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Differential TGF-β Signaling in Retinal Vascular Cells: A Role in Diabetic Retinopathy?

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Abstract

Purpose.: An early hallmark of preclinical diabetic retinopathy is thickening of the capillary basal lamina (BL). TGF-β, a multipotent cytokine acting through its receptors ALK5 and -1, has been postulated to be involved in this phenomenon. In light of this possible role, TGF-β signaling and its downstream molecular effects were characterized in cultured vascular endothelial cells and pericytes of the retina.

Methods.: Bovine retinal endothelial cells and pericytes were stimulated with TGF-β1 in the presence or absence of SD-208, a specific inhibitor of the TGF-β type I receptor ALK5, or ALK5 small interfering (si)RNA. TGF-β-signaling pathways were characterized by analysis of phosphorylated Smad2 or -1/5/8 proteins and TGF-β target genes (PAI-1, fibronectin, CTGF, Smad7, and Id1) and protein (fibronectin).

Results.: ALK5 was expressed in both cell types, whereas ALK1 was exclusively expressed in endothelial cells. In endothelial cells, TGF-β induced Smad2 phosphorylation at high concentrations, which was efficiently blocked by ALK5 inhibition. In contrast, in pericytes, Smad2 phosphorylation was rapidly induced at low concentrations of TGF-β. The ALK1-Smad1/5/8 pathway was activated by TGF-β in endothelial cells only. TGF-β caused ALK5-mediated upregulation of PAI-1, Smad7, and fibronectin and in pericytes at lower TGF-β concentrations than in endothelial cells. CTGF mRNA expression was induced only in pericytes. Fibronectin protein was confirmed to be regulated by TGF-β in both cell types.

Conclusions.: TGF-β signaling in retinal endothelial cells and pericytes show that these cells, and in particular the pericytes, have the essential characteristics to allow for a role of TGF-β in BL thickening in preclinical diabetic retinopathy.

Diabetic retinopathy (DR) is a leading cause of blindness [1,2] for which no known effective preventive therapy is available. To develop such a treatment, a better understanding of the asymptomatic preclinical phase (PCDR) of the disease is mandatory. In PCDR, one of the earliest changes is thickening of the basal lamina (BL) that surrounds the endothelial cells and pericytes of the retinal capillaries. [3–6] BL thickening is believed to be a crucial step in the progression of DR, suggested by findings in an in vivo experimental model of diabetes in rats, where downregulation of fibronectin (FN) synthesis partly prevented retinal BL thickening, but also reduced other more advanced features of PCDR, such as apoptosis of pericytes and the development of acellular capillaries. [7] In another study, downregulation of the extracellular matrix (ECM) components FN, laminin, and collagen type IV reduced vascular leakage in streptozotocin-induced diabetes. [8] These findings indicate that prevention of BL thickening may be an effective strategy for preventing the development of sight-threatening DR in patients with diabetes mellitus.

BL thickening is thought to result from the disturbance of the balance between synthesis and degradation of ECM components, [9–11] induced by the metabolic consequences of high intracellular glucose. [12]

However, various growth factors, such as vascular endothelial growth factor (VEGF) [13] and transforming growth factor (TGF)-β, may play a more direct role in the disturbance of this balance, which has been described as an inappropriate response to injury. [14] A causal role of TGF-β in capillary BL thickening and diabetic nephropathy has been clearly demonstrated in mouse brain and human kidney, [15–17] respectively. However, convincing evidence of such a role in the retina is lacking. Recently, Gerhardinger et al. [18] showed that two drugs that are effective in the suppression of experimental DR had in common that upregulation of genes of the TGF-β pathway were suppressed, suggesting that TGF-β signaling plays a major role in the early pathogenesis of this disease.

We have shown that an important downstream mediator of the profibrotic effects of TGF-β, connective tissue growth factor (CTGF), is upregulated early in the diabetic retina. [6,19] Furthermore, we found that in a transgenic mouse model lacking one allele of CTGF, BL thickening was attenuated. [6] Although CTGF can be induced in cultured cells by factors other than TGF-β (e.g., high glucose levels and advanced glycation end products [AGEs]), our findings could also be explained by involvement of TGF-β in diabetic retinal BL thickening.

TGF-β belongs to the TGF-β superfamily of growth factors, which comprises more than 30 members, including bone morphogenetic proteins (BMPs) and activins. Three isoforms of TGF-β have been identified in mammals (i.e., TGF-β1, -β2, and -β3). [20] TGF-β has multiple functions, which, in addition to its role as a main inducer of ECM production, include regulation of cell proliferation and differentiation, immune cell modulation, and cytokine production. [21,22] TGF-β can induce expression of CTGF, platelet-derived growth factor, fibroblast growth factors, and VEGF. [23]

TGF-β superfamily signaling pathways are shown in Figure 1. TGF-β signals via complexes of type I and II receptors [20] (Fig. 1A). Type I receptor ALK5 activates the Smad2 and -3 proteins, which regulate transcription of specific target genes. Inhibitory Smad7 is a natural inhibitor of TGF-β signaling that prevents the activation of R- and co-Smads, acting as a negative feedback regulation mechanism (Fig. 1). [24] Plasminogen activator inhibitor-1 (PAI-1) is one of the specific targets of TGF-β-ALK5-Smad2/3 signaling. [25] FN is also induced specifically through ALK5, resulting in ECM deposition. [26] Thus, the profibrotic effects of

TGF-β are mediated by the ALK5-Smad2/3 pathway.

In most cell types, TGF-β signals via ALK5. However, endothelial cells (ECs) express a second type I receptor, ALK1. ECs can therefore respond to TGF-β through two distinct pathways [25] (Figs. 1A, 1B). TGF-β can stimulate angiogenesis through ALK1, whereas ALK5 has opposite effects on EC behavior. The final effect is dependent on context, TGF-β concentration and endoglin expression. [27]

BMPs antagonize the profibrotic effect of TGF-β. [21] BMPs can specifically induce inhibitor of differentiation-1 (Id1), which is a nuclear factor that is involved in cell cycle progression and prevents cell differentiation [28] through Smad1/5/8. [29] In human epithelial cells, TGF-β has been shown to repress transcription of Id1 by a Smad3-dependent mechanism. [28] In ECs, however, TGF-β signaling via ALK1-Smad1/5/8 can also specifically induce Id1, and this stimulates migration and proliferation (a proangiogenic effect). [26]

Thus, TGF-β is a key profibrotic factor and is associated with diabetic complications such as diabetic nephropathy. In the eye, in both embryonic development and in adult tissues, expression of TGF-β and its receptors has been demonstrated. [30] We hypothesize that TGF-β is involved in thickening of the BL in retinal vascular cells in PCDR. In light of this hypothesis, we set out to characterize TGF-β signaling in cultured bovine retinal endothelial cells and pericytes as well as its effects on the expression of ECM-related proteins.

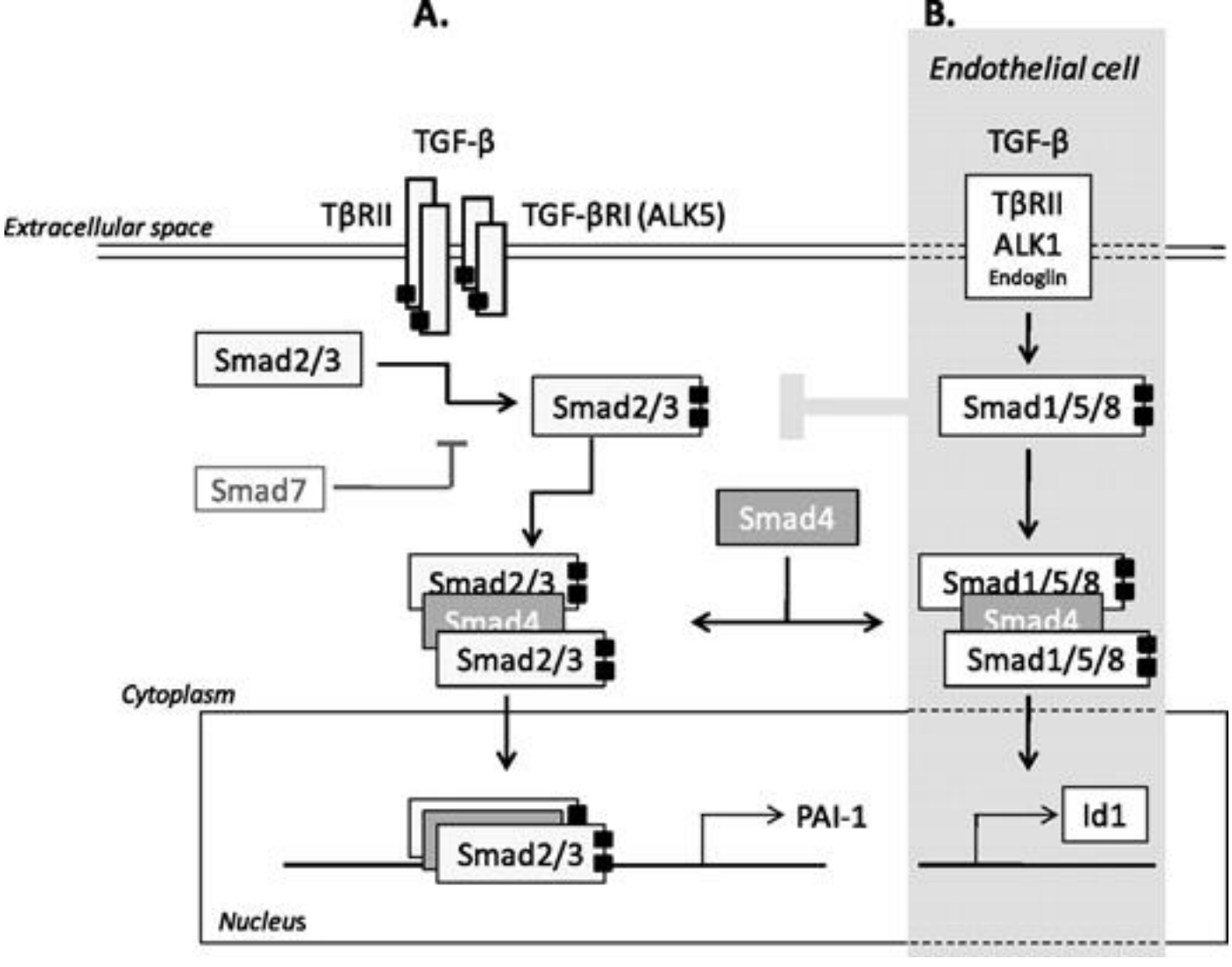
Materials and Methods

Bovine Retinal Vascular Cell Cultures

Bovine retinal endothelial cells (BRECs) and pericytes (BRPCs) were isolated from freshly enucleated calf eyes by using a differential filtration method, as described previously. [13] Freshly isolated BRECs were grown in bovine endothelial cell growth medium (Cell Applications, San Diego, CA) and were used in passage 1. BRPCs were cultured in Dulbecco's modified Eagle's medium (DME; Invitrogen-Gibco, Carlsbad, CA) containing 10% fetal calf serum (FCS) and penicillin/streptomycin and were used in passage 3 or 4. Before the experiments were performed, the cells were plated in six-well culture plates

coated with collagen and fibronectin (BRECs) or collagen alone (BRPCs). Purity of cell cultures was checked microscopically and by PCR, with von Willebrand factor and NG2 as markers for BRECs and BRPCs, respectively. At 80% confluence, the cells were serum-starved before each experiment. During the experiments, BRECs and BRPCs were cultured in DMEM in the presence of FCS, because these cells go into apoptosis in the complete absence of serum. [31] BRECs and BRPCs were stimulated with low (0.25 ng/mL) or high (5 ng/mL) concentrations of recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ), in the presence or absence of 1 μM of the specific ALK5 kinase inhibitor SD-208 (Sigma-Aldrich, St. Louis, MO). [32,33] The cells were incubated with SD-208 for 1 hour before addition of TGF-β. Solvent only was added to control samples (1 μL DMSO/mL medium). In addition, ALK5 was inhibited by transfection with ALK5 siRNA. Therefore, BRECs and BRPCs grown to 60% to 70% confluence in six-well plates in DMEM containing FCS, but without antibiotics, were transfected (Dharma FECT-1; Dharmacon Inc., Lafayette, CO) plus siRNA at a concentration of 2 μM in reduced-serum medium (Opti-Mem I; Invitrogen-Gibco) according to the manufacturer's recommendations. Bovine ALK5 siRNA duplexes were designed by Eurogentec (Maastricht, The Netherlands), with the sequence (5′→3′) as follows: duplex 1-CCAGCUGCCUUAUUAUGAU; duplex 2-GCAUGUGUAUAGCUGAAAU; duplex 3-GAAUGGAACUUGCUGUAUU. As a negative control, on-target plus nontargeting (NT)siRNA (Dharmacon, Inc.) was used. In addition, cells with transfection medium without siRNA and cells with regular medium were used as the control.

Figure 1.



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TGF-β signaling pathways. TGF-β signals via a heteromeric complex of type I and II transmembrane serine/threonine kinase receptors. (**A**) On ligand binding, the type II receptor (TβRII) recruits and activates the TGF-β type I receptor (TGF-βRI /ALK5). ALK5 propagates the signal to the nucleus by phosphorylating receptor-regulated (R-)Smad2 and (R-)Smad3. Phosphorylated Smad2 and -3 form complexes with the common-mediator (co-)Smad4, which regulate transcription of specific target genes in the nucleus. Smad7 is an inhibitor of TGF-β signaling and prevents activation of R- and co-Smads. TGF-β can specifically induce PAI-1 expression via ALK5-Smad2/3 signaling. (**B**) Exclusively in endothelial cells, TGF-β can also signal via the type I receptor ALK1 and phosphorylate Smad-1, -5, and -8, which form complexes with Smad4 and translocate to the nucleus. Smad1/5/8 activation specifically induces Id1 expression. The TGF-β/ALK5 and TGF-β/ALK1 pathways have opposite effects on endothelial cell behavior. Smad1/5/8 phosphorylation has been suggested to inhibit Smad2/3 phosphorylation (*gray stop-line*). [26] Endoglin is an accessory TGF-β receptor and promotes signaling via ALK1.

Three hours after transfection, medium was replaced by complete DMEM with FCS. Cells were grown for 24 hours and harvested for ALK5 gene and protein expression or stimulated with 5 ng/mL TGF-β1.

Protein Extraction and Western Blot Analysis

Protein lysates (three samples per experimental condition) were collected in 100 µL lysis buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1× phosphatase inhibitors, and 1× Complete Protease Inhibitors; Roche Biochemicals, Almere, The Netherlands).

Western blot analyses were performed as described previously.[34] Twenty-five micrograms of protein was separated on a 7.5% SDS-PAGE gel, and anti-phospho-Smad2 (cat. no. 3101; Cell Signaling Technology, Beverly, MA) and anti-phospho-Smad1/5/8 (cat. no. 9511; Cell Signaling Technology) were used to detect endogenous levels of phosphorylated Smad proteins. Smad2/3 antibody (cat. no. 3102; Cell Signaling Technology) was used to check for total Smad protein content. The antibodies were diluted 1:750 in 3% nonfat dry milk (Bio-Rad, Hercules, CA) in TBS/0.05% Tween-20 and incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit secondary antibody (Sigma-Aldrich) was diluted 1:10,000. Other antibodies used were anti-ALK5 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-fibronectin (1:5000; Dako, Cambridgeshire, UK). Anti-actin antibody (1:10.000; Sigma) was used as a protein loading control, with HRP-rabbit-anti-mouse secondary antibody (1:10.000; DakoCytomation, Glostrup, Denmark). Detection of protein bands was performed by digital scanning of the enhanced chemoluminescence (ECL) signal (Roche) with an image-capturing device (LAS-3000; Fujifilm Corp., Tokyo, Japan). All Western blots experiments were performed at least twice.

RNA Isolation and mRNA Quantification

Total RNA (six samples per experimental condition) was isolated (TRIzol; Invitrogen) according to the manufacturer's instructions. The amount of total RNA was approximately 3 µg/sample. A 1-µg aliquot of total RNA was DNase-I treated (amplification grade; Invitrogen) and reverse transcribed into first-strand cDNA (Superscript III and oligo$(dT)_{12-18}$; Invitrogen). The specificity of the primers was confirmed by a nucleotide-nucleotide

BLAST (http://www.ncbi.nlm.nih.gov/blast.cgi/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) search. Primer details are given in Table 1. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on a 3% agarose gel. Real-time quantitative (q)PCR was performed (iCycler iQ system; Bio-Rad). For each primer set, a mastermix was prepared, consisting of 1× fluorescein (Absolute SYBR Green Fluorescein; ABgene, Epsom, UK) and 2 pM primers with RNase-free water. One microliter of cDNA (diluted 1:20) in 19 μL mastermix was amplified using the following PCR protocol: an activation step at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds and at 60°C for 45 seconds, followed by 95°C for 1 minute and a melting program (60–95°C). Relative gene expression (R) was calculated by using the equation: $R = E^{-Ct}$, where E is the mean efficiency of all samples for the gene being evaluated and Ct is the cycle threshold for the gene as determined during real-time PCR. The qPCR data were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin together, as determined by geNorm. [35]

Table 1.

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Primers

Expression of Id1 mRNA was measured with a gene expression assay with predesigned probe and primers (*Taq*Man; Applied Biosystems, Foster City, CA). Five microliters of diluted cDNA was amplified by using the same PCR protocol as just described, but without the melting program. For visualization of mRNA expression levels of TGF-β receptors in BRECs and BRPCs, a shortened PCR protocol was used (25–30 cycles), predetermined to avoid a plateau effect. The specific PCR products were separated on a 3% agarose gel and stained (SYBR Safe; Invitrogen). All PCR experiments were performed at least twice.

Statistical Analysis

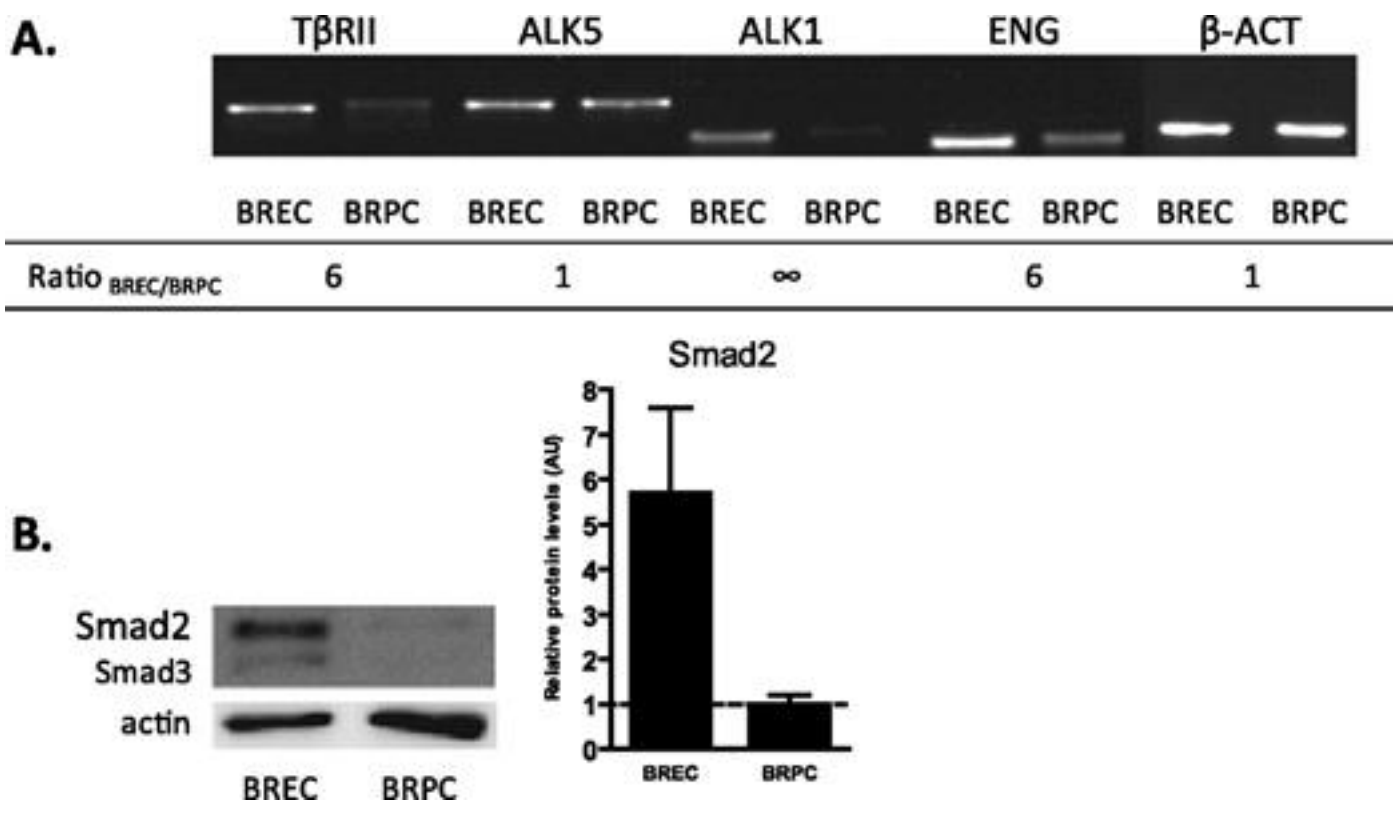
Gene expression data showed a normal distribution. Differences in gene expression levels between groups were calculated by using single ANOVA with a value of $P < 0.05$ considered to indicate significant differences (two-tailed).

Results

Steady State mRNA Expression Levels of TGF-β Receptors and Smad2 Protein in BRECs and BRPCs

Expression of ALK5 and TβRII was detected in both cell types, but TβRII was expressed at much lower levels in BRPCs than in BRECs. ALK1 was expressed only in BRECs (Fig. 2A). Expression levels of endoglin were higher in BRECs than in BRPCs. A quantitative comparison between receptor expression levels is given as a ratio of BRECs over BRPCs in Figure 2. Taken together, the results show that the various TGF-β receptors have different steady state expression levels in BRECs and BRPCs. Total Smad2 and -3 protein levels were considerably higher in BRECs compared to BRPCs (Fig. 2B).

Figure 2.



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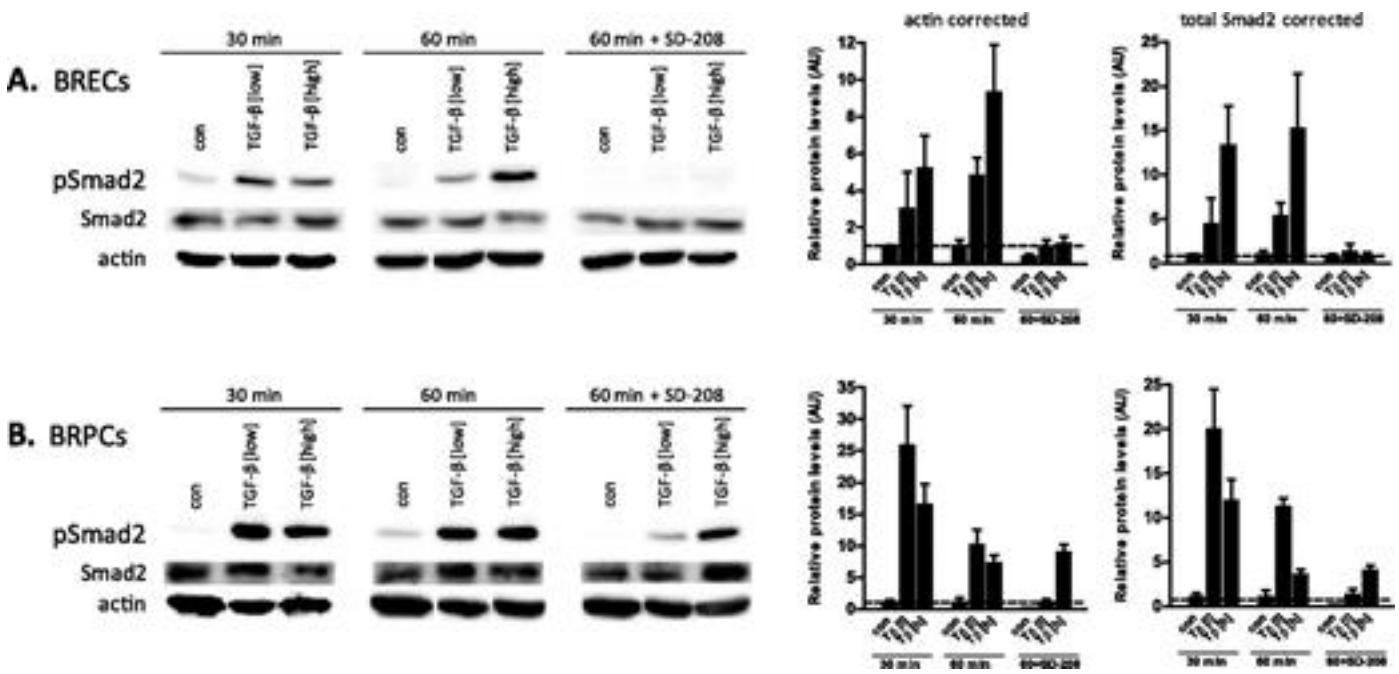
Steady-state TGF-β receptor expression and Smad2 protein levels in BRECs and BRPCs. (**A**)

The expression of TβRII, ALK5, ALK1, and endoglin (ENG) was analyzed in BREC and BRPC cultures by RT-PCR. PCR products were visualized on a 3% agarose-gel after 25 to 30 cycles. Ratios of β-actin-normalized gene expression levels in BRECs and BRPCs are shown. (**B**) Total Smad2 and -3 protein levels in BRECs and BRPCs were analyzed by Western blot. β-Actin was used as a loading control. The graph shows quantitative data of total Smad2, corrected for β-actin and relative to BRPCs.

Induction of Smad2 Phosphorylation in BRECs and BRPCs

Phosphorylation of endogenous Smad2 was analyzed by Western blot. Both stimulation with a low (0.25 ng/mL) and a high (5 ng/mL) concentration of TGF-β1 resulted in phosphorylation of Smad2 protein in both cell types at 30 and 60 minutes (Fig. 3). However, the strongest induction in BRECs was observed after 60 minutes with a high concentration of TGF-β (Fig. 3A). In comparison, induction of Smad2 phosphorylation was more pronounced in BRPCs, both at the earlier time point and at the lower concentration of TGF-β (Fig. 3B). Pretreatment with the specific ALK5 kinase inhibitor SD-208 effectively blocked activation of Smad2 in BRECs and reduced the induction moderately in BRPCs. No relevant differences in total Smad2 levels were observed with TGF-β and/or SD-208 in both cell types. Normalization of phosphorylated Smad2 levels with actin or total Smad2 showed similar effects (graphs). These results suggest that the ALK5-mediated TGF-β pathway is differentially activated in BRECs and BRPCs.

Figure 3.



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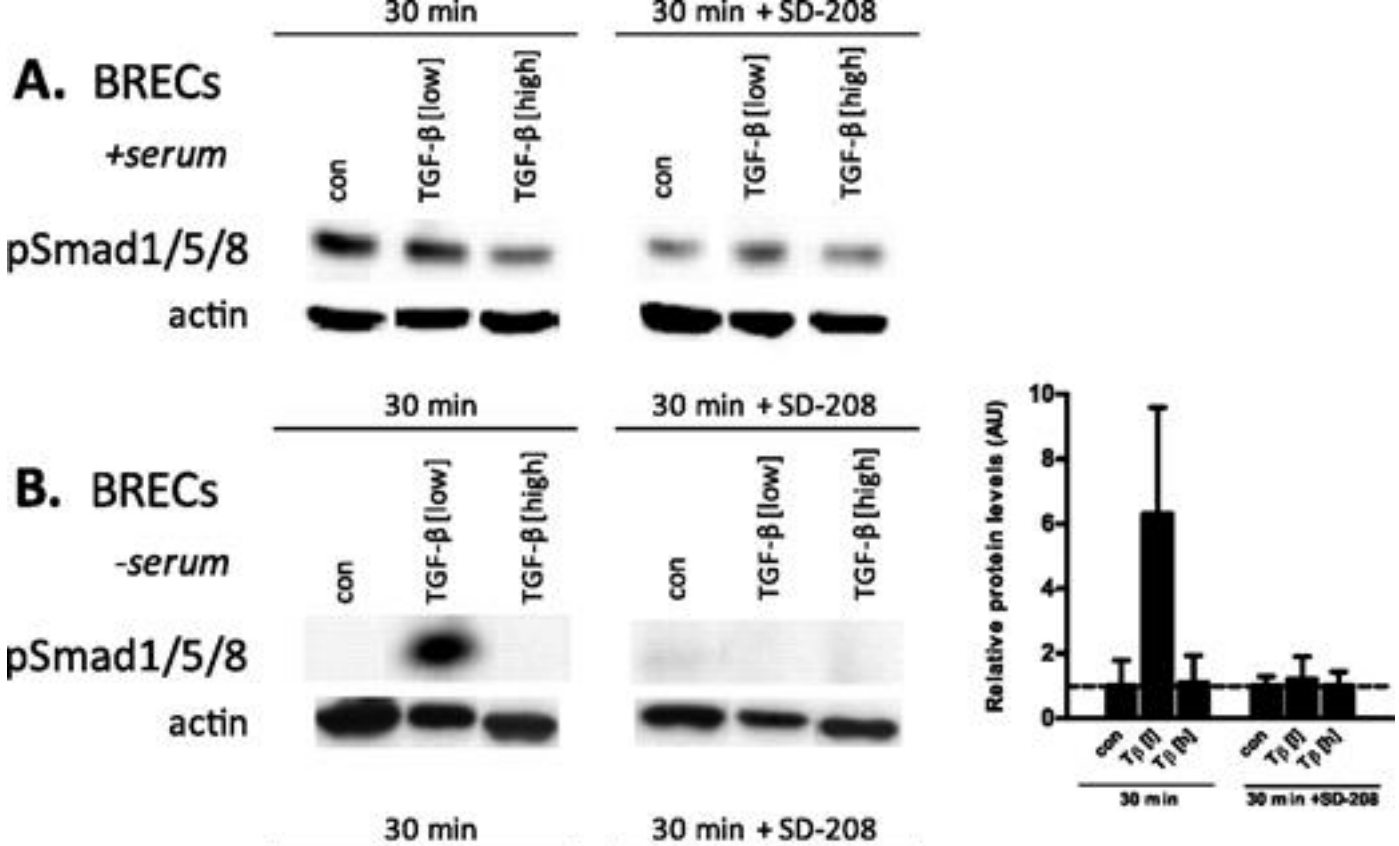
Differential induction of Smad2 phosphorylation in BRECs and BRPCs. The effect of TGF-β on phosphorylation of Smad2 protein was determined by Western blot. BRECs (**A**) and BRPCs (**B**) were incubated with low (0.25 ng/mL) or high (5 ng/mL) concentrations of TGF-β1 for 30 and 60 minutes in the presence or absence of the ALK5 inhibitor SD-208. Total Smad2 levels are also shown. β-Actin was used as a loading control. Graphs show quantitative data of phosphorylated Smad2 relative to control samples without SD-208, corrected for β-actin and total Smad2. Data are expressed as the mean ± SD.

Induction of Smad1/5/8 Phosphorylation in BRECs

Besides Smad2, TGF-β can also activate Smad1/5/8 signaling through ALK1 in endothelial cells. In BRECs cultured in the presence of FCS, Smad1/5/8 phosphorylation was observed both in control and TGF-β-containing medium, and preincubation with SD-208 moderately reduced the levels of phosphorylated Smad1/5/8 (Fig. 4A). This finding suggests that an unknown factor in FCS caused low levels of Smad1/5/8 phosphorylation in the absence of exogenous TGF-β. Therefore, we also cultured BRECs under serum-free conditions. In the absence of FCS, unstimulated BRECs did not show Smad1/5/8 phosphorylation, whereas low but not high concentrations of TGF-β rapidly induced it (Fig. 4B). In the absence of FCS, preincubation with SD-208 completely inhibited activation of Smad1/5/8. In BRPCs cultured in the presence or absence of FCS with or without TGF-β, Smad1/5/8 phosphorylation was not detected (Fig. 4C). These results show that the Smad1/5/8 signaling pathway can be activated by low levels of TGF-β in BRECs only, most likely via ALK1, but that activated ALK5 is necessary for this response.

Figure 4.



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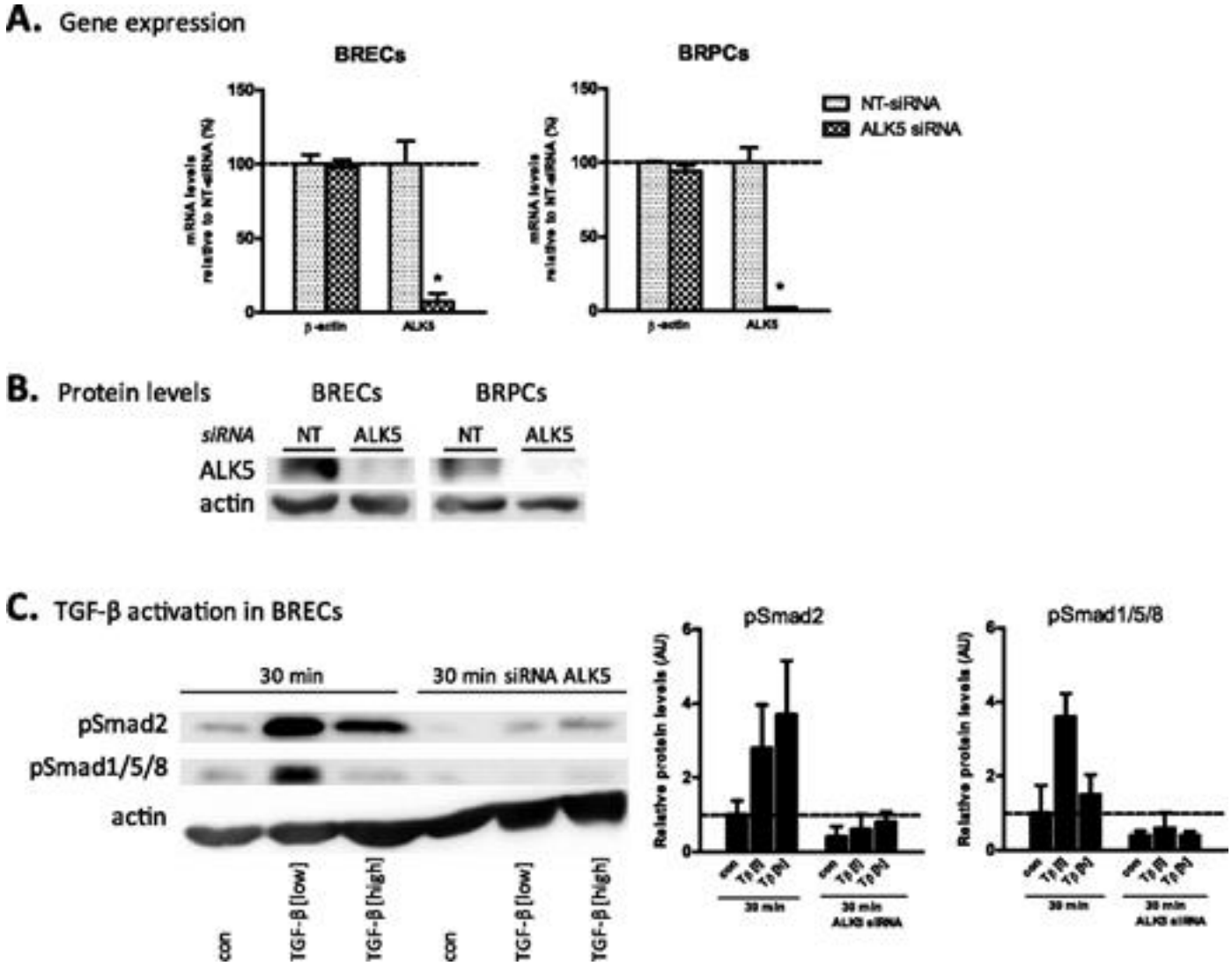
Induction of Smad1/5/8 phosphorylation in BRECs, but not BRPCs. The effect of TGF-β on phosphorylation of Smad1/5/8 protein was determined by Western blot. (**A**, **B**) BRECs were incubated with low (0.25 ng/mL) or high (5 ng/mL) concentrations of TGF-β1 for 30 minutes with or without the ALK5 inhibitor, SD-208, in the presence (**A**) or absence (**B**) of serum. (**C**) BRPCs were incubated with low and high concentrations of TGF-β1 with or without the ALK5 inhibitor in the presence of serum. β-Actin was used as a loading control. The graph shows quantitative data of phosphorylated Smad1/5/8, corrected for β-actin and relative to control samples in BRECs without serum. Data are expressed as the mean ± SD.

Inhibition of TGF-β Pathway Activation by ALK5 siRNA

To confirm the specificity of SD-208 for inhibiting ALK5 and subsequent Smad phosphorylation, we stimulated BRECs and BRPCs with TGF-β after transfection with

siRNA against ALK5. First, ALK5 knockdown efficiency was analyzed on the mRNA and protein level (Fig. 5). Transfection with nontargeting (NT) siRNA did not have an effect on ALK5 levels compared with nontransfected cells and cells treated with transfection medium alone (data not shown). In both BRECs and BRPCs, ALK5 gene expression was effectively and specifically decreased with ALK5 siRNA, compared with NT siRNA (Fig. 5A). In line with mRNA expression, ALK5 protein levels were inhibited by ALK5 siRNA (Fig. 5B). In BRECs, TGF-β-induced Smad2 phosphorylation was almost completely prevented in cells transfected with ALK5 siRNA compared with cells transfected with NT siRNA (Fig. 5C). Moreover, phosphorylation of Smad1/5/8, induced by low concentrations of TGF-β in BRECs, was inhibited by ALK5 siRNA. These results confirm that Smad1/5/8 phosphorylation in BRECs is dependent on ALK5.

Figure 5.



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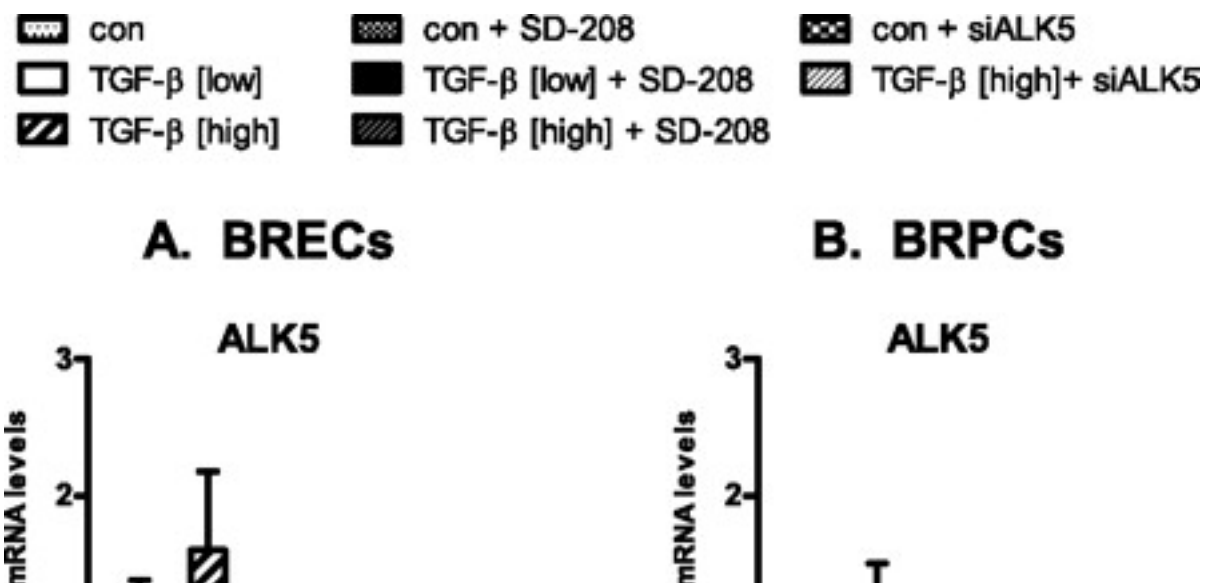
TGF-β pathway activation is inhibited by ALK5 siRNA. ALK5 mRNA was silenced by transfection with ALK5 siRNA in BRECs and BRPCs. (**A**) Twenty-four hours after

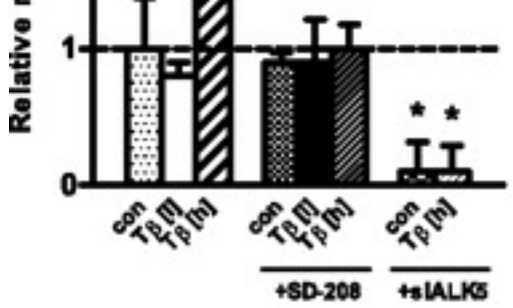
transfection, mRNA expression of the ALK5 gene was evaluated. Nontargeting (NT)-siRNA was used as a control. (**B**) ALK5 protein levels after transfection were determined by Western blot. (**C**) NT- and ALK5-siRNA–transfected cells were stimulated with 5 ng/mL TGF-β1 for 30 minutes, and Smad2 and -1/5/8 phosphorylation in BRECs was determined. β-Actin was used as the loading control. The graphs show quantitative data of phosphorylated Smad2 and -1/5/8, corrected for β-actin and relative to control samples in BRECs without serum. Data are expressed as the mean ± SD.

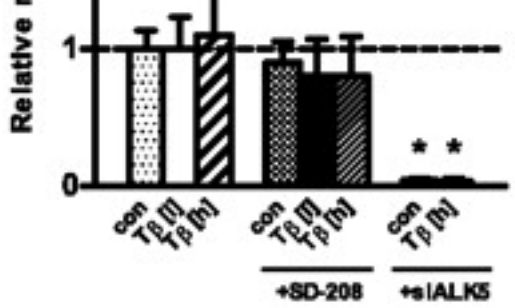
Differential mRNA Levels of Profibrotic Genes Downstream of TFG-β Signaling in BRECs and BRPCs

Expression levels of ECM-related molecules that act downstream of TGF-β signaling were analyzed at 24 hours after TGF-β stimulation. ALK5 mRNA levels were not significantly changed by TGF-β or SD-208 in both cell types, whereas ALK5 siRNA-transfected cells showed decreased ALK5 levels (Fig. 6). In BRECs, a high concentration of TGF-β induced mRNA expression of PAI-1, FN, and Smad7, but not of CTGF. In BRPCs, both low and high concentrations of TGF-β induced expression of all genes, including CTGF (Fig. 6). Thus, CTGF expression was induced only in BRPCs and was not decreased by SD-208 in BRECs. SD-208 significantly decreased expression levels of TGF-β-induced genes, in the presence and absence of TGF-β stimulation. Likewise, ALK5 siRNA decreased expression of all TGF-β-induced genes. Together, this shows the dependence of the TGF-β pathway on ALK5. These results show that TGF-β has differential effects on profibrotic gene expression in BRECs and BRPCs.

Figure 6.




Relative
1
0
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5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Tβ [l]
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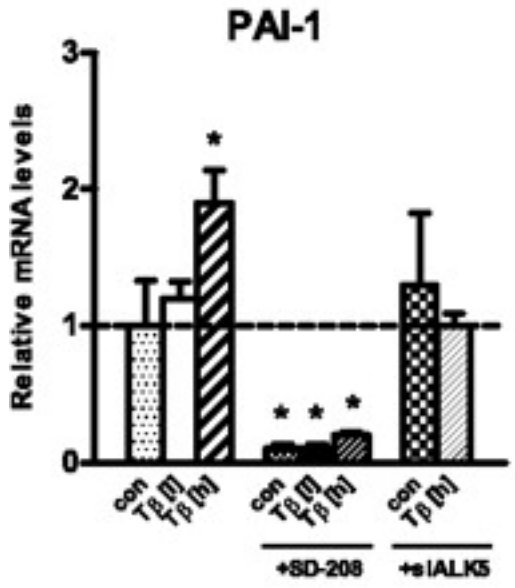
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Relative mRNA levels
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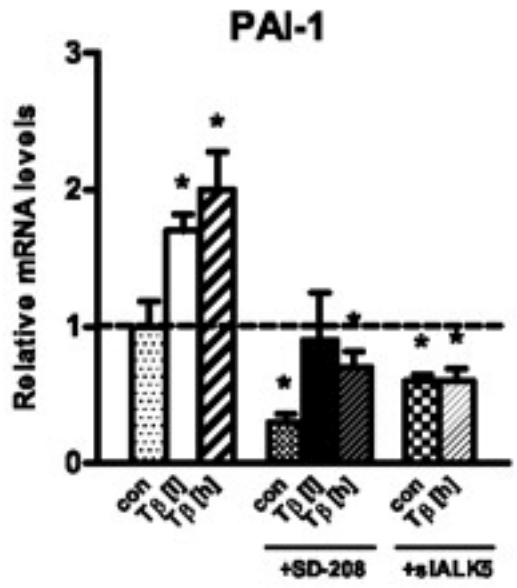
Tβ [l]
Tβ [h]
+SD-208
+sIALK5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Relative mRNA levels
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Tβ [l]
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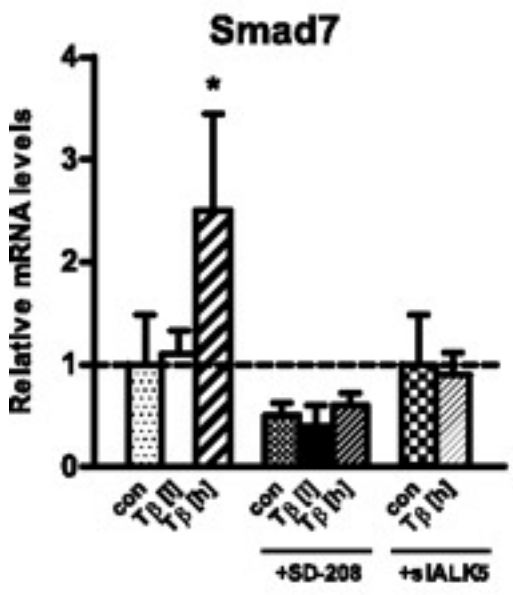
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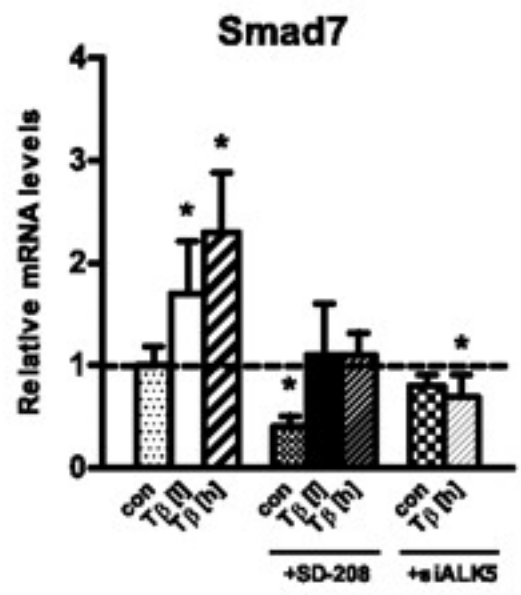
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5


Smad7
Relative mRNA levels
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Tβ [h]
+SD-208
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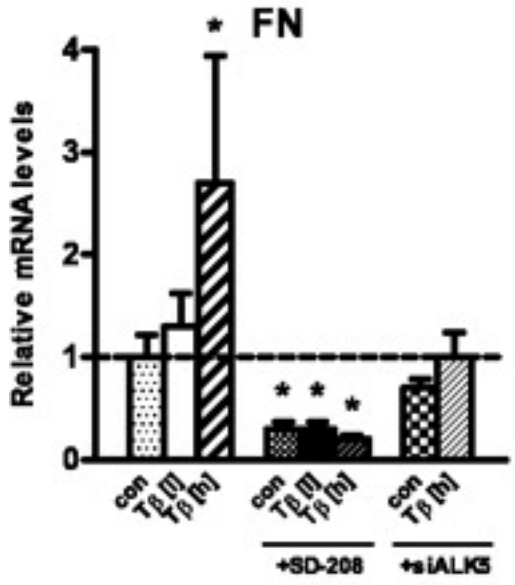
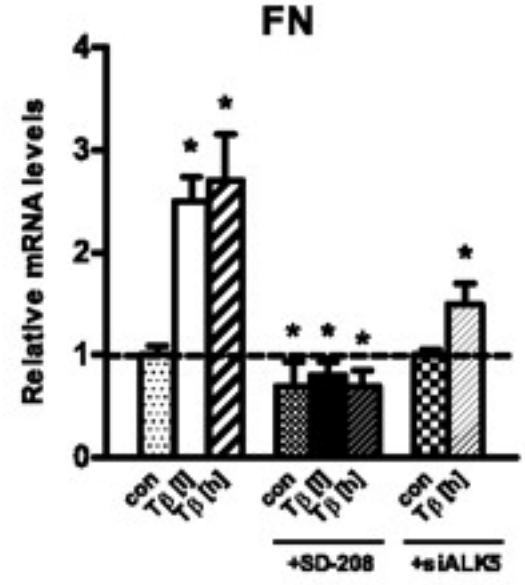
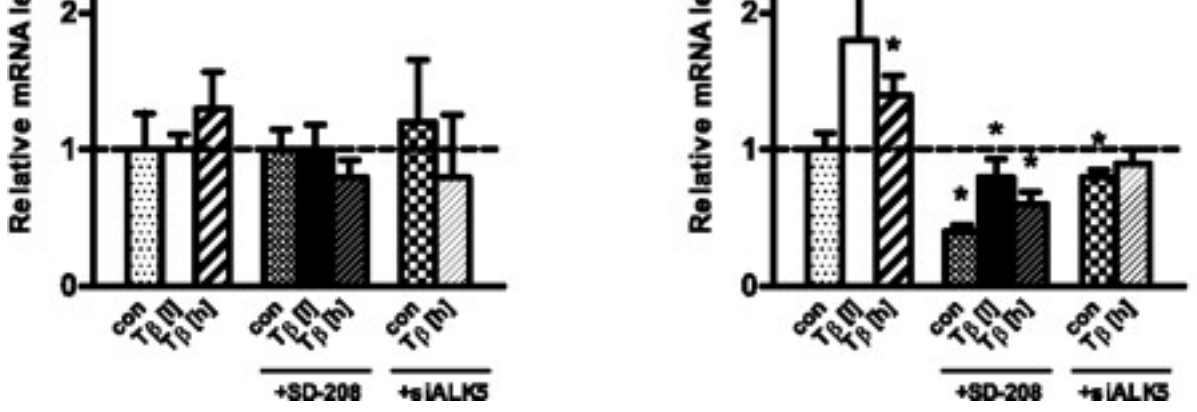

FN
Relative mRNA levels
4
3
2
1
0
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5


FN
Relative mRNA levels
4
3
2
1
0
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5

CTGF
3
2
1
0
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5

CTGF
3
2
1
0
con
Tβ [l]
Tβ [h]
+SD-208
+sIALK5



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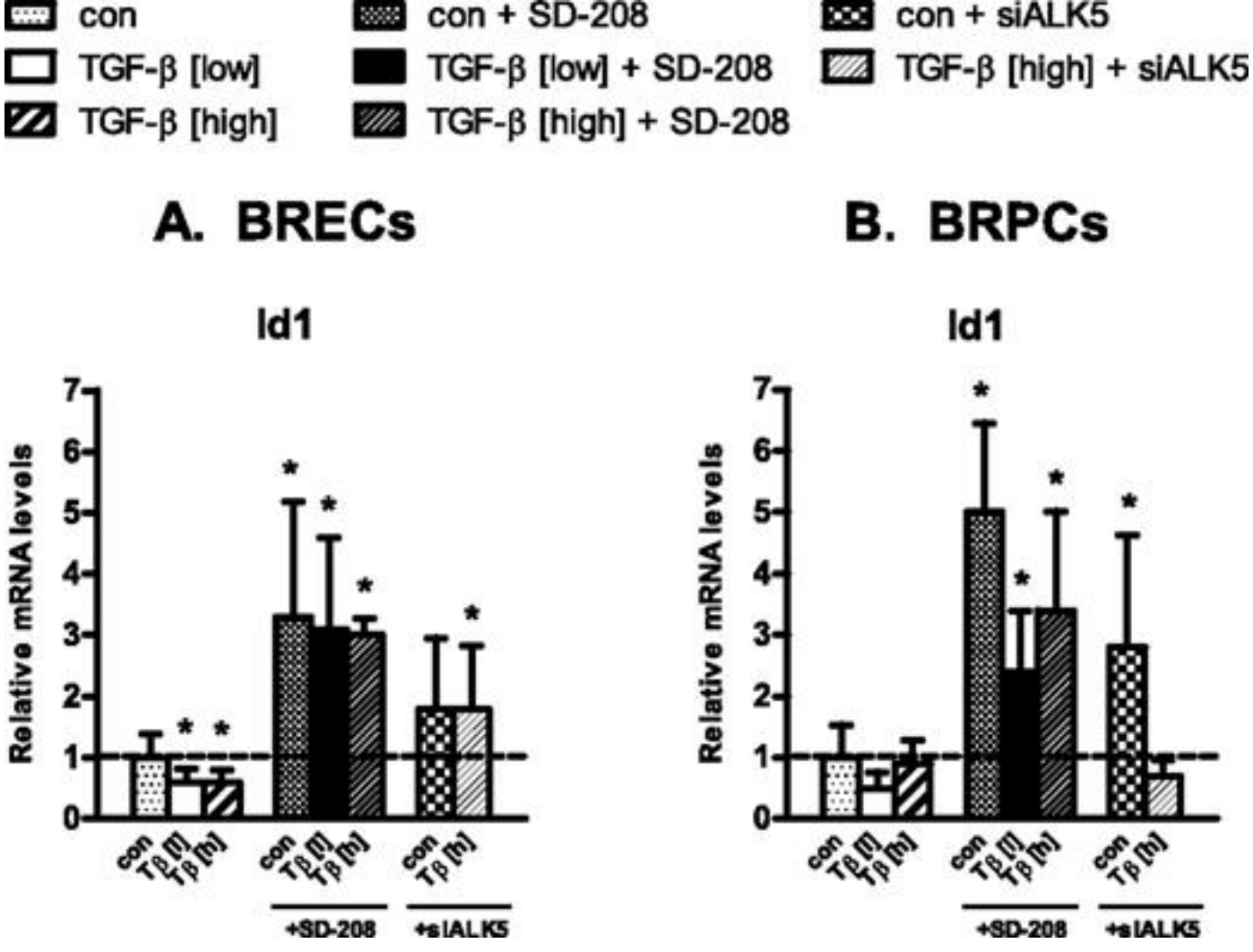
TGF-β-induced profibrotic gene expression is repressed by inhibition of TGF-β/ALK5 signaling in BRECs and BRPCs. In BRECs (**A**) and BRPCs (**B**), mRNA expression of ALK5, PAI-1, Smad7, FN, and CTGF was analyzed in response to stimulation with low (0.25 ng/mL) or high (5 ng/mL) concentrations of TGF-β1 in the presence or absence of SD-208 and in response to high concentrations of TGF-β1 after ALK5 siRNA transfection. Values represent expression levels (mean ± SD) after a 24-hour incubation relative to untreated control cells, which have been set to one (*dashed line*). *Significant change ($P < 0.05$).

Increase in Id1 mRNA Levels by Inhibition of TGF-β/ALK5 Signaling in BRECs and BRPCs

Id1 is a downstream target gene of the Smad1/5/8 pathway. Although TGF-β at low concentrations activated Smad1/5/8 in BRECs in our study (Fig. 4B), mRNA levels of Id1 were not induced after 24 hours of TGF-β stimulation in BRECs, but rather were decreased (Fig. 7). In both cell types and under all conditions, inhibition of the ALK5 pathway by preincubation with SD-208, strongly increased mRNA levels of Id1. ALK5 inhibition with siRNA had a similar effect. These results suggest that the presence and activity of ALK5 represses the expression of Id1 in both BRECs and BRPCs.

Figure 7.



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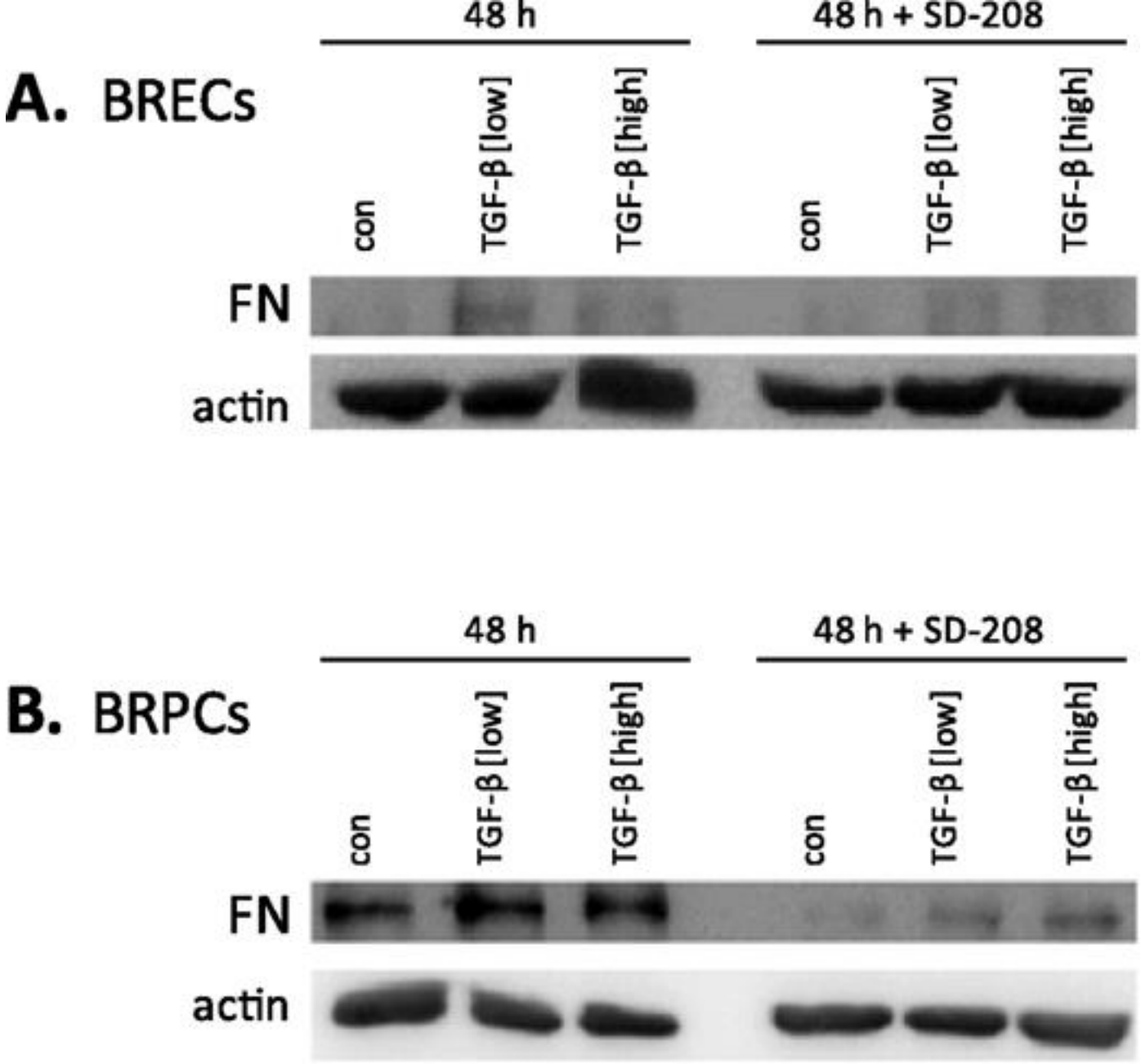
Increased mRNA levels of Id1 by inhibition of TGF-β/ALK5 signaling in BRECs and BRPCs. In BRECs (**A**) and BRPCs (**B**), mRNA expression of Id1 was analyzed in response to stimulation with low (0.25 ng/mL) or high (5 ng/mL) concentrations of TGF-β1 in the presence or absence of SD-208 and in response to high concentrations of TGF-β1 after ALK5 siRNA transfection. Values represent expression levels (mean ± SD) after a 24-hour incubation relative to untreated control cells, which have been set to one (*dashed line*). *Significant change ($P < 0.05$).

Differential FN Protein Levels in BRECs and BRPCs

FN protein levels were determined at 48 hours after TGF-β stimulation by Western blot. FN protein levels were much higher in BRPCs than in BRECs. FN levels were increased by TGF-β in BRECs and BRPCs and expression was effectively inhibited by SD-208 (Fig. 8). These data confirm the TGF-β/ALK5 pathway-dependent induction of FN mRNA.

Figure 8.



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TGF-β induced FN protein levels are repressed after ALK5 inhibition in BRECs and BRPCs. FN protein levels in BRECs (**A**) and BRPCs (**B**) were determined by Western blot 48 hours after stimulation with low (0.25 ng/mL) or high (5 ng/mL) concentrations of TGF-β1 in the presence or absence of SD-208.

Discussion

In the present study, TGF-β signaling in BRECs and BRPCs was investigated at different levels: receptors, Smad proteins, and downstream targets. Overall, activation of the ALK5 pathway and induction of downstream profibrotic molecules by TGF-β was much more pronounced in BRPCs than in BRECs. Transcript analysis of TGF-β receptors revealed that BRECs and BRPCs express different levels of TGF-β receptors. Of importance, BRPCs do

not express ALK1, and do not show Smad1/5/8 phosphorylation in response to TGF-β. This may at least partly explain the differential effects of TGF-β that were observed in BRECs and BRPCs. Our results suggest that pericytes could play a major role in the process of BL thickening which occurs early in the pathogenesis of diabetic retinopathy (DR) by upregulation of profibrotic genes. [36] Recent evidence shows that TGF-β is also necessary for normal retinal vascular barrier function. [37] In the retina, the pericyte-to-endothelial ratio is relatively high (1:1) compared with other microvascular beds (1:10), [38] suggesting that pericytes largely contribute to effects of TGF-β on the retinal microvasculature.

TGF-β differentially induced specific downstream genes and ECM-related molecules in BRECs and BRPCs through activation of the ALK5-Smad2/3 pathway. First of all, activation of this pathway, as demonstrated by phosphorylation of Smad2, differed between the two cell types: BRPCs responded earlier and at lower TGF-β concentrations than BRECs, despite lower levels of total Smad2 protein. Second, in BRPCs, downstream effector genes PAI-1, Smad7, FN, and CTGF were induced at low TGF-β concentrations, whereas in BRECs only high TGF-β concentrations increased expression of three of these genes, but not of CTGF. Expression of CTGF was not regulated by the TGF-β/ALK5 pathway in BRECs, suggesting that specifically in these cells, CTGF is regulated by other pathways, acting independently of TGF-β. At the protein level, FN was present at higher levels and more distinctly regulated by TGF-β/ALK5 in BRPCs. Specific inhibition of ALK5 using two separate techniques, showed that the effects are dependent on ALK5.

An explanation for the difference in activation of the TGF-β/ALK5 pathway that we observed in both cell types may be the presence (in BRECs) or absence (in BRPCs) of the TGF-β type I receptor ALK1, although a contribution of the different expression levels of TβRII and endoglin cannot be ruled out. Total Smad2/3 levels do not seem to explain the higher TGF-β-responsiveness of BRPCs, as these levels were distinctly lower compared with those in BRECs. In endothelial cells, ALK5 and -1 can both act as co-receptors of TβRII in TGF-β signaling, upstream of the Smad2/3 and -1/5/8 pathways, respectively. Smad phosphorylation in each pathway activates its own set of downstream effector genes, with opposite effects. In the present study, we found that phosphorylation of Smad1/5/8 is inducible in BRECs, but unresponsive to TGF-β in BRPCs. At low TGF-β concentrations in BRECs, both pathways are activated and may keep each other in balance. Others have

shown that at low TGF-β concentrations, ALK1 activation can inhibit the ALK5 pathway, [26] possibly through suppression of Smad2/3 by phosphorylated Smad1/5/8. Smad1/5/8 (and Smad2) phosphorylation was also detected in control BRECs cultured in the presence of serum, suggesting that serum, containing TGF-β and also other growth factors, has an effect on TGF-β signaling.

Our results support the idea that TGF-β and other growth factors can regulate the activation state of endothelial cells by a fine-tuned balance between ALK5 and -1 signaling. [25] The balance in endothelial cells between ALK5 and -1 signaling represents two opposing states, quiescent and proliferative, respectively. [26] Above a certain threshold of TGF-β, the balance may shift toward predominant ALK5-Smad2/3 signaling. Most likely because of the absence of ALK1, BRPCs respond to much lower concentrations of TGF-β by ALK5-Smad2/3 pathway activation and subsequent induction of downstream profibrotic effectors.

In the present study, it was shown that phosphorylation of Smad1/5/8 in BRECs, besides induction via ALK1, is dependent on ALK5, since it was abrogated with two separate methods of ALK5 inhibition. This result is in agreement with those in studies by others that have shown that the presence and activity of ALK5 is necessary for the activation of the ALK1-Smad1/5/8 pathway by TGF-β in endothelial cells. [25,31]

The net effect of activation of TGF-β pathways in BRECs and BRPCs was demonstrated on the mRNA expression level. In control samples, the Smad2/3-ALK5 pathway appeared to be active, as downstream PAI-1 expression levels were downregulated with inhibition of ALK5. ALK5 activity in the presence of serum may be relevant in physiological conditions, where TGF-β most probably has a role in the stabilization of blood vessels [39] mediated by the proteinase inhibitor PAI-1. [40]

Moreover, ALK5 activity also seems to have a negative regulatory effect in BRECs on the Smad1/5/8 pathway farther downstream, since Id1 mRNA expression was not increased on TGF-β stimulation, whereas inhibition of ALK5 markedly elevated Id1 expression. In BRPCs, inhibition of ALK5 also resulted in increased Id1 mRNA expression levels, suggesting cross-talk between the ALK5-Smad2/3 pathway and Id1 expression in these

cells as well, independent of ALK1. An explanation for this finding may be a TGF-β-induced transcriptional repression of Id1, mediated by a Smad2/3-dependent mechanism, as was shown in other types of cells. [28] Alternatively, inhibition of profibrotic TGF-β signaling may cause the anti-fibrotic BMP-pathway to become more active, resulting in a shift in the TGF-β/BMP balance.

The role of TGF-β in the pathogenesis of PCDR has not been clearly established. In contrast, TGF-β is generally accepted as the main causal factor in BL thickening in the glomerulus in diabetic nephropathy. [15,41,42] The present study shows that TGF-β can regulate ECM synthesis in (bovine) retinal vascular cells. BRPCs were more responsive to TGF-β with respect to upregulation of profibrotic factors compared to BRECs. Pericytes are supportive cells embedded in the BL of capillaries and contribute to stabilization of the vascular tube formed by the endothelial cells by synthesis of ECM. If TGF-β has a role in the excessive ECM accumulation in the BL of retinal capillaries in PCDR, it may therefore mainly involve the pericytes. This is in line with our previous work where we have shown that profibrotic factors involved in BL thickening of retinal capillaries in diabetes are mainly expressed in pericytes. [6,19,36,43–45]

In eyes of STZ-induced diabetic rats and in rats injected intraocularly with VEGF, gene expression of CTGF, FN, collagen, and TIMP1 was increased, but we found that in vitro, FN and collagen were only induced by VEGF in BRPCs and not in BRECs. Furthermore, we have previously reported that CTGF is not only involved in the angiofibrotic switch in proliferative DR, [43,44] but is also necessary for diabetic retinal BL thickening in PCDR, since $CTGF^{+/-}$ mice failed to develop this response. [6] This finding was supported by the observation in human PCDR that CTGF staining is localized mainly in retinal pericytes, whereas control eyes demonstrated a predominant microglial staining pattern. [36] Of note, it is at present unclear whether in PCDR, CTGF is a downstream mediator of the profibrotic effects of TGF-β, or acts as an inducer of ECM synthesis independently of TGF-β. [46,47] Retinal vessels from diabetic rats showed both increased TGF-β pathway activation and CTGF mRNA expression, [18] suggesting CTGF may act downstream of TGF-β in PCDR. This finding may only concern the retinal pericytes, because we observed that CTGF expression is differentially regulated in both cell types and is not responsive to TGF-β in endothelial cells.

In summary, the TGF-β responsiveness and resulting expression of downstream effectors observed in BRECs and BRPCs in our study show that these cells, and in particular the pericytes, have the essential characteristics to allow for a role of TGF-β in BL thickening in PCDR.

Footnotes

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