Loss of SPDEF and gain of TGFBI activity after androgen deprivation therapy promote EMT and bone metastasis of prostate cancer

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Androgen deprivation therapy (ADT) targeting the androgen receptor (AR) is a standard therapeutic regimen for treating prostate cancer. However, most tumors progress to metastatic castration-resistant prostate cancer after ADT. We identified the type 1, 2, and 4 collagen-binding protein transforming growth factor–β (TGFβ)–induced protein (TGFBI) as an important factor in the epithelial-to-mesenchymal transition (EMT) and malignant progression of prostate cancer. In prostate cancer cell lines, AR signaling stimulated the activity of the transcription factor SPDEF, which repressed the expression of TGFBI. ADT, AR antagonism, or overexpression of TGFBI inhibited the activity of SPDEF and enhanced the proliferation rates of prostate cancer cells. Knockdown of TGFBI suppressed migration and proliferation in cultured cells and reduced prostate tumor growth and brain and bone metastasis in xenograft models, extending the survival of tumor-bearing mice. Analysis of prostate tissue samples collected before and after ADT from the same patients showed that ADT reduced the nuclear abundance of SPDEF and increased the production of TGFBI. Our findings suggest that induction of TGFBI promotes prostate cancer growth and metastasis and can be caused by dysregulation or therapeutic inhibition of AR signaling.

INTRODUCTION
Targeting androgen receptor (AR) functions by androgen deprivation therapy (ADT) is initially clinically effective; however, most tumors become resistant within a few years and progress to castration-resistant prostate cancer (CRPC) (1–3). In a significant percentage of CRPC cases, AR functions are still active because of different mechanisms (4), which justifies inhibition of AR signaling remaining a key therapeutic strategy (5). The epithelial-to-mesenchymal transition (EMT) is emerging as one factor promoting metastatic CRPC (6, 7) and is involved in resistance to chemotherapy (8). Several lines of evidence show the consequential effect of decreasing AR content to EMT (9, 10) and support its role in metastasis progression in prostate cancer patients after ADT (11–13). Therefore, understanding cross-talk between AR signaling and the EMT can lead to novel therapeutic strategies for CRPC.

Transforming growth factor–β (TGFβ) pathway is a master signaling for the EMT, and many studies indicate a negative cross-talk between the AR and the TGFβ signaling (14–16). TGFβ-induced protein (TGFBI) is one downstream effector of the TGFβ signaling pathway (17) and is involved in extracellular matrix (ECM) remodeling that promotes the EMT and metastasis in several types of cancer (18, 19). It has been suggested that TGFBI regulates the metastatic process by promoting adhesion to lymphatic endothelial cells under hypoxic conditions (20) and by regulating secretion of metalloproteinases (21). However, whether TGFBI is involved in the development of metastatic CRPC after ADT remains unclear.

The transcription factor SPDEF is a member of the ETS (E26 transformation–specific) transcription factor family (22). There is a mutual regulation loop between SPDEF and the AR (23), and SPDEF also interacts with homeobox protein NKX-3.1 to regulate the expression of the gene encoding prostate-specific antigen (24). SPDEF is believed to be a tumor suppressor (25), and loss of SPDEF is associated with induction of the EMT (26). In addition, the TGFβ pathway reportedly inhibits the transcription of SPDEF (27), suggesting cross-talk between TGFβ and AR signaling during prostate tumor progression. We hypothesized that inactivated AR signaling after ADT is linked to a TGFβ-signaling–associated program that enables prostate cancer epithelial cells to undergo the EMT, thus facilitating the development of metastatic CRPC. Herein, we investigated the mechanism that links inactivated AR signaling after ADT to a TGFβ-associated program and report an inhibitory role of the AR on TGFβ signaling through SPDEF-mediated suppression of TGFBI.

RESULTS
TGFβ-induced TGFBI is positively associated with the EMT in prostate cancer
To investigate EMT regulation during cancer progression, we used the AC3 prostate cancer cell line, which was derived from a prostate-specific Pten/Tp53-null mouse (28) and showed self-reinforcing TGFβ signaling leading to the EMT (29, 30). We thus compared the TGFβ/BMP gene signature profile of mesenchymal type cells (EpCAM negative, AC3E–) to that of epithelial type cells (EpCAM positive, AC3E+) by polymerase chain reaction (PCR) array analysis (fig. S1A), and confirmed that both messenger RNA (mRNA) and protein of TGFBI were enriched in mesenchymal type AC3E– cells (Fig. 1A and fig. S1B). Moreover, in the parental AC3 line, the expression of Tgfb1 and two other mesenchymal markers (Vim and Slug) was induced after TGFβ treatment (Fig. 1B) but suppressed by a TGFβ inhibitor (SB431542) (Fig. 1C). We further examined the TGFBI response in human prostate cancer cells and found that AR-negative cells (PC3 and RasB1) were more sensitive to TGFβ modulation than were AR-positive cells (LNCaP and 22Rv1) (Fig. 1, D and E). Given that TGFβ is a “master regulator” of EMT,
we demonstrated that overexpressing TGFBI activated the expression of EMT markers in AR-positive cells (Fig. 1F and fig. S1, C and D) but reduced the abundance of an epithelial marker (CDH1) (Fig. 1F). In contrast, TGFBI knockdown decreased VIM and increased CDH1 in RasB1 cells regardless of exogenous TGFβ (Fig. 1G). These results indicated that induction of TGFBI is associated with TGFβ signaling and the EMT in prostate cancer.

**AR signaling inhibits TGFBI**

Given that AR-negative cells were more responsive to TGFβ than AR-positive cells after treatment with TGFβ or a TGFβ inhibitor (Fig. 1, D and E), we hypothesized that it was possible that TGFBI is repressed by AR signaling. To address this issue, we performed AR knockdown and observed increased expression of TGFBI mRNA in AR-positive 22Rv1 and LNCaP cells (Fig. 2A), and abundance of the induced TGFBI protein was unresponsive to the TGFβ inhibitor (Fig. 2B). In contrast, reconstitution of the AR in AR-negative PC3 and RasB1 cells reduced TGFBI abundance (Fig. 2C), counteracting the inductive effects of TGFβ (Fig. 2D). These results suggested that TGFBI is regulated by AR signaling in addition to TGFβ signaling. We further tested whether TGFBI can be regulated by AR signaling modulators using the agonist dihydrotestosterone (DHT) and an inhibitor (MDV3100). DHT treatment reduced TGFBI (Fig. 2, E and F), whereas MDV3100 induced them (Fig. 2, G and H). We further confirmed that suppression of TGFBI was dependent on the AR using a small interfering RNA (siRNA) in LNCaP cells (Fig. 2I) and through reconstitution of AR in PC3 cells (Fig. 2J). Next, we analyzed TGFBI expression in The Cancer Genome Atlas (TCGA) prostate cancer data set, which was grouped on the basis of relative AR activities defined by the levels of androgen-responsive signatures (31, 32). We found that RasB1 cells had a better survival rate (Fig. 3C) and a lower extravasation and seeding frequency of tumor cells into both the bone and the brain (Fig. 3, D and E). We confirmed the lower metastasis incidence by a histopathological examination (Fig. 3F). In addition to decreased lesion formation in the metastasis model, TGFBI knockdown reduced proliferation (Fig. 3G) and colony formation (Fig. 3H) in cultured cells and suppressed tumor growth (Fig. 3, I to K) in a subcutaneous xenograft model. Finally, in cultured AR-positive cells (LNCaP and LNCaP-AR), TGFBI promoted cell migration, invasion, and proliferation (fig. S2, A to D). Overexpression of TGFBI was confirmed by Western blotting (fig. S2E). Thus, TGFBI critically promotes malignant progression in prostate cancer.

**TGFBI is required for metastasis and tumor growth of prostate cancer cells**

Because TGFBI induced an EMT (Fig. 1 and fig. S1), we examined its role in metastasis-related activities in AR-negative prostate cancer cells. We found that TGFBI knockdown in two stable cell lines expressing TGFBI-targeting short hairpin RNA (shRNA) reduced migratory activities (Fig. 3A). Knockdown efficiencies of TGFBI were confirmed by a Western blot assay (Fig. 3B). Next, we examined metastasis effects of TGFBI using the experimental metastasis model of intracardiac injection of tumor cells in mice. We found that mice injected with stable TGFBI-knockdown AR activities were negatively associated with TGFBI expression (Fig. 2K). Moreover, the relationship between TGFBI expression and AR signaling was confirmed by a gene set enrichment analysis (GSEA) using the Taylor prostate cancer database (33) with another androgen-response signature (34), showing that increased TGFBI was associated with the gene set inactivated by AR signaling (fig. S1E). These results support that AR signaling suppresses TGFBI in prostate cancer.

**SPDEF is activated by AR signaling and suppresses TGFBI**

To identify upstream factors that suppress TGFBI expression upon AR signaling activation, we analyzed the inverse association between TGFBI expression and androgen-activated gene sets (31, 32) in TCGA prostate data set by a GSEA (fig. S3, A and B) and then identified SPDEF in the core enrichment group as a possible regulator (fig. S3B). We confirmed an inverse relationship between the expression of SPDEF and that of TGFBI in a panel of prostate cancer cells (Fig. 4A). We showed that TGFBI expression was associated with AR-negative cells, whereas SPDEF was expressed in AR-positive cells (Fig. 4A). We next examined whether AR activity stimulates SPDEF. As we expected, DHT increased SPDEF abundance on both mRNA and protein levels, whereas an AR inhibitor (MDV) decreased both in AR-positive cells (Fig. 4, B and C). We thus studied the role of SPDEF in TGFBI expression using an SPDEF-knockdown approach (Fig. 4D). We found that SPDEF knockdown increased the abundance of endogenous TGFBI mRNA even in cells treated with DHT (Fig. 4, E and F). Furthermore, a correlation analysis using two

![Figure 1](http://stke.sciencemag.org/)

*Fig. 1. TGFBI is associated with TGFβ signaling and EMT markers in prostate cancer. (A) Western blotting for TGFBI in AC3 cells in the presence (E+) or absence (E−) of EpCam. (B and C) Quantification of TGFBI, VIM, and SLUG mRNAs in AC3 cells after 24 hours treatment with TGFβ (B) or a TGFβ inhibitor (SB431542) (C) relative to vehicle (Veh)– or dimethyl sulfoxide (DMSO)–treated cells. (D and E) Quantification of TGFBI mRNA in a panel of prostate cancer cells after treatment with TGFβ (D) or SB431542 (E) as in (B) and (C). (F) Western blotting of lysates from cells after expression by transient transfection with TGFBI or empty vector (EV). (G) Western blotting of lysates from RasB1 cells transfected with control (Luc) or TGFBI-targeted shRNA and treated with vehicle (−) or TGFβ. Blots are representative of three independent experiments. Data are means ± SEM from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.*
clinical data sets [TCGA and Taylor et al. (33)] confirmed the inverse correlation between TGFBI and SPDEF (Fig. 4G). These data suggest that SPDEF is involved in androgen-mediated suppression of TGFBI in prostate cancer.

**SPDEF transcriptionally suppresses TGFBI**

Using two SPDEF-specific shRNAs in AR-positive cells, we observed induction of TGFBI at both the mRNA (Fig. 5A) and protein (Fig. 5B) levels. Conversely, overexpression of SPDEF in AR-negative cells resulted in reduced TGFBI abundance (Fig. 5, C and D). We hypothesized that SPDEF acts as a transcriptional repressor of the TGFBI promoter. Analysis of chromatin immunoprecipitation (ChIP)-seq data (35) revealed that SPDEF appeared to bind multiple sites of the TGFBI gene (fig. S3C), and a search for putative SPDEF response elements (SREs) identified five candidate SREs in the TGFBI promoter (SRE1 to SRE5; Fig. 5E). ChIP assays indicated that two SRE sites (SRE1 and SRE5) were enriched with SPDEF after DHT treatment (Fig. 5F), supporting that binding is site-specific and AR-dependent. Consistently, the endogenous binding of SPDEF was reduced only at SRE1 and SRE5 after MDV treatment (Fig. 5G). Using a reporter assay in which we mutated SRE1 or SRE5 in the TGFBI promoter (Fig. 5E), we found that DHT decreased wild-type reporter activity, but inclusion of MDV rescued activity (Fig. 5H). Mutating either SRE increased reporter activity (Fig. 5I) and disrupted the inhibitory effects of DHT (Fig. 5F) or SPDEF overexpression (Fig. 5K), which supports a site-specific repressive interaction between SPDEF and the two SREs. Thus, we conclude that reduction of TGFBI by AR signaling is dependent on SPDEF-mediated transcriptional repression.

**TGFBI compensates for the tumor suppressor effects of SPDEF and promotes proliferation in an androgen deprivation condition**

Earlier studies showed that SPDEF is a tumor suppressor (21). Stable SPDEF knockdown in nonmetastatic LNCaP-AR cells significantly increased their proliferation (Fig. 6A) and migratory activity (Fig. 6B). Conversely, overexpression of SPDEF in AR-negative RasB1 cells inhibited proliferation (Fig. 6C). Although SPDEF can transcriptionally suppress endogenous TGFBI expression, coexpression of exogenous TGFBI overcame the inhibitory effects of SPDEF and increased cell proliferation (Fig. 6, C and D). In addition, we monitored metastasis-related functions (migration and invasion) and found that SPDEF significantly suppressed both; however, coexpression of TGFBI rescued the inhibitory effects of SPDEF (Fig. 6, E and F). Likewise, SPDEF

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Fig. 2. AR signaling negatively regulates TGFBI. (A) TGFBI mRNA expression in 22Rv1 or LNCaP cells treated with a control (NC) or AR-targeted siRNA (siAR) for 48 hours. (B) Western blotting of LNCaP cells after AR knockdown and TGFβ inhibition (SB431542) for 24 hours. (C) Western blotting of AR-negative cells stably transduced with either a control (EV) or AR-expressing (AR) vector. (D) Western blotting of RasB1 cells stably expressing AR (or empty vector) and treated with TGFβ for 24 hours. (E and F) Abundance of TGFβ at the mRNA (E) or protein (F) level in control or AR-overexpressing LNCaP cells and treated with DHT or vehicle (−) for 24 hours. (G and H) As in (E) and (F) in cells treated with AR inhibitor MDV3100 (MDV) or vehicle for 24 hours. (I) Western blotting of LNCaP cells transfected with AR-targeted siRNA (siAR) for 48 hours then DHT for 24 hours. (J) Western blotting of parental cultures of PC3 cells and those transiently transfected with AR then treated with DHT. (K) Mean TGFβ expression in TCGA prostate cancer data set grouped by relative AR activity based on a gene set [Nelson et al. (31) or Wang et al. (32); n = 207 per group]. Significance was determined by Student’s t test. Western blots are representative of three independent experiments. Data are means ± SEM. n = 3 biological replicates. **P < 0.01, ***P < 0.001.
knockdown increased TGFBI abundance and rendered it nonresponsive to the inducible effect of AR inhibition (fig. S4A). On the basis of a few traditional protein markers, SPDEF knockdown promoted an EMT switch, reducing the abundance of epithelial markers (CK18 and CDH1; fig. S4B) and increasing that of mesenchymal markers (SLUG and VIM; fig. S4C). This EMT effect of SPDEF loss was at least partly due to consequently increased TGFBI, given that TGFBI knockdown prevented the effects of SPDEF knockdown (fig. S4, B and C). We further tested whether TGFBI is involved in the development of ADT resistance by mimicking androgen deprivation with charcoal-stripped serum (CSS). Under this condition, overexpression of TGFBI enhanced the proliferation rate of LNCaP-AR cells (Fig. 6, G and I). Even when cells were cultured in both CSS and MDV, overexpression of TGFBI still enhanced proliferation rates (Fig. 6, H and J). These results support our hypothesis that the abundance of TGFBI contributes to ADT resistance and promotes the progression of prostate cancer.

DISCUSSION

Our findings support the tumor promoter functions of TGFBI and a molecular mechanism that SPDEF is a key transcriptional factor repressing TGFBI under androgen regulation (Fig. 7H). Reducing the activity of AR signaling by ADT or pharmacological AR inhibitors suppressed SPDEF, which activates EMT and TGFβ signaling, facilitating the development of metastatic CRPC (Fig. 7H). This model is consistent with the antitumor effects of SPDEF (25) and its role in controlling the metastatic process (36). In addition, because it was shown that SPDEF can be repressed by TGFβ signaling (22), our finding on the AR-SPDEF-TGFBI axis further

Prostate cancers in patients receiving ADT have reduced SPDEF abundance and increased TGFβ signaling

Our results suggested that the AR-SPDEF axis serves as a gatekeeper to maintain TGFBI abundance below a threshold. We confirmed the inverse correlation between TGFBI and SPDEF in a database of clinical tissue samples (Fig. 7A) and a prostate cancer tissue array (Fig. 7, B and C). Low-grade tumors had greater abundance of SPDEF and lower abundance of cytoplasmic TGFBI, whereas the opposite was seen in high-grade tumors (Fig. 7, B and C). Induction of TGFβ signaling is implicated in tumor progression and metastasis after ADT in prostate cancer patients (9–11). ADT-induced TGFβ signaling, as well as correlation with the SPDEF-TGFBI axis, was confirmed through analysis of consecutive tissue sections of prostate tumor samples isolated from patients before and after therapy, and phosphorylated SMAD2 as a readout for TGFβ signaling.

We found that nuclear SPDEF abundance was reduced (Fig. 7, D and E), and both TGFBI abundance and TGFβ signaling were increased in prostate tumors from patients who had received ADT compared to those from hormone-naïve patients (Fig. 7, D and F). Furthermore, using the TCGA prostate cancer data set, we found that patients with both low TGFBI and high SPDEF abundance correlated with better survival (Fig. 7G). Thus, TGFBI abundance is positively correlated with disruption of AR-SPDEF signaling and is associated with a poor prognosis.
from extracellular TGFβ are likely very different compared to earlier stages (intracellular AR-SPDEF axis). Therefore, in the early stages of prostate cancer, it is possible that many invasion or early stages of cancer, whereas it promotes malignancy (invasion and metastases) in late phases (40–42). It is possible that prostate cancer cells acquire new mutations resulting in different cellular contexts and that the AR and TGFβ signaling alternate at different stages of prostate cancer. Thus, normal epithelial cells rely on AR signaling to survive and mature, but TGFβ signaling can prevent its proliferation; however, primary prostate cancer may have increased AR activities or mutations countering the antitumor effects of TGFβ signaling (15, 16). Therefore, in the early stages of prostate cancer, it is possible that many downstream factors of TGFβ signaling, like TGFβI, are not only blocked from extracellular TGFβ ligand stimulation but also suppressed by the intracellular AR-SPDEF axis.

The cellular contexts of prostate cancer at later stages of progression are likely very different compared to earlier stages (43). In addition to AR-targeted therapies as proposed in our model (Fig. 7H), genes involved in the EMT or TGFβ signaling activation could be derepressed due to mutations of other tumor suppressor genes. This idea raises a question as to whether the frequent reduction of SPDEF in advanced prostate cancer, as demonstrated by our results and other reports (44), is due to genetic or epigenetic changes. Further investigation is required to confirm whether the genomic locus covering SPDEF is a mutation hotspot. However, even if the genome near SPDEF is intact, we might need to consider the possibility that the AR can no longer activate SPDEF in CRPC because transcription profiles of the AR greatly differ before and after ADT (43); therefore, genes involved in metastasis can still be derepressed due to the absence of the AR-SPDEF axis in CRPC.

The AR gene structure consists of three major domains: an N-terminal domain, a DNA binding domain, and a ligand-binding domain (LBD). Currently, more than 20 splice variants of the AR have been identified, and many lack the LBD (45). It is possible that selection of AR variants could account for reactivation of TGFβ signaling. It was shown that the AR can suppress TGFβ signaling through a physical interaction with Smad3 (16). The LBD of the AR is essential and sufficient for the association with the TGFβ effector Smad3, and DHT can enhance the interaction (16). Therefore, prostate cancers containing the AR variants that lack the LBD may be prone to malignant progression because TGFβ signaling is constitutively active even when cells cannot respond to the TGFβ ligand due to a lack of its cognate receptor. Supporting this idea, a transgenic mouse model expressing the AR splice variant, AR3 (loss of LBD), in the prostate epithelium showed increased tumor-promoting autocrine/paracrine factors, including TGFβ2 and EMT markers (46).

This AR-SPDEF-TGFβI axis provides one explanation of the common progression to metastatic CRPC after ADT in patients. In addition, there are several studies showing that inhibition of AR signaling activates other oncogenic programs (such as the pathway mediated by the phosphatidylinositol 3-kinase) that mediate progressively malignant outcomes (47, 48). Therefore, these findings indicate a clinical dilemma for targeting AR signaling in the management of prostate cancer. A combination of AR-based therapeutics with agents that inhibit AR-repressed tumor promoters should be considered in clinical trial settings.
Fig. 5. AR signaling inhibits TGFBI through SPDEF-dependent transcription. (A and B) Abundance of SPDEF and TGFBI mRNA (A) and protein (B) in the LNCaP-AR cells transfected with control (shLuc) or SPDEF-targeted shRNA (S1, shSPDEF-1; S2, shSPDEF-2). (C and D) Abundance of SPDEF and TGFBI mRNA (C) and protein (D) in PC3 and RasB1 cells transiently transfected with a control (EV) or SPDEF-expressing vector. (E) Top: Schematic of the predicted SREs in the promoter of human TGFBI. Bottom: Schematic of TGFBI expression reporter constructs showing the wild-type and mutant sequences of SRE1 and SRE5. (F and G) ChIP assays in LNCaP-AR cells treated with DHT (F) or MDV3100 (G). Antibody against GAPDH served as the control. Enrichment is given as a percentage of the total input and then normalized to immunoglobulin G (IgG). (H) Relative MFI of the TGFBI reporters (SRE1 and SRE5) in LNCaP-AR cells after treatment with DHT, MDV, and their combination (DHT + MDV). (I) Relative MFI of wild-type or mutant-SRE (M1, M5) TGFBI reporters in LNCaP-AR cells. (J and K) TGFBI reporter activity in response to DHT in LNCaP-AR cells (J) or transient SPDEF overexpression in RasB1 cells (K). Western blots are representative of three independent experiments. Data are means ± SEM from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 6. TGFBI promotes malignant phenotypes in prostate cancer cells. (A and B) Effect of SPDEF knockdown in LNCaP-AR cells on proliferation (A) and migration (B) (shLuc, control; S1, shSPDEF-1; S2, shSPDEF-2). (C) Proliferation of RasB1 cells cotransfected with control vector (EV/con), SPDEF (SPDEF/con), and/or TGFBI (SPDEF/TGFBI) (D) Western blotting of samples from cells assessed in (C), representative of three independent experiments. (E and F) Migration (E) and invasion (F) abilities in RasB1 cells described in (C). Representative images are shown. (G to J) Effect of transient expression of TGFBI on proliferation in LNCaP-AR cell lines grown in CSS-containing medium (G and I) and treated with MDV3100 (H and J). Images are representative of n = 6 experiments. Data are means ± SEM from at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
MATERIALS AND METHODS

Cells, reagents, and treatments

A mouse prostate cancer cell line (AC3) was isolated from 4-month-old PbCre4+;Ptenfl/fl;TP53fl/fl;Luc+ prostate tumors and cultured in PrEGM (Lonza), as previously described (29). AC3-subcloned cell lines were directly sorted from AC3 cells expressing markers characteristic of CD24+/EpCAM+ (AC3E+) and CD24+/EpCAM− (AC3E−), as previously described (29). Human prostate cancer cell lines (DU145, PC3, and LNCaP) were obtained from the American Type Culture Collection. The LNCaP-AR and RasB1 cell lines were provided by K. Kelly [National Cancer Institute/National Institutes of Health (NCI/NIH)]. Human cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). RPMI medium was also supplemented with 1 mM sodium pyruvate/10 mM Hepes buffer. For DHT or TGFβ treatment, cells were starved for 24 hours in 10% CSS-containing RPMI 1640 medium before incubation with 10 nM DHT (Sigma-Aldrich) or TGFβ (2 ng/ml) (R&D Systems) overnight. In experiments with an AR inhibitor or TGFβ inhibitor, cells were incubated with 10 μM MDV3100 (Selleckchem) or 10 μM SB431542 (Sigma-Aldrich) for 24 hours in 10% FBS-containing RPMI 1640 medium.

PCR array analysis

The amplified complementary DNA (cDNA) from AC3E+ and AC3E− cells was diluted with nuclease-free water and added to the RT² quantitative PCR (qPCR) SYBR green Master Mix (SA Biosciences). Then, 25 μl of the experimental cocktail was added to each well of a Mouse TGFβ/BMP Signaling Pathway PCR Array (SA Biosciences). A qPCR was performed as described below for the real-time qPCR. All data from the PCR were collected by MxPro software (provided by Stratagene with purchase of the Mx3000 Multiplex Quantitative PCR System) and analyzed through SA Biosciences PCR Array Data Analysis Web Portal.

Construction, transfection, and lentiviral transduction

The SPDEF-binding site was located upstream of human TGFBI on chromosome 5: 136027619 (SRE1), 136028086 (SRE2), 136028143 (SRE3), 136028432 (SRE4), and 136028492 (SRE5), respectively at GRCh38. These promoters with response element–green fluorescent protein (GFP) reporter vectors were constructed using the Clone-it Enzyme free Lentiviral kit (System Biosciences). Response element mutations were made using a Site-Directed Mutagenesis System kit (Invitrogen). TGFBI, AR, and SPDEF cDNA–expressing constructs used in this study were cloned into a pcDNA3.1 or pCDH-CMV-MCS-EF1-Puro (System Biosciences) vector. Plasmid DNA was prepared for transfection using the Qiagen Plasmid Midi Kit (Qiagen). All primers used for these constructs are listed in table S1. All constructs were verified by a DNA sequence analysis. siRNAs (NC, siAR, and siTGFBI) were obtained from ON-TARGETplus (System Biosciences). Response element mutations were made using a Site-Directed Mutagenesis System kit (Invitrogen). TGFBI, AR, and SPDEF cDNA–expressing constructs used in this study were cloned into a pcDNA3.1 or pCDH-CMV-MCS-EF1-Puro (System Biosciences) vector. Plasmid DNA was prepared for transfection using the Qiagen Plasmid Midi Kit (Qiagen). All primers used for these constructs are listed in table S1. All constructs were verified by a DNA sequence analysis. siRNAs (NC, siAR, and siTGFBI) were obtained from ON-TARGETplus.
SMARTpool siRNA (Thermo Scientific Dharmacon). Transient transfections of plasmids and siRNAs were carried out using the X-tremeGENE HP DNA Transfection Reagent (Roche) or Lipofectamine 2000 (Invitrogen), respectively. Lentiviral constructs expressing a TGFBI vector and an SPDEF shRNA or control (shLuc or shLacZ) vector were packaged using the pLKO.1-puro lentiviral vector packaging system (RNAi Core Lab) and used to infect PC3, RasB1, and LNCaP-AR prostate cancer cells. Infected cells were selected with puromycin for 1 month to establish cells with constitutive knockdown of SPDEF or TGFBI.

**Real-time qPCR analysis**

Cell lysis and RNA isolation were performed using the mirVana PARIS RNA isolation system (Ambion). RNA was quantitated by spectrophotometry (Thermos Fisher Scientific) and used to prepare cDNA with the SuperScript III kit (Invitrogen). The amplification step used the SYBR green PCR master mix (Applied Biosystems), and assays were performed as previously described (49). All primers used for the PCR are listed in table S2. Fifteen clinical samples were collected from the Taipei Medical University Joint Human Biological Database, and approval was obtained from the Taipei Medical University–Joint Institutional Review Board (no. 201311034). RNA was extracted from dissected tissues containing >70% tumor cell content.

**Western blot analysis**

Total protein was harvested by directly lysing 10^6 cells in 150 μl of radioimmunoprecipitation assay buffer containing complete protease inhibitors (Roche) and phosphatase inhibitors (Roche). Twenty micrograms of protein lysates was resolved on SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane (Millipore), and probed with primary antibodies overnight followed by incubation with secondary horseradish peroxidase–conjugated antibodies for 1 hour. Protein bands were visualized using Western Bright ECL (Advansta) and detected on x-ray film. All antibodies are listed in table S3.

**Clinical outcomes and human gene expression data set analyses**

The mRNA expression data were retrieved from the Taylor human prostate cancer data set (33) and TCGA. The Taylor data set of 98 primary and 13 metastatic prostate cancer samples was under Memorial Sloan-Kettering Cancer Center Institutional Review Board approval. TCGA data sets consisted of 414 primary prostate cancer samples from patients treated with a radical prostatectomy under NCI/NIH Review Board approval. For the z score analysis, gene sets were scored by summing expression z scores per tumor within the cohort. Tumors were mean-stratified by TGFBI expression, and the mean expression of each of these genes was determined in each group. For expression data analyses, a gene signature was activated or inactivated by the indicated pathway relative to all tumor samples. Expression data (and resulting z scores) were log2–normalized. GSEA software was available from the Broad Institute and was implemented in GenePattern v3.6.1, which provides an enrichment score (ES) with respect to a priori defined gene set signatures for each individual tumor sample. Gene sets of AR-dependent responsive gene (31, 32) signatures were used to determine correlations with TGFBI levels. Total human prostate cancers from the Taylor and TCGA were assigned to two groups based on the median TGFBI expression. The correlation between each of these genes and the gene set was validated by values of the normalized ES and false discovery rate.

**Migration and invasion assay**

Cell migration and invasion were determined using a two-chamber migration assay (8-μm pore size; BD Biosciences) or invasion assay [membrane coated with a layer of Matrigel (BD Biosciences) ECM proteins] according to the manufacturer’s instructions. In total, 10^5 cells were seeded in serum-free medium in the upper chamber and allowed to migrate/invade toward the bottom chamber containing 10% FBS medium for 16 hours, and assays were performed as previously described (49).

**Metastasis and survival assays in mice**

The animal experiment was performed in accordance with a protocol approved by the Taipei Medical University Animal Care and Use Committee. For the metastasis analysis, human prostate cancer cells harboring a luciferase expression vector with or without encoding gene expression were subjected to intracardiac injections into 5-week-old male nude mice (National Laboratory Animal Center (NLAC); six mice per group) at 10^5 cells per mouse. BLI was performed on day 30 after the injection as previously described (50).

**Proliferation assay**

Cells were stably transfected with a gene encoding an SPDEF or a TGFBI expression vector, or an SPDEF shRNA vector and seeded at a density of 2 × 10^3 cells per well in 96-well plates. To mimic ADT conditions, LNCaP-AR cells were stably delivered with the pcDNA3.1 and pcDNA3.1/TGFBI plasmids for 24 hours in CSS-containing medium. Cells were seeded at a density of 5 × 10^3 cells per well in six-well plates. TGFBI-overexpressing and control group cells were respectively treated with DMSO or 10 μM MDV3100 in CSS-containing medium, and assays were performed as previously described (49).

**Colony-formation assay**

In total, 5 × 10^4 cells were seeded in 0.3% agarose (Affymetrix) on top of a 0.6% agarose layer. Assays were performed in triplicate and incubated for 2 days at 37°C in a humidified incubator. Every 2 to 3 days, half of the medium was gently aspirated out and replaced with fresh medium in soft agar. After 3 weeks, colonies were fixed in methanol and stained with crystal violet. For quantification, crystal violet–positive colonies larger than 50 μm in diameter were counted from 100× microscope fields in triplicate.

**Tumorigenicity assays in mice**

Animal work was performed in accordance with a protocol approved by the Taipei Medical University Animal Care and Use Committee. For the tumorigenicity assays, 6- to 7-week-old male nude mice (NLAC) were subcutaneously injected with 10^5 RasB1/shLacZ and RasB1/shTGFBI human prostate cancer cells in 50% Matrigel (BD Biosciences). The tumor size was measured weekly with calipers, and the tumor volume was calculated using the following formula: tumor volume = (4/3) × (L/2) × (W/2)^2, where L is the length and W is the width.

**ChIP assay**

ChIP assays were performed with the EZ-Magna ChIP A kit (Millipore) with a modified protocol as previously described (49). Samples were purified through real-time qPCR purification kit columns, and 2 μl of eluted chromatin was used as a template in the real-time qPCR. ChIP antibodies and real-time qPCR primers are listed in table S4.

**Promoter reporter assay**

LNCaP-AR and RasB1 cells were plated in six-well plates and then transfected with constructs or mock control. Three days after transfection,
median fluorescent intensity (MFI) values for the GFP were detected using fluorescence-activated cell sorting (FACS) and measured using FACSDiva software (BD Biosciences). Results are expressed as the relative MFI normalized to the value of the molecule (the difference of the determination).

**IHC staining**

A tissue array containing 40 cases of prostatic carcinoma was purchased from US Biomax. The 17 cases of clinical tissue samples from prostate cancer patients who were treated before and after ADT were collected from Taipei Medical University–Wan Fang Hospital. Tissue samples were obtained and used according to protocols approved by the Taipei Medical University–Joint Institutional Review Board (no. N201610023). IHC staining was performed as previously described (49) using antibodies and antigen-retrieval methods indicated in table S5. For the histomorphometric analysis of tissue sections, 10 bright-field microscopic images were collected in each core under ×200 magnification using an Axioplan microscope system (Zeiss). TGFBI-, p-SMAD2 bodies and antigen-retrieval methods indicated in table S5. For the histomorphometric analysis of tissue sections, 10 bright-field microscopic images were collected in each core under ×200 magnification using an AxioVision software (Zeiss). The association between the clinicopathological characteristics of prostatic adenocarcinoma and gene expression was determined by a $\chi^2$ test or Fisher’s exact test.

**Statistical analysis**

All data are means ± SEM for a minimum of three independent experiments. Statistical calculations were performed with GraphPad Prism analytical tools. Differences between individual groups were analyzed by two-tailed Student’s t test or a one-way analysis of variance (ANOVA) followed by Bonferroni’s post-test for comparisons among three or more groups. A log-rank test was used for the survival curve analysis. The method for determining cutoffs was predicated by half of the number of patients. $P < 0.05$ was considered significant.

**SUPPLEMENTARY MATERIALS**

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**Fig. S1.** Induction of TGFβ1 is involved in the activated EMT, activated TGFβ1 signaling, and inactivated AR signaling in prostate cancer.

**Fig. S2.** The effect of TGFβ1 in AR-positive prostate cancer cells.

**Fig. S3.** Induction of TGFβ1 is associated with decreased androgen-activated SPDEF expression in prostate cancer patient samples.

**Fig. S4.** Role of TGFβ1 in EMT after SPDEF knockdown in AR-inhibited prostate cancer cells.

**Table S1.** Primer sequences of the promoter reporter constructs.

**Table S2.** Real-time qPCR primer sequences.

**Table S3.** Western blotting antibodies.

**Table S4.** ChIP antibodies and primer sequences.

**Table S5.** IHC staining antibodies.

**REFERENCES AND NOTES**


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Loss of SPDEF and gain of TGFBI activity after androgen deprivation therapy promote EMT and bone metastasis of prostate cancer
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Metastatic effects of primary prostate cancer therapy
Metastatic progression in patients with prostate cancer is common despite pharmacological inhibition of androgen receptor signaling. This drug resistance is associated with increased signaling through the transforming growth factor –β (TGFβ) signaling pathway, and Chen et al. have identified why. In both cultured prostate tumor cells and in tumor-bearing mouse models, the authors found that activation of the androgen receptor induces a transcriptional repressor (called SPDEF) of TGFβ-induced protein (TGFBI), which mediates cell adhesion to the extracellular matrix, a behavior that can facilitate metastasis. However, androgen deprivation therapy (ADT), a common clinical strategy, decreases the abundance of SPDEF and consequently increases that of TGFBI, thereby promoting the metastasis of prostate tumor cells. These findings are another example of how therapies that prevent growth in the primary tumor can inadvertently promote metastasis.