C), phosphorylated NF- κ B (Figs. 6D–6F), phosphorylated p38 MAPK (Figs. 6G–6I), phosphorylated GSK-3 (Figs. 6J–6L), phosphorylated AKT (Fig. 6M–6O), and phosphorylated SMAD2 (Figs. 6P–6R). In concert with the *in vitro* signal blocking results (Figs. 2 and 5), these findings suggested that Müller glial activation of signaling molecules related to TGF- β autoinduction and TGF- β –induced VEGF-A production during diabetic fibrovascular proliferation.

DISCUSSION

In contrast with the subretinal space attached with the RPE cell monolayer, no cells of epithelial origin are present at the epiretinal (i.e., intravitreal) space where fibrovascular proliferation occurs secondary to PDR; therefore, the cellular source of myofibroblasts and the mechanism of transdifferentiation remain incompletely understood. The present study demonstrated the following novel findings on the multifaceted role of Müller glial TGF- β together with its regulatory signaling pathways in the pathogenesis of diabetic epiretinal fibrovascular proliferation. First, TGF- β 1/2–initiated Müller GMT, recently recognized as the etiology of idiopathic epiretinal membrane,⁹ could also apply to epiretinal fibrovascular proliferation in patients with PDR (Fig. 1). Second, TGF- β 1/2 autoinduction, as seen in RPE cells during EMT for subretinal fibrovascular proliferation owing to nAMD,⁶ would also be appropriated to the amplifying process of Müller GMT in diabetic fibrovascular proliferation (Figs. 2 and 6). Third, screening of PDR-associated profibrotic cytokines identified TGF- β 1/2 as the exclusive inducer of VEGF-A production in Müller glial cells (Fig. 3). Finally, Müller glial autoinduction of TGF- β 1/2 concurrently accelerated autocrine signal transduction for VEGF-A production (Figs. 4–6). Collectively, complicated cross-talk existed between multiple signaling pathways in Müller glial cells, implying the possible modification of balance between fibrogenic Müller GMT and angiogenic VEGF-A production (Fig. 7).

The currently observed TGF- β –initiated Müller GMT in diabetic fibrovascular proliferation was originally identified as the molecular mechanism of idiopathic epiretinal membrane,⁹ a clinical entity characterized by avascular fibrocellular proliferation with much lower levels of VEGF-A in the vitreous compared with PDR.²⁴ One possible explanation for the PDR-specific potent induction of VEGF-A in TGF- β –stimulated Müller glial cells would be much higher levels of angiotensin II in the vitreous of eyes with PDR.²⁵ Because angiotensin II was shown to upregulate TGF- β 1 expression in Müller glial cells,²⁶ the increased concentration of angiotensin II in PDR eyes could thus reach and promote the vicious cycle of TGF- β 1/2 autoinduction, leading to sufficiently augmented levels of TGF- β 1/2 for Müller glial VEGF-A production. Indeed, T β RI/II-positive Müller glial cells were immunoreactive for VEGF-A in our patient tissues, in accordance with the positive correlation between VEGF-A and TGF- β 1/2² as well as angiotensin II²⁵ in PDR eyes. Consistently, our previous data with diabetic mice demonstrated that angiotensin II signaling contributed to retinal expression of VEGF-A,²⁷ the concentration of which reasonably correlated with renin enzymatic activity²⁸ and soluble (pro)renin receptor²⁴ in the vitreous of patients with PDR. Independently of TGF- β , angiotensin II was also shown to directly induce VEGF-A expression in pericytes²⁹ and macrophages,³⁰ further enhancing the angiogenic activity of PDR.

The cellular sources of myofibroblasts with their molecular markers in diabetic fibrovascular tissues were previously suggested to include Müller glial cells positive for GS, GFAP, and α -SMA¹⁰; vascular endothelial cells positive for CD31 and α -SMA³¹; and bone marrow-derived fibrocytes positive for CD45, CD11b, and α -SMA,³¹ as well as CD45 and C-C chemokine receptor 2 and type I collagen.³² Additionally, the mesenchymal transcription factor SNAIL was shown to be immunoreactive despite no demonstration of its cellular origin in diabetic fibrovascular tissues.³⁵ As concerns avascular fibrocellular proliferation associated with diabetic macular edema, the cellular source of myofibroblasts with its molecular markers in epiretinal membranes was shown to be hyalocytes positive for CD45, CD64, and α -SMA,³⁴ which would possibly be involved in more advanced fibrovascular tissues as resident macrophages at the epiretinal space. Resultant phenotypes in these kinds of cells stimulated with TGF- β were reported to include collagen gel hypercontraction,³⁵ increased cell migration and proliferation, and upregulation of SNAIL, SM22, α -SMA, type I collagen, and fibronectin⁹ in Müller glial cells; upregulation of SNAIL, SM22, and fibroblast-specific protein-1 in vascular endothelial cells³¹; upregulation of α -SMA in fibrocytes³²; and collagen gel hypercontraction and upregulation of α -SMA in hyalocytes.³⁶ In contrast, TGF- β –induced phenotypic changes in contractility and α -SMA expression were only marginal in astrocytes,³⁶ the major gliosis-related cellular component in non-neovascular epiretinal membranes associated with diabetic macular edema³⁷ as well as PDR.³⁸ In addition to Müller GMT, diabetic fibrovascular proliferation would therefore result from myofibroblastic differentiation from other cellular sources, including vascular endothelial cells (commonly known as EndoMT or endothelial-mesenchymal transition), fibrocytes, and possibly hyalocytes. Notably, high glucose stimulation to Müller cells was shown to induce SNAIL-mediated CTGF and fibronectin expression,³³ which would imply the potential involvement of

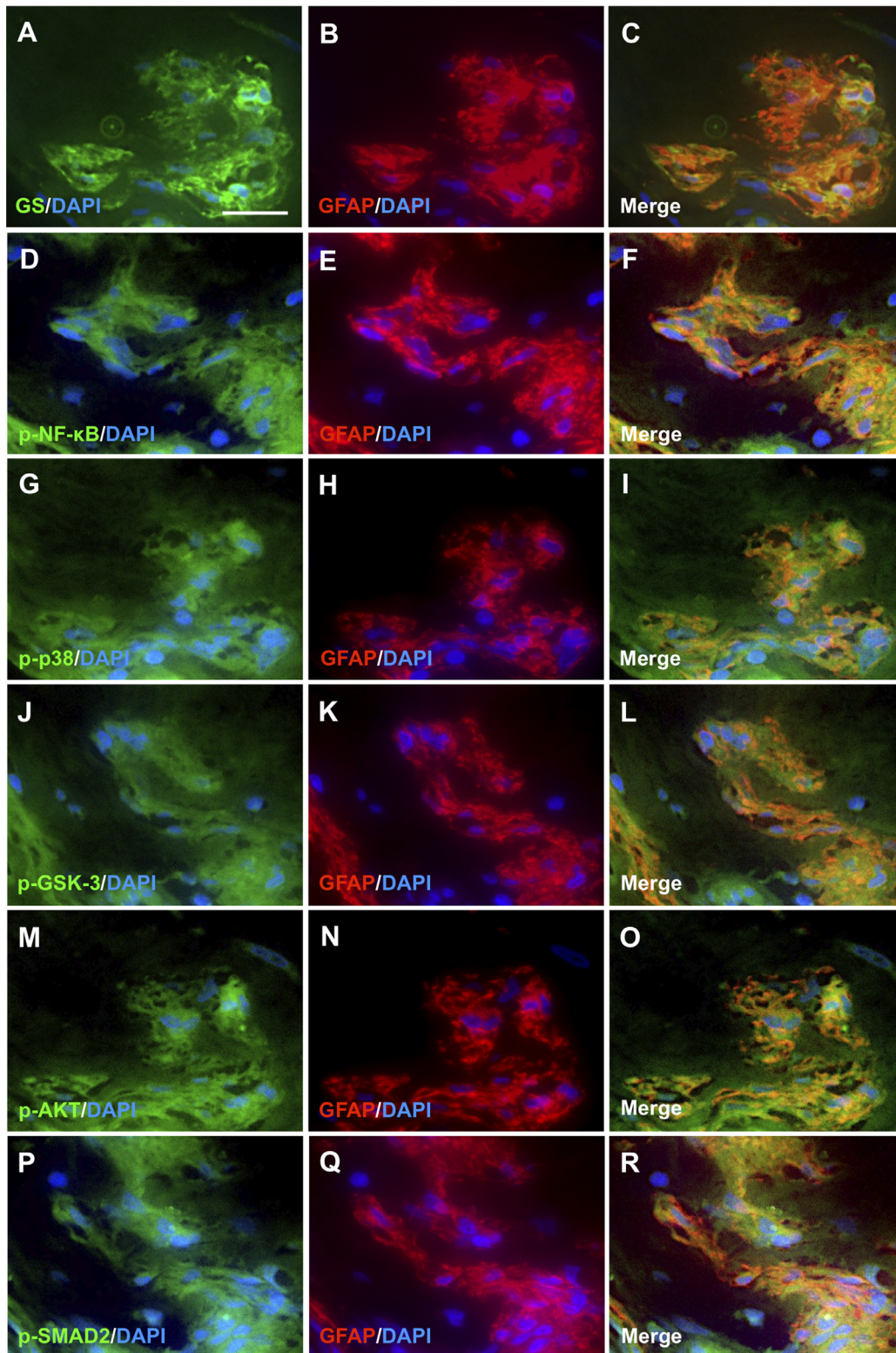


FIGURE 6. Activation of TGF- β 1/2-induced signaling molecules in Müller glial cells migrated into diabetic fibrovascular tissues. (A–R) Double labeling of GS (green) and GFAP (red) (A–C), phosphorylated NF- κ B (green) and GFAP (red) (D–F), phosphorylated p38 (green) and GFAP (red) (G–I), phosphorylated GSK-3 (green) and GFAP (red) (J–L), phosphorylated AKT (green) and GFAP (red) (M–O) and phosphorylated SMAD2 (green) and GFAP (red) (P–R) in diabetic fibrovascular tissue specimens with DAPI (blue) counterstain to nuclei. Scale bar = 20 μ m.

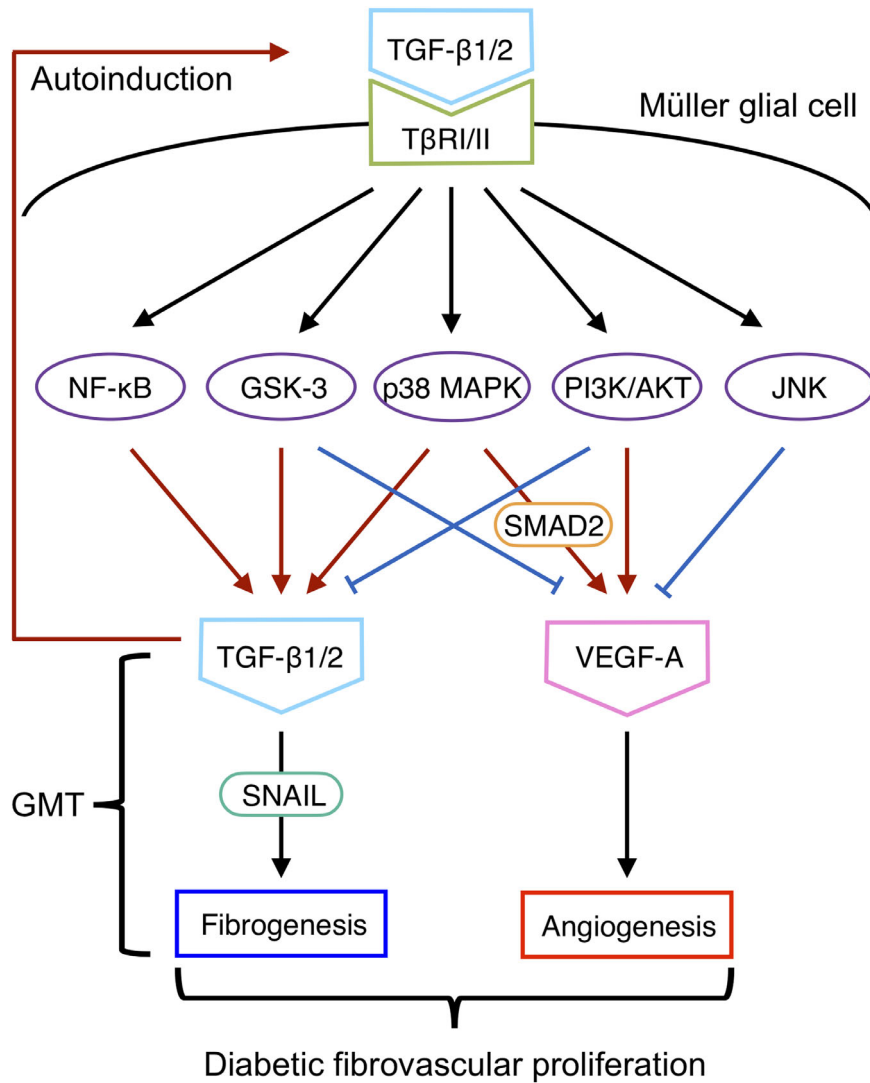


FIGURE 7. Dual involvement of Müller glial autoinduction of TGF- β 1/2 in diabetic fibrovascular proliferation via GMT and VEGF-A production. Müller glial autoinduction of TGF- β 1/2 dually causes the pathogenic cue to facilitate and amplify diabetic fibrovascular proliferation via TGF- β 1/2-driven fibrogenic GMT and VEGF-A-driven angiogenesis.

TGF- β -independent Müller GMT in diabetic fibrovascular proliferation.

In the present study, Müller glial VEGF-A production was achieved exclusively by stimulation with TGF- β 1/2 alone out of various profibrotic cytokines. Similarly, TGF- β -specific induction of VEGF-A was also found in RPE cells via p38 MAPK and PI3K/AKT, although ERK and JNK dependencies were different compared with Müller glial VEGF-A production.^{21,22} Interestingly, TGF- β solely was shown to induce, in an exclusive manner as well, Müller GMT⁹ and EMT from RPE cells,⁵ both of which were mediated via SNAIL. In the laser-induced model of choroidal neovascularization or subretinal fibrovascular proliferation, TGF- β inhibition in mice reduced SMAD2/3 phosphorylation, VEGF-A production, and the size of lesions, all of which were otherwise augmented.³⁹ SMAD3-deficient mice in this model, also mitigating the size of lesions, suppressed the expression of VEGF-A and TGF- β compared with wild-type animals,⁷ suggesting the facilitatory role of TGF- β -SMAD2/3 canonical signaling in the expression of both

VEGF-A and TGF- β (i.e., autoinduction of TGF- β) in RPE cells. In contrast, this study revealed the activation of SMAD2 canonical signaling in Müller glial VEGF-A production but not TGF- β autoinduction. Subretinal and epiretinal fibrovascular proliferations, although the signaling pathways were somewhat different between RPE and Müller cells, would consequently share the resultant common process whereby self-amplified TGF- β signaling dually governs angiogenesis and fibrosis. In this sense, nAMD and PDR, both of which are the main cause of blindness worldwide, could reasonably be regarded as TGF- β -induced fibrovascular proliferation disorders targeting RPE and Müller cells, respectively.

Anti-VEGF-A treatments have hitherto brought a limited or negligible efficacy to ocular fibrosis in the clinical setting. Although preclinical investigation would be quite a hurdle at present owing to a lack of appropriate animal models of diabetic fibrovascular proliferation, TGF- β signaling is considered a promising candidate to be targeted for the management of PDR on the basis of the current and previ-

ous data.^{2,3,9} In contrast, TGF- β signaling was shown to be required for pericyte differentiation and vascular integrity in the retina of neonatal mice,⁴⁰ thus leading to the notion that TGF- β would rather be protective in eyes with early stage non-PDR, which begins with pericyte loss.⁴¹ These accumulated piles of evidence warrant further research to seek for future therapeutic strategy, given that TGF- β may function in a stage-specific manner. Nevertheless, TGF- β would thus be an important molecular candidate targeted alone or in combination with anti-VEGF-A treatment in the long-term management of diabetic patients with PDR as well as severe non-PDR to inhibit fibrovascular proliferation.

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