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Journal of Cellular Biochemistry 118:2009–2016 (2017)

Journal of Cellular Biochemistry

Transforming Growth Factor-β_1 Modulates the Expression of Syndecan-4 in Cultured Vascular Endothelial Cells in a Biphasic Manner

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ABSTRACT

Proteoglycans are macromolecules that consist of a core protein and one or more glycosaminoglycan side chains. Previously, we reported that transforming growth factor-β_1 (TGF-β_1) regulates the synthesis of a large heparan sulfate proteoglycan, perlecan, and a small leucine-rich dermatan sulfate proteoglycan, biglycan, in vascular endothelial cells depending on cell density. Recently, we found that TGF-β_1 first upregulates and then downregulates the expression of syndecan-4, a transmembrane heparan sulfate proteoglycan, via the TGF-β receptor ALK5 in the cells. In order to identify the intracellular signal transduction pathway that mediates this modulation, bovine aortic endothelial cells were cultured and treated with TGF-β_1. Involvement of the downstream signaling pathways of ALK5—the Smad and MAPK pathways—in syndecan-4 expression was examined using specific siRNAs and inhibitors. The data indicate that the Smad3–p38 MAPK pathway mediates the early upregulation of syndecan-4 by TGF-β_1, whereas the late downregulation is mediated by the Smad2/3 pathway. Multiple modulations of proteoglycan synthesis may be involved in the regulation of vascular endothelial cell functions by TGF-β_1. J. Cell. Biochem. 118: 2009–2016, 2017.

KEY WORDS: ENDOTHELIAL CELL; PROTEOGLYCAN; SYNDECAN-4; TGF-β

Vascular endothelial cells cover the luminal surface of blood vessels in a monolayer and are thus a unique cell type because they have direct contact with blood. The cells regulate the blood coagulation-fibrinolytic system by synthesizing and secreting several physiological substances, including von Willebrand factor [Jaffe et al., 1974], which facilitates blood coagulation; prostacyclin [Revtyak et al., 1987], which inhibits platelet aggregation; and plasminogen activator [Levin and Loskutoff, 1982], which converts plasminogen to plasmin, which then degrades the fibrin clot. Proteoglycans are macromolecules that consist of a core protein and glycosaminoglycan side chain(s) as a common feature and are a component of the extracellular matrix or are found on the cell surface. Although proteoglycans exhibit multiple functions such as extracellular matrix assembly, lipid metabolism, permeability and thrombosis [Camejo, 1981; Berenson et al., 1984] in vascular tissue, one of the most important functions appears to be the anticoagulant activity of glycosaminoglycan chains such as heparan sulfate and dermatan sulfate. The heparan sulfate and dermatan sulfate chains activate antithrombin III [Mertens et al., 1992] and heparin cofactor II [Tollefsen et al., 1983], respectively, and contribute to the anticoagulant property of vascular endothelium.

The major types of heparan sulfate proteoglycans synthesized by vascular endothelial cells are perlecan, which is the major extracellular matrix proteoglycan [Saku and Furthmayr, 1989];

Conflict of interest: The authors declare no competing financial interests.
Grant sponsor: The Japan Society for the Promotion of Science; Grant numbers: 15K14992, 15K08047.
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Manuscript Received: 11 November 2016; Manuscript Accepted: 22 December 2016
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 26 December 2016
DOI 10.1002/jcb.25861 • © 2016 Wiley Periodicals, Inc.

members of the syndecan family of transmembrane proteoglycans such as syndecan-1 and syndecan-4 [Kojima et al., 1992]; and the cell surface-associated proteoglycans such as glypican-1 [Mertens et al., 1992]. The cells also synthesize small leucine-rich dermatan sulfate proteoglycans such as biglycan and decorin [Järveläinen et al., 1991; Yamamoto et al., 2005]. It has been revealed that some cytokines and growth factors regulate proteoglycan synthesis in vascular endothelial cells. For example, fibroblast growth factor-2 and vascular endothelial growth factor-165 enhance the expression of biglycan and perlecan, respectively [Kinsella et al., 1997; Kaji et al., 2006]. Transforming growth factor-β_1 (TGF-β_1) induces the expression of biglycan and perlecan in a cell density-dependent manner [Kaji et al., 2000]. In addition, connective tissue growth factor suppresses the synthesis of biglycan but newly induces the synthesis of decorin in the cells when cell density is low [Kaji et al., 2004].

Recently, we found that TGF-β_1 regulates the expression of syndecan-4 in vascular endothelial cells in a biphasic manner [Hara et al., 2016]. The cytokine first upregulates and then downregulates endothelial syndecan-4 expression. Two receptors for TGF-β_1 are expressed in vascular endothelial cells: activin receptor-like kinase (ALK) 1 and ALK5 [Goumans et al., 2002; Shi and Massagué, 2003]. ALK1 activates its downstream transcriptional factor Smad1/5/8 [Goumans et al., 2003], whereas ALK5 can activate both the canonical Smad2/3 pathway and the non-Smad pathway composed of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase1/2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38 MAPK [Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Macias et al., 2015]. It is possible that the biphasic regulation of endothelial syndecan-4 expression by TGF-β_1 is mediated by the Smad pathway, non-Smad pathway, or both.

The present study used a culture system of bovine aortic endothelial cells to clarify the intracellular signal transduction pathways that mediates the early upregulation and the late downregulation of endothelial syndecan-4 expression by TGF-β_1.

MATERIALS AND METHODS

MATERIALS

Vascular endothelial cells derived from bovine aorta were obtained from Cell Applications (San Diego, CA). Dulbecco's modified Eagle's medium and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline were obtained from Nissui Pharmaceutical (Tokyo, Japan). Tissue culture dishes and plates were obtained from Iwaki (Chiba, Japan). Fetal bovine serum was obtained from HyClone Laboratories (Waltham, MA). Recombinant human TGF-β_1 was purchased from Wako (Osaka, Japan). Heparinase II (derived from *Flavobacterium heparinum*), heparinase III (EC 4.2.2.8, derived from *Flavobacterium heparinum*), and diethylaminoethyl-Sephacel (DEAE-Sephacel) were purchased from Sigma-Aldrich (St Louis, MO). PD98059, SP600125, and SB203580 were purchased from Cayman Chemical (Ann Arbor, MI). Goat polyclonal antibody against syndecan-4 (N-19) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Smad2/3 (#8685), anti-phospho-Smad2/3 (#8828), anti-ERK1/2 (#9102), anti-phospho-ERK1/2 (#9101), anti-JNK (#9252), anti-phospho-JNK (#9255), anti-p38 MAPK (#9212), anti-phospho-p38 MAPK (#9211), horseradish peroxidase (HRP)-conjugated anti-rabbit (#7074), and HRP-conjugated anti-mouse (#7076) IgG antibodies were obtained from Cell Signaling Technology (Beverly, MA). HRP-conjugated anti-goat IgG antibody (ab6885) was obtained from Abcam (Bristol, UK). Mouse monoclonal antibody against GAPDH (015–25473) was purchased Wako Purechemical Industries (Osaka, Japan). Lipofectamine RNAiMAX and Opti-MEM were obtained from Invitrogen (Carlsbad, CA). Other reagents, which were of the highest grade, were obtained from Nacalai Tesque (Kyoto, Japan).

CELL CULTURE AND TREATMENT

Vascular endothelial cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum until confluent. The cells were transfected with siRNAs for knockdown of Smad2, Smad3, or both and then stimulated with TGF-β_1 (1 or 5 ng/mL) for 6 or 24 h at 37°C. In another experiment, confluent cultures of the cells were treated with PD98059 (20 μM), SP600125 (10 μM), or SB203580 (10 μM) for 1 h and then stimulated with TGF-β_1 (5 ng/mL) for 6 or 24 h. The expression levels of syndecan-4, ERK1/2, JNK, p38 MAPK, and Smad2/3 were determined by real-time RT-PCR or Western blot analysis as described below.

siRNA TRANSFECTION

The transient transfection of siRNAs was performed using Lipofectamine RNAiMAX, according to the manufacturer's protocol. Briefly, an annealed siRNA duplex and Lipofectamine RNAiMAX were dissolved in Opti-MEM in separate tubes and incubated for 5 min at room temperature. They were then mixed and incubated for 20 min at room temperature. Vascular endothelial cells were grown to about 80% confluence in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and incubated for 24 h at 37°C in fresh Dulbecco's modified Eagle medium supplemented with both 10% fetal bovine serum and the siRNA/Lipofectamine RNAiMAX mixture. The final concentrations of siRNA and Lipofectamine RNAiMAX were 40 nM and 0.2%, respectively. The siRNAs were purchased from FASMAC (Kanagawa, Japan) and the sequences of the sense and antisense strands of the siRNAs were as follows: bovine Smad2 siRNA (siSmad2), 5′-UUCAAAACCCUGAUUAACGdTdT-3′ (sense) and 5′-CGUUAAUCAGGGUUUUGAAdTdT-3′ (antisense), and bovine Smad3 siRNA (siSmad3), 5′-UGUUUUCGGGGAUGGAAUGdTdT-3′ (sense) and 5′-CAUUCCAUCCCCGAAAACAdTdT-3′ (antisense). Negative control siRNA (siControl) (Qiagen, Valencia, CA) was used as a nonspecific sequence.

REAL-TIME RT-PCR

A monolayer of vascular endothelial cells was washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and lysed with QIAzol lysis reagent (Qiagen). A quarter volume of chloroform was mixed with the lysate, and the mixture was centrifuged. The supernatant was collected, 70% ethanol was added to a concentration of 52.5%, the suspension was centrifuged at 20,000 × *g*, and the supernatant was discarded. The precipitate was suspended again in 70% ethanol and centrifuged at 20,000 × *g*, and the obtained

precipitate containing the total RNA was dried. Complementary DNA was synthesized from the mRNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA). Real-time PCR was performed using GeneAce SYBR qPCR Mix α (Nippon Gene, Tokyo, Japan) with 1 ng/μL cDNA and 0.1 μM primers on a StepOnePlus Real-Time PCR System (Applied Biosystems). The levels of syndecan-4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were quantified by the relative standard curve method. The fold change of the intensity value of syndecan-4 was normalized by that of GAPDH. The sequences of the bovine gene-specific forward and reverse primers were as follows: syndecan-4, 5′-TTGC CGTCTTCCTCGTGC-3′ (forward) and 5′-AGGCGTAGAACTCATT GGTGG-3′ (reverse), Smad2, 5′-CAGAATACCGAAGGCAGACG-3′ (forward) and 5′- TGAGCAACGCACTGAAGG −3′ (reverse), Smad3, 5′-ACTACAGCCATTCCATCC-3′ (forward) and 5′-ATCTGGTGGT-CACTGGTCTC-3′ (reverse), TSP-1, 5′–3′ (forward) and 5′–3′ (reverse), PAI-1, 5′-CCGTGGAACAAGGATGAG-3′ (forward) and 5′- CGGAA-CAGCCTGAAGAAG-3′ (reverse), and GAPDH, 5′-AACACCCTCAA-GATTGTCAGCAA-3′ (forward) and 5′-ACAGTCTTCTGGGTGGCAGT GA −3′(reverse).

PROTEOGLYCAN CORE PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Proteoglycans were extracted from the cell layer and the conditioned medium of vascular endothelial cells under dissociative conditions. Specifically, the conditioned medium was harvested, and solid urea was added to a final concentration of 8 M. The cell layer was washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and lysed with 8 M urea cell extract solution (pH 7.5) containing 120 mM 6-aminohexanoic acid, 12 mM benzamidine, 10 mM N-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100. The extracts were applied to DEAE-Sephacel (0.3 mL of resin) columns and washed four times with 8 M urea buffer (pH 7.5) containing 0.25 M NaCl, 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base. Proteoglycans were eluted with 0.9 mL 3 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol for 2 h at −20°C; this precipitation step was repeated three times. Precipitated proteoglycans were digested with heparinase II and III (13.3 U/mL each) in 100 mM Tris-HCl buffer (pH 7.0) containing 10 mM calcium acetate and 18 mM sodium acetate for 3 h at 37°C to determine core proteins of syndecan-4. Separately, total proteins from vascular endothelial cells were prepared by lysis in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl buffer solution containing 2% SDS and 10% glycerol, pH 6.8) followed by incubation at 95°C for 10 min. The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA) before adding 2-mercaptoethanol and bromophenol blue to the samples. The proteoglycans and cellular proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred to a polyvinyl difluoride membrane (Immobilon-P, Millipore, Billerica, MA) at 2 mA/cm^2 for 1 h following the method of Kyhse-Andersen [Kyhse-Andersen, 1984]. Membranes were blocked with 2% BSA solution or 5% skim milk in 20 mM Tris-HCl buffer solution (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 and then incubated overnight with a primary antibody against syndecan-4, Smad2/3, phosphorylated Smad2/3, ERK1/2, phosphorylated ERK1/2, JNK, phosphorylated JNK, p38 MAPK, or phosphorylated p38 MAPK at 4°C. The membranes were washed and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence western blotting detection reagents (Chemi-Lumi One L, Nacalai, Kyoto, Japan) and scanned with a LAS 3000 Imager (Fujifilm, Tokyo, Japan). Representative blots from three independent experiments are shown.

STATISTICAL ANALYSIS

Data were analyzed for statistical significance by analysis of variance and Student's *t*-test or Tukey's method, as appropriate. *P* values of less than 0.05 were considered statistically significantly different.

RESULTS

TGF-β_1 ACTIVATES p38 MAPK AND Smad2/3 IN VASCULAR ENDOTHELIAL CELLS

Figure 1 shows the expression of syndecan-4 mRNA in vascular endothelial cells treated with TGF-β_1. The expression of syndecan-4

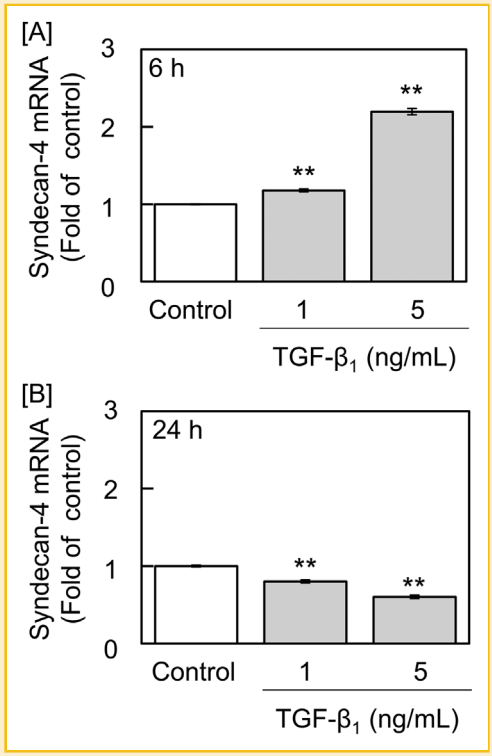

Fig. 1. Effects of TGF-β_1 on the expression of syndecan-4 mRNA in vascular endothelial cells. Bovine aortic endothelial cells were treated with 1 and 5 ng/mL TGF-β_1 at 37°C for 6 or 24 h ([A] and [B], respectively). Values are the mean ± S.E. of four samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

mRNA was elevated at 6 h and reduced at 24 h by the cytokine at 1 and 5 ng/mL. This result is consistent with our recent study [Hara et al., 2016], showing that TGF-β_1 modulates endothelial syndecan-4 expression in a biphasic manner.

Figure 2 shows the phosphorylation of MAPKs (ERK1/2, JNK, and p38 MAPK) and Smad2/3, which may be involved in the modulation of syndecan-4 expression by TGF-β_1 as the downstream signaling pathways of the cytokine. For the MAPKs, the phosphorylation of only p38 MAPK was elevated by 1 and 5 ng/mL TGF-β_1 after 1 h and longer. On the other hand, the phosphorylation of Smad2/3 was increased by 1 ng/mL TGF-β_1 after 1h and the increase disappeared after 2 h and longer. TGF-β_1 at 5 ng/mL the phosphorylation of Smad2/3 after 1 h and longer.

THE p38 MAPK PATHWAY MEDIATES THE EARLY UPREGULATION OF ENDOTHELIAL SYNDECAN-4 EXPRESSION BY TGF-β_1

To examine the involvement of MAPKs in the modulation of syndecan-4 mRNA expression by TGF-β_1, vascular endothelial cells were pretreated with the MEK1 inhibitor PD98059, JNK inhibitor SP600125, or p38 MAPK inhibitor SB203580 and then stimulated with TGF-β_1 (Fig. 3). Syndecan-4 mRNA expression was upregulated after 6 h and downregulated after 24 h by TGF-β_1. PD98059 (Fig. 3A) and SP600125 (Fig. 3B) did not influence the early upregulation or the late downregulation. In contrast, SB203580 suppressed the early upregulation of syndecan-4 mRNA expression by TGF-β_1, although the late downregulation was unaffected by the inhibitor (Fig. 3C). In addition, TGF-β_1 increased syndecan-4 core protein expression in the cell layer, and this increase was completely diminished by SB203580 (Fig. 3D); the core protein was not detected in the conditioned medium. The intensity of nonspecific bands was almost same after treatment with heparinase II/III. These results suggest that TGF-β_1 activates p38 MAPK, which mediates the early upregulation of syndecan-4 expression by the cytokine in vascular endothelial cells.

Smad2/3 PATHWAYS MEDIATE THE LATE DOWNREGULATION OF SYNDECAN-4 EXPRESSION BY TGF-β_1

To examine the involvement of Smad2 and Smad3 in the modulation of syndecan-4 expression by TGF-β_1, vascular endothelial cells were transfected with siSmad2 or siSmad3 and then treated with 5 ng/mL TGF-β_1 (Fig. 4). The expression of Smad2 (Fig. 4A) and Smad3 (Fig. 4B) was markedly reduced by treatment with siSmad2 and siSmad3, respectively. Additionally, we measured the expression of plasminogen activator inhibitor-1 (PAI-1) mRNA to confirm the functional knockdown of Smad2 and Smad3 (Figs. 4C and 4D). After a 24-h transfection of either siSmad2 or siSmad3 in the absence of

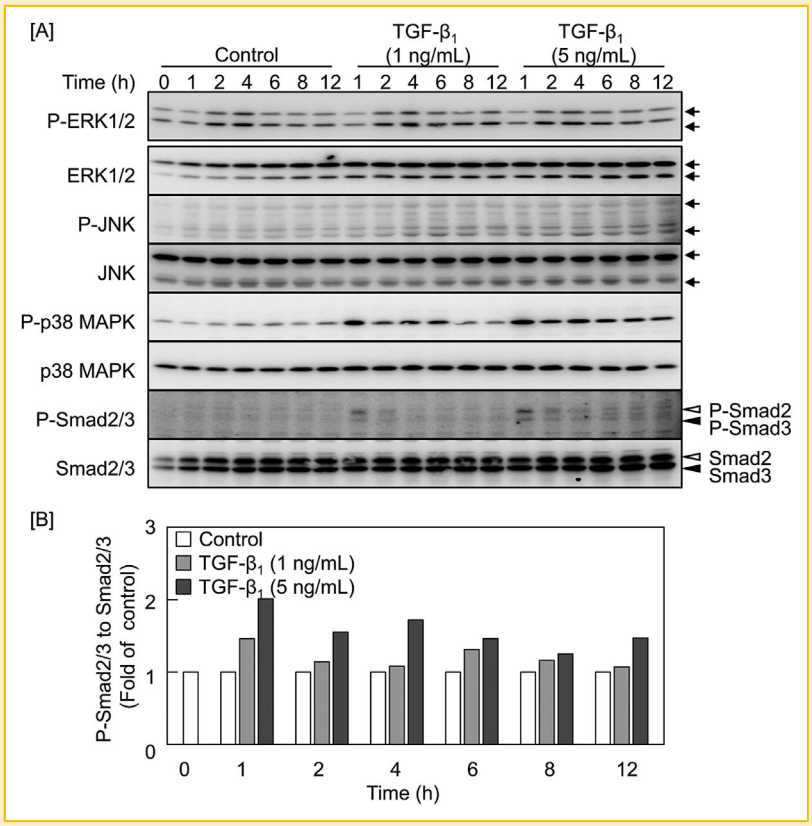

Fig. 2. Effects of TGF-β_1 on the activation of ERK1/2, JNK, p38 MAPK, and Smad2/3 in vascular endothelial cells. Bovine aortic endothelial cells were treated with 1 and 5 ng/mL TGF-β_1 at 37°C for 1, 2, 4, 6, 8, or 12 h. [A] Expression of P-ERK1/2, ERK1/2, P-JNK, JNK, P-p38 MAPK, p38 MAPK, P-Smad2/3, and Smad2/3 proteins. Open and filled arrowheads indicate the position of Smad2 and Smad3, respectively. Arrows indicate the positions of P-ERK1/2, ERK1/2, P-JNK, and JNK. [B] The ratio of the intensity of P-Smad2/3 to that of Smad2/3 in [A].

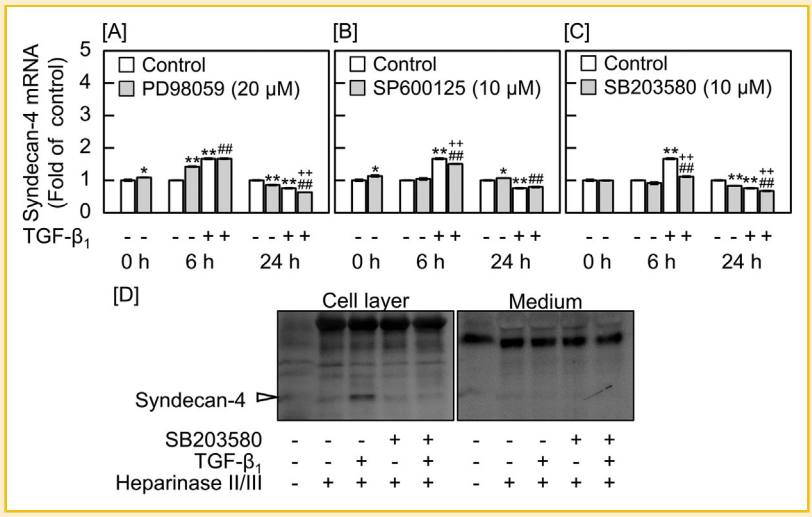

Fig. 3. Effects of the MAPK pathway inhibitors PD98059, LY364947, and SB203580 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were pretreated with [A] the MEK1 inhibitor PD98059 at 20 μM, [B] JNK inhibitor SP600125 at 10 μM, or [C] p38 MAPK inhibitor SB203580 at 10 μM at 37°C for 1 h and then treated with 5 ng/mL TGF-β_1 for 6 or 24 h. Values are the mean ± S.E. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{-}P < 0.01$ versus MAPK inhibitor without TGF-β_1; $^{++}P < 0.01$ versus TGF-β_1. [D] The expression of syndecan-4 core protein. Arrowhead indicates the position of syndecan-4. Bovine aortic endothelial cells were pretreated with 10 μM SB203580 at 37°C for 1 h and then treated with 5 ng/mL TGF-β_1 for 6 h.

TGF-β_1, the PAI-1 mRNA expression was markedly reduced. This suggests that the basal level of PAI-1 mRNA was suppressed by either Smad2 or Smad3 knockdown. After a 6-h incubation with TGF-β_1, the PAI-1 mRNA expression was markedly induced; siSmad3 but not siSmad2 significantly suppressed the induction, suggesting that TGF-β_1-induced PAI-1 mRNA expression was suppressed only by siSmad3. These results were consistent with previous reports [Yingling et al., 1997; Xu et al., 2000] and showed a specific knockdown of Smad2 and Smad3 by siSmad2 and siSmad3, respectively. siRNA-mediated knockdown alone of either Smad2 (Fig. 4E) or Smad3 (Fig. 4F) significantly increased the expression of endothelial syndecan-4 mRNA without treatment with TGF-β_1, suggesting that both Smad2 and Smad3 are involved in the reduction of the basal expression of endothelial syndecan-4. The early upregulation and late downregulation of syndecan-4 mRNA expression by TGF-β_1 were both not affected by siRNA-mediated knockdown of Smad2 (Fig. 4E). On the other hand, siRNA-mediated knockdown of Smad3 diminished the upregulation of syndecan-4 mRNA expression by TGF-β_1 (Fig. 4F), suggesting that Smad3 is a member of the signaling pathway that mediates the TGF-β_1-induced upregulation of syndecan-4 expression. The late downregulation of syndecan-4 core protein expression by TGF-β_1 was observed in cells treated with or without siRNA for Smad2, but this downregulation was reduced by siRNA for Smad3 (Fig. 4G). This result indicates that Smad3 is involved in the late downregulation of syndecan-4 core protein expression by TGF-β_1. These results indicate that the early upregulation of syndecan-4 mRNA expression by TGF-β_1 is mediated by Smad3, whereas Smad2 or Smad3 alone is not involved in the late downregulation of syndecan-4 mRNA expression by TGF-β_1; the late downregulation of syndecan-4 core protein expression by TGF-β_1 is mediated by Smad3.

To clarify the role of Smad2/3 in the late downregulation, the effects of the knockdown of both Smad2 and Smad3 on the expression of syndecan-4 mRNA and the core protein were investigated. The results indicated that the siRNA-mediated knockdown of both Smad2 and Smad3 diminished the late downregulation of syndecan-4 mRNA expression by TGF-β_1, although the knockdown of just one could not diminish the downregulation (Fig. S1, upper panel). In addition, the results again indicated that siSmad3 with or without siSmad2 diminished the decrease in syndecan-4 core protein expression by TGF-β_1 after a 24-h treatment (Fig. S1, lower panel). These results suggest that the late downregulation of syndecan-4 expression by TGF-β_1 is mediated by Smad2/3 at the mRNA level and by Smad3 at the protein level.

ACTIVATION OF p38 MAPK IS MEDIATED BY Smad3

In order to examine whether the phosphorylation of p38 MAPK by TGF-β_1 is induced by Smad2 or Smad3, vascular endothelial cells transfected with siSmad2 or siSmad3 were stimulated with TGF-β_1, and the phosphorylation of p38 MAPK was investigated. As shown in Figure 5, TGF-β_1 increased the phosphorylation of p38 MAPK after 4 h and 8 h; the increase was markedly suppressed by siSmad3 but not siSmad2, suggesting that the activation of p38 MAPK is mediated by Smad3.

DISCUSSION

Recently, we reported that biglycan intensifies the ALK5-Smad2/3 signaling induced by TGF-β_1 and downregulates syndecan-4 in cultured vascular endothelial cells [Hara et al., 2016]. In this study, we observed that TGF-β_1 first upregulates and then downregulates

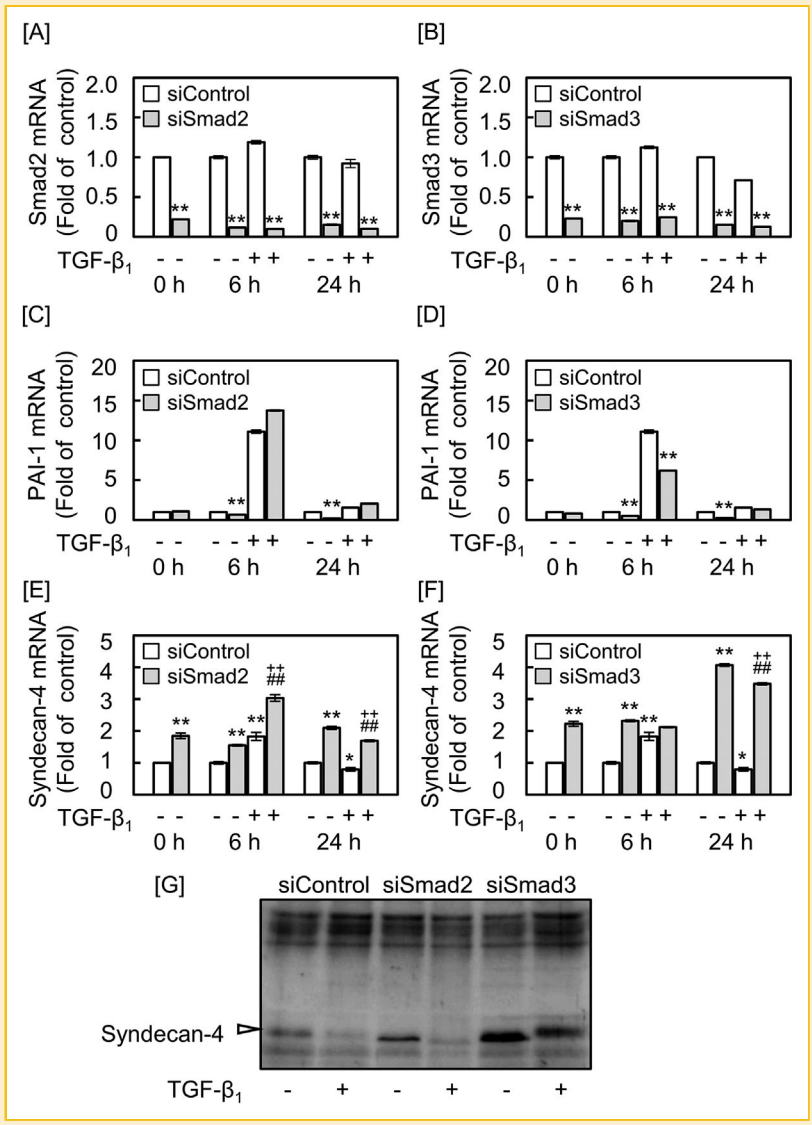

Fig. 4. Effects of siRNA-mediated knockdown of Smad2 or Smad3 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siSmad2 or siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 6 or 24 h. The expression of mRNAs for [A] Smad2, [B] Smad3, [C and D] PAI-1, and [E and F] syndecan-4. Values are the means ± S.E. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus siControl; $^{-}P < 0.01$ versus siSmad2 or siSmad3 without TGF-β_1; $^{++}P < 0.01$ versus TGF-β_1. [G] The expression of syndecan-4 core protein. Arrowhead indicates the position of syndecan-4. Bovine aortic endothelial cells were transfected with siControl, siSmad2, or siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 24 h.

endothelial syndecan-4 expression. In the present study, we investigated the signaling pathways that mediate the biphasic regulation of endothelial syndecan-4 expression, and the following results were obtained: (1) The biphasic regulation by TGF-β_1 was confirmed. (2) TGF-β_1 activated MAPKs, including ERK1/2, JNK, and p38 MAPK, as well as Smad2/3. (3) The early upregulation of syndecan-4 by TGF-β_1 was diminished by either an inhibitor of p38 MAPK or siRNA for Smad3 at both the mRNA and syndecan-4 core protein expression levels. (4) The late downregulation of syndecan-4 mRNA expression was reduced when cells were treated with siRNAs for both Smad2 and Smad3, whereas the late downregulation of syndecan-4 core protein expression was reduced only by siRNA for Smad3. (5) The activation of p38 MAPK was reduced by siRNA for Smad3 but not by that for Smad2. Together, these results suggest that the early upregulation of endothelial syndecan-4 expression by TGF-β_1 is mediated by the ALK5–Smad3–p38 MAPK pathway, whereas the late downregulation is mediated by the ALK5–Smad2/3 pathway. Specifically, the downregulation of syndecan-4 expression by TGF-β_1 is mediated by the interaction of Smad3 with Smad2 at the mRNA expression level, whereas only Smad3 is crucial at the core protein expression level. The present data showed for the first time that TGF-β_1 and p38 MAPK serve as regulatory molecules for endothelial syndecan-4 expression.

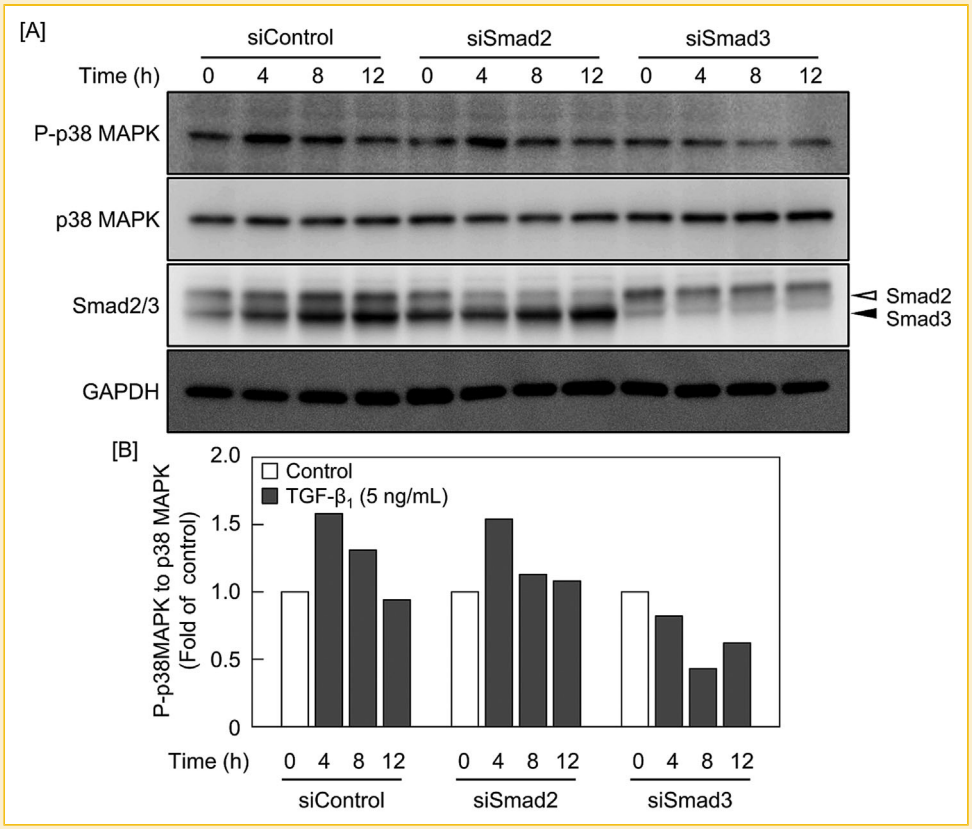

Fig. 5. Effects of siRNA-mediated knockdown of Smad2 or Smad3 on the activation of p38 MAPK in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siSmad2 or siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 4, 8, or 12 h. [A] Expression of P-p38 MAPK, p38 MAPK, Smad2/3, and GAPDH proteins. Open and filled arrowheads indicate the position of Smad2 and Smad3, respectively. [B] The ratio of the intensity of P-p38 MAPK to that of p38 MAPK in [A].

The TGF-β_1 that vascular endothelial cells are exposed to is mainly released from aggregated platelets. When vascular endothelial cells are injured, platelets aggregate at the damaged site, and TGF-β_1 is released from α-granules in the platelets [Assoian and Sporn, 1986]. At that time, TGF-β_1 activates p38 MAPK and upregulates plasminogen activator inhibitor type 1 expression to maintain the fibrin clot that prevents bleeding [Woodward et al., 2006]. TGF-β_1-activated p38 MAPK increases permeability [Goldberg et al., 2002] and induces the apoptosis of vascular endothelial cells [Hyman et al., 2002]. In addition to these inflammatory responses, the TGF-β_1–p38 MAPK pathway also increases the synthesis and activity of focal adhesion kinase, which is crucial for cell survival, motility, and proliferation [Walsh et al., 2008]. Syndecan-4 molecules have three heparan sulfate chains on the extracellular domain of their core proteins that are used for focal adhesion; the heparan sulfate chains play a critical role in stress fiber formation [Gopal et al., 2010]. It has also been reported that syndecan-4 is an anti-inflammatory molecule. Specifically, a large number of neutrophils, increased expression of neutrophil chemotactic factors, and higher mortality were reported in syndecan-4 knockout mice injected with lipopolysaccharide [Ishiguro et al., 2001; Tanino et al., 2012]. These results and our data, which showed that the induction of endothelial syndecan-4 expression occurs in the early stage of the exposure to TGF-β_1, suggest that the upregulation of endothelial syndecan-4 expression by TGF-β_1 may be a defense mechanism against an acute injury to vascular endothelial cells.

It has been reported that the atherosclerotic vascular wall has fewer heparan sulfate proteoglycans and more dermatan sulfate proteoglycans compared to the normal vascular wall [Stevens et al., 1976]. In addition, the lack of syndecan-4 disrupts the alignment of vascular endothelial cells along the direction of blood flow, resulting in the formation of a wide range atherosclerotic lesions, including near vascular branching points where jet laminar flow is impeded to create disturbed flow [Baeyens et al., 2014]. The disturbed flow increases thrombogenicity and triggers chronic inflammation [Brooks et al., 2002]. Additionally, vascular endothelial cells exposed to disturbed flow exhibit a lower expression of focal adhesion molecules such as VE-cadherin and β-catenin than cells exposed to pulsatile flow [Miao et al., 2005]. The functional damage of vascular endothelial cells caused by the disturbed flow induces chronic TGF-β_1 accumulation in the vascular wall, and the accumulated TGF-β_1 suppresses the expression of syndecan-4 by activation of Smad2/3, which contributes to the progression of atherosclerosis [Tull et al., 2006; Nesbitt et al., 2009; Popovic et al., 2009]. Together with the results from our previous study, it is suggested that

biglycan, a proteoglycan whose synthesis is induced by TGF-β_1 [Kaji et al., 2000], intensifies the TGF-β_1–ALK5 signaling via the core protein and that activated Smad2/3 in the signaling pathway suppresses the expression of syndecan-4 at the late stage of TGF-β_1 exposure in vascular endothelial cells. In other words, TGF-β_1 induces endothelial biglycan synthesis; as a result, syndecan-4 expression is suppressed by enhanced TGF-β_1 signaling. This regulation of endothelial proteoglycan synthesis by TGF-β_1 may be a component of the mechanisms underlying the histopathological changes in the types of proteoglycans—fewer heparan sulfate proteoglycans and more dermatan sulfate proteoglycans [Stevens et al., 1976]—seen in atherosclerotic vascular walls.

The data from the present study revealed the signaling pathways that mediate the regulation of syndecan-4 expression by TGF-β_1 in vascular endothelial cells. They indicated that TGF-β_1 upregulates syndecan-4 expression via the ALK5–Smad3–p38 MAPK pathway at the early stage, whereas downregulation of the expression occurs via the ALK5–Smad2/3 pathway. The mechanism responsible for switching the signaling pathway from the Smad3–p38 MAPK pathway to the Smad2/3 pathway for syndecan-4 expression appears to be important. It was reported that the signal transduction of Ask1, which is a MAP kinase kinase kinase that regulates the stress responses, is regulated in a biphasic manner by ubiquitination. Ubiquitinated Ask1 is transiently activated and transduces a proliferation signal [Maruyama et al., 2014]; however, when Ask1 is not ubiquitinated, the molecule is activated continuously and induces an apoptotic signal [Ichijo et al., 1997]. Because Smad3 is also ubiquitinated [Fukuchi et al., 2001; Moren et al., 2003], it may be possible that one of the mechanisms that regulates the signaling pathway for endothelial syndecan-4 is the ubiquitination of Smad3. Further studies are required to clarify the detailed mechanisms by which the signaling pathway for endothelial syndecan-4 expression is switched from the Smad3–p38 MAPK pathway for upregulation at the early stage to the Smad2/3 pathway for downregulation at the late stage.

ACKNOWLEDGMENTS

We would like to thank Dr. Thomas N. Wight of the Benaroya Research Institute at Virginia Mason, Seattle, WA, USA, for his helpful comments and suggestions on this study and manuscript. This work was supported by a Grant-in-Aid for Challenging Exploratory Research #15K14992 (to T. K.) and a Grant-in-Aid for Scientific Research (C) #15K08047 (to C. Y.) from the Japan Society for the Promotion of Science.

REFERENCES

Assoian RK, Sporn MB. 1986. Type beta transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. J Cell Biol 102:1217–1223.

Baeyens N, Mulligan-Kehoe MJ, Corti F, Simon DD, Ross TD, Rhodes JM, Wang TZ, Mejean CO, Simons M, Humphrey J, Schwartz MA. 2014. Syndecan 4 is required for endothelial alignment in flow and atheroprotective signaling. Proc Natl Acad Sci USA 111:17308–17313.

Berenson GS, Radhakrishnamurthy B, Srinivasan SR, Vijayagopal P, Dalferes ER, Jr., Sharma C. 1984. Recent advances in molecular pathology. Carbohydrate-protein macromolecules and arterial wall integrity-a role in atherogenesis. Exp Mol Pathol 41:267–287.

Brooks AR, Lelkes PI, Rubanyi GM. 2002. Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. Physiol Genomics 9:27–41.

Camejo G. 1981. The interaction of lipids and lipoproteins with the intercellular matrix of arterial tissue: Its possible role in atherogenesis. Adv Lipid Res 19:1–53.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425:577–584.

Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, Miyazono K. 2001. Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol Biol Cell 12:1431–1443.

Goldberg PL, MacNaughton DE, Clements RT, Minnear FL, Vincent PA. 2002. P38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. Am J Physiol Lung Cell Mol Physiol 282:L146–L154.

Gopal S, Bober A, Whiteford JR, Multhaupt HA, Yoneda A, Couchman JR. 2010. Heparan sulfate chain valency controls syndecan-4 function in cell adhesion. J Biol Chem 285:14247–14258.

Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, ten Dijke P. 2003. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol Cell 12:817–828.

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten dijke P. 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. Embo J 21:1743–1753.

Hara T, Yoshida E, Shinkai Y, Yamamoto C, Fujiwara Y, Kumagai Y, Kaji T. 2016. Biglycan intensifies ALK5-Smad2/3 signaling by TGF-beta1 and downregulates syndecan-4 in cultured vascular endothelial cells. J Cell Biochem. http://onlinelibrary.wiley.com/doi/10.1002/jcb.25721/full

Hyman KM, Seghezzi G, Pintucci G, Stellari G, Kim JH, Grossi EA, Galloway AC, Mignatti P. 2002. Transforming growth factor-beta1 induces apoptosis in vascular endothelial cells by activation of mitogen-activated protein kinase. Surgery 132:173–179.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275:90–94.

Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Iwase M, Yoshikai Y, Yanada M, Yamamoto K, Matsushita T, Nishimura M, Kusugami K, Saito H, Muramatsu T. 2001. Syndecan-4 deficiency leads to high mortality of lipopolysaccharide-injected mice. J Biol Chem 276:47483–47488.

Järveläinen HT, Kinsella MG, Wight TN, Sandell LJ. 1991. Differential expression of small chondroitin/dermatan sulfate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. J Biol Chem 266:23274–23281.

Jaffe EA, Hoyer LW, Nachman RL. 1974. Synthesis of von Willebrand factor by cultured human endothelial cells. Proc Natl Acad Sci USA 71:1906–1909.

Kaji T, Yamada A, Miyajima S, Yamamoto C, Fujiwara Y, Wight TN, Kinsella MG. 2000. Cell density-dependent regulation of proteoglycan synthesis by transforming growth factor-β_1 in cultured bovine aortic endothelial cells. J Biol Chem 275:1463–1470.

Kaji T, Yamamoto C, Oh-i M, Fujiwara Y, Yamazaki Y, Morita T, Plaas AH, Wight TN. 2006. The vascular endothelial growth factor VEGF165 induces perlecan synthesis via VEGF receptor-2 in cultured human brain microvascular endothelial cells. Biochim Biophys Acta 1760:1465–1474.

Kaji T, Yamamoto C, Oh-i M, Nishida T, Takigawa M. 2004. Differential regulation of biglycan and decorin synthesis by connective tissue growth factor in cultured vascular endothelial cells. Biochem Biophys Res Commun 322:22–28.

Kinsella MG, Tsoi CK, Jarvelainen HT, Wight TN. 1997. Selective expression and processing of biglycan during migration of bovine aortic endothelial cells. The role of endogenous basic fibroblast growth factor. J Biol Chem 272:318–325.

Kojima T, Shworak NW, Rosenberg RD. 1992. Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. J Biol Chem 267:4870–4877.

Kyhse-Andersen J. 1984. Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polycrylamide to nitrocellulose. J Biochem Biophys Methods 10:203–209.

Levin EG, Loskutoff DJ. 1982. Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activators. J Cell Biol 94:631–636.

Macias MJ, Martin-Malpartida P, Massague J. 2015. Structural determinants of Smad function in TGF-beta signaling. Trends Biochem Sci 40:296–308.

Maruyama T, Araki T, Kawarazaki Y, Naguro I, Heynen S, Aza-Blanc P, Ronai Z, Matsuzawa A, Ichijo H. 2014. Roquin-2 promotes ubiquitin-mediated degradation of ASK1 to regulate stress responses. Sci Signal 7:ra8.

Mertens G, Cassiman JJ, Van den Berghe H, Vermylen J, David G. 1992. Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. J Biol Chem 267:20435–20443.

Miao H, Hu Y-L, Shiu Y-T, Yuan S, Zhao Y, Kaunas R, Wang Y, Jin G, Usami S, Chien S. 2005. Effects of flow patterns on the localization and expression of VE-cadherin at vascular endothelial cell junctions: In vivo and in vitro investigations. J Vasc Res 42:77–89.

Moren A, Hellman U, Inada Y, Imamura T, Heldin CH, Moustakas A. 2003. Differential ubiquitination defines the functional status of the tumor suppressor Smad4. J Biol Chem 278:33571–33582.

Moustakas A, Heldin CH. 2005. Non-Smad TGF-beta signals. J Cell Sci 118:3573–3584.

Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. 2009. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nat Med 15:665–673.

Popovic N, Bridenbaugh EA, Neiger JD, Hu JJ, Vannucci M, Mo Q, Trzeciakowski J, Miller MW, Fossum TW, Humphrey JD, Wilson E. 2009. Transforming growth factor-beta signaling in hypertensive remodeling of porcine aorta. Am J Physiol Heart Circ Physiol 297: H2044–H2053.

Revtyak GE, Johnson AR, Campbell WB. 1987. Prostaglandin synthesis in bovine coronary endothelial cells: Comparison with other commonly studied endothelial cells. Thromb Res 48:671–683.

Saku T, Furthmayr H. 1989. Characterization of the major heparan sulfate proteoglycan secreted by bovine aortic endothelial cells in culture. Homology to the large molecular weight molecule of basement membranes. J Biol Chem 264:3514–3523.

Shi Y, Massagué J. 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113:685–700.

Stevens RL, Colombo M, Gonzales JJ, Hollander W, Schmid K. 1976. The glycosaminoglycans of the human artery and their changes in atherosclerosis. J Clin Invest 58:470–481.

Tanino Y, Chang MY, Wang X, Gill SE, Skerrett S, McGuire JK, Sato S, Nikaido T, Kojima T, Munakata M, Mongovin S, Parks WC, Martin TR, Wight TN, Frevert CW. 2012. Syndecan-4 regulates early neutrophil migration and pulmonary inflammation in response to lipopolysaccharide. Am J Respir Cell Mol Biol 47:196–202.

Tollefsen DM, Pestka CA, Monafo WJ. 1983. Activation of heparin cofactor II by dermatan sulfate. J Biol Chem 258:6713–6716.

Tull SP, Anderson SI, Hughan SC, Watson SP, Nash GB, Rainger GE. 2006. Cellular pathology of atherosclerosis: Smooth muscle cells promote adhesion of platelets to cocultured endothelial cells. Circ Res 98:98–104.

Walsh MF, Ampasala DR, Hatfield J, Vander Heide R, Suer S, Rishi AK, Basson MD. 2008. Transforming growth factor-beta stimulates intestinal epithelial focal adhesion kinase synthesis via Smad- and p38-dependent mechanisms. Am J Pathol 173:385–399.

Woodward RN, Finn AV, Dichek DA. 2006. Identification of intracellular pathways through which TGF-beta1 upregulates PAI-1 expression in endothelial cells. Atherosclerosis 186:92–100.

Xu W, Angelis K, Danielpour D, Haddad MM, Bischof O, Campisi J, Stavnezer E, Medrano EE. 2000. Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor. Proc Natl Acad Sci USA 97:5924–5929.

Yamamoto C, Deng X, Fujiwara Y, Kaji T. 2005. Proteoglycans predominantly synthesized by human brain microvascular endothelial cells in culture are perlecan and biglycan. J Health Sci 51:576–583.

Yingling JM, Datto MB, Wong C, Frederich JP, Liberati NT, Wang XF. 1997. Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein. Mol Cell Biol 17:7019–7028.

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