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Prostate tumor progression is mediated by a paracrine TGF- β /Wnt3a signaling axis

Xiaohong Li¹, Veronica Placencio¹, Juan M. Iturregui², Consolate Uwamariya¹, Ali-Reza Sharif-Afshar¹, Tatsuki Koyama³, Simon W. Hayward¹, and Neil A. Bhowmick^{1,*}

¹The Vanderbilt-Ingram Cancer Center and Department of Urologic Surgery, Vanderbilt University School of Medicine, Nashville, TN 37232

²Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN 37232. Department of Veterans Affairs Medical Center, Nashville, TN 37212, USA

³Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, TN 37232

Abstract

TGF- β is an important paracrine factor in tumorigenesis. Ligand binding of the type I and II TGF- β receptors initiate downstream signaling. The role of stromal TGF- β signaling in prostate cancer progression is unknown. In mice the conditional stromal knockout of the TGF- β receptor type II expression (Tgfbr2^{fspKO}) resulted in the development of prostatic intraepithelial neoplasia and progression to adenocarcinoma within seven months. Clinically, we observed a loss of TGF- β receptor type II expression in 69% of human prostate cancer-associated stroma compared to 15% of stroma associated with benign tissues (n =140, p value < 0.0001). To investigate the mechanism of paracrine TGF- β signaling in prostate cancer progression, we compared the effect of the prostatic stromal cells from Tgfbr2^{fspKO} and Tgfbr2^{floxE2/floxE2} mice on LNCaP human prostate cancer cells in vitro and tissue recombination xenografts. Induction of LNCaP cell proliferation and tumorigenesis was observed by Tgfbr2^{fspKO} prostate stroma as a result of elevated Wnt3a expression. Neutralizing antibodies to Wnt3a reversed LNCaP tumorigenesis. The TGF- β inhibition of Wnt3a expression was in part through the suppression of Stat3 activity on the Wnt3a promoter. In conclusion, the frequent loss of stromal T β RII expression in human prostate cancer can relieve the paracrine suppression of Wnt3a expression.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death in men in the United States. The understanding of stromal-epithelial interactions in the prostate will help determine the etiology of prostate cancer progression. Many factors including TGF- β and Wnt signaling molecules are found to be mutated or deregulated in prostate cancer. Conventionally, TGF- β plays a tumor suppressive role in normal and preneoplastic epithelia, yet paradoxically can promote motility and resistance to cell death in transformed epithelia (Bierie and Moses, 2006; Biswas *et al.*, 2007; Roberts and Wakefield, 2003). Further data have emerged that TGF- β also has additional roles in the tumor microenvironment born out by mouse models with conditional ablation of TGF- β signaling in a subset of stromal fibroblasts (Bhowmick *et al.*, 2004a; Bhowmick *et al.*, 2004b). These studies suggest TGF- β can be a paracrine tumor suppressor as the stromal loss of TGF- β responsiveness initiated prostatic intraepithelial neoplasia (PIN) and squamous cell carcinoma in the forestomach

*Address correspondence to: Neil A. Bhowmick, Department of Urologic Surgery, A1302 Medical Center North, 1161 21st Ave South, Nashville, TN 37232, Tel.: 615-343-7140, Fax: 615-322-5869, neil.bhowmick@vanderbilt.edu.

(Bhowmick *et al.*, 2004a). In contrast, Wnt ligands are long known oncogenes (Nusse *et al.*, 1991; Nusse *et al.*, 1984; Nusse and Varmus, 1982). Canonical Wnt signaling, through β -catenin stabilization, has been of specific interest in cancer biology as a regulator of cell proliferation, adhesion, survival, movement, and polarity. Together, the TGF- β and Wnt pathways have both opposite and synergistic roles in cancer progression with evidence of crosstalk in cytoplasmic signaling (Labbe *et al.*, 2007; Nishita *et al.*, 2000).

TGF- β signaling involves the binding of the ligand isoforms, TGF- β 1, 2, and 3 to the extracellular domain of the TGF- β receptor type II (T β RII), followed by the transphosphorylation of the TGF- β receptor type I (T β RI), and the subsequent activation of downstream signaling molecules including Smad2, Smad3, and Smad4 (Shi and Massague, 2003). TGF- β receptor type III (T β RIII) expression is specifically important for TGF- β 2 signaling. Proposed mechanisms for resistance to TGF- β include decreased expression of T β RI, T β RII, or T β RIII on the cell surface of melanoma, pancreas, breast, and colon cancers (Buck *et al.*, 2004; Calin *et al.*, 2000; Goggins *et al.*, 1998; Grady *et al.*, 1999; Schmid *et al.*, 1995). Most epithelial-derived tumors (> 85% of all human cancers) become resistant to the growth-inhibitory effect of TGF- β by different mechanisms (Elliott and Blobe, 2005; Pasche, 2001). However, both the loss and elevation of T β RII expressions are associated with prostate cancer (Gerdes *et al.*, 1998; Kim *et al.*, 1998; Kim *et al.*, 1996; Royuela *et al.*, 1998). The consequence of TGF- β signaling in the prostatic stromal cells in man has not been studied.

As many Wnt ligands are stromally secreted with cognate receptors expressed by the epithelia, Wnt proteins are good candidates for mediating stromal-epithelial interactions. Nineteen closely related but distinct Wnt ligands are reported to date. In vertebrates, Wnt signaling can involve canonical and/or non-canonical pathways (Clevers, 2006; Johnson and Rajamannan, 2006). The canonical or β -catenin-dependent pathway, rely on specific Wnt ligands to bind a transmembrane receptor complex for cytoplasmic accumulation of β -catenin, and eventual nuclear translocation with lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF) transcription factors. Mutations in the canonical Wnt signaling pathway components, such as APC and β -catenin, are common in carcinoma of the colon, but are relatively rare in prostate cancer (Morin, 1999; Voeller *et al.*, 1998). Yet, nuclear β -catenin accumulation in prostate cancer specimens is comparatively frequent (Chen *et al.*, 2004; Chesire *et al.*, 2002; Morin, 1999; Verras and Sun, 2006; Yardy and Brewster, 2005). The report of epithelial expression of Wnt/ β -catenin target gene, Twist, in 90% of human prostate cancer tissues further suggests the role of deregulated Wnt signaling pathway (Kwok *et al.*, 2005; Kwok *et al.*, 2007). Thus, other mechanisms, besides mutations, need to be considered to explain the pervasive β -catenin/TCF activity documented in human prostate cancer.

This study was designed to address the role of stromal T β RII in prostatic tumorigenesis. The mouse with conditional knockout for T β RII in the stromal compartment (Tgfbr2^{fspKO}) was previously reported to develop PIN (Bhowmick *et al.*, 2004a). Here, we report the Tgfbr2^{fspKO} mouse prostates can in fact progress to adenocarcinoma. Stromal cells deficient in TGF- β responsiveness were further found to promote tumorigenesis in a Wnt3a dependent manner. Finally, the loss of T β RII expression in stromal cells was recapitulated in human prostate cancer, in contrast to stromal cells associated with benign prostate epithelia.

MATERIALS AND METHODS

Animals

Tgfbr2^{floxE2/floxE2} and Tgfbr2^{fspKO} mice bred on the C57BL/6 background were generated and maintained as previously described (Bhowmick *et al.*, 2004a). Adult male severe

combined immunodeficient (SCID) mice and C57BL/6 mice were purchased from Harlan (Indianapolis, IN). Vanderbilt Institutional Animal Care and Use Committee approved the animal procedures.

Cell Culture and production of conditioned medium

Primary mouse prostate stromal cell cultures were generated from 6–8 week old $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ mice. Briefly, the prostates were dissected, cut into 2mm³ pieces, plated on 6-well plates in high glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 10⁻⁸ M testosterone, and 5 µg/mL insulin (Sigma-Aldrich, St. Louis, MO). Once the cells reached confluence, they were subcultured up to five passages and then discarded. LNCaP cells were purchased from ATCC, routinely passaged in RPMI medium containing 10% fetal bovine serum and 0.1% penicillin/streptomycin. Conditioned $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ stromal media were collected after two passages, from approximately 1×10⁶ cells plated on a 100mm dish for 3 days in culture media and centrifuged prior to application on LNCaP cells. The conditioned media was normalized by the cell numbers at the time of collection.

Tissue rescue and tissue recombination

Tissue rescues were performed by direct grafting of prostates dissected from 6 week old $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ mice into the renal capsule of SCID mice, as previously described (Wang *et al.*, 2000). The rescued tissues were harvested after 7 months. Prostate tissue recombinants were generated by combining 250,000 $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ prostatic stromal cells with either prostate epithelial organoids from wild type mice, or 100,000 cultured LNCaP cells, in 50 µl of rat-tail collagen. The prostate epithelial organoids were obtained from C57BL/6 mice (8–12 weeks) by collagenase digestion as previously reported (Hayward *et al.*, 1992; Turner *et al.*, 1990). The tissue recombinants were grafted under the renal capsule of C57BL/6 mice (for recombinants of prostate organoids) or SCID mice (for LNCaP cell recombinants) (Cunha, 1994; Fujii *et al.*, 1982; Hayward *et al.*, 2001; Wong *et al.*, 1992). Host mice were sacrificed 2 months or 7 weeks following grafting. The grafts were removed, photographed, and processed for histologic evaluation. The dimensions were measured, and the tumor volume was calculated by the formula: volume = width × length × 0.52 using Image J program. Note that the formula underestimates the volume of invasive tumors, and accordingly underestimate the significance of size differences between small noninvasive and larger invasive tumors.

Measurement of cell proliferation and luciferase assay

LNCaP cells were passaged at 10⁵ cells per well in 24-well plates. After overnight growth and attachment, the culture media were changed to either $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ stromal conditioned media. The cells in $Tgfr2^{fspKO}$ stromal condition medium were treated with or without Wnt3a neutralization antibody at 1 ng/ml to 15 ng/ml, final concentration. Cell numbers were counted by hemocytometer after 72 hours.

The cells passaged in the 24-well plates were otherwise transfected with c-myc luciferase construct (He *et al.*, 1998) with Lipofectamine LTX (Invitrogen, Carlsbad, CA), according to the users' manual. Next day the cells were treated with conditioned media with/without Wnt3a neutralizing antibody, or control rat IgG for 24hrs before the cells were harvested, analyzed using Luciferase assay system (Promega, Madison, WI), normalized by total protein content.

Antibodies and immunohistochemistry

Paraffin-embedded tissue sections (5 μ m) were de-paraffinized and hydrated through xylene and graded alcohols using a standard protocol. 1% antigen unmasking solution (Vector laboratories, Burlington, CA) was used for antigen retrieval according to manufacturer instructions. Subsequent immunohistochemical staining used antibodies against phosphorylated-histone H3 (1:1000, Upstate), TGF β type II receptor or p63 (1:1000, Santa Cruz Biotech, Santa Cruz, CA), and Twist (1:500, Santa Cruz). Appropriate HRP-conjugated secondary antibodies and DAB incubation (Dako North America, Carpinteria, CA) was used for visualization. The ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA) was used to detect apoptotic cells, according to manufacturer's instructions.

The host SCID mice grafted with prostate tissue recombinants were injected intraperitoneally with mouse monoclonal Wnt3a or isotype control IgG (R&D Systems), at 100 μ g per mouse in a 0.2 ml volume. Injection was started two weeks after grafting and subsequently twice weekly until sacrifice on the fourth week post grafting.

SiRNA transfection and Western analysis

Primary cultured mouse prostate stromal cells were transfected with Stat3 siRNA or the control siRNA using siRNA transfection reagent (Santa Cruz Biotech), the cells were harvested after 48 hours. Samples were separated by 10% SDS-PAGE gel and electro-transferred to nitrocellulose membrane. Membranes were blocked and incubated with primary antibodies for Wnt3a (R&D systems) or anti-actin (Santa Cruz) overnight at 4°C. Respective horseradish peroxidase conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) were applied to the blots for 1 hour. Protein bands were visualized using ECL plus Western blotting detection system (GE Healthcare).

Real-time quantitative reverse transcription-PCR and ChIP analysis

RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription and quantitative real-time PCR used iScript and iQ SYBR Green supermix, respectively (Bio-Rad, Hercules, CA). Relative quantitation was calculated by $\Delta\Delta C_t$ method normalized to 18s rRNA. See supplemental material for sequences of RT-PCR primers.

Tgfr2^{floxE2/floxE2} and Tgfr2^{fspKO} prostate stromal cells were grown to confluence prior to cross-linked with 1% formaldehyde, lysed, and sonicated as described previously (Nissen and Yamamoto, 2000). Chromatin fragments were immunoprecipitated with Stat3 antibody and normal mouse IgG as negative control (Santa Cruz Biotech) overnight at 4°C. After immunoprecipitation, 60 μ L protein G Agarose were added to pull down the chromatin/protein-antibody complex, and further steps following the protocol from the EZ Chip kit (Upstate, Temecula, CA). PCRs were performed with for 30 cycles with the primers: forward-TGGACTCAGGAGGAATCAATGTGG and reverse-CGAGGTAGTTGTTAGGGAGTCCAA for the -854 Stat3 binding site of Wnt3a promoter and forward-AGAGGGACCCATTCCTCTGTGAAA and reverse-TGGAGCTGGAAGCAAGGAAGTGT for the -3542 Stat3 binding site of Wnt3a promoter. PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide.

Human prostate tumor samples and tissue microarray

Human prostate samples were collected from radical prostatectomy tissue, with prostate cancer and adjacent normal areas indicated by a pathologist consistent with Institutional Review Board approval. The tissues were evaluated following 4% paraformaldehyde

fixation, paraffin embedding, and hematoxylin and eosin staining of 5 μ m sections. Fifteen such benign prostate samples in addition to the samples from tissue microarray (TMA, Imgenex, San Diego, CA) that included 125 tissue cores with benign and cancer histology were immunohistochemically stained. Information on all tissue samples including Gleason score, prostate specific antigen (PSA) expression, clinical stage, and H&E were available.

Statistical Analysis

The univariate associations between the clinical correlates and the stromal TGF- β were tested using Wilcoxon rank-sum tests. To express the relationship between the proportions of stromal TGF- β and Gleason scores, log-linear models were considered. Odds of TGF- β expression were computed for benign and malignant samples, and the results were reported in terms of the odds ratio. All reported P-values were two-sided.

RESULTS

The loss of T β RII expression in the prostate stroma leads to adenocarcinoma

The Tgfr2^{fspKO} mouse model was used to study the contribution of the loss of T β RII in the prostatic stroma to prostatic adenocarcinoma progression. The Tgfr2^{fspKO} mouse prostates develop PIN lesions by six weeks of age with 100% penetrance, as previously reported (Figure 1A) (Bhowmick *et al.*, 2004a). Electron microscopy further supported the failure of epithelial differentiation, as there were no secretory vesicles in the six-seven week old Tgfr2^{fspKO} mouse prostate epithelial cells, compared their presence in Tgfr2^{floxE2/floxE2} mouse prostates (Figure 1B). If PIN is assumed to be a precursor of prostate adenocarcinoma, the progression of the disease was predicted in under long-term observation (Davidson *et al.*, 1995; Qian *et al.*, 1995). Since the Tgfr2^{fspKO} mice die by seven weeks of age, the prostates were rescued at six weeks, and allografted to the renal capsule of immunocompromised male SCID mice. Twenty five percent of the Tgfr2^{fspKO} prostates developed into adenocarcinoma by seven months following grafting (Figure 1C). Tissue-rescued prostates from six-week old Tgfr2^{floxE2/floxE2} mice under the same conditions were histologically normal by H&E staining. Immunohistochemistry for the expression of T β RII confirmed a significant decrease in T β RII expression in the stromal compartment of Tgfr2^{fspKO} prostates (Figure 1D).

Epithelial proliferation and differentiation markers were used to evaluate development of prostate adenocarcinoma. The mitotic rate of the prostatic epithelium of Tgfr2^{fspKO} mice was four-fold greater than that from Tgfr2^{floxE2/floxE2} mice in the seven-month progression model, as determined by quantitating phosphorylated-histone H3 expression (Figure 2A). The mouse dorsolateral prostate (mDLP) antibody was used to localize secretions found in differentiated prostatic epithelium, was present in the Tgfr2^{floxE2/floxE2} tissues but was focally absent in Tgfr2^{fspKO} tissues (Figure 2B) (Donjacour *et al.*, 1990). Then mDLP staining in both normal and malignant rescued tissues confirmed the prostatic origin of the tissues. P63 expression was basally localized in the Tgfr2^{floxE2/floxE2} tissues, as expected (Figure 2C) (Kurita *et al.*, 2004). In contrast, p63 positive cells were rare and scattered in the Tgfr2^{fspKO} prostate rescues, supportive of the progression of adenocarcinoma and indicating a disruption of the basal cell layer. Another reported marker for prostatic adenocarcinoma progression, Twist, was detected only in the Tgfr2^{fspKO} tissue rescues that progressed to adenocarcinoma (Figure 2D) (Hotz *et al.*, 2007; Kwok *et al.*, 2005; Zhang *et al.*, 2007). The loss of T β RII expression in the stroma resulted in the progression of prostate tumorigenesis in mice.

Wnt3a mediates the progression of prostate tumors by $Tgfr2^{fspKO}$ prostate stroma

In light of the observed expression of Twist in $Tgfr2^{fspKO}$ prostate adenocarcinoma, and recent publications on the importance of Wnt signaling in prostate cancer progression, we hypothesized that Wnt ligand isoforms are paracrine mediators of the loss TGF- β responsiveness in the stromal cells. Reverse transcription real-time PCR analysis of $Tgfr2^{floxE2/floxE2}$ and $Tgfr2^{fspKO}$ prostatic stromal cells revealed that four out of nineteen Wnt ligands had elevated expression in $Tgfr2^{fspKO}$ cells relative to $Tgfr2^{floxE2/floxE2}$ cells (Figure 3A). Among them, $Tgfr2^{fspKO}$ stromal cells had a median Wnt3a elevation of 4-fold over $Tgfr2^{floxE2/floxE2}$ cells. Wnt 5a, Wnt 6, and Wnt 10b were also expressed higher by $Tgfr2^{fspKO}$ cells, however, viable antibodies for these Wnt isoforms were not available to perform further confirmatory studies. Elevated Wnt3a expression was confirmed at the protein level by Western blot detection (Figure 3B). This indicated TGF- β signaling as a suppressor of Wnt3a expression in prostate stromal cells and potential paracrine mediator prostate epithelial Wnt signaling.

To test the specific role of Wnt3a on epithelial transformation and proliferation, LNCaP cells were incubated with conditioned media from either $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ mouse prostatic stromal cells in the presence or absence of a Wnt3a neutralizing antibody. The transcriptional activity of a canonical Wnt oncogenic target, c-myc, was measured by a luciferase reporter assay. The LNCaP cells incubated with $Tgfr2^{fspKO}$ conditioned media consistently had three-fold greater c-myc reporter activity than those cells incubated with $Tgfr2^{floxE2/floxE2}$ conditioned media (Figure 4A). Under the same conditions, the addition of the Wnt3a neutralizing antibody down regulated reporter activity of the LNCaP cells incubated with $Tgfr2^{fspKO}$ conditioned media to levels similar to that found when associated with $Tgfr2^{floxE2/floxE2}$ conditioned media alone. Further, $Tgfr2^{fspKO}$ conditioned media induced 2.7-fold increase in proliferation of LNCaP cells relative to $Tgfr2^{floxE2/floxE2}$ conditioned media (Figure 4B). Wnt3a neutralizing antibody reduced the proliferative effect of $Tgfr2^{fspKO}$ conditioned medium in a dose dependent manner. The presence of 10 ng/ml Wnt3a neutralizing antibody decreased LNCaP cell proliferation to comparable levels as cells grown in $Tgfr2^{floxE2/floxE2}$ conditioned medium.

Next, the mechanism of transcriptional up regulation of Wnt3a in the $Tgfr2^{fspKO}$ prostatic stromal cells was analyzed. A transcription factor binding site search 5 kb upstream of the Wnt3a transcription start site suggested putative Stat DNA binding elements at -863/-854 bp and -3551/-3542 bp (<http://www.cbrc.jp/research/db/TFSEARCH.html>, <http://genome-www.stanford.edu/cgi-bin/genecards/carddisp.pl?gene=Wnt3a>, <http://www.cbil.upenn.edu/cgi-bin/tess/tess>). ChIP analysis indicated that *in vivo*, the -854 bp consensus site had elevated Stat3 binding in $Tgfr2^{fspKO}$ prostate stromal cells compared to $Tgfr2^{floxE2/floxE2}$ stroma. However, the -3542 bp consensus site was specifically occupied by Stat3 in $Tgfr2^{fspKO}$ prostatic stromal cells but not detectible in the $Tgfr2^{floxE2/floxE2}$ prostatic stromal cells (n = 4, Figure 4C). Accordingly, we focused on the role of Stat3 on Wnt3a expression, in $Tgfr2^{fspKO}$ prostate stromal cells. Western blotting resolved reduced Wnt3a expression when Stat3 was knocked-down by siRNA compared to scrambled siRNA control (Figure 4D). Together, the data indicate stromal TGF- β responsiveness regulates Wnt3a expression in a Stat3-dependant mechanism to induce Wnt activity in adjacent epithelial cells.

$Tgfr2^{fspKO}$ prostatic stromal cells promote human prostate cancer progression

To determine if the tumorigenic effects of the prostatic stroma were a result of paracrine signaling, tissue recombination techniques were used to combine cultured $Tgfr2^{floxE2/floxE2}$ or $Tgfr2^{fspKO}$ prostatic stromal cells with adult wild type mouse prostatic epithelial organoids. Following allografting the tissue recombinants into syngenic C57BL/6 male mice

for eight weeks, we found that the $Tgfr2^{floxE2/floxE2}$ stroma-associated prostatic grafts were comparable to intact prostates of wild type mice. The $Tgfr2^{fspKO}$ stroma-associated prostatic grafts developed PIN lesions, recapitulating the intact $Tgfr2^{fspKO}$ prostates (Figure 5A). These results revealed that paracrine factors affected prostatic epithelial differentiation and established the tissue recombination methodology as a viable model for subsequent grafting experiments. To enable the study of stromal-epithelial signaling in adenocarcinoma, we developed a chimeric model of mouse prostatic stromal cells with LNCaP cells, an established human prostate cancer epithelial line. A five-fold increase in gross tumor volumes was observed in recombinants of LNCaP/ $Tgfr2^{fspKO}$ compared to tumors of LNCaP/ $Tgfr2^{floxE2/floxE2}$ recombinants (Figure 5B). Although the histology of the two chimeric tissues was not appreciably different (Figure 5C), immunohistochemistry of phosphorylated-histone H3 revealed increased mitosis in LNCaP/ $Tgfr2^{fspKO}$ tissue recombinants compared to LNCaP/ $Tgfr2^{floxE2/floxE2}$ recombinants (Figure 5D). Together, the loss of $T\beta RII$ expression in the stroma initiated paracrine transformation of normal epithelia and supported tumor progression of prostate cancer.

Next, host mice harboring the chimeric LNCaP/ $Tgfr2^{fspKO}$ tissue recombinants were treated with the Wnt3a neutralizing antibody or isotype IgG control. The antibodies were injected intra-peritoneally two weeks following xenografting. Although the gross tumor size between the isotype IgG control and the Wnt3a neutralizing antibody were not appreciably different, the histology of the tumors revealed areas of necrosis in the tumors when mice were treated with the neutralizing antibody compared to control (Figure 6A). Further immuno-localization for cells under going apoptosis confirmed 2.3-fold greater number in tumors treated with the Wnt3a neutralizing antibody compared to the control (p value = 0.001, Figure 6B). There was a lack of mitotic cells in the areas of necrosis, as determined by phosphorylated-histone 3 immuno-localization (Figure 6B). The mitotic rate in the surviving tumors from neutralizing antibody treated mice was significantly less than that in isotype control mice (p value = 0.002, Figure 6C). Thus, down regulation of TGF- β signaling in the prostatic stromal compartments is associated with canonical Wnt signaling in the adjacent epithelia to support tumor initiation and further tumor progression. Inhibiting paracrine Wnt3a activity was effective in reducing growth of tumors deficient in stromal TGF- β signaling.

T βRII expression is lost in stromal cells of human prostate adenocarcinomas

Finally, to explore the role of stromal TGF- β signaling in human prostate cancer progression, we localized $T\beta RII$ expression by immunohistochemistry of 140 benign and malignant prostate tissues. Prostate samples from patients who underwent radical prostatectomy were obtained from Vanderbilt University and Imgenex Co. These patients received no documented treatment before surgery. The tissues were grouped based on Gleason score and compared based on the staining for $T\beta RII$ in the prostatic stroma (Figure 7). The $T\beta RII$ antibodies that tested in the respective $Tgfr2^{floxE2/floxE2}$ and $Tgfr2^{fspKO}$ prostates in Figure 1D were used for these immunohistochemical studies. $T\beta RII$ was highly expressed in epithelial cells of all prostate samples examined. The stromal $T\beta RII$ staining pattern, albeit less intense, was representative of >95% of the tissue in each array spot based on blinded pathology scoring (Figure 7). Stromal $T\beta RII$ was expressed in 85% of the tissues associated with benign epithelia. In contrast, an average 31% of the prostate cancer tissues with Gleason scores 6–10 maintained stromal $T\beta RII$ staining. Further the clinical correlates, pre-surgical serum PSA expression and age (p value=0.97, 0.31, respectively), did not statistically distinguish between benign and prostate cancer in this population. There was no correlation of stromal TGF- β expression and a specific Gleason score. As the proportions of stromal TGF- β expression for malignant samples were relatively similar to each other than that of benign samples, counts in malignant samples were combined and compared to the

benign group. Multivariate analysis suggested the odds of positive stromal T β RII is 11.5 times as high in the benign group compared to the malignant group (with 95% power; confidence interval: 4.2 to 31.3; p value < 0.0001).

DISCUSSION

The progression of the grafted prostate tissue rescues from Tgfbr2^{fspKO} mouse to adenocarcinoma led us focus on the consequence of the loss of TGF- β signaling in the stroma on malignant progression. Although PIN lesions spontaneously developed in the Tgfbr2^{fspKO} mouse prostates by 5–7 weeks of age, it was not clear if the model supported further progression to adenocarcinoma (Bhowmick *et al.*, 2004a). Due to early lethality of the Tgfbr2^{fspKO} mice, we used tissue rescue and recombination grafting techniques to reveal the long-term role of stromal TGF- β signaling in tumor progression. As only 25% of the Tgfbr2^{fspKO} mouse prostates progressed to adenocarcinoma, we chose to use an established human prostate cancer cell line, LNCaP, to further study the paracrine impact of the loss of TGF- β responsiveness in the stroma. LNCaP cells do not express functional TGF- β receptors (Guo and Kyprianou, 1999). Thus, the resulting differences in the tumor size between Tgfbr2^{floxE2/floxE2} and Tgfbr2^{fspKO} prostatic stroma associated tissue recombinants (Figure 5) were due to TGF- β signaling differences in the tumor microenvironment. It is likely stromally derived factors normally suppressed by TGF- β signaling, accelerated LNCaP tumor progression. A candidate approach identified Wnt3a as one such TGF- β regulated cytokine, subsequently was shown to have an important role in tumor survival. Elevated Wnt signaling is attributed in the initiation and progression of prostate cancer with relatively infrequent mutations in the pathway (Yardy and Brewster, 2005). This study provides a mechanism for the elevated Wnt activity in prostate epithelia. The loss of T β RII expression in the stroma of 69% of human prostatic cancer tissues and the resulting signaling repercussions suggests the relatively frequent evidence of elevated Wnt signaling in prostate cancer can be a result of paracrine activity. The data further supports stromal TGF- β signaling to be a tumor suppressor in the prostate (Bhowmick and Moses, 2005; Bhowmick *et al.*, 2004b).

The expression of T β RII in mature prostate epithelial cells is thought to contribute to the relatively low proliferative state. Accordingly, previous studies hypothesized that a loss of epithelial T β RII expression played a role in the process of high-grade prostate cancer progression. However, immunohistochemical staining for T β RII expression are ambiguous with reports of both decreased and elevated T β RII expression in prostate adenocarcinomas (Cardillo *et al.*, 2000; Gerdes *et al.*, 1998; Kim *et al.*, 1998; Kim *et al.*, 1996; Royuela *et al.*, 1998). Our focus was on stromal T β RII expression in prostate cancer progression. We do not make any conclusions regarding the status of epithelial T β RII expression, as the immunohistochemical development was extended to enable visualization of stromal expression. The apparent histochemical staining for T β RII was significantly higher in epithelial cells than that found in the stromal compartment at any pathologic state (Figure 7). Stromal staining associated with PIN lesions in the human tissues were not examined, since it was impossible to determine which lesions would progress to cancer. The question of T β RII antibody specificity in our immunohistochemistry study was addressed by the positive and negative stromal staining pattern in the Tgfbr2^{floxE2/floxE2} and Tgfbr2^{fspKO} prostatic tissues, respectively (Figure 1).

We report on TGF- β regulation of paracrine Wnt signaling, rather than the better-studied intracellular interaction between the TGF- β and Wnt signaling pathways. Incidentally, many Wnt ligands are stromally secreted with cognate receptors expressed by the epithelia, such as human prostate cancer tissues (Ellwood-Yen *et al.*, 2003; Kwok *et al.*, 2005). Wnt target genes, like Twist (Figure 2) and c-myc (Figure 4) were up regulated in the epithelium

associated with $Tgfr2^{fspKO}$ prostate stroma (Bhowmick *et al.*, 2004a). In a comparison of primary cultured prostatic stromal cells from $Tgfr2^{loxE2/floxE2}$ and $Tgfr2^{fspKO}$ mice, we found that the expression of Wnt ligands were up regulated in $Tgfr2^{fspKO}$ stromal cells (Figure 3). There was a pronounced up regulation of Wnt3a at both RNA and protein levels. Ectopic expression has shown that Wnt3a to be a secreted form of Wnt ligand, unlike other Wnt proteins that associate with the cell membrane (Shibamoto *et al.*, 1998). Although no Smad binding elements were found in the Wnt3a gene promoter, Stat binding sites were predicted. Interestingly, TGF- β signaling is reported to inhibit Stat3 in T lymphocytes (Bright and Sriram, 1998) and corroborated here in the prostate stroma. We showed that the knockout of TGF- β signaling in prostate stromal fibroblasts resulted in the up regulation of Stat3 binding of the Wnt3a promoter by ChIP analysis (Figure 4). The neutralization of Wnt3a in $Tgfr2^{fspKO}$ stromal conditioned media reduced LNCaP proliferation *in vitro*. The neutralization of Wnt3a in mice with LNCaP tumor xenografts also resulted in greater areas of cell death due to necrosis and apoptosis in LNCaP tumors accompanied by lower rate of mitosis compared to those treated with the IgG isotype control (Figure 6). The mechanism for the TGF- β paracrine cross-talk with Wnt signaling seems to be mediated in part through Stat3 signaling.

Previously, we reported that paracrine HGF signaling is involved in the tumorigenesis observed in $Tgfr2^{fspKO}$ mice. Here, the data suggests that disruption of TGF- β signaling in the prostatic stromal cells up regulates the expression of Wnt3a to promote tumorigenesis in a Stat3 dependent manner. Clearly, tumor initiation and progression is not a single signaling pathway event. Interestingly, the induction of HGF and Wnt3a, resulting from the disruption of stromal $Tgfr2$, is tied by elevated activation of Stat3. We showed in this study increased Stat3 binding with Wnt3a promoter activity. Stat3 activation can also induce HGF expression by co-operation with Src (Qiao *et al.*, 2002; Sam *et al.*, 2007; Wojcik *et al.*, 2006). In the context of a tumor many growth factors, including IL-6, HGF and IGF-1, are known stimulators of Stat proteins. Thus there are multiple potential mechanisms that would support paracrine Wnt3a signaling in prostate cancer. Like other targeted monoclonal antibody-based therapies in the clinic, the Wnt3a neutralizing antibody was well tolerated by the host mice. This is the first attempt of using Wnt3a neutralizing antibody to inhibit prostate tumor growth. The promising outcome suggests such Wnt antagonists can be effective for prostate cancer patients, specifically those with undetectable stromal T β RII expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TGF-β	Transforming growth factor beta
TβRII	TGF- β type II receptor
$Tgfr2^{loxE2/floxE2}$	floxed TGF- β type II receptor
$Tgfr2^{fspKO}$	TGF- β type II receptor conditional fibroblast knockout

PIN	Prostatic intraepithelial neoplasia
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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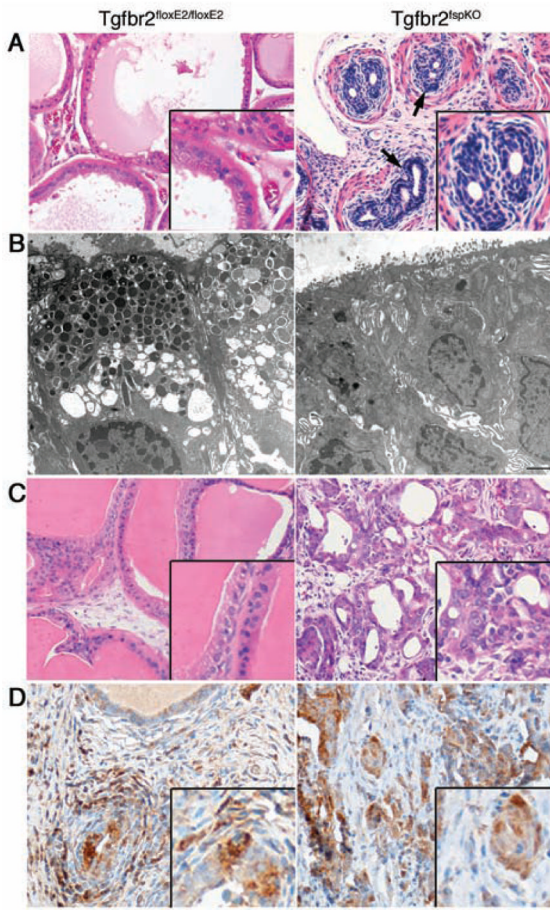


Figure 1.

The loss of TβRII expression in the prostatic stroma of mice leads to transformation of adjacent epithelia. **A.** Histologic comparison of $Tgfb2^{floxE2/floxE2}$ (left) and $Tgfb2^{fspKO}$ (right) mouse prostates by H&E staining suggest $Tgfb2^{fspKO}$ mouse prostates develop PIN by 6 weeks of age. **B.** Electron microscopy indicates the absence of secretory vesicles in $Tgfb2^{fspKO}$ mouse prostates compared to their presence in $Tgfb2^{floxE2/floxE2}$ prostates. Scale bar represents 2 μm . **C.** Following tissue rescue of $Tgfb2^{floxE2/floxE2}$ and $Tgfb2^{fspKO}$ prostates for seven months, the histology of the $Tgfb2^{fspKO}$ prostates progressed to adenocarcinoma while the $Tgfb2^{floxE2/floxE2}$ prostates maintained a wild type phenotype. **D.** Immunohistochemistry for TβRII expression of the rescued tissues showed positive stromal and epithelial staining in the $Tgfb2^{floxE2/floxE2}$ prostates, yet only epithelial staining in the $Tgfb2^{fspKO}$ prostates. Scale bar represents 50 μm for panels A, C, and D; 25 μm for insets.

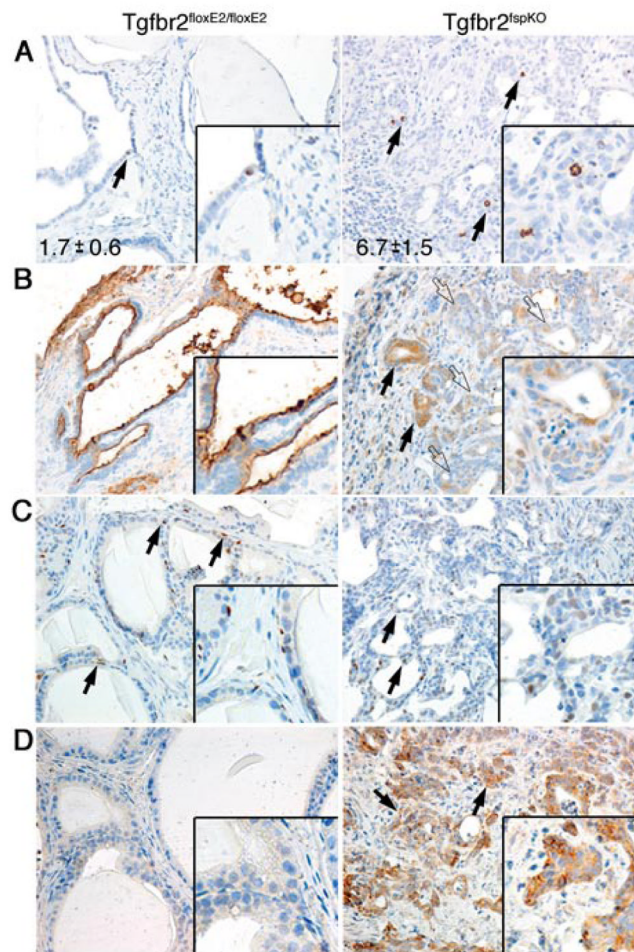


Figure 2. *Tgfr2*^{flxKO} prostates develop adenocarcinoma in seven months. **A.** Phosphorylated-histone H3 staining of the mitotic cells suggested higher proliferation rate in the *Tgfr2*^{flxKO} prostates rescues than the normal *Tgfr2*^{flx} prostates rescues. The mean ± standard deviation of positive staining is indicated in each panel ($P < 0.01$, $n = 6$ for both test and control). **B.** Immunohistochemistry for mouse dorsolateral prostate secretory proteins (mDLP) was detectable control *Tgfr2*^{flx} prostates, but often lost in the *Tgfr2*^{flxKO} tissue rescues. **C.** Immunohistochemistry for p63 revealed disorganized staining pattern in the prostates of *Tgfr2*^{flxKO} compared to *Tgfr2*^{flx}. **D.** Immunohistochemistry for Twist expression was positive in the adenocarcinoma *Tgfr2*^{flxKO} tissues, but not expressed in the *Tgfr2*^{flx} prostates. The sections were nuclear counterstained with hematoxylin (blue). Scale bar represents 50 μm for panels and 25 μm for insets.

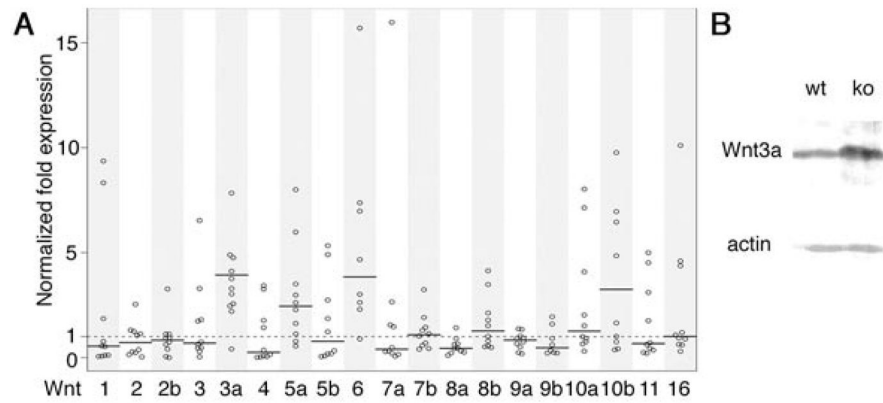


Figure 3. $Tgfr2^{fspKO}$ prostatic stromal cells have elevated Wnt3a expression. **A.** The screening of 19 Wnt isoforms by real-time PCR revealed specific Wnt isoforms to have greater mRNA expression by cultured $Tgfr2^{fspKO}$ prostatic stromal cells relative to control, $Tgfr2^{floxE2/floxE2}$ stromal cells. Each dot represents a comparative expression level of a $Tgfr2^{fspKO}$ sample relative to the average expression level of the $Tgfr2^{floxE2/floxE2}$ samples (baseline). The data were normalized to 18s ribosomal RNA expression. The dotted horizontal line is at the value of 1 representing no difference from the $Tgfr2^{floxE2/floxE2}$ average. The thick horizontal lines indicate the medians within each group. (There are 3 data (83.9, 88.0, 410.1) in Wnt3a and 1 (51.5) in Wnt10b that are out of the plot range.) **B.** Western blot confirmed specifically Wnt3a protein expression was greater in $Tgfr2^{fspKO}$ prostatic stromal cells compared to that from $Tgfr2^{floxE2/floxE2}$ cells.

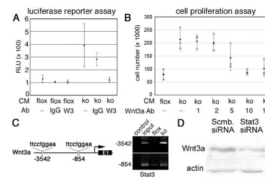


Figure 4.

Wnt3a expression is down regulated by TGF- β in prostate stromal cells. **A.** Conditioned media from Tgfbr2^{floxE2/floxE2} or Tgfbr2^{fspKO} stromal cells were incubated with LNCaP cells in the presence or absence of Wnt3a neutralizing antibody and isotype control IgG. Subsequently, luciferase reporter assay for the c-myc promoter was used as readout for canonical Wnt activity in LNCaP cells. **B.** Cell counting was used to measure LNCaP cell proliferation following incubation with Tgfbr2^{floxE2/floxE2} or Tgfbr2^{fspKO} prostatic stromal conditioned media. The addition of Wnt3a neutralizing antibody inhibited LNCaP cell proliferation in a dose dependent manner. The graphs in panels **A** and **B** indicate mean \pm standard deviation ($P < 0.01$, $n = 12$). **C.** Wnt3a promoter analysis suggested Stat transcription factor binding consensus sites. ChIP analysis suggested greater Stat3 binding to the Wnt3a -854 and -3542 promoter elements in Tgfbr2^{fspKO} stromal cells compared to Tgfbr2^{floxE2/floxE2} stromal cells. **D.** Western blotting for Wnt3a following siRNA-knockdown of Stat3 in Tgfbr2^{fspKO} stromal cells showed inhibition of Wnt3a expression. The scrambled (Scmb.) siRNA did not affect Wnt3a expression. Actin expression served as a loading control.

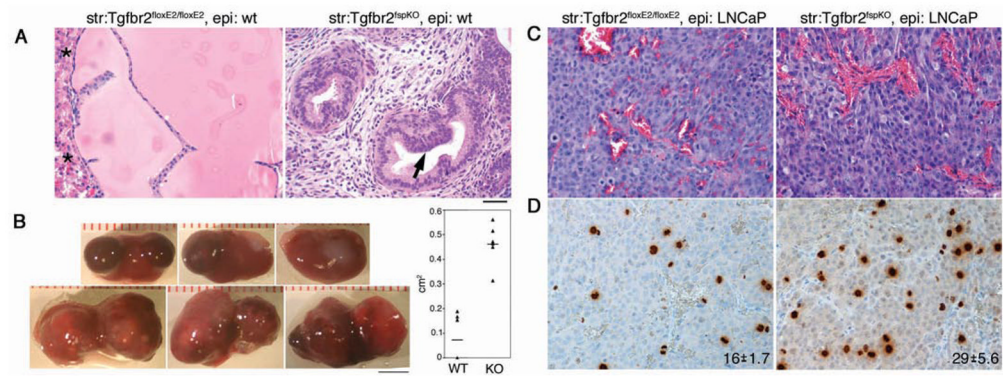


Figure 5. $Tgfb2^{fspKO}$ prostatic stromal cells increase tumorigenicity of prostate epithelial cells. **A.** Tissue recombinant of $Tgfb2^{floxE2/floxE2}$ or $Tgfb2^{fspKO}$ mouse prostate stromal cells with wild type mouse prostate organoids recapitulated the histology of the respective intact mice. Arrow indicates PIN lesion development in the tissue recombinant associated with $Tgfb2^{fspKO}$ stromal cells. Asterisks indicate kidney parenchyma. **B.** The gross representations of the LNCaP/ $Tgfb2^{fspKO}$ tumors in renal xenografts were larger than control, LNCaP/ $Tgfb2^{floxE2/floxE2}$ tumors. Tumor volumes calculated using Image J software were graphed as mean \pm standard deviation ($P < 0.01$, $n = 6$). Scale bar represents 4 mm. **C.** H&E for the LNCaP/ $Tgfb2^{floxE2/floxE2}$ and LNCaP/ $Tgfb2^{fspKO}$ recombinant tumors histology showed little difference. **D.** Immunohistochemistry for phosphorylated-histone H3, indicated the mitotic index of LNCaP/ $Tgfb2^{fspKO}$ tumors to be greater than LNCaP/ $Tgfb2^{floxE2/floxE2}$ tumors. The mean positive staining is indicated in each panel \pm standard deviation ($P < 0.01$, $n = 6$). The scale bar in panel A represents 50 μ m for panels A, C, and D.

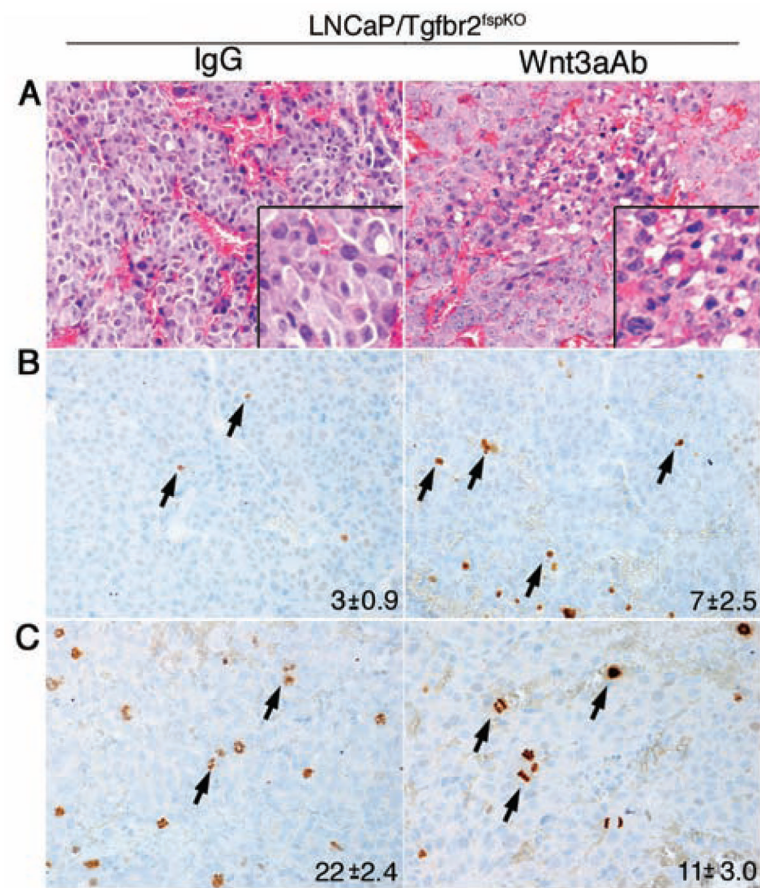
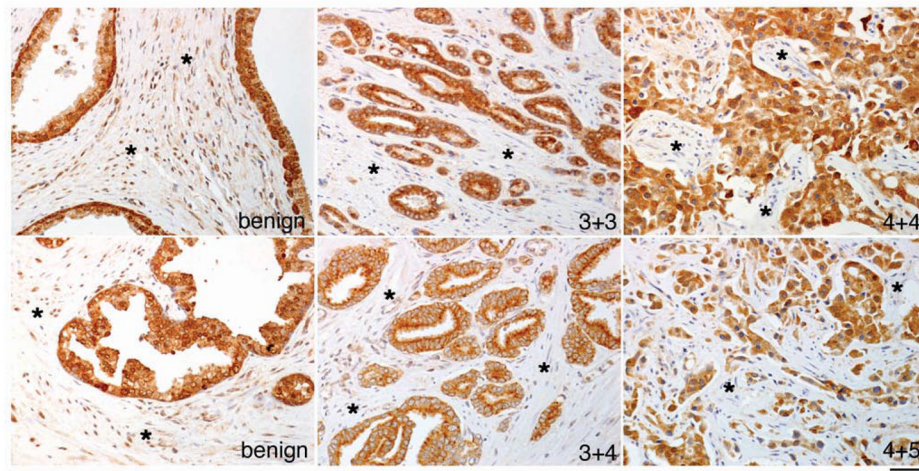


Figure 6.

Wnt3a neutralizing antibody inhibits tumorigenic progression of LNCaP/Tgfr2^{fspKO} tissue recombinants. Wnt3a neutralizing antibody or isotype control IgG was i. p. injected to the hosted SCID mice twice a week for two weeks, started two weeks post-grafting. **A.** Histology by H&E showed increased necrotic areas in the tumors from Wnt3a antibody injected mice, compared to the ones from control mice (indicated by dashed line). **B.** Greater number of apoptotic cells was localized in the tumors treated with Wnt3a neutralizing antibody compared to control by Apop-tag immunohistochemistry ($P < 0.01$, $n = 8$). **C.** Immunohistochemistry of phosphorylated-histone H3 showed less mitotic cells in the tumors from Wnt3a treated mice compared to control ($P < 0.01$, $n = 6$). The mean \pm standard deviation is indicated in each panel. The scale bar represents 50 μ m for panels and 25 μ m for insets.



Histopathology of Specimen	Total	Stromal TβRII (+)	
		Number	Percentage
Benign	33	28	84.8%
Gleason 6	4	1	25.0%
Gleason 7	38	11	28.9%
Gleason 8	18	2	11.1%
Gleason 9	42	19	45.2%
Gleason 10	5	0	0%
Gleason 6-10	107	33	30.8%

Figure 7.

Immunohistochemistry for TGF- β type II receptor (T β RII) expression is not detectable in stromal cells of human prostate adenocarcinomas. The pathologic grade of the representative immunohistochemistry images is indicated as benign or Gleason score. Note T β RII was consistently expressed in epithelial cells, but often lost in stromal cells of neoplastic tissues. Scale bar represents 50 μ m. The table indicates the distribution of tissue pathology with positive histochemical T β RII staining in the stromal compartment.