NEOPLASIA
www.neoplasia.com

Volume 13 Number 8 August 2011 pp. 758–770 758

Type III TGF-β Receptor Enhances Colon Cancer Cell Migration and Anchorage-Independent Growth[1,2]

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Abstract

The type III TGF-β receptor (TβRIII or betagylcan) is a TGF-β superfamily coreceptor with emerging roles in regulating TGF-β superfamily signaling and cancer progression. Alterations in TGF-β superfamily signaling are common in colon cancer; however, the role of TβRIII has not been examined. Although TβRIII expression is frequently lost at the message and protein level in human cancers and suppresses cancer progression in these contexts, here we demonstrate that, in colon cancer, TβRIII messenger RNA expression is not significantly altered and TβRIII expression is more frequently increased at the protein level, suggesting a distinct role for TβRIII in colon cancer. Increasing TβRIII expression in colon cancer model systems enhanced ligand-mediated phosphorylation of p38 and the Smad proteins, while switching TGF-β and BMP-2 from inhibitors to stimulators of colon cancer cell proliferation, inhibiting ligand-induced p21 and p27 expression. In addition, increasing TβRIII expression increased ligand-stimulated anchorage-independent growth, a resistance to ligand- and chemotherapy-induced apoptosis, cell migration and modestly increased tumorigenicity *in vivo*. In a reciprocal manner, silencing endogenous TβRIII expression decreased colon cancer cell migration. These data support a model whereby TβRIII mediates TGF-β superfamily ligand-induced colon cancer progression and support a context-dependent role for TβRIII in regulating cancer progression.

Neoplasia (2011) 13, 758–770

Introduction

Transforming growth factor β (TGF-β) plays a dichotomous role in human cancers, functioning both as a tumor suppressor and as a tumor promoter [1]. Many human tumors downregulate or exhibit mutations in components of the TGF-β signaling pathway, resulting in functional resistance to the homeostatic functions of the pathway [2]. Conversely, many late-stage human tumors increase TGF-β expression, which has a tumor-promoting effect by suppressing immune surveillance, inducing epithelial to mesenchymal transition, and promoting tumor invasiveness, angiogenesis, and metastasis [2]. This dual role of TGF-β as both a tumor suppressor and tumor promoter remains a fundamental roadblock to effectively targeting the TGF-β pathway for the treatment of human cancers.

Abbreviations: TβRIII, type III TGF-β receptor; sTβRIII, soluble TβRIII

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[1]This work was supported by the National Institutes of Health/National Cancer Institute (RO1-CA135006 and RO1-CA136786 to G.C.B. and F32-CA1361252 to C.E.G).

[2]This article refers to supplementary materials, which are designated by Figures W1 to W3 and are available online at www.neoplasia.com.

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Received 13 April 2011; Revised 18 June 2011; Accepted 20 June 2011

DOI 10.1593/neo.11528

TGF-β superfamily signaling is regulated and mediated by a co-receptor, the type III TGF-β receptor (TβRIII or betagylcan), which binds all three TGF-β isoforms, multiple bone morphogenetic proteins (BMPs), and inhibin [3–6]. TβRIII functions by presenting ligand to the respective type II and type I TGF-β superfamily receptors, which, upon ligand binding, transphosphorylate to activate the respective type I TGF-β superfamily receptor [4,7]. This leads to the phosphorylation and subsequent activation of the Smad transcription factors, which then interact with Smad4, translocate to the nucleus, and regulate the transcriptional activity of a variety of TGF-β superfamily responsive genes in a cell type–specific manner [8]. TβRIII also mediates ligand-dependent and -independent p38 pathway signaling [4,9,10], inhibits nuclear factor κB signaling [11,12], and activates Cdc42 to regulate cell proliferation and migration [13]. TβRIII inhibits TGF-β superfamily signaling through ectodomain shedding–mediated generation of soluble TβRIII (sTβRIII), which has been demonstrated to bind and sequester TGF-β away from its receptors [4,14].

In normal intestinal epithelium, TGF-β functions as a tumor suppressor through the regulation of cell growth and the induction of apoptosis [15]. TGF-β superfamily signaling is commonly disrupted in colon cancer with frequent alterations in components of the TGF-β superfamily signaling pathways [16–20]. However, studies have demonstrated that response to TGF-β is dependent on tumor stage, with TGF-β functioning to inhibit proliferation in early stages of colon cancer and promoting growth and invasion in later stages and during tumor progression [21,22]. Colon cancer cells have been demonstrated to secrete TGF-β and elevated levels of TGF-β1 are significantly correlated with metastatic disease, disease recurrence, and decreased survival [24–26]. In addition, TGF-β1 has been demonstrated to promote colon cancer cell proliferation in a Ras-dependent but Smad-independent manner [27] and to promote Ras-mediated invasiveness in intestinal epithelial cells in a TβRII-dependent manner [28]. Therefore, in late stages of colon carcinogenesis, TGF-β may function as a tumor promoter, supporting a dual role for TGF-β signaling in colon cancer progression.

Similarly to TGF-β, BMP expression is increased in colorectal tumors and correlates with poor prognosis and metastasis [29]. BMPs have diverse biologic roles in colon cancer, regulating proliferation, migration, invasion, apoptosis, and differentiation [30–33]. Overexpression of BMP4 in the Smad4-deficient cell line, SW480, enhances cell proliferation, migration, invasiveness, and adhesion [34]. These studies demonstrate that alterations in TGF-β and BMP signaling play a dual role in colon carcinogenesis, both inhibiting and promoting colon cancer.

TβRIII is expressed in normal intestinal goblet cells; however, it does not undergo proper posttranslational modification and is unable to bind TGF-β1, resulting in insensitivity to TGF-β1–mediated growth inhibition [35]. In contrast, neighboring absorptive cells, which express functional TβRIII, are growth-inhibited by TGF-β1, demonstrating that TβRIII can modulate TGF-β1 signaling in normal colon cells [35]. Expression of oncogenic Ras in goblet cells restores posttranslational modification of TβRIII and causes these cells to become growth-stimulated in response to TGF-β1 treatment, suggesting that K-Ras confers a more aggressive phenotype through alterations in TβRIII posttranslational modifications [35,36]. These data suggest that TβRIII may play an important role in mediating TGF-β signaling in colon cancer.

Recently, TβRIII expression has been demonstrated to be lost or decreased in multiple human cancers, including breast, prostate, ovarian, pancreatic, and non–small cell lung cancers [37–41]. TβRIII has been demonstrated to be an important regulator of cell migration, invasion, cell growth, and angiogenesis, with restoration of TβRIII expression inhibiting cancer progression [13,37]. Taken together, these data support a role for TβRIII as a mediator of TGF-β superfamily signaling during cancer progression. Here, we examined the role of TβRIII in human colon cancer.

Materials and Methods

TβRIII Gene Expression Analysis on Complementary DNA Array

An array containing normalized complementary DNA (cDNA) from colon carcinomas and matched normal tissues (n = 37) (Cancer Profiling Array; Clontech; Takara Bio Co, Madison, WI) was probed with a [^{32}P]-labeled cDNA probe for TβRIII following methods recommended by the manufacturer. The TβRIII cDNA probe was amplified by polymerase chain reaction (PCR) using the forward primer 5′ GTAGTGGGTTGGCCAGATGGT 3′ and reverse primer 5′ CTGCTGTCTCCCCTGTGTG 3′. Twenty-five nanograms of purified PCR products was labeled by random primed DNA labeling using [α^{32}P]dCTP as per the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). The labeled cDNA probe was purified on a BD CHROMA SPIN+STE-100 column (BD Biosciences, Clontech; Takara Bio Co). Images were acquired using a phosphorimager, and subsequent data analysis was performed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD; http://rsb.info.nih.gov/ij/). The TβRIII array was normalized to a ubiquitin-probed array.

Tissue Microarray

A custom polyclonal TβRIII antibody (820) was created by immunizing rabbits with a GST-fusion protein of the human TβRIII cytoplasmic domain [37]. Immunohistochemistry for TβRIII was performed on a colon cancer tissue microarray (Cooperative Human Tissue Network, National Cancer Institute, Bethesda, MD) containing colon carcinomas (n = 323), normal colon epithelium (n = 60), and adenomatous polyps (n = 34). The array was deparaffinized, rehydrated, treated with 3% hydrogen peroxide, blocked with 10% normal goat serum, incubated with the 820 TβRIII custom polyclonal antibody at 4°C overnight, and incubated with antirabbit IgG-HRP antibody (Vector Laboratories, Burlingame, CA). Cells were counterstained using hematoxylin. The immunoreactivity for TβRIII was semiquantitatively scored by two independent observers in a blinded manner, with staining intensity defined as 0 to 1 (no or weak staining), 2 (moderate staining), and 3 (intense staining). All images were acquired at a magnification of ×20.

Cell Culture, Stable Cell Lines, and Adenoviral Infection

Human colon cancer cell lines HT29, SW480, and SW620 were cultured in McCoy 5A + 10% fetal bovine serum (FBS) and high-glucose Dulbecco modified Eagle medium + 10% FBS, respectively. HT29 stable cell lines, representing a pool of stable clones, were derived as previously described and maintained in 500 μg/ml G418 [37,40]. Adenoviral infections were performed as previously described [39]. All adenoviral infections were performed at a multiplicity of infection of 25 for all constructs. Cells were treated with the Ras antagonist farnesyl thiosalicylic acid (FTS) at 200 μM or dimethyl sulfoxide (DMSO) control for 3 days before harvest. Media and FTS were changed daily to maintain the correct concentration of FTS.

TGF-β Binding and Cross-linking

TGF-β binding and cross-linking experiments were performed as previously described [37,39].

Western Blot Analysis

A total of 2.5×10^5 cells were plated in six-well dishes and allowed to recover. Cells were serum-starved overnight and then treated with 100 pM TGF-β or 20 nM BMP-2 for the indicated times. The cells were lysed in boiling sample buffer and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted for the proteins of interest. Primary antibodies (p-Smad1/5/8 [no. 9511], Smad1 [no. 9743], p-Smad2 [no. 3101], Smad2 [no. 3103], pp44/42 [no. 9101], p44/42 [no. 9102], p-p38 [no. 4511], and p38 [no. 9212]) were purchased from Cell Signaling Technology (Danvers, MA), and a 1:1000 dilution was used for immunoblot analysis. Primary Ras antibody (no. OP40) was purchased from EMD Chemicals (Gibbstown, NJ), and a 1:2000 dilution was just for immunoblot analysis. Protein levels were determined by immunoblot analysis followed by densitometric analysis, including background subtraction and normalization to β-actin using ImageJ software (NIH).

Proliferation Assay

HT29-Neo and HT29-TβRIII cells were plated at 3000 cells per well in a 96-well plate and grown overnight with ligand stimulation (20 nM BMP-2, 40 nM BMP-2, 50 pM TGF-β, or 100 pM TGF-β). The next day, cells were pulsed with 1 μCi of ^{3}H per well for 4 hours at 37°C. Cells were washed in cold PBS and 10% trichloroacetic acid (TCA) and then incubated for 1 hour at 4°C with 10% TCA. Cells were then washed with cold 10% TCA and lysed overnight with 0.2M NaOH. Lysates were then read on a scintillation counter.

Soft Agar Assay

Six-well plates were coated with a 0.8% base layer of agarose in McCoy 5A medium with 10% FBS, L-glutamine, and 500 μg/ml G418. HT29-Neo and HT29-TβRIII cells were counted and plated at 6×10^3 cells/ml in 0.4% agar in McCoy 5A medium with 10% FBS, L-glutamine, and 500 mg/ml G418. Cells were incubated at 37°C for 21 days and fed every 3 days with McCoy 5A + 10% FBS + ligand. Cells were fixed and stained with 0.005% crystal violet in 10% neutral buffered formalin solution, then washed with PBS. Colonies were counted and quantified using Bio-Rad Quantity One software (Hercules, CA).

Fibronectin Transwell Motility Assay

To assess migration, 2.5×10^5 cells were seeded in serum-free medium in the upper chamber of a transwell filter, coated both at the top and bottom with 50 μg/ml fibronectin (Calbiochem, La Jolla, CA). Cells were untreated, treated with 100 pM TGF-β or 20 nM BMP-2 and were allowed to migrate for 12 hours at 37°C through the fibronectin toward the lower chamber containing medium plus 10% FBS. Cells on the upper surface of the filter were removed, and cells that migrated to the underside of the filter were fixed and stained using the 3-Step Stain Set (Richard-Allan Scientific, Kalamazoo, MI). Each assay was performed in duplicate, and each experiment was conducted at least three times with three random fields from a 20× magnification analyzed for each membrane. Data analysis was performed using ImageJ software (NIH).

Monolayer Wound Healing Motility Assay

HT29-Neo, HT29-TβRIII, HT29-NTC, and HT29-shRNA-TβRIII cells were plated to confluence and then scratched to cause a wound. Cells were untreated, treated with 40 nM BMP-2, 100 pM TGF-β, with or without 5 μM SB431542 or 15 μM SB203580, for 24 hours. Images were taken at 0- and 24-hour time points with a Nikon inverted microscope (Melville, NY) at a magnification of ×10. Cells were maintained in their selection medium at 37°C, 5% CO_2 during incubation. The percentage of wound closure was calculated ± SEM.

Immunofluorescence

For actin staining, the cells were fixed in a 4% parformaldehdye and permeabilized with 0.1% Triton X for 5 minutes. Blocking was performed with 1% bovine serum albumin, and cells were incubated with a 1:50 dilution of phalloidin conjugated to Texas red (Molecular Probes, Carlsbad, CA). For E-cadherin staining, the cells were fixed with a 1:1 solution of methanol and acetone at −20°C. Blocking was performed with 1% bovine serum albumin, and cells were incubated with a 1:250 dilution of E-cadherin antibody (BD Biosciences, Madison, WI), followed by incubation with an antimouse antibody conjugated to Texas Red (Molecular Probes). Immunofluorescence images were obtained using a Nikon inverted microscope at a magnification of ×60. Line scan analysis was performed using ImageJ software (NIH).

HT29 Xenograft Model

All animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University. HT29-Neo or TβRIII cells were grown in McCoy 5A + 10% FBS under the selection of G418 (500 μg/ml). Twenty-four hours before injection, cells were transferred to selection-free media. TβRIII expression was confirmed by [^{125}I]TGF-β binding and cross-linking. A total of 1×10^6 low–passage number (P10) HT29-Neo or HT29-TβRIII cells were injected subcutaneously into the right and left flanks of BALB/cAnNCr *nu/nu* mice. Mice were weighed, and tumor width (W) and length (L) were measured every 3 days. Tumor volume was determined using the formula: $V = 0.5 \times L \times W^2$. Mice were followed for 21 days, when some mice reached humane end points. On sacrifice, tumors were excised, weighed, and fixed in 4% paraformaldehyde for 2 hours followed by 70% ethanol. In addition, the lungs, liver, and axillary lymph nodes were harvested and fixed in 4% paraformaldehyde for 2 hours followed by 70% ethanol to determine the sites of metastases. Tumors, lungs, livers, and lymph nodes were stained with hematoxylin and eosin to examine histology and to determine metastatic incidence.

Results

TβRIII Expression Is Increased in Human Colon Cancer

A decrease in TβRIII expression, both at the mRNA and protein levels, has been demonstrated in multiple human cancer types, including breast, kidney, non–small cell lung, ovarian, pancreatic, and prostate cancers [37–42]. As alterations in both TGF-β and BMP signaling have been demonstrated to contribute to colon carcinogenesis, we examined the expression of TβRIII in human colon cancer using a cDNA array with matched normal and colon carcinoma tissue (Figure 1*A*). In contrast to what has been reported in other tumor

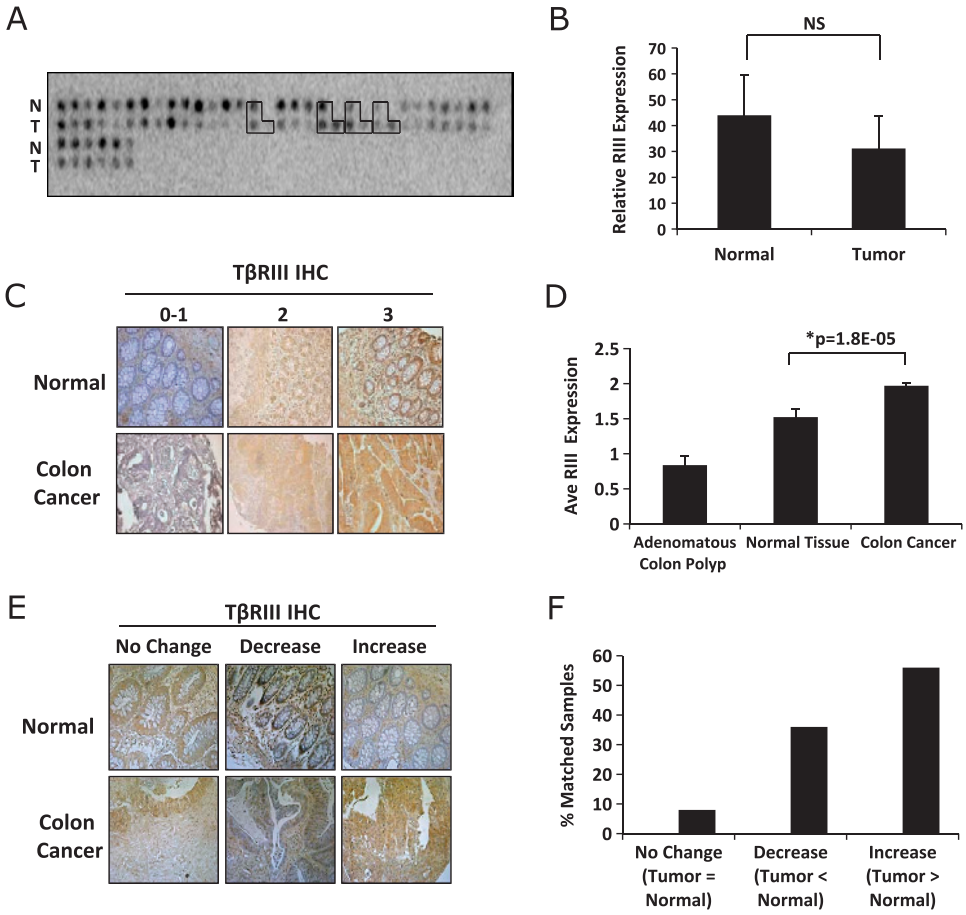

Figure 1. TβRIII expression increases during colon cancer. (A) A cDNA array with matched normal and colon tumors (n = 37) was hybridized with a [^{32}P]-labeled probe for TβRIII. The signal intensity for each spot was determined using ImageJ software. Boxed areas represent paired normal, tumor, and metastases. (B) Graphical representation of the mean signal intensity ± SD of the intensity. NS indicates not significant. (C) TβRIII IHC was performed on a human tissue microarray of normal colon and tumor specimens. The tissue array was scored on a scale of 0 (no staining) to 3 (highest). Adenomatous colon polyp (n = 34), normal tissue (n = 60), and colon cancer (n = 323). Original magnification, ×20. (D) Graphical representation of the average intensity score of TβRIII protein expression in normal and tumor tissues ± SEM. (E) TβRIII IHC was performed on a human tissue microarray of normal colon and tumor specimens. Representative matched normal and tumor sample pairs from the same patient are shown, demonstrating no change, an increase, or a decrease in TβRIII expression. Original magnification, ×20. (F) Graphical representation of the percentage of matched pairs (n = 25) that demonstrate an increase (n = 14), no change (n = 2), or decrease (n = 9) in TβRIII protein expression in tumor *versus* normal tissue.

types, there was no significant difference in average TβRIII mRNA expression between normal tissue and colon carcinomas (Figure 1*B*). In addition, no difference in TβRIII expression was observed with regard to stage or grade of tumors (data not shown). We then examined TβRIII expression at the protein level in human colon cancer specimens by performing immunohistochemistry (IHC) for TβRIII on human tissue microarrays containing normal colon epithelium (n = 60), adenomatous polyp tissue (n = 34), and colon carcinomas (n = 323; Figure 1, *C* and *D*). When comparing TβRIII protein expression between normal colon epithelium and colon carcinomas, a modest but significant increase in TβRIII protein levels was observed (Figure 1*D*). Comparison between matched normal and tumor pairs (n = 25) demonstrates that whereas 8% of the matched pairs had no change and 36% had a decrease in TβRIII expression, the majority (56%) had an increase in TβRIII protein expression (Figure 1, *E* and *F*). These data demonstrate that TβRIII expression is not significantly altered at the mRNA level in human colon cancer, whereas TβRIII protein expression increases in a large subset of colon tumors, suggesting posttranscriptional regulation of TβRIII expression in human colon cancer.

TβRIII Enhances Signaling in Colon Cancer Cells

To investigate the role of TβRIII in colon cancer, several colon cancer cell lines were analyzed for TβRIII expression. HT29, a Smad4-positive cell line derived from a primary colon carcinoma, and SW480 and SW620 cells, Smad4-deficient cell lines that are derived from the primary colon cancer (SW480) and metastatic lymph node (SW620) from the same patient, all express TβRIII, albeit with different patterns of posttranslational modification (Figure 2*A*). To examine the effects of an increase in TβRIII expression on colon cancer, HT29 colon cancer cell lines stably expressing Neo or TβRIII were created, and TβRIII expression was verified by binding and cross-linking ^{125}I-TGF-β (Figure 2, *A* and *B*). The expression of both membrane-bound TβRIII and sTβRIII was increased in HT29-TβRIII cells relative to HT29-Neo cells (Figure 2*B*).

Restoring TβRIII expression has been demonstrated to inhibit TGF-β responsiveness in other cancer types, including in breast cancer, as well as inhibiting BMP responsiveness in pancreatic cancer [37,43]. To determine whether increased expression of TβRIII alters TGF-β or BMP-2 responsiveness, phosphorylation of Smad2, Smad1/5/8, p38, and ERK in response to ligand stimulation was examined in HT29-Neo and HT29-TβRIII Smad4-expressing colon cancer cells. There was a significant increase in basal levels of p-Smad1/5/8 in HT29-TβRIII cells in the absence of exogenous ligand treatment (Figure 2*C*). In response to TGF-β1, p-Smad2, p-Smad1/5/8, and p-p38 levels were increased in HT29-TβRIII cells relative to HT29-Neo cells, with an earlier onset of Smad2 phosphorylation (Figure 2*C*). In response to BMP-2, HT29-TβRIII cells exhibited an earlier onset of Smad1/5/8 phosphorylation and increased p-p38 signaling (Figure 2*C*). No change in ERK signaling was observed, basally or with ligand treatment. These data suggest that TβRIII has both ligand-independent and ligand-dependent effects on signaling because Smad1/5/8 phosphorylation was enhanced both basally and in response to exogenous ligand treatment, and Smad2 and p38 enhancement was ligand specific. Together, these data demonstrate that TβRIII increases TGF-β– and BMP-2–mediated canonical and noncanonical signaling in colon cancer cells, in stark contrast to the inhibitory effects of TβRIII on TGF-β superfamily signaling in other cancer contexts [37,39].

TβRIII Increases Ligand-Stimulated Proliferation in Colon Cancer Cells by Decreasing Basal and Ligand-Stimulated p21 and p27 Expression

TGF-β treatment in the presence of oncogenic K-Ras, which regulates posttranslational modification of TβRIII, has been demonstrated to induce proliferation and downregulate p21 in colon cancer cells [27,36]. To examine the effects of TβRIII expression on proliferation, HT29-Neo and TβRIII cells were treated with BMP-2 or TGF-β and proliferation was examined by ^{3}H-thymidine incorporation. In the absence of ligand stimulation, no significant difference was observed in proliferation between HT29-Neo and TβRIII cells (Figure 3, *A* and *B*). However, TGF-β1 or BMP-2 treatment resulted in moderate growth arrest in HT29-Neo cells (10%-15% reduction in growth with TGF-β1 treatment and 7%-15% reduction with BMP-2 treatment) (Figure 3, *A* and *B*). In contrast, treatment of HT29-TβRIII cells with either BMP-2 or TGF-β resulted in resistance to ligand-induced growth arrest and a modest, but significant increase in cell growth (≥5% increase in growth with both TGF-β1 and BMP treatment;

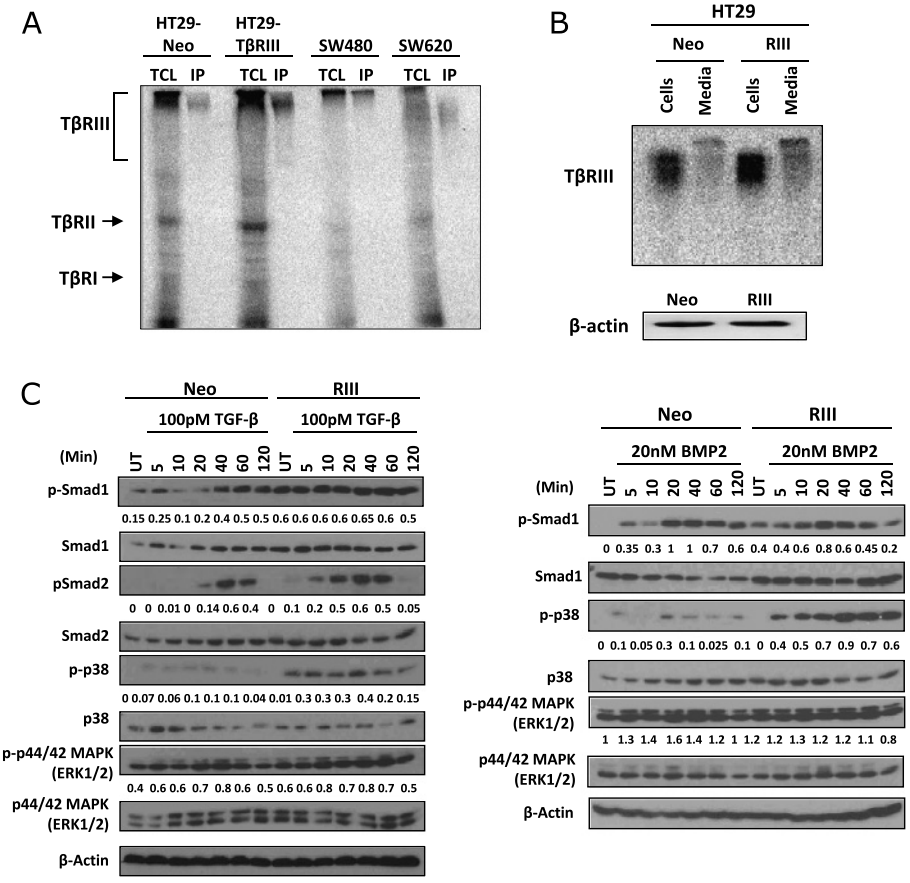

Figure 2. TβRIII is expressed in colon cancer cell lines and enhances BMP-2– and TGF-β–mediated signaling. (A) Binding and cross-linking of total cell lysates (TCL) (left lanes) and immunoprecipitation (IP) for TβRIII (right lanes) from four different cell lines: HT29 stable cell lines, namely, HT29-Neo and HT29-TβRIII; SW480 cells, derived from a primary colorectal adenocarcinoma; and SW620 cells, derived from the metastatic lymph node site of a colorectal adenocarcinoma from the same patient as the SW480 line. (B) Expression of sTβRIII in HT29 stable cell lines. Cells were grown in 10% FBS McCoy 5A for 24 hours. TβRIII and sTβRIII expression was examined in cells and medium by binding and cross-linking. β-Actin serves as a total protein control (bottom panel). (C) HT29-Neo and TβRIII cells were serum-starved overnight and then treated with 100 pM TGF-β or 20 nM BMP-2 during the indicated time course (minutes). Western blot analyses were performed for the indicated proteins. Densitometric analysis is shown normalized to β-actin.

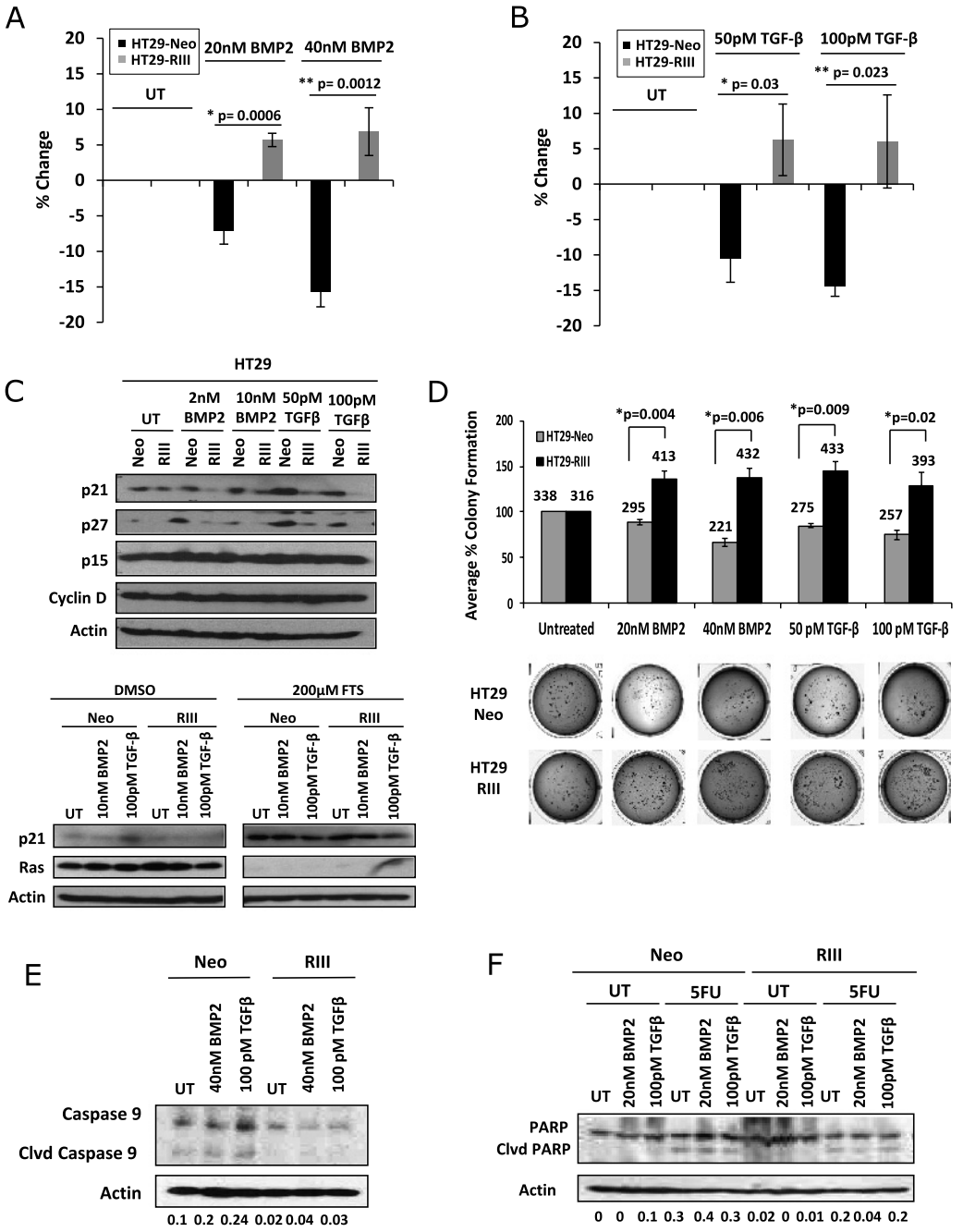

Figure 3. TβRIII expression increases colon cancer *in vitro* tumorigenicity. (A) HT29-Neo and TβRIII cells were treated with 20 or 40 nM BMP-2 for 24 hours. Proliferation was analyzed by a ^{3}H incorporation assay. The percent proliferation was determined by normalizing the counts to those of untreated samples. (B) HT29-Neo and TβRIII cells were treated with 50 or 100 pM TGF-β1 for 24 hours. Proliferation was analyzed by a ^{3}H incorporation assay. (C) HT29-Neo and TβRIII cells were treated with 2 or 10 nM BMP-2 and 50 or 100 pM TGF-β1 for 24 hours. HT29-Neo and TβRIII cells were treated with 200 μM FTS or DMSO for 3 days. Western blot analyses were performed to analyze protein levels of p21, p27, p15, cyclin D, and Ras with β-actin as a total protein control. (D) HT29-Neo and TβRIII cells were plated in a soft agar assay untreated or treated with 20 nM BMP-2, 40 nM BMP-2, 50 pM TGF-β, or 100 pM TGF-β for 21 days. The mean percent colony formation ± SEM is shown normalized to the untreated Neo or TβRIII. Average colony number is shown above the bar graph. (E) HT29-Neo and TβRIII cells were treated with 40 nM BMP-2 or 100 pM TGF-β for 48 hours and examined for apoptosis by Western blot analysis of caspase 9 levels. Densitometric analysis is shown normalized to β-actin. (F) HT29-Neo and TβRIII cells were treated with 50 μM 5-fluorouracil for 48 hours. Cells were concurrently treated with 20 nM BMP-2 or 100 pM TGF-β and examined for apoptosis induction by Western blot analysis of PARP cleavage. Densitometric analysis is shown normalized to β-actin.

Figure 3, *A* and *B*). Although there is a modest increase in proliferation in the HT29-TβRIII ligand–treated cells, these differences are significant (Student's *t* test, $P < .05$) in comparison to the reduction in proliferation observed in the HT29-Neo ligand–treated cells. These data demonstrate that TβRIII can inhibit ligand-mediated growth arrest and stimulate proliferation in response to TGF-β or BMP-2 in colon cancer cells. Similar results were obtained in the SW480 cells in response to BMP treatment (Figure W1*C*).

TGF-β and BMP regulate proliferation through induction of a number of cell cycle proteins, including the cyclin-dependent kinase inhibitor, p21 [44]. To explore the mechanism by which TβRIII regulates colon cancer cell proliferation, the effect of TβRIII on p21, p27, cyclin D, and p15 levels was examined. In response to treatment with either BMP-2 or TGF-β1, p21 and p27 protein levels increased with no change in cyclin D1 or p15 levels in HT29-Neo cells (Figure 3*C*). SW480-GFP cells exhibit a modest increase in levels of p27 in response to TGF-β treatment and a modest decrease in p21 levels in response to BMP-2 ligand stimulation (Figure W1*B*). In contrast, HT29-TβRIII cells exhibit a reduction in basal p21 levels relative to HT29-Neo cells and neither BMP-2 nor TGF-β induced p21 or p27 expression (Figure 3*C*). Similarly, SW480-TβRIII cells exhibited a reduction in basal p21 levels relative to SW480-GFP cells, and TGF-β treatment failed to stimulate p27 expression (Figure W1*B*). Interestingly, no significant alterations in p21 mRNA levels were observed in the HT29-Neo or HT29-TβRIII cell lines in response to TGF-β or BMP-2 treatment (Figure W1*A*), suggesting that TβRIII promotes proliferation in colon cancer cells through the down-regulation of p21 at the protein level.

As oncogenic K-Ras, which regulates posttranslational modification of TβRIII, has been demonstrated to induce proliferation and downregulate p21 in colon cancer cells, we examined the contribution of Ras signaling to TβRIII-mediated decreases in p21 levels by using the Ras inhibitor, FTS [27,36,45]. As previously observed, treatment with FTS inhibits Ras and significantly upregulates p21 protein levels in HT29-Neo and HT29-TβRIII cells (Figure 3*C*) [45]. Inhibition of Ras by FTS treatment also attenuates TβRIII-mediated inhibition of ligand-induced p21 levels, suggesting that TβRIII-mediated regulation of p21 occurs at least partially in a Ras-dependent manner in colon cancer cells (Figure 3*C*).

TβRIII Increases Colon Cancer Cell Tumorigenicity In Vitro

To examine the effect of TβRIII on anchorage-independent colon cancer cell growth, a soft agar assay was performed with HT29-Neo and HT29-TβRIII cells. In the absence of ligand stimulation, no difference in colony formation was observed between HT29-Neo and TβRIII cells (Figure 3*D*). In response to either BMP-2 or TGF-β treatment, HT29-Neo cells had a modest but significant decrease in colony formation. In contrast, in HT29-TβRIII cells colony formation significantly increased in response to ligand stimulation (Figure 3*D*). These data demonstrate that TβRIII expression enhances ligand-stimulated anchorage-independent growth, a hallmark of tumorigenicity, in colon cancer cells.

TGF-β signaling has been demonstrated to regulate apoptosis, with resistance to apoptosis being another characteristic of tumorigenicity. Although increasing TβRIII expression only modestly decreased anoikis in HT29 cells (data not shown), increasing TβRIII expression significantly reduced ligand-induced apoptosis, as demonstrated by decreased levels of caspase 9 in HT29 cells (Figure 3*E*). In addition, the expression of TβRIII inhibited chemotherapy (5-fluorouracil)-induced apoptosis in HT29 cells (Figure 3*F*). These data demonstrate that TβRIII can inhibit ligand-induced and chemotherapy-induced apoptosis and may inhibit anoikis. This resistance to apoptosis supports a protumorigenic role for TβRIII in colon cancer.

TβRIII Increases Colon Cancer Cell Motility

TβRIII has been demonstrated to regulate cell motility and invasion in both epithelial and cancer cells [13,37,38]. In the context of other cancers, the ability to inhibit cell motility and invasion plays an important role in TβRIII's function as a suppressor of cancer progression. The increased tumorigenicity observed in colon cancer cells with increased TβRIII expression *in vitro* suggests that TβRIII may regulate motility in colon cancer cells as well. A fibronectin transwell migration assay demonstrated a trend toward an increase in basal migration of SW480-TβRIII and SW620-TβRIII cells in the absence of ligand stimulation, whereas TGF-β– and BMP-2–treated SW480-TβRIII and SW620-TβRIII cells had a significant increase in motility in comparison to GFP-infected control cells (Figures 4*A* and W3). However, no further increase in migration is observed between untreated and ligand-stimulated TβRIII cells, suggesting that TβRIII mediates an increase in basal migration. In addition, HT29-TβRIII cells consistently migrated faster than the HT29-Neo cells in response to ligand treatment in a monolayer wound healing assay (Figure 4*B*). In a reciprocal manner, short hairpin RNA (shRNA)–mediated silencing of endogenous TβRIII significantly decreased both basal and ligand-induced migration in HT29 cells (Figure 4*B*). In addition, knockdown of TβRIII in SW480 and SW620 cells demonstrated a trend toward a decrease in migration in a fibronectin transwell migration assay, with a significant reduction in migration on BMP ligand stimulation in SW620 cells (Figures 4*A* and W3).

As TβRIII expression in colon cancer cells enhanced both Smad and p38 signaling (Figure 2), we examined the contribution of these pathways to TβRIII-mediated stimulation of migration. HT29-Neo or TβRIII cells were treated with the ALK5 inhibitor SB431542 or the p38 inhibitor SB203580, and effects on migration were assessed in a monolayer wound healing assay. The ALK5 inhibitor and the p38 inhibitor both inhibited TβRIII-mediated stimulation of migration (Figure 4*C*). On treatment with either the ALK5 inhibitor SB431542 or the p38 inhibitor SB203580, HT29-TβRII cells migrated at a rate comparable to HT29-Neo cells and did not demonstrate a ligand-induced increase in migration. This suggests that TβRIII regulates migration in colon cancer cells through both the canonical (ALK5) and noncanonical (p38) TGF-β signaling pathways.

Reorganization of the actin cytoskeleton occurs during most types of cell migration. TGF-β has been demonstrated to induce activation of the actin cytoskeleton [46,47]. Loss of cell-cell junctions can also occur during cell migration, which can be followed by the loss or decreased expression of the cell junction protein E-cadherin [47]. Consistent with an increased migratory phenotype, HT29-TβRIII cells exhibit alterations in actin and E-cadherin staining relative to HT29-Neo cells (Figure 5, *A* and *B*). Although HT29-Neo cells demonstrated organized actin and E-cadherin staining with cuboidal cell morphology, sharply defined cell-cell contacts and a smooth edge along the wound and in confluent areas of the culture (Figure 5, *A* and *B*), HT29-TβRIII cells exhibited a more elongated cell phenotype, with disorganized actin and E-cadherin staining, more diffuse localization and a decrease in staining at cell-cell junctions (Figure 5, *A* and *B*). In addition, HT29-TβRIII cells formed lamellipodia with membrane ruffling along

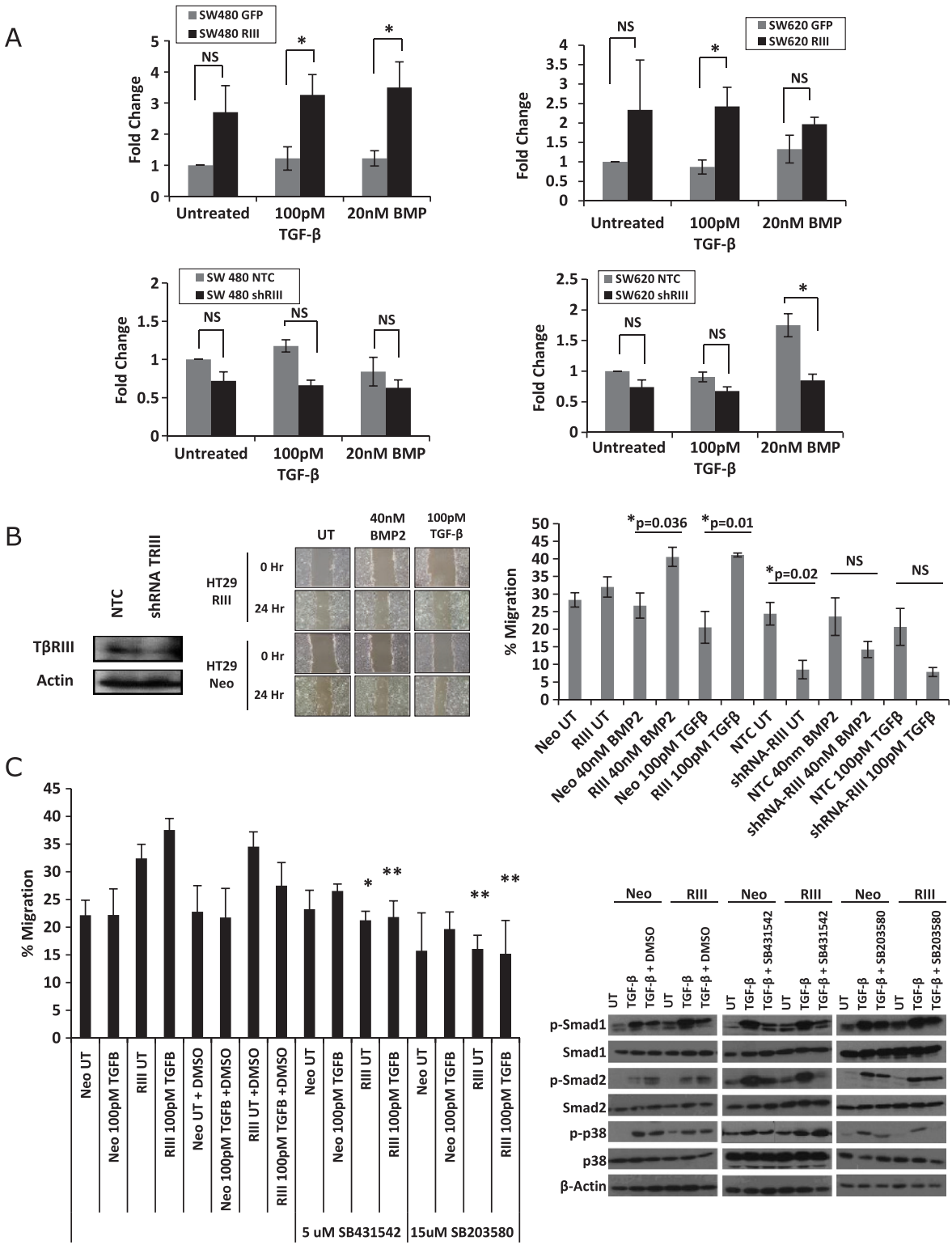

Figure 4. TβRIII increases colon cancer cell migration. (A) SW480 and SW620 GFP, TβRIII, NTC, or shTβRIII adenovirally infected cells were plated in a fibronectin transwell migration assay. Cells were plated in serum-free conditions on a fibronectin-coated transwell (50 μg/ml) with and without ligand treatment. Migration toward serum was measured by counting the number of cells on the filter after 12 hours. Fold change ± SEM is demonstrated. *$P < .05$. NS indicates not significant. (B) HT29-Neo, TβRIII, and NTC (nontargeting control) or shRNA TβRIII adenovirally infected cells were plated in a monolayer scratch wound assay. Cells were grown to confluence and then wounded by scratching and treated with 40 nM BMP-2 or 100 pM TGF-β. The percent migration was calculated by measuring the wound closure over time (0 and 24 hours). (C) Scratch wound assay with HT29-Neo and TβRIII cells treated with ligand and the ALK5 inhibitor SB431542 (5 μM) or the p38 inhibitor SB203580 (15 μM). The percent migration was calculated by measuring the wound closure over time (0 and 24 hours). *$P = .02$ RIII UT *versus* RIII UT + SB431542. **$P = .01$ RIII 100 pM TGF-β *versus* RIII 100 pM TGF-β + SB431542, RIII UT *versus* RIII UT + SB203580, RIII 100 pM TGF-β *versus* RIII 100 pM TGF-β + SB203580. Western blot analysis showing inhibition of TGF-β signaling with inhibitor treatment. Cells were treated with 100 pM TGF-β for 40 minutes, with or without DMSO, 5 μM SB431542 or 15 μM SB203580 treatment.

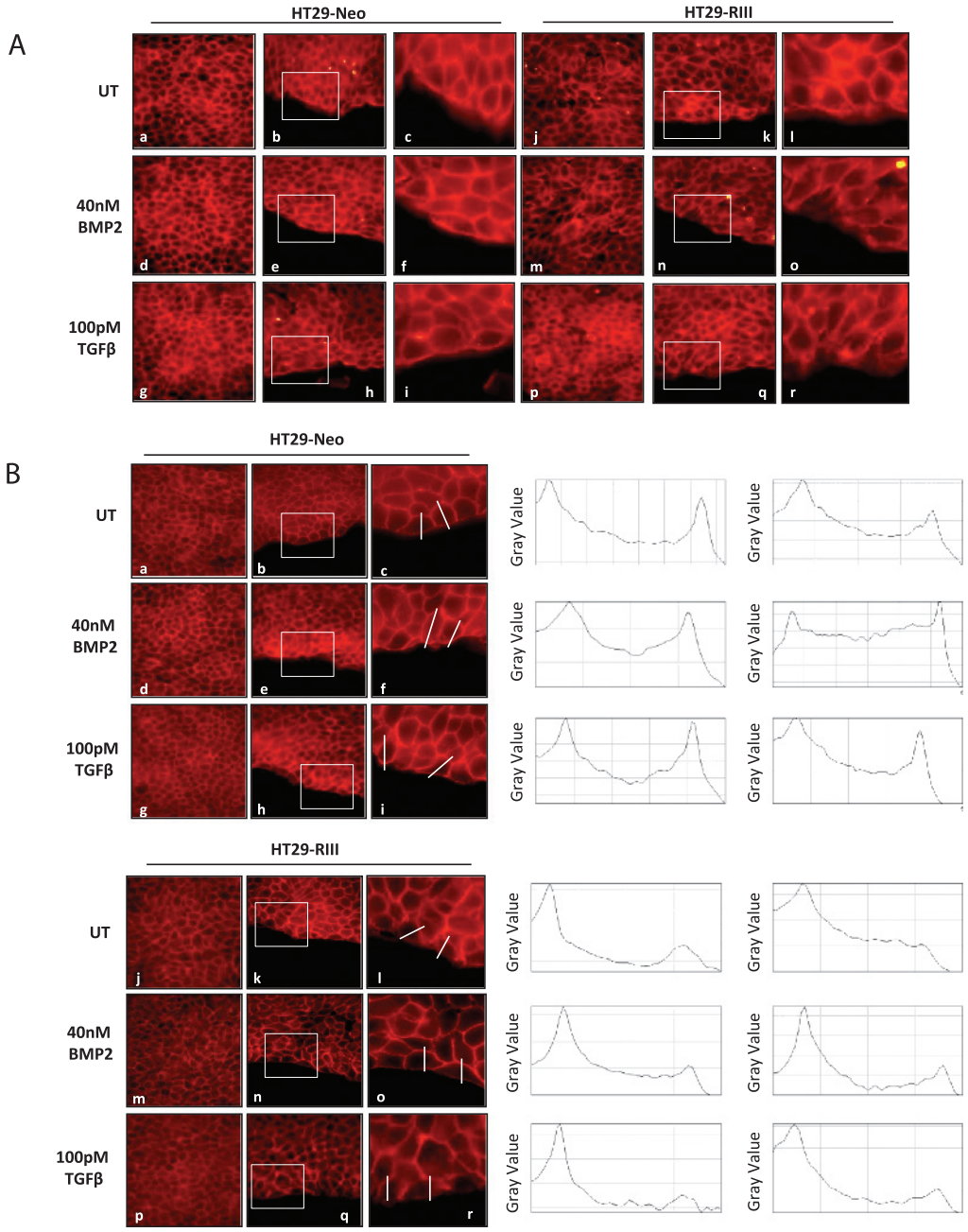

Figure 5. TβRIII alters actin and E-cadherin localization in colon cancer cells. (A) Actin (phalloidin) immunofluorescent staining. HT29-Neo and TβRIII cells were grown to confluence and then wounded by scratching and treated with 40 nM BMP-2 or 100 pM TGF-β. At 18 hours after scratch, cells were fixed and stained for actin. Images show actin staining in confluent culture (a, d, g, j, m, p) and along the wound edge (b, e, h, k, n, q). Original magnification, ×60. Boxed area (b, e, h, k, n, q) is shown enlarged in c, f, i, l, o, r. (B) E-cadherin immunofluorescent staining. HT29-Neo and TβRIII cells were grown to confluence and then wounded by scratching and treated with 40 nM BMP-2 or 100 pM TGF-β. At 18 hours after scratch, cells were fixed and stained for E-cadherin. Images show E-cadherin staining in confluent culture (a, d, g, j, m, p) and along the wound edge (b, e, h, k, n, q). Original magnification, ×60. Boxed area (b, e, h, k, n, q) is shown enlarged in c, f, i, l, o, r. Line scan analysis of individual cells (ImageJ software; NIH) shows E-cadherin staining intensity in a single cell from the interior toward the wound edge. The line demonstrates the area of measured intensity.

the scratch edge, which was not observed in the HT29-Neo cells. Whereas HT29-Neo cells demonstrated E-cadherin staining along the edge of the wound, HT29-TβRIII cells lacked E-cadherin staining in cells lining the edge of the wound, as demonstrated by line scan analysis of E-cadherin intensity across individual cells (Figure 5*B*). Collectively, these data support a TβRIII-mediated increase in colon cancer cell migration, which stands in contrast to the effect of TβRIII in other cancer cell lines where TβRIII expression significantly inhibits both motility and invasiveness [13,37,38].

TβRIII Enhances Early Colon Cancer Tumorigenicity In Vivo

The expression of TβRIII in colon cancer cells increased tumorigenicity *in vitro*, with increased proliferation and cell migration and a

reduction in apoptosis in response to ligand. To investigate the *in vivo* effects of TβRIII on tumorigenicity, we performed xenograft studies with the HT29 stable cell lines. HT29-Neo and HT29-TβRIII cells were injected subcutaneously into the flanks of female Balb/c *Nu/Nu* mice, and tumor volume was measured every 3 days. Early in the study, HT29-TβRIII tumors were significantly larger than the HT29-Neo tumors (day 9; Figure 6*B*). However, by day 21, no significant difference in tumor volume or tumor mass was observed between HT29-Neo and TβRIII tumors (Figure 6, *B* and *C*), and no metastases were observed in either HT29-Neo or HT29-TβRIII xenograft mice. TβRIII expression was examined by IHC (day 21 tumors), and all HT29-TβRIII tumors maintained TβRIII overexpression in comparison to HT29-Neo tumors (Figure 6*A*). These results suggest that TβRIII enhances early tumorigenicity *in vivo* but that other factors may compensate to ameliorate these effects during cancer progression in this model system.

Discussion

In this study, we demonstrate that, in contrast to cancers of the breast, kidney, lung, ovary, pancreas, and prostate where TβRIII expression is decreased, in human colon cancer, TβRIII expression is not significantly altered at the mRNA level and is increased at the protein level. In colon cancer cells, increasing TβRIII expression enhanced both TGF-β– and BMP-2–induced signaling, including phosphorylation of p38, Smads 1/5/8, and Smad2. Further, TβRIII induced resistance to ligand-mediated growth arrest, increased proliferation through a decrease in p21 induction and increased *in vitro* tumorigenicity in response to either BMP or TGF-β treatment as well as *in vivo* tumorigenicity at early time points. The increase in tumorigenicity is due to a TβRIII-mediated resistance to ligand- and chemotherapy-induced apoptosis, increased anchorage-independent cell growth, and increased cell migration. Collectively, these data suggest a role for TβRIII as a mediator of TGF-β superfamily function during colon cancer progression.

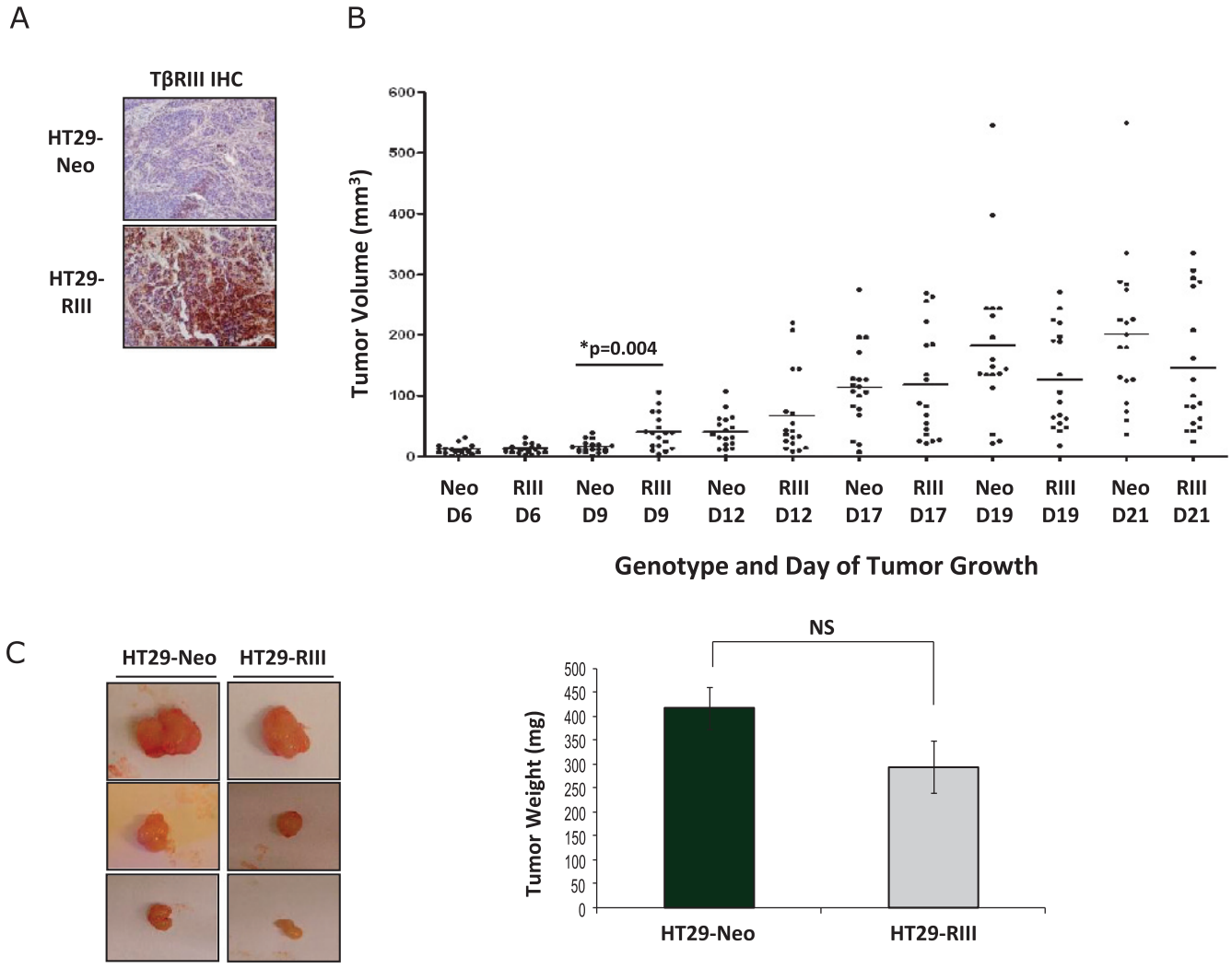

Figure 6. TβRIII enhances early colon cancer tumorigenicity *in vivo*. A total of 1×10^6 HT29-Neo or HT29-TβRIII colon cancer cells were injected subcutaneously into the right and left flanks of BALB/cAnNCr *nu/nu* mice. Mice were weighed, and tumor width (W) and length (L) were measured every 3 days. Tumor volume was determined using the formula: $V = 0.5 \times L \times W^2$. (A) TβRIII IHC of HT29-Neo and TβRIII tumors at day 21. (B) Graphical representation of HT29-Neo and TβRIII tumor volume over time (D indicates day). D9, $^*P = .004$. (C) Representative images of HT29-Neo and TβRIII xenograft tumors and graphical comparison of final tumor mass ± SEM of HT29-Neo and TβRIII xenografts at day 21. NS indicates not significant.

The maintenance and increase in TβRIII expression observed in colon cancer in comparison to normal colon tissue is in striking contrast to what has been previously observed in multiple human cancer types, including breast, lung, ovarian, pancreatic, and prostate cancers [37–41], where TβRIII expression is lost early in cancer progression and significantly inhibits metastasis, motility, invasion, and angiogenesis through sTβRIII-mediated down-regulation of TGF-β signaling [37,38,48,49]. In colon cancer, there is no significant change in TβRIII expression at the mRNA level, whereas TβRIII protein expression is significantly increased in 56% of matched normal and tumor pairs (Figure 1*E*), suggesting posttranscriptional regulation of TβRIII expression. Indeed, oncogenic K-Ras–dependent posttranscriptional regulation of TβRIII has been reported in colon cancer, resulting in a more tumorigenic phenotype [36]. As K-Ras is mutated in up to 50% of colon cancer patients, and these patients have a worse prognosis [50], the current results suggest that TβRIII could function downstream of oncogenic K-Ras to mediate this effect. Further supporting this hypothesis is the attenuation of TβRIII-mediated inhibition of ligand-induced p21 up-regulation in the presence of the Ras inhibitor, FTS (Figure 3*C*). The interaction of TβRIII and the Ras pathway remains to be further explored.

How might sustained or increased TβRIII expression promote colon cancer progression? We demonstrate here that increasing TβRIII expression in colon cancer cells enhances both canonical and noncanonical TGF-β superfamily signaling in colon cancer cells, with an increase in the basal phosphorylation of Smad1/5/8 and both BMP-2 and TGF-β ligand–enhanced phosphorylation of Smad2, Smad1/5/8, and p38 (Figure 2). These results are consistent with the well-established ligand presentation role of TβRIII, where TβRIII binds TGF-β superfamily ligands and increases ligand binding to respective type I and type II TGF-β superfamily receptors to enhance signaling [6,51]. The enhancement of p38 signaling, as well as the ability of TβRIII to regulate the biology of Smad4 deficient colon cancer lines, both suggest that TβRIII has both Smad4-dependent and -independent effects on signaling and biology in colon cancer cells. As increased levels of TGF-β have been demonstrated to correlate with increased proliferation and invasion of colon cancer cells *in vitro*, disease progression, and a poorer prognosis for human colon cancer patients [24,26–28], the ability for TβRIII to enhance TGF-β superfamily signaling provides one potential mechanism for TβRIII promoting colon cancer progression. These results are also consistent with the observation that markers of BMP (i.e., ID1) and TGF-β signaling (i.e., PAI-1) are increased in colon cancer tissue relative to matched normal tissue (Figure W2). In contrast, TβRIII has been previously demonstrated to downregulate Smad signaling in other tumor types, in part through sTβRIII-mediated sequestration of ligand [37]. Interestingly, in colon cancer, whereas TβRIII is shed to produce sTβRIII (Figure 2*B*), and increasing TβRIII expression does increase the level of sTβRIII (Figure 2*B*), the level of sTβRIII relative to TβRIII is relatively low compared with other cancer cell lines, including breast cancer lines (data not shown) [37]. Thus, TβRIII may exert a different role in colon cancer than in other solid tumors owing to the maintenance of expression during colon cancer progression and the relative preservation of cell surface TβRIII relative to sTβRIII in colon cancer.

Increased TβRIII-mediated TGF-β superfamily signaling seems to promote colon cancer progression by altering numerous aspects of colon cancer biology. Increased TβRIII expression enhances proliferation in response to treatment with either BMP-2 or TGF-β, largely through TβRIII-mediated down-regulation of both basal and ligand-induced p21 and p27 levels in HT29 cells and a decrease in basal p21 levels and a lack of ligand-mediated increase in p27 levels in SW480-TβRIII cells (Figures 3*C* and W1*B*). This alteration in p21 levels may occur through Smad4-mediated mechanisms or through the TβRIII-mediated regulation of the noncanonical p38 pathway. Treatment with the Ras inhibitor FTS attenuated this TβRIII-mediated repression of ligand-induced p21, suggesting that this occurs, at least partially, in a Ras-dependent manner (Figure 3*C*). Oncogenic K-Ras has also been demonstrated to induce proliferation in response to TGF-β, associated with down-regulation of p21 and PTEN [36], in part through posttranscriptional regulation of TβRIII, further supporting a role for TβRIII downstream of oncogenic K-Ras in colon cancer. Expression of TβRIII in colon cancer cells also confers an increase in anchorage independent growth. Although this may be due in part to increased proliferation, we also noted TβRIII-mediated resistance to ligand-induced apoptosis, which may contribute to the enhanced colony formation (Figure 3).

The effects of TβRIII on promoting resistance to apoptosis in colon cancer contrasts with the reported role for TβRIII in prostate and renal cell cancer, where expression of TβRIII or treatment with sTβRIII has been demonstrated to enhance apoptosis *in vivo* [9,42,52]. Similar to the differences noted above, some of these alterations could be due to the relative effect of sTβRIII *versus* full-length TβRIII. However, at least in the context of renal cell cancer, the effects of TβRIII on enhancing apoptosis were due to the cytoplasmic domain and mediated through p38 phosphorylation [42]. Although the precise mechanism by which TβRIII regulates apoptosis remains to be defined, the divergent effects of TβRIII on apoptosis in different contexts further highlights the context-dependent nature of TβRIII in regulating cancer biology.

The context-dependent effects of TβRIII are perhaps most striking when examining the effects of TβRIII on migration. We have previously demonstrated that restoring TβRIII expression in breast, ovarian, lung, pancreatic, and prostate cancer cells inhibits ligand-induced migration [37–41,53], with robust effects on decreasing directional persistence through activation of Cdc42 and increasing filopodia formation [13]. Here we report that, in the context of colon cancer, increasing TβRIII expression may enhance basal and significantly enhances ligand-induced migration, whereas shRNA-mediated silencing of endogenous TβRIII expression inhibits both basal and ligand-mediated cell migration, suggesting a role for TβRIII in both ligand-dependent and -independent migration in colon cancer cells (Figures 4 and W3). We further demonstrate that TβRIII-mediated increases in colon cancer cell migration are dependent on TβRIII-mediated enhancement of ALK-5 and/or p38 signaling, suggesting both Smad4-dependent and -independent effects (Figure 4*C*). Consistent with an effect of increasing cell migration, increasing TβRIII expression in colon cancer cells reorganized the actin cytoskeleton from predominantly cell-cell junction localization to a more diffuse localization, increased lamellipodia formation along a scratch wound edge, and decreased E-cadherin staining at cell-cell junctions (Figure 5). Whereas TβRIII seems to regulate cell migration through effects on the actin cytoskeleton in multiple cellular contexts, this seems to be mediated through different pathways. Indeed, the ability of TβRIII to either increase or decrease Smad-dependent signaling, to contribute to non-Smad signaling, and to regulate signaling through the production of sTβRIII provides multiple mechanisms by which TβRIII could differentially regulate cell migration in different contexts [4]. Current investigations are

focused on elucidating the mechanistic basis for the context-dependent effects of TβRIII on migration.

On the basis of the robust effects of TβRIII on enhancing ligand-stimulated colony formation in soft agar (Figure 3*D*) and migration (Figure 4), we were surprised to note only a modest, yet significant, enhancement of colon cancer xenograft growth by TβRIII at early time points (Figure 6), with no significant differences observed at later time points. This enhancement is consistent with our *in vitro* observations in colon cancer models and remains in stark contrast to the robust inhibition of xenograft growth by TβRIII in lung and prostate cancer models [38,41] and of breast cancer metastasis in a syngeneic model [37]. The modest effect on colon cancer here may be due to alterations in ligand-mediated effects occurring during *in vivo* tumorigenesis, a lack of significant ligand stimulation in the xenograft model, the use of a nonorthotopic xenograft model, or our inability to monitor the effect on metastasis in the model system used. As the HT29 cell line has been demonstrated to have a low engraftment rate with limited metastasis when implanted orthotopically [54], an orthotopic study may be limited in its ability to provide additional data on the effects of TβRIII on colon cancer metastasis. Indeed, we have recently defined important contributions of the host immune system in defining the effects of TβRIII on cancer progression *in vivo* (B. Hanks and G.C. Blobe, unpublished observations). Future studies will further explore the context-dependent effects of TβRIII on cancer progression in orthotopic, syngeneic murine models.

In conclusion, in contrast to other tumor types, TβRIII expression is maintained and enhanced in human colon cancers and functions to promote colon cancer progression through promotion of proliferation, migration, anchorage-independent growth, and resistance to apoptosis. Taken together, these data suggest that similar to the role of TGF-β superfamily signaling pathways, the role of TβRIII in regulating/mediating cancer biology is cell type and context dependent. As such, targeting this axis will require a more detailed understanding of the role TβRIII and the entire TGF-β superfamily signaling pathway in human cancer biology.

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Supplemental Methods

p21 Reverse Transcription–PCR

A total of 3×10^5 HT29-Neo and TβRIII cells were plated in a six-well plate and allowed to recover. Cells were then treated with 10 nM BMP-2, 40 nM BMP-2, 100 pM TGF-β, or 400 pM TGF-β for 24 hours. Total RNA was extracted using the RNeasy Mini Kit per the manufacturer's instructions (Qiagen, Valencia, CA). Half a microgram of RNA was reverse transcribed using the SuperScript First-Strand Synthesis System for reverse transcription–PCR (Invitrogen, Carlsbad, CA). Each PCR contained 1 μg of cDNA along with p21 primers: hp21, forward 5′ CAGGGGACAGCAGAGGAAGA 3′ and reverse 5′ TTAGGGCTTCCTCTTGGAGAA 3′; or hGAPDH, forward 5′ GAGTCAACGGATTTGGTCGT 3′ and reverse 5′ TTGATTTTGGAGGGATCTCG 3′ primers.

Western Blot Analyses

A total of 2.5×10^5 SW480 cells were plated in six-well plate and allowed to recover, then adenovirally infected with GFP or FL-TβRIII-GFP as described previously. At 36 hours after infection, cells were treated with BMP-2 (2 and 10 nM) or TGF-β1 (50 or 100 pM) for 24 hours. Western blot analyses were performed to analyze protein levels of p21 (no. 2946; Cell Signaling Technology) and p27 (no. 2552; Cell Signaling Technology) protein level with β-actin as a total protein control. Densitometric analysis, including background subtraction and normalization to β-actin, was performed using ImageJ software (NIH).

Proliferation Assay

SW480 cells adenovirally infected with GFP or FL-TβRIII-GFP as described previously and were plated at 3×10^3 cells per well in a 96-well plate and grown overnight with ligand stimulation (20 nM BMP-2, 40 nM BMP-2, 50 pM TGF-β, or 100 pM TGF-β). The next day, cells were pulsed with 1 μCi of ^3H per well for 4 hours at 37°C. Cells were washed in cold PBS and 10% TCA and then incubated for 1 hour at 4°C with 10% TCA. Cells were then washed with cold 10% TCA and lysed overnight with 0.2 NaOH. Lysates were then read on a scintillation counter.

ID1 and PAI-1 Expression Analysis

Matched normal colon mucosa and colon adenocarcinoma gene expression data (Affymetrix U133 Plus 2) from 32 patients were obtained from the Gene Expression Omnibus (GEO) GSE8671. Raw expression data (.CEL) files were MAS5 normalized using Affymetrix Expression Console Version 1.0. MAS5 data were then $\log_2$ transformed using the log transform function in MATLAB (release R2009a). To examine the differences in expression level between normal and tumor tissue, the Affymetrix U133 Plus 2 probe set annotation file (release 24) was acquired from the Affymetrix Web site, and probe sets were identified for each gene of interest. When multiple probes were present for a given gene, probe expression levels were averaged for each sample. A paired *t* test (GraphPad Prism 4.0; Graph Pad, Inc, La Jolla, CA) was used to calculate differences in gene expression between matched normal mucosa and colon adenocarcinoma samples.

Gene Name	Affymetrix Probe ID
TGFβRIII	204731_at
	226625_at
ID1	208937_s_at
PAI-1 (*SERPINE1*)	202627_s_at
	202628_s_at
	1568765_at
β-actin	2008937_x_at
	213867_x_at
	2245954_x_at

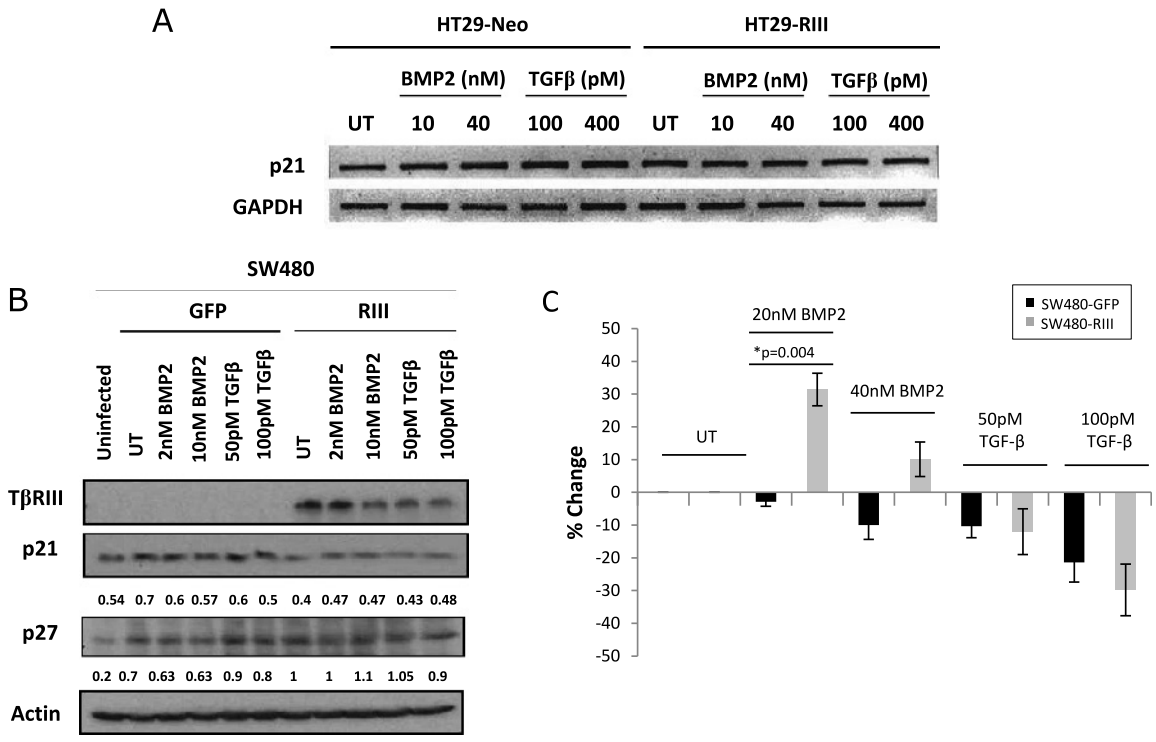

Figure W1. TβRIII does not alter p21 mRNA levels. (A) Reverse transcription–PCR analysis of p21 mRNA levels in HT29-Neo and TβRIII cells. HT29-Neo and TβRIII cells were treated with 10 nM BMP-2, 40 nM BMP-2, 100 pM TGF-β, or 400 pM TGF-β for 24 hours. (B) SW480 cells were adenovirally infected with GFP or FL-TβRIII-GFP. At 36 hours after infection, cells were treated with BMP-2 (2 and 10 nM) or TGF-β1 (50 or 100 pM) for 24 hours. Western blot analyses were performed to analyze levels of p21 and p27 proteins with β-actin as a total protein control. Densitometric analysis is shown normalized to β-actin. (C) SW480 cells were adenovirally infected with GFP or TβRIII-GFP. At 36 hours after infection, SW480 cells were treated with 20 and 40 nM BMP-2 or 50 and 100 pM TGF-β1 for 24 hours. Proliferation was analyzed by a ^{3}H incorporation assay. The percent proliferation was determined by normalizing the counts to those of untreated samples.

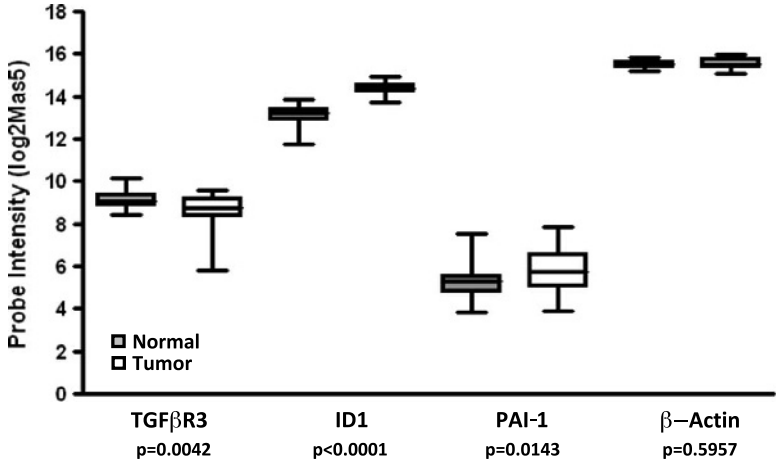

Figure W2. TGF-β and BMP response is enhanced in colon cancer. Box-and-whisker plot showing patterns of gene expression in patient matched normal colon mucosa (gray) and colon adenocarcinoma (white). Colon adenocarcinoma samples show significantly lower levels of TGF-β RIII ($P = .0042$, paired t test), but significantly higher levels of the BMP and TGF-β transcriptional targets ID1 ($P < .0001$) and PAI-1 ($P = .0143$), respectively. No statistically significant differences in β-actin ($P = .5957$) levels were identified.

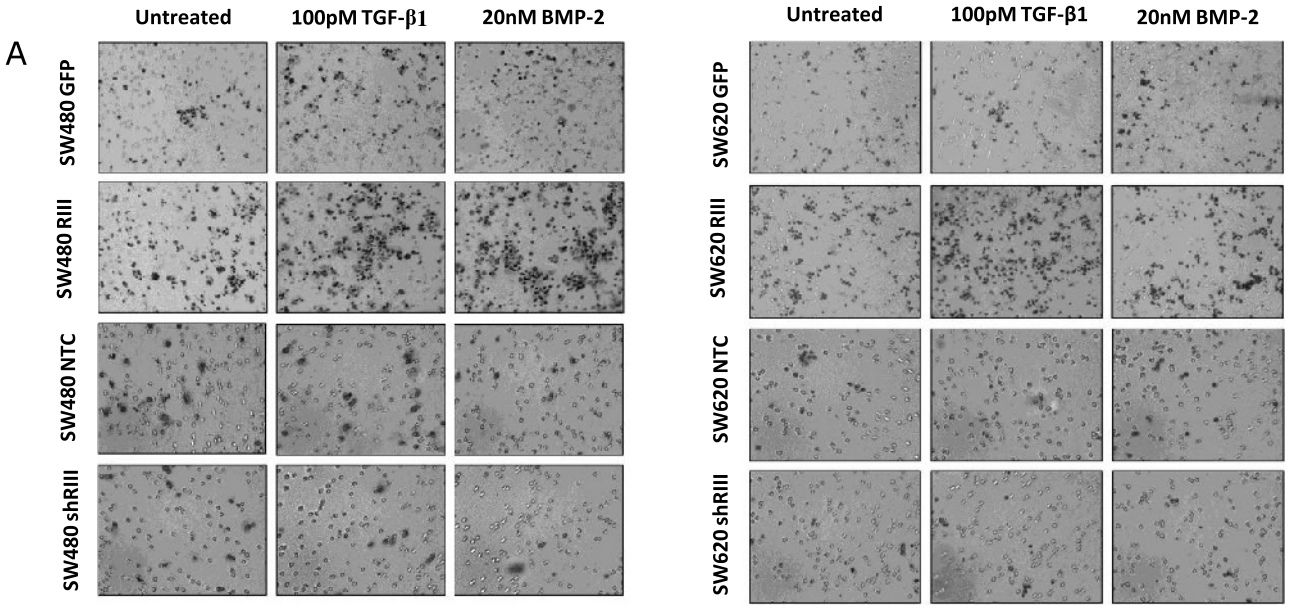

Figure W3. TβRIII increases colon cancer cell migration in a transwell migration assay. (A) Representative images of SW480 and SW620 GFP, TβRIII, NTC, or shTβRIII adenovirally infected cells were plated in a fibronectin transwell migration assay. Cells were plated in serum-free conditions on a fibronectin-coated transwell (50 μg/ml) with and without ligand treatment. Original magnification, $\times$20.