

Gremlin mediates the TGF- β -induced induction of profibrogenic genes in human retinal pigment epithelial cells

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Abstract. Proliferative vitreoretinopathy (PVR) is characterised by the contraction and growth of fibrotic membranes on the retina and within the vitreous body. Retinal pigment epithelial (RPE) cells, a major cellular component of the fibrotic membrane, is one of the cell types that have been previously reported to associate with PVR pathogenesis. During PVR, RPE cells undergo increased cell proliferation, migration and the secretion of extracellular matrix molecules, such as fibronectin and type I collagen. A variety of cytokines and growth factors are involved in the formation of the fibrotic membrane. Although gremlin has been reported to serve an important role in the regulation of epithelial-to-mesenchymal transition in PVR, the relationship between gremlin and the expression of profibrogenic factors in human RPE cells remains unclear. In the present study, gremlin promoted RPE cell proliferation and the expression of type I collagen and fibronectin. In addition, knocking down gremlin expression by siRNA significantly suppressed the transforming growth factor (TGF)- β 1- and TGF- β 2-induced expression of type I collagen and fibronectin in RPE cells. These findings suggest that gremlin may serve an important role in the development of PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is a serious complication that is caused by rhegmatogenous retinal detachment and is the leading cause of retinal detachment surgery failure (1,2). It is physiologically characterised by increased cell proliferation, migration and secretion of extracellular matrix (ECM) proteins, which results in the formation of fibrotic membranes in response to retinal detachment. Retinal

pigment epithelial (RPE) cells are one of the major cellular components of the fibrotic membrane. Of the number of cytokines and growth factors that have been previously reported to contribute to PVR pathogenesis, transforming growth factor (TGF)- β is of particular importance (3).

Gremlin is a highly conserved 184-amino-acid protein that contains a cysteine-rich region (4-7). Structurally, it is a member of the cysteine knot superfamily, which can be present in both soluble and cell-associated forms (8-11). Gremlin belongs to a family of bone morphogenetic protein antagonists that participate in a number of physiological processes, including cell survival, differentiation, growth and development (4,8-12). Gremlin is predominantly localised to the outer retina, and high levels of its expression have been demonstrated in bovine retinal pericytes in response to elevated glucose levels compared with control treated pericytes (13). The dysfunction of gremlin has also been observed to be associated with a number of diseases, such as diabetic fibrotic disease (4,8-12,14).

Although it has been previously shown that, during PVR, gremlin induces epithelial-to-mesenchymal transition (EMT) in RPE cells (15), information on the potential link between gremlin and the expression of pro-fibrogenic factors in human RPE cells remain limited. The present study demonstrated that gremlin increased the proliferation and expression of fibronectin and type I collagen in human RPE cells, whereas gremlin knockdown by small interfering (si)RNA expression significantly reduced TGF- β 1- and TGF- β 2-induced expression of fibronectin and type I collagen in human RPE cells.

Materials and methods

Reagents. Gremlin-1 (cat. no. SRP3285) and DAPI (cat. no. D9542) were purchased from Sigma-Aldrich; Merck KGaA. Recombinant human TGF- β 1 and TGF- β 2 were obtained from Cell Signaling Technology, Inc. Anti-fibronectin (cat. no. ab2413) and anti-type I collagen (cat. no. ab34710) antibodies, type I collagen (human pro-collagen I α , cat. no. ab229389) and fibronectin (cat. no. ab219046) ELISA kits were purchased from Abcam. TRITC conjugated goat anti-rabbit IgG secondary antibody (cat. no. ZF-0316) was obtained from Zhongshan Golden Bridge Biotechnology Co., Ltd. Human gremlin siRNA and siRNA control

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(cat. no. siP06969473939) were purchased from Guangzhou RiboBio Co., Ltd. The Gremlin siRNA sequence is CCACCTACCAAGAAGAAGA.

Cell culture. Human RPE cells (ARPE-19; CRL-2302) were purchased from the American Type Culture Collection. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 ng/ml streptomycin at 37°C and 5% CO₂ in a humidified atmosphere. ARPE-19 cells were transfected as described below. Transfected ARPE-19 cells were incubated with recombinant human TGF-β1 or TGF-β2.

Gremlin siRNA transfection. The cells were transfected with gremlin siRNA or control siRNA (50 nM each). Transfections were performed using the riboFECT™ CP reagent according to the manufacturer's protocol. Assays were performed 48 h after transfection, including assessment of mRNA and protein levels and immunofluorescent staining.

Cell viability and proliferation assay. Cell viability and proliferation was measured using MTT and 5-bromo-2-deoxyuridine (BrdU) assays. For MTT assay, RPE cells were cultured in 96-well plates at 1.0x10⁴ cells/well for 24 h and were partially starved in DMEM supplemented with 1% FBS for 12 h, following which they were treated with various concentrations (25, 50 and 100 ng/ml) of gremlin at various time points (12, 24 and 48 h). MTT (5 mg/ml; 20 μl) was then added to the culture medium, and the cells were incubated for an additional 4 h at 37°C. DMSO was then added to each well to dissolve the formazan crystals formed. Absorbance in each well was measured at 490 nm using a microplate reader. The BrdU assay was performed according to the manufacturer's protocol. A total of 5.0x10³ cells were seeded in a 96-well plate for 24 h and were partially starved in DMEM supplemented with 1% FBS for 12 h, and then treated with or without tested substances for 48 h at 37°C. A 10 μM concentration of BrdU was added to the medium. Measurement of cell proliferation was quantified by BrdU ELISA kit (Roche Diagnostics). Absorbance in each well was measured at 450 nm with a microplate reader (Bio-Rad Laboratories, Inc.)

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from the RPE cells. cDNA synthesis was performed with 2 μg total RNA using RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. The reaction mixtures contained the following: 1 μl oligo(dT) primer, 4 μl 5x reaction buffer, 1 μl Ribolock™ RNase Inhibitor, 2 μl 10 mM dNTP mix and 1 μl RevertAid™ M-MuLV Reverse Transcriptase in a total volume of 20 μl. Incubation was for 60 min at 42°C before the reaction was terminated by heating at 70°C for 5 min. The ABI Sequence Detector System 7500 (Thermo Fisher Scientific, Inc.) was used to perform RT-qPCR. The PCR solution consisted of specific primers (0.3 μM each), 2.5 μl cDNA, and 12.5 μl Maxima SYBR-Green qPCR Master Mix (Fermentas; Thermo Fisher Scientific, Inc.) to a final reaction volume of 25 μl. The sequences of the primers used were as follows: Human

fibronectin forward, 5'-GATAAATCAACAGTGGGAGC-3' and reverse, 5'-CCCAGATCATGGAGTCTTTA-3'; human type I collagen forward, 5'-TGGTGGTTATGACTTTGGTTACGAT-3 and reverse, 5'-TGTGCGAGCTGGGTTCTTTCTA-3'; human GAPDH forward, 5'-TGTTTCGACAGTCAGCCGCAT-3' and reverse, 5'-ACTCCGACCTTCACCTTCCC-3'; and human gremlin forward, 5'-AAGCGAGACTGGTGCAAAC-3' and reverse, 5'-CTTGCAGAAGGAGCAGGACT-3'. The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 39 cycles of 95°C for 15 sec, 61°C for 30 sec and 72°C for 30 sec. The relative mRNA expression levels were calculated as the difference between target and GAPDH expression levels using the 2^{-ΔΔCq} method (16).

Immunofluorescence staining. Human RPE cells were fixed with 4% paraformaldehyde for 20 min at 23°C, washed three times with PBS and permeabilised using 0.5% Triton X-100 for 15 min at 23°C. The cells were subsequently blocked with 10% goat serum (Zhongshan Golden Bridge Biotechnology) for 1 h at 23°C before incubation with rabbit anti-fibronectin (dilution, 1:100) or rabbit anti-collagen I (dilution, 1:100) primary antibodies, which were diluted in PBS supplemented with 10% goat serum, overnight at 4°C. The next day, the cells were incubated with TRITC-conjugated goat anti-rabbit IgG (dilution, 1:200) secondary antibodies at room temperature for 1 h. The samples were counterstained with DAPI (1:1,000; cat. no. D9542, Sigma-Aldrich; Merck KGaA) at room temperature for 10 min. The samples were examined using a fluorescence microscope (DS-Ri1-U2; Nikon Corporation) and photographed at x200 magnification in five random fields (DS-U2; Nikon Corporation).

ELISA. The measurement of fibronectin protein levels of using ELISA have been previously reported (17-19). Cells were seeded at 4x10³ cells per well in 12-well plates. After reaching a confluency of approximately 85-90%, the medium was changed to a serum-free medium for 12 h. Cells were transfected with control siRNA or gremlin siRNA using the riboFECT™ CP reagent. After transfection, cells were incubated with recombinant human TGF-β1 or TGF-β2 for 48 h at 37°C. The samples were collected after the cells were treated. Fibronectin and type I collagen levels in the culture supernatants of RPE cells were measured using fibronectin and type I collagen ELISA kits according to manufacturer's protocols.

Statistical analysis. All statistical differences were evaluated using one-way analysis of variance followed by Tukey's test. All data are presented as the mean ± SD and were analysed using SPSS 17.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Gremlin induces human RPE cell proliferation and increases fibronectin and type I collagen expression. To examine the role of gremlin in the proliferation and expression of fibronectin and type I collagen in human RPE cells, cells were treated with ascending concentrations of gremlin (0, 25, 50 and 100 ng/ml) for 48 h. At a concentration of 50 ng/ml or 100 ng/ml, gremlin significantly increased RPE cell proliferation compared with the control group (Fig. 1A and C). In

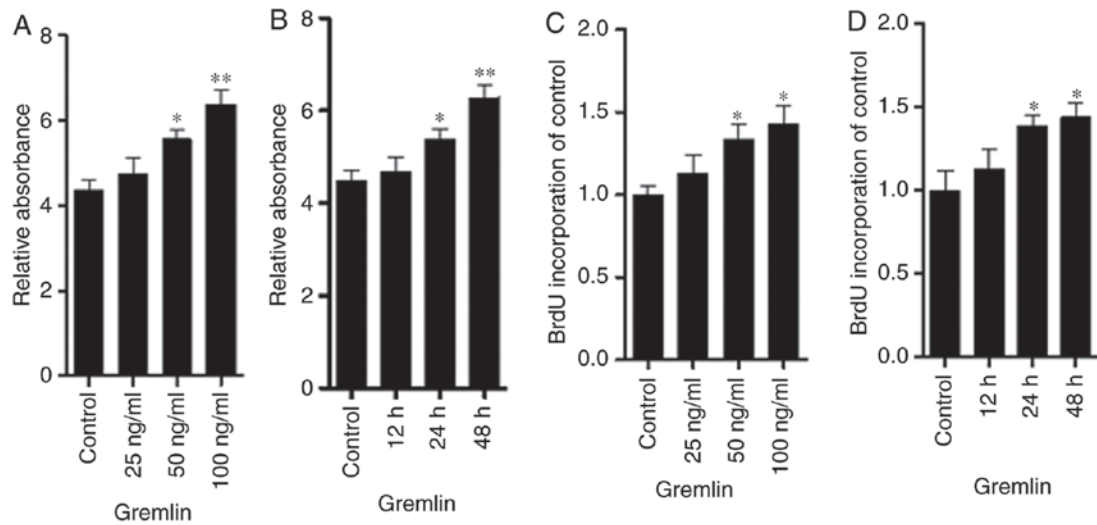


Figure 1. Gremlin induces RPE cell proliferation. (A) RPE cell viability was analysed using MTT assay following treatment with 0, 25, 50 or 100 ng/ml gremlin for 48 h. (B) RPE cell viability was analysed by MTT assay following treatment with 100 ng/ml gremlin for 12, 24 and 48 h. (C) RPE cell proliferation was analysed using BrdU assay following treatment with 0, 25, 50 or 100 ng/ml gremlin for 48 h. (D) RPE cell proliferation was analysed using BrdU assay following treatment with 100 ng/ml gremlin for 12, 24 and 48 h. The data shown represent the mean \pm SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control. BrdU, 5-bromo-2-deoxyuridine; RPE, retinal pigment epithelial cells.

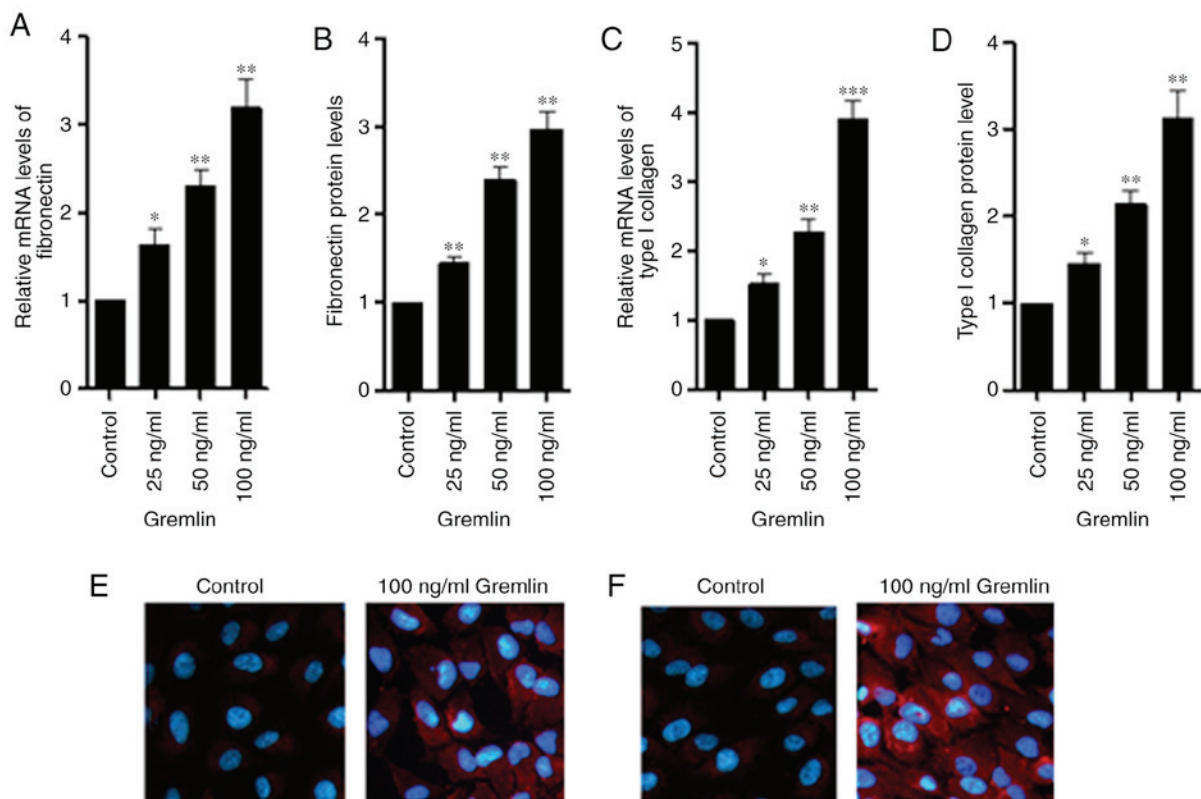


Figure 2. Gremlin promotes the expression and secretion of type I collagen and fibronectin in RPE cells. The expression of type I collagen and fibronectin in RPE cells was determined using reverse transcription-quantitative PCR, ELISA and immunofluorescence staining. (A) Relative levels of fibronectin mRNA expression and (B) fibronectin secretion were measured using ELISA following treatment with 25, 50 or 100 ng/ml gremlin for 48 h. (C) Relative levels of type I collagen mRNA expression and (D) type I collagen secretion were measured using ELISA following treatment with 25, 50 and 100 ng/ml gremlin for 48 h. (E) Immunofluorescence staining of fibronectin and (F) type I collagen in RPE cells following treatment with 100 ng/ml gremlin for 48 h. Magnification, x200. The data represent the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. RPE, retinal pigment epithelial cells.

addition, RPE cell proliferation was significantly enhanced following incubation with 100 ng/ml gremlin for 24 and 48 h compared with the control group (Fig. 1B and D). Subsequently, the mRNA and protein levels of fibronectin and type I collagen

were measured using RT-qPCR, ELISA and immunofluorescence staining. At a concentration of 25, 50 or 100 ng/ml, recombinant gremlin significantly increased the expression of fibronectin (Fig. 2A and B) and type I collagen (Fig. 2C and D)

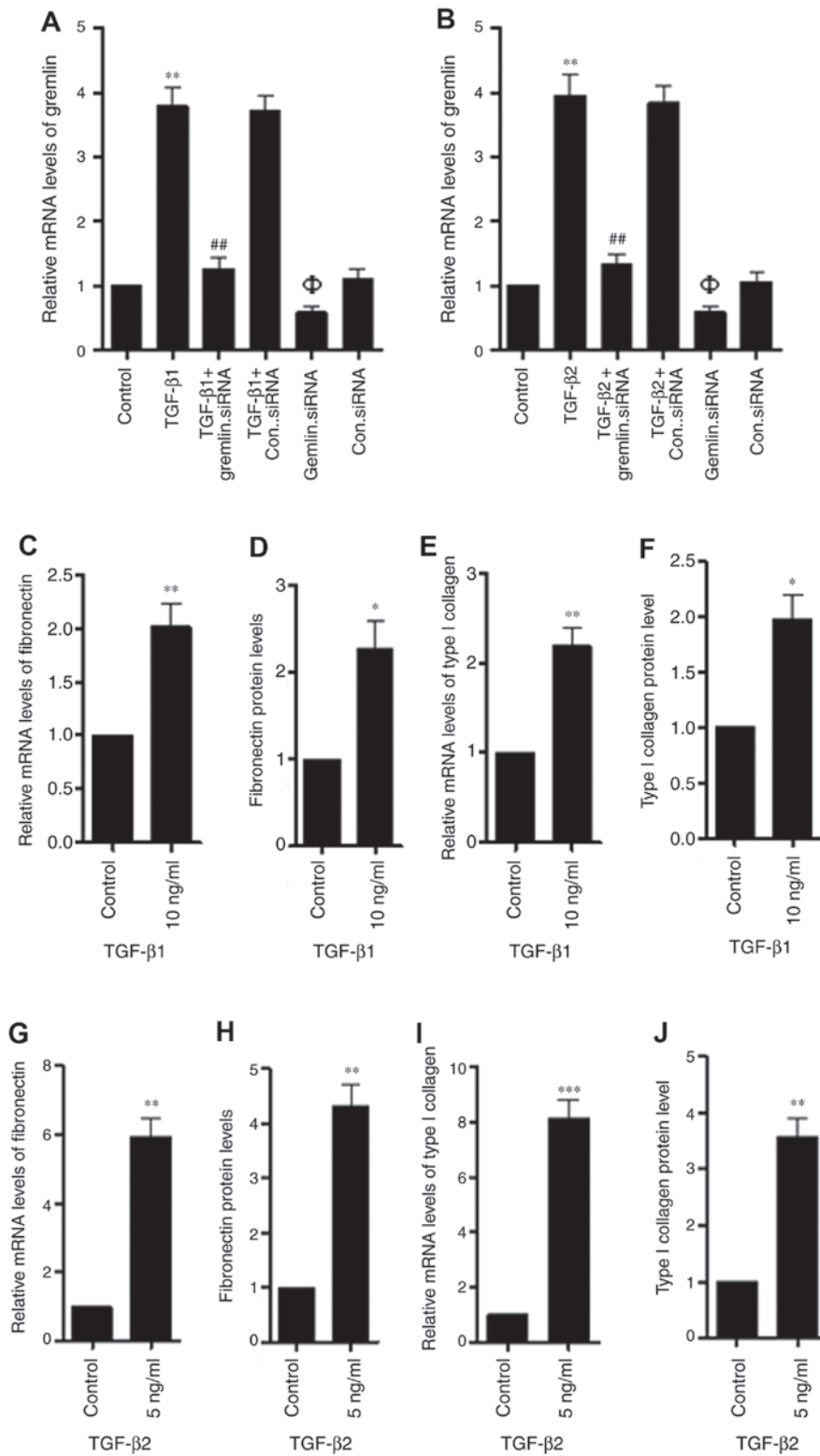


Figure 3. TGF-β1 and TGF-β2 increase the expression and secretion of gremlin, fibronectin and type I collagen in human RPE cells. (A) RPE cells were subjected to TGF-β1 and/or gremlin siRNA transfection or (B) TGF-β2 and/or gremlin siRNA transfection for 48 h, following which the relative expression of gremlin mRNA was measured using reverse transcription-quantitative PCR. (C) RPE cells were first treated with 10 ng/ml TGF-β1 for 48 h before fibronectin mRNA expression, (D) fibronectin secretion, (E) type I collagen mRNA expression and (F) type I collagen secretion were measured. (G) RPE cells were first treated with 5 ng/ml TGF-β2 for 48 h before fibronectin mRNA expression, (H) fibronectin secretion, (I) type I collagen mRNA expression and (J) type I collagen secretion were measured. The data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control; ##P<0.01 vs. respective TGF-β1 or TGF-β2 group; ΦP<0.05 vs. Control group. TGF-β, transforming growth factor-β; RPE, retinal pigment epithelial cells; siRNA, small interfering RNA. Con. represents Control.

compared with the control group. In addition, strong positive staining of fibronectin (Fig. 2E) and type I collagen (Fig. 2F)

was observed following treatment of RPE cells with gremlin (100 ng/ml, 48 h).

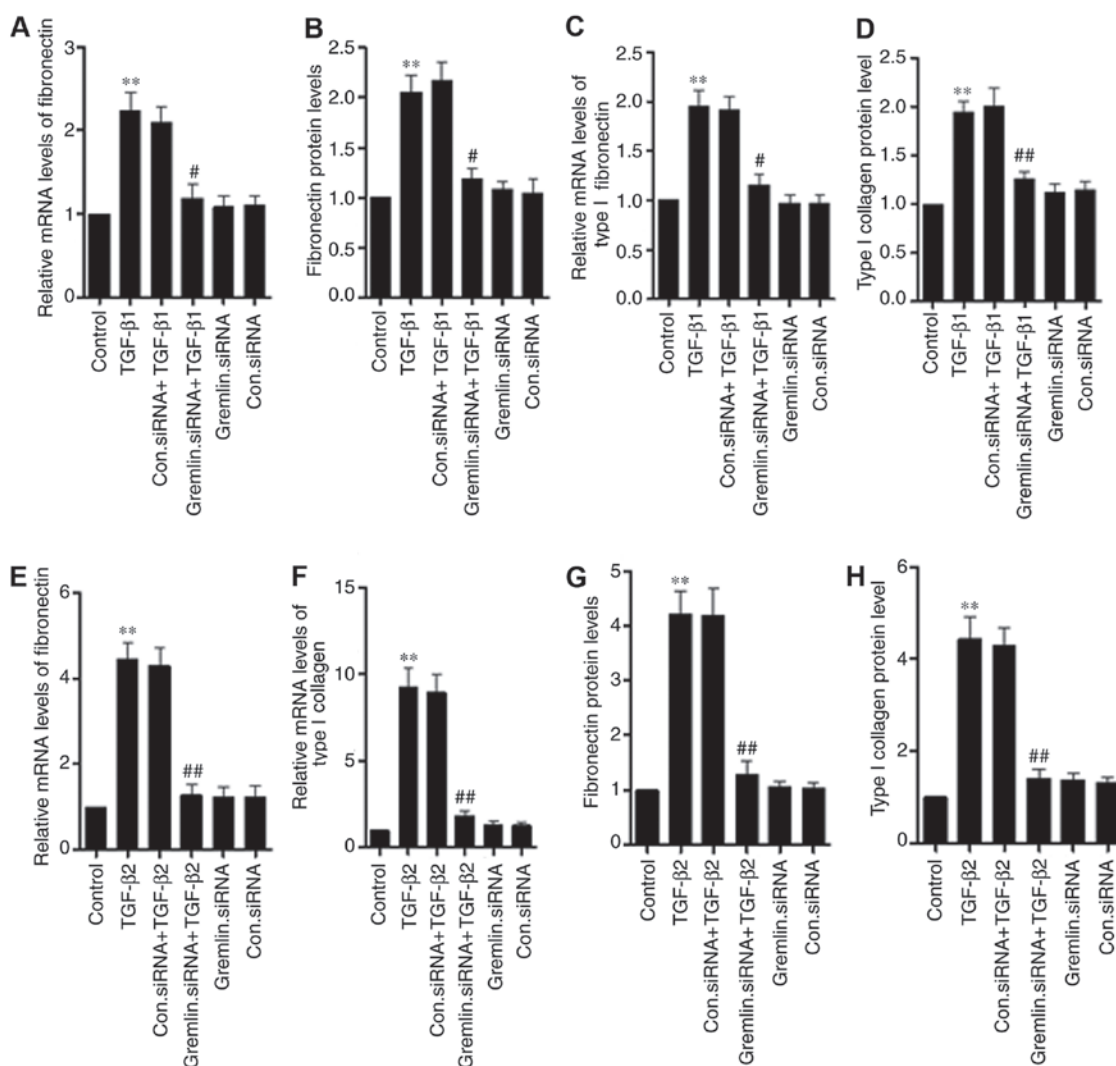


Figure 4. Gremlin mediates the TGF- β -induced induction of fibronectin and type I collagen expression and secretion in human RPE cells. The expression and secretion of fibronectin and type I collagen were measured using reverse transcription-quantitative PCR and ELISA, respectively. (A) Following treatment with TGF- β 1 and/or gremlin knockdown, fibronectin mRNA expression, (B) fibronectin secretion, (C) type I collagen mRNA expression and (D) type I collagen secretion were measured. (E) Following treatment with TGF- β 2 and/or gremlin knockdown, fibronectin mRNA expression, (F) fibronectin secretion, (G) type I collagen mRNA expression and (H) type I collagen secretion were measured. The data shown represent the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. respective TGF- β 1 or TGF- β 2 group. TGF- β , transforming growth factor- β ; RPE, retinal pigment epithelial cells. Con. represents Control.

TGF- β 1 and TGF- β 2 increase the expression of gremlin, fibronectin and type I collagen in human RPE cells. The protein and mRNA levels of gremlin, fibronectin and type I collagen were measured using ELISA and RT-qPCR following treatment of RPE cells with TGF- β . TGF- β 1 and TGF- β 2 treatment increased the expression of gremlin in human RPE cells, which was significantly reversed by gremlin siRNA (Fig. 3A and B). TGF- β 1 (10 mg/ml) increased fibronectin (Fig. 3C and D) and type I collagen (Fig. 3E and F) expression in human RPE cells and their levels in the cell supernatant. Additionally, TGF- β 2 (5 ng/ml) significantly upregulated the levels of fibronectin (Fig. 3G and H) and type I collagen (Fig. 3I and J) in human RPE cells and RPE cell supernatant.

Gremlin mediated TGF- β induces the expression of fibronectin and type I collagen in human RPE cells. Gremlin knockdown following TGF- β -treatment decreased the

expression of fibronectin and type I collagen in human RPE cells. RT-qPCR and ELISA were used to measure the expression or secretion of fibronectin and type I collagen. Gremlin knockdown significantly reversed the TGF- β 1-induced expression and secretion of fibronectin (Fig. 4A and B) and type I collagen (Fig. 4C and D) compared with the TGF- β 1-treated group. Compared with the TGF- β 2-treated group, gremlin knockdown also significantly reversed TGF- β 2 induced expression and secretion of fibronectin (Fig. 4E and F) and type I collagen (Fig. 4G and H).

Discussion

Fibrotic membrane contractions can result in severe visual impairment or blindness (20). RPE cell proliferation, migration and ECM deposition are some of the main physiological changes that occur during PVR, which is under the influence of a variety of cytokines and growth factors (21-23).

In PVR, RPE cells undergo EMT and begin losing epithelial characteristics whilst adopting a more mesenchymal, fibroblast-like phenotype, resulting in increased proliferation and migration, resistance to apoptosis and production of ECM proteins (24,25). However, the relationship between gremlin and the expression of proteins associated with the ECM in human RPE cells currently remains unclear. In the present study, the role of gremlin in the expression of fibronectin and type I collagen, and in RPE cell proliferation, was investigated. It was found that gremlin significantly increased the proliferation and expression of fibronectin and type I collagen in RPE cells, suggesting that gremlin may serve a role in the development of PVR.

TGF- β is a major cytokine involved in the regulation of cell proliferation, migration, cell death, differentiation, protein synthesis, and the pathological and physiological processes associated with tissue repair and development (26). Elevated TGF- β levels have been reported in the subretinal fluids, vitreous and epiretinal membranes of patients with PVR (27-30). TGF- β serves a vital role in PVR formation since it regulates cell proliferation, enhances ECM protein synthesis and induces ECM deposition (23,31). It has been previously demonstrated that the inhibition of TGF- β expression may prevent PVR progression (32). In addition, silencing of gremlin expression has been shown to significantly reduce TGF- β 1-induced ECM expression in human tubular epithelial cells and TGF- β 2-induced ECM synthesis in human lens epithelial cells (33,34). However, the relationship between gremlin and TGF- β 1 and TGF- β 2 in human RPE cells remains currently unclear. To clarify this, the results of the present study showed that TGF- β 1 and TGF- β 2 increased gremlin expression in human RPE cells, whilst gremlin knockdown significantly reversed TGF- β 1- and TGF- β 2-induced ECM expression in human RPE cells. This observation suggested that TGF- β 1 and TGF- β 2 can both induce the expression of ECM proteins by increasing gremlin expression, further implicating gremlin to be a mediator in PVR pathogenesis associated with TGF- β .

The present study does have a number of limitations. Apart from fibronectin and collagen type I, epithelial markers such as E-cadherin and zonula occludens-1, and mesenchymal markers including N-cadherin and α -smooth muscle actin, are also involved in the development of PVR (35,36). In the present study, the migration of RPE cells was not studied. In addition, the levels of fibronectin and collagen I were measured using ELISA and not by western blotting.

In conclusion, the data from the present study suggested that gremlin can increase RPE cell proliferation and induce fibronectin and collagen I expression in human RPE cells. Silencing of gremlin expression significantly reduced TGF- β 1- and TGF- β 2-induced fibronectin and collagen I expression in human RPE cells. Thus, TGF- β induced fibronectin and collagen I expression via regulation of gremlin. Therefore, gremlin may serve a role in the development of PVR, which potentially extends our knowledge regarding the role of gremlin in PVR pathogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DQ performed the experiments and wrote the paper. YJ analysed the data and revised the paper. XJ designed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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