SMAD3 Represses Androgen Receptor-mediated Transcription

Steven A. Hayes, Mark Zarnegar, Manju Sharma, Fajun Yang, Donna M. Peehl, Peter ten Dijke, and Zijie Sun

ABSTRACT

The androgen-signaling pathway is important in the growth and progression of prostate cancer. Androgen ablation therapy, which may result in programmed cell death, is often used to treat advanced prostate cancer. The growth-promoting effects of androgen are mediated mostly through the androgen receptor (AR). Transforming growth factor β (TGF-β) plays critical roles in controlling prostate cell proliferation, differentiation, and apoptosis. Normal transcripts and proteins of TGF-β receptors are frequently lost in prostate cancer cells, especially in advanced stages of the disease. However, the mechanisms by which TGF-β inhibits proliferation and induces apoptosis in prostate cancer cells is not clear. We investigated the molecular mechanism by which TGF-β inhibits transcriptional activation mediated by AR. Using transient transfection systems, we demonstrated that Smad3 specifically represses transcriptional activation mediated by AR on two natural androgen-responsive promoters. This repression is transmitted through TGF-β signaling and can be regulated by other Smad proteins. A protein-protein interaction between AR and Smad3 was demonstrated both in vitro and in vivo, and the transcription activation domain of AR and the MH2 of Smad3 were identified as being responsible for binding. Additional functional experiments showed that the repression of AR by Smad3 is mediated solely through the MH2 domain. These results provide fresh insight for understanding the mechanism by which TGF-β regulates the androgen-signaling pathway in prostate cancer cells.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (1). Like most cancers, prostate carcinogenesis likely involves a multistep progression from precancerous cells to cells that proliferate locally and eventually metastasize. The androgen-signaling pathway is important for the growth and progression of prostate cancer (reviewed in Ref. 2). Androgen withdrawal by castration has been commonly used to treat advanced prostate cancer. The growth-promoting effects of androgen are mediated mostly through the androgen receptor (AR)3 (3, 4). Like other steroid hormone receptors, AR is a ligand-activated transcription factor that modulates diverse biological effects through regulation of downstream target genes (5, 6). Upon activation by ligand, the AR translocates to the nucleus, where it binds to the androgen response element and recruits other cofactors to regulate transcription (2, 7). The signals mediated by transforming growth factor β (TGF-β) play fundamental roles in the determination of cell fate and growth control (8, 9). Of importance to note for our study here, TGF-β is a potent growth inhibitor of prostatic epithelial cells (10, 11). In prostate cancer, the TGF-β pathway is often inactive, with the loss of normal RNA transcripts and proteins of TGF-β receptors (11–13). Restoration of the TGF-β pathway in prostate cancer cells that are originally refractory to TGF-β can suppress in vitro tumorigenic growth by inhibiting cell proliferation (14, 15).

TGF-β signals are transduced through a group of intracellular signal transducers, the Smad protein family (8, 16). The Smad proteins can be divided into three distinct subgroups, the receptor-regulated Smads (R-Smads; 1, 2, 3, 5, and 8), the common mediator Smads (Co-Smads; 4), and the inhibitory Smads (I-Smads; 6 and 7), that antagonize the signaling function of the R- and Co-Smads. Upon stimulation by TGF-β or related growth factors, receptor-activated Smads become phosphorylated by the kinase activity of activated TGF-β receptors, oligomerize with the common Smads, and move into the nucleus. These activated Smad protein complexes exert their transcriptional function by binding to DNA and forming active complexes with other transcription factors. Smads have been found to complex with transcriptional activators and repressors, such as AP-1 (17), CBP/P300 (18, 19), and TGFIF (20), to regulate a variety of cellular processes. The activities of nuclear hormone receptors are mediated, at least in part, through the Smad proteins. For example, the vitamin D receptor (VDR) interacts with Smad3 to enhance ligand-dependent transcription (21), and the glucocorticoid receptor (GR) inhibits TGF-β-responsive promoters through a physical interaction with Smad3 (22).

In general, Smad proteins consist of three domains including an NH2-terminal MH1 domain, a COOH-terminal MH2 domain, and an intervening linker between these two domains (8, 9, 16). The MH1 domain is very similar in R-Smads and Smad4, but only weakly conserved in I-Smads (23). Although the functions of MH1 are not clear, it has been shown that the MH1 domain has intrinsic affinity to the MH2 domain and inhibits the MH2 functions. The MH2 domain has been identified as involved in protein-protein interaction (8). However, in the case of I-Smads, the MH2 domain was shown to be sufficient for their inhibitory effect (24).

Androgen promotes the growth and proliferation of prostatic epithelial cells and TGF-β negatively regulates this process, suggesting that the cross-talk between TGF-β and androgen-signaling pathways may play significant roles in regulating prostate cancer growth and progression. Expression of Smad proteins has been observed in the prostate tissues (25). However, the mechanism by which TGF-β/Smads inhibit proliferation and induce apoptosis in prostate cancer cells is not clear. Moreover, it is also unclear whether the cellular effects of TGF-β in prostate are mediated through AR. We therefore investigated the molecular mechanism by which TGF-β inhibits transcriptional activation mediated by AR and identified the region responsible as the MH2 domain. Protein-protein interaction between AR and Smad3 was demonstrated both in vitro and in vivo. These results provide new information for understanding how TGF-β regulates the androgen-signaling pathway.

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2 To whom requests for reprints should be addressed, at Departments of Surgery and Genetics, Stanford University, 300 Pasteur Drive, R135, Stanford, CA 94305-5328. E-mail: zsun@stanford.edu.

3 The abbreviations used are: AR, androgen receptor; TGF-β, transforming growth factor β; R-Smad, receptor-regulated Smad; Ca-Smad, common mediator Smad; I-Smad, inhibitory Smad; VDR, vitamin D receptor; GR, glucocorticoid receptor; aa, amino acid; GST, glutathione S-transferase; ER, estrogen receptor; TR, thyroid receptor; FBUS, fetal bovine serum; RELU, relative light unit; TGFIF, 5′TG3′ interacting factor; DHT, dihydrotestosterone; PSA, prostate-specific antigen; TAD, transactivation domain; DBD, DNA-binding domain; LBD, ligand-binding domain; MMTV, murine mammary tumor virus.
MATERIALS AND METHODS

Plasmids. Smad expression constructs have been described previously (26). The truncated Smad3 expression constructs containing the MH1 and MH2 domains were constructed by subcloning the N-amino acids (aa) 1–212 or COOH-terminal (aa 212–426) fragments into the pcDNA3 vector (Invitrogen, Carlsbad, CA) in-frame with an N-terminal flag epitope tag. The same MH1 and MH2 fragments of Smad3 were also subcloned into the pVP16 vector for mammalian two-hybrid analysis. Various deletion mutants of AR were cloned into pGEX-2TK (Amersham, Arlington Heights, IL) for making glutathione S-transferase (GST) fusion proteins (27, 28). These AR-truncated fragments were also cloned downstream of the GAL4 DNA-binding domain (DBD) in the pM expression vector (Clontech, Palo Alto, CA). Luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (−41 to +61), with or without four GAL4-binding sites, were provided by Dr. Donald Ayer (29). pVP16AD-Smad2 and 3 proteins were constructed by subcloning the full-length Smad2 and Smad3 cDNAs into pVP16 (Clontech).

The AR expression vector pSV-ArO was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). pMMTV-Apa3-luc was provided by Dr. Richard Pestell (Albert Einstein College of Medicine, New York, NY). The reporter plasmids, pPSA7kb and pPSA500bp-luc, with the luciferase gene under the control of promoter fragments of the human prostate-specific antigen were obtained from Trapman (30) and Bellegard (31). pSV-β-gal, an SV40-driven β-galactosidase reporter plasmid (Promega, Madison, WI) was used in this study as an internal control. The pSOG-ARA70 plasmid, containing the full-length ARA70 cDNA, and the reporter plasmid pARE-luc were kind gifts from Dr. Chang (32), pCMV-VDR and pVDRE-luc plasmids were provided by Dr. David Feldman (Stanford University, Stanford, CA). A human estrogen receptor (ER) expression construct (pcDNA3-ER) and a luciferase reporter plasmid with three estrogen-responsive elements were kindly provided by Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human tyrosine kinase receptor β (TRβ) expression vector driven by the SV40 promoter and a luciferase reporter controlled by two TRES were kindly provided by Dr. Anthony Hollenberg (Beth Israel Deaconess Medical Center, Boston, MA).

Cell Cultures and Transient Transfections. Both a monkey kidney cell line, CV-1, and a human prostate cell line, PC-3, were maintained in DMEM supplemented with 5% fetal bovine serum (FBS) (HyClone, Denver, CO). CV-1 cells have low levels of endogenous steroid hormone receptor activity and are capable of supporting TGF-β signaling (33). Transient transfections were carried out by using a LipofectAMINE transfection kit (Life Technologies, Inc., Gaithersburg, MD). Approximately 3 × 10⁶ cells were seeded into a 24-well plate 16 h before transfection. About 300 ng of total plasmid DNA and 1.2 μl of LipofectAMINE/well were used in the transfection. Following transfection, the cells were washed and fed medium containing 5% charcoal-stripped FBS (steroid hormone free; HyClone) in the presence or absence of steroid hormones. Cells were incubated for another 24 h, and luciferase activity was measured as relative light units (RLU) in a Monolight 3010 luminometer (PherMingen, San Diego, CA) according to the manufacturer’s protocol. The RLU from individual transfections were normalized by β-galactosidase activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean RLU/β-galactosidase (±SD) from representative experiments.

GST-Pull-Down Assay. Expression and purification of GST fusion proteins were performed as described previously (34). The full-length and truncated Smad3 proteins were generated and labeled in vitro by the TnT-coupled reticulocyte lysate system (Promega). Equal amounts of GST-AR fusion proteins coupled to glutathione-Sepharose beads were incubated with radiolabeled Smad3 proteins at 4°C for 2 h in a modified binding buffer (20 mM Tris-HCl (pH 7.8), 180 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 50 μM ZnCl₂, 10% glycerol, 0.1% NP-40, 0.05% dry nonfat milk, 1 mM DTT, and 0.5 mM PMSF). Beads were carefully washed three times with 500 μl of binding buffer and then analyzed by SDS-PAGE followed by autoradiography.

Immunoprecipitation and Western Blotting. The human AR expression vector pARO, alone or with a Flag-tagged pcDNA3-Smad3 expression plasmid, was transfected into CV-1 cells. Transfected cells were cultured in the presence of 10 nm R1881 for 48 h and then harvested in a buffer containing 0.5% NP-40, 150 mM NaCl, 2 mM MgCl₂, 50 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.5 mM PMSF, and 25 mM NaF. Lysates were clarified by incubation on ice and centrifugation for 5 min; 400 μl of clarified lysate from each sample were preclarified for 20 min with 10 μl of protein A-Sepharose beads bound to 1 μg of antigo IgG (Pharmacia, Arlington Heights, IL). Preclarified lysates were incubated with prequillibrated protein A-Sepharose beads with either mouse normal IgG or Flag monoclonal antibody (Sigma, St. Louis, MO) at 4°C for 3 h. The beads were washed three times in 500 μl of lysis buffer and were eluted by boiling in SDS-PAGE sample buffer. Following SDS-PAGE, proteins were transferred to nitrocellulose (BA85; Schleicher & Schuell, Keene, NH) and blocked overnight at 4°C in TBST (50 mM Tris-HCl, 150 mM NaCl, and 0.08% Tween 20) with 5% low-fat milk. The first antibody was a polyclonal antibody against the NH₂ terminus of AR and was used at 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was antimouse IgG conjugated to horseradish peroxidase and was used at 1:2000 dilution (Bio-Rad). Detection was performed with ECL reagents according to manufacturer’s protocol using ECL Hyperfilm (Amersham).

RESULTS

Smad3 Represses Ligand-dependent Activation of the AR. TGF-β has been shown to be a negative proliferation regulator for prostate cells. To determine whether TGF-β signaling in prostate cells is mediated through Smad proteins, we investigated a possible role for Smad proteins in regulating androgen-dependent transcription. Plasmids capable of expressing AR, various Smad proteins, and a luciferase reporter plasmid regulated by the androgen-responsive elements in the MMTV-LTR (MMTVpA3-Luc) were transfected into CV-1 cells. The cells were cultured with FBS containing various growth factors, including TGF-β. An approximately 18-fold induction of AR-mediated transcriptional activity was observed in the presence of 10 nm DHT (Fig. 1). Ligand-dependent AR activation was repressed nearly 6-fold by cotransfection of the Smad3 expression construct. Expression of other Smad family members, including the R-Smad2 and the Co-Smad4 as well as the I-Smad6 and I-Smad7, showed no effect (Fig. 1). The positive control, the known AR coactivator ARA70, brought about an additional 2-fold increase in DHT-dependent transcription (32). Thus, Smad3 specifically repressed transcription from an AR-dependent promoter.

The human prostate-specific antigen (PSA) is an AR-regulated target gene that has been widely used as a prostate-specific tumor marker in the clinic (30, 35). To determine whether the repression of AR activity by Smad3 could be reproduced on a natural AR-dependent promoter, transient transfections were repeated in CV-1 cells with a luciferase reporter driven by the 7-kb PSA promoter (31). A similar dose-dependent inhibitory effect of Smad3 was observed on AR-mediated transcription from the PSA promoter, whereas Smad2 had no effect (Fig. 2A). Smad3 also repressed a minimal PSA promoter, containing only the proximal 330 bp of the PSA promoter (Fig. 2B). The 330 bp contains the two androgen-responsive regions and little else. To ensure that the Smad3-mediated inhibition did not reflect toxic or other nonspecific effects of the cotransfected plasmids, luciferase expression in all experiments was normalized using β-galactosidase production from a cotransfected plasmid. We examined the intracellular steady-state levels of AR protein in the transfectants and found them to be similar, indicating that the Smad3-mediated repression was not due to lowered levels of expression of AR from the cotransfected plasmid (data not shown). The results strongly suggest that Smad3 inhibited AR-regulated transcription from multiple, naturally occurring androgen-responsive promoters.

Repression of Smad3 Is Induced by TGF-β and Regulated by Other Smad Proteins. Previous studies have demonstrated that TGF-β signals are transmitted by Smad proteins. However, the presence of other Smad family members, including the R-Smad2 and the Co-Smad4, did not significantly affect the Smad3-mediated repression (Fig. 3). In contrast, repression of Smad3 by TGF-β was strongly inhibited by the Co-Smad4 (Fig. 3B). Smad3 repression by TGF-β was also significantly inhibited by the I-Smad6 and I-Smad7 (data not shown). These results suggest that Smad3 inhibition by TGF-β requires the presence of other Smad family members, including the R-Smad2 and the Co-Smad4.
Smads are the only TGF-β receptor substrates with a demonstrated ability to propagate TGF-β signals. To determine whether repression of AR by Smad3 is induced by TGF-β, we repeated the transfection experiments with serum-free medium, with or without TGF-β (36). In this experiment, there was a 3-fold decrease in AR-dependent transcription when Smad3 was cotransfected with the MMTV-AR-Luc construct (Fig. 3A). Addition of TGF-β1 reduced the level of luciferase expression to almost background levels (Fig. 3A). TGF-β1 by itself had no effect on transcription from the MMTV promoter (Fig. 3A). The results suggest that the repression mediated by Smad3 is a downstream effect of TGF-β signaling.

To further evaluate the repression by TGF-β/Smad3 of AR-dependent transcription in a physiologically relevant cellular context, transient transfections were repeated in PC-3 cells, a human prostate cancer cell line. Promoter activity was measured in the presence and absence of TGF-β. As seen in Fig. 3B, either TGF-β or Smad3 alone did not significantly alter PSA promoter activity. However, in the presence of TGF-β, Smad3 was able to reduce AR induction to almost baseline levels (Fig. 3B). Smad3 proteins were below detection limit in the whole-cell lysates prepared from both CV-1 and PC-3 cells as determined by Western blotting with a specific Smad3 antibody (data not shown). Again, this demonstrates that Smad3 can repress androgen signaling in a prostate-derived cell type, and this repression requires activation of Smad3 by TGF-β.

As a receptor-activated Smad protein, Smad3 activity is modulated by other Smad proteins including the Co-Smad4 and I-Smads. Previous studies have shown that Smad4 can form a heterodimer with Smad3 and be translocated into the nucleus to activate the transcriptional response (37–39). The I-Smads are believed to function by competing with the R-Smads for phosphorylation by the TGF-β receptors (24, 40). Although neither the Co-Smad4 nor I-Smads alone affected AR-dependent transcription (Fig. 1), we needed to test whether they could modulate the ability of Smad3 to repress AR function. As seen in Fig. 3C, while expression of Smad3 alone affected AR-dependent transcription approximately 8-fold, coexpression of Smad4 and Smad3 resulted in greater repression than expression of Smad3 alone. When Smad7 was coexpressed with Smad3, Smad7 alleviated the repression by Smad3 of the MMTV promoter (Fig. 3D). This effect was specific to Smad7, as expression of Smad6 showed no significant impact (Fig. 3D). Taken together, these data are consistent with our observation that no endogenous Smad3 protein was detected in CV-1 cells and suggest that Smad3-mediated repres...
cells were cultured with $10^{-2}$ for 24 h, respectively. The cells were harvested and luciferase activities were measured.

B

with $10^{-2}$ were performed in CV-1 cells. Ten nanograms of Smad3 and 5 or 10 ng of pCMV-Smad2 or pCMV-Smad4 were added to the transfections where indicated. The cells were treated with $10^{-8}$ M DHT or left untreated as indicated. D, 20 ng of Smad7 or Smad6 expression constructs were cotransfected with 10 ng of pCMV-Smad3 as well as the reporter constructs.

Fig. 3. Repression of Smad3 on AR is a downstream effect of TGF-β. A, CV-1 cells were transfected with the same amount of different reporters and expression constructs as described in the legend to Figs. 1 or 2. After 6 h of transfection, cells were incubated with complete serum-free medium (MCDB105) and treated with $10^{-8}$ M DHT and 1 ng/ml TGF-β for 24 h, respectively. The cells were harvested and luciferase activities were measured. B, a prostate cancer cell line, PC-3, was transfected as described in A. Following transfection, cells were cultured with $10^{-8}$ M DHT and 1 ng/ml TGF-β. Fold induction represents the ratio of luciferase values in the presence versus absence of DHT. C, The transient transfections were performed in CV-1 cells. Ten nanograms of Smad3 and 5 or 10 ng of pCMV-Smad2 or pCMV-Smad4 were added to the transfections where indicated. The cells were treated with $10^{-8}$ M DHT or left untreated as indicated. D, 20 ng of Smad7 or Smad6 expression constructs were cotransfected with 10 ng of pCMV-Smad3 as well as the reporter constructs.

Fig. 4. The repressive effect of Smad3 is selective. For each sample, 50 ng of pSV-β-gal, 200 ng of luciferase reporter vectors (pVDRE-luc, pERE-luc, and pTRE-luc), and 20 ng of the corresponding receptor expression constructs were transfected into CV-1 cells, with or without 20 ng of pCMV-Smad3. Eighteen hours posttransfection, the specific ligands to each receptor were added in the following concentrations: $10^{-8}$ M 1α,25-dihydroxyvitamin D$_3$ (VDR), $10^{-7}$ M β-estradiol (ER), and $10^{-8}$ M triiodothyronine (T$_3$).
construct was used (Fig. 6B), consistent with previous evidence of an activation domain within aa 1–563 of AR. Transcription was enhanced when either the full-length or the MH2 domain of Smad3 was added, but not when the MH1 domain of Smad3 or full-length Smad2 was used. Furthermore, in agreement with the results of the in vitro binding assays and coimmunoprecipitation, the MH2 domain of Smad3 was shown to have an even stronger interaction with AR than full-length Smad3 in this experiment. On the basis of the data from multiple lines of experimentation, we conclude that Smad3 specifically interacts with AR and that this interaction is mediated by the MH2 domain of Smad3 and the TAD domain of AR.

The MH2 Domain of Smad3 Contributes to Inhibition of AR.

Results from both in vitro and in vivo experiments have shown that the MH2 domain of Smad3 directly mediates interaction with AR. To determine whether the MH2 domain alone could repress an AR-dependent promoter, full-length Smad3 was compared to MH1 and MH2 in a transcription repression experiment. Expression of full-length Smad3 led to the expected ligand-dependent repression of AR (Fig. 7). Full repression of AR-induced activation was also achieved by transfection with the MH2 domain alone. In contrast, the MH1 domain had little effect on transcription. Thus, the MH2 domain of Smad3 is sufficient for repression of AR activation.

**DISCUSSION**

Numerous studies have demonstrated that TGF-β functions as a negative growth regulator and inhibits androgen-promoted growth in prostate cells. Although a potential cross-talk between TGF-β and androgen pathways has been suggested, there were no data identifying the link. The data we present here serve to identify a convergent point between these pathways. Specifically, Smad3 physically and functionally interacts with AR. Our results are consistent with the previous studies on the physiological relationship between TGF-β and AR in the regulation of normal prostate tissue growth and in the development and progression of prostate cancer (12, 13, 41).

Loss of the TGF-β-signaling pathway has been frequently observed in prostate cancer cells (42, 43). The evidence provided in this study has shown Smad3 repressing AR-mediated transcription on both MMTV and PSA promoters. PSA has been broadly used as a clinical marker to monitor the progression of prostate cancer. Notably, repression was seen with an intact natural promoter from the PSA gene as well as with a mini-promoter containing little more than the androgen response elements. In addition, this Smad3-mediated repression was enhanced by Smad4 but diminished by Smad7. Taken together, these findings indicated a direct role of Smad3 to repress the AR-mediated transcription and suggest a potential link between TGF-β/Smads signaling and suppression of human prostate cancer progression.

The Smad3-induced repression of AR-mediated transcription is quite novel and involves a mechanism that is distinct from previous reports on the cross-talk between Smads and nuclear hormone receptors. Smad3 was found to enhance VDR-mediated, ligand-dependent transactivation through interaction of the MH1 domain of Smad3 and
the LBD of VDR. This complex then recruits the steroid receptor coactivator (21). In contrast, the GR has been shown to repress Smad3-mediated transcription, illustrating that regulation can also occur in the opposite direction (22). Our finding that Smad3 represses AR-mediated transcription thus represents a new molecular mechanism for TGF-β/Smad regulation of nuclear receptors.

The MH2 domain of Smad3 was found to mediate its binding to AR and to be responsible for repressing AR transactivation. These data are consistent with previous studies of Smad structure-function where the MH2 domain was shown to be involved in many biological processes through interaction with other regulatory proteins (8, 20). The MH2 domain mediates both homomeric and receptor-induced heteromeric interactions between Smad4 and R-Smads (37, 39). The MH2 of Smad3 also interacts with GR, suggesting an important role for the MH2 domain in regulating nuclear receptors (22). The biological activity of the MH2 domain may be modulated by interaction with the MH1 domain, when the protein is not phosphorylated. Upon receptor-mediated phosphorylation, this interaction may be altered and each domain may display the DNA and protein interactions required for the proper activity of the transcriptional complex (44). Our results demonstrating that deletion of the MH1 domain was required to detect AR-Smads interaction by coimmunoprecipitation are consistent with previous studies of Smad structure-function where the MH1 domain is dispensable for the repression, it seems unlikely that repression by Smad3 is attributable to competition with AR for DNA binding. Since the TAD of AR has been mapped to interact with Smad3 protein, one possible mechanism for repression by Smad3 could be attributable to disrupting the conformation of AR and recruiting other coactivators in the complex. Supporting this hypothesis are data showing that upon ligand binding an intrinsic interaction of AR between the NH2- and COOH-terminal fragments occurs to form an active transcriptional complex (51).

In conclusion, this study demonstrates for the first time that Smad3 selectively represses AR-mediated transcription. The protein-protein interaction between AR and Smad3 may present a novel mechanism for cross-talk between the TGF-β and androgen-signaling pathways. Given that prostate cancer cells are generally androgen dependent but have defects in TGF-β receptor expression, these data provide important new information into the molecular consequences of the loss of TGF-β signaling. Further study of the regulation by TGF-β and Smad3 of androgen-induced transcription will provide fresh insights into the biology of prostate cancer and may lead to the development of new treatments.

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