

## Smad3 Is Overexpressed in Advanced Human Prostate Cancer and Necessary for Progressive Growth of Prostate Cancer Cells in Nude Mice

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**Abstract** **Purpose:** The purpose of this study was to investigate the potential role of Smad3, a key mediator of transforming growth factor- $\beta$  signaling, in progression of prostate cancer. **Experimental Design:** Expression of Smad proteins was determined in human prostate cancer tissue array and cell lines. Growth and metastasis of cells overexpressing dominant-negative Smad3 (Smad3D) were studied to determine its role in tumor progression in mice. Cell growth, apoptosis, and expression of angiogenic molecules in tumor lesions were studied to determine potential pathways that Smad3 promotes tumor progression. **Results:** Smad3 was overexpressed in human prostate cancer, which correlated with Gleason score and expression of proliferating cell nuclear antigen. Androgen-independent PC-3MM2 and DU145 cells expressed much higher levels of Smad3 than did androgen-dependent LNCaP, 22Rv1, and LAPC-4 cells. Overexpression of Smad3D in PC-3MM2 cells (PC-3MM2-Smad3D) had minimal direct effects on cell growth but attenuated effects of transforming growth factor- $\beta$ 1 on gene expression and cell growth. Overexpression of Smad3D did not significantly alter tumor incidence but reduced tumor growth rate and metastasis incidence. Most cells in the control tumors, but not PC-3MM2-Smad3D tumors, were positively stained by an antibody to proliferating cell nuclear antigen. Microvessels and expression of angiogenic molecule interleukin-8 were significantly reduced in tumors from PC-3MM2-Smad3D cells. PC-3MM2-Smad3D tumors also expressed lower levels of vascular endothelial growth factor and platelet-derived growth factor. **Conclusions:** These data suggest that Smad3, through regulating angiogenic molecule expression in tumor cells, is critical for progression of human prostate cancer.

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is overexpressed in prostate cancer, especially in advanced disease. It has been shown that overexpression of TGF- $\beta$ 1 in prostate cancer tissues and high urinary and serum levels of TGF- $\beta$ 1 are associated with enhanced tumor angiogenesis and tumor metastasis, and poor clinical outcome (1–3). TGF- $\beta$ 1 induces biological effects through a family of type I and type II transmembrane serine/threonine kinase receptors. On interaction with TGF- $\beta$ 1, TGF- $\beta$  receptor (T $\beta$ R) II recruits T $\beta$ RI, leading to phosphorylation and activation of T $\beta$ RI. With the assistance of adapter proteins, T $\beta$ RI

binds the receptor-regulated Smads (Smad2 and/or Smad3) and phosphorylates the Smads at their COOH-terminal SSXS motif. The phosphorylated Smad2 or Smad3 will then associate with the common Smad4, translocate into the nucleus, bind to concatamers of a CAGA sequence (Smadbox), and activate target gene expression through interaction with other transcription factors, such as activator protein-1 and forkhead activin signal transducers (4, 5). TGF- $\beta$ 1 also induces gene transcription through Smad-independent pathways (6). As such, TGF- $\beta$ 1 stimulates TGF- $\beta$ -activated kinase 1, via TGF- $\beta$ -activated kinase 1 binding protein 1, and leads to the activation of transcription factors activator protein-1, nuclear factor- $\kappa$ B, and activating transcription factor-2 (7–10). In addition, intracellular signaling of TGF- $\beta$  can be mediated by stimulation of Ras, which induces the activation of mitogen-activated protein kinases and stress-activated protein kinases, leading to the activation of activator protein-1 (11).

Our previous studies showed that overexpression of a dominant-negative type II T $\beta$ R (T $\beta$ RII-DN) does not alter tumorigenicity (incidence) but inhibits growth and metastasis of orthotopic PC-3 tumors, which correlates with poor angiogenesis and reduced interleukin-8 (IL-8) expression in the tumor lesions (12). Overexpression of the T $\beta$ RII-DN has minimal direct effects on cell growth in culture and attenuates TGF- $\beta$ -induced cell growth inhibition and IL-8 production (12). Moreover, we found that TGF- $\beta$ 1 up-regulates IL-8 expression at the transcription level in human prostate cancer

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cells (13). These data suggest that TGF- $\beta$ -regulated IL-8 expression is involved in angiogenesis and the progression of human prostate cancer. In the present study, we investigated potential roles of Smad3 in prostate cancer progression. We found that Smad3 protein is overexpressed in surgical specimens of human prostate cancer, which correlates with expression of proliferating cell nuclear antigen (PCNA) and Gleason scores. Smad3 expression levels are much higher in two lines of androgen-independent prostate cancer cell compared with those in three lines of androgen-dependent cells. Overexpression of dominant-negative Smad3 (Smad3D) did not alter the frequency of tumor development but significantly reduced the rate of tumor growth. In addition, cells harboring Smad3D failed to grow in the tibia of nude mice. IL-8 expression in both cell culture and orthotopic tumors, and microvessel density in orthotopic tumors derived from PC-3MM2 cells harboring Smad3D were significantly reduced. These data suggest that Smad3-mediated TGF- $\beta$  signaling may play a crucial role in the progression of human prostate cancer cells.

## Materials and Methods

**Mice.** Specific pathogen-free male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The mice were used according to institutional guidelines when they were 8 to 10 weeks of age.

**Reagents.** Eagle's minimal essential medium, Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS, and fetal bovine serum (FBS) were purchased from M.A. Bioproducts. A mRNA isolation kit was purchased from Invitrogen. Human IL-8 ELISA kits were purchased from BioSource International. Antibodies against Smad2, Smad3, Smad4, and Smad7 were purchased from Santa Cruz Biotechnology; phosphorylated Smad2 was from Chemicon International, Inc.; phosphorylated Smad3 was from Calbiochem (EMD Biosciences, Inc.); PCNA was from BD Bioscience-PharMingen; and  $\beta$ -actin was from Sigma Chemical Co. Smad3 antibody used in immunohistochemistry was from Zymed Laboratories, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma. The paraffin-embedded tissue sections of human prostate cancer tissue arrays were obtained from U.S. Biomax, Inc. The plasmid p3TP-lux, a luciferase reporter in which the expression of luciferase is driven by a composite promoter, consisting of three TPA response elements of the human collagenase gene and one TGF- $\beta$  response element of the human plasminogen activator inhibitor-1 promoter (14), was obtained from Addgene, Inc. The pcDNA3 vector-based plasmid encoding a cDNA for FLAG-tagged human Smad3 mutant (FLAG-Smad3D) in which three COOH-terminal serine residues were changed to alanine residues (15) was generously provided by Dr. Yasuko Yamamura (Department of Retroviral Regulation, Tokyo Medical and Dental University Medical Research Division, Tokyo, Japan).

**Tumor cells and culture.** The highly metastatic PC-3MM2 human prostate carcinoma cells (16) were derived from the well-documented PC-3 cells (17). LNCaP cells (18), DU145 cells (19), and 22Rv1 cells (20) were all obtained from American Type Culture Collection. Cells were maintained as a monolayer culture in Eagle's minimal essential medium supplemented with 5% FBS (for PC-3MM2 and DU145 cells) or 10% FBS (for LNCaP and 22Rv1 cells), nonessential amino acids, sodium pyruvate, vitamin A, and glutamine. In addition, we were

kindly provided with LAPC-4 cells (21) by Dr. Karen Knudson (Department of Cancer and Cell Biology, University of Cincinnati, Cincinnati, OH) with the approval of Dr. Robert Reiter (Department of Urology, University of California at Los Angeles, Los Angeles, CA). LAPC-4 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 10 nmol/L dihydrotestosterone. Based on cell morphology, growth rate and androgen dependence, and expression of androgen receptor and prostate-specific antigen, the cell lines used in this study did not undergo hybridization. Cells in their exponential growth phase were harvested by a 1-min treatment with a 0.25% trypsin-0.02% EDTA solution. The flasks were tapped to detach the cells, MEM-10% FBS was added, and the cell suspension was gently agitated to produce a single-cell suspension. The cells were washed and resuspended in HBSS. Only suspensions of single cells with viability exceeding 95% (ascertained by trypan blue exclusion) were used.

PC-3MM2 cells were transfected with pcDNA3 vector or pcDNA3-FLAG-Smad3D using Fugene 6 transfection reagent (Roche Applied Science) at 37°C for 24 h. Transfected cells were selected in medium containing 800  $\mu$ g/mL G418. After 2 weeks of continuous culture, drug-resistant clones were isolated and expanded to derive PC-3MM2-Neo and PC-3MM2-Smad3D cells. The cells were maintained in medium supplemented with 400  $\mu$ g/mL G418 and cultured for approximately 2 to 3 weeks in the absence of G418 before being used.

**Tumor cell inoculation.** Mice were anesthetized with Nembutal before tumor cell inoculation. The intraprostatic injection ( $10^5$  per mouse) of tumor cells was done as detailed in our previous study (12). For bone injection, a procedure described by others was used (22). Briefly, tumor cells ( $2 \times 10^5$  per mouse in 10  $\mu$ L HBSS) were directly injected into the proximal end of the tibia of nude mice through a 26-gauge needle. Prostatic tumors (including the prostate) were excised and weighed after the mice were killed by cervical dislocation at days 28 to 35 after tumor inoculation. Regional lymph node metastasis was assessed by microscopic examination of H&E-stained serial paraffin sections. The tumor samples were collected for H&E staining, mRNA extraction, and immunohistochemical analysis. Growth of tumor cells in the bone was monitored in an X-ray machine (Image Station In-Vivo FX system, Kodak).

**RNA isolation and quantitative real-time reverse transcription-PCR.** Total RNA was extracted and analyzed by real-time reverse transcription-PCR (RT-PCR) as detailed in our previous studies (12). Briefly, total RNA in cell cultures was extracted with Trizol reagent. For RNA extraction from tumors, fresh tumor tissues were treated with RNALater immediately after their removal from mice, transferred into Trizol, and homogenized using a Polytron homogenizer, and total tissue RNA was extracted. After treatment with DNase, cDNA was synthesized from the RNA using reverse transcriptase (Stratagene) with oligo(dT) as the primer, followed by cDNA amplification in a 7300 Real-time PCR System (Applied Biosystems) using the Brilliant SYBR Green QPCR Master Mix (Stratagene). The cycle threshold values were used to calculate the normalized expression of target genes against  $\beta$ -actin using the Q-Gene software (23).

**Western blot analysis.** The expression of Smad proteins was determined by using immunoblotting as described in our previous studies (13).

**Doubling time.** Tumor cells were plated at  $5 \times 10^3$  cells per well of 24-well plates. After incubation for various lengths of time (1-5 days), the cells were harvested by trypsinization and counted. Doubling time was calculated from the growth curve of the cultures.

**Effects of TGF- $\beta$ 1 on cell growth in vitro.** Effects of TGF- $\beta$ 1 on cell growth *in vitro* were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as detailed in our previous studies (24). Cells were cultured for 96 h in Eagle's minimal essential medium-1% FBS containing different concentrations of human TGF- $\beta$ 1 at 37°C in 5% CO<sub>2</sub>. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2 mg/mL in PBS) was added to the cultures at 50  $\mu$ L/well during the final 2 h of incubation. After removing the medium, the dark blue formazan was dissolved in DMSO and the absorbance was measured

with a FluoStar Optima multidetection microplate reader (BMG Lab Technologies) at 570 nm. The percentages of growth inhibition were calculated according to the following formula: growth inhibition (%) =  $(1 - A_{570} \text{ of treated group} / A_{570} \text{ of control group}) \times 100$ .

**Immunohistochemical staining.** Immunohistochemical staining was done as detailed in our previous studies (12). For immunohistochemistry of paraffin-embedded sections of tissue arrays and tumor samples from mice, sections (4-5  $\mu\text{m}$ ) were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were treated with a citric acid-based antigen retrieval buffer (DAKO Co.), followed by 3%  $\text{H}_2\text{O}_2$  in methanol, and incubated in blocking buffer (5% bovine serum albumin and 5% horse serum in PBS) and then with the blocking buffer containing antibodies against Smad2, Smad3, or IL-8. After washing, the slides were incubated with a biotinylated secondary antibody (BioGenex Laboratories), followed by washing and incubation with the streptavidin-conjugated peroxidase (BioGenex Laboratories). A positive reaction was visualized by incubating the slides with stable diaminobenzidine and counterstaining with Gill's hematoxylin (BioGenex Laboratories) and mounted with Universal Mount mounting medium (Fisher Scientific). The intensity and extent of cytoplasm-positive labeling for Smad2 and Smad3 in tissue arrays were assessed semi-quantitatively and scored as 0 (no staining), 1+ (weak and focal staining in <25% of tissue), 2+ (moderate intensity in 25-50% of tissue), and 3+ (moderate intensity in >50% of tissue), and 4+ (strong and diffused staining in >50% of tissue). For CD31 staining, frozen sections (8-10  $\mu\text{m}$ ) were fixed in cold acetone, treated with 3%  $\text{H}_2\text{O}_2$  in methanol, incubated in blocking solution, and then reacted with an antibody to CD31. Slides were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was visualized as described above.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** Apoptosis in paraffin-embedded tissue sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method using a kit from Promega Co. as detailed in our previous studies (12, 25).

**ELISA.** PC-3MM2 tumors were collected and homogenized in 1% Tween 20-PBS containing a proteinase inhibitor cocktail (Roche Applied Science). Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories) and IL-8 in the lysates was measured by using an ELISA kit according to the manufacturer's instructions.

**Statistical analysis.** The tumor growth studies were done with four to eight mice per group and repeated at least once. Differences in the immunohistochemical staining of surgical specimens of human prostate cancer and the frequency of tumor development among groups were analyzed with the  $\chi^2$  test. Differences in tumor weight, effects of TGF- $\beta$ 1 on cell growth, and IL-8 production in tumors among groups were compared by ANOVA.

## Results

**Expression of Smad2 and Smad3 in human prostate cancer specimens and cell lines.** The receptor-regulated Smad2 and/or Smad3 are essential for TGF- $\beta$  signaling (4, 5). To determine their potential roles in prostate cancer progression, we evaluated the expression of Smad2 and Smad3 in surgical specimens of human prostate cancer by immunohistochemistry. Sixty-seven tumor tissues and six normal tissues from patients of 38- to 87-year-old men were analyzed (Table 1). A basal level of Smad2 and Smad3 immunoreactivity was detected in all normal prostate tissue sections (Fig. 1). Smad2 expression was moderately elevated in tumor sections and there was no significant correlation between Smad2 expression level and Gleason score (Fig. 1). In contrast, Smad3 expression, detected in both the cytoplasm and nucleus, was significantly

**Table 1.** Relationship between the expression of Smad3 and Gleason score

Gleason score	Smad3 staining				Intensity (>1+)
	1+ (basal)	2+	3+	4+	
Normal	6	0	0	0	0/6
6-7	12	8	8	7	23/35
8-10	2	7	7	16	30/32
Tumor vs normal					$P = 0.0002$
Gleason 8-10 vs Gleason 6-7					$P = 0.0062$

elevated in 53 tumor tissue sections (53 of 67, 79%;  $P = 0.0002$ ). Among the tumor sections, Smad3 staining was elevated in ~66% (23 of 35) of the specimens with Gleason scores 6 to 7 and 94% (30 of 32) of those with Gleason scores 8 to 10, respectively ( $P = 0.0062$ ). Moreover, the intensity of the staining was significantly increased in the specimens with Gleason scores 8 to 10 (Table 1). Next, we correlated the expression of Smad proteins with cell growth and apoptosis in consecutive sections of the same samples. Cell growth was estimated by immunohistochemical staining with an antibody against PCNA that is expressed in replicating cells (26) and apoptosis determined by TUNEL assay. Whereas the majority of cells in the specimens of Gleason scores 8 to 10 stained strongly positive by the PCNA antibody, both the portion of PCNA positively stained cells and the intensity of the staining were significantly reduced in tumor samples of Gleason 6 to 7 and normal prostate tissue (Fig. 1). Very few cells were positively stained by the TUNEL method in all the specimens examined. These data indicate that overexpression of Smad3 correlates with cell proliferation in human prostate cancer cells.

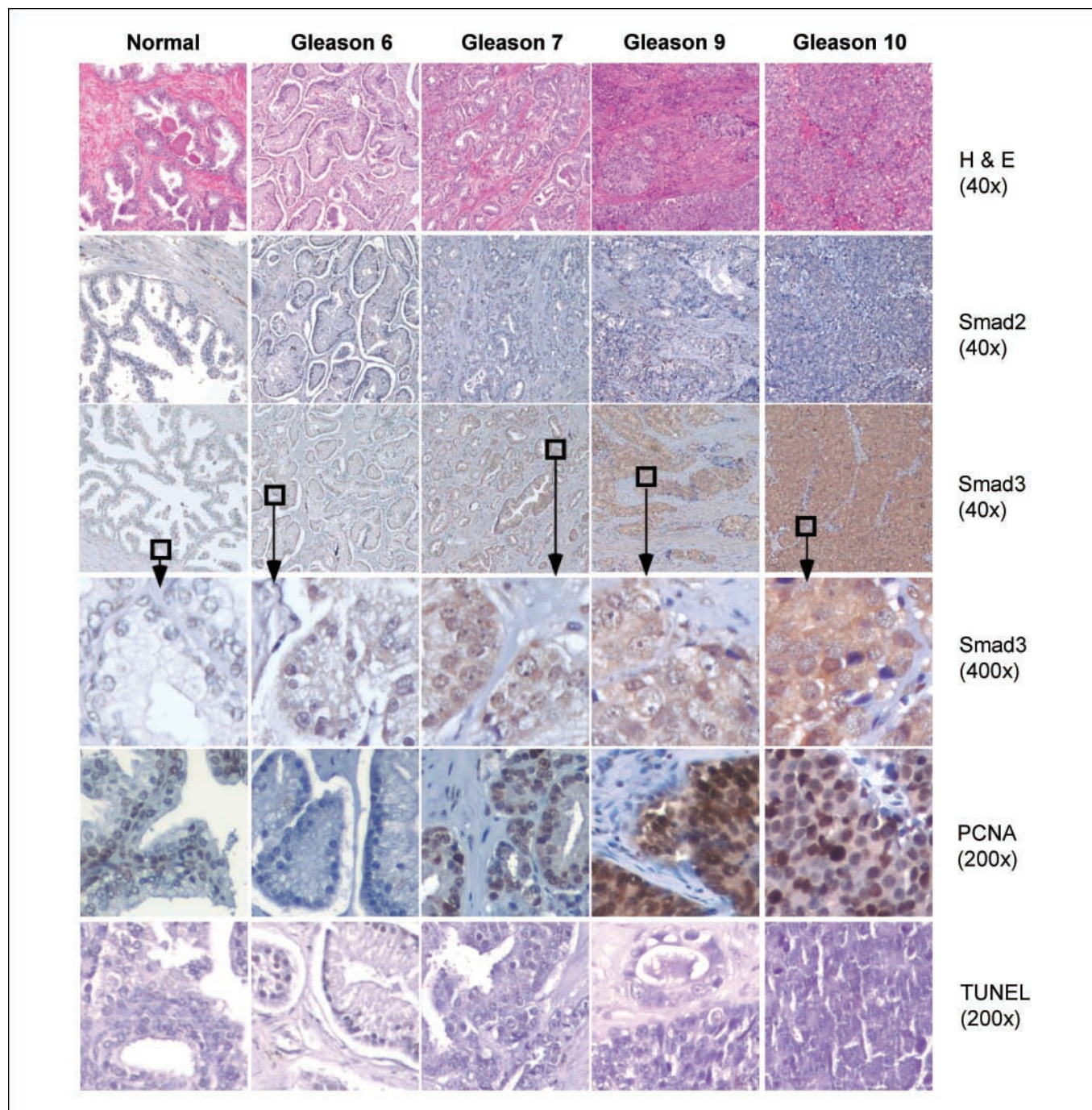
Next, we determined expression of Smad proteins in five most commonly used prostate cancer cell lines, PC-3MM2, 22Rv1, LAPC-4, LNCaP, and DU145 cells. As shown in Fig. 2A, the androgen-independent cells (PC-3MM2 and DU145) expressed significantly higher levels of Smad3 than did the androgen-dependent cells (LNCaP, LAPC-4, and 22Rv1). Although expression levels of Smad2, Smad4, and Smad7 also varied among the five cell lines, the extent of variation was much modest compared with that of Smad3 (Fig. 2A). Focusing on the two cell lines that expressed higher levels of Smad3, we determined effects of TGF- $\beta$ 1 on the phosphorylation of Smad2 and Smad3. As shown in Fig. 2B, Smad2 was constitutively phosphorylated in both PC-3MM2 and DU145 cells and the phosphorylation was further elevated on the exposure to 10 ng/mL TGF- $\beta$ 1 for 30 min (Fig. 2B). On the other hand, a very low basal level of phosphorylated Smad3 was detected in PC-3MM2 and DU145 cells and the treatment with TGF- $\beta$ 1 induced phosphorylation of Smad3 at a magnitude significantly higher than that of Smad2 in both cell lines (Fig. 2B). In contrast, the androgen-dependent cell lines showed very low levels of constitutive Smad2 phosphorylation and the same treatment with TGF- $\beta$ 1 did not significantly enhance the phosphorylation of Smad2 or Smad3 (Fig. 2B).

Taken together, data from the immunohistochemical analysis of prostate cancer specimens and the immunoblotting analysis of prostate cancer cell lines strongly suggest that Smad3 may

play an important role in progression of human prostate cancer.

**Establishment of cell lines that overexpress Smad3D.** To directly investigate the role of Smad3 in regulating growth and progression of prostate cancer, we determined effects of overexpression of a Smad3D on TGF- $\beta$ -regulated gene expression and cell growth in the highly metastatic PC-3MM2 cells, which was used in our previous studies to reveal the effects of T $\beta$ RII-DN on tumor progression (12) and TGF- $\beta$ 1-regulated IL-8 expression (13). PC-3MM2 cells were stably

transfected with a Smad3D and cloned to derived PC-3MM2-Smad3D-C1 and PC-3MM2-Smad3D-C2 cells: PC-3MM2-Smad3D-C1 cells expressed relatively higher Smad3D than PC-3MM2-Smad3D-C2 cells. Parental and control vector (pcDNA3)-transfected cells served as controls. Overexpression of the transgene was confirmed by quantitative RT-PCR (Fig. 3A) and Western blotting (Fig. 3B). Moreover, overexpression of Smad3D did not significantly alter expression levels of Smad2, Smad4, and Smad7 in PC-3MM2-Smad3D-C1 cells (Fig. 3B).



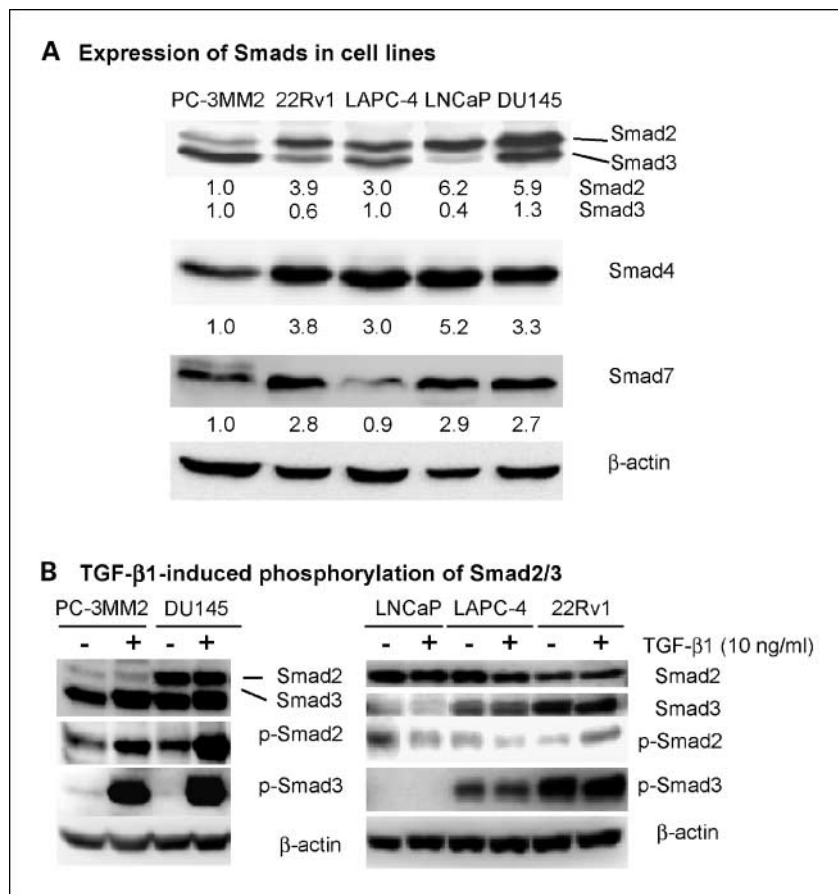
**Fig. 1.** Immunohistochemistry and TUNEL staining of the tissue arrays of human prostate cancer specimens. The expression of Smad3 and PCNA, but not Smad2, correlates with Gleason score.

To determine whether transfection of Smad3D or control vector had any direct effect on the growth of PC-3MM2 cells, we measured the doubling times of the variants in their exponential phase of growth in culture and found that there were no discernable differences in the doubling time among parental, control vector-transfected, and Smad3D-transfected cells (data not shown). In addition, we found that there was no significant difference in growth rates among the variants when cultured under various concentrations of serum (data not shown). Therefore, overexpression of Smad3D had no direct effects on cell growth in culture.

To test whether overexpression of Smad3D compromised TGF- $\beta$  signaling, we determined its effects on TGF- $\beta$  stimulation of 3TP-lux activity, a plasminogen activator inhibitor-1 promoter-based luciferase reporter that is widely used and highly TGF- $\beta$  responsive (14, 27). As shown in Fig. 3C, in PC-3MM2 and PC-3MM2-Neo cells treated with 10 ng/mL TGF- $\beta$ 1 for 24 h, the 3TP-lux activity was enhanced by ~4-fold. This response was diminished in PC-3MM2-Smad3D-C1 and significantly reduced in PC-3MM2-Smad3D-C2 cells, respectively (Fig. 3C). Similarly, TGF- $\beta$ 1-induced expression of endogenous plasminogen activator inhibitor-1 (Fig. 3D) and urokinase-type plasminogen activator (Fig. 3E), another TGF- $\beta$ -responsive gene (28, 29), was diminished in PC-3MM2 overexpressing Smad3D. Last, we determined effects of overexpression of Smad3D on inhibition of PC-3MM2 cell growth by TGF- $\beta$ 1 (12, 30, 31). Growth of parental and control vector-transfected PC-3MM2 cells was suppressed by TGF- $\beta$ 1 in a dose-

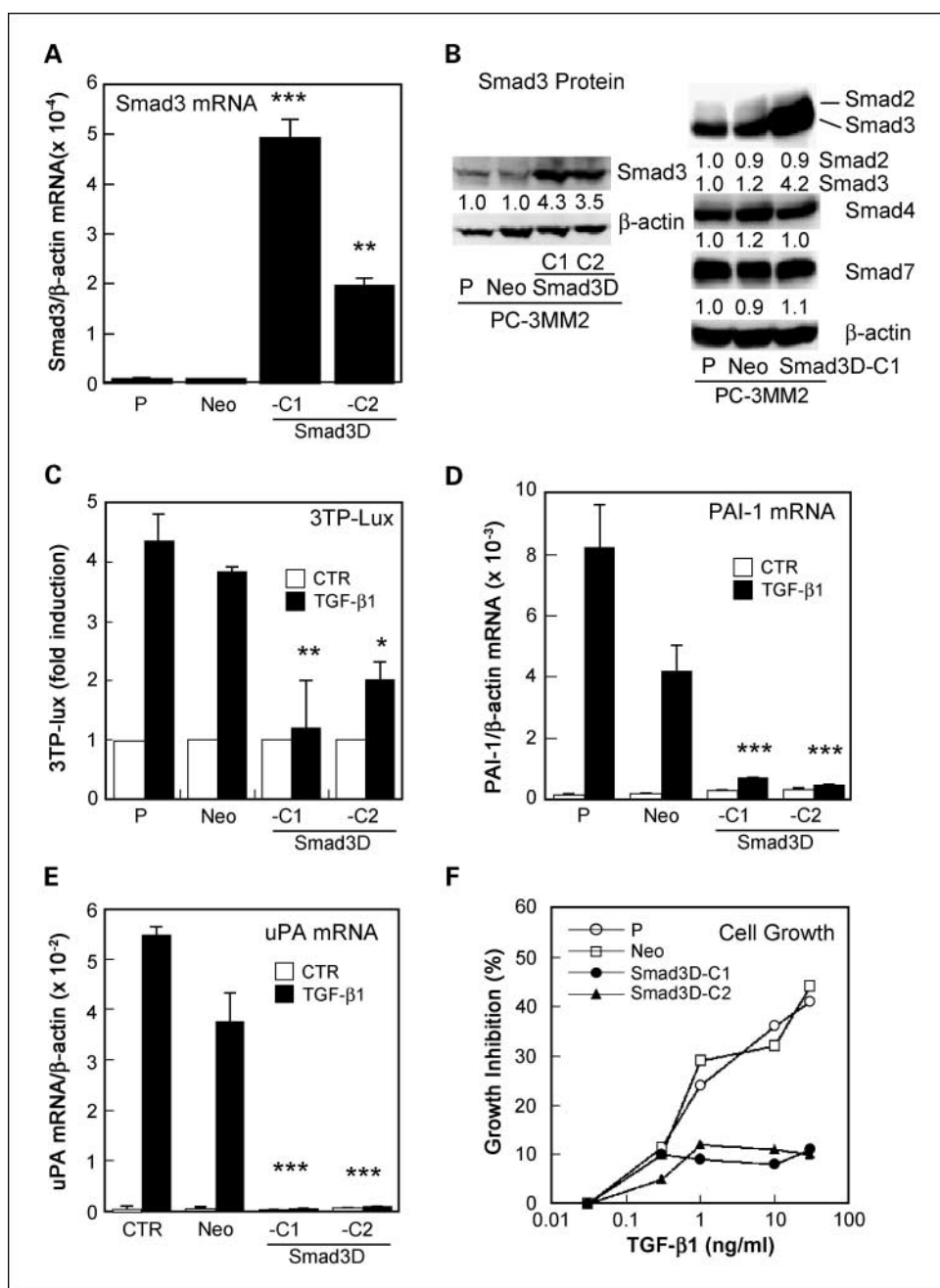
dependent manner and the growth was inhibited up to 40% when the cells were exposed to 30 ng/mL TGF- $\beta$ 1. In contrast, PC-3MM2-Smad3D-C1 and PC-3MM2-Smad3D-C2 cells became resistant to the inhibitory effects of TGF- $\beta$ 1 (Fig. 3F). Taken together, these data clearly showed that overexpression of Smad3D compromised the responses of PC-3MM2 cells to TGF- $\beta$ 1 for gene expression and cell growth inhibition and there was no significant difference in these responses between PC-3MM2-Smad3D-C1 and PC-3MM2-Smad3D-C2 cells.

**Effects of overexpression of Smad3D on tumorigenicity and tumor progression.** Next, we investigated whether overexpression of Smad3D could affect growth of prostate cancer cells in nude mice. PC-3MM2, PC-3MM2-neo, PC-3MM2-Smad3D-C1, or PC-3MM2-Smad3D-C2 cells ( $10^5$  per mouse in 20  $\mu$ L HBSS) were inoculated into the prostate of nude mice. On day 28 after tumor cell inoculation, mice were sacrificed and tumor incidence and weight were measured. Paraffin sections of aortic lymph node were stained by H&E to identify metastases. As shown in Fig. 4A, all mice (16 of 16) inoculated with PC-3MM2, PC-3MM2-neo, or PC-3MM2-Smad3D-C2 cells and 14 of 16 mice injected with PC-3MM2-Smad3D-C1 cells developed orthotopic tumors. Therefore, overexpression of Smad3D did not significantly alter the frequency of tumor development (tumorigenicity) of PC-3MM2 cells. Tumors formed by PC-3MM2-Smad3D-C1 and PC-3MM2-Smad3D-C2 cells were, however, much smaller than those formed by parental and control vector-transfected cells (Fig. 4A). Para-aortic lymph node metastases were found in 8 of 11 and 8 of 8 mice inoculated



**Fig. 2.** Expression of Smad proteins in human prostate cancer cell lines. *A*, expression of Smad2, Smad3, Smad4, and Smad7 in prostate cancer cells. Densitometric quantitation, relative to PC-3MM2 cells, on expression of Smad proteins was done on the bands of immunoblotting and shown below each lane. *B*, effects of TGF- $\beta$ 1 on Smad2 and Smad3 phosphorylation. Cells were in starved in serum-free medium for 24 h and stimulated with 10 ng/mL of TGF- $\beta$  for 30 min.

**Fig. 3.** Characterization of Smad3D-transfected PC-3MM2 cells. **A**, expression of Smad3 mRNA Smad3D-transfected cells determined by real-time RT-PCR. **B**, expression of Smad2, Smad3, Smad4, and Smad7 protein in Smad3D-transfected cells determined by immunoblotting. Densitometric quantitation, relative to PC-3MM2 cells, on expression of Smad proteins was done on the bands of immunoblotting and shown below each lane. **C**, effects of TGF- $\beta$ 1 on 3TP-lux activity in control and Smad3D-transfected cells determined by the luciferase assay. **D**, effects of TGF- $\beta$ 1 on PAI-1 mRNA expression in control and Smad3D-transfected cells determined by real-time RT-PCR. **E**, effects of TGF- $\beta$ 1 on  $\mu$ PA mRNA expression in control and Smad3D-transfected cells determined by real-time RT-PCR. **F**, effects of TGF- $\beta$ 1 on growth of control and Smad3D-transfected cells determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with control vector-transfected cells.



with PC-3MM2 and PC-3MM2-neo cells, respectively. In contrast, 0 of 12 and 1 of 8 mice injected with PC-3MM2-Smad3D-C1 and PC-3MM2-Smad3D-C2 cells, respectively, developed lymph node metastases. It has been shown that TGF- $\beta$ 1 and TGF- $\beta$  signaling are critical for development of bone metastasis by prostate cancer (32–34). We therefore determined effects of overexpression of Smad3D on growth of PC-3MM2 cell in the bone of mice. PC-3MM2 or PC-3MM2-Smad3D-C1 cells ( $2 \times 10^5$  per mouse in 10  $\mu$ L HBSS) were directly injected into the tibia of nude mice. The injection of PC-3MM2 cells produced osteolytic bone lesions (Fig. 4B) in 8 of 11 mice. In contrast, PC-3MM2-Smad3D-C1 cells failed to produce X-ray detectable tumors (Fig. 4B) in all mice (0 of 12). These data indicate that a blockade of TGF- $\beta$  signaling with

Smad3D significantly compromised tumor growth in both the prostate and bone.

**Cell growth, apoptosis, and angiogenesis in orthotopic tumors of PC-3MM2 cells overexpressing Smad3D.** Immunohistochemistry and real-time RT-PCR analyses were carried out on orthotopic tumors sampled on day 10 after tumor cell inoculation. Smad3 protein, detectable in both PC-3MM2 and PC-3MM2-Neo tumors, was significantly elevated in tumors formed by PC-3MM2-Smad3D-C1 cells (Fig. 5). Consistently, Smad3 mRNA levels in PC-3MM2-Smad3D-C1 tumors were  $\sim$ 8-fold higher than that in PC-3MM2-Neo tumors ( $P < 0.001$ ; Fig. 6B). Therefore, cells overexpressing Smad3D did not grow in the prostate of mice. Most cells in tumors of PC-3MM2 parental and PC-3MM2-Neo cells were stained

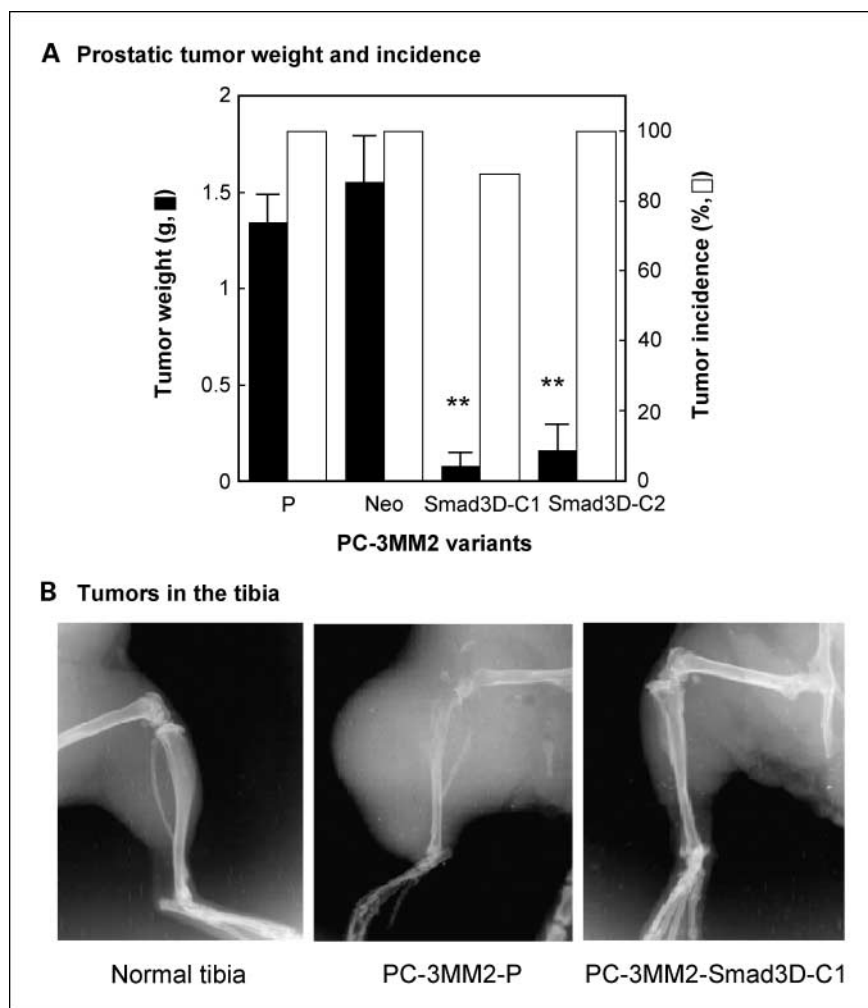
strongly positive by an antibody against PCNA, a nuclear antigen expressed in proliferating cells (26). In contrast, both the portion of PCNA-positive cell and the intensity of PCNA staining were significantly reduced in PC-3MM2-Smad3D-C1 tumors (Fig. 5). In consistent with immunohistochemical staining, the quantitative RT-PCR showed that expression of PCNA and Ki67 mRNA (Fig. 6B), another nuclear protein expressed exclusively in proliferating cells (35), was reduced by 75% ( $P < 0.05$ ) and 89% ( $P < 0.01$ ), respectively, in PC-3MM2-Smad3D-C1 tumors compared with that in tumors of PC-3MM2-Neo cells. The number of TUNEL-positive cell was also moderately increased in PC-3MM2-Smad3D-C1 tumors; they, however, constituted  $<1\%$  of cells in a tumor lesion (Fig. 5).

Because our previous study showed that microvessel density in orthotopic tumors formed by PC-3MM2 cells overexpressing TBR1-DN construct is significantly reduced (12), we determined whether overexpression of Smad3D altered angiogenesis in PC-3MM2 tumors. As shown in Fig. 5, microvessels, revealed by immunohistochemical staining with CD31 antibody, were evident in tumors formed by PC-3MM2 and PC-3MM2-Neo cells but not tumors formed by PC-3MM2-Smad3D-C1 cells. Indeed, the real-time RT-PCR analysis showed that expression of CD31 mRNA in PC-3MM2-Smad3D tumors was reduced by  $\sim 82\%$  compared with that in PC-3MM2-Neo tumors ( $P < 0.05$ ; Fig. 6B).

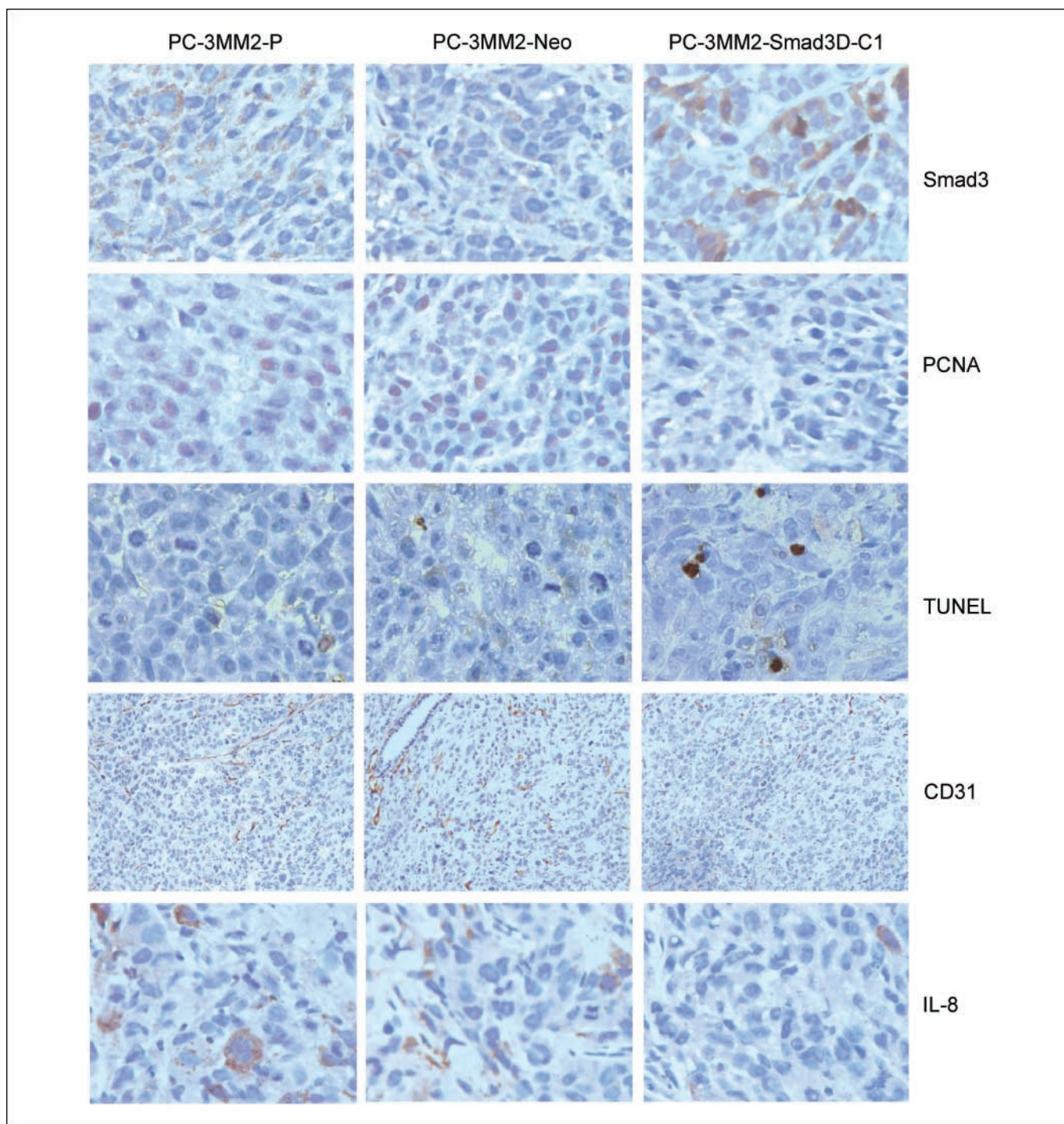
**Expression of angiogenic molecules in tumors of PC-3MM2 cells overexpressing Smad3D.** To elucidate potential mechanisms responsible for the reduction of angiogenesis in PC-3MM2-Smad3D tumors, we determined expression of several proangiogenic molecules that have been reported to be involved in angiogenesis in prostate cancer (36–38). As shown in Fig. 5, PC-3MM2-Smad3D-C1 tumors contained fewer IL-8-positive cells. We further quantitatively analyzed IL-8 expression in the tumors by real-time RT-PCR and ELISA (Fig. 6A). These analyses clearly showed that IL-8 expression in PC-3MM2-Smad3D-C1 tumors was significantly lower than that in PC-3MM2-P or PC-3MM2-Neo tumors. In addition, expression of vascular endothelial growth factor and platelet-derived growth factor (Fig. 6B) was also significantly reduced in PC-3MM2-Smad3D-C1 tumors compared with those in PC-3MM2-Neo tumors. In contrast, the levels of matrix metalloproteinases 2 and 9 were not significantly varied among the tumors formed by PC-3MM2 cell variants (Fig. 6B).

## Discussion

The role of TGF- $\beta$  signaling in carcinogenesis and progression of prostate cancer is currently under intensive investigation. Most studies, including clinical specimen analysis and studies in preclinical cellular and animal models, were focused mainly on



**Fig. 4.** Growth of PC-3MM2 variants in the prostate and tibia of nude mice. PC-3MM2-P, PC-3MM2-Neo, PC-3MM2-Smad3D-C1, and PC-3MM2-Smad3D-C2 cells were injected into the prostate ( $10^5$  per mouse) or tibia ( $2 \times 10^5$  per mouse). Overexpression of Smad3D did not alter tumorigenicity but retarded growth of cells in the prostate (A) and abolished tumor formation in the tibia (B).



**Fig. 5.** Immunohistochemistry and TUNEL staining of orthotopic tumors of PC-3MM2 cell variants. Tumor cells were inoculated into the prostate of nude mice. On day 10 after tumor cell inoculation, tumors were sampled for the staining. The staining of PCNA, CD31, and IL-8 was reduced in PC-3MM2-Smad3D-C1 tumors. The number of TUNEL-positive cell was moderately higher in PC-3MM2-Smad3D-C2 tumors compared with that in PC-3MM2-P and PC-3MM2-Neo tumors.

expression and function of T $\beta$ R $\beta$ s. These studies conclude that TGF- $\beta$  signaling in most types of cancer, including prostate cancer, inhibits carcinogenesis and promotes tumor progression. Indeed, our previous studies showed that overexpression of a T $\beta$ R $\beta$ -DN compromised progression of PC-3MM2 tumors in the prostate of nude mice (12). Because TGF- $\beta$  signaling is mediated by several intracellular pathways and there are cross-talks among

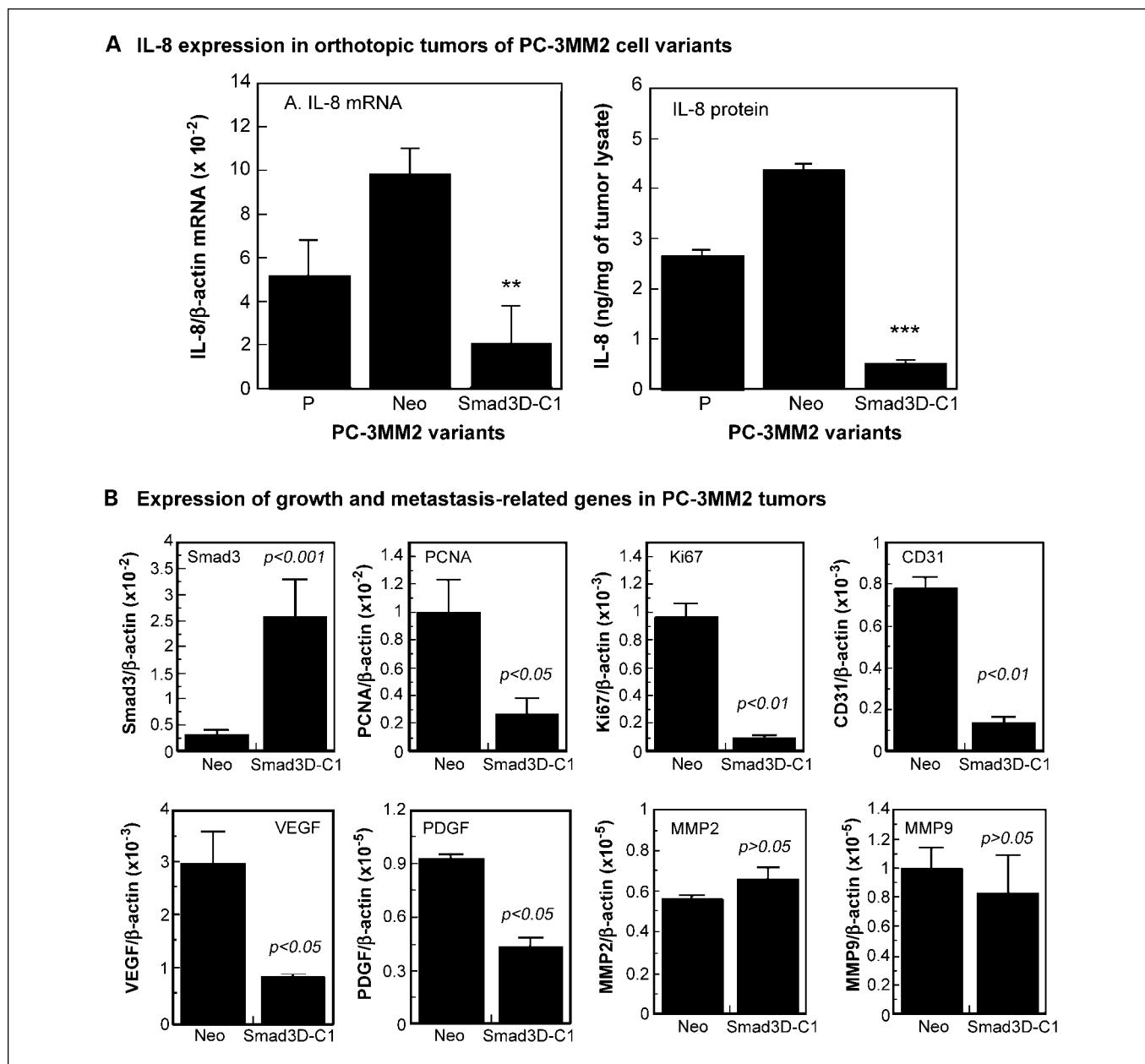
different pathways (11, 39, 40), it is necessary to further delineate the contribution of each of TGF- $\beta$  signaling pathways in tumor suppression and promotion. The focus of this research was to determine the role of Smad3 in prostate cancer progression. We showed that Smad3 expression, but not Smad2, is significantly elevated in surgical specimens of human prostate cancer and the expression levels of Smad3 correlate with PCNA



expression and Gleason score. Among the five commonly used prostate cancer cell lines, Smad3 expression levels were significantly higher in the two androgen-independent and androgen receptor-negative cell lines (PC-3MM2 and DU145) compared with that in the three lines of androgen-dependent cells (LNCaP, LAPC-4, and 22Rv1). On the other hand, although the expression levels of Smad2, Smad4, and Smad7 also vary among the five lines of cells, the magnitude of variation is not as significant as that of Smad3. Enforced overexpression of Smad3D in the highly metastatic PC-3MM2

human prostate cancer cells had minimal, if any, effects on tumorigenicity (tumor incidence) but significantly retarded growth of primary tumors, reduced metastases, and abolished formation of tumor lesions in the bone of mice. These data suggest that Smad3 may be critical for growth and progression of human prostate cancer.

Androgen ablation therapy is the mainstay treatment for advanced prostate cancer (41, 42). It has been shown that expression of Smad3 is elevated in the prostate and tumors of rats under androgen ablation therapy, in addition to the



**Fig. 6.** Analysis of gene expression in orthotopic tumors of PC-3MM2 cell variants. **A**, IL-8 expression. PC-3MM2 cell variants were inoculated into the prostate of nude mice. On day 10 after tumor inoculation, tumors were sampled for *in vitro* analysis of IL-8 expression by real-time RT-PCR and ELISA. The expression of IL-8 in tumors formed by PC-3MM2-Smad3D-C1 tumors was significantly reduced compared with that in PC-3MM2-Neo tumors. **B**, differential expression of growth- and metastasis-related genes in PC-3MM2 tumor variants. PC-3MM2-Neo or PC-3MM2-Smad3D-C1 cells were inoculated into the prostate of nude mice. Ten days later, tumors were sampled and total RNA was analyzed by real-time RT-PCR. VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9.

up-regulation of Smad2 and the induction of apoptosis (43). Our immunohistochemical staining data on the surgical specimens of human prostate cancer are the first to describe the relationship of Smad3 expression and Gleason score. The positive correlation between Smad3 expression level and Gleason score as well as PCNA expression strongly suggests that Smad3 promotes progression of human prostate cancer. Because of the lack of detailed clinical information on the specimens, one cannot rule out that the differential expression of Smad3 is caused by therapies, such as androgen ablation therapy. However, three lines of evidence argue against this notion. First, we did not detect significant difference in Smad2 expression, which is also up-regulated by androgen ablation (43). Second, expression of PCNA, a proliferating cell marker, correlates with Smad3 level and Gleason score in the tumor samples. Third, apoptosis, revealed by TUNEL staining, is not detected in the tumor expressing high level of Smad3. Nevertheless, further studies with detailed clinical information are necessary for validating the finding of Smad3 expression in human prostate cancer.

The progressive growth of primary tumors and metastases depends on an adequate blood supply (44). Microvessel density in invasive tumors of human prostate cancer directly correlates with their metastasis potential (45). In general, tumors smaller than 1 to 2 mm in diameter can receive nutrients by diffusion, but further growth of the lesions must be preceded by the formation of new blood vessels (46). The prevascular stage of a tumor is associated with local, noninvasive, benign tumors, whereas the vascular stage is associated with aggressive, invasive, and metastatic tumors (46). The *in vitro* growth of PC-3MM2-Smad3D cells does not significantly differ from that of parental or control vector-engineered cells. Tumors formed by Smad3D-engineered cell was, however, much smaller with less vasculature. These data indicate that Smad3 regulates expression of angiogenic molecules in tumor

cells and angiogenesis in tumor lesions and suggest that Smad3 may be one of key components in the critical angiogenesis switch in prostate cancer. The observations in this report are consistent with the findings from our previous study in cells expressing a T $\beta$ RII-DN (12). On the other hand, we did observe a significant difference between tumors formed by PC-3MM2 cells overexpressing T $\beta$ RII-DN (12) and Smad3D reported here. In contrast to that in tumors formed by PC-3MM2-DNR cells, no massive apoptosis was observed in tumors formed by PC-3MM2-Smad3D cells. Because the activation of T $\beta$ R triggers multiple signaling pathways, which can be compromised by overexpression of T $\beta$ RII-DN but not Smad3D, these data suggest that other pathways, such as TGF- $\beta$ -activated kinase 1- and/or Ras-related ones, are also involved in TGF- $\beta$  signaling-regulated tumor growth and metastasis. This possibility is currently under investigation in our laboratory.

In summary, data presented in this report show that overexpression of a Smad3D in PC-3MM2 cells did not alter tumorigenicity of the cells in the prostate of mice but compromised progression of orthotopic tumors formed by the cells. IL-8 expression and angiogenesis in tumors formed by the dominant-negative receptor-engineered cells were significantly reduced. These data indicate that the TGF- $\beta$ -regulated IL-8 expression cascade is critical for the progression of orthotopic tumors formed by PC-3MM2 cells in nude mice and suggest a potential mechanism by which TGF- $\beta$  signaling promotes tumor angiogenesis and tumor progression in human prostate cancer.

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