

# Thrombin Induces Epithelial-Mesenchymal Transition and Collagen Production by Retinal Pigment Epithelial Cells via Autocrine PDGF-Receptor Signaling

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**PURPOSE.** De-differentiation of RPE cells into mesenchymal cells (epithelial-mesenchymal transition; EMT) and associated collagen production contributes to development of proliferative vitreoretinopathy (PVR). In patients with PVR, intraocular coagulation cascade activation occurs and may play an important initiating role. Therefore, we examined the effect of the coagulation proteins factor Xa and thrombin on EMT and collagen production by RPE cells.

**METHODS.** Retinal pigment epithelial cells were stimulated with factor Xa or thrombin and the effect on zonula occludens (ZO)-1,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen, and platelet-derived growth factor (PDGF)-B were determined by real-time quantitative-polymerase chain reaction (RQ-PCR), immunofluorescence microscopy, and HPLC and ELISA for collagen and PDGF-BB in culture supernatants, respectively. PDGF-receptor activation was determined by phosphorylation analysis and inhibition studies using the PDGF-receptor tyrosine kinase inhibitor AG1296.

**RESULTS.** Thrombin reduced ZO-1 gene expression ( $P < 0.05$ ) and enhanced expression of the genes encoding  $\alpha$ -SMA and the pro- $\alpha 1$  chain of collagen type-1 ( $P < 0.05$ ), indicating EMT. Also, ZO-1 protein expression declined on thrombin stimulation, whereas production of  $\alpha$ -SMA and collagen increased. In contrast to thrombin, factor Xa hardly stimulated EMT by RPE. Thrombin clearly induced PDGF-BB production and PDGF-R $\beta$  chain phosphorylation in RPE. Moreover, AG1296 significantly blocked the effect of thrombin on EMT and collagen production.

**CONCLUSIONS.** Our findings demonstrate that thrombin is a potent inducer of EMT by RPE via autocrine activation of PDGF-receptor signaling. Coagulation cascade-induced EMT of RPE may thus contribute to the formation of fibrotic retinal membranes in PVR and should be considered as treatment target in PVR.

**Keywords:** factor Xa, thrombin, proliferative vitreoretinopathy (PVR), epithelial-mesenchymal transition (EMT),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), zonula occludens (ZO)-1, collagen, fibrosis, retinal pigment epithelium (RPE), platelet-derived growth factor (PDGF)

Proliferative vitreoretinopathy (PVR) is a complication that is characterized by the formation of fibroproliferative membranes in the retina and develops in approximately 10% of patients who undergo surgery for rhegmatogenous retinal detachment. The fibroproliferative membranes, which primarily contain differentiated RPE cells, fibroblasts, glial cells, immune cells, and extracellular matrix (ECM), have contractile properties that cause re-detachment of the retina.<sup>1–5</sup>

Epithelial-mesenchymal transition (EMT) is a de-differentiation process in which epithelial cells lose typical epithelial features and acquire mesenchymal features that promote migratory capacity, invasiveness, elevated resistance to apoptosis, and strongly enhanced capacity to produce ECM components.<sup>5,6</sup> Epithelial-mesenchymal transition is, for instance,

associated with reduced expression of epithelial proteins, such as the tight junction proteins (e.g., zonula occludens [ZO]-1) and adherent junction proteins (e.g., E-cadherin) and enrichment of mesenchymal proteins, such as  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), fibronectin, and collagen.<sup>6,7</sup> Epithelial-mesenchymal transition or EMT-like processes have been implicated in the development of organ fibrosis, for instance in lungs, liver, and kidney, but also the lens of the eye.<sup>6,8</sup> It has been recognized that RPE cells undergo EMT in PVR membranes and as such are major contributors to the excessive ECM deposition in these membranes.<sup>4,9–11</sup> Moreover, the associated upregulation of  $\alpha$ -SMA contributes to membrane contractility.<sup>2,4</sup> Although these data point at an important role for EMT by RPE cells in the fibrotic process of PVR, there are only limited data available on

factors that drive EMT by human RPE. It has been demonstrated that platelet-derived growth factor (PDGF) induces  $\alpha$ -SMA expression by human RPE and that PDGF-receptor inhibition prevents EMT and PVR development in experimental animal models.<sup>12-15</sup> A better insight into the process of EMT by human RPE is not only crucial to delineate PVR pathology but is also required to improve treatment strategies, especially because satisfying treatment to prevent PVR development is currently lacking.

Tissue damage results in activation of the extrinsic coagulation pathway with tissue factor-dependent activation of factor X to factor Xa. Factor Xa is subsequently involved in the conversion of prothrombin into thrombin, which in turn converts soluble fibrinogen into insoluble fibrin.<sup>16</sup> In addition to their role in coagulation, factor Xa and thrombin influence several cellular responses that have important roles in inflammation, tissue repair, and wound healing, but that also contribute to fibrogenesis. They can, for instance, activate endothelial cells; promote chemotaxis of inflammatory cells; stimulate proliferation of fibroblasts, smooth muscle cells, and epithelial cells; and induce the production of ECM components, such as collagen.<sup>16-21</sup> These cellular effects of factor Xa and thrombin are mostly mediated via activation of the cell surface-expressed protease activated receptor (PAR)1 and PAR2.<sup>16</sup>

PVR development is associated with breakdown of the blood-retinal barrier and activation of coagulation, as evidenced by the deposition of fibrin in the retina and vitreous in PVR patients.<sup>22-24</sup> In line with this, Ricker et al.<sup>25</sup> demonstrated that subretinal fluids from patients with rhegmatogenous retinal detachment exhibited high capacity to generate thrombin activity in a tissue factor-dependent manner. Taken together, this suggests that RPE cells are likely to encounter direct contact with coagulation proteins during PVR development. Previous studies by us and others have demonstrated that factor Xa and thrombin can stimulate the production of cytokines/chemokines and profibrotic and proangiogenic factors by human RPE via PAR1 activation.<sup>26-30</sup> Moreover, there are indications that thrombin induces EMT by rat RPE through transcriptional repression of the epithelial marker E-cadherin and induction of actin stress fiber formation.<sup>31,32</sup> However, so far, it is unknown whether factor Xa and thrombin can induce EMT and collagen production by human RPE.

In this study, we examined the effects of factor Xa and thrombin on EMT by the human RPE cell line ARPE-19, as well as in primary human RPE cells. We demonstrate that thrombin stimulates EMT by RPE, as reflected by reduced expression of ZO-1 and enhanced  $\alpha$ -SMA expression and collagen production. Furthermore we demonstrate that thrombin stimulates PDGF receptor (PDGF-R)  $\beta$  activity in RPE, most likely via autocrine release of PDGF-BB, and that inhibition of this reverses the effects of thrombin on ZO-1,  $\alpha$ -SMA, and collagen production. Our data indicate that thrombin is a strong inducer of EMT by RPE via activation of PDGF-receptor signaling.

## MATERIALS AND METHODS

### Cell Cultures

The human retinal pigment epithelial cell line ARPE-19 was obtained from ATCC (Manassas, VA). ARPE-19 cells between passages 23 and 30 were used for experiments. ARPE-19 and primary RPE cells<sup>26</sup> were cultured in RPE medium (Dulbecco's modified Eagle's medium/Ham's F-12 1:1 medium; HyClone, Logan, UT), containing 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium). The cells were maintained

under standard cell culture conditions at 37°C in humidified air with 5% CO<sub>2</sub>.

### Real-Time Quantitative PCR Analysis

ARPE-19 cells were seeded into six-well plates at a density of  $5 \times 10^5$  cells per well in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently, the cells were incubated for 0, 12, 24, 36, or 48 hours in the presence or absence of human factor Xa (1 U/mL) or thrombin (5 U/mL) (Calbiochem, La Jolla, CA) (the concentrations used are based on findings in a previous study<sup>26</sup>) in RPE medium containing 1% FCS for analysis of *ACTA2* (encoding for  $\alpha$ -SMA) and *COL1A1* (encoding for the pro-alpha1 chain of type I collagen) transcript levels. For analysis of *TJPI* (encoding for ZO-1) transcript levels, ARPE-19 cells were seeded into six-well plates at a density of  $5 \times 10^5$  cells per well in RPE medium containing 1% FCS. Cells were allowed to adhere overnight, followed by an additional 7 to 9 days so as to enable tight junction formation, while refreshing medium containing 1% FCS every 3 to 4 days. Subsequently, the cells were incubated for 0, 12, 24, 36, or 48 hours in the presence or absence of human factor Xa (1 U/mL) or thrombin (5 U/mL) in RPE medium containing 1% FCS. RNA was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and reverse transcribed into cDNA.<sup>33</sup> Transcript levels of *ACTA2*, *COL1A1*, and *TJPI* were determined by real-time quantitative-polymerase chain reaction (RQ-PCR) (7700 PCR system; Applied Biosystems, Foster City, CA). *PDGFRA* (PDGF-R $\alpha$  chain) and *PDGFRB* (PDGF-R $\beta$  chain) mRNA expression levels were determined in unstimulated ARPE-19 cells. In addition, ARPE-19 cells were stimulated with factor Xa (1 U/mL) or thrombin (5 U/mL) for 0, 1, 2, 4, 6, or 8 hours and the effect on *PDGFB* mRNA expression level was determined. Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* (Abelson).<sup>33</sup> Primer and probe combinations used are listed in the Table.

### Fluorescent Immunocytochemistry

For analysis of  $\alpha$ -SMA protein expression, ARPE-19 cells were cultured on 12-mm cover slips for 2 to 3 days in RPE medium containing 0.5% FCS. The cells were stimulated with factor Xa (1 U/mL) or thrombin (5 U/mL), or remained unstimulated for an additional 48 hours. Thereafter, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 minutes. After washing twice with PBS, fixed cells were incubated with glycine (300 mM) for 5 minutes and then permeabilized with PBS containing 0.1% Triton X-100 for 10 minutes. Cells were then washed twice with PBS containing 0.1% Tween 20, incubated with PBS containing 1% fatty acid free bovine serum albumin (BSA) and 0.1% Tween 20 for 1 hour and then incubated overnight with rabbit anti-human- $\alpha$ -SMA primary antibody (clone E184, 04-1094; Millipore, Billerica, MD) at 4°C. Thereafter, cells were washed three times with PBS containing 0.1% Tween 20 and subsequently incubated for 1 hour with Alexa Fluor594-conjugated chicken anti-rabbit secondary antibody (Invitrogen, Paisley, UK) in PBS containing 1% BSA, 0.1% Tween 20, and 10% human serum. Following secondary antibody incubation, cells were washed six times with PBS containing 0.1% Tween 20 and then incubated with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 10 minutes. Cells were analyzed by fluorescence microscopy.

For analysis of ZO-1 protein expression, ARPE-19 cells were cultured on 12-mm cover slips for 7 to 9 days in RPE medium containing 1% FCS to allow formation of tight junctions. Stimulation and staining of the cells were conducted as

described above, using overnight incubation with Alexa Fluor488-conjugated mouse anti-human ZO-1 primary antibody (clone ZO-1-1A12, 339188; Invitrogen) at 4°C, followed by DAPI staining.

For analysis of collagen type 1 expression, ARPE-19 cells were cultured on 12-mm cover slips for 2 to 3 days in RPE medium containing 0.5% FCS. Twenty-four hours before stimulation and during the 48-hour stimulation period, 50 µg/mL ascorbic acid and 200 µM L-Proline (Sigma-Aldrich) was added to the culture medium. After the first 24 hours, 50 µg/mL ascorbic acid and 200 µM L-Proline were added to the cell cultures again. Here, the cells were incubated overnight with a mouse anti-human collagen type 1 primary antibody (clone 5D8-G9, MAB3391; Millipore) at 4°C, 1-hour incubation with FITC-conjugated rabbit anti-mouse secondary antibody (Dako, Heverlee, Belgium), and finally stained with DAPI.

### Collagen Measurement in Culture Supernatants

ARPE-19 cells were seeded into six-well plates at a density of  $1 \times 10^5$  cells per well in RPE medium containing 1% FCS and allowed to grow till 100% confluence. Twenty-four hours before stimulation, 50 µg/mL ascorbic acid and 200 µM L-proline were added to the culture media. Subsequently the cells were incubated in fresh RPE medium containing 1% FCS, 50 µg/mL ascorbic acid, and 200 µM L-proline for 24 or 48 hours in the presence or absence of factor Xa (1 U/mL) or thrombin (5 U/mL). In case of 48-hour incubation, 50 µg/mL ascorbic acid and 200 µM L-proline were again added to the cell cultures after the first 24 hours of incubation. Following stimulation, the cells and medium were harvested together and proteins were ethanol-precipitated in 67% ethanol.<sup>21</sup> Collagen was assessed by measuring hydroxyproline levels in proteins by HPLC, as previously described.<sup>21</sup> The amount of collagen present in the culture supernatants was calculated based on the fact that collagen contains 12.2% wt/wt hydroxyproline and expressed relative to the amount produced by unstimulated cells.<sup>34</sup>

### Detection of PDGF Receptors and PDGF Receptor Phosphorylation

To determine protein expression levels of the PDGF-R $\alpha$  and PDGF-R $\beta$  chains in ARPE-19 cells, unstimulated cells were lysed in protein lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol, and protease inhibitors). Equal amounts of protein (25 µg) were loaded on SDS-PAGE and blotted onto a nitrocellulose membrane. Western blots were stained with rabbit anti-human PDGF-R $\alpha$  (clone C-20, SC-338), rabbit anti-human PDGF-R $\beta$  (clone P-20, SC-339) (Santa Cruz Biotechnologies, Heidelberg, Germany), or mouse anti-human  $\beta$ -actin (clone AC-15, AB6276; Abcam, Cambridge, UK) antibodies. To assess PDGF-receptor phosphorylation, cultures were stimulated with factor Xa (1 U/mL), thrombin (5 U/mL), PDGF-BB (50 ng/mL) (R&D Systems, Minneapolis, MN) as positive control, or remained unstimulated for 8 hours in RPE medium containing 1% FCS. Hereafter cells were lysed in protein lysis buffer and cell lysates (~500 µg) were used for immunoprecipitation using an anti-PDGF-R $\beta$  antibody as described.<sup>35</sup> Immune complexes were collected with a mixture of protein A- and G-Sepharose (Sigma-Aldrich) and the level of PDGF-R $\beta$  phosphorylation was determined by using an anti-phosphotyrosine antibody (#9411; Cell Signaling, Danvers, MA) as described<sup>35</sup> and quantified by densitometric analysis (ImageQuant TL; GE Healthcare, Freiburg, Germany).

### PDGF-BB Detection in Culture Supernatants

ARPE-19 cells were seeded into six-well plates at a density of  $5 \times 10^5$  cells per well in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently, the cells were incubated for 24 hours in the presence or absence of human factor Xa (1 U/mL) or thrombin (5 U/mL) in RPE medium containing 1% FCS. Supernatants were harvested and PDGF-BB levels were measured using a PDGF-BB ELISA (R&D Systems) according manufacturer's instructions.

### PDGF Receptor Inhibition Studies

RPE cells were seeded into six-well plates at a density of  $5 \times 10^5$  cells per well in RPE medium containing 1% FCS and allowed to adhere overnight. Hereafter the cells were incubated with RPE medium containing 10 µM of the PDGF-receptor tyrosine kinase inhibitor AG1296 (Calbiochem) for 60 minutes and subsequently stimulated for 12 hours with factor Xa (1 U/mL), thrombin (5 U/mL), or PDGF-BB (50 ng/mL) as positive control. To determine the effect of AG1296 on *TJP1* mRNA expression, the seeded cells were cultured for 7 to 9 days in RPE medium containing 1% FCS. Hereafter, the cells were incubated with fresh RPE medium containing 10 µM AG1296 for 60 minutes and subsequently stimulated for 24 hours with factor Xa (1 U/mL), thrombin (5 U/mL), or PDGF-BB (50 ng/mL). The effect of AG1296 on *ACTA2*, *TJP1*, and *COL1A1* mRNA expression was determined by RQ-PCR. The AG1296 concentration used was nontoxic to RPE cells as determined by lactate dehydrogenase (LDH) release (Roche, Mannheim, Germany) and microscopic appearance of the cells.

### Statistical Analysis

Data were analyzed using the Kruskal-Wallis (one-way ANOVA) test followed by the paired Student's *t*-test when applicable. A *P* value less than 0.05 was considered significant.

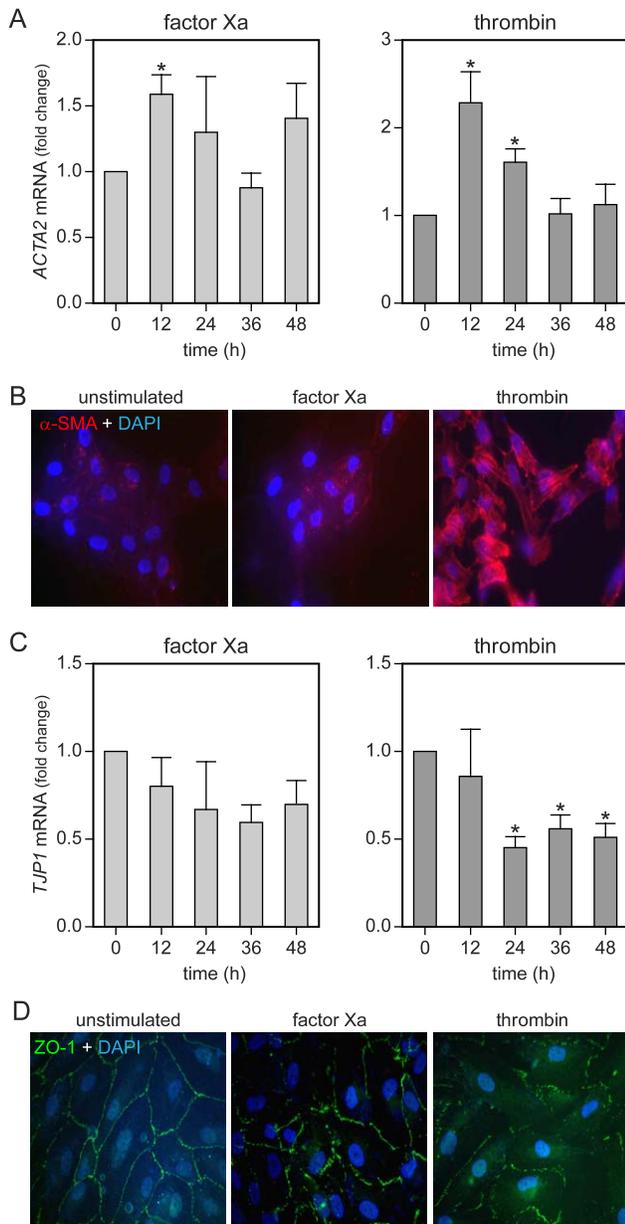
## RESULTS

### The Effect of Factor Xa and Thrombin on $\alpha$ -SMA and ZO-1 Expression by RPE

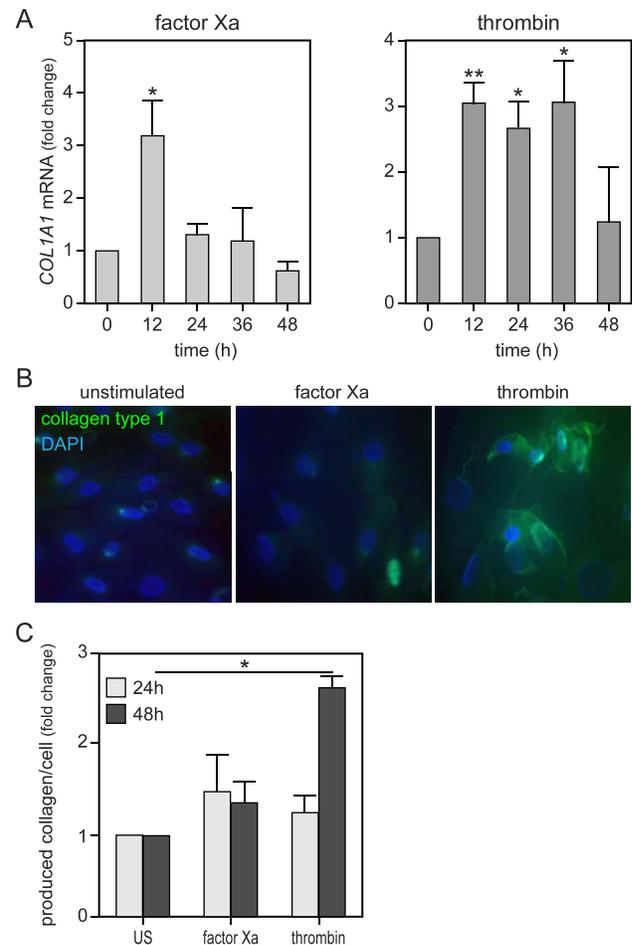
Factor Xa and thrombin enhanced *ACTA2* mRNA expression by ARPE-19, with maximal levels at 12 hours following stimulation and with thrombin being significantly ( $P < 0.05$ ) more potent (Fig. 1A). In line with this, 48-hour stimulation with thrombin clearly enhanced the expression of  $\alpha$ -SMA filaments in ARPE-19, whereas this was less evident for factor Xa (Fig. 1B). *TJP1* mRNA expression was significantly ( $P < 0.05$ ) reduced 24 hours after thrombin stimulation and was still reduced at 48 hours. Factor Xa also appeared to decrease *TJP1* mRNA expression, but this effect did not reach significance (Fig. 1C). Forty-eight-hour stimulation with thrombin also clearly reduced ZO-1 protein expression by ARPE-19, although this was not observed with factor Xa (Fig. 1D). Taken together, these results show that thrombin strongly enhances  $\alpha$ -SMA expression and reduces ZO-1 expression by ARPE-19 cells, whereas the expression of these proteins is hardly influenced by factor Xa.

### The Effect of Factor Xa and Thrombin on Collagen Production by RPE

Factor Xa and thrombin stimulation resulted in significantly ( $P < 0.05$  and  $P < 0.01$ , respectively) elevated levels of *COL1A1* mRNA 12 hours after stimulation. For thrombin, this

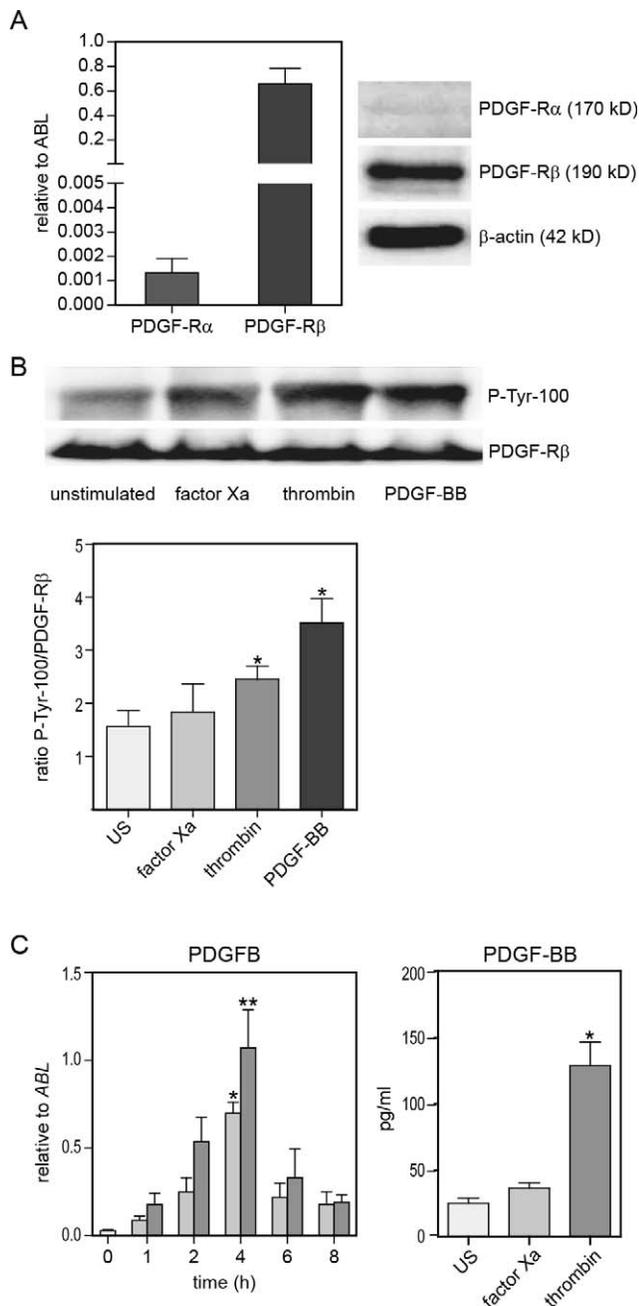


**FIGURE 1.** (A) ARPE-19 cells were stimulated for 0, 12, 24, 36, and 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL), and the *ACTA2* mRNA expression level was determined by RQ-PCR and normalized against the control gene *ABL*. (B) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL), and the cells were fixed and stained with anti- $\alpha$ -SMA (primary antibody (rabbit anti-human) and Alexa Fluor594-conjugated secondary antibody (chicken anti-rabbit)). The nuclei were stained with DAPI. (C) ARPE-19 cells were stimulated for 0, 12, 24, 36, and 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL), and the *TJP1* mRNA expression level was determined by RQ-PCR and normalized against the control gene *ABL*. (D) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL) and the cells were fixed and stained with Alexa Fluor488-conjugated anti-ZO-1 antibody (mouse anti-human). The nuclei were stained with DAPI. RQ-PCR data are presented as the mean value from four independent experiments  $\pm$  SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the paired Student's *t*-test when applicable.  $P < 0.05$  was considered significant. \* $P < 0.05$  compared with  $t = 0$  hours. Immunofluorescence pictures are representative for three independent experiments.



**FIGURE 2.** (A) ARPE-19 cells were stimulated for 0, 12, 24, 36, and 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL) and the *COL1A1* mRNA expression level was determined by RQ-PCR and normalized against the control gene *ABL*. (B) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL) and the cells were fixed and stained with anti-collagen type 1 primary antibody (mouse anti-human) and FITC-conjugated secondary antibody (rabbit anti-mouse). The nuclei were stained with DAPI. (C) ARPE-19 cells were stimulated for 24 or 48 hours with factor Xa (1 U/mL) or thrombin (5 U/mL) and after stimulation the cell numbers were determined and hydroxyproline content in the culture supernatants was determined by HPLC. RQ-PCR and HPLC data are presented as the mean value from four to five independent experiments  $\pm$  SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the paired Student's *t*-test when applicable.  $P < 0.05$  was considered significant. \* $P < 0.05$  and \*\* $P < 0.01$  compared with  $t = 0$  hours or unstimulated (US). Immunofluorescence pictures are representative for three independent experiments.

elevation was sustained up to at least 36 hours after stimulation and returned to baseline level 48 hours after stimulation (Fig. 2A). Moreover, collagen type 1 protein expression was clearly enhanced after 48 hours of thrombin stimulation, although this was not the case for factor Xa (Fig. 2B). These findings were confirmed by the collagen levels detected in the culture supernatants, demonstrating a significant ( $P < 0.05$ ) increase in collagen secretion by RPE after 48 hours of thrombin stimulation (Fig. 2C). Collectively, these data demonstrate that thrombin strongly enhances collagen production by RPE cells.



**FIGURE 3.** (A) *PDGFRA* and *PDGFRB* mRNA expression levels in unstimulated ARPE-19 cells were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value of four independent experiments  $\pm$  SEM. PDGF-R $\alpha$ , PDGF-R $\beta$ , and  $\beta$ -actin protein expression levels in unstimulated ARPE-19 cells were determined by Western blot. (B) ARPE-19 cells were stimulated for 8 hours with or without factor Xa (1 U/mL), thrombin (5 U/mL), or PDGF-BB (50 ng/mL). Total lysates from these stimulated cells were used for immunoprecipitation using an anti-PDGF-R $\beta$  antibody. Immune complexes were collected with a mixture of protein A- and G-Sepharose and the PDGF-R $\beta$  levels were determined. Levels of PDGF-R $\beta$  phosphorylation were determined by using an anti-phosphotyrosine antibody. The depicted Western blot is representative of three independent experiments. Densitometric analysis was performed and the P-Tyr-100/PDGF-R $\beta$  ratio was determined. Data are presented as the mean value from three independent experiments  $\pm$  SEM. (C) ARPE-19 cells were stimulated for 0, 1, 2, 4, 6, and 8 hours with factor Xa (1 U/mL) or thrombin (5 U/mL) and the *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean values from four independent

### The Effect of Factor Xa and Thrombin on PDGF Receptor Phosphorylation

PDGF-isoforms can stimulate  $\alpha$ -SMA expression and collagen production by various cell types, including RPE.<sup>13,36,37</sup> Previously we found that factor Xa, but thrombin much more strongly, stimulated the production of PDGF-AA, PDGF-AB, and PDGF-BB by RPE cells (ARPE-19 and primary RPE),<sup>26</sup> and here we identified thrombin as a potent inducer of EMT by RPE. Collectively, these data suggest that thrombin may exert its EMT effect in RPE in an indirect manner that involves activation of PDGF-receptor signaling. PDGF molecules signal via homodimeric or heterodimeric receptors consisting of PDGF-R $\alpha$  and/or PDGF-R $\beta$  chains.<sup>38</sup> Therefore, we first examined PDGF-receptor chain expression in ARPE-19. Analysis of mRNA and protein expression of the PDGF-R $\alpha$  and PDGF-R $\beta$  chains revealed that ARPE-19 cells abundantly express PDGF-R $\beta$  chains, but hardly express PDGF-R $\alpha$  chains (Fig. 3A). Similar results were found in primary RPE (data not shown). This suggests that PDGF signaling in ARPE-19 will most likely predominantly occur via PDGF-R $\beta$  homodimers. PDGF-BB can signal via PDGF-R $\beta$ -homodimers, whereas PDGF-AB and -AA signal via PDGF-R $\alpha$  containing dimers.<sup>38</sup> As expected, stimulation of ARPE-19 with PDGF-BB resulted in significant ( $P < 0.05$ ) phosphorylation of PDGF-R $\beta$  chains, indicating receptor activation (Fig. 3B). In ARPE-19 cells, factor Xa hardly stimulated PDGF-R $\beta$  chain phosphorylation, whereas thrombin significantly ( $P < 0.05$ ) stimulated PDGF-R $\beta$  chain phosphorylation (Fig. 3B).

### The Effect of Factor Xa and Thrombin on PDGF-BB Production

Next we examined whether the lower level of PDGF-R $\beta$  phosphorylation induced by factor Xa as compared with thrombin related to kinetic differences in PDGF-BB induction. Hereto, ARPE-19 cells were stimulated with factor Xa and thrombin for different time points and the effect on *PDGFB* mRNA expression was determined. This revealed that factor Xa and thrombin enhanced *PDGFB* mRNA expression in a time-dependent manner, being maximal and significant (thrombin  $P < 0.01$ , factor Xa  $P < 0.05$ ) 4 hours after stimulation, with thrombin being the most potent (Fig. 3C). In line with this, 24-hour thrombin stimulation resulted in a significant ( $P < 0.05$ ) increase in PDGF-BB secretion by ARPE-19 cells, whereas the increase induced by factor Xa was not significant (Fig. 3C). These data combined demonstrate that thrombin is the more potent inducer of PDGF-BB expression.

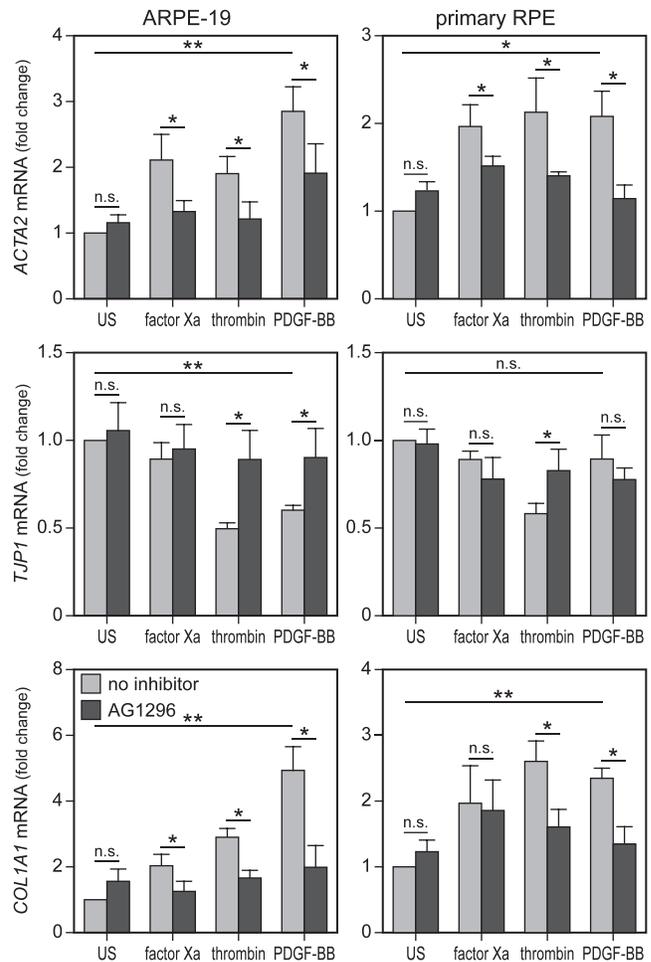
### The Effect of PDGF-Receptor Inhibition on the Regulation of $\alpha$ -SMA, ZO-1, and Collagen Type 1 Expression by Factor Xa and Thrombin

To examine whether the effect of thrombin on  $\alpha$ -SMA, ZO-1, and collagen type 1 expression by RPE depends on PDGF-receptor activation, we performed inhibition studies with the PDGF-receptor tyrosine kinase inhibitor AG1296, which potently inhibits signaling of human PDGF-R $\alpha$  and -R $\beta$ . PDGF-

experiments  $\pm$  SEM. ARPE-19 cells were stimulated for 24 hours with or without factor Xa (1 U/mL) or thrombin (5 U/mL) and PDGF-BB protein expression levels were determined. Data are presented as the mean value from four independent experiments  $\pm$  SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the paired Student's *t*-test when applicable.  $P < 0.05$  was considered significant. \* $P < 0.05$  and \*\* $P < 0.01$  compared with  $t = 0$  hours or US.

TABLE. PCR Primer and Probe Sequences

| Gene                        | Sequence Forward Primer, 5'-3' | Sequence Reverse Primer, 5'-3' | Sequence Probe, 5'-3'                    |
|-----------------------------|--------------------------------|--------------------------------|--|
| <i>ABL</i> <sup>26</sup>    | TGGAGATAACATCTAAGCATAACTAAAGGT | GATGTAGTTGCTTGGGACCCA          | FAM-CCATTTTGGTTTGGGCTTCACACCATT-TAMRA    |
| <i>ACTA2</i>                | CCGACCGAATGCAGAAGGA            | ACAGAGTATTTGCGTCCGGA           | FAM-ATCACGGCCCTAGCACCCAGCA-TAMRA         |
| <i>COL1A1</i>               | ACTGGCCCCCTGGTCC               | GGGCTTCCAGCAGCACCTT            | FAM-CCGGACCCCGAGCCACCT-TAMRA             |
| <i>PDGFB</i> <sup>26</sup>  | TCCCGAGGAGCTTTATGAGATG         | CGGGTCATGTTCAAGTCCAAC          | FAM-AGTGACCACTCGATCCGCTCTTTG-TAMRA       |
| <i>PDGFR</i> <sup>58</sup>  | TGAAGGCAGGCACATTTACATCTA       | TACAGGAGTCTCGGATCAGTTG         | FAM-TGCCAGACCCAGATGTAGCTTTGTACCT-TAMRA   |
| <i>PDGFRB</i> <sup>58</sup> | GGGACAGGGAGGTGGATT             | ATTTCCGATCACAATGCACA           | FAM-TCTACAGACTCCAGGTGTCTCCATCACGTC-TAMRA |
| <i>TJP1</i>                 | GAGACGCTGGAACCTGACCAA          | TGTTTGTCTTGATCTATGATTTGCTT     | FAM-AGCTCTGGCAATTAATTCGCCCTGCATACA-TAMRA |

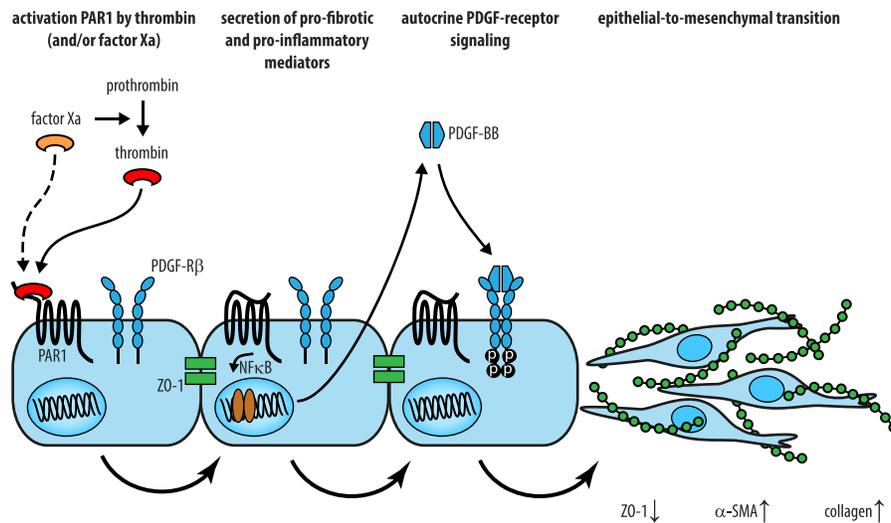


**FIGURE 4.** ARPE-19 cells and primary RPE were stimulated for 12 (*ACTA2* and *COL1A1*) or 24 hours (*TJP1*) with factor Xa (1 U/mL), thrombin (5 U/mL), or PDGF-BB (50 ng/mL) in the absence or presence of the inhibitor AG1296 (10  $\mu$ M). *ACTA2*, *TJP1*, and *COL1A1* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from five to six independent experiments  $\pm$  SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the paired Student's *t*-test when applicable.  $P < 0.05$  was considered significant. \* $P < 0.05$  and \*\* $P < 0.01$  compared with US.

BB significantly ( $P < 0.05$ ) enhanced *ACTA2* and *COL1A1* mRNA expression by both ARPE-19 and primary RPE. PDGF-BB significantly ( $P < 0.05$ ) reduced *TJP1* mRNA levels in the ARPE-19 cells, but not in the primary RPE cells. The effects of PDGF-BB on *ACTA2*, *TJP1*, and *COL1A1* mRNA levels were completely reversed ( $P < 0.05$ ) by AG1296 (Fig. 4). In line with previous results (Figs. 1, 2), thrombin significantly ( $P < 0.05$ ) enhanced *ACTA2* and *COL1A1* mRNA expression and reduced *TJP1* mRNA expression by ARPE-19. Comparable effects for thrombin on *ACTA2*, *COL1A1*, and *TJP1* mRNA levels were observed in primary RPE (Fig. 4). These effects of thrombin were significantly ( $P < 0.05$ ) blocked by AG1296. Also, the factor Xa stimulated increase in *ACTA2* and *COL1A1* mRNA levels in ARPE-19 and primary RPE cells was blocked by AG1296.

## DISCUSSION

EMT contributes to fibrotic processes, including those occurring in the retina of patients with PVR.<sup>2,13,39,40</sup> Blood-



**FIGURE 5.** Schematic summary of the activation of RPE by factor Xa and thrombin resulting in EMT. Factor Xa and thrombin activate PAR1, which results in NF- $\kappa$ B signaling and the secretion of a broad panel of proinflammatory cytokines and growth factors, including PDGF-BB.<sup>26</sup> The secreted PDGF-BB binds a PDGF-R $\beta$  chain resulting in the formation of PDGF-R $\beta$  dimers, autophosphorylation, and activation of PDGF-receptor signaling. Signaling through the PDGF-receptor results in differentiation of RPE into a mesenchymal cell type characterized by decreased ZO-1 expression and increased  $\alpha$ -SMA expression and collagen production. The *dashed line* between factor Xa and PAR1 indicates a weaker effect compared with thrombin.

retinal barrier breakdown and associated activation of the coagulation cascade contributes to PVR development.<sup>25,41</sup> Here, we demonstrate that the coagulation protein thrombin is a strong inducer of EMT by human RPE and that factor Xa does this to a far lesser extent.

EMT occurs in response to injury to promote wound closure and tissue regeneration. However, in fibrosis, which can be regarded as a dysregulated healing attempt, EMT programs are not attenuated, thereby contributing to excessive matrix production.<sup>42</sup> We found that thrombin downregulated the expression of epithelial tight junction protein ZO-1, while expression of the contractile protein  $\alpha$ -SMA was enhanced. This transformation of RPE cells into contractile myofibroblast-like cells allows migration into the vitreous where they contribute to PVR membrane formation and the contractile properties of these membranes.<sup>5,7</sup> Myofibroblast-like cells appear to be the main cell type responsible for collagen accumulation in fibrosis.<sup>43,44</sup> In line with this, we found that thrombin-induced downregulation of ZO-1 and upregulation of  $\alpha$ -SMA was accompanied by enhanced production of collagen, which is one of the main constituents of PVR membranes.<sup>45</sup> The effects of factor Xa on EMT and collagen production were less pronounced. We regard this as unlikely to be related to the concentration used, as we previously observed that 1 U/mL factor Xa generated maximal PAR1 activation in RPE.<sup>26</sup> Activation of RPE by thrombin may thus represent a major route of coagulation factor-driven EMT in PVR.

Fibrin clots have been suggested to contribute to fibrosis in general by providing ECM-producing cells with a scaffold for adherence, proliferation, and ECM production.<sup>22,28</sup> Along this hypothesis, retinal fibrin deposition has been suggested to promote RPE cell proliferation, differentiation, and collagen synthesis in PVR.<sup>25,41</sup> However, experiments in fibrinogen knockout mice revealed that bleomycin-induced lung fibrosis was not diminished,<sup>46</sup> whereas direct blockade of PAR1 did inhibit bleomycin-induced lung fibrosis in mice.<sup>47</sup> These data indicate that fibrin deposition is not per se required for fibrosis development and that especially the cell-mediated effects of thrombin and factor Xa contribute to fibrosis development in this model. Here we demonstrate that especially thrombin

induces EMT and collagen production by human RPE, whereas others have shown that thrombin stimulates human RPE cell migration and rat RPE cell proliferation.<sup>28,48</sup> Moreover, we and others found that factor Xa and thrombin stimulate the production of several cytokines and growth factors by RPE via PAR1 activation.<sup>26–30</sup> Therefore, the direct receptor-mediated cellular effects that thrombin and factor Xa elicit in RPE by activation of PAR1 may play an important role in PVR development.

PDGF-receptor activity has been observed in human PVR membranes<sup>10,49,50</sup> and PDGF has been found to stimulate EMT by RPE.<sup>13</sup> PDGF-receptor chains dimerize into functional signaling units on ligand binding.<sup>38</sup> Here we found that ARPE-19, as well as primary RPE, predominantly express the PDGF-R $\beta$  chain, which is in line with previous observations.<sup>51</sup> Consequently, RPE cells can be expected to predominantly form PDGF-R $\beta$  homodimers, which is activated by PDGF-BB.<sup>38</sup> In our current study, we confirm our previous finding<sup>26</sup> that thrombin induces PDGF-BB production by RPE more potently than factor Xa. In line with this, we here demonstrate that thrombin clearly enhances PDGF-R $\beta$  chain phosphorylation (activation) in ARPE-19, whereas factor Xa does this to a far lesser extent. Moreover, the effects of factor Xa and thrombin on *ACTA2*, *TJP1*, and *COL1A1* mRNA expression were blocked by the PDGF-receptor tyrosine kinase inhibitor AG1296. Thus, thrombin, and to a lesser extent factor Xa, most likely induce EMT by RPE via PDGF-R $\beta$  signaling initiated via autocrine release of PDGF-BB. Despite low expression of PDGF-R $\alpha$  by RPE cells, activation of this receptor is considered important to PVR development.<sup>50,52</sup> We cannot exclude involvement of PDGF-R $\alpha$  in our study, as it can be activated by PDGF-A and PDGF-B chain containing PDGF-dimers<sup>38</sup> that are induced by factor Xa and thrombin in RPE (this study),<sup>26</sup> while AG1296 also blocks PDGF-R $\alpha$  activation. Transactivation of PDGF-R $\alpha$  via other growth factor receptors, for instance the FGF- or epidermal growth factor (EGF)-receptor also cannot be excluded.<sup>53,54</sup> Although we consider this unlikely, as we previously demonstrated that neither thrombin nor factor Xa stimulate FGF and EGF production by RPE.<sup>26</sup> Nevertheless, our

data clearly indicate that the stimulatory effects of thrombin on EMT of RPE involves PDGF-R $\beta$  activation.

Factor Xa had a far less pronounced effect on EMT, collagen production, and PDGF-R $\beta$  phosphorylation by RPE than thrombin in our studies. Whether this relates to the fact that factor Xa requires cofactors, such as tissue factor, endothelial protein C receptor, or annexin 2 for efficient PAR1 cleavage<sup>55-57</sup> and a relative absence of such factors in our culture system, is unclear. However, we found that factor Xa stimulates PDGF-BB secretion by ARPE-19 to a lesser extent than thrombin and that factor Xa less potently but with comparable kinetics to thrombin enhanced *PDGFB* mRNA expression in ARPE-19. This indicates that production of a certain amount of PDGF and subsequent level of PDGF-receptor activation has to be achieved by the coagulation factor to efficiently induce EMT by RPE.

Collectively, our previous<sup>26</sup> and current findings demonstrate that coagulation cascade activity can induce EMT of RPE via autocrine PDGF-receptor signaling (Fig. 5). Inhibition of thrombin activity or its activator factor Xa by using clinically applicable anticoagulants may therefore be considered in the treatment of PVR. Alternatively, targeting of the downstream PDGF-receptor signaling cascade with specific PDGF-receptor targeting tyrosine kinase inhibitors or the use of PAR1 antagonists may also be of therapeutic interest.

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