Novel Regulation of Vascular Endothelial Growth Factor-A (VEGF-A) by Transforming Growth Factor β₁

**Requirement for Smads, β-catenin, and GSK3β**

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Vascular endothelial growth factor (VEGF) is a vital angiogenic effector, regulating key angiogenic processes. Vascular development relies on numerous signaling pathways, of which those induced by transforming growth factor-β (TGFβ) are critical. The Wnt/β-catenin signaling pathway is emerging as necessary for vascular development. Although VEGF, TGFβ, and Wnt signal transductions are well studied individually, it has not been demonstrated previously that all three can interact or be dependent on each other. We show that regulation of VEGF by TGFβ₁, in human pulmonary artery smooth muscle cells (PASMCs), depends on a direct interaction between TGFβ signaling proteins, Smads, and members of the Wnt/β-catenin signaling family. VEGF promoter reporter constructs identified a region of the VEGF promoter containing two T cell factor (TCF)-binding sites as necessary for TGFβ₁-induced VEGF transcription. Mutation of TCF sites and expression of dominant negative TCF4 abolished TGFβ₁-induced VEGF promoter activity. Studies in Smad2 and Smad3 knock-out mouse embryonic fibroblasts demonstrated that one or both are required for VEGF regulation by TGFβ₁, with transfection of dominant negative Smad2 or Smad3 into PASMCs confirming this. Chromatin immunoprecipitation assays showed in cell interactions of Smad2 and Smad3 with TCF4 and β-catenin at the VEGF promoter, whereas co-immunoprecipitation showed a direct physical interaction between Smad2 and β-catenin in the nucleus of PASMCs. Finally, we demonstrate that TGFβ₁ regulates TCF by modifying β-catenin phosphorylation via regulation of glycogen synthase kinase 3β. These results provide new insight into the molecular regulation of VEGF by two interacting pathways necessary for vascular development, maintenance, and disease.

The mammalian vascular endothelial growth factor (VEGF) family consists of five members as follows: VEGF-A, -B, -C, and -D and placental growth factor (1). VEGF-A was the first of the VEGF family to be identified and has been studied in the greatest detail (2). VEGF-A exists as at least six different splice variants of differing amino acid numbers, VEGF-A₁₂₅, VEGF-A₁₄₅, VEGF-A₁₆₅, VEGF-A₁₈₅, VEGF-A₁₈₉, and VEGF-A₂₀₆ (2). VEGF-A has a central role in angiogenesis and plays an essential role in embryonic vasculogenesis. Loss of a single VEGF allele causes embryonic lethality by embryonic day 11 because of defective vasculogenesis/angiogenesis (3). Physiological angiogenesis in the adult is restricted to wound healing and the female reproductive cycle (3); however, its pathological role in diseases, including cancer, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, and rheumatoid and osteoarthritis (4–8), is well documented and has attracted an intense research focus. Regulation of VEGF is known to occur at transcription, post-transcription, translation, and differential cellular localization of various isoforms (9). Post-transcriptional regulation involves stabilization of VEGF mRNA by binding of the RNA-binding protein HuR to AU-rich elements in the 3′-untranslated region of VEGF mRNA (9), whereas translational regulation depends on the presence of internal ribosome entry sites in the 5′-untranslated regions (10). Transcriptional regulation of VEGF involves a plethora of external factors. Its promoter contains binding sites for numerous transcription factors, including Sp1, AP-2, Egr-1, p53, TCF, and HIF-1α (11).

The involvement of the TGFβ superfamily in vascular development, homeostasis, and disease is increasingly reported. Its importance is illustrated by mouse models in which loss of TGFβ receptors stalling angiogenesis at an early stage resulting in fatality (12). The TGFβ superfamily consists of three TGFβ isoforms (TGFβ₁, TGFβ₂, and TGFβ₃) in addition to the activins, inhibins, and bone morphogenetic proteins. Although all TGFβ isoforms are involved in angiogenesis, mice lacking TGFβ₂ and TGFβ₃ have a relatively normal vasculature compared with mice lacking TGFβ₁ (12). Furthermore, TGFβ₁ is known to regulate cell proliferation and migration, associated with new vessel formation, extracellular matrix synthesis, required for vessel differentiation, and the recruitment of smooth muscle cells or pericytes for vessel stabilization (12).
TGFβ Regulation of VEGF in PASMCs

TGFβ1 is sequestered in an inactive complex in an unstimulated environment. Following activation, TGFβ1 binds a type I serine/threonine kinase receptor, which results in recruitment of a type II receptor and subsequent transphosphorylation of the type I receptor, by the constitutively active type II receptor. Type I receptor phosphorylation allows association and phosphorylation of R-Smad transcription factor proteins, Smad2 or Smad3 (Smad derived from Smad homologues in Caenorhabditis elegans and Drosophila melanogaster). Following phosphorylation, Smad2 or Smad3 can associate with Smad4, which promotes cytoplasm to nucleus shuttling of the Smad complex. Once in the nucleus, the Smad complex associates directly with DNA or binds DNA in association with other transcription factors, such as Sp1 and AP-1, and co-regulators, such as the histone acetyltransferases p300 and CREB-binding protein, to regulate gene transcription (13).

Here we investigated the molecular mechanisms involved in the regulation of VEGF-A by TGFβ1, in pulmonary artery smooth muscle cells, a key cell type in the maintenance and structure of the pulmonary vasculature. TGFβ1 has been shown previously to regulate VEGF in some cell types (14, 15) via Smad protein signaling (15), but the mechanisms downstream of Smad activation have not been clearly defined. Smads are known to bind DNA with low specificity and generally require a complex to be formed with other transcription factors. The Wnt/β-catenin signaling pathway is emerging as a crucial component of vascular development and pathology. For example, the Wnt receptor, fzd5 causes defects in yolk sac angiogenesis and embryonic death at embryonic day 10.75 (16), whereas the loss of Wnt7b results in severe defects in the smooth muscle component of the major pulmonary vessels (17). Furthermore, β-catenin is known to play dual roles, regulating cell proliferation when in the nucleus and cell adhesion when at the cell surface, both key angiogenic processes (16).

Here we show the novel and critical requirement for interaction of the canonical TGFβ signaling pathway with members of the Wnt/β-catenin signaling pathway in the regulation of VEGF. Transfection studies using a VEGF promoter deletion series and mutated VEGF promoter reporters showed a requirement for the transcription factor canonically regulated by Wnt/β-catenin signaling, T cell factor (TCF). A TCF requirement for TGFβ1-induced VEGF promoter activity was confirmed using a dominant negative TCF4 construct. Evidence from studies with dominant negative Smad constructs and a Smad3 inhibitor in PASMC and Smad2 and Smad3 knock-out mouse embryonic fibroblasts (MEFs) showed an involvement of conventional Smad signaling in VEGF regulation. In addition studies with GSK3β inhibitors, overexpression constructs, and GSK3β knock-out MEFs showed a role for GSK3β in the process. Furthermore, chromatin immunoprecipitation (ChIP) for TCF4, β-catenin, GSK3β and Smad2, Smad3, and Smad4 suggested a transcriptional complex containing all six proteins was involved in VEGF regulation.

TGFβ1 has been shown to regulate β-catenin whole cell levels (18) and translocation (19). In contrast to these findings we found that TGFβ1-mediated induction of VEGF transcription requires TCF activity with no increase in whole cell levels of β-catenin or a change in β-catenin nuclear levels, but rather a TGFβ1-mediated change in β-catenin phosphorylation status. Using a combination of pharmacological inhibitors, overexpression constructs, and knock-out MEFs, we are able to show TGFβ1 acts via glycogen synthase kinase to regulate β-catenin phosphorylation. Collectively, these studies show how integration of two vital vascular signaling pathways converge at the VEGF promoter in a Smad-TCF4-β-catenin-GSK3β complex.

EXPERIMENTAL PROCEDURES

Cell Culture—Proximal human PASMCs were purchased at passage 3 from Clonetics® (Lonza Group Ltd., Basel, Switzerland) and cultured to passage 6 in Smooth Muscle Cell Growth Medium (TCS Cellworks, Buckingham, UK). Experiments were set up in 24- or 6-well plates at 37 °C in a 5% CO2 humidified incubator. Wild type, homozygous Smad2 knock-out, and homozygous Smad3 knock-out MEF cells were a kind gift from Erwin Böttinger (20). MEFs were grown to confluence in DMEM, containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, and 2.5 μg/ml amphotericin B (Sigma). Once confluent, MEFs were seeded to 24 well plates at a density of 5 × 10⁴ cells/well and cultured to the density required for the specific experimental procedure. Cells at the required confluence were growth-arrested by serum withdrawal in serum-free DMEM, containing 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, and 2.5 μg/ml amphotericin B. For transfection experiments only, penicillin, streptomycin, and amphotericin B were excluded from serum-free media as per the transfection reagent manufacturer’s protocol.

Medium was replaced with serum-free with or without TGFβ1 or Wnt3a (R & D Systems, Abingdon, Oxon, UK). Wild type and homozygous GSK3β knock-out MEFs were a kind gift from Jim Woodgett (21). MEFs were grown to confluence in DMEM (Invitrogen) containing 10% fetal calf serum, 0.11 g/liter sodium pyruvate, 3.7 g/liter sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, and 2.5 μg/ml amphotericin B (Sigma). Once confluent MEFs were growth-arrested by serum withdrawal in serum-free DMEM.

VEGF Assay—The concentration of VEGF in culture medium supernatants was determined by enzyme-linked immunosorbent assay (ELISA). The human VEGF-A and mouse VEGF ELISA kits were purchased from R & D Systems. The assay was performed according to the manufacturer’s protocol and has been described in detail previously (22). MEF VEGF concentrations were normalized to cell number, as counted at 24 h.

RNA Isolation and Reverse Transcription (RT)-PCR—Cells in 6-well plates were treated with and without TGFβ1 and collected at the specified time. Total RNA was isolated using the RNeasy plus mini kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol. 1 μg of total RNA was reverse-transcribed in a total volume of 25 μl, including 132 units of Moleney murine leukemia virus reverse transcriptase, 26.4 units of RNase inhibitor, 0.6 μg of (dT)₁₅ primer, 2 μM dNTPs, and 1X Moleney murine leukemia virus RT buffer provided by Promega (Madison, WI). The reaction was incubated at 42 °C for 90 min. Aliquots of the RT products were subsequently used for
PCR amplification. 2 μl of RT products was brought to a volume of 50 μl containing 1.5 mM MgCl₂, 0.25 mM of each dNTPs, 0.5 μM of both the upstream and downstream PCR primers, and 1× Green GoTaq® Reaction Buffer and 1.25 units of GoTaq® DNA polymerase, provided by Promega. Amplification was carried out with a PTC-100 programmable thermal controller (Bio-Rad) after an initial denaturation at 94 °C for 3 min. This was followed by 30 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The following primers were used: VEGF sense 5’-ATCTGCAATGTTGATGTGTTGGA-3’ and VEGF antisense 5’-GGGCGAATACCTACGGAATG-3’; GAPDH sense 5’-CCGAGTCGAGGATTGGTCTGATTGG-3’ and GAPDH antisense 5’-GCTCTTGGAAGATGGTGATGCGATTTCCC-3’.

The PCR products were visualized by electrophoresis on a 2% agarose gel in 0.5× TBE buffer after staining with 0.5 μg/ml ethidium bromide. The UV-illuminated gels were photographed.

Quantitative Real Time RT-PCR—Human VEGF-A expression was determined using the primer sequences listed above. β₂-Microglobulin was used as the housekeeping gene using the following primer sequences: sense 5’-ATATCAAGATATGCGCC-ATCT-3’ and antisense 5’-AGATATGCTGCGCCTGTG-3’. Murine VEGF-A expression was determined using the following primer sequences: sense 5’-CTGTGCAAGGCTGCTTGATACC-3’ and antisense 5’-GTTCCCAGAACCCTCTGAGGAG-3’. Murine β-actin was used as a housekeeping gene using the following primer sequence: sense 5’-AAATCTGTGCCTGACATCAA-3’ and antisense 5’-AAGGAAAGCTGTGA-AAAAGACC-3’. 1.6 μl of reverse transcribed cDNA was subjected to real time PCR using Excite real time mastermix with SYBR green (Biogene, Cambridge, UK) and the Mx3000P® quantitative PCR system (Stratagene). Each reaction consisted of 1× Excite mastermix, SYBR green (1:60,000 final concentration), 40 nm of both sense and antisense primers, 1.6 μl of DNA (or distilled H₂O), and H₂O to a final volume of 20 μl. Thermal cycler conditions included incubation at 95 °C for 10 min followed by 50 cycles of 95 °C for 30 s and 59 °C for 1 min. Integration of the fluorescent SYBR green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melting curve analysis, where a single melting peak eliminated the possibility of primer-dimer association. For melting curve analysis to be performed, the products were heated from 55 to 95 °C after the 50 cycles.

Transfections with VEGF Promoter-driven Luciferase Constructs and Transcription Factor Reporter Constructs—PASMCs were cultured in 24-well plates to 90–95% confluence, growth-arrested for ~16 h in serum and antibiotic-free DMEM. Cells were transfected at a ratio of 1:2 DNA/Lipofectamine 2000 (LF2000, Invitrogen) according to the manufacturer’s instructions. Cells were always co-transfected with the internal control plasmid pRL-SV40 (Promega, Southampton, UK) containing the Renilla luciferase gene. Media containing LF2000 were left on the cells for 2 h and then removed and replaced with either 500 μl of serum and antibiotic-free media or media containing a stimulus. Cells were incubated with stimulus for the required time, washed in phosphate-buffered saline, harvested using 1× passive lysis buffer (Promega, Southampton, UK), and stored at −20 °C for short term storage or −80 °C for longer term storage. Firefly and Renilla luciferase activities were measured using the dual luciferase assay system kit (Promega, Southampton, UK) and Microplate Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The VEGF promoter-driven luciferase constructs were a kind gift from Professor Dieter Marme, Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany (23). The Sp-1 reporter construct containing 6 Sp-1-binding sites was a kind gift from Professor Jeffrey E. Kudlow, School of Medicine, University of Alabama at Birmingham (24). The TCF reporter, TOPglow, and negative control, FOPglow, were purchased from Millipore. The AP2-reporter construct was a kind gift from Professor Helen Hurst, CRUK, London, UK (25). The p53 cis-reporting system was purchased from Stratagene.

Transfections with Dominant Negative and Overexpression Constructs—Human PASMC co-transfections were performed using FuGENE HD (Roche Diagnostics) according to the manufacturer’s instructions and using a 1:2 DNA/FuGENE HD ratio. PASMCs were cultured to ~90% confluence, serum-starved for 7–8 h in serum and antibiotic DMEM, and transfected for 15–16 h. Cells were then stimulated for the stated time and harvested as above. Dominant negative Smad2 and Smad3 constructs were a kind gift from Anne Sturrock (Division of Respiratory, Critical Care, and Occupational Pulmonary Medicine, University of Utah Medical Center) (26). Dominant negative TCF4 was a kind gift from Thilo Hagen (Wolfson Digestive Diseases Centre, University of Nottingham, UK) (27). GSK3β wild type expression construct and associated empty vector was a kind gift from Professor Xiao-Fan Wang (Pharmacology and Cancer Biology, Duke University Medical Center).

Mouse Embryonic Fibroblast Transfections—MEFs were cultured in 24-well plates to 50–80% confluence, growth-arrested for ~8 h in serum and antibiotic free DMEM. Cells were transfected at a ratio of 1:3 DNA/FuGENE 6 (Roche Diagnostics), according to the manufacturer’s instructions, for 15–16 h. Cells were stimulated for 24 h and harvested as above. Smad2 and Smad3 expression constructs were a kind gift from Elizabeth J. Robertson (Department of Molecular and Cellular Biology, Harvard University) (28).

Site-directed Mutagenesis—All site-directed mutagenesis was conducted with the Stratagene QuikChange® II site-directed mutagenesis kit according to the kit protocol. TCF-binding sites were mutated individually at residue 110 bp from the transcription start site and 142 bp from the transcription start site. The 110 sense and antisense primer sequences were as follows: CGC GTGTGG AAG GGC C GAG GCT CGC CTG TCC, GGACAG GCGAGC CTC G GCC CTT CCA CAC GCG. The 142 sense and antisense primers were as follows: GCTCCTGGAAGAATGGTGATGCGATTTCCC-3’. The 110 sense and antisense primer sequences were as follows: CGC GTGTGG AAG GGC C GAG GCT CGC CTG TCC, GGACAG GCGAGC CTC G GCC CTT CCA CAC GCG.
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Cells were cultured to confluence in 6-well plates, serum-starved, and treated using the drug or reagent at the concentrations and time courses stated. At the end of treatment, cells were washed with phosphate-buffered saline and treated with an extraction buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate (Na3VO4), 0.1 mM dithiothreitol, 0.4 μg/ml leupeptin, and pepstatin) with cell scraping. The cell extract was stored at −20 °C until required.

Protein samples were subject to electrophoresis in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted to PVDF membranes, and the blot was blocked for 1 h at room temperature with blocking buffer (0.1% TBST with 5% fat-free dried milk powder). The blot was then incubated with primary antibody (1:1000 dilution), at 4 °C overnight. The blot was washed with 0.1% TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Cambridge, UK) (1:2000 dilution with 5% fat-free dried milk powder). The blot was then incubated with ECL Western blotting detection reagent (GE Healthcare). If stripping was required the following procedure was carried out: the membrane was incubated in stripping buffer (100 mM Tris-HCl, pH 6.8) at 50 °C for 30 min with occasional agitation, followed by washing the membrane in a large volume of 0.1% TBST, and blocking of the membrane for 1 h in blocking reagent at room temperature.

The antibodies used were as follows: β-catenin (H-102, sc-7199, Santa Cruz Biotechnology); GAPDH (AbD Serotec, Oxford, UK); total Smad2/3 (catalog number 3102, Cell Signaling Technology); phospho(serine 465/467); Smad2 (catalog number 3102, Cell Signaling Technology); phospho-Ser-9-GSK3β (catalog number 9315, Cell Signaling Technology); normal rabbit IgG (AB-105-C, R & D Systems); dephosphorylated β-catenin (ab19451, Abcam, Cambridge, UK); phospho-β-catenin (Ser-33/37/Thr-41) (catalog number 9561, Cell Signaling Technology); Lamin A/C (sc-7292, Santa Cruz Biotechnology); GAPDH (AbD Serotec, Oxford, UK); total Smad2/3 (catalog number 3102, Cell Signaling Technology); dephosphorylated β-catenin (ab19451, Abcam, Cambridge, UK); phospho-β-catenin (Ser-33/37/Thr-41) (catalog number 9561, Cell Signaling Technology); Lamin A/C (sc-7292, Santa Cruz Biotechnology); total GSK3β (catalog number 9315, Cell Signaling Technology); and phospho-Ser-9-GSK3β (catalog number 9336, Cell Signaling Technology).

Cytoplasm/Nuclear Extraction and Separation—Cells were grown to confluence in 100-mm dishes, serum-starved, and incubated for the specified times with or without 1 ng/ml TGFβ1 or 10 μM SB216763 (Toc’s Bioscience, Bristol, UK). Cytoplasmic and nuclear fractions were separated using Cellytic™ NuCLEAR™ extraction kit (Sigma).

Chromatin Immunoprecipitation Assay—PASMCs were cultured to confluence in 75-cm2 flasks (2 per condition), growth-arrested, and incubated with 1 ng/ml TGFβ1 for 0 and 2.5 h. The ChIP assay was performed using the ChIP-IT Express kit (Active Motif, Rixensart, Belgium) following the manufacturer’s protocol. Briefly, cells were fixed using 37% formaldehyde in minimal cell culture medium, removed from the flask by scraping, lysed, and sheared by sonication. Chromatin was split into 50-μl aliquots. 10 μl of chromatin from each condition was removed for use as “input control DNA.” The remainder of the chromatin aliquot was incubated overnight at 4 °C in a 100-μl solution containing protein G magnetic beads and 4 μg of target antibody or associated IgG control. The magnetic beads were washed prior to elution of immunoprecipitated DNA. All samples, including inputs, were then incubated at 65 °C for 2.5 h and then for 1 h at 37 °C in the presence of proteinase K. Immunoprecipitated DNA was ready for use in PCR following the addition of a solution to stop proteinase K activity. Input DNA underwent a further phenol/chloroform extraction before being used in PCR.

The VEGF production was measured using ELISA. A, time course of VEGF production in PASMCs treated with 1 ng/ml TGFβ1 for 0, 2, 4, 8, 16, and 24 h compared with unstimulated controls. (*, p < 0.05; **, p < 0.01; and ***, p < 0.001 by ANOVA). The VEGF primers yielded a 161-bp pair product corresponding to −262 to −101 of the VEGF gene promoter and were as follows: sense 5’-GGCTGTTCATCTGGACAGACTTT-3’, and antisense 5’-AGCCTCAGCCCTTCCACA-3’. The “upstream VEGF” primers yielded a 232-bp product corresponding to −1589 to −1357 of the VEGF gene promoter and...
**TGFβ Regulation of VEGF in PASMCs**

**RESULTS**

**TGFβ Increases VEGF-A165 Protein Production**—There was a concentration-dependent increase in VEGF release above control in cells cultured with 1 or 10 ng/ml TGFβ, which was significant at 24 h (Fig. 1A). 1 ng/ml TGFβ caused a time-dependent increase in VEGF release, significant at all time points after 8 h (Fig. 1B).

**TGFβ Increases VEGF Transcription**—Reverse transcriptase and real time PCR showed that 1 ng/ml TGFβ, replaced with 250 μl of serum-free media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide (Sigma), and then incubated for 20 min at 37 °C. This medium was removed, and the plates were dried overnight. 250 μl of dimethyl sulfoxide (DMSO) was then added to dissolve the blue-colored tetrazolium. The absorbance was read at 550 nm in a TECAN GENios (Tecan UK Ltd., Theale, Reading, UK) microplate reader. Viability was set as 100% in control cells.

**Inhibitor Studies**—Cells were serum-starved for 24 h. Cells were preincubated for 30 min with the stated concentrations of Smad3 inhibitor, SIS3 (Merck) or GSK3β inhibitor SB216763 (Tocris Cookson Ltd., Avonmouth UK), followed by a 24-h TGFβ stimulation. Supernatants were collected for analysis by ELISA.

**Cell Viability**—The toxicity of all the chemicals and vehicles used in this study was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide assay. At the end of the experiment culture media were removed and replaced with 250 μl of serum-free media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide (Sigma), and then incubated for 20 min at 37 °C. This medium was removed, and the plates were dried overnight. 250 μl of dimethyl sulfoxide (DMSO) was then added to dissolve the blue-colored tetrazolium. The absorbance was read at 550 nm in a TECAN GENios (Tecan UK Ltd., Theale, Reading, UK) microplate reader. Viability was set as 100% in control cells.

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**TGFβ Increases VEGF-A165 Protein Production**—There was a concentration-dependent increase in VEGF release above control in cells cultured with 1 or 10 ng/ml TGFβ, which was significant at 24 h (Fig. 1A). 1 ng/ml TGFβ, caused a time-dependent increase in VEGF release, significant at all time points after 8 h (Fig. 1B).

**TGFβ Increases VEGF Transcription**—Reverse transcriptase and real time PCR showed that 1 ng/ml TGFβ, caused a 1.6-fold increase in VEGF-A mRNA levels over control, at 1 h (Fig. 2A, panels i and ii). To confirm increases in mRNA levels were because of VEGF-A gene transcription, cells were cul-
tured for 30 min with 5 μg/ml actinomycin D, an inhibitor of RNA polymerase II, followed by 1 ng/ml TGFβ1. Pretreatment with actinomycin D prevented the TGFβ1-induced increase in both VEGF-A mRNA, by real time and RT-PCR (Fig. 2B, panels i and ii), and VEGF-A protein (Fig. 2C). The cells were transfected with a 2068-bp VEGF promoter fragment (~2018 to +50) ligated to firefly luciferase. There was an average 1.65-fold increase in luciferase expression in PASMCs treated with 10 ng/ml TGFβ1 for 3.5 h compared with unstimulated cells (Fig. 2B, panel ii). Pretreatment with actinomycin D resulted in a 2-fold increase in luciferase activity (Fig. 2B, panel i). Similarly, the AP2 reporter showed no response to 10 ng/ml TGFβ1 (Fig. 4A, panel i). A p53 reporter showed no response to TGFβ1; however, co-transfection with the positive control plasmid pFC-P53 resulted in a 1.7-fold increase in luciferase activity (Fig. 4A, panel iii). Finally, the TCF reporter, TOPglow, was transfected into cells and showed a 2-fold increase in firefly luciferase activity in response to 3.5 h of stimulation with 10 ng/ml TGFβ1, whereas the negative control construct, containing mutated TCF-binding sites, FOPglow, did not respond to TGFβ1 (Fig. 4A, panel iv). Although this implicated a role for TCF and showed only the TCF reporter responded directly to TGFβ1, it is possible that TCF may act in association with other proteins at non-TCF-binding sites within the VEGF-318 reporter, in response to TGFβ1. To investigate this further, each of the four transcription factor luciferase reporters were assayed for response to direct TCF activation by Wnt3a. As shown in Fig. 4B, only the TCF reporter, TOPglow, responded to direct TCF stimulation suggesting the transcriptional complex formed does not interact with the Sp-1, AP2, or p53 sites within the region of the VEGF promoter.

To provide evidence for a direct requirement for the VEGF promoter-specific TCF-binding sites, site-directed mutants were created of the two TCF-binding sites in the VEGF promoter, using the wild type 318-bp VEGF promoter reporter as a template. In addition a double mutant was created in which both TCF sites were mutated. Transfection of the mutant plasmids followed by 3.5 h of TGFβ1 stimulation showed loss of either individual binding site was sufficient to prevent TGFβ1-mediated VEGF promoter activity (Fig. 4C). Transfection of a double TCF mutant also showed no response to TGFβ1; however, an increase in basal reporter activity was seen, suggesting a possible role for the presence of both sites in basal regulation of VEGF.

To further confirm TCF involvement, a dominant negative TCF4 construct was co-transfected into PASMCs with the 318-bp VEGF promoter reporter. In the presence of empty vector, pcDNA3.1, TGFβ1 was able to induce VEGF-318-Luc activity. However, in the presence of the dominant negative TCF4 construct, induction of VEGF-318-Luc was prevented (Fig. 4D).
FIGURE 4. A, levels of luciferase expression in PASMC following transient transfection with the following: panel i, the Sp-1 luciferase reporter and control plasmid (0.5 µg of DNA and 1 µl of LF2000 per well for 2 h); panel ii, AP2 luciferase reporter (0.5 µg of DNA and 1 µl of LF2000 per well for 2 h) and p53 expression construct, pFC-p53 (0.5 µg of pFC-p53 was co-transfected with 0.5 µg of p53 reporter construct and 2 µl of LF2000 per well for 3 h); and panel iv, TCF luciferase reporter, TOPglow, and control plasmid, FOPglow (0.5 µg of DNA and 1 µl of LF2000 per well for 2 h). An increase in luciferase expression in response to 3.5 h of TGFβ stimulation was only seen with the TOPglow TCF reporter.

B, levels of luciferase expression in PASMCs following a 2-h transient transfection with 0.5 µg/well TCF, Sp-2, AP-2, and p53 luciferase reporters with 1 µl/well LF2000. An increase in luciferase expression in response to 3.5 h of Wnt3a (75 ng/ml) stimulation was only seen with the TOPglow TCF reporter.

C, levels of luciferase expression in PASMCs following transient transfection with wild type 318-bp fragment of the VEGF promoter (−267/+50) ligated to a luciferase reporter construct, or site-directed mutants of the TCF-binding site at the −110-bp position, the −142-bp position, or both sites of the VEGF 318-luciferase construct. Cells were grown to 90% confluence, growth-arrested, and transfected for 2 h with 0.5 µg of DNA and 2 µl of LF2000 per well. Cells were then stimulated for 3.5 h with 10 ng/ml TGFβ1. Increases in luciferase expression from the wild type (wt) construct were abolished by mutation of the TCF sites. D, levels of luciferase expression in PASMC transiently transfected with VEGF-318 luciferase when co-transfected with either control plasmid, pcDNA3.1, or dominant negative TCF4 plasmid. Cells were grown to 90% confluence, growth-arrested, and transfected with 0.4 µg of DNA and 2 µl of LF2000 per well for 16–20 h. There was an increase in promoter activity in cells transfected with control plasmid, pcDNA3.1, or dominant negative TCF4 plasmid. Cells were grown to 90% confluence, growth-arrested, and transfected with 0.4 µg of DNA and 2 µl of LF2000 per well for 16–20 h. There was an increase in promoter activity in cells transfected with control plasmid, pcDNA3.1, or dominant negative TCF4 plasmid. Cells were grown to 90% confluence, growth-arrested, and transfected with 0.4 µg of DNA and 2 µl of LF2000 per well for 16–20 h. There was an increase in promoter activity in cells transfected with control plasmid, pcDNA3.1, or dominant negative TCF4 plasmid. Cells were grown to 90% confluence, growth-arrested, and transfected with 0.4 µg of DNA and 2 µl of LF2000 per well for 16–20 h. There was an increase in promoter activity in cells transfected with control plasmid, pcDNA3.1, or dominant negative TCF4 plasmid.
Finally, ChIP was used to determine whether TGFβ1 treatment increased TCF4 binding to the native VEGF promoter. Protein-DNA complexes were immunoprecipitated with a TCF4 antibody followed by DNA isolation and purification and PCR. Nonimmunoprecipitated chromatin was used as an “input” control, and an IgG antibody control was performed on all occasions. Incubation with TGFβ1 for 2.5 h resulted in increased binding of TCF4 to the VEGF promoter over IgG antibody control and 0-h control (Fig. 4E). PCR primers (VEGF −262 to −161) were designed to cover only the 182-bp fragment established as important by deletion series experiments and known to contain the two TCF-binding sites implicated by

**Figure 4E**

A: 
- TGFβ IgG - TGFβ IgG

**VEGF -262 to -161**

- VEGF upstream

β Catenin IP Inputs

B: 

| Time (Mins) | Control | 15 | 30 | 60 | 120 | 240 | 480 |
|------------|---------|----|----|----|-----|-----|-----|
| 0          | -       | -  | +  | -  | +   | -   | -   |
| 15         | -       | +  | -  | -  | -   | +   | -   |
| 30         | +       | -  | +  | -  | -   | +   | -   |
| 60         | +       | +  | -  | +  | -   | +   | -   |
| 120        | +       | +  | +  | +  | -   | +   | -   |
| 240        | +       | +  | +  | +  | +   | +   | -   |
| 480        | +       | +  | +  | +  | +   | +   | +   |

C: 

| Condition | β-catenin | GAPDH |
|-----------|-----------|-------|
| cytoplasm | -         | +     |
| nucleus   | +         | -     |

D: 

| Condition | β-catenin | GAPDH |
|-----------|-----------|-------|
| cytoplasm | -         | +     |
| nucleus   | +         | -     |

E: 

| Condition | β-catenin | GAPDH |
|-----------|-----------|-------|
| cytoplasm | -         | +     |
| nucleus   | +         | -     |

**Figure 4F**

F: 
- TGFβ IgG - TGFβ IgG

**VEGF -262 to -161**

- VEGF upstream

β Catenin IP Inputs

**Figure 4G**

G: 
- TGFβ IgG - TGFβ IgG

**VEGF -262 to -161**

- VEGF upstream

β Catenin IP Inputs
site-directed mutagenesis. A second set of primers covering an irrelevant stretch of the VEGF promoter (VEGF upstream) were used as a control for nonspecific binding of proteins to an irrelevant section of the VEGF promoter. These primers gave a band at 232-bp in the input samples, but no amplification was seen in the immunoprecipitated samples (Fig. 4E).

**TGFβ Regulates β-Catenin by Affecting Its Phosphorylation Status but Not Its Cellular Levels or Location**—Canonically, TCFs are known to be an end signal to Wnt signaling. A key component of the Wnt signaling pathway is the protein β-catenin. In an unstimulated cell, β-catenin is phosphorylated by constitutively active glycogen synthase kinase 3β (GSK3β), ubiquitinated, and degraded by the proteosome. In the presence of Wnt, GSK3β is inhibited, and β-catenin is hypophosphorylated and stabilized, allowing its translocation to the nucleus and association with TCF (29). With this in mind, ChIP was performed to assess whether β-catenin was able to associate with the VEGF promoter in response to TGFβ, at the same region shown to bind TCF4 in response to TGFβ. As shown in Fig. 5A, β-catenin association with the VEGF promoter is increased upon 2.5 h of TGFβ stimulation. As with TCF4, no amplification is seen using the upstream primers. We sought to establish how TGFβ, was regulating β-catenin to result in its increased association with the VEGF promoter. Initially Western blots were performed to determine whether GBF affected β-catenin cellular levels. A time course across 8 h using 1 ng/ml TGFβ, was performed, and whole cell lysate was extracted. 10 μM SB216763, a GSK3 inhibitor, was used as a positive control for β-catenin build up. TGFβ, had no effect on β-catenin whole cell levels, and little further induction over basal was seen with SB216763 (Fig. 5B). Consequently we were interested in establishing if β-catenin translocated between cytoplasm and nucleus in response to TGFβ, as has been shown previously in bone marrow-derived adult human mesenchymal stem cells (19). Thus, cells were stimulated for 4 h with 1 ng/ml TGFβ, or 10 μM SB216763 and nuclear, and cytoplasmic fractions were separated. Western blotting for total β-catenin showed that β-catenin was constitutively present in the nucleus, and its levels did not significantly change in response to TGFβ, or SB216763 (Fig. 5C).

It has been shown that β-catenin activity can be regulated by its phosphorylation status (30). As a result the nuclear/cytoplasmic extracts were re-blotted using an antibody specific for unphosphorylated β-catenin (amino acids 27–37) which is stable and active. These blots showed a significant difference between β-catenin phosphorylation in control cells and β-catenin phosphorylation in TGFβ,- and SB216763-stimulated cells (Fig. 5D). Unstimulated cells had a low level of dephosphorylated β-catenin that was significantly increased by both TGFβ, stimulation and inhibition of GSK3β. To confirm this observation, samples were probed for phosphorylated β-catenin. As expected, phosphorylation was high in unstimulated cells and reduced in response to TGFβ, stimulation and GSK3β inhibition (Fig. 5E). Subsequently, we wanted to establish that the unphosphorylated form of β-catenin, created upon TGFβ, stimulation, was able to bind the VEGF promoter. A ChIP assay was performed and showed a 2.5-h incubation with 1 ng/ml TGFβ,-induced binding of unphosphorylated β-catenin to the relevant region of the VEGF promoter (Fig. 5F). Furthermore, ChIP analysis showed no binding of the phosphorylated form of β-catenin to the VEGF promoter (Fig. 5G). These data suggest TGFβ,-induced binding of VEGF is dependent on dephosphorylation of β-catenin followed by its association with TCF4 at the VEGF promoter.

**GSK3β Links TGFβ to β-Catenin Phosphorylation Status**—By having established that TGFβ, was able to reduce β-catenin phosphorylation, it was necessary to establish if this was via negative regulation of GSK3β, a kinase well characterized for its ability to phosphorylate β-catenin. Initially whole cell lysates from a 1 ng/ml TGFβ, time course were probed for total GSK3β to establish if TGFβ, was regulating the expression of the kinase. GSK3β was present constitutively in control PASMCs, and no decrease in expression was seen in response to TGFβ, (Fig. 6A). We then went on to ascertain whether GSK3β was present in the nucleus of PASMCs, and whether TGFβ, was capable of changing the localization of GSK3β within the cell, as has been shown previously in response to proapoptotic stimuli in neuroblastoma cells (31) and partial hepatectomy in rats (32). Nuclear and cytoplasmic extracts were taken from control PASMCs and those receiving 1 and 2 h of TGFβ, stimulation. Equal cytoplasmic and nuclear protein concentrations were loaded onto a Western blot, and the resulting membrane was probed for total GSK3β, with lamin A/C used as nuclear marker. Interestingly a large proportion of GSK3β was found to be constitutively present in the nucleus; however, no TGFβ,-mediated regulation of GSK3β translocation was seen (Fig. 6B). Subsequently we wished to measure GSK3β phosphorylation at serine 9. GSK3β is a constitutively active kinase with phospho-
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Phosphorylation at serine 9 known to reduce its kinase activity. As such, we hypothesized that TGFβ1 would cause an increase in serine 9 phosphorylation, indicating a reduction in kinase activity and thus reduced phosphorylation of β-catenin. Nuclear and cytoplasmic extracts were probed using an antibody specific to phosphorylated serine 9 on GSK3β. As shown in Fig. 6B, as little
as 1 h after TGFβ₁ stimulation an increase in phospho-GSK3β-Ser-9 was visible indicating that TGFβ₁ is able to reduce GSK3β kinase activity via serine 9 phosphorylation. Having provided a link between TGFβ₁ and GSK3β, it was necessary to provide evidence that modulation of GSK3β was sufficient to affect VEGF production by PAMScs. Initially this was indicated by a simple dose response to the GSK3β inhibitor compound SB216763, in the presence and absence of TGFβ₁. GSK3β inhibition caused an increase in basal levels of VEGF, which as the dose of SB216763 increased was sufficient to result in basal VEGF levels, in the presence of inhibitor, equal to TGFβ₁-induced levels in the absence of inhibitor (Fig. 6C). Subsequently, the VEGF-318-luciferase reporter was tested for its response to SB216763 to give an indication of VEGF promoter activity in response to GSK3β inhibition. SB216763 was able to induce VEGF-318-luc activity providing further evidence for the role of GSK3β in TGFβ₁ regulation of VEGF (Fig. 6D).

In addition to using the pharmacological inhibitor of GSK3β, we were able to obtain mouse embryonic fibroblasts with GSK3β homozygously knocked out as a kind gift from Dr. James Woodgett (Samuel Lunenfeld Research Institute, Toronto, Canada). A time course across 8 h in both the wild type and GSK3β⁻/⁻ MEFs was performed with 1 ng/ml TGFβ₁. In the wild type MEFS a significant TGFβ₁-induced increase in VEGF protein production was seen by 4 h and was maintained up to 8 h (Fig. 6E, panel i, dark lines). In the GSK3β⁻/⁻ MEFS, the basal level of VEGF was increased compared with the wild type, and no significant TGFβ₁-induced effect was measured (Fig. 6E, panel i, light lines). This is in agreement with the data produced using the SB compound in PAMScs. Furthermore, we were able to measure VEGF mRNA levels, by real time PCR, in the wild type and GSK3β⁻/⁻ MEFS. As with the protein data generated by ELISA, TGFβ₁ was able to significantly induce VEGF mRNA levels in the wild type MEFS (Fig. 6E, panel ii, dark lines), whereas in the GSK3β⁻/⁻ MEFS the basal levels of VEGF mRNA were increased to such an extent that no significant further induction was seen in response to TGFβ₁ (Fig. 6E, panel ii, light lines).

Having established the effect of GSK3β inhibition or loss, we wanted to ascertain if the opposing effect was seen when GSK3β activity was increased. Because of GSK3β existing as a constitutively active kinase, it is difficult to increase its activity alone. Instead it requires physically increasing the level of active GSK3β protein by overexpression. Therefore, we used an expression plasmid to force overexpression of wild type GSK3β in PAMScs, in combination with both the largest VEGF-luciferase reporter available to us and the VEGF-318-Luc reporter. As GSK3β is a negative regulator of β-catenin, and therefore of the TGFβ₁-induced VEGF expression in PAMScs, we expected its overexpression to reduce or abolish the TGFβ₁ effect. As shown in Fig. 6F, panels i and ii, overexpression of wild type GSK3β was sufficient to prevent TGFβ₁ induction of both VEGF-2068-Luc and VEGF-318-Luc activity.

Finally, we wanted to assess if GSK3β was able to incorporate into the TGFβ₁-induced transcriptional complex formed at the VEGF promoter, already shown to contain β-catenin and TCF4. ChIP was performed using an anti-GSK3β antibody and showed an increase in GSK3β association with the VEGF promoter after 2.5 h of TGFβ₁ stimulation (Fig. 6G). No association was seen when using the upstream VEGF primers suggesting GSK3β associates in the same region of the promoter as TCF4 and β-catenin.

Regulation of VEGF Production by TGFβ₁, Involves Smad2, Smad3, and Smad4—The canonical signaling of TGFβ₁ involves phosphorylation of Smad2/3 by the serine/threonine kinase TGFβ type I receptor, upon TGFβ₁ binding. To initially confirm this pathway is active in PAMScs, Western blots were performed on whole cell lysates from PAMScs stimulated with 1 ng/ml TGFβ₁ across a time course of 0–8 h. Smad2 phosphorylation was seen at all time points in response to TGFβ₁ compared with little phosphorylation in unstimulated controls (Fig. 7A). Total Smad2/3 was used as a loading control following membrane stripping.

To establish if Smad2 and Smad3 were involved in TGFβ₁ induction of VEGF in PAMScs, dominant negative forms of the proteins were co-transfected with the 318-bp VEGF promoter reporter. Co-transfection of the 318-bp VEGF promoter reporter with the empty vector control, pCMV5, resulted in a 1.75-fold increase in luciferase activity (Fig. 7B). Co-transfection with either dominant negative Smad2 or dominant negative Smad3 abolished the ability of the 318-bp VEGF promoter reporter to respond to TGFβ₁ (Fig. 7B). This suggested a role for both Smad2 and Smad3 in TGFβ₁-induced VEGF release from PAMScs; however, it is understood that these dominant negative constructs can give an unclear image as they may interfere with other Smads. A chemical inhibitor of Smad3 is available (SIS3) (33), which abrogates Smad3 phosphorylation and prevents its association with Smad4. In the presence of increasing concentrations of this compound, TGFβ₁ induction of VEGF dose-dependently decreased to basal levels (Fig. 7C).
This inhibitor has been shown to have no effect on Smad2 and indicates that although Smad2 may be involved in a TGFβ1-induced transcriptional complex required for VEGF transcription, loss of Smad3 is critical to the complex. To confirm a native transcriptional role for the Smads and to establish a role for Smad4, chromatin immunoprecipitation was performed using antibodies against Smad2, -3, and -4. RT-PCR showed an increase in binding of Smad2, -3, and -4 in response to 1 ng/ml TGFβ1 (Fig. 7D) at the VEGF promoter. No amplification was seen when using “upstream” primers.

Smad2 and -3 Null Mouse Embryonic Fibroblasts Further Confirm a Role for Smad Proteins in TGFβ1-induced VEGF—Wild type (WT), homozygous Smad2 (2/−/−) knockout, and homozygous Smad3 (3/−/−) knock-out MEFs were a kind gift from Professor Erwin Böttinger. ELISAs were used to measure the response of WT, 2/−/−, and 3/−/− MEFs across a TGFβ1 concentration range and a time course. At 48 h of stimulation with 1, 5, and 10 ng/ml TGFβ1, there was a significant increase in VEGF production compared with unstimulated controls in all cell types. However, the basal and TGFβ1-stimulated VEGF levels were greatly reduced in the Smad2/−/− and Smad3/−/− MEFs (Fig. 8A, panel i). Of the three cell lines, only the WT MEFs had a significant TGFβ1-induced increase in VEGF following 24 h of stimulation with 1 ng/ml TGFβ1 (Fig. 8A, panel ii). TGFβ1 induction of VEGF in the Smad2/−/− MEFs is noticeably greater than in the Smad3/−/− MEFs. To establish if this is because of Smad3 compensation, dose responses to the Smad3 inhibitor SIS3 in wild type, Smad2/−/−, and Smad3/−/− MEFs were performed. As shown in Fig. 8B, TGFβ1-induced VEGF was decreased by the inhibitor in both wild type (Fig. 8B, panel i) and Smad2/−/− (Fig. 8B, panel ii) MEFs. This suggests that loss of Smad3 alone is sufficient to
prevent TGFβ₁-induced VEGF in wild type MEFs and that loss of Smad2 alone only partially reduces TGFβ₁-induced VEGF because of compensation by Smad3. Only upon Smad3 inhibition in Smad2⁻⁻⁻⁻ MEF cells is the TGFβ₁-induced VEGF completely prevented. As expected no significant induction of VEGF is seen in the Smad3⁻⁻⁻⁻ MEFs, and there is no significant effect of the inhibitor (Fig. 8B, panel iii).

To provide evidence for the effects of Smad loss on VEGF transcription, the VEGF promoter reporter constructs, VEGF-318-Luc wild type, and the mutants Δ110, Δ142, and Δ110/142 were transfected into each of the three MEF cell lines. Following a 24-h 10 ng/ml TGFβ₁ stimulation, a 1.64-fold increase in luciferase activity was seen in the WT MEFs compared with unstimulated controls. Furthermore, in agreement with data in the PAMSCs, no TGFβ₁ stimulation was observed when the TCF site mutants were transfected (Fig. 8C, panel i). Transfection of the Smad2⁻⁻⁻⁻ and Smad3⁻⁻⁻⁻ cells with the VEGF-318-Luc wild type and mutant constructs in the presence of a control vector gave no response to TGFβ₁ (Fig. 8C, panel ii). However, when co-transfection was performed to "replace" the knocked out Smad protein, the luciferase response to TGFβ₁ was recovered (1.66-fold increase in Smad2⁻⁻⁻⁻; 1.93-fold increase in Smad3⁻⁻⁻⁻) when using the wild type VEGF-318-Luc construct (Fig. 8B, panel iii). However, in agreement with data generated in the wild type MEFs, Smad recovery in the knock-out MEFs had no effect on TGFβ₁ induction of the mutated constructs. Together data in the PAMSCs and MEFs show a strong role for Smads in TGFβ₁-induced VEGF production in both cell types.

**DISCUSSION**

This study is the first to demonstrate that transcriptional regulation of VEGF by TGFβ₁ can involve the transcription factor TCF, the multifunctional protein β-catenin, and the kinase GSK3β, in addition to canonical TGFβ signaling proteins Smad2, -3, and -4. We also report the novel finding that TGFβ₁ is capable of modulating the phosphorylation status of amino acids 27–37 of β-catenin.

TGFβ₁, VEGF, and β-catenin/TCF signaling are all increasingly viewed as critical for vascular development. Although TGFβ₁ regulation of VEGF has been studied previously, primarily in endothelial cells, it is previously unreported in vascular smooth muscle cells. Endothelial cells alone can only initiate angiogenesis and are not sufficient to maintain vessel stability. Smooth muscle cells are critical to provide vessel stability and prevent vessel regression (34). Furthermore, interaction and paracrine signaling between smooth muscle and endothelial cells play a key role in vessel maintenance.

Our initial studies showed that TGFβ₁ stimulation of normal human pulmonary artery smooth muscle cells increased VEGF-A protein release and VEGF-A mRNA expression. Previous studies have shown TGFβ₁ regulation of VEGF to occur transcriptionally via distinct and specific transcription factors, depending on cellular context. For example, Sp-1, HIF1α, and SAF-1 were required in human cholangiocellular carcinoma cells, mouse myelomonocytes, and human chondrocytes, respectively (14, 35, 36). Thus it appears that VEGF regulation by TGFβ₁ can show large transcriptional variation between cell types, and as such we were interested in determining the transcriptional mechanisms required in PAMSCs. Initially transcriptional regulation was suggested by prevention of TGFβ₁-induced VEGF production with actinomycin D and confirmed by studies using VEGF promoter luciferase constructs. To determine the key transcription factor–binding sites involved in our system, we used a series of deletions of the VEGF promoter ranging from 2068 to 102 bp. We found promoter activity to be maintained down to the 318-bp construct. However, all luciferase activity was lost using the 135- and 102-bp constructs, suggesting that the main regulatory sites were contained within the 135–318-bp region. This region contains two Sp-1, one AP-2, one p53, and two TCF transcription factor–binding sites, among others. To demonstrate which one of these transcription factors were required, we used luciferase reporter constructs for each of the four different transcription factors. Both the Sp-1 and p53 reporters did not respond to TGFβ₁; however, luciferase levels were increased in response to positive controls. The AP-2 reporter construct did not respond to TGFβ₁; however, despite the sampling of a number of agents published as inducing AP-2 activity (37, 38), a positive control was not found. The TCF reporter TOPglow did respond to TGFβ₁, suggesting TCF is important in TGFβ₁-induced VEGF. This observation was supported by transient transfection data using site-directed mutants of the 318-bp VEGF promoter reporter. Mutation of either individual TCF site abolished the ability of TGFβ₁ to induce the 318-bp reporter without affecting basal levels of luciferase activity. Mutation of both TCF-binding sites also resulted in an inability of TGFβ₁ to induce luciferase levels;
However, an increase in basal luciferase activity was seen suggesting that TCF may regulate basal VEGF transcription by interaction with only a single TCF-binding site, whereas TGFβ1-induced VEGF requires the presence of both functional TCF-binding sites. Transient co-transfection of a dominant negative TCF4 construct, with the 318-bp VEGF reporter, was also able to prevent TGFβ1-induced increases in luciferase. Chromatin immunoprecipitation using a TCF4-specific antibody confirmed in vivo binding of TCF4 to the VEGF promoter, within the region identified as important by reporter assays.

Ours are the first studies to show TCF4 is involved in TGFβ1-induced VEGF production, although TCF4 has been shown to transcriptionally regulate VEGF in response to other stimuli in cells other than smooth muscle cells, for example, K-ras induction of VEGF in HeLa and Caco-2 cells (39) and prostaglandin E2 induction of VEGF in LS-174T and HCA-7 cell lines (40). Additionally, we show TGFβ1 is able to regulate VEGF via two specific TCF4-binding sites (−143 to −138 and −111 to −106) within the VEGF promoter, and we are the first to show in vivo association of TCF4 with the VEGF promoter by ChIP.

TCF4 is known to be a key component of the Wnt signaling pathway. Canonical Wnt/β-catenin signaling maintains low cytoplasmic β-catenin levels in the absence of stimulation through continuous ubiquitin-proteasome-mediated degradation. Wnt signaling results in inhibition of the constitutively active GSK3β. Consequently, phosphorylation of β-catenin is prevented; β-catenin is stabilized; its levels increase; and nuclear translocation is initiated. Once in the nucleus β-catenin is able bind and activate transcription factors of the TCF/liter EF family (41). TCF has been shown previously to regulate VEGF via the canonical β-catenin accumulation pathway in response to Ginsenoside-Rg1, the most prevalent active constituent of ginseng. This pathway utilized inhibition of GSK3β by its phosphorylation by PKB/AKT (42). Thus we went on to establish if TGFβ1 was able to regulate whole cell β-catenin levels. We showed that this was not the case and that SB216763, an inhibitor of glycogen synthase kinase, was also unable to induce an increase in β-catenin levels. This suggests that β-catenin may be expressed at high levels or be intrinsically stable in pulmonary artery smooth muscle cells making it difficult to induce increased expression. This is in agreement with high basal levels of β-catenin seen in Fig. 5B and the lack of β-catenin degradation over the 8 h time course. TGFβ1 has been shown previously to be capable of inducing
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β-catenin nuclear translocation independently of whole cell β-catenin levels (19). As such, we wanted to establish whether TGFβ1 was regulating the cellular location of β-catenin in PASMCs. We showed by Western blot, following nuclear/cytoplasm extraction, that β-catenin is present in the nucleus of unstimulated PASMCs and that nuclear levels are not increased in response to TGFβ1. This suggests TGFβ1 does not regulate TCF4 via induction of β-catenin translocation.

Whereas β-catenin levels have frequently been shown to be important in regulating its activity (43), it has also been shown, especially in cellular contexts where β-catenin is stable, that phosphorylation of β-catenin can affect its activity irrespective of cellular levels (30). As a result, nuclear/cytoplasm extractions were reprobed with an antibody specific for dephosphorylated amino acids 27–37 of β-catenin. β-Catenin in a dephosphorylated state has been shown to have increased signaling activity (30).

Here, we see the novel ability of TGFβ1 to increase the levels of dephosphorylated (amino acids 27–37) β-catenin (and decrease the levels of phosphorylated β-catenin). Furthermore, TGFβ1 is able to do this to a similar extent as the GSK3β inhibitor, SB216763. We then went on to show that dephosphorylated β-catenin (but not phosphorylated β-catenin) was able to bind the...
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VEGF promoter in response to TGFβ suggests the dephosphorylated form of β-catenin is required for transcriptional regulation of VEGF. β-Catenin overexpression plasmids with mutated phosphorylation sites are available, and future work involves transfecting these constructs into PAMSCs to establish the effect of phosphorylation site mutation on VEGF reporter activity.

The next obvious question was the following: how is TGFβ1 regulating β-catenin phosphorylation levels? Any previous increases in levels of active (unphosphorylated) β-catenin in response to TGFβ1 have correlated with an increase in total levels of β-catenin (18). We saw a steady level of total β-catenin and modulation of phosphorylation alone. We hypothesized that this regulation was occurring via modulation of GSK3β as this kinase can phosphorylate β-catenin, and TGFβ1 has been shown previously to regulate GSK3β activity (18). We show that TGFβ1 does not have any effect on total GSK3β levels. Regulation of cellular localization is known to regulate access to substrates of GSK3β and thus effectively regulate its ability to phosphorylate these substrates. We found that the level of nuclear GSK3β is high in PASMCs in comparison with cytoplasmic levels; however, no shuttling out of the nucleus was evident in response to TGFβ1. Subsequently we assessed the ability of TGFβ1 to regulate the kinase activity of GSK3β by altering its phosphorylation status at serine 9, a site which when phosphorylated is thought to auto-inhibit the enzyme by looping into its active site. We showed that TGFβ1 was able to increase phosphorylation of GSK3β at serine 9. We then went on to show that modulation of GSK3β by pharmacological inhibition, overexpression, and MEK knock-out was able to affect the TGFβ1-induced VEGF levels. In addition we were able to perform successful chromatin immunoprecipitation of GSK3β with the VEGF promoter in response to TGFβ1. It is unlikely that GSK3β is binding directly to the VEGF promoter, but ChIP confirms its presence in the transcriptional complex at the VEGF promoter in response to TGFβ1. To our knowledge, ChIP of GSK3β has not been previously performed in association with any promoter, and TGFβ1 regulation of VEGF via GSK3β modulation is also previously unreported.

It is well documented that TGFβ signals through a conserved family of intracellular signal transducers, termed Smads (44). Binding of TGFβ1 to its receptor results in phosphorylation of Smad2 or Smad3. To initially establish that signaling via Smads was active in PASMCs, Smad2 phosphorylation was detected by Western blot. As expected, TGFβ1-induced Smad2 phosphorylation was performed transfection studies, in PASMCs, using dominant negative Smad2 and Smad3 constructs to establish if either of these so-called co-Smads were involved in the transcriptional regulation of VEGF. These studies showed that the presence of either Smad2 or Smad3 dominant negative protein was sufficient to abolish the response of the 318-bp VEGF construct to TGFβ1. This may either be a direct effect of loss of Smad2 or -3 or because of sequestration of the “receptor activated” Smad, Smad4 by the dominant negative protein. An inhibitor specific to Smad3 (i.e. shown to have no effect on Smad2) was used to help clarify the role of Smad2 and Smad3 individually and showed that Smad3 inhibition alone was sufficient to inhibit TGFβ1 induction of VEGF in PAMSCs. This suggests that Smad2 is not able to compensate for a loss of Smad3 in PASMCs. This is strengthened by data generated in Smad2 and Smad3−/− MEFs which show a more pronounced loss of TGFβ1-induced VEGF in Smad3−/− MEFs than in Smad2−/− MEFs. Furthermore, it is possible to prevent the TGFβ1-induced VEGF response in Smad2−/−/MEFs by inhibiting Smad3 with the pharmacological inhibitor, suggesting the TGFβ1-induced response in these cells is because of Smad3 compensation. Slight confusion may arise from data generated using luciferase reporters in the knock-out MEFs as Smad2−/− MEFs were unable to generate a response to a wild type VEGF-luciferase reporter until Smad2 was recovered by overexpression. However, transfection of these false constructs does not incorporate them into the native transcriptional complexes, and it is likely that compensation by Smad3 requires interaction with the full native VEGF promoter under the full transcriptional regulation of the cell. Importantly, the presence of both Smad2 and -3, in addition to Smad4, within the transcriptional complex at the VEGF promoter, in PASMCs, in response to TGFβ1 was confirmed by ChIP and implies both Smad2 and -3 contribute to the signaling complex under native conditions.

To provide further evidence of the transcriptional complex formed in response to TGFβ1, in addition to the ChIP data, and to give an idea of whether TGFβ1 causes protein associations or an association of a constitutively formed complex with the VEGF promoter, co-immunoprecipitations were performed. The data generated show a constitutive complex containing Smad2, β-catenin, and GSK3β exists within PASMCs. Co-immunoprecipitation of Smad3 and TCF4 was attempted but was unsuccessful partly, in the case of TCF4, because of the low abundance of the proteins and partly because of difficulty detecting the proteins by Western blot because of molecular masses close to 60 kDa, at which level, despite using a light chain-specific secondary detection antibody, a large nonspecific band was always present.

Finally to show Smads were capable of interfering with β-catenin/TCF4 transcriptional activity, we performed...
co-transfection of dominant negative Smad2 and -3 with the TCF reporter, TOPglow. Expression of either Smad2 or -3 dominant negative protein prevented reporter activity and suggests Smads are able to directly affect TCF/β-catenin transcriptional activity.

In conclusion, our studies provide evidence that TGFβ1 induces VEGF via two TCF-binding sites on the VEGF promoter and a complex consisting of TCF4, unphosphorylated β-catenin, GSK3β, and Smad2, -3, and -4 (Fig. 10). TGFβ signaling, β-catenin/TCF4/GSK3β signaling, and VEGF are implicated in vascular development and protection, and this interaction between the three pathways may have important widespread implications for vascular maintenance in development and disease.

REFERENCES

1. Olsson, A. K., Dimberg, A., Kreuger, J., and Claesson-Welsh, L. (2006) Nat. Rev. Mol. Cell Biol. 7, 359–371
2. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000) Nature 407, 242–248
3. McColl, B. K., Stacker, S. A., and Achen, M. G. (2004) APMIS 112, 463–480
4. Kanazawa, H., Asai, K., Hirata, K., and Yoshikawa, J. (2003) J. Biol. Chem. 278, 36280–36288
5. Kanazawa, H., Hirata, K., and Yoshikawa, J. (2002) Thorax 57, 885–888
6. Clavel, G., Bessis, N., and Boissier, M. C. (2003) Joint Bone Spine 70, 321–326
7. Ballara, S. C., Miotla, J. M., and Paleolog, E. M. (1999) Int. J. Exp. Pathol. 80, 387–389
8. Beck, P. L., and Podolsky, D. K. (1999) Inflamm. Bowel Dis. 5, 44–60
9. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E. P., and Roberts, A. B. (2001) J. Biol. Chem. 276, 19945–19953
10. Zhang, F., Phiel, C. J., Spece, L., Gurvich, N., and Klein, P. S. (2003) J. Biol. Chem. 278, 33067–33077
11. Amini Nik, S., Ebrahim, R. P., Van Dam, K., Cassiman, J. J., and Teijpar, S. (2007) Exp. Cell Res. 313, 2887–2895
12. Liu, L., and Simon, M. C. (2004) Cancer Res. 64, 114, 359–371
13. Shi, Y., and Massague, J. (2003) J. Biol. Chem. 278, 19945–19953
14. Sanchez-Elsner, T., Botella, L. M., Velasco, B., Corbi, A., Attisano, L., and Bernabeu, C. (2001) J. Biol. Chem. 276, 38527–38535
15. van Ginneken, B., De Vos, P. T., Langdon, M., and Lemke, H. (2000) PNAS 97, 3333–3338
16. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000) Nature 407, 242–248
17. O’Neill, L. A., Nairn, R., and McVey, D. K. (2003) J. Biol. Chem. 278, 38527–38535
18. Luster, A. D., and Tschopp, J. (2003) Nat. Immunol. 4, 103–109
19. Amini Nik, S., Ebrahim, R. P., Van Dam, K., Cassiman, J. J., and Teijpar, S. (2007) Exp. Cell Res. 313, 2887–2895
20. Liu, L., and Simon, M. C. (2004) Cancer Res. 64, 114, 359–371
21. Shi, Y., and Massague, J. (2003) J. Biol. Chem. 278, 38527–38535
22. Santisteban, P., Santisteban, M., and Garcia-Cao, I. (2000) J. Leukocyte Biol. 68, 564–573
23. Finkenzeller, G., Technau, A., and Marone, D. (1995) Biochem. Biophys. Res. Commun. 208, 432–439
24. Biggs, J. R., Kudlow, J. E., and Kraft, A. S. (1996) J. Biol. Chem. 271, 152–163
25. Van Gijn, M. E., Daemen, M. J., Smits, J. F., and Blankesteijn, W. M. (2002) Exp. Cell Res. 276, 19945–19953