Novel Role of Thromboxane Receptors β Isoform in Bladder Cancer Pathogenesis

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Abstract

These studies were undertaken to determine the potential role of thromboxane receptors (TP) in bladder cancer. The data reported herein show that expression of the TP-β receptor protein is increased in tissue obtained from patients with bladder cancer and associated with a significantly poorer prognosis ($P < 0.005$). Bladder cancer cell lines express the TP-β isoform, unlike immortalized nontransformed urothelial cells (SV-HUC) that express only the TP-α isoform. TP-β receptor expression, but not TP-α, promoted cell proliferation, migration, and invasion *in vitro*, and also resulted in malignant transformation of SV-HUC cells *in vivo*. Agonist-mediated phosphorylation of extracellular signal-regulated kinase and FAK was dependent on the expression of TP-β. Furthermore, TP-β mediated multiple biological effects by signaling through either G-protein α subunit 12 or β-arrestin 2. Treatment of mice with the TP receptor antagonist GR32191, alone or in combination with cisplatin, significantly delayed tumor onset and prolonged survival of mice transplanted with TCC-SUP bladder cancer cells compared with vehicle or cisplatin alone. These results support the model that the TP-β receptor isoform plays a unique role in bladder cancer progression and its expression may have predictive value and provide a novel therapeutic target. [Cancer Res 2008;68(11):4097–104]

Introduction

Bladder cancer is the fifth most common cancer in the United States with ~60,000 new cases each year (1). Approximately 25% of the patients will die of the disease. Early diagnosis and a better understanding of the mechanisms of carcinogenesis may result in improved survival. We have previously found that thromboxane synthase was overexpressed in patients with bladder cancer and was associated with a significantly poorer prognosis (2). Thromboxane synthase catalyzes the formation of thromboxane A_2 (TP) from prostaglandin H_2, which is derived from the precursor arachidonic acid (3). Thromboxane A_2, although very labile, stimulates it receptors to produce a myriad of pharmacologic events (4–6).
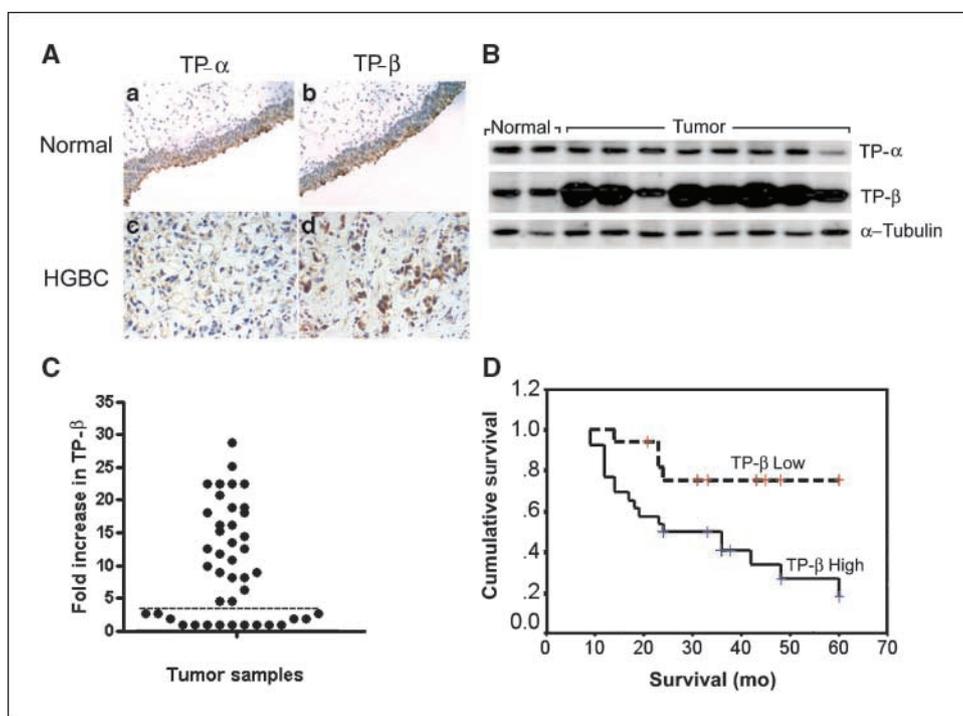
TP receptors are G protein–coupled receptors (GPCR) and are expressed as two different isoforms, TP-α (7) and TP-β (8), that arise by alternative mRNA splicing (9). The two isoforms share both common and different intracellular signaling mechanisms and different trafficking patterns. Both isoforms signal through $G\alpha_q$, resulting in transient increases in intracellular calcium and IP3 formation and through $G\alpha_{12}$ to stimulate sodium-hydrogen exchange (6, 10, 11). When stimulated by ligand, TP-β, but not TP-α, couples to $G\alpha_i$, which decreases cyclic AMP production (12, 13). Stimulation of TP receptors is associated with a mitogenic response (14). Stimulation of both receptors results in phosphorylation of extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK; ref. 15, 16). Collectively, these observations lead to the current studies that were designed to determine the expression levels of TP-α and TP-β receptors in bladder cancer and their potential role in cancer phenotypes.

Materials and Methods

Patients and tumor specimens. Samples from 43 tumors were obtained from untreated patients who underwent surgery for bladder cancer at the Urology and Nephrology Center in Mansoura, Egypt between August 1998 and April 2000. The bladder cancer tissue bank used in this study was established in 1992 and contains fresh frozen bladder cancer tissues and all the clinical data, follow-up data, and longitudinal outcome data on patients presenting with bladder cancer and their tumors stored in this bank. Tumor stage and grade were defined according to Unio Internationale Contra Cancrum and WHO classification, as previously described (17). In all cases, tumor and adjacent nonneoplastic bladder tissue were available for the study. Before surgery at the Center, all patients provided written informed consent to allow any excess tissue for research studies. These samples were used to provide the data presented in Fig. 1B to D. Human bladder cancer paraffin blocks were obtained from the Hollings Cancer Center Tumor Bank, Medical University of South Carolina (MUSC) for the immunohistochemical studies. All specimens were formalin fixed and paraffin embedded. For those samples, tumor grade and stage were available; however, no demographic or follow-up data were available for those patients. These samples were used to generate the data presented in Fig. 1A.

Cell culture and chemicals. Bladder cancer cell lines T24, TCC-SUP, UM-UC-3, SW780, HT-1376, 5637, J82, and RT4 were obtained from American Type Culture Collection and cultured in RPMI 1640 with 10% fetal bovine serum (FBS). The Simian virus 40–immortalized human uroepithelial (SV-HUC), nontransformed urothelial cell line was provided Dr. Santhanam Swaminathan (University of Wisconsin, Comprehensive Cancer Center, Madison, WI) and cultured in Ham's F-12 (Life Technologies/Invitrogen) supplemented with 1% FBS. All cell lines were propagated at 37°C in an atmosphere containing 5% CO_2. The receptor antagonists Pinane TP (PTXA2) and SQ29548 were purchased from Cayman Chemical Company and another receptor antagonist (GR32191) was a gift from the GlaxoSmithKline pharmaceutical company.

Generation of human TP isoform–specific antipeptide antibodies. Rabbit polyclonal antibodies were raised against peptides representing

Figure 1. Expression of TP receptors and cumulative survival in patients with bladder cancer. *A,* representative immunohistochemical localization of TP receptor isoforms in human bladder tissue. Tissue sections were stained for the presence of TP-α and TP-β isoforms using antibodies specific for the two isoforms. TP-α and TP-β are present in the epithelial cell layer of normal tissue (*a* and *b*; magnification, ×200). In the high-grade bladder cancer tissue (*HGBC*), TP-β is overexpressed but TP-α is not (*c* and *d*; magnification, ×400). The samples are representative of seven cases. *B,* immunoblot analysis of normal and tumor tissue for TP-α and TP-β protein levels. *C,* scatter plot analyses of quantified TP-β receptor levels in bladder cancer tissues. The Western blots were scanned and quantitated. Protein levels were normalized to α-tubulin and are expressed as a fold increase over nontumor bladder tissue. *D,* Kaplan-Meier survival curve for patients with bladder cancer expressing either high (>3-fold; *n* = 28) or low levels (*n* = 15) of TP-β. There was a statistically significant difference (*P* < 0.005) in survival for patients expressing high (*TP-β High*) versus low (*TP-β Low*) levels of TP-β protein.

residues 329 to 343 of human TP-α and residues 394 to 407 of human TP-β. Peptides were synthesized and conjugation to bovine serum albumin was performed using the Imject EDC kit (Pierce). Antisera were assessed for reactivity by ELISA using the corresponding TP receptor peptides. The TP-α-Ab and TP-β-Ab antisera had titers of 1/15,000 to 1/20,000 and 1/5,000 to 1/10,000, respectively. The antibodies were specific, reacting only with the epitopes they were raised against, with no crossreactivity against other peptides derived from divergent regions of TP receptors. Active bleeds from the same rabbit were pooled and, after sterilization, were stored at −20°C in the presence of 0.01% sodium azide. Antisera were further purified on Affigel 10-peptide affinity columns before use in immunohistochemistry and immunoblotting. By Western blot analyses, each antibody recognizes a single band of the expected size for each isoform. In addition, immunoreactivity is not observed in cell lines that do not express the appropriate mRNA (Supplementary Fig. S1*A* and *B*). Analysis of protein extracts from normal and tumor tissues also showed the single band with molecular weights corresponds to TP-α and TP-β (Supplementary Fig. S1*C* and *D*).

Immunohistochemistry and terminal deoxynucleotidyl-transferase–mediated dUTP nick-end staining. Antigen retrieval was done by heating in a microwave oven for 5 min on high power in 10 mmol/L citrate (pH 6.0). Sections were washed and nonspecific binding was blocked with 10% horse serum in TBS [50 mmol/L Tris-HCl, 0.9% NaCl (pH 8.0)] for 20 min and then incubated overnight at 4°C with the TP isoform–specific primary antibodies at a 1:200 dilution in the blocking solution. Anti-CD31 antibody (DAKO) was used at dilution 1:25. Proliferating cell nuclear antigen (PCNA) staining was done using mouse monoclonal antibody against human PCNA (Santa Cruz) at dilution 1:200. After incubation at 4°C, followed by three 10-min washes in TBST, Immpress horse anti-rabbit or horse anti-mouse secondary was incubated (Vector Laboratories) for 45 min at room temperature. After washing with TBS containing Tween 20 (TBST), 3,3′-diaminobenzidine substrate (Sigma) was added for 30 s followed by washing in TBST. Slides were counterstained with hematoxylin. For terminal deoxynucleotidyl-transferase–mediated dUTP nick-end (TUNEL) staining, sections were stained using the Situ Cell Death Detection kit (Roche Applied Science). For all antibodies used, isotype-matched control antibodies were used as negative control (Supplementary Fig. S2).

Western blot analysis. Tumor and normal bladder tissues were snap frozen in liquid nitrogen within 30 min after the surgery. Frozen sections were stained with H&E, and samples identified that had at least 70% tumor

cells were selected for further studies. Pulverized tissue powders were lysed in radioimmunoprecipitation assay buffer [RIPA; 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1% Triton X100, 0.1% SDS, and 1% deoxycholate and protease inhibitor cocktail (Complete Protease Inhibitors, Roche)] for 15 min on ice. Total proteins were extracted from bladder cancer cell lines. Cells at 90% confluence were washed twice with ice cold PBS and were lysed in RIPA buffer containing protease inhibitors. Equal amounts of total protein (40 μg) were resolved by 12% SDS-PAGE and subjected to Western blot analyses using enhanced chemiluminescence system (Amersham-Pharmacia). Screening Western blots for TP-α and TP-β isoform expression in bladder cancer cell lines was done using rabbit polyclonal antibodies against TP-α or TP-β (described above). SV-HUC cells stably transfected with TP-α or TP-β plasmids were examined for PCNA, tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FAK, pFAK, ERK, pERK (Santa Cruz), and intercellular adhesion molecule 1 (ICAM-1; Abcam) protein expression.

Reverse transcriptase-PCR. Total RNA was extracted from the bladder cancer cell lines (Supplementary Fig. S1) with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was reversed transcribed using Superscript II (single strand synthesis RT; Invitrogen). Aliquots from this reaction were subjected to PCR using 11-F (5′-ACGGA-GAAGGAGCTGCTCATCT-3′; complement of nucleotides 1070–1091 of the published sequences) and α-C-R (5′-CCAGCCCCTGAATCCTCA-3′; residues 1337–1320 of TP-α), and β-11-R (5′-CAAAAGGAAGCAACTGTACCCC-3′; residues 1399–1378 of TP-β) were used as primers to amplify a 268-bp fragment of TP-α and a 330-bp fragment of TP-β. The reaction mixture contained 2 mmol/L Mg^{2+}, 0.2 mmol/L deoxynucleotide triphosphates, 1× Taq Gold buffer, 0.88 pmol/μL Primers, and 0.02 U/μL Taq Gold (Applied Biosystems). The basic PCR reaction conditions were as follows: 95° for 10 min; then 34 cycles of 95°C for 30 s, 57 cycles for 45 s, 72°C for 1 min, followed by 72° for 7 min. Hypoxanthine phosphoribosyl transferase (HPRT) was used as PCR control. HPRT was amplified with the conditions described in TP reverse transcription-PCR using the primers HPRT-F (5′-CTTGCTCGAGATGTGAT-GA-3′) and HPRT-R (5′-GTCTGCATTGTTTTGCCAGTG-3′) corresponding to nucleotides 145 to 165 and 414 to 434, respectively, of the HPRT mRNA sequence. PCR products were analyzed by electrophoresis on a 1% Tris-borate EDTA gel containing Ethidium bromide and visualized under UV light.

Transfection of cells with TP-α and TP-β DNA. SV-HUC cells were grown to 80% confluence and transfected with 2 μg of pcDNA3 vector

encoding either the TP-α or TP-β receptor isoforms. Transfection was performed in 6-well plates using the Fugene reagent (Roche) according to the manufacturer's instructions. After 72 h posttransfection, cells were selected with 500 μg/mL G418 (Roche) and stable pools were isolated after 14 d.

Cell growth assay. SV-HUC and T24 cells were seeded at 5,000 cells per well in 96-well plates and then treated with either compound or vehicle (ethanol) alone. The number of viable cells were quantified at the indicated time points, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT blue) assay, performed in quadruplicate, according to the manufacturer's instructions (Sigma-Aldrich). Absorbance was measured at wavelength of 550 nm with reference wavelength of 690 nm.

Migration and invasion assays. Cell migration experiments were carried out using 8-μm pore size migration chambers (Falcon; Becton Dickinson) precoated at 4°C overnight with fibronectin (Becton Dickinson) at a concentration of 5 μg per square centimeter in PBS. The following day, the fibronectin solution was aspirated and the migration chambers were rinsed once with water and allowed to air dry before the migration experiment. Cell invasion experiments were carried out using rehydrated 8-μm pore size invasion chambers precoated with Matrigel (Becton Dickinson). Cells at 80% confluence were trypsinized, harvested, and counted. For each condition, cells were seeded at 25,000 cells per well (T24) or 100,000 cells per well (SV-HUC) in 500 μL serum-free medium, then added to each migration and invasion chamber. Medium (750 μL) containing 10% serum was used as a chemoattractant in the lower chamber. Both upper and lower chambers contained the indicated compound. Cells were allowed to migrate for 8 h or invade for 24 h at 37°C in the presence of 5% CO_2. Cells that did not migrate or invade were removed by wiping the top of the membrane with a cotton swab, and the migrating and invading cells were fixed and stained with Diff-Quik per the manufacturers protocol (Dade Behring). Migrating and invading cells in 10 high-power fields in each chamber were counted, and the mean cell number was calculated. Each experiment was conducted in triplicate and repeated thrice.

Cell morphology and immunofluorescence studies. Cell morphology and immunofluorescence analyses were performed on glass chamber slides precoated with 5 μg/mL fibronectin. T24 cells were seeded at low density (~ 4 cells/mm^2) in normal growth medium and incubated for a period of 12 h. For U46619, or PTXA$_2$ treatment and solvent control, cells were incubated for another 12 h with either U46619 (1 μmol/L), PTXA$_2$ (1 μmol/L), or solvent control (water). Cells were examined using phase contrast microscopy and bright field pictures were taken. For actin cytoskeletal reorganization, cells were fixed with 2% formaldehyde for 10 min. After washing twice with cold PBS, cells were permeabilized with 0.1% Triton X-100. Cover slips were blocked in 2% BSA and actin distribution was examined by phalloidin staining as per the manufacturer's instructions (Molecular Probes). Slides were examined using an Olympus Fluoview IX70 confocal microscope.

Cell viability assay and analysis of apoptosis. Cell viability was analyzed under the microscope at ×200 using trypan blue staining. Flow cytometric measurements of apoptosis were done after 12 h of treatment with solvent control or TP receptor antagonists. Cells were washed twice in ice-cold PBS and resuspended in binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L CaCl$_2$), at 1×10^6 cells in a 400 μL volume of binding buffer, in duplicate. Cells were incubated with 10 μL of human Annexin V-phycoerythrin (BD Bioscience Inc.) for 15 min at room temperature in dark. The cells were kept on ice and analyzed with a BD FACSAria Flow Cytometer flow cytometer within 1 h after staining.

Xenograft mouse model. TCC-SUP tumorigenic human bladder cancer cells were selected as they express TP-β receptor and were used for the drug combination studies. Immortalized nontransformed normal urothelial SV-HUC cells were selected because they express the TP-α. These cells were stably transfected with pcDNA3, TP-α, or TP-β for cell transformation studies. Both cell lines were used in a s.c. model in immunocompromised (*nu/nu*) mice. TCC-SUP cells (5×10^6) or SV-HUC cells (5×10^7) in Matrigel (BD Bioscience, Inc.) were injected s.c. into the right and left flanks of anesthetized mice. Tumor growth was monitored in these mice twice a week. For mice injected with TCC-SUP, GR32191 or vehicle control was administered daily (20 mg/kg) by gavage with treatment initiated 24 h after

initial injection. Two cycles of cisplatin [single high dose (5 mg/kg) or single low dose (0.5 mg/kg)] were administrated at day 4 and day 11 posttumor cell injection.

Statistical analysis. Kaplan-Meier survival curves were constructed for overall patient survival for two groups with and without overexpressed TP-β receptor protein. ANOVA was used to analyze the *in vitro* drug treatments and drug combination data. Data are presented as the mean ± SD. For all statistical tests, a two-sided *P* value of <0.05 was used to reject the null hypothesis. Kaplan-Meier curves were used to describe time to tumor onset in each mouse group, and a Cox proportional hazards model was used to estimate and test for differences across treatment groups. Hazard ratios and their 95% confidence intervals were to describe rates of tumor onset and *P* values were used to compare tumor onset across groups. The proportional hazards assumption was tested using Schoenfeld residuals. Statistical analyses were performed using R statistical software and using the survival library.

Results

Expression of TP-β receptors in bladder cancer tissue. Our initial screening of tissues taken from patients with bladder cancer showed an increased expression of TP receptors compared with control tissue (2). Because TP receptors exist in two isoforms, we next sought to determine whether there was a differential expression of the two in bladder cancer tissue compared with normal tissue. In normal tissue, TP-α and TP-β were both expressed in the transitional epithelial cells (Fig. 1*Aa* and *b*). In the tissue of patients with high-grade bladder cancer, there was a significant overexpression of TP-β without concomitant significant overexpression of TP-α in the epithelial and stromal compartments (Fig. 1*Ac* and *d*).

TP-β receptor expression in human bladder cancer tissue and survival. Because TP-β receptor protein was overexpressed in high-grade bladder cancer tissue, the question arose as to whether it was associated with survival. Tissue from patients with known outcomes were analyzed for the presence of TP-β receptor protein and categorized as overexpressers if there was greater than a 3-fold higher level of expression compared with control tissue (Fig. 1*B* and *C*). Patients in the overexpresser category had a significantly poorer outcome compared with those that were categorized as low expressers (Fig. 1*D*; *P* < 0.005). In contrast, there was no observed overexpression of TP-α in tumor tissue and no correlation with the expression of TP-β (data not shown).

Expression of TP-β receptors in bladder cell lines. Because the expression of TP-β was enhanced in the tissue of several bladder cancer patients, we examined the complement of TP isoforms expressed in a series of established bladder cancer–derived cell lines. TP-β protein is expressed at varied amounts in all the bladder cancer cell lines examined (Fig. 2*A*). In contrast, the SV-HUC cell line, an immortalized nontransformed bladder cell line, expressed TP-α but no detectable TP-β. The expression of TP-α in the bladder cancer cell lines was quite variable and below the limits of detection in approximately half of the cell lines examined (Fig. 2*A*).

Effects of TP-β receptors on cell growth, migration, and invasion. Because TP-β receptors were found to be overexpressed in bladder cancer cells, we sought to determine if they played a role in pathophysiologic events associated with cancer. SV-HUC cells were transfected with either control vector, or TP-β or TP-α vector DNA, and the effects on *in vitro* proliferation, migration, and invasion were determined. Transfection of SV-HUC cells with TP-α did not significantly alter the growth rate compared with the control vector–transfected cells (Fig. 2*B*). However, transfection of the SV-HUC cells with TP-β resulted in a significantly greater

growth rate compared with TP-α–transfected cells and control vector–transfected cells, in the absence (data not shown) or presence of TP agonist U46619 (Fig. 2*B*; *P* < 0.05). Similar to the effects seen on proliferation, transfection of TP-β receptor DNA into SV-HUC cells resulted in a significantly enhanced migration and invasion compared with vehicle control or TP-α receptor–transfected cells in the presence of U46619 (Fig. 2*C*; *P* < 0.05). To determine if the presence of the TP-β receptor is associated with molecular events known to mediate proliferation and migration, Western blot analysis of the transfected cells was performed for PCNA, associated with proliferation, and ICAM-1, associated with migration. TP-α–transfected cells, stimulated with the TP receptor agonist U46619, did not show any increase in either PCNA or ICAM-1 compared with the vector alone–transfected cells (Fig. 2*B*, *right*). In contrast, cells transfected with TP-β and stimulated with U46619 showed significant increases in PCNA and ICAM-1 compared with either pcDNA3 or TP-α–transfected cells (Fig. 2*B*, *right*). These differential effects were independent of agonist stimulation (data not shown).

Given these effects of TP-β, we sought to determine if TP-β could contribute to malignant transformation of SV-HUC cells *in vivo*. SV-HUC cells were stably transfected with pcDNA3, pcDNA3-TP-α, or pcDNA3-TP-β and were injected s.c. into *nu/nu* mice. Only cells transfected with TP-β were able to form tumors in the mice (4 of 10). Histologic examination of the tumors confirmed a well-differentiated malignant phenotype (Fig. 2*E*).

We previously have shown that bladder cancer cell migration and invasion was dependent on the presence of functional TP receptors

in T24 cells (2). Consistent with the TP receptor–stimulated migration of the T24 cells were classic morphologic changes that were stimulated by U46619. U46619 stimulation resulted in elongation of the cells with a polarized distribution of filamentous (F)-actin (Fig. 3*A*). The addition of PTXA$_2$, a TP receptor antagonist, to T24 cells in their basal state resulted in a change in cellular morphology from a somewhat stellate-like appearance to a circular shape (Fig. 3*A*). To determine if the altered cell morphology observed in PTXA$_2$-treated cells reflected an increase in apoptosis, T24 cells were treated with PTXA$_2$ for 12 hours, and flow cytometric analyses were used to measure Annexin-V labeling to phosphatidylserine, a membrane phospholipid exposed at the surface of apoptotic cells. Treatment with GR32191 and PTXA$_2$ resulted in a statistically significant 2- and 2.4-fold increase in Annexin V–positive cells (*P* = 0.03 and 0.007, respectively; Fig. 3*B*). The percentage of Annexin V and propidium double positive was also significantly higher in cells treated with GR32191 (1.9-fold; *P* = 0.002) and cells treated with PTXA$_2$ (3.9-fold; *P* = 0.04) compared with the control (Fig. 3*B*). Total protein was extracted from treated and control cells and activated caspase-3 was measured using an anticaspase-3 rabbit polyclonal antibody. Treatment with PTXA$_2$ decreased the levels of procaspase-3 and increased the levels of active caspase-3 (Fig. 3*C*).

Because ERK and FAK phosphorylation are associated with proliferation and migration, we sought to determine if stimulation of TP receptors could mediate these events. In T24 cells, stimulation with U46619 resulted in increased phosphorylation of ERK and FAK in a concentration-dependent manner (Fig. 3*D*). Antagonism of the TP receptors by PTXA$_2$ in T24 cells resulted in a
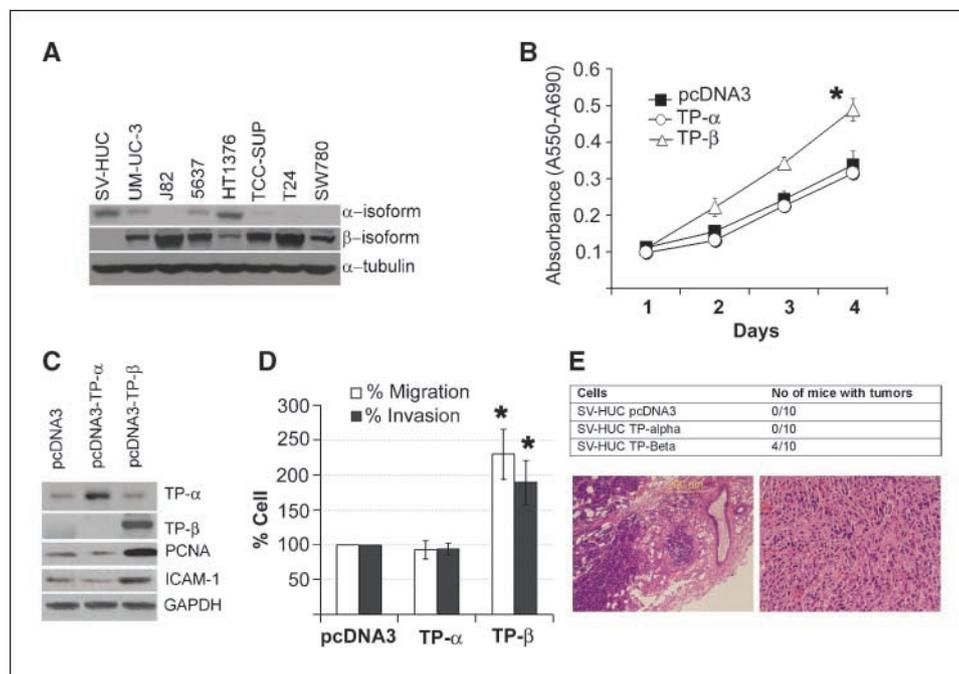
Figure 2. Distribution and biological effects of TP receptor isoforms in bladder cells. *A,* immunoblot analysis of TP-α and TP-β isoform expression in human bladder cancer cell lines. SV-HUC is an immortalized nontransformed bladder epithelial cell line. All the other cell lines are derived from bladder cancer patient tissue. Western blots were performed using isoform-specific antibodies. α-tubulin was used as a loading control. *B to D,* effect of transfection of TP-α and TP-β isoforms into SV-HUC cells on proliferation (*B*) migration and invasion (*D*); *, statistically significant (*P* < 0.05) differences compared with control and TP-α. *B,* SV-HUC cells were transfected with TP-α, TP-β, or pcDNA3 vector DNA, and G418-resistant stable pooled clones were selected. *Points,* mean for triplicate determinations; *bars,* SD. *C,* Western blot analyses for expression of TP-α, TP-β, PCNA, and ICAM-1. GAPDH was used as a loading control. Figure is representative of three experiments. Cell growth was determined using the MTT assay. *D,* the cells were stimulated with U46619 (1 μmol/L) and allowed to migrate or invade for 8 or 24 h, respectively, before being counted. *Columns,* mean for three experiments conducted in triplicate; *bars,* SD. *E,* effect of different TP isoforms on SV-HUC cell transformation *in vivo*; SV-HUC stably transfected with TP-α, TP-β, or vector alone were injected s.c. into nude mice. Tumor growth was monitored over time. *Top,* summary table for tumor growth. *Bottom,* representative H&E staining for tumors derived from SV-HUC-TP-β injected mice.

concentration-dependent decrease in pERK and pFAK (Fig. 3D). In contrast, in SV-HUC cells, neither PTXA$_2$ nor U46619 had any significant effect on pERK or pFAK levels (Fig. 4A). SV-HUC cells transfected with TP-β had higher basal levels of pFAK and pERK, and treatment with U46619 increased pERK and pFAK (Fig. 4B), whereas addition of PTXA$_2$ to the U46619-treated cells restored the basal levels of pERK and pFAK, indicating that the observed agonist-mediated stimulation was TP-β receptor dependent. In cells treated with PTXA$_2$ alone, the elevated basal levels of pFAK and pERK were reduced, indicating that the increased levels were due to the TP-β receptor and/or increased TXA$_2$ endogenous synthesis (data not shown).

β-arrestins bind to activated transmembrane-spanning receptors and regulate their signaling and internalization. For example, β-arrestin 2 binds to and down-regulates the transforming growth factor β–dependent inhibition of cell proliferation (18). Recent data showed that TP-β interacts with β-arrestin 2 (19). We determined that bladder cancer cell lines express high levels of β-arrestin 2 (data not shown). The TP receptor agonist U46619 produced a significant ($P < 0.05$) increase in T24 cell migration (Fig. 4D). RNAi-mediated knockdown of β-arrestin 2 (Fig. 4C) resulted in a reduction of the response to the U46619 (Fig. 4D). In contrast, knockdown of β-arrestin 1 (Fig. 4C) failed to inhibit the response to U46619 (Fig. 4D).

Tumor metastasis is associated with remodeling of the actin cytoskeleton. One class of the proteins that have been reported to affect actin remodeling and play a role in tumor metastases is G$_{\alpha 12}$ (20, 21). Northern blot analysis showed high levels of G$_{\alpha 12}$ expression in bladder cancer cell lines and tissues (data not shown). shRNAi knockdown of G$_{\alpha 12}$ resulted in a decreased U46619-mediated stimulation of the cell migration (Fig. 4D). Thus, it seems that cellular response to TP receptor agonist stimulation is mediated through both β-arrestin 2 and G$_{\alpha 12}$ signaling.

Effects of TP receptor antagonists on tumor cell growth *in vitro* and *in vivo*. Cisplatin is part of the standard of care for patients with bladder cancer. The results of the *in vitro* studies raised the possibility that a TP receptor antagonist could have an additive or supra-additive effect to cisplatin on tumor cell growth. T24 cells were grown in the presence of increasing concentrations of cisplatin and two different TP receptor antagonists, GR32191 or SQ29548, and the presence of dead cells was assessed by trypan blue staining and direct cell counts. Both GR32191 and SQ29548 alone produced a significant ($P < 0.05$) increase in the percent cell death (Fig. 5A, right). When combined with cisplatin, they had a synergistic effect ($P < 0.05$).

Due to the observed change in F-actin distribution expression after treatment with PTXA$_2$ (Fig. 2B and C, right), it was possible that TP antagonist treatment would enhance the cytotoxicity of paclitaxel, which mediates its effects in large part via premature stabilization of microtubule assembly with disruption of the cytoskeletal framework. T24 cells were treated with solvent control or paclitaxel in the absence or presence of PTXA$_2$. Combined treatment with GR32191 or SQ29548 and paclitaxel resulted in increased cytotoxicity (Fig. 5A, left).

Based on these *in vitro* results, we proceeded to examine the effect of a TP receptor antagonist and cisplatin on tumor onset and growth *in vivo*. Mice were transplanted with TCC-SUP cells and treated with vehicle, GR32191, cisplatin, or cisplatin plus GR32191. Two different doses (low, 0.5 mg/kg and high, 5 mg/kg given twice) of cisplatin were studied. Kaplan-Meier curves showing time to tumor are shown in Fig. 5C. The median time to tumor incidence was 8 days in the vehicle alone group compared with 17 days in the
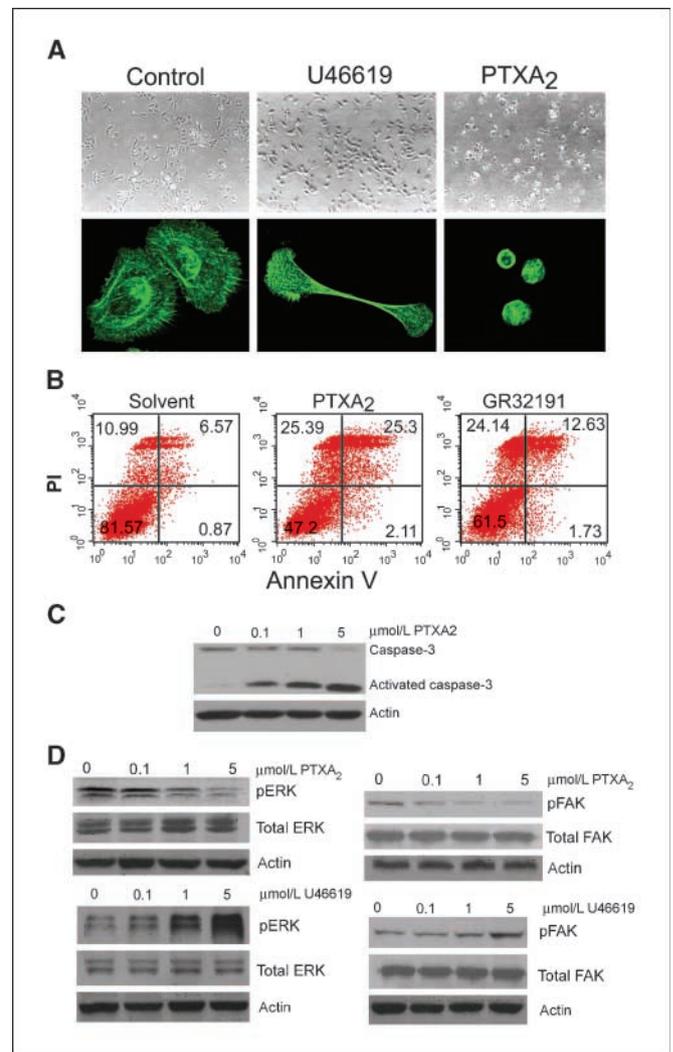
Figure 3. Effects of TP receptor agonists and antagonists on morphology, apoptosis, and phosphorylation of ERK and FAK in T24 cells. *A*, TP receptor agonist (U46619, 1 μmol/L) or antagonists (PTXA$_2$, 1 μmol/L) alter cell morphology and F-actin distribution. Data represent those observed in >80% of the cell population in 100 fields. *B*, flow cytometric analysis of cells treated with PTXA$_2$ for 12 h and double stained with Annexin V-FITC and propidium iodide (PI). Percentage of cells in early or late apoptosis is provided in the lower right and upper right quadrants, respectively. *C*, effect of TP receptor antagonists on caspase-3 activation. T24 bladder cancer cells were treated with PTXA$_2$ (1 μmol/L) or GR32191 (2 μmol/L). Levels of caspase-3 and active caspase-3 were determined by Western blot analysis. GAPDH served as the loading control. *D*, immunoblot analysis of pERK and pFAK. PTXA$_2$ decreased the basal levels of pERK and pFAK and U46619 increased the levels, respectively. There were no significant changes in total ERK and FAK. Actin was used as a loading control. Figure is representative of three experiments.

GR32191-treated group. Median time to tumor onset in mice treated with the single low dose of cisplatin was 12 days and addition of GR32191 delayed median tumor onset up to 24 days. Mice treated with the single high dose of cisplatin alone had a median tumor onset of 42 days, compared with 56 days when used in combination with GR32191. Using Cox proportional hazards model, highly significant differences were found between mice treated with vehicle alone versus GR32191 ($P < 0.02$) and mice treated with single low-dose (0.5 mg/kg) cisplatin alone versus single low-dose cisplatin+GR32191 ($P < 0.001$). No statistically significant difference was found between time to tumor in mice treated with single high-dose (5 mg/kg) cisplatin in the absence

or presence of GR32191 ($P = 0.19$). Treatment with GR32191 alone significantly slowed tumor growth compared with the vehicle control group ($P = 0.004$). Both doses of cisplatin significantly delayed tumor growth compared with the vehicle alone ($P = 0.0001$ and 0.0006, respectively). The addition of GR32191 to single low-dose cisplatin had no significant effect on tumor growth rate, whereas GR32191 in combination with single high-dose cisplatin had a significant effect on tumor growth rate ($P = 0.03$).

To further characterize the tumors derived from vehicle-treated mice versus GR32191-treated mice, immunohistochemical analysis and H&E staining were performed to assess the phenotypes of the tumor. There was a significantly higher percentage ($P < 0.01$) of undifferentiated tumors detected by H&E staining of the tumors xenografts (Fig. 5Cd) and significantly increased ($P < 0.05$) cell proliferation as indicated by PCNA staining in vehicle-treated mice (Fig. 5Cc) compared with GR32191-treated mice. There was a significantly higher ($P < 0.05$) microvessel density, measured by staining with the endothelial marker CD31 in tumors derived from mice treated with vehicle control compared with mice treated with GR32191 (Fig. 5Ca). In contrast, a significant increase in apoptosis (TUNEL staining, $P < 0.05$) was observed in tumors derived from mice treated with GR32191 (Fig. 5Cb) compared with vehicle-treated mice. Thus, the reduced tumor growth observed after treatment with TP receptor antagonist was due to decreased proliferation, increased apoptosis, and diminished angiogenesis.

Discussion

This study provides the first evidence that TP-β receptors are overexpressed in some patients with bladder cancer, and that in those patients, it is associated with a poorer prognosis. In addition, survey of multiple bladder cancer cell lines revealed that TP-β receptors were highly expressed. In contrast, immortalized normal bladder cell line SV-HUC expresses the TP-α isoform only. TP-α

was either not expressed or present in lower levels in the bladder cancer cell lines.

Previous studies implicated a potential role of TP in the pathogenesis of several different types of cancer. Specifically, thromboxane synthase was increased in these cancers and/or cell lines derived from them (22–25). We have previously found that thromboxane synthase is elevated in bladder cancer and carries a poor prognosis (2). Inhibition of TP synthesis results in a decrease in proliferation and migration (2). These studies helped to establish a potential role for TP in the cancer phenotype. The final step in the stimulation of the cell by TP relies on the presence of TP receptors.

The transfection of the immortalized but non-transformed SV-HUC cell line with TP-β receptor recapitulated a potential cancer phenotype as shown by an increased rate of proliferation, migration, and invasion compared with both the control pcDNA3 and TP-α DNA–transfected cells. The observation that TP-α did not have an increased rate of proliferation and migration compared with pcDNA3 shows that the effect seen with TP-β is specific for this isoform and not a nonspecific effect of transfection of a TP receptor or empty vector. Stimulation of T24 but not SV-HUC cells with U46619 was associated with an increased phosphorylation of ERK and FAK. Also, treatment with the TP receptor antagonist PTXA$_2$ had no effect on phospho-ERK and phospho-FAK in the SV-HUC cells but significantly reduced these levels in the T24 cells. Increased phospho-ERK and phospho-FAK were observed in SV-HUC cells stably transfected with TP-β. This increase was restored to the normal level after addition of PTXA$_2$. U46619 treatment did not alter phospho-ERK and phospho-FAK in SV-HUC stably transfected with pcDNA3 vector or TP-α, which further prove the TP-β–specific effects. Of greater significance was the observation that TP-β–transfected SV-HUC cells formed tumors in nude mice. Collectively, these observations support the notion that the elevated expression of the TP-β receptor plays a role in the phenotype of bladder cancer cells.
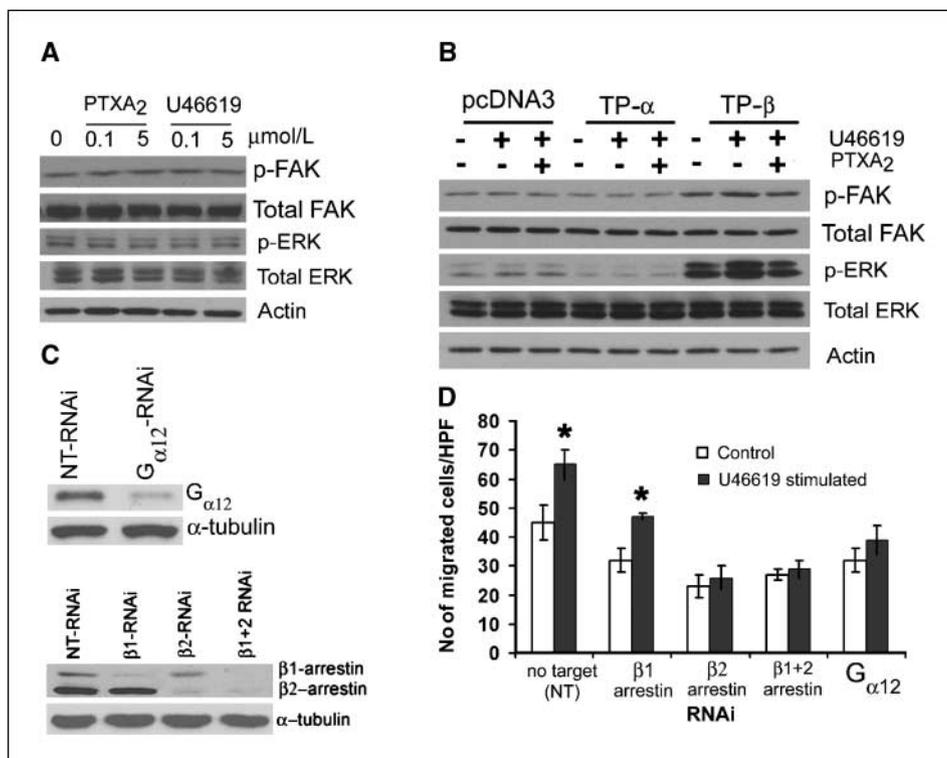
Figure 4. Receptor isoform–specific effects after treatment with either a TP receptor agonist or antagonist. *A* to *D,* effects of U46619 and PTXA2 on phosphorylation of ERK and FAK, and cell migration in wild-type SV-HUC cells (*A*) or SV-HUC cells transfected with pcDNA3, TP-α, or TP-β (*B*). Figure is representative of three experiments. *C,* Western blot analysis for cells stably transfected with shRNAi vector for β arrestins or no target shRNAi (*bottom*) and cells stably transfected with shRNA for G$_{α12}$ or shRNA vector with no target shRNA (*top*). *D,* cells were stimulated with 1 μmol/L U46619 for 12 h, and cell migration was measured by transwell migration assay. *, statistically significant differences compared with the control.

Multiple intracellular signaling pathways mediate the cancer phenotype. TP-α and TP-β receptors are G protein–coupled and couple to some of the same G proteins. TP-β but not TP-α has also been shown to interact with β-arrestins 1 and 2 (26–29). Once thought to only enhance desensitization, β-arrestins have been shown to also act as scaffolding proteins and also to activate mitogen-activated protein kinase and phosphorylate ERK. In further support of a role for β-arrestins in mediating the effects of TP-β are the observations of Buchanan and colleagues (30) who found that β-arrestin 1 may play an important role in prostaglandin E_2 activation of *Src* and transactivation of epidermal growth factor receptor in colorectal cancer cells. In addition, β-arrestin was shown to enhance the metastatic potential of colorectal cancer cells *in vivo* (30). However, in contrast, we found that β2-arrestin and not β1-arrestin mediated TP-β–induced migration. We further showed that $G\alpha_{12}$ also plays a role in TP-β–induced cell migration, consistent with the previous observations of its role in metastases (20, 21). Thus, there are several intracellular signaling pathways unique to TP-β that could be responsible for the increased proliferation and migration seen in the bladder cancer cells and the SV-HUC cells transfected with TP-β. Further studies will be needed to sort out the critical intracellular signaling pathways responsible for the cancer phenotype.

Of significant interest is the observation that there was a differential up-regulation of only one of the two TP receptor isoforms. The mechanism(s) for this isoform-specific up-regulation are currently unknown. This is the first report of altered expression of a specific TP receptor isoform occurring in cancer cells.

The addition of GR32191 or SQ29548 to paclitaxel *in vitro* synergistically increased tumor cell death. Paclitaxel is a widely used anticancer drug with shown activity in epithelial cell solid tumors including bladder cancers (31). Although paclitaxel is one of the most successfully used chemotherapeutic agents, the success rate for previously untreated bladder cancer patients is only 40% to 50%. Therefore, combination of paclitaxel and a TP receptor antagonist may improve efficacy.

Cisplatin has been used as a chemotherapeutic agent for the treatment of bladder cancer (32), but its use is associated with renal toxicity (33) and increased renal synthesis of thromboxane A_2 (34, 35). Increased intrarenal synthesis of TP has been shown to be associated with impaired function (36, 37). Thus, the use of a TP receptor antagonist as adjunctive therapy has the potential advantage of enhancing the chemotherapeutic effects of cisplatin, coupled with reducing the untoward side effects. Previous studies have shown that TP receptor antagonist treatment increases Na^+, K^+-ATPase activity along with intracellular accumulation and sensitivity to cisplatin in non–small cell lung cancer (38). Furthermore, TP receptor antagonists enhanced cisplatin-induced apoptosis in lung cancer cell lines by up-regulating ICH-1L (39). Our results further support the notion that TP receptor antagonists augment *in vitro* and *in vivo* responses to cisplatin.

Treatment with the TP receptor antagonist reduced cell proliferation *in vitro* and also increased the time of tumor onset and reduced the rate of tumor growth *in vivo*. Patients with the 3-fold or greater overexpression of TP-β in their tumor tissue had a significantly poor prognosis. These results coupled with the cellular studies raise the possibility that the TP-β receptor could serve as a novel therapeutic target in bladder cancer. There have been previous clinical studies with the TP receptor antagonist GR32191 in healthy individuals (40) and in patients with asthma (41) or cardiovascular diseases (42), and was shown to be safe. Not only
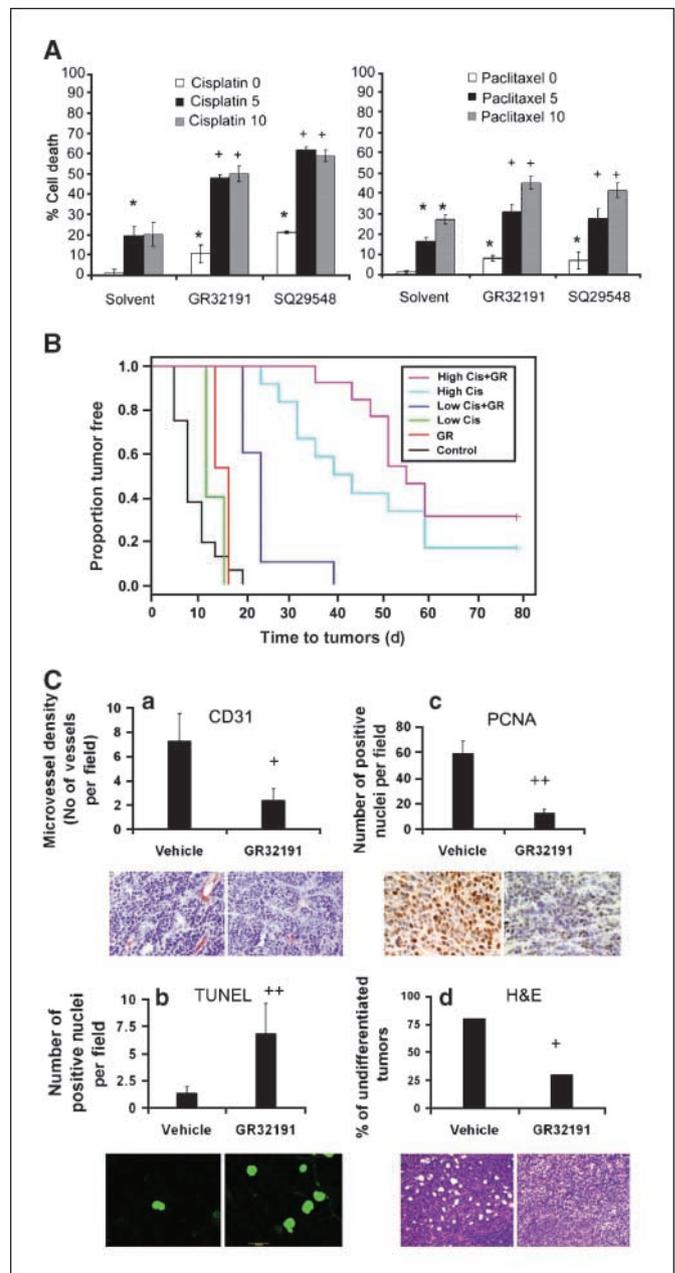
Figure 5. Antitumor effects of TP receptor antagonists. *A*, effects of TP receptor antagonists on cisplatin- (*left*) or paclitaxel- (*right*) treated T24 cells. T24 cells were incubated with vehicle, GR32191 (1 μmol/L), or SQ29548 (1 μmol/L), and increasing concentrations of cisplatin for 12 h. Cell death was determined using trypan blue staining. *Columns,* mean for three experiments determined in triplicate; *bars,* SD. *, $P < 0.05$ for TP receptor antagonist compared with solvent. +, $P < 0.05$ cisplatin or paclitaxel plus TP receptor antagonist compared with cisplatin or paclitaxel alone. *B*, antitumor effects of GR32191 and cisplatin treatment in immunocompromised mice. Subcutaneous tumors from TCC-SUP human bladder cancer cells were treated with vehicle control (12 mice), GR32191 (15 mice), 5 mg/Kg cisplatin (cisplatin high; 12 mice), 5 mg/kg cisplatin in combination with GR32191 (13 mice), or 0.5 mg/kg (single low-dose cisplatin) alone (10 mice), or 0.5 mg/kg cisplatin in combination with GR32191 (10 mice). Tumor size was measured over time. Kaplan-Meier curves showing time to tumor onset across the treatment groups. *C*, pathologic evaluation of tumors derived from mice treated with GR32191 ($n = 15$) and vehicle-treated mice ($n = 12$). Representative images of CD31 immunohistochemical staining (*Ca*), TUNEL immunofluorescence staining (*Cb*), PCNA immunohistochemical staining (*Cc*), and H&E staining (*Cd*) are shown. Graphs on the top of each panel indicate the quantitative analysis for each of these markers. Five fields from 5 independent tumors (total 25) were counted and scored blindly. Statistically significant ($P < 0.05$) differences compared with vehicle alone were indicated (++, $P < 0.001$; +, $P < 0.05$).

could the TP-β receptor be a therapeutic target but its presence and/or overexpression could be used as a predictor of prognosis and dictate therapy. Clearly, further studies are needed to delineate the role of TP-β receptors in bladder cancer. Although the role of GPCRs in tumor progression has been extensively studied, to our knowledge, this is the first report that a GPCR alone was able to transform primary cells (Fig. 2). Significantly, we have found that TP-β is highly expressed in multiple cancer–derived cell lines (prostate, breast, colon, and renal cell carcinoma) and is absent in additional immortalized cell lines (e.g., PrEC prostate cells). Collectively, these findings coupled with its correlation with disease progression, metastatic potential, and overall patient survival make TP-β an ideal candidate for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Novel Role of Thromboxane Receptors β Isoform in Bladder Cancer Pathogenesis

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