The Role of Gremlin, a BMP Antagonist, and Epithelial-to-Mesenchymal Transition in Proliferative Vitreoretinopathy

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PURPOSE. Proliferative vitreoretinopathy (PVR), a major reason for failure of retinal detachment surgery, is characterized by the formation of scarlike tissue that contains transdifferentiated retinal pigment epithelial (RPE) cells. The scar tissue occurs in response to growth factors such as transforming growth factor (TGF)-β and epidermal growth factor (EGF). The authors postulate that transdifferentiation of RPE cells may arise via epithelial-to-mesenchymal transition (EMT). Bone morphogenetic proteins (BMPs) are expressed in the retina and have an anti-proliferative role. Gremlin is expressed in the outer retina and is a BMP antagonist. The study was conducted to establish a model of PVR by inducing EMT in the human RPE cell line ARPE-19, using TGF-β and EGF and to establish the contribution of gremlin to EMT.

METHODS. ARPE-19 cells were cultured and stimulated with TGF-β1, EGF, and gremlin. The expression of α-smooth muscle actin (α-SMA), vimentin, and zona occludens (ZO)-1 were examined via PCR, Western blot analysis, and immunofluorescence. Zymography was performed for matrix metalloproteinase (MMP) activity. Scratch assays were performed to assess migration.

RESULTS. A model of EMT was established in the ARPE-19 cell line. The characteristics of EMT include gain of α-SMA, loss of ZO-1, upregulation of MMP activity and enhanced migration. Gremlin plays an important role in this process, contributing to the gain of α-SMA, loss of ZO-1, and upregulation of MMP activity.

CONCLUSIONS. EMT occurs in vitro in the ARPE-19 cell line in response to the growth factors TGF-β1 and EGF. EMT is also induced by Gremlin. (Invest Ophthalmol Vis Sci. 2007;48: 4291–4299) DOI:10.1167/iovs.07-0086

Proliferative vitreoretinopathy (PVR) is a major complication of rhegmatogenous retinal detachment surgery. It is one of the main causes of failure of retinal detachment surgery. The incidence of PVR has remained unchanged since the 1980s, and although retinal reattachment rates have increased, visual outcomes remain quite poor after surgery, suggesting the need for adjunctive therapy. PVR is characterized by the growth and contraction of cellular membranes on both surfaces of the retina and within the vitreous cavity. This intraocular proliferation and contraction of the cellular membrane causes subsequent tractional retinal detachment resulting in serious vision complications. Charteris et al. suggest several targets for adjunctive agents in PVR: the initial deposition of fibrin, the proliferating cells involved, the production of extracellular matrix (ECM), and the growth factors involved in PVR. Several studies have revealed that numerous types of cells are involved in the development of PVR. These cells include RPE, Müller glial cells, macrophages, and fibrocytes. Vidinova et al. demonstrated that, in the early stages of PVR, RPE cells and fibroblasts are mainly present. In the later stages, the cellular makeup is altered, and mainly fibroblasts are found, with occasional RPE cells. Müller cells have been shown to function as an effector cell type in traction retinal detachment associated with PVR. However, RPE cells have a central role in the early stages of inducing PVR. RPE cells alone from epiretinal membranes can induce PVR in the rabbit eye. Other cells, such as fibroblasts and glial cells, also have roles in the later stages of this disease process, in forming the contractile epiretinal membrane.

RPE cells are known to secrete, and be modulated by, a wide range of growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF).

In vitreous and standard cultures, RPE cells involved in PVR undergo phenotypic changes and thus no longer resemble the normal cell populations from which they originate. This transdifferentiation is a hallmark of epithelial-to-mesenchymal transition (EMT), by which epithelial cells lose their epithelial phenotype and acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility. It has been studied extensively in the tubule epithelial cell of the kidney and forms a basis on which a model of RPE EMT can be established. More specifically, cell morphology changes from a cuboidal to a fibroblastic shape. Epithelial cell adhesion is diminished and is characterized by the loss of epithelial markers such as E-cadherin and zona occludens protein (ZO)-1 and the replacement by mesenchymal markers such as α-smooth muscle actin (α-SMA) and vimentin. In addition to changes in cell morphology, disruption of the tubular basement membrane occurs by means of matrix metalloproteinases (MMPs), which in turn facilitates enhanced cell migration and invasion. This process is responsible for diversifying tissues during normal embryologic development and also produces fibrosis in adult tissues. Examples of EMT in adult tissues include lens EMT in cataract formation and renal tubulointerstitial fibrosis.

Various growth factors and cytokines contribute to the induction of EMT, these include TGF-β, CTGF, EGF, IGF-I, and FGF-2. Among them, the profibrotic cytokine TGF-β has been shown to play a pivotal role in initiating and maintaining EMT in a variety of physiological and pathophysiological systems. EGF triggers EMT in the kidney, highlighted by the induction of several
mesenchymal markers, including vimentin, and decreases expres-
sion of epithelial markers such as E-cadherin.\textsuperscript{15} The importance of TGF-\(\beta\) in PVR is highlighted by its abundant expression in the vitreous and subretinal fluid of patients with PVR.\textsuperscript{16} TGF-\(\beta\) has also been demonstrated to have a direct effect on RPE cells and may contribute to the development of PVR.\textsuperscript{19–22} Indeed, the characteristic functions of TGF-\(\beta\), such as the stimulation of extracellular matrix production, contraction of cellular membrane, and induction of inflammation, are all negative factors in the development and progression of PVR.

Gremlin, a member of the differential screening-selected gene \textit{alternative} in the neuroblastoma (DAN) family of bone morphogenetic protein (BMP) antagonists, is commonly thought to affect different processes during growth, differentiation, and development by heterodimerizing with various BMPs.\textsuperscript{23,24} Once bound to BMPs, particularly BMP-2, -4, and -7,\textsuperscript{25,26} gremlin prevents their interacting with BMP receptors and thus inhibiting subsequent downstream signaling. Both BMP-2 and -4 have been shown to be important in late embryonic development, in which they stimulate apoptosis and regression of tissue.\textsuperscript{27,28} They are also expressed in the adult retina where they are postulated to play an antiproliferative role.\textsuperscript{29} Gremlin has an important role in the organization of the developing retina. Moreover, the detrimental outcome of aberrant overexpression of gremlin in the chick optic vesicle results in a wide range of eye defects, including coloboma, microphthalmia, and disorganizations of the neuroretina.\textsuperscript{29} Recently, gremlin expression has been shown in bovine retinal pericytes in response to elevated glucose and was also localized to the outer retina.\textsuperscript{30} It may have a role to play in diabetic retinopathy. It has been shown to play a role in diabetic fibrotic disease\textsuperscript{31,32} and tubulointerstitial fibrosis.\textsuperscript{33} Of interest, TGF-\(\beta\) has also been demonstrated to have a direct effect on RPE cells and may contribute to the development of PVR.\textsuperscript{19–22} Indeed, the characteristic functions of TGF-\(\beta\), such as the stimulation of extracellular matrix production, contraction of cellular membrane, and induction of inflammation, are all negative factors in the development and progression of PVR.

**Materials and Methods**

**Cell Culture and EMT**

Human adult RPE cells\textsuperscript{34} (ARPE-19; LGI Promochem, Teddington, UK) were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium supplemented with 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 2 mM l-glutamine (Invitrogen). Cells were maintained at 37°C in a humidified atmosphere. The culture medium was replaced twice weekly. For experiments, cells were serum starved in K-1 medium: DMEM/ Ham’s F12 medium supplemented with 50 U/ml penicillin; 50 \(\mu\)g/ml streptomycin; 2 mM l-glutamine; 5 \(\mu\)g/ml insulin, 5 \(\mu\)g/ml transferrin, and 5 ng/ml selenium (ITS; Sigma-Aldrich, St. Louis, MO); and 36 ng/ml hydrocortisone. Induction of cells to undergo EMT was performed according to the method of Okada et al.\textsuperscript{15} with 10 ng/ml TGF-\(\beta\)1 and 10 ng/ml EGF for 24, 48, and 72 hours. RNA, or protein, was isolated or immunofluorescence was performed.

Cells were also treated with TGF-\(\beta\)-2 (10 ng/ml) or gremlin (50 or 100 ng/ml) to assess their effects on RPE cells. RNA or protein was isolated or immunofluorescence was performed.

**RNA Isolation and Reverse Transcription-PCR**

Total RNA was isolated from cultured cells (RNeasy mini kit; Qiagen, Valencia, CA), according to the manufacturers’ instructions. RT-PCR was performed as follows: 2 \(\mu\)g of total RNA was treated with DNase1 (Invitrogen, Carlsbad, CA), according to the manufacturers’ instructions, to remove chromosomal DNA. Reverse transcription was performed with random primer (Invitrogen) and reverse transcriptase (Superscript II; Invitrogen), according to the manufacturers’ protocols. Limited-cycle PCR was performed with the following primers, designed with Primer3 software:\textsuperscript{35} vimentin; sense 5’-GAG AAC TTT GCC GTT GAA GC-3’, antisense 5’-CGT GAT GCT GCT GAGT TTC G3-3’; ZO-1, sense 5’-CCA GAA TCT CGG AAA AGT GC-3’, antisense 5’-ACC GTG TAA TGG CAG ACT CC-3’; \(\alpha\)-SMA, sense 5’-ATC ACC ATC GGA AAT GAA CG-3’, antisense 5’-CTG GAA GGT GGA CAG AGA GG-3’; and 18S, sense 5’-GTT GAG CGA CGA TGG TT-3’, antisense 5’-GCC TGA GCC AGT CAG TGT AG-3’.

**Real-Time PCR**

Transcript levels were determined by real-time PCR (Taqman PCR, with a 7700 analyzer; Applied Biosystems, Inc. [ABI] Foster City, CA). The sequence-specific primers and probe mixtures for human vimentin (Hs00185858_m1), human ZO-1 (Hs00268480_m1), and 18S (Hs910893E) were from predeveloped assays (ABI). All results were normalized to 18S rRNA.

**Preparation of Cell Lysates and Western Blot Analysis**

Whole-cell extracts were prepared from cultured cell monolayers as follows: the culture medium was drained off the cells, and the adherent cells were washed twice with ice-cold PBS. The cells were lysed with ice-cold modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich). The cell suspension was transferred into a centrifuge tube, left on ice, and vortexed every 5 minutes, for 15 minutes to lyse the cells. The lysate was then centrifuged at 14,000 g in a precooled centrifuge for 15 minutes. The supernatant was collected and the concentration of protein in extracts was determined by using a protein assay concentrate (Bio-Rad, Hercules, CA). Protein samples (10 \(\mu\)g) were dissolved in sample buffer (100 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 50 \(\mu\)L/\(\mu\)L \(\beta\)-mercaptoethanol) at a concentration of 1:1. Samples were resolved by SDS-PAGE according to the method of Laemmli\textsuperscript{36} and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The blot was placed in blocking solution (5 wt/vol nonfat dried milk in TBST) for 60 to 120 minutes at room temperature on a shaking table. The blots were incubated with primary antibody overnight at 4°C with rabbit Anti-ZO-1 (Zymed Laboratories. S. San Francisco, CA) 1:1000, monoclonal anti-\(\alpha\)-smooth muscle actin (Sigma-Aldrich) 1:2000, or anti-\(\beta\)-actin (Sigma-Aldrich) 1:50,000. This procedure was followed by incubation with polyclonal rabbit anti-mouse or anti-rabbit IgG-HRP-conjugated immunoglobulins (1:1000; Cell Signaling; Beverly, MA) for a further hour at room temperature. Antigen detection was performed with an enhanced chemiluminescence system (Supersignal West Dura; Pierce Biotechnology, Rockford, IL) and exposed to x-ray film.

**In-gel Zymography**

After experimentation, conditioned medium was collected and centrifuged at 5000 g for 5 minutes to remove the cells. Equal volumes of conditioned media from each experimental condition were mixed with loading buffer (25 M Tris [pH 6.8], 20% glycerol, 2% SDS, and 10 \(\mu\)g/ml bromophenol blue) and resolved by SDS-PAGE according to the method of Laemmli,\textsuperscript{29} on 10% polyacrylamide gels containing 1 \(\mu\)g/ml gelatin substrate. SDS-polyacrylamide gels were washed twice in 2.5% Triton X-100 and once in deionized \(\text{H}_2\text{O}\), incubated overnight at 37°C in incubation buffer (50 mM Tris [pH 8.0], 0.5 mM NaCl, and 10 mM CaCl\(_2\)), and stained with 0.25 mg/ml brilliant blue R in a solution of 30% acetic acid and 10% isopropanol. Gelatinolytic activity was subsequently visualized as clear bands on a blue background.

**Immunocytochemistry**

ARPE-19 were seeded onto eight-well slides (Permanox; Labtec, Nalgene, Cairo, Egypt). Cells were treated with TGF-\(\beta\)-1 (10 ng/mL; R&D Systems, Minneapolis, MN), EGF (10 ng/mL; Sigma-Aldrich),
and gremlin (25 ng/mL; R&D Systems) as indicated and subsequently stained for ZO-1 and vimentin by using standard techniques. Briefly, chamber slides were rinsed in PBS and fixed in 3.7% paraformaldehyde in TBS for 20 minutes. After they were rinsed in TBS, the cells were permeabilized with 0.01% Triton X-100 for 3 minutes and then rinsed three times in TBS. The slides were incubated with primary antibody to ZO-1 (1:100 dilution) and vimentin (1:200 dilution) overnight at 4°C. The slides were rinsed and incubated with Oregon green–conjugated secondary antibody (Invitrogen-Molecular Probes, Eugene, OR) for 1 hour at room temperature. Control incubation included the nonimmune IgG and the secondary antibody alone (data not shown). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Stained cells were visualized with a microscope (Leitz DM40; Leica Microsystems, Wetzlar, Germany), and images were captured (AxioCam system, with AxioVision 3.0.6 software; Carl Zeiss Meditec, Inc., Thornwood, NY).

**Migration Assays**

In all migration assays, the ARPE-19 cells were grown to confluent monolayers. They were then serum deprived for 24 hours, before being treated for 72 hours with the growth factor that was being examined. At the end of 72 hours, a scratch wound was inflicted on the monolayer, with a p20 pipette tip. The ability of the ARPE-19 cells to close the wound space was used to assess the migratory ability of the cells. Two time points, 24 and 48 hours after the application of the scratch, were used to assess the migratory ability of the cells. Light microscope images were taken at three time points: time point 0,
when the scratch was first applied, and then 24 and 48 hours after the scratch was applied.

**Statistical Analysis**

Each result is representative of at least three independent experiments. All values are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used to determine significant differences between growth factor–treated groups and the control group. Results were significant when \( P < 0.05 \).

**RESULTS**

**Change in Morphology of ARPE-19 Cells Treated with TGF-\( \beta \)1 and EGF**

The morphology of the ARPE-19 cells was examined, after 72 hours of the following six treatments: control, TGF-\( \beta \)1 (5 ng/mL), TGF-\( \beta \)1 (10 ng/mL), EGF (10 ng/mL), TGF-\( \beta \)1 (5 ng/mL)+EGF (10 ng/mL), and TGF-\( \beta \)1 (10 ng/mL)+EGF (10 ng/mL). Normally, confluent ARPE-19 cells demonstrate a classic epithelial morphology and form a cobblestone-like monolayer (Fig. 1A). After treatment with TGF-\( \beta \)1 and EGF, the cells become more elongated and demonstrated a mesenchymal, fibroblastic morphology (Figs. 1B–D). The effects were most notable with the combination of TGF-\( \beta \)1 and EGF at the 72-hour time point (Figs. 1E, 1F).

**Loss of Epithelial Markers and Acquisition of Mesenchymal Markers in ARPE-19 Cells after Treatment with TGF-\( \beta \)1 and EGF**

The mRNA levels of the mesenchymal markers \( \alpha \)-SMA and vimentin and the epithelial marker ZO-1 were examined by RT-PCR and quantitative PCR. 18S RT-PCR was used as a control for equal amounts of RNA in the RT reaction. Lane 1, control; lane 2, TGF-\( \beta \)1 5 ng/mL; lane 3, TGF-\( \beta \)1 10 ng/mL; lane 4, EGF 10 ng/mL; lane 5, TGF-\( \beta \)1 5 ng/mL + EGF 10 ng/mL; and lane 6, TGF-\( \beta \)1 10 ng/mL + EGF 10 ng/mL. (B) Quantitative PCR results for vimentin expression, normalized to 18S. (C) Quantitative PCR results for ZO-1 expression, normalized to 18S. ZO-1 was maximally depressed at the 72-hour time point. *\( P < 0.05 \); **\( P < 0.01 \).
control for equal amounts of RNA in the RT reaction. Up-regulation of the mRNAs for α-SMA and vimentin (Fig. 2A) were noted with each of the six treatments described earlier, after 72 hours. Real-time PCR for vimentin mRNA demonstrated a clear increase in vimentin levels, which reached its maximum at the 48-hour time point (Fig. 2B). There was a decrease in expression of ZO-1 mRNA, with each treatment, especially after 72 hours (Fig. 2A). This result was verified by real-time PCR, which showed a maximum loss in ZO-1 at 72 hours (Fig. 2C).

Western blot analyses were also performed to examine the protein levels of α-SMA and ZO-1, after treatment with TGF-β1 and EGF, for 72 hours. These blots confirm the increased expression of α-SMA and the decreased expression of ZO-1 that was previously seen on RT-PCR and quantitative PCR (Fig. 3A). Figure 3B illustrates the immunofluorescence results for ZO-1 and vimentin expression after 72 hours of treatment with TGF-β1 (10 ng/mL) compared with control. The downregulation of ZO-1 and the upregulation of vimentin expression are clearly evident. 

**Figure 3.** Induction of EMT characteristics in ARPE-19 cells treated with TGF-β1 and EGF. (A) Western blot analysis of whole-cell extracts prepared from ARPE-19 cells, treated for 72 hours with TGF-β1 and EGF. Induction of α-SMA expression and loss of ZO-1 expression reached its maximum with the combination of TGF-β1 and EGF treatment for 72 hours. Treatments are as shown in Figure 2. β-Actin is also shown as a control. (B) Immunocytochemistry of ARPE-19 cells was used to illustrate altered expression in ZO-1 and vimentin after the induction of EMT by TGF-β1 (10 ng/mL) treatment for 72 hours. Cells were fixed with paraformaldehyde and stained with antibodies against ZO-1 and vimentin. Nuclei were counterstained with DAPI. (C) Comparison of effects of TGF-β1 and β2 on α-SMA and ZO-1 expression. Western blot analysis of whole cell extracts prepared from ARPE-19 cells, treated for 72 hours with TGF-β1 and β2. TGF-β2 caused a greater increase in α-SMA expression and a greater loss of ZO-1. (D) Zymography of media obtained from ARPE-19 cells after 72 hours of incubation with TGF-β1 alone, TGF-β1+EGF, and TGF-β2 alone. There was an increase in MMP activity in the cells treated with both TGF-β1 and EGF. The increase is less dramatic in the cells treated with TGF-β1 and β2.
Increased MMP Activity in ARPE-19 Cells Treated with TGF-β1 and EGF

The breakdown of basement membrane to facilitate migration is the final characteristic of EMT. The membrane breakdown occurs secondary to increased MMP activity. MMP activity was assessed through zymography (Fig. 3D). Control (no treatment) was compared to treatment with TGF-β1 (10 ng/mL) and EGF (10 ng/mL) for 72 hours. There was a clear increase in MMP activity.

Enhanced Migration of ARPE-19 Cells

The most dramatic changes induced in epithelial and mesenchymal marker expression was with the combination of TGF-β1 (10 ng/mL) and EGF (10 ng/mL). Based on this result, the migration of the transformed ARPE-19 cells was examined with scratch assays for 48 hours. Figure 4 shows the enhanced migratory ability of the cells treated with the combination of TGF-β1 and EGF, compared with the control.

Characteristics of EMT Induced by TGF-β2 in ARPE-19 Cells

A comparison was performed between TGF-β1 and -β2. On Western blot, there was a greater effect of TGF-β2 on α-SMA expression. There was also a greater downregulation of ZO-1 associated with TGF-β2 compared with TGF-β1 (Fig. 3C). TGF-β1 and -β2 induced a similar increase in MMP activity (Fig. 3D). There was a similar increase in migratory ability in cells treated with TGF-β1 compared to TGF-β2 (Fig. 4).

Gremlin-Induced Characteristics of EMT in ARPE-19 Cells

Western blot analyses were performed to examine the protein levels of α-SMA and ZO-1, after treatment with gremlin 50 ng/mL for 72 hours. Figure 5A shows the upregulation of α-SMA expression and the downregulation of ZO-1 with gremlin treatment compared with the control, although not to the same extent as with TGF-β1 treatment. Figure 5B illustrates the immunofluorescence results after 72 hours of treatment with gremlin 25 ng/mL. There was a clear reduction in ZO-1 expression and an increase in vimentin expression.

Gremlin-Induced MMP Activity in ARPE-19 Cells

Figure 5C illustrates the zymogram produced in the ARPE-19 cells in response to 2 different doses of Gremlin; 50 ng/mL and 100 ng/mL. The intensities of the bands of MMP-2 activity were greater in gremlin-treated cells than in the control cells. The effect appeared to reach its maximum at a dose of 50 ng/mL.

Migratory Ability of ARPE-19 Cells Treated with Gremlin

Figure 6 shows the migration assays performed over a period of 48 hours in ARPE-19 cells pretreated with either 50 or 100 ng/mL gremlin. Gremlin did not induce enhanced migratory ability compared with the control with either dose.

DISCUSSION

The presence of the growth factors TGF-β and EGF has been confirmed in the subretinal fluid of patients with retinal detachment, indicating a role for these factors in the pathogenesis of PVR. We examined the effect of these growth factors on a human retinal pigment cell line (ARPE-19) and then, using the model established by Liu for renal fibrogenesis, established a model of EMT in the retina. Similar to the renal model, we showed a loss of epithelial markers, a gain of mesenchymal markers, and enhanced migration. Instead of demonstrating basement membrane breakdown, we showed increased MMP activity.

We demonstrated the loss of ZO-1 in ARPE-19 cell EMT. ZO-1 forms a part of tight junctional complexes. Its downregulation in EMT resulted in the loss of transepithelial barrier function. ZO-1 was downregulated after treatment with TGF-β1 and EGF. The effect was most notable after 72 hours of treatment with TGF-β1 (10 ng/mL)+EGF (10 ng/mL). The loss of ZO-1 in RPE EMT has also been noted in EMT occurring in other organic diseases, such as in pulmonary fibrosis.

The normal RPE expresses only a small amount of the mesenchymal markers α-SMA and vimentin. α-SMA is necessary to enhance cell motility. Vimentin is the major intermediate filament of mesenchymal cells and has viscoelastic properties that allow it to stabilize cell structure in migration. Both α-SMA and vimentin were upregulated after...
treatment with TGF-β1 and EGF. This change in expression was most obvious in the combined treatment with TGF-β1 and EGF. These results are consistent with previous work demonstrating the expression of α-SMA in human RPE and bovine RPE in response to stimulation by TGF-β.44,45 Similarly, Casaroli-Marano et al.8 demonstrated the upregulation of vimentin in human RPE cells stimulated by vitreous from patients with proliferative retinal disorders and subretinal fluid from patients with retinal detachment.

It has been demonstrated that TGF-β1 and EGF have synergistic effects in cultured renal proximal tubular (MCT epithelial cell) EMT.15 In keeping with this, application of TGF-β1 and EGF to the cultured ARPE-19 cell line showed that TGF-β1 and EGF also have synergistic effects in transforming the RPE from the organized epithelial cobblestone-like monolayer to a more mesenchymal morphology, downregulating epithelial markers and upregulating mesenchymal markers.

MMPs are a family of zinc-dependent endopeptidases that are capable of degrading all components of the extracellular matrix and basement membrane.46 We have demonstrated a clear increase in MMP activity with treatment with TGF-β and EGF. The synergistic effect of TGF-β1 and EGF on the upregulation of MMP activity was clear. This finding is in agreement with the idea that MMPs are involved in vitreoretinal disorders.47

Tanihara et al.48 demonstrated that RPE cells express both TGF-β1 and -β2. TGF-β2 is likely to be the predominant TGF-β isoform in the posterior segment of the eye in PVR.49 Therefore, a comparison was performed between TGF-β1 and -β2. We show that TGF-β2 has a greater effect than TGF-β1 on the upregulation of α-SMA and the downregulation of ZO-1. MMP activation and migratory ability appear to be similar between TGF-β1 and -β2. An explanation for the greater effect of TGF-β2 on the induction of EMT in the ARPE-19 cells may be its ability to activate other growth factors such as TGF-β1 and PDGF. The presence of multiple growth factors may allow a synergy of action, similar to that shown by Okada et al.15 with TGF-β1 and EGF and that shown by Stutz et al.17 with TGF-β1 and FGF-2.

It is known that gremlin expression is enhanced in profibrotic diseases such as diabetic retinopathy,30 diabetic nephropathy,51,52 and tubulointerstitial fibrosis.53 Other work has demonstrated that Gremlin expression colocalizes with TGF-β expression in both in vivo and in vitro models of diabetic nephropathy.53 High glucose triggers expression of both TGF-β and gremlin in the bovine retina, and the expression is
modulated by the use of anti-TGF-β antibody in vitro, which suggests that the TGF-β released by the RPE in injury such as retinal detachment and diabetic retinopathy may act in part by activating gremlin.

Expression of constitutively active EGF receptor has been shown to induce gremlin in developing chick limbs, resulting in polydactyl. During the normal development of limb buds, BMP signaling is necessary to induce apoptosis and thus regression of tissue between digits. Gremlin acts as the counter force in this, blocking the antiproliferative effect of the BMPs. The increased expression of gremlin in this situation causes in a pro-proliferative situation, resulting in polydactyl. Once the development of the embryo is complete, gremlin becomes quiescent. In the adult retina, it is possible that the release of growth factors such as TGF-β and EGF in injury acts to produce EMT and subsequent fibrosis, in part by reactivating gremlin. It is also possible that the synergistic action of the combination of TGF-β and EGF may be due to a greater ability to induce the expression of gremlin.

Therefore, in our model of ARPE-19 cell EMT, the ability of gremlin to fulfill the requirements of EMT was examined. Gremlin appeared to contribute to EMT in the RPE. It fulfilled three of the four characteristics of EMT: It upregulated α-SMA, downregulated ZO-1, and increased MMP activity. It did not enhance migration.

The exact mechanism behind the ability of gremlin to induce EMT is not clear. What is known is that gremlin antagonizes the antiproliferative effect of BMPs, by binding to BMPs and preventing them from interacting with their receptors. Of note, the administration of BMP-7 reverses EMT in the kidney model and antagonizes TGF-β1-induced E-cadherin downregulation. It has also been shown that BMP-7 inhibits the release of proinflammatory cytokines. It may be that BMP-7 reverses EMT by preventing the release of growth factors, and the subsequent activation of gremlin.

We successfully established a model of EMT in the RPE by using TGF-β1 and EGF. Gremlin is a major participant in EMT. It fulfills all the characteristics of EMT except for the enhancement of migration. Much work remains to be done, including the examination of intracellular signaling. The ability of BMPs to reverse EMT induced by growth factors and gremlin should be examined. Smad signaling especially Smad 3, which is known to be involved in EMT should be examined in detail. Finally, the ability of TGF-β antibody, MMP inhibitors, and the various Smad inhibitors, such as halofuginone, to inhibit EMT should be studied. These studies may provide potential novel therapies for the prevention and treatment of PVR.

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**References**

Gremlin and EMT in Proliferative Vitreoretinopathy


