Research Article

The Type III Transforming Growth Factor-β Receptor as a Novel Tumor Suppressor Gene in Prostate Cancer

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Abstract

The transforming growth factor-β (TGF-β) signaling pathway has an important role in regulating normal prostate epithelium, inhibiting proliferation, differentiation, and both androgen deprivation–induced and androgen-independent apoptosis. During prostate cancer formation, most prostate cancer cells become resistant to these homeostatic effects of TGF-β. Although the loss of expression of either the type I (TβRI) or type II (TβRII) TGF-β receptor has been documented in ~30% of prostate cancers, most prostate cancers become TGF-β resistant without mutation or deletion of TβRI, TβRII, or Smads2, 3, and 4, and thus, the mechanism of resistance remains to be defined. Here, we show that type III TGF-β receptor (TβRIII or betaglycan) expression is decreased or lost in the majority of human prostate cancers as compared with benign prostate tissue at both the mRNA and protein level. Loss of TβRIII expression correlates with advancing tumor stage and a higher probability of prostate-specific antigen (PSA) recurrence, suggesting a role in prostate cancer progression. The loss of TβRIII expression is mediated by the loss of heterozygosity at the TGFBR3 genomic locus and epigenetic regulation of the TβRIII promoter. Functionally, restoring TβRIII expression in prostate cancer cells potently decreases cell motility and cell invasion through Matrigel *in vitro* and prostate tumorigenicity *in vivo*. Taken together, these studies define the loss of TβRIII expression as a common event in human prostate cancer and suggest that this loss is important for prostate cancer progression through effects on cell motility, invasiveness, and tumorigenicity. [Cancer Res 2007;67(3):1090–8]

Introduction

Transforming growth factor-β (TGF-β) is a ubiquitous, pleiotropic growth factor that regulates many cell processes including cell proliferation, differentiation, and apoptosis (1–4). There are three TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, that exert their cellular effects by binding to cell surface receptors, the type I (TβRI), type II (TβRII), and type III (TβRIII) TGF-β receptors (5, 6). TβRII is a constitutively active serine/threonine kinase that, upon ligand binding, recruits and phosphorylates TβRI to stimulate its serine/threonine kinase activity. TβRI then phosphorylates and activates the transcription factors Smad2 or Smad3, which form a complex with Smad4, that translocates to the nucleus to initiate transcription of TGF-β target genes in a cell-specific manner.

TβRIII, also known as betaglycan, is a 849-amino-acid proteoglycan with a short 41-amino-acid cytoplasmic domain (7, 8). TβRIII is the most abundantly expressed TGF-β receptor and binds all three TGF-β isoforms with high affinity. Due to its structural characteristics and lack of obvious signaling motifs, TβRIII was originally thought to function primarily as a TGF-β coreceptor, serving to sequester and present ligand to TβRII (7–9), with ectodomain shedding producing a soluble extracellular domain (sTβRIII) that antagonizes TGF-β signaling (10). This assumption has been challenged by recent studies demonstrating that mice lacking TβRIII die at gestational day 13.5 due to defects in the heart and liver (11), and that blocking TGF-β2 binding specifically to TβRIII inhibits TGF-β2–induced mesenchymal transformation in chick embryonic hearts (12). Recent studies have also expanded the role of TβRIII in mediating/regulating TGF-β signaling through its short, but highly conserved cytoplasmic domain. Although the cytoplasmic domain of TβRIII is not required for ligand presentation, this domain is required for full promotion of TGF-β signaling (13). TβRIII interacts through this cytoplasmic domain with the scaffolding proteins Gα-interacting protein, C terminus (14), and β-arrestin 2 (15), as well as with the autophosphorylated, active form of TβRII (13). The interaction of TβRIII and TβRII results in the phosphorylation of the cytoplasmic domain of TβRIII by TβRII, mediating the binding of β-arrestin 2 to TβRIII and potentially the disassociation of TβRIII from TβRII (13). Whereas Gα-interacting protein, C terminus, binding stabilizes TβRIII on the cell surface to increase TGF-β responsiveness (14), β-arrestin 2 binding results in the internalization of both TβRIII and TβRII and down-regulation of TGF-β signaling (15).

The TGF-β signaling pathway has an important role in regulating normal prostate epithelium, inhibiting proliferation, differentiation, and both androgen deprivation–induced and androgen-independent apoptosis (16–18). During prostate cancer formation, as in other cancers, prostate cancer cells become resistant to the antiproliferative effects of TGF-β (19). Although the loss of expression of either TβRI or TβRII has been documented in 31% of prostate cancers (20–22), most prostate cancers become resistant to the homeostatic effects of TGF-β without mutation or deletion of TβRI, TβRII, or Smads2, 3, and 4. Thus, other mechanisms must enable prostate cancer cells to escape the tumor-suppressive properties of TGF-β. Recently, loss of TβRIII expression has been reported in renal cell carcinoma (23) and endometrial cancer (24). Moreover, exogenous administration of sTβRIII suppresses tumorigenicity *in vivo* (25–27). Given that TβRIII has emerging roles in mediating and regulating TGF-β signaling, and the expression and functional significance of TβRIII

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in prostate cancer has not been established, we investigated whether the loss of TβRIII expression is a mechanism through which prostate cancer cells escape the tumor-suppressive effects of TGF-β.

Materials and Methods

Immunohistochemistry. Immunohistochemical studies were done on paraffin sections provided by the Duke University Research Foundation and the Cooperative Prostate Cancer Tissue Resource Prostate Tissue Microarray (TMA 2 Gleason). Before analyzing the prostate tissue microarray, the purified TβRIII-specific antibody (Sigma, St. Louis, MO) was tested at various dilutions with prostate cancer specimens obtained from the Duke Tissue Bank to identify the optimal antibody concentration required for reproducible immunohistochemical staining with minimum background. Consecutive tumor sections with the primary antibody substituted with a normal rabbit immunoglobulin G served as negative controls. All paraffin sections were deparaffinized in xylene and hydrated by immersing in increasing dilutions of ethanol (100–70%) followed by distilled water. The samples were then immersed in 1% hydrogen peroxide in PBS for 30 min to block the endogenous peroxidase activity. Nonspecific binding from serum components was blocked by covering samples in 10% normal goat serum diluted in PBS-Brij solution (625 μL 30% Brij in 1 L PBS) for 1 h. Samples were then blocked using an avidin-biotin blocking kit (Vector, Burlingame, CA). TβRIII-specific antibodies were diluted in 10% normal goat serum in PBS-Brij solution and applied at a concentration of 1:200 overnight at 4°C. After the overnight incubation with the primary antibody, the samples were immersed thrice in PBS-Brij solution for 5 min each. The samples were then probed with the secondary antibody (biotinylated anti-rabbit immunoglobulin G) at a concentration of 1:100 at room temperature for 1 h. Samples were then incubated with Vectastain ABC reagent (Vector) for 30 min at room temperature. Samples were washed between each incubation using a PBS-Brij solution (625 μL 30% Brij in 1 L PBS). Reactivity was visualized with an avidin-biotin complex immunoperoxidase system using diaminobenzidine as the chromagen and Mayer's hematoxylin as the counterstain (Vector). All slides were reviewed independently by two investigators (R. Turley and E. C. Finger) who were blinded to clinical and pathologic data and reconfirmed by a second set of evaluations by a board-certified pathologist (T. Fields) blinded to the interpretations of the first set of evaluations. Immunostaining results were compared, and discrepancies were reviewed. There was significant agreement between the three observers (99.6% correspondence); thus, the pathologist's scores are presented.

Oncomine microarray data and meta-analysis. Six independent gene profiling studies (28–33) publicly available on the Oncomine Cancer Profiling Database[4] were used to investigate TβRIII mRNA levels in prostate cancer. For each study, the mean TβRIII expression and the SD for both tumor and normal prostate tissue were calculated. Using the statistical program, Comprehensive Meta-analysis (Biostat, Inc., Englewood, NJ), the standard difference in means between the normal and tumor TβRIII from the mean TβRIII expression, the SD, and sample number for each individual study were calculated, and the combined meta-analysis of all six studies, along with each 95% confidence interval, was plotted. A negative value indicates a decrease in TβRIII expression in tumor versus normal.

Cell culture and reagents. DU145 cells were purchased from the American Type Culture Collection (Manassas, VA). DU145 cells were maintained in modified Eagle's medium supplemented with 10% FCS and 1 mmol/L sodium pyruvate. Cells were kept incubated at 37°C in 5% CO_2.

Stable cell line formation. DU145 cells were plated in six-well plates and grown to 80% confluency. Regular cell media was then replaced with Opti-MEM for transfection. In three wells of the six-well plate, 2 μg full-length, hemagglutinin-tagged rat TβRIII was transfected with 4 μL LipofectAMINE 2000 following the manufacturer's protocol. The empty vector pcDNA3.1 was transfected in the other three wells in the same fashion of TβRIII. The cells were incubated in the Opti-MEM containing the DNA and LipofectAMINE 2000 for 4 h, then the media was changed back to each cell line's regular media. After 48 h, cells were trypsinized, pooled, and replated in a 75-cm^2 flask. Selection was started by supplementing cell media with 500 μg/mL G418 72 h after transfection. Cells were selected for 4 weeks until the flask becomes 100% confluent with G418-resistant stables. After five passages, TβRIII expression was confirmed with affinity labeling. DU145 cells stably expressing full-length TβRIII and the empty vector pcDNA3.1 were maintained by supplementing their media with 500 μg/mL G418.

Affinity labeling. Cells were labeled with 100 pmol/L ^{125}I-TGFβ_1 in Krebs-Ringer-HEPES [50 mmol/L HEPES (pH 7.5), 130 mmol/L NaCl, 5 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 5 mmol/L KCl] plus 0.5% bovine serum albumin for 3 h at 4°C. Cells were rinsed with Krebs-Ringer-HEPES four times, then surface proteins were cross-linked with 0.5 mg/mL disuccinimidyl suberate for 15 min. The reaction was quenched by incubating with 20 mmol/L glycine for 10 min. Cells were again rinsed with Krebs-Ringer-HEPES, and then lysed in 100 μL 2× sample buffer. Proteins were resolved on 7.5% SDS-PAGE and detected by a phosphoimager.

Proliferation studies. Twenty thousand DU145 cells stably expressing the empty vector pcDNA3.1 or TβRIII were plated in triplicate in six-well plates and grown at 37°C in 5% CO_2. Cells were counted in triplicate using a Coulter counter every 24 h for 6 days. New media supplemented with 10% fetal bovine serum was added to plates every 48 h. About 100 pmol/L TGF-β treatment (or media supplemented with 10% fetal bovine serum) was added to select samples every 48 h. Cells were counted in triplicate using a Coulter counter every 24 h.

DNA extraction. Eight different patient prostate tumor samples and eight matched patient normal prostate tissue were obtained from the Duke Tissue Resource Network. Each sample was paraffin embedded and mounted on glass microscope slides. The samples were deparaffinized by immersion in xylene. The tumor tissue was lifted with a scalpel blade, transferred into a microfuge tube, and digested overnight at 65°C in buffer containing proteinase K. The lysate was heated at 95°C for 10 min to inactivate proteinase K. Lysate volumes sufficient to provide 4 μg genomic DNA was used directly for PCR. Control DNA was obtained by digesting matched normal prostate tissue.

Microsatellite PCR loss of heterozygosity analysis. Genomic DNA extracted from human prostate cancer specimens and matching normal prostate tissue was kindly provided by the Duke Prostate Cancer Tissue Bank. Microsatellite markers, D1S1588, D1S2804, and D1S435, were used in PCR reactions in which the forward primer was synthesized with a 5′ fluorescent tag (Integrated DNA Technology, Inc., Coralville, IA). PCR products were visualized using an ABI sequencer, and data was analyzed using GeneScan software. To determine the loss of heterozygosity (LOH), a ratio for each normal sample was calculated by dividing the total area calculated by the GeneScan software under the first peak by the total area under the second peak. The ratio of the peak areas for the tumor sample was calculated in the same fashion. Finally, the peak-area ratio for the normal prostate tissue was divided by the peak-area ratio for the tumor tissue. Criteria for LOH was a ratio >1.5 or <0.67.

Methylation and histone acetylation analysis. Prostate cancer cell lines DU145 and PC3 were treated with either 10 μmol/L 5-aza-2-deoxycytidine for 96 h and/or 500 nmol/L trichostatin A (TSA) for 24 h or with vehicle (no treatment). RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed. Quantitative SYBR Green real-time reverse transcription-PCR was done using TβRIII-specific or control glyceraldehyde-3-phosphate dehydrogenase primers. Data were normalized against glyceraldehyde-3-phosphate dehydrogenase levels and expressed relative to basal levels of TβRIII message levels from cell lines without treatment.

Wound-healing assay. DU145 cells were plated to confluence in six-well plates and scratched with a p200 tip to cause a wound. Images were taken using a Nikon Eclipse TE2000-U microscope under 10× magnification at multiple time points from 0 to 24 h. Cells were maintained in their selection media at 37°C in 5% CO_2 during this time period. The percent of the wound

[4] http://www.oncomine.org

remaining open ± SEM was calculated using Adobe Photoshop, with the 0-h time point being set to 100% for each sample type.

Fibronectin motility assay. A total of 50,000 DU145 cells stably expressing the empty vector pcDNA3.1 or TβRIII were seeded in serum-free media + 500 μg/mL G418 ± 150 pmol/L TGF-β1 in the upper chamber of fibronectin-coated transwell filters. The lower chamber was loaded with regular media supplemented with 10% fetal bovine serum. After a 24-h incubation at 37°C in 5% CO_2, the tops of the filters were scrapped, and cells that migrated through the fibronectin were fixed and stained using 3 Step Stain Set (Richard-Allan Scientific, Kalamazoo, MI). Cells that migrated to the lower surface of the membrane were counted in three fields under 10× magnification.

Matrigel invasion assay. Matrigel invasion chambers were purchased from BD Biosciences (San Jose, CA). The BD BioCoat Matrigel Invasion chambers included Falcon Cell Culture inserts containing an 8-μm pore size PET membrane with a thin layer of Matrigel basement membrane matrix. DU145 cells lines (25,000–50,000 cells per well) were resuspended in 500 μL serum-free media with 500 μg/mL G418 ± 150 pmol/L TGF-β1 and then seeded onto the upper compartment of each chamber. The lower compartment was filled with 700 μL of medium containing normal serum with 10% fetal bovine serum and 500 μg/mL G418. The chambers were then incubated for 48 h at 37°C. Noninvading cells were removed from the upper surface of the filter, and cells that had migrated through the filter were fixed and stained using the 3 Step Stain Set (Richard-Allan Scientific). Filters were allowed to dry and then were mounted onto a normal microscope slide. Cells that migrated to the lower surface of the filter were considered to have invaded through the overlying matrix and were counted in three fields using light microscopy.

Human prostate cancer xenograft model. Animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University. A total of 1×10^6 DU145-Neo or DU145-RIII cells were injected s.c. into both flanks of athymic BALB/cAnNCr-*nu/nu* mice. The DU145-Neo group contained six mice, and the DU145-RIII contained five mice. Mice were weighed, and tumor width (W) and length (L) were measured every 3 days. Tumor volume was approximated using the formula $V = 0.5 \times L \times W^2$. Tumor volume was followed for 36 days when some of the mice were reaching humane end points, at which time the mice were sacrificed. Upon autopsy, tumors were excised and weighed.

Results

Decreased TβRIII expression in prostate cancer. To assess a large number of prostate cancer specimens simultaneously, we did immunohistochemical analysis for TβRIII expression on a prostate cancer tissue array specifically designed to define grade-specific markers for prostate cancer. The prostate cancer tissue array contains prostate cancer tissue cores obtained from radical prostatectomy specimens from 250 patients (mean age, 63), with 3 cases of Gleason 4, 31 cases of Gleason 5, 53 cases of Gleason 6, 54 cases of Gleason 7, 52 cases of Gleason 8, 54 cases of Gleason 9, and 2 cases of Gleason 10. There are also 48 cases of separately represented high-grade prostate intraepithelial neoplasia (HGPIN) cores (mean age, 63) from among these 250 patients and 32 samples of control non-neoplastic tissue [18 benign prostatic hyperplasia cases (mean age, 63) obtained from radical retropubic prostatectomy and 14 organ donor prostates (mean age, 71)]. Organ donor prostates were obtained during total organ harvest, perfused until the time of donor kidney removal, with the donor prostate tissue immediately transported and fixed in a similar fashion to the radical prostatectomy specimens. Additional data available for these cases included age at diagnosis, PSA at diagnosis, tumor size, tumor-node-metastasis stage, time to recurrence, and vital status. TβRIII staining for each core prostate tissue sample was scored on a scale of 0 to 3 by three independent observers, including a board-certified pathologist, where a score of

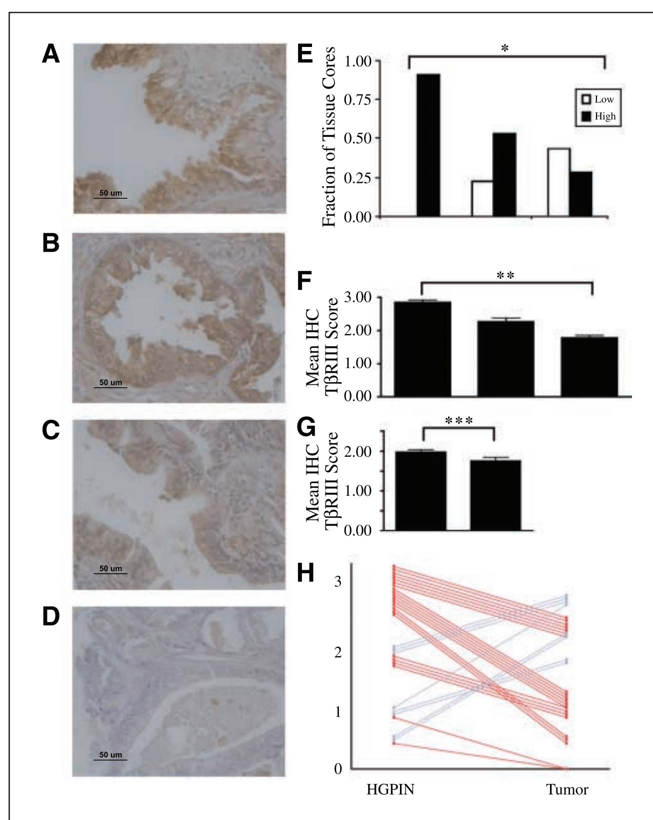


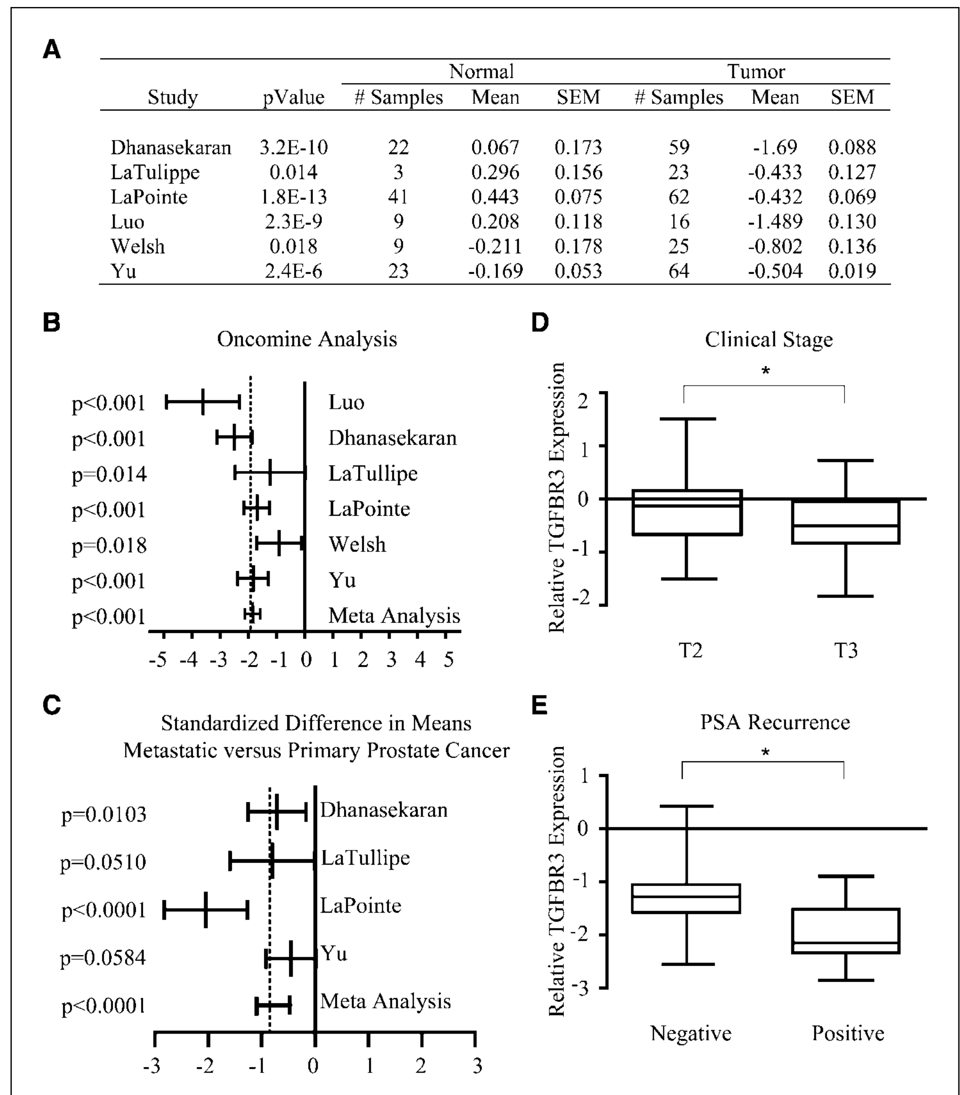
Figure 1. Decreased TβRIII expression in prostate cancer. *A* to *D,* a prostate cancer tissue array containing samples from 342 patients was stained with a TβRIII-specific antibody, and immunohistochemical (*IHC*) staining was scored on a 0 to 3 scale by a board-certified pathologist, 0 corresponding to no staining and 3 representing high levels of staining. Representative staining of non-neoplastic prostate tissue scored as 3 (*A*), benign prostatic hyperplasia scored as 3 (*B*), HGPIN scored as 2 (*C*), and invasive prostate cancer scored as 0 (*D*). *E* to *F,* IHC staining scores of non-neoplastic prostate tissue and BPH were combined to represent a normal tissue control. The proportion of high versus low staining in each sample type, as well as the mean TβRIII IHC individual scores of each group are represented graphically. *, $P < 0.001$, Fisher's exact probability test; **, $P < 0.0001$, ANOVA analysis; ***, $P = 0.0546$, independent two-tailed *t* test. *G*, correlation of the mean TβRIII IHC score to clinical stages T2 and T3. *H,* patient-matched HGPIN and prostate cancer IHC TβRIII scores.

0 corresponds to absent staining, 0.5 corresponds to trace staining, 1 corresponds to low levels of staining, 2 corresponds to medium levels of staining, and 3 corresponds to high levels of staining. Either prostatic epithelium or tumor was graded for each core, with emphasis not to include staining of stromal or smooth muscle elements that usually stained intensely for TβRIII (data not shown). TβRIII expression decreased progressively from non-neoplastic prostate tissue (Fig. 1*A, B, E*, and *F*) to HGPIN (Fig. 1*C, E*, and *F*) to invasive prostate cancer (Fig. 1*D–F*) with the proportion with abundant TβRIII expression decreasing from 91% in control non-neoplastic prostate tissue to 53.2% in HGPIN to 27.8% in invasive prostate cancer (Fig. 1*E*; $P = 0.0019$, two-tailed Fisher's exact probability test). In contrast, the proportion with little to no TβRIII expression increased from 0% in control non-neoplastic prostate tissue to 22.6% in HGPIN to 45% in invasive prostate cancer (Fig. 1*E*; $P = 0.0019$, two-tailed Fisher's exact probability test). The mean immunohistochemistry TβRIII score also decreased from 2.85 ± 0.08 for control non-neoplastic prostate tissue to 2.27 ± 0.11 for HGPIN to 1.79 ± 0.06 for prostate cancer (Fig. 1*F*; P value <0.0001, ANOVA).

To evaluate whether progressive loss of TβRIII expression occurred in matched patient specimens, we analyzed the 48 specimens with matched HGPIN and invasive prostate cancer from the same patient. A total of 22 of the 48 (46%) patient-matched sets showed a decrease in TβRIII expression from HGPIN to invasive prostate cancer, whereas only 8 of the 48 (17%) showed an increase in TβRIII expression (Fig. 1*G* and *H*). Seven of the 18 (39%) remaining specimens exhibiting no change in TβRIII expression already exhibited little to no expression in the HGPIN specimen. The mean immunohistochemistry TβRIII score also decreased from 2.24 $\pm$ 0.14 for HGPIN to 1.78 $\pm$ 0.14 for prostate cancer in these matched patient specimens (P = 0.021, two-tailed independent t test). These data indicate that TβRIII expression was significantly decreased in prostate cancer, with loss of TβRIII expression correlating with prostate cancer progression.

We also analyzed correlations of our mean immunohistochemistry TβRIII score with clinical stage, primary Gleason grade of the core, and total Gleason score of the prostate tumor. Although there was no correlation between immunohistochemistry TβRIII score and total Gleason score or primary Gleason grade (data not shown), there was a trend for decreased TβRIII expression between clinical T stages 2 and 3 (Fig. 1*G*; P = 0.0546). Clinical stages 1 and 4 were omitted due to insufficient sample number. These data suggest that the loss of TβRIII expression is a rather early event in prostate cancer progression, with loss beginning during the formation of high-grade intraepithelial neoplasia and increasing as these progress to invasive prostate cancer.

We then investigated whether there was also a loss of TβRIII expression at the mRNA level. Using six previously published gene profiling studies for benign prostate and prostate cancer specimens publicly available through the Gene Expression Omnibus repository and Oncomine Gene Profiling Databases, we established that the mean TβRIII mRNA levels were significantly decreased in prostate tumor tissue as compared with normal prostate tissue in all six studies (Fig. 2*A*). Because TβRIII expression is high in the stroma, this decrease could potentially be due to a decrease in stromal contribution to the mRNA expression in tumor versus benign specimens. However, one of these studies, Yu et al. (30), used only normal peripheral zone prostate tissue with at least 60% glandular components and tumor tissues with at least 70% tumor, and another study, Lapointe et al. (31), found very few genes in which expression correlated with epithelial content, making this



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Study	pValue	Normal # Samples	Normal Mean	Normal SEM	Tumor # Samples	Tumor Mean	Tumor SEM
Dhanasekaran	3.2E-10	22	0.067	0.173	59	-1.69	0.088
LaTulippe	0.014	3	0.296	0.156	23	-0.433	0.127
LaPointe	1.8E-13	41	0.443	0.075	62	-0.432	0.069
Luo	2.3E-9	9	0.208	0.118	16	-1.489	0.130
Welsh	0.018	9	-0.211	0.178	25	-0.802	0.136
Yu	2.4E-6	23	-0.169	0.053	64	-0.504	0.019

Figure 2. Decreased TβRIII mRNA in prostate cancer versus normal prostate. *A,* data from six independent gene profiling studies identified by the study's first author, and the corresponding mean TβRIII expression in normal versus tumor samples, with the number of samples and each individual study's P value indicated. *B,* meta analysis was done using the Comprehensive Meta-analysis program. The standard difference in means between normal and prostate cancer TβRIII expression for each individual study and the combined meta-analysis of all six studies, along with the 95% confidence interval, is plotted (negative values indicate a decrease in TβRIII expression in cancer versus normal). *C* to *E,* meta-analysis was done as outlined above, comparing TβRIII mRNA expression levels in the primary tumor compared with metastasis. *C,* T2 compared with (*D*) T3 clinical stage (*, P = 0.0339, independent two-tailed t test) and (*E*) the presence or absence of PSA recurrence (*, P = 0.0062, independent one-tailed t test).

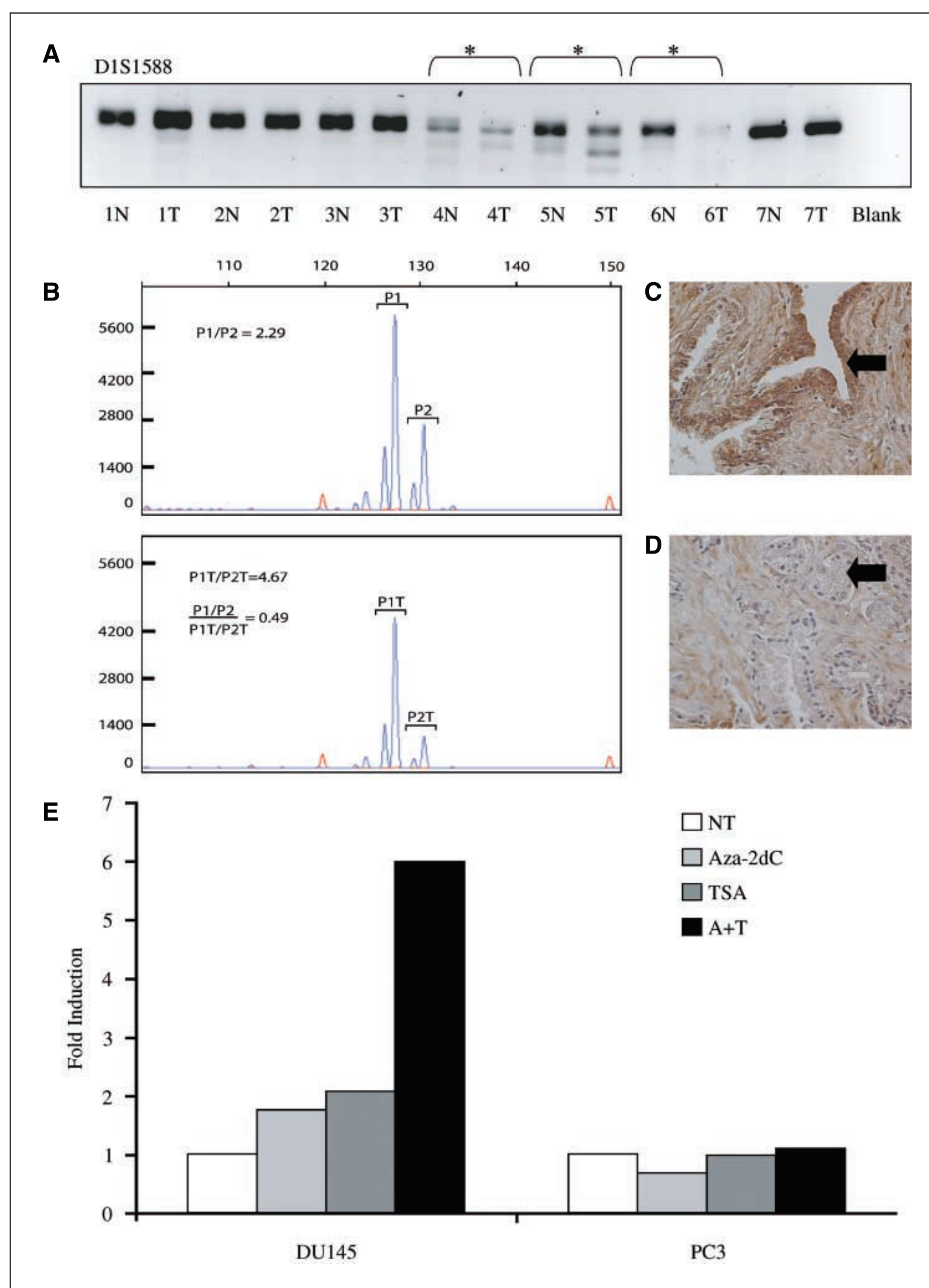

Figure 3. Loss of heterozygosity of the TGFBR3 genomic locus in prostate cancer. *A,* representative PCR samples for microsatellite marker D1S1588 were run on a 3% MetaPhor agarose gel to verify LOH. *, LOH. *B,* a representative matched tumor (*bottom*)/normal (*top*) sample demonstrating LOH. *C* and *D,* representative immunohistochemical analysis of prostate cancer tissues on which LOH studies were done, with (*C*) high TβRIII expression in normal prostate tissue and (*D*) loss of expression in a representative prostate cancer specimen (*arrows*). *E,* DU145 cells were treated with 5-aza-2-deoxycytidine (*Aza-2dC*, 10 mmol/L, 96 h), TSA (500 nmol/L, 24 h), with both (*A* + *T*), or with vehicle (*NT*). Expression of TβRIII is shown as measured by SYBR green real-time reverse transcription-PCR with TβRIII-specific primers. Data are normalized against glyceraldehyde-3-phosphate dehydrogenase levels and expressed relative to basal levels of TβRIII message levels from cell lines without treatment.

explanation less likely. Moreover, combining all six studies in a meta-analysis, we calculated the combined standard difference in means to be −1.855 ± 0.140 (95% confidence interval, −2.218 to −1.581; $P < 0.001$; Fig. 2*B*), confirming the loss of TβRIII expression in prostate cancer as compared with benign prostate.

In four of the six gene profiling studies, relative TβRIII mRNA expression was available for metastases as well as the primary tumors. In all four studies, TβRIII mRNA was further decreased in metastases as compared with the primary tumor. A meta-analysis of all four studies resulted in a standard difference in means between primary prostate cancers and metastases to be −0.814 ± 0.152 (95% confidence interval, −1.112 to −0.515; $P < 0.001$; Fig. 2*C*), supporting further loss of TβRIII expression in metastatic disease.

Analysis of data from Lapointe et al. (31) also confirmed a statistically significant decrease in TβRIII mRNA levels in clinical stage T3 as compared with clinical stage T2 ($P = 0.0339$; Fig. 2*D*) and established a decrease in TβRIII mRNA levels in patients with known PSA recurrence relative to those who did not have PSA recurrence ($P = 0.0062$; Fig. 2*E*). Taken together, these results show that TβRIII expression is decreased in prostate cancer at both the message and protein level, with decreased expression correlating with increasing clinical stage, metastatic disease, and PSA recurrence, but not Gleason score or grade.

LOH of the TGFBR3 genomic locus in human prostate cancer. Previous studies have shown that chromosome 1p, where TGFBR3 is located, exhibits a loss of heterozygosity in a number of human cancers, including prostate cancer (34). To examine

whether LOH represents a mechanism for decreased TβRIII expression in human prostate cancer, we did a preliminary study using PCR-based LOH analysis on eight patient prostate cancer specimens with matching normal adjacent prostate tissue using three microsatellite markers (D1S1588, D1S2804, and D1S435) that are informative for the TGFBR3 genomic locus at 1p32. Using these three microsatellite markers, we were able to establish that 37.5% (3:8) of our samples showed LOH at the TGFBR3 locus (Fig. 3*A* and *B*). In addition, by examining TβRIII expression by immunohistochemistry in these specimens, we were able to confirm that the loss of TβRIII expression correlated with LOH in all three cases (Fig. 3*C* and *D*). Thus, these studies confirm that LOH of the TGFBR3 locus is one mechanism through which TβRIII expression is lost during prostate cancer progression.

Because only 37.5% of the samples in this preliminary LOH analysis showed loss of allelic DNA as a mechanism for decreased TβRIII expression, we investigated other mechanisms through which TβRIII might be down-regulated at the mRNA level. Epigenetic regulation via promoter hypermethylation and/or histone acetylation is another potential mechanism to account for the decreased TβRIII mRNA levels in prostate cancer. To test whether epigenetic regulation of TβRIII could be involved in prostate cancer, we treated the prostate cancer cell lines, PC3 and DU145 cells, with the DNA methylation inhibitor 5-aza-2-deoxycytidine and/or the histone deacetylase inhibitor TSA. PC-3 cells, derived from a bone metastasis from a stage IV adenocarcinoma of the prostate, express high endogenous levels of TβRIII, whereas DU145 cells, derived from a brain metastasis from a patient with adenocarcinoma of the prostate, express very low levels of TβRIII. RIII. 5-Aza-2-deoxycytidine and TSA treatment induced the expression of TβRIII in the DU145 cells with low basal TβRIII expression 6-fold, while having no significant effect on the PC3 cells with high basal TβRIII expression (Fig. 3*E*). These studies suggest that TβRIII expression may be decreased in prostate cancer not only through the loss of the TGFBR3 genomic locus, but also though epigenetic silencing.

TβRIII does not alter prostate cancer cell proliferation. To explore the functional significance of TβRIII in prostate cancer, we analyzed TGF-β receptor expression in the PC-3 and DU145 prostate cancer cells lines and the immortalized prostate epithelial cells LHS and LHSR (35). The LHS and LHSR cells were immortalized by infecting prostate epithelial cells with retroviruses expressing LT, *hTERT*, and ST and SV40 LT, *hTERT*, ST, and H-*ras*, respectively (35). Both LHS and LHSR cells expressed high levels of TβRIII, suggesting that prostate epithelial cells normally express high levels of TβRIII (data not shown). Although the PC-3 cell line also expressed high-level TβRIII, the DU145 prostate cell line expressed low levels of TβRIII and relatively normal levels of TβRII and TβRI (Supplementary Fig. S1*A*, data not shown). Thus, we chose the DU145 cell line to investigate the functional consequences of the loss of TβRIII expression. We established a DU145 stable cell line re-expressing TβRIII (DU145-RIII) and a control DU145 cell line expressing empty vector, pcDNA3.1 (DU145-Neo). Expression of TβRIII was verified by performing ^{125}I-TGFβ1 binding and cross-linking studies, with DU145-RIII expressing all three TGF-β receptors and DU145-Neo cells expressing very low levels of TβRIII (Supplemental Fig. S1*A*). TβRII and TβRI levels were equivalent in both DU145-RIII and DU145-Neo cells. We then tested whether TβRIII effects cell proliferation in the presence and absence of TGFβ1. Exogenous expression of TβRIII had no effect on basal DU145 cell proliferation because the growth curves of DU145-Neo and DU145-RIII were similar (Supplemental Fig. S1*B*). In addition, in the presence of 100 pmol/L TGFβ1, exogenous expression of TβRIII had no effect on TGFβ1-regulated DU145 cell

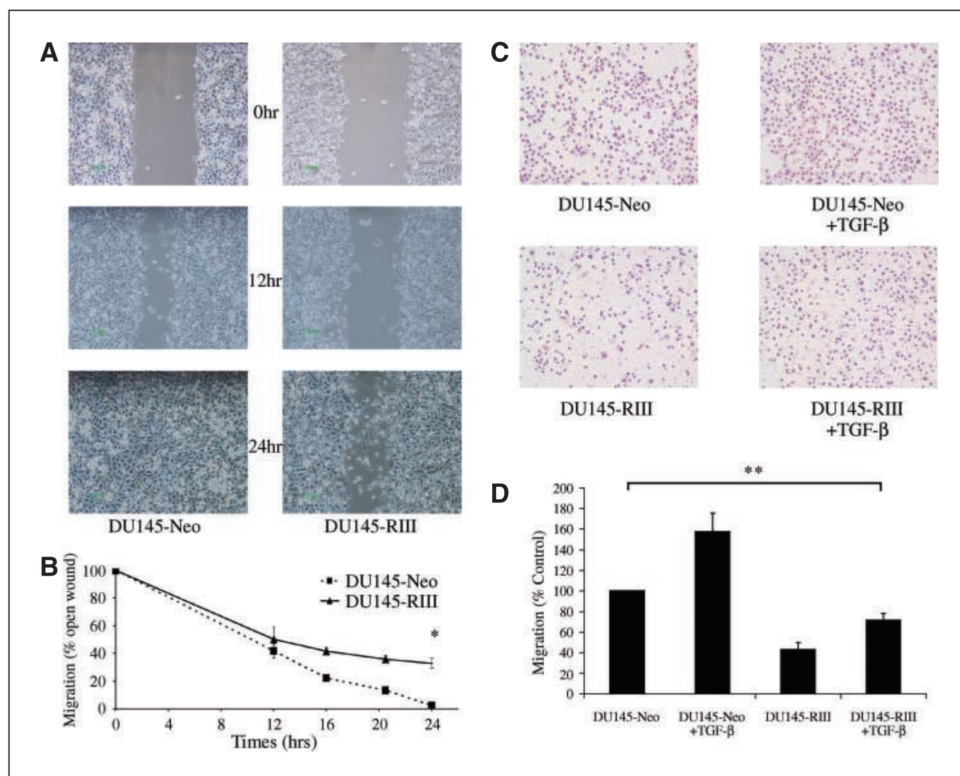

Figure 4. TβRIII decreases prostate cancer cellular motility. *A,* DU145-Neo or DU145-RIII cells were plated to confluence in six-well plates and scratched with a p200 tip to form a uniform wound. Images were taken using a Nikon Eclipse TE2000-U microscope under 10× magnification at the indicated times over a 24-h time course at 37°C in 5% CO_2. Images are representative of three separate experiments. *B,* the percent of the wound remaining open ± SEM was calculated using Adobe Photoshop, with the 0-h time point being set to 100% for each sample type. *, $P = 0.028$, independent two-tailed *t* test. *C,* 100,000 DU145-Neo or DU145-RIII cells were seeded in serum-free media ± 150 pmol/L TGF-β1 in the upper chamber of fibronectin-coated transwell filters. The lower chamber was filled with regular media supplemented with 10% fetal bovine serum. After a 24-h incubation at 37°C in 5% CO_2, the top of the filters were scraped, and cells that migrated through the fibronectin were fixed and stained. *D,* cells that migrated to the lower surface of the membrane were counted in three fields under 10× magnification with data representative of three separate experiments. **, $P = 0.0004$, Kruskal-Wallis test.

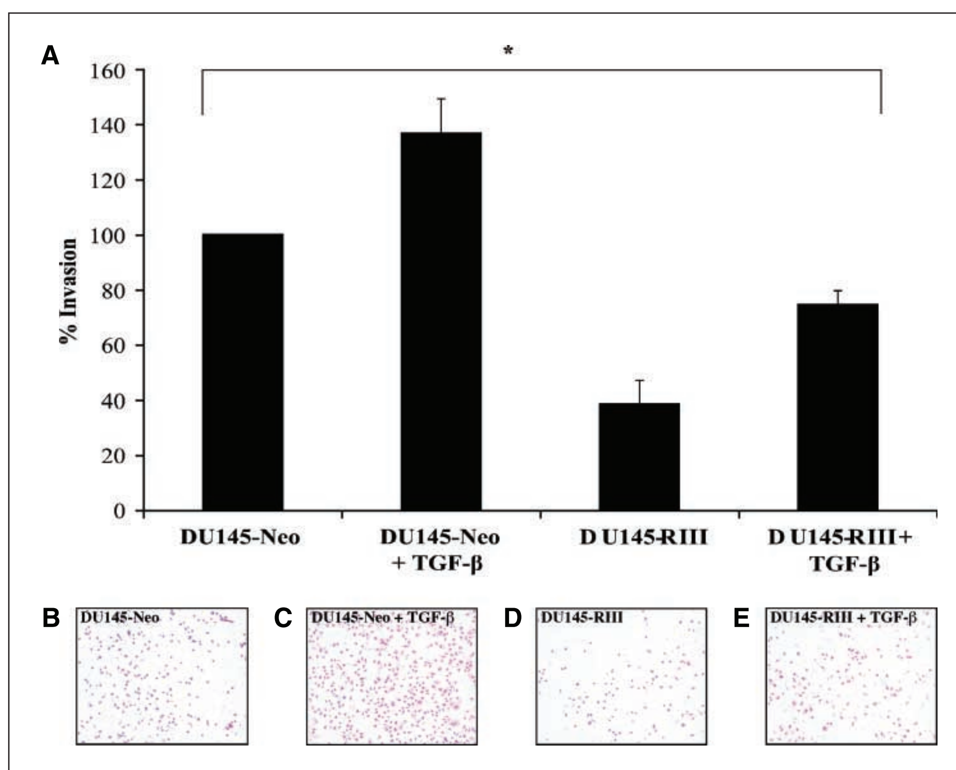

Figure 5. TβRIII decreases prostate cancer cellular invasion through Matrigel. 75,000 DU145-Neo or DU145-RIII cells were seeded in serum-free media ± TGF-β1 in the upper chamber of Matrigel transwells. The lower chamber was loaded with regular media supplemented with 10% fetal bovine serum. After 48 h at 37°C in 5% CO_2, the top filter was scraped, and invading cells were fixed and stained. *A,* cells were counted in three fields under 10× magnification. Invasion index was calculated as the mean ± SEM of invading cells from four separate experiments, normalized within each experiment to empty vector control invasion; *, $P = 0.0143$, Kruskal-Wallis test. *B* to *E,* representative images from four separate experiments.

proliferation because, again, the growth curves of DU145-Neo and DU145-RIII were similar (Supplemental Fig. S1*C*). These results show that expression of TβRIII had no effect on cellular proliferation *in vitro* and did not alter responsiveness to TGFβ1 in the DU145 cell line.

TβRIII decreases prostate cancer cell migration. The decrease in TβRIII expression in prostate cancer relative to benign prostate and in higher stage and metastatic prostate cancers relative to lower stage and nonmetastatic prostate cancers all suggested a potential role for TβRIII in regulating cell migration and/or invasion. To examine the role of TβRIII in regulating cell migration, we first used a monolayer wound-healing assay and monitored wound closure over a 24-h time course (36). DU145 prostate cancer cells stably expressing TβRIII consistently migrated slower than control DU145-Neo cells (Fig. 4*A* and *B*). Although wounds in DU145-Neo cells closed completely by 24 h, wounds in confluent DU145-RIII cells remained 32.7 ± 3.7% open at 24 h (Fig. 4*A* and *B*). These studies suggested that expression of TβRIII was sufficient to inhibit cellular migration.

To further establish the effects of TβRIII on prostate cancer cell migration, we did cell migration assays using fibronectin-coated transwells seeded with either DU145-RIII cells or control DU145-Neo cells ± 150 pmol/L TGF-β1 ligand. TβRIII significantly reduced migration through fibronectin-coated transwells, with only 42.5 ± 6.5% migration of the DU145-RIII cells at 24 h relative to the DU145-Neo cells. Addition of TGF-β1 increased the migration of DU145-Neo cells 57.5 ± 17.9% and migration of DU145-RIII cells 79.5 ± 31.7%, relative to their untreated counterparts (Fig. 4*C* and *D*). However, in the presence of 150 pmol/L TGF-β1 ligand, there was only 55.7 ± 2.7% migration of the DU145-RIII cells at 24 h relative to the DU145-Neo cells (Fig. 4*C* and *D*; $P = 0.0004$, Kruskal-Wallis test). Importantly, these differences were not due to differences in proliferation at this time point as established by our growth curves (Supplement Fig. S1*B* and *C*). Taken together, these studies establish a role for TβRIII in decreasing prostate cancer cellular motility in the presence and absence of exogenous TGF-β1 *in vitro*.

TβRIII decreases prostate cancer cell invasiveness. Although alterations in motility are necessary for prostate cancer cells to metastasize, they also need to invade through the basement membrane. To examine the role of TβRIII in regulating prostate cancer cell invasiveness, we used the reconstituted basement membrane, Matrigel, to model the basement membrane (37). In a manner similar to the fibronectin motility assays, the addition of 150 pmol/L TGF-β1 increased invasion of DU145-Neo stable cells by 36.6 ± 12% and DU145-RIII cells by 108.8 ± 57.1% relative to the same cell lines without TGF-β1 treatment. Strikingly, expression of exogenous TβRIII in DU145 prostate cancer cells significantly inhibited their ability to invade Matrigel by 61.6 ± 14.8% in the absence of TGF-β1 and 54.9 ± 2.1% in the presence of TGF-β1 (Fig. 5; $P = 0.0143$, Kruskal-Wallis test). Again, these differences were not due to differences in proliferation as established by our growth curves (Supplement Fig. S1*B* and *C*). Taken together, these results suggest that one potential consequence of the progressive loss of TβRIII expression from preinvasive to invasive to metastatic prostate cancer is the ability for these cells to become more motile and invasive.

TβRIII decreases prostate tumor growth *in vivo*. To further define TβRIII as a tumor suppressor gene in prostate cancer, we tested whether the restoration of TβRIII expression in DU145 prostate cancer cells decreased tumor growth in a mouse xenograft model. Athymic mice were injected s.c. with either DU145-Neo or DU145-RIII cells and followed. Although tumors formed in both groups and the overall weight of the mice in both groups was similar, tumors from DU145-Neo cells consistently grew faster and to greater volumes as compared with tumors from DU145-RIII cells

(Fig. 6*A*). At the day of sacrifice, mice with tumors created from DU145-Neo cells had a mean calculated volume 3.48 times larger than tumors created from DU145-RIII cells (331.3 ± 58.4 mm^3 versus 95.5 ± 22.1 mm^3; $P = 0.022$, two-tailed independent t test). Given that tumor volume was a calculated estimate, we also measured tumor mass at excision. Mean tumor mass, measured at the time of sacrifice, was significantly greater in the control DU145-Neo group as compared with the DU145-RIII group (Fig. 6*B*; $P = 0.039$, two-tailed independent t test). These studies strongly support a tumor suppressor role for TβRIII in prostate cancer *in vivo*.

Discussion

Here, for the first time, we provide evidence that TβRIII is a tumor suppressor gene in prostate cancer. Through immunohistochemical analysis of 350 prostate tissue cores from more than 250 patients, we show that TβRIII expression is frequently decreased or lost in human prostate cancers, with 22.6% of HGPIN and 45% of invasive prostate cancer specimens expressing little to no TβRIII, whereas all of the control non-neoplastic prostate tissue expressed medium to high levels of TβRIII. Thus, the loss of TβRIII expression is the most frequent alteration in the TGF-β signaling pathway in prostate cancer described to date. TβRIII expression is also decreased at the message level in prostate cancer, with LOH at the TβRIII genomic locus correlating with decreased TβRIII expression. The loss of TβRIII expression correlates with increasing clinical disease, metastatic disease, and PSA recurrence, suggesting a role for the loss of TβRIII expression in tumor progression. Functionally, loss of TβRIII expression results in increased cell motility and invasiveness *in vitro* and increased tumorigenicity *in vivo*. Taken together, these studies define TβRIII as a tumor suppressor gene in prostate cancer, with loss of expression correlating with prostate cancer progression.

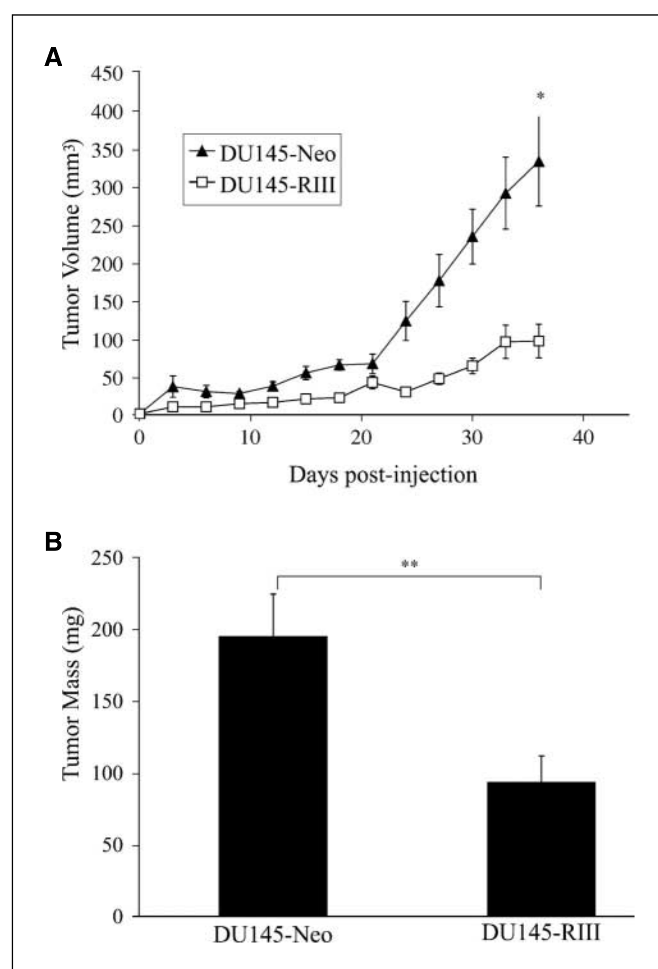

Figure 6. TβRIII decreases prostate cancer tumorigenicity *in vivo. A,* 1×10^6 DU145-Neo or DU145-RIII cells were injected s.c. into both flanks of athymic BALB/cAnNCr-*nu*/*nu* mice. The DU145-Neo group contained six mice, and the DU145-RIII contained five mice. Mice were weighed, and tumor width (W) and length (L) were measured every 3 days. Tumor volume was approximated using the formula $V = 0.5 \times L \times W^2$. *, $P = 0.004$, one-way ANOVA. *B,* mice were sacrificed at day 36 postinjection, with tumors forming in 11:12 of sites inoculated with DU145-Neo cells and 10:10 sites inoculated with DU145-RIII cells. Tumors from both groups were excised and weighed. The mean tumor mass ± SEM was calculated for each mouse group. **, $P = 0.039$, two-tailed independent t test.

How might the loss of TβRIII expression result in increased cell motility and invasiveness? TβRIII functions as a coreceptor for the TGF-β signaling pathway, enhancing ligand binding to TβRII and increasing TGF-β signaling. In addition to this role, TβRIII through its cytoplasmic domain interacts with the scaffolding proteins Gα-interacting protein, C terminus (14) and β-arrestin 2 (15) which have the potential to scaffold TβRIII to Smad-independent signaling pathways, including the mitogen-activated protein kinase pathways (38). TβRIII is also proteolytically cleaved in the extracellular domain to produce soluble TβRIII (sTβRIII) that can sequester TGF-β ligand and antagonize TGF-β signaling (10). In terms of prostate cancer, sTβRIII has been shown to decrease tumor growth and angiogenesis in a xenograft model (27). Thus, decreasing TβRIII expression could result in decreased TGF-β–mediated Smad-dependent or Smad-independent signaling or in decreased expression of sTβRIII. The mechanism by which reduced TβRIII expression enhances prostate cancer cell motility and invasiveness remains an active area of investigation.

TβRIII is on the short arm of chromosome 1, 1p32, a region that is frequently deleted in human cancers, including breast, colon, endometrial, gastric, kidney, lung, ovarian, and testicular cancer (34, 39). Although there are other potential tumor suppressor genes in this region, including TP73 (40) and RUNX3 (41), previous studies have not established these as the tumor suppressors in these cancers. In the present study, we established LOH at the TβRIII locus in 37% of the patients, with LOH correlating with decreased TβRIII expression, supporting TβRIII as a tumor suppressor gene in human prostate cancer. TβRIII has also been reported to be lost at an early stage in renal cell carcinogenesis (23). Whether TβRIII is the tumor suppressor gene on chromosome 1p32 in renal cell and other human cancers remains to be defined.

Although most cases of early prostate cancer can be cured with localized approaches and locally advanced and metastatic prostate cancer can often be controlled with hormonal therapy, many patients ultimately become hormone refractory and succumb to their disease, with 27,350 U.S. deaths expected in 2006. Thus, better treatment options are required for advanced hormone refractory prostate cancer. Given the role of the TGF-β signaling pathway in regulating normal prostate epithelial homeostasis, including androgen depletion–induced and androgen-independent apoptosis, this pathway is a promising target for treatment of advanced-stage, hormone refractory prostate cancer. To effectively target this pathway, increased understanding of the mechanisms through which prostate cancer cells become resistant to the tumor suppressor effects of TGF-β is needed. Our current data support

that loss of TβRIII receptor expression as a common mechanism through which prostate cancer cells escape TGF-β–mediated tumor suppression. Given that treatment of the TβRIII-deficient cell line DU145 with the TSA and 5-aza-2'-deoxycytidine resulted in increased TβRIII expression, our data suggest that TβRIII levels could be restored in some cases via histone deacetylase inhibitors and 5-aza-2'-deoxycytidine analogs. In addition, because the ectodomain of TβRIII is normally shed, some endogenous properties of TβRIII could be restored by the administration of exogenous sTβRIII, as was recently validated in a preclinical model of prostate cancer (27). The role of epigenetic regulation of TβRIII expression and the role of soluble TβRIII are currently being further defined.

TβRIII expression may also have a prognostic value in prostate cancer. We have shown that TβRIII mRNA and protein levels decrease with increased clinical stage and correlate with higher PSA recurrence and metastatic disease. Whether immunohistochemical staining for TβRIII on tissue specimens obtained from radical prostatectomy could direct physicians in planning the quality and aggressiveness of adjunctive chemotherapy or radiotherapy requires prospective validation.

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