

CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

TGF- β induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways¹

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TGF- β induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways.

Background. Angiogenesis has a key role in numerous disease processes. One of the most important angiogenic factors is vascular endothelial growth factor (VEGF-A), whereas thrombospondin-1 (TSP-1) is a major antiangiogenic factor. Recent studies have shown that VEGF-A as well as TSP-1 is regulated by transforming growth factor- β 1 (TGF- β 1), but the mechanism remains unclear.

Methods. We examined the role of TGF- β 1 and its signaling pathways in mediating expression of these two molecules. Rat proximal tubular cells (NRK52E) were stimulated with TGF- β 1 to induce VEGF-A and TSP-1 synthesis. To clarify roles of receptor-activated Smads (R-Smads), we blocked Smad signaling using overexpression of the inhibitory Smad, Smad7, and by using fibroblasts from wild-type or knockout mice. To confirm the antiangiogenic role of Smads, soluble Flt-1 regulation in response to TGF- β 1 was also examined. In addition, the effect of conditioned media from NRK52E and Smad knockout cells was examined on endothelial cell proliferation.

Results. Induction of VEGF-A and TSP-1 by TGF- β 1 in NRK52E cells was associated with activation of pathway-restricted R-Smads (Smad2 and 3) and blocking these Smads by overexpression of Smad7 blocked their induction. By using of Smad knockout cells, Smad3 was shown to have a key role in the stimulation of VEGF-A expression whereas Smad2 was critical for TSP-1 expression. Consistent with the hypothesis that Smad2 has an antiangiogenic function, we also demonstrated that Smad2, but not Smad3, mediated the expression of VEGF-A antagonist, soluble VEGF-A receptor sFlt-1, in response to TGF- β 1. Conditioned media from NRK52E, which was stimulated by TGF- β 1 for 24 hours, did not induce endothelial cell

proliferation. However, conditioned media from Smad2 knockout induced endothelial cell proliferation, whereas endothelial cell proliferation was inhibited by Smad3 knockout-derived conditioned media.

Conclusion. R-Smads have distinct roles in mediating the expression of pro- and antiangiogenic growth factors in response to TGF- β 1.

Angiogenesis has a critical role in development, normal health, and disease. Impaired angiogenesis has been linked to renal disease associated with aging [1] and with renal progression [2], whereas excessive angiogenesis has been found in diabetic retinopathy [3] and in tumor metastasis [4]. One of the most important proangiogenic factors is vascular endothelial growth factor (VEGF-A) [5–7], while thrombospondin-1 (TSP-1) is antiangiogenic and has been shown to oppose VEGF-A actions [1, 2, 8, 9]. TSP-1 expression has also been linked to impaired angiogenesis and aging-associated renal disease and progressive renal damage [1, 2, 10, 11]. Recently, a soluble form of the VEGF-A receptor, sFlt-1, has been recognized as the true natural inhibitor of VEGF-A [12, 13]. This soluble receptor (sFlt-1) has now been linked to conditions associated with endothelial dysfunction, including hypertension, heart failure, and preeclampsia [14, 15]. In the latter condition, circulating sFlt-1 is thought to have a key role in the pathogenesis of disease [14]. Thus, an understanding of the mechanisms regulating these growth factors may provide insight into the prevention and treatment of these conditions.

Transforming growth factor- β 1 (TGF- β 1) is known to have a key role in many disease processes, including diabetes [16, 17], cancer, aging [18–20], preeclampsia [21, 22], and renal disease [23]. TGF- β 1 is also known to have both angiogenic and antiangiogenic actions depending on the specific condition. TGF- β 1 acts by stimulating a complicated intracellular signaling pathway

¹See Editorial by Woolf, p. 862.

Key words: VEGF, TSP-1, sFlt-1, Smad KO cell, endothelial cell proliferation.

Received for publication December 2, 2003
and in revised form January 29, 2004
Accepted for publication February 20, 2004

consisting of receptor-activated Smads (R-Smads2 and 3) and inhibitory Smads (Smad6 and 7). Distinct roles for each R-Smad have only been identified in the past few years. In this paper we provide evidence that a major difference between Smad2 and Smad3 signaling relates to their role in mediating the expression of pro- and antiangiogenic molecules in response to TGF- β 1. Smad3 has a proangiogenic role and stimulates VEGF-A expression, whereas Smad2 has an antiangiogenic role in mediating TSP-1 and sFlt-1 expression. These data may provide an explanation for the paradoxical roles of TGF- β 1 in angiogenesis in various disease states.

METHODS

Materials

TGF- β 1 was ordered from R&D systems (Minneapolis, MN, USA). Antibody reagents included mouse monoclonal antibody to Smad2 (Transduction Laboratories, Lexington, KY, USA), thrombospondin (TSP Ab-4; A6.1) (NeoMarkers) (Lab Vision Co., Fremont, CA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA, USA) and polyclonal antibodies to Smad7 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Smad3 antibody (Zymed Laboratory, Inc., South San Francisco, CA, USA), p-Smad2 antibody (Cell Signaling, Beverly, MA, USA), and thrombospondin (TSP Ab-8) (NeoMarkers) (Lab Vision Co.). NP40208 is a novel 2,4-disubstituted pteridine that inhibits the intracellular kinase domain of the type I TGF- β receptor (T β R-I) (Scios, Inc., Sunnyvale, CA, USA).

Cell culture

Rat proximal tubular epithelial cell line (NRK52E) or fibroblasts derived from mouse embryo deficient in Smad2 or Smad3 [24] were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 60 μ g/mL penicillin, and 100 μ g/mL streptomycin in 5% CO₂ at 37°C and media was changed every 3 days. Confluent cells were incubated for 24 hours with 0.5% FBS prior to stimulation. TGF- β 1 was then added to confluent cell at the designated concentrations for 0 to 24 hours. Endothelial basal media (EBM) media with endothelial growth media (EGM)-MV (Cambrex, East Rutherford, NJ, USA) was used to culture human umbilical venous endothelial cell (HUVEC) (Cambrex).

Establishing doxycycline-regulated Smad7-expression NRK52E cell lines

The doxycycline-regulated Smad7-expressing cell line was established as described previously [25]. Briefly, mouse Smad7 cDNA was subcloned using a tetracycline

(Tet)-inducible vector, pTRE (Clontech, Palo Alto, CA, USA). An improved pTet-on vector, pEFpurop-Tet-on [26], was used, in which the gene encoding the "reverse" Tet repressor was subcloned into a pEF-BOS vector, pEFr-PGKpuropAv18, thereby conferring puromycin resistance [26]. To obtain doxycycline (a Tet derivative)-induced Smad7-expressing NRK52E cell lines, pTRE-Smad7 and pEFpurop-Tet-on were cotransfected into NRK52E cells by electroporation, and then the stable transfected cells were selected in the presence of puromycin (4 μ g/mL). Doxycycline (4 μ g/mL) was used to induce Smad7. At least three experiments were performed for each experiment. Cell viability in each experimental condition was examined by lactate dehydrogenase (LDH) assay with TOX-7 LDH Assay Kit (Sigma Chemical Co., St. Louis, MO, USA).

RNAse protection assay (RPA)

Riboprobes were prepared as previously described [27]. Briefly, rat VEGF-A (327 bp) was subcloned into Bluescript SK+ (Stratagene, La Jolla, CA, USA). After linearization, an antisense riboprobe was synthesized with T7 polymerase in the presence of α -³²P-labeled uridine triphosphate (UTP). Total RNA was isolated with RNeasy Kit (Qiagen, Valencia, CA, USA). Three micrograms of total RNA samples were hybridized for 30 minutes at 90°C with a mixture of ³²P-UTP-labeled riboprobes of rat VEGF-A and the housekeeping gene (L32) (1 \times 10⁵ cpm for each probe), and RPA was performed as described previously [27]. The protected hybridized RNA was denatured at 85°C and electrophoresed on 10% polyacrylamide gels. The gels were transferred to Whatman filter paper, dried, and exposed to Kodak X-O mat film overnight at -70°C.

Real time-polymerase chain reaction (PCR)

To quantify mRNA expression, real-time PCR was performed. After 0.5 μ g of total RNA was converted to cDNA with Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA), PCR was performed with rat VEGF-A [28], TSP-1 [29] or GAPDH primers [30] mixed with SYBR Green JumpStat Taq ReadyMix (Sigma Chemical Co.) using a DNA Engine Opticon (MJ Research, Waltham, MA, USA) as follows: 94°C for 2 minutes, then 44 cycles of denaturation at 94°C for 15 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 20 seconds. Amplicon sizes were 492 bp (rat 164 VEGF-A), 360 bp (rat 120 VEGF-A), 379 bp (rat TSP-1), and 299 bp (rat GAPDH). Reaction specificity was confirmed by electrophoretic analysis of products prior to real-time reverse transcription (RT)-PCR and bands of expected size were detected. Ratios for either VEGF-A/GAPDH mRNA or TSP-1/GAPDH mRNA were calculated for each sample and expressed as mean \pm SD.

Enzyme-linked immunosorbent assay (ELISA) for VEGF-A and sFlt-1 protein synthesis in the supernatant of culture media

NRK52E cells were grown in six-well plates and culture supernatants were analyzed by Quantikine mouse ELISA kit for VEGF-A or soluble Flt-1 (R&D Systems), which cross-reacts with rat samples [31]. Total VEGF-A or sFlt-1 protein (pg) in the supernatant was corrected by cell protein content (μg) (Bio-Rad Protein Assay) (Bio-Rad, Richmond, CA, USA).

Western blotting

Cells were washed in phosphate-buffered saline (PBS) and then lysed in 100 μL of cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethyleneglycol tetraacetate (EGTA), 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, and 1 mmol/L Na_3VO_4], containing a 1:50 dilution of a protease inhibitor cocktail (Pharmingen, San Diego, CA, USA) for 30 minutes on ice. Samples were centrifuged at 14,000g for 5 minutes to pellet cell debris. To isolate nuclear proteins, cells from 100 mm dishes were resuspended in 100 μL 10 mmol/L Tris-HCl, 2 mmol/L MgCl_2 , 5 mmol/L KCl, 10% glycerol, 1 mmol/L EDTA, and 1 mmol/L dithiothreitol. NP40 (1%) was added and cells allowed swelling on ice then vortexed. Lysates were centrifuged (700g, 4°C) and nuclear pellets resuspended in 30 μL 20 mmol/L HEPES, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L dithiothreitol before sonication. After determination of the protein concentration using the Bio-Rad Protein Assay (Bio-Rad), 30 μg of protein samples were mixed with sample buffer (Invitrogen, Carlsbad, CA, USA), boiled, resolved on NuPAGE Bis-Tris Gel (4% to 12%) gel, and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with 5% (wt/vol) bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Triton (TBST) for 60 minutes at room temperature. Each primary antibody was incubated at 4°C overnight. After washing with TBST, the membrane was rocked with secondary antibody (antimouse IgG or anti rabbit IgG, horseradish peroxidase-linked antibody) (Cell Signaling, Beverly, MA, USA) for 60 minutes at room temperature. The blot was then developed using the enhanced chemiluminescence (ECL) detection kit (Amersham International, Piscataway, NJ, USA) to produce a chemiluminescence signal, which was captured on x-ray film. Positive immunoreactive bands were quantified by densitometry.

Endothelial cell proliferation assay

To further investigate the functional role of TGF- β 1/Smads in angiogenesis, we examined the angio-

genic effect of conditioned media of TGF- β 1-stimulated NRK52E cells or Smad2 and Smad3 knockout cells on HUVEC. HUVEC (1×10^4) were cultured for 24 hours with conditioned media from either NRK52E cell or Smad knockout cells, and endothelial cell number was determined using the methylthiazolotetrazolium (MTT) Assay Kit (Sigma Chemical Co.). To block the function of exogenous TGF- β 1 in the conditioned media, 50 nmol/L NP40208, a T β R-I inhibitor, (Scios Inc.) was added to the conditioned media prior to stimulation of HUVEC. In an in vitro kinase assay NP40208 inhibits the T β R-I kinase with an IC_{50} of 0.048 $\mu\text{mol/L}$. When tested at 50 nmol/L the compound has no effect on type II T β R kinase (data provided by Scios Inc.). In our preliminary data, 50 nmol/L NP40208 inhibited VEGF-A expression in response to TGF- β 1 (data not shown).

Statistical analysis

All values presented are expressed as mean \pm SD. Analysis of variance (ANOVA) followed by Bonferroni correction was used in all instances. Significance was defined as $P < 0.05$.

RESULTS

The effect of TGF- β 1 on VEGF-A and TSP-1 induction in NRK52E cell

To examine the effects of TGF- β 1 on the expression of mRNA for VEGF-A or TSP-1 in NRK52E cells, either RNase protection assay for VEGF-A or real-time PCR for TSP-1 were used. Five nanograms per milliliter TGF- β 1 increased both VEGF-A and TSP-1 mRNA in a time-dependent manner and peaked at 24 hours (Fig. 1A and B).

Both VEGF-A and TSP-1 protein were also increased as early as 4 hours after incubation with 5 ng/mL TGF- β 1 and progressively reached a maximum after 24 hours or 32 hours incubation, respectively (Fig. 1C and 1).

Dose-dependent effect of TGF- β 1 on VEGF-A or TSP-1 protein synthesis

To investigate the dose dependency of TGF- β 1 on VEGF-A or TSP-1 protein synthesis, NRK52E cells were stimulated by TGF- β 1 at concentrations of 0 to 5 ng/mL for 24 hours, respectively, and VEGF-A protein in the supernatant was measured by ELISA (Fig. 1E) while Western blotting was used for TSP-1 synthesis (Fig. 1F). VEGF-A protein expression was increased by 1 ng/mL TGF- β 1 in the supernatant of NRK52E cells. Higher doses of TGF- β 1 (5 ng/mL) enhanced more VEGF-A protein expression (Fig. 1E). TSP-1 synthesis was also stimulated in a dose-dependent manner (Fig. 1F).

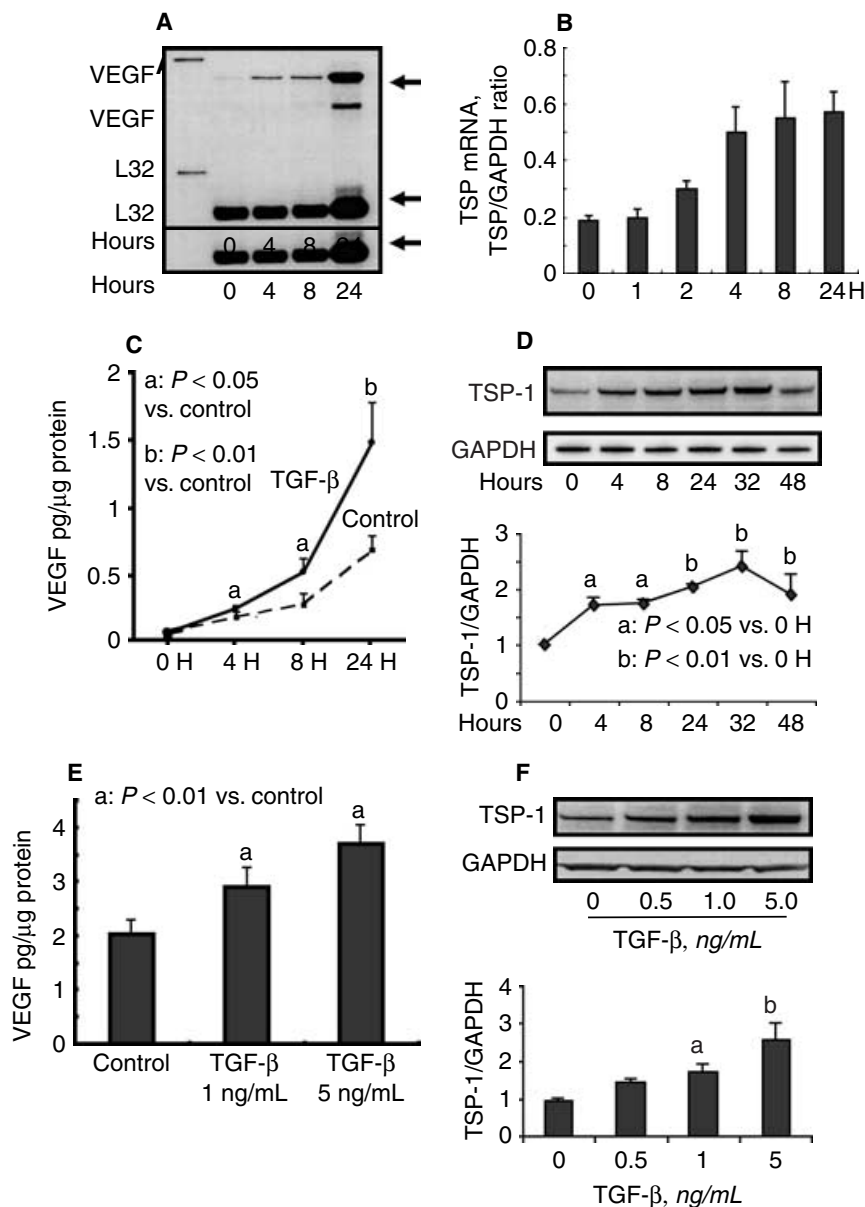


Fig. 1. Vascular endothelial growth factor (VEGF-A) and thrombospondin-1 (TSP-1) mRNA expression/protein synthesis induced by transforming growth factor- β 1 (TGF- β 1) in NRK52E cells. VEGF-A protein in supernatant of culture media was measured by enzyme-linked immunosorbent assay (ELISA). TSP-1 protein was examined by Western blotting. The protein expressions were quantified by densitometry. (A) Time course of VEGF-A mRNA expression by 5 ng/mL TGF- β 1. L32 was used as a control. (B) Time course of TSP-1 mRNA expression. (C) Time course of VEGF-A protein synthesis in supernatant of culture media by 5 ng/mL TGF- β 1. (D) Time course of TSP-1 protein synthesis by 5 ng/mL TGF- β 1. (E) Dose dependency of VEGF-A protein synthesis in supernatant of cultured media at 24 hours. (F) Dose dependency of TSP-1 protein synthesis at 24 hours. ^a $P < 0.05$ vs. 0 ng/mL TGF- β 1; ^b $P < 0.01$ vs. 0, 0.5, and 1 ng/mL TGF- β 1. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

R-Smads are critical for the expression of VEGF-A and TSP-1 induced by TGF- β 1

The R-Smads, Smad2 and Smad3, are known to play a critical role in TGF- β 1 signaling. To examine the collective role of R-Smads on VEGF-A and TSP-1 expression by TGF- β 1, we inhibited TGF- β -induced Smad2 and Smad3 activation by overexpressing the inhibitory Smad, Smad7, using a doxycycline-inducible system in the NRK52E cells. As shown in Figure 2A, TGF- β 1-induced Smad2 phosphorylation (determined by Western blotting of whole cell lysates) was blocked by overexpression of Smad7. Similarly, TGF- β 1-induced Smad3 activation (determined by Western blotting of nuclear protein as Smad3 translocates to the nucleus when activated) was also blocked by Smad7 overexpression

(Fig. 2B). The inhibition of both R-Smads was associated with an inhibition of VEGF-A mRNA and protein (Fig. 2C and E) and TSP-1 mRNA and protein (Fig. 2D and F). The inhibition of mRNA expression for VEGF-A and TSP-1 were observed as early as 2 hours (Fig. 2C and D), and the reduction in protein was noted at 24 hours (Fig. 2E and F). LDH assay and trypan blue staining did not show any difference with doxycycline-Smad7 treatment (data not shown). These findings suggest that TGF- β 1 directly stimulates VEGF-A and TSP-1 production, which could be regulated by either Smad2 or Smad3, or both.

We next examined whether the effects of TGF- β 1 to induce VEGF-A and TSP-1 were mediated by individual R-Smads. To investigate the role of Smad3 in

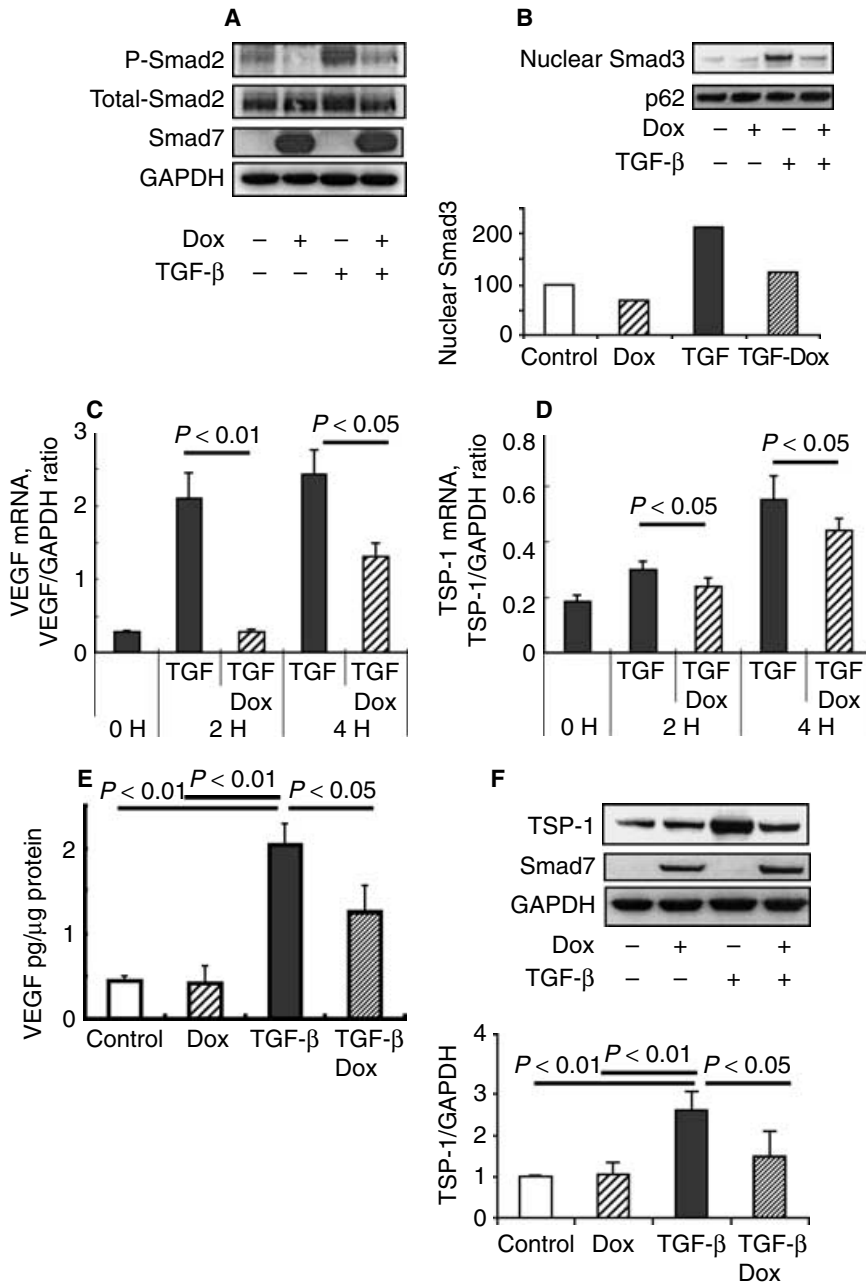


Fig. 2. The effect of overexpressing Smad7 in vascular endothelial growth factor (VEGF-A)/thrombospondin-1 (TSP-1) expression induced by transforming growth factor-β1 (TGF-β1) in NRK52E cells. (A) Doxycycline (Dox)-induced Smad 7 inhibits the phosphorylation of Smad2 induced by TGF-β1 at 60 minutes. **(B)** Doxycycline-induced Smad7 inhibits the nuclear translocation of Smad3 induced by TGF-β1 at 60 minutes. p62 was used for control nuclear protein. **(C)** Doxycycline-induced Smad7 inhibits the induction of VEGF-A mRNA. **(D)** Dox-induced Smad7 suppresses TSP-1 mRNA expression induced by TGF-β1. **(E)** Doxycycline-induced Smad7 inhibits VEGF-A protein synthesis at 24 hours. **(F)** Doxycycline-induced Smad7 inhibits TSP-1 protein synthesis induced by TGF-β1 at 24 hours. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

VEGF-A/TSP-1 regulation, we examined the effect of TGF-β1 on VEGF-A/TSP-1 in mouse fibroblasts obtained from Smad3 knockout or wild-type mice. While wild-type mouse fibroblasts synthesized VEGF-A protein in response to TGF-β, this was totally abrogated in Smad3 knockout cells (Fig. 3A). In contrast, TSP-1 synthesis was stimulated independent of Smad3 (Fig. 3B).

On the other hand, the absence of Smad2 abrogated TSP-1 expression, whereas TSP-1 expression was increased in Smad2 wild-type cell (Fig. 4B). In contrast, TGF-β1-induced VEGF-A synthesis was not prevented in Smad2 knockout cell (Fig. 4A).

These studies suggested a key role for Smad3 in mediating TGF-β1-induced VEGF-A expression (a proangiogenic molecule) whereas Smad2 was critical for TGF-β1-induced TSP-1 expression (an antiangiogenic molecule). To determine if this reflected a general mechanism that separated the actions of these two Smads, we further investigated whether Smad2 also mediated the production of soluble VEGF-AR-1 (sFlt-1), which is now recognized as the natural antagonist of VEGF-A and which may have a key role in preeclampsia and other conditions [12, 13]. sFlt-1 was also stimulated by TGF-β in NRK52E cell in a time- and dose-dependent manner

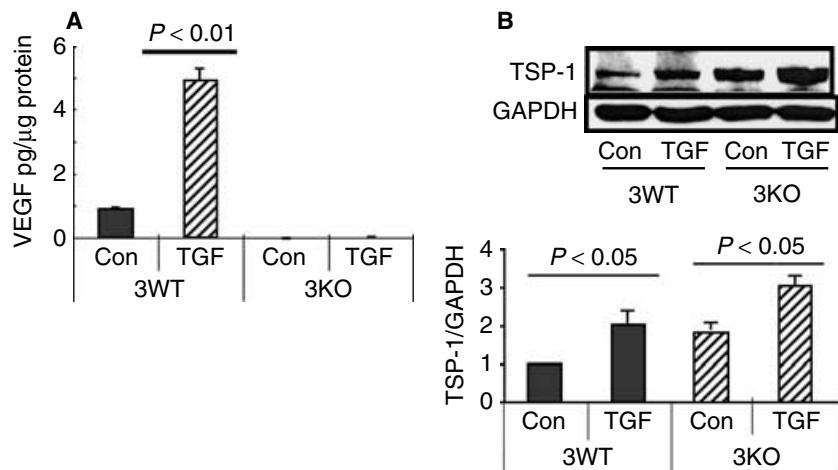


Fig. 3. Role of Smad3 in regulation of vascular endothelial growth factor (VEGF-A) or thrombospondin-1 (TSP-1). (A) VEGF-A protein synthesis in fibroblasts derived from mouse embryo deficient in Smad3 [3 knockout (3KO) or wild-type (3WT)] under basal condition (con) or transforming growth factor- β 1 (TGF- β 1) stimulation at 8 hours. (B) TSP-1 protein synthesis in 3KO or 3WT under basal condition or TGF- β 1 stimulation at 24 hours. TSP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions were quantified by densitometry.

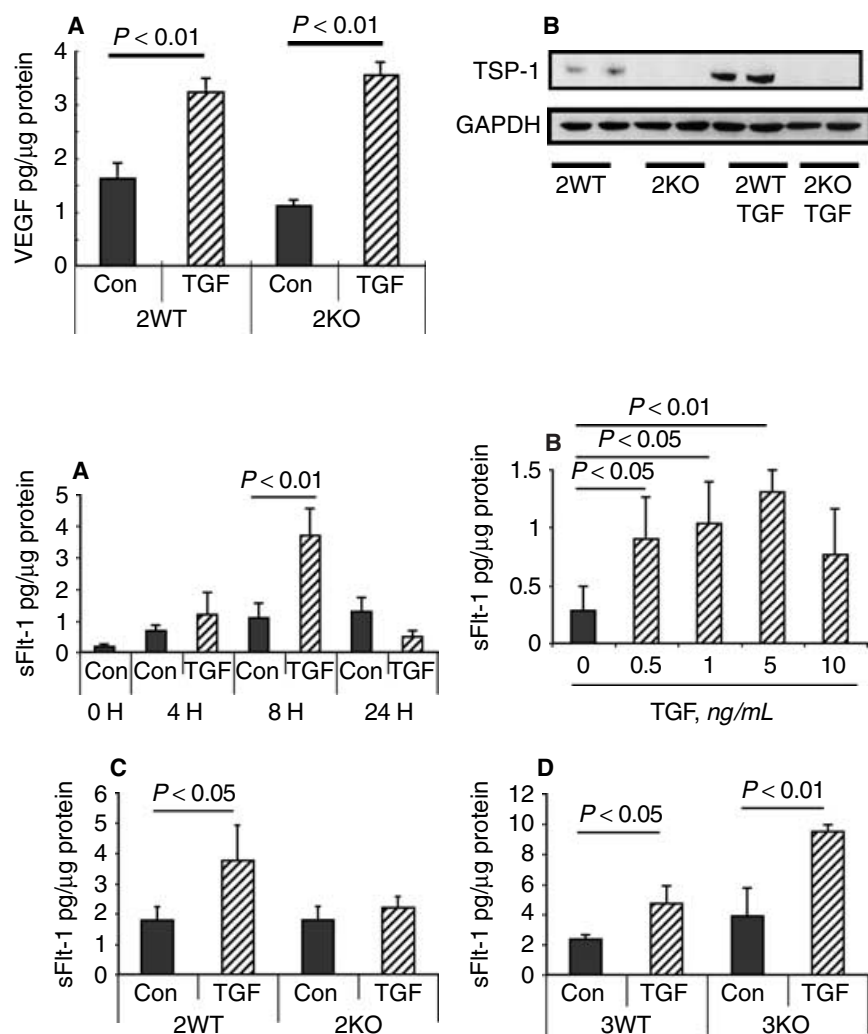


Fig. 4. Role of Smad2 in regulation of vascular endothelial growth factor (VEGF-A) or thrombospondin-1 (TSP-1). (A) VEGF-A protein synthesis in fibroblasts derived from mouse embryo deficient in Smad2 [2 knockout (2KO) or wild-type (2WT)] under basal condition (con) or transforming growth factor- β 1 (TGF- β 1) stimulation at 8 hours. (B) TSP-1 protein synthesis in 2KO or 2WT under basal condition or TGF- β 1 stimulation at 24 hours. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

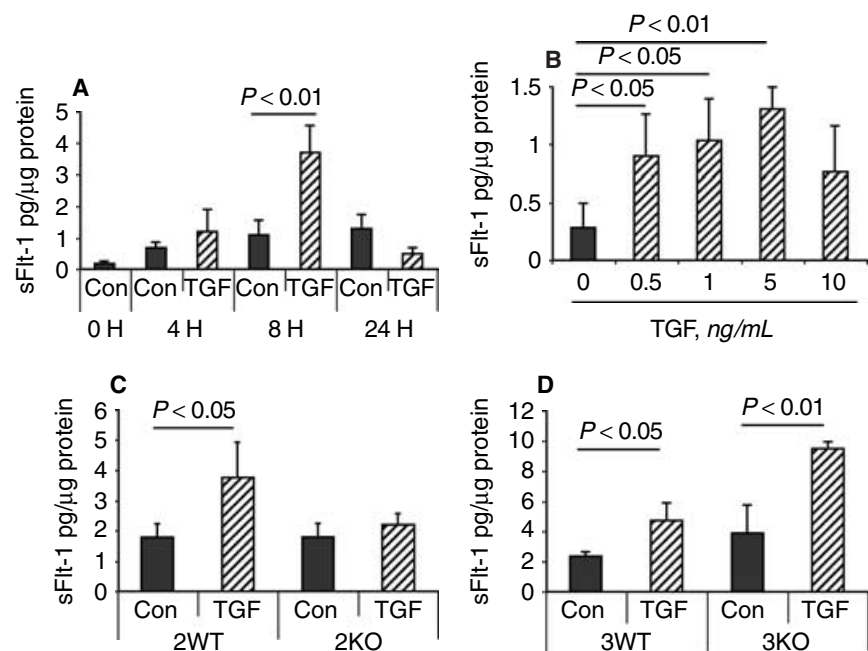


Fig. 5. sFlt-1 protein synthesis induced by transforming growth factor- β 1 (TGF- β 1) in NRK52E cell and mouse fibroblast. sFlt-1 protein was measured by enzyme-linked immunosorbent assay (ELISA). (A) Time course of sFlt-1 protein synthesis under 5 ng/mL TGF- β 1 stimulation at 8 hours. (B) Dose dependency of sFlt-1 protein synthesis under TGF- β 1 stimulation at 8 hours. (C) sFlt-1 protein synthesis in Smad2 [2 knockout (2KO) or wild-type (2WT)] fibroblasts under basal condition (con) or TGF- β 1 stimulation at 8 hours. (D) sFlt-1 protein synthesis in 3KO or 3WT fibroblasts with or without TGF- β 1 stimulation at 8 hours. GAPDH is glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 5A and B). Consistent with the hypothesis, TGF- β 1 failed to enhance sFlt-1 production in Smad2 knockout cells (Fig. 5C), whereas the normal enhancement of sFlt-1 was observed in Smad3 knockout cells (Fig. 5D).

Finally, to examine the functional roles of Smads in angiogenesis/antiangiogenesis, we tested the effect of conditional media, in which NRK52E, wild-type, Smad2 knockout, or Smad3 knockout fibroblasts had been

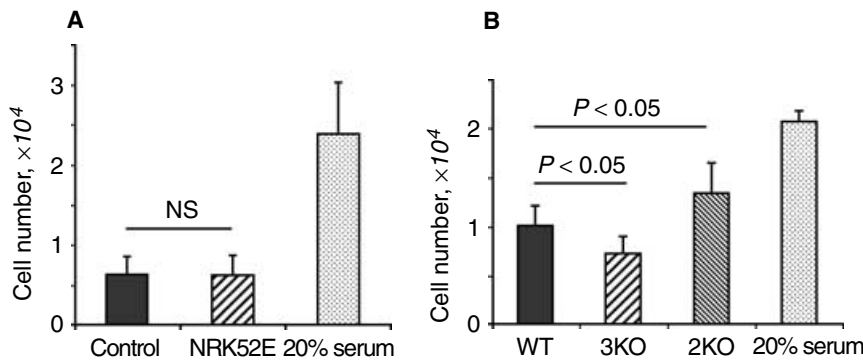


Fig. 6. Endothelial cell proliferation assay. (A) Human umbilical venous endothelial cells (HUVEC) was stimulated by 20% serum media or conditioned media (24 hours) from transforming growth factor- β 1 (TGF- β 1)-stimulated NRK52E cells. (B) HUVEC were stimulated by 20% serum media or conditioned media (24 hours) from TGF- β 1-stimulated wild-type cell (WT), Smad3 knockout cell (3KO), and Smad2 knockout cell (2KO). At 24 hours, cell number was measured by MTT assay ($N = 8$).

stimulated by TGF- β 1 for 24 hours, on endothelial cell proliferation at 24 hours. As shown in Figure 6A, conditioned media from NRK52E cells did not induce HUVEC proliferation. In contrast, Smad2 knockout-conditioned media stimulated HUVEC proliferation while Smad3 knockout-conditioned media failed to stimulate proliferation compared to wild-type-conditioned media (Fig. 6B).

DISCUSSION

In this study, we report that TGF- β 1 induces the synthesis of angiogenic (VEGF-A) and antiangiogenic (TSP-1 and sFLT-1) growth factors in proximal tubular cells and fibroblasts. To dissect out the mechanism responsible for this stimulation, we investigated the roles of the R-Smads (Smad2 and 3), which are known to be activated by TGF- β 1 in a variety of cell types. Both R-Smads were shown to be activated in response to TGF- β 1. To determine if the R-Smads played a critical role in TGF- β 1 mediated synthesis of the growth factors, we blocked Smad2 and Smad3 phosphorylation by overexpressing the inhibitory Smad, Smad7, using a doxycycline-inducible system. Overexpression of Smad7 blocked both the expression of VEGF-A and TSP-1 in proximal tubular cells in response to TGF- β 1. While these studies demonstrated a key role for the R-Smads in this response, they did not distinguish between Smad2 and Smad3. Using knockout strategies, we were able to show that Smad2 and Smad3 play distinct but parallel roles in the stimulation of these growth factors in response to TGF- β 1. VEGF-A expression was regulated by Smad3, whereas Smad2 regulated the expression of TSP-1 and sFlt-1. We have also recently demonstrated similar patterns of regulation of VEGF-A and TSP-1 in NRK52E cells using antisense strategies (data not shown).

Interestingly, the conditioned media from NRK52E did not show any proliferative effect on endothelial cells, which could relate to the release of both angiogenic and antiangiogenic factors that neutralize the effect of each other. To dissect this mechanism further, we examined the effect of conditioned media

from Smad3 and Smad2 knockout fibroblasts following TGF- β 1 stimulation. Smad2 knockout-conditioned media stimulated endothelial cell proliferation compared to wild-type-conditioned media whereas Smad3 knockout-conditioned media resulted in less proliferation than wild-type-conditioned media. These data are thus consistent with the observations that Smad3 knockout media has minimal VEGF-A but more TSP-1 and sFlt1, whereas Smad2 knockout media has less TSP-1 and sFLT-1 and more VEGF-A.

These findings provide the first demonstration, to our knowledge, that the R-Smads have distinct and opposite effects on angiogenesis, with Smad3 stimulating the production of angiogenic factors, whereas Smad2 stimulated the production of antiangiogenic factors. This could have important implications in the often conflicting data related to the role of TGF- β 1 on angiogenesis and antiangiogenesis [32–37].

One of the major signaling pathways through which TGF- β 1 acts is by activating Smads. In this regard, both Smad2 and Smad3 are known to be activated by TGF- β 1, and it has been previously thought that they work in union to stimulate extracellular matrix production, apoptosis, and the development of fibrosis [23, 25, 38, 39]. On the other hand, Smad7, inhibitory Smad, can inhibit the activation of Smad2 and 3. In this study, overexpression of Smad7 blocked the expression of both VEGF-A and TSP-1 in response to TGF- β 1, consistent for a role for Smad 2 and/or Smad 3 in their regulation. However, the inhibition of VEGF-A and TSP-1 by overexpression of Smad 7 was incomplete, suggesting that other pathways, such as mitogen-activated protein kinases (MAPKs), may be involved. Indeed, our preliminary data have shown that both the extracellular signal-related kinase (ERK) as well as p38 MAPKs also partially regulate the expression of these two growth factors in response to TGF- β 1 (data not shown).

In this study, we report that Smad3 is proangiogenic. Specifically, we found that Smad3 is a critical intracellular signaling molecule mediating TGF- β 1-stimulated VEGF-A expression. Smad3 has been previously reported to regulate VEGF-A expression in COS cells [40].

However, it is also important to clarify VEGF-A regulation in resident renal cells since VEGF-A expression in the kidney has a critical role in maintaining capillary structure and viability, and loss of VEGF-A results in glomerular injury [41] and progressive renal damage [1, 2, 42]. Interestingly, TSP-1 as well as sFlt-1 expression were enhanced in Smad3 knockout cell in response to TGF- β 1, suggesting that Smad3 could also inhibit the expression of these antiangiogenic factors, in addition to enhance VEGF-A expression.

In contrast, we found that Smad2 was critical in the production of antiangiogenic factors in response to TGF- β 1. Both TSP-1 and sFLT-1 were induced through Smad2-dependent pathways. This could provide an additional mechanism by which TGF- β 1 could accelerate progressive renal disease, as production of these growth factors have been shown to be highly linked with renal injury [14] and progression [10, 11, 29].

Conditioned media from NRK52E cells did not induce endothelial proliferation. It could be because both VEGF-A, an angiogenic factor, and TSP-1, an antiangiogenic factor, were contained in the conditioned media. Consistent with this possibility was the observation that conditioned media from Smad2 knockout cells but not Smad3 knockout cells stimulated endothelial proliferation, as Smad2 knockout cells produce more VEGF-A and less TSP-1 than the Smad3 knockout cells. In addition to the divergent effect of TGF- β 1 on angiogenic and antiangiogenic growth factors produced by the tubular cell and the fibroblast, TGF- β 1 also regulates angiogenesis by direct effects on endothelial cell proliferation and also by modulating expression of the VEGF receptor on the endothelial cell. Indeed, it has been shown that TGF- β 1 down-regulates kinase-inserted domain receptor (KDR) in endothelial cell [43] while hypoxia induces KDR [44] or Flt-1 [45]. These data suggest that TGF- β 1 is a complex molecule with multiple mechanisms by which it can regulate angiogenesis.

Only recently has it been appreciated that the R-Smads may have distinct functions. Smad2 knockout mice are embryonically lethal [46], whereas Smad3 knockout mice remain viable [47]. Smad2 has also been shown to mediate production of matrix metalloproteinase 2, whereas the induction of c-fos, Smad7, and TGF- β 1 autoinduction are dependent on Smad3 [24].

With respect to other Smads, it has been recently reported that Smad4 may also have an antiangiogenic role [48]. Overexpression of Smad4 in pancreatic adenocarcinoma cells led to decreased expression of VEGF and increasing expression of TSP-1 [48]. Interestingly, although TGF- β could increase expression of both factors, this Smad4-mediated difference in expression levels was maintained in cell overexpressing Smad4 [48]. These data suggest Smad4 may also have antiangiogenic actions. Future studies will be needed to investigate the interrelation of Smad4 with the R-Smads in the angiogenic response.

CONCLUSION

Our results demonstrate that R-Smads play distinct and opposing roles in regulating angiogenic factors. Further studies are necessary to determine if selective activation of these Smads may account for the different angiogenic responses observed in conditions in which TGF- β 1 is expressed.

ACKNOWLEDGMENTS

Support for these studies was provided by National Kidney Fellowship grant (to Takahiko Nakagawa) by NIH DK-52121 (R.J.), and by a George O'Brien Center grant (P50D4-064233-01) (H.Y.L. and R.J.).

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