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TITLE: Identify the Impact of TGF- $\beta$  Signaling on the Stroma in the Progression of Prostate Cancer

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> As a result of androgen ablation TGF-β1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical in understanding and ultimately combating androgen-non-responsive prostate cancer. Studying the conditional TGF-β type II receptor fibroblast knockout mouse model we developed (FβKO), we found that TGF-β signaling in the prostate stromal fibroblasts regulate both stromal and epithelial differentiation in the prostate. As proposed we attempted to develop mice that are stromally knocked out for TGF-β signaling and express the large T antigen in the prostate epithelia, but was unsuccessful. Thus we made tissue recombinants of prostatic epithelia with FβKO stromal cells. This resulted in the development of poorly differentiated adenocarcinoma compared to when the same epithelia was combined with control stromal cells. Moreover, we found that the FβKO associated epithelia was refractile to androgen ablation. The mechanism of these observations seems to be due to stromally derived paracrine Wnt5a activating the epithelial frizzled receptor 2 that enabled prostate epithelial survival in an androgen deficient environment. We hope to gain permission to progress with these experiments to further address the mechanism of stromal TGF-β signaling impact on prostate cancer androgen responsiveness and differentiation.					
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## INTRODUCTION

The prostate epithelial and stromal compartments interact to regulate prostate development and function, in part through the tight regulation of glandular apoptosis and proliferation. These interactions are mediated by various factors that include hormones and cytokines. Specific signaling by androgens is required for prostate development and maintenance of function through the stimulation of proliferation and inhibition of apoptosis of prostatic epithelial cells (Hayward and Cunha, 2000; Montgomery et al., 2001). Cytokines such as EGF, IGF, and TGF- $\beta$  isoforms can also in-turn stimulate the expression of the androgen receptor (AR) in an androgen-independent fashion (Byrne et al., 1996; Culig et al., 1996). Often the development and progression of prostate cancer is dependent on androgens and their receptor for prostate cellular proliferation and differentiation. As a result, its inhibition has been the primary therapy for metastatic prostate cancer and much effort has been devoted to elucidating the role of the androgen receptor in prostate cancer. As a result of androgen ablation TGF- $\beta$ 1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical since androgen-signaling antagonists are currently used in treating patients with malignant prostate cancer, benign prostate hyperplasia, and as a chemoprevention of prostate cancer in clinical trials. However populations of hormone-non-responsive cancer cells unfortunately frequently arise. The central hypothesis of this proposal is that TGF- $\beta$  signaling in the prostatic fibroblasts contributes to normal prostatic epithelial differentiation; when this signal is altered in the case of some cancers the differentiation status of the epithelia is altered.

TGF- $\beta$  isoforms (TGF- $\beta$ 1,  $\beta$ 2,  $\beta$ 3) have long been established as physiological regulators of prostate growth because of their ability to inhibit cell proliferation and mediate apoptosis (Kyprianou and Isaacs, 1989; Martikainen et al., 1990). TGF- $\beta$ s exert their effects through binding to the TGF- $\beta$  type II receptor (T $\beta$ RII) and subsequent recruitment of the type I receptor (T $\beta$ RI) for downstream cytoplasmic signaling through multiple parallel signaling pathways (Attisano and Wrana, 2002). TGF $\beta$  plays a key role in the steroidal regulation of tissues and in the important growth regulation axis existing between androgenic signaling, smooth muscle differentiation and epithelial proliferation. The present proposal seeks to address the role of TGF $\beta$  signaling in mouse prostate tissue under in vivo conditions in light of signaling pathways identified through studies in cell lines. In order to understand the role of TGF- $\beta$  in the prostate we have developed and studied mouse models, that employ the Cre-lox methodology to conditionally ablate T $\beta$ RII expression in the stromal fibroblasts (Bhowmick et al., 2004) and those that express the large SV40 T antigen transgene (TAG) in the prostate epithelia (from collaborator, Dr. Robert Matusik, Vanderbilt U., TN) (Kasper et al., 1998). The proposal was based on preliminary data that ablation of T $\beta$ RII in fibroblasts results in preneoplastic prostate intraepithelial neoplasia (PIN) lesions and prostate-specific TAG expression results in PIN and progression of focal adenocarcinoma (Kasper et al., 1998). We proposed to develop mice that expressed both TAG in the prostate epithelia and concomitant loss of T $\beta$ RII in the stromal fibroblasts (mouse model termed TNT) to examine epithelial and stromal differentiation (Task1). Since these TNT mice were not thought not to be able to live past 8 weeks of age, we also proposed to rescue the prostatic tissues from these mice as xenografts and further study differences in cellular differentiation and androgen responsiveness (Task2).

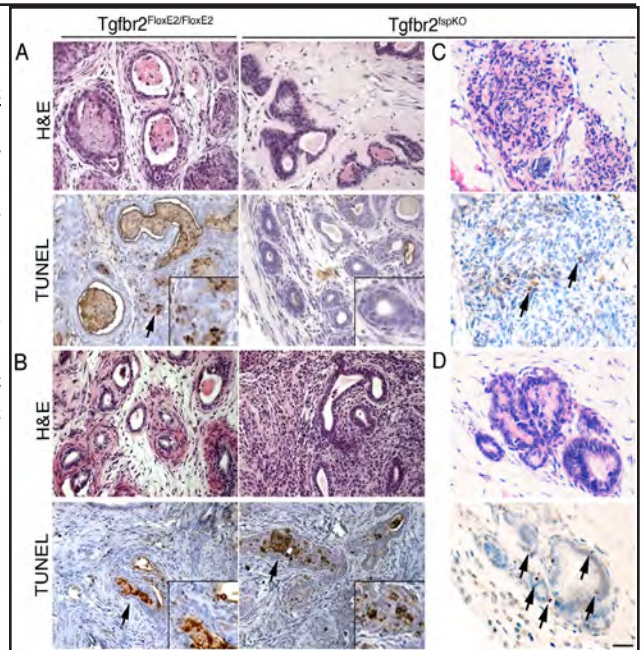
**BODY****Stromal TGF- $\beta$  responsiveness is associated with prostatic tumor progression**

We focused on how the TGF $\beta$  signaling pathway components may be involved in prostate cancer progression and subsequent regression. It was not feasible to study prostate cancer progression in the mouse model that expressed both SV40 large T-antigen (TAg) in the prostate epithelia and concomitant loss of T $\beta$ RII in the stromal fibroblasts (mouse model termed TNT) due to early lethality (Task1). The expression of the SV40 large T antigen by the prostatic epithelia, driven by the probasin promoter in transgenic mice, results in primarily in PIN lesions by 12-15 week of age with occasional foci of adenocarcinoma. Initial studies in recombining prostatic stromal cells from Flox and F $\beta$ KO mice with the 12T7f epithelial organoids proved to be interesting. The recombined tissues were xenografted under the renal capsule for 8 weeks. After which time, some of the host mice were castrated. This phenotype was reminiscent of that observed in the F $\beta$ KO mice. Through tissue recombination xenografting, we were able to show that TAg expressing prostatic epithelia also become refractile to androgen ablation when grafted with prostate stromal cells deficient in TGF- $\beta$  signaling (Figure 1). Additionally, the phenotype of the TAg prostatic epithelia, that normally form preneoplastic PIN lesions, develop normal prostatic morphology when recombined with wild type or Tgfr2<sup>floxE2/floxE2</sup> (control) stromal cell. However, the combination of TAg epithelia with Tgfr2<sup>fspKO</sup> stromal cells developed into adenocarcinoma reproducibly. Thus together it would suggest that the stromal cells mediate the size and androgen responsiveness of the prostate as well as prostate cancer.

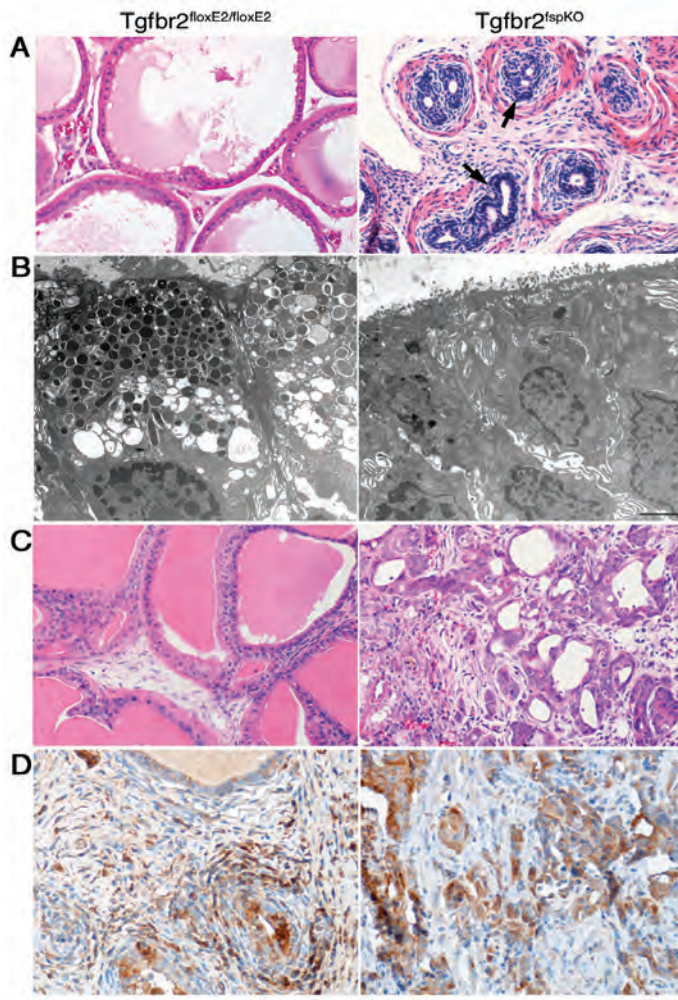
**The loss of T $\beta$ RII expression in the prostate stroma can lead to adenocarcinoma**

The Tgfr2<sup>fspKO</sup> mouse model was used to study if the loss of T $\beta$ RII in the prostatic stroma contributes to prostatic adenocarcinoma progression. The Tgfr2<sup>fspKO</sup> mouse prostates develop PIN lesions by six weeks of age, as previously reported (Figure 2A) (Bhowmick et al., 2004). Electron microscopy further revealed the loss of epithelial differentiation, as there were no secretory vesicles in the six-seven week old Tgfr2<sup>fspKO</sup> mouse prostate epithelial cells, compared their presence in Tgfr2<sup>floxE2/floxE2</sup> mouse prostates (Figure 2B). If PIN is assumed to be a precursor of prostate adenocarcinoma, the progression of the disease was expected under long-term observation. Since the Tgfr2<sup>fspKO</sup> 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<sup>fspKO</sup> prostates developed into adenocarcinoma by seven months following grafting (Figure 2C). Tissue rescued prostates from six-week old Tgfr2<sup>floxE2/floxE2</sup> mice under the same conditions were histologically normal by H&E staining. Immunohistochemistry for the expression of T $\beta$ RII confirmed a significant decrease in T $\beta$ RII expression in the stromal compartment of Tgfr2<sup>fspKO</sup> prostates (Figure 2D).

Epithelial proliferation and differentiation markers were used to evaluate development of prostate adenocarcinoma. The mitotic rate of the prostatic epithelium of Tgfr2<sup>fspKO</sup>



**Figure 1. Inhibition of Wnt signaling restores Tgfr2<sup>fspKO</sup> prostate responsiveness to androgen ablation.** (A) Tgfr2<sup>floxE2/floxE2</sup> or Tgfr2<sup>fspKO</sup> mature mouse prostates were transduced with GFP adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). (B) Tgfr2<sup>floxE2/floxE2</sup> or Tgfr2<sup>fspKO</sup> mature mouse prostates were transduced with SFRP-2 adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining was not statistically different between GFP-Tgfr2<sup>floxE2/floxE2</sup> and SFRP-2-Tgfr2<sup>floxE2/floxE2</sup> allografts (P value = 0.2819). Percent positive epithelial TUNEL positive staining in SFRP-2-Tgfr2<sup>fspKO</sup> allografts was statistically greater than GFP-Tgfr2<sup>fspKO</sup> allografts (P value = 0.0373). (C) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfr2<sup>fspKO</sup> prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given GFP adenovirus through out the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). (D) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfr2<sup>fspKO</sup> prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given SFRP-2 adenovirus through out the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining in tissue recombinations of 12T7f LADY epithelial organoids and SFRP-2-Tgfr2<sup>fspKO</sup> allografts was statistically greater than those in tissue recombinations of 12T7f LADY epithelial organoids and GFP-Tgfr2<sup>fspKO</sup> allografts (P value = 0.0472). Scale bar indicates 25  $\mu$ m.



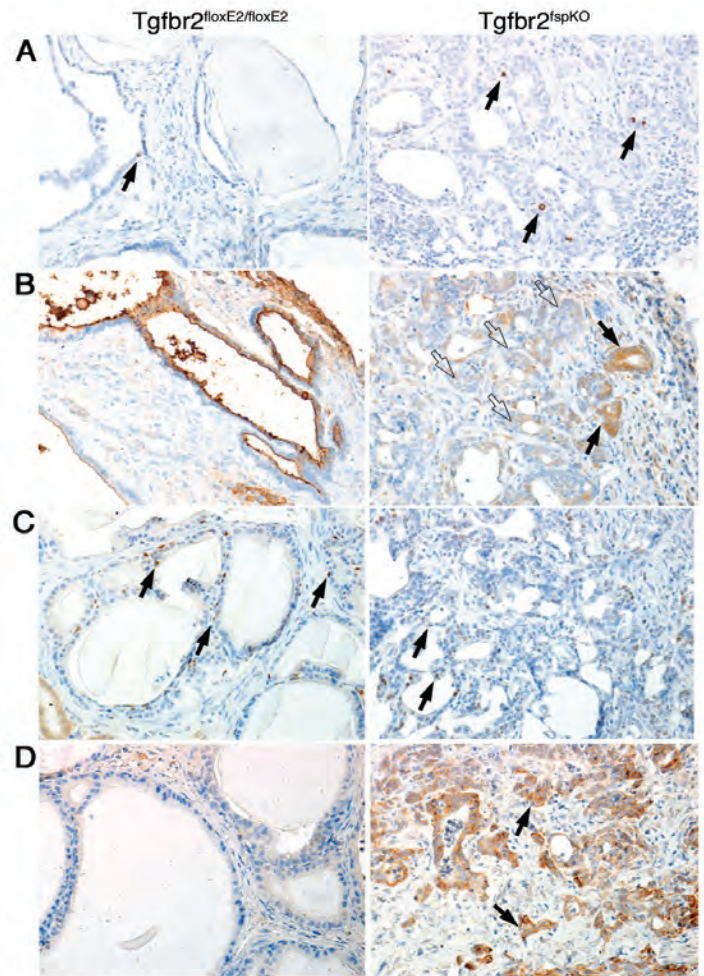
**Figure 2. The loss of TβRII expression in the prostatic stroma of mice leads to transformation of adjacent epithelia.** A. Histologic comparison of *Tgfr2*<sup>flxE2/flxE2</sup> (left) and *Tgfr2*<sup>fspKO</sup> (right) mouse prostates by H&E staining suggest *Tgfr2*<sup>fspKO</sup> mouse prostates develop PIN by 6 weeks of age. B. Electron microscopy indicates the absence of secretory vesicles in *Tgfr2*<sup>fspKO</sup> mouse prostates compared to their presence in *Tgfr2*<sup>flxE2/flxE2</sup> prostates. Scale bar represents 2 μm. C. Following tissue rescue of *Tgfr2*<sup>flxE2/flxE2</sup> and *Tgfr2*<sup>fspKO</sup> prostates for seven months, the histology of the *Tgfr2*<sup>fspKO</sup> prostates progressed to adenocarcinoma while the *Tgfr2*<sup>flxE2/flxE2</sup> prostates maintained a wild type phenotype. D. Immunohistochemistry for TβRII expression of the rescued tissues showed positive stroma and epithelial staining in the *Tgfr2*<sup>flxE2/flxE2</sup> prostates, yet only epithelial staining in the *Tgfr2*<sup>fspKO</sup> prostates. Scale bar represents 50 μm for panels A, C, and D.

basal cell layer. Another reported marker for prostatic adenocarcinoma progression, Twist, was detected only in the *Tgfr2*<sup>fspKO</sup> tissue rescues that progressed to adenocarcinoma (Figure 3D) (Hotz et al., 2007; Kwok et al., 2005; Zhang et al., 2007). Our data showed for the first time that loss of TβRII expression in the stroma induced prostate tumorigenesis in mice.

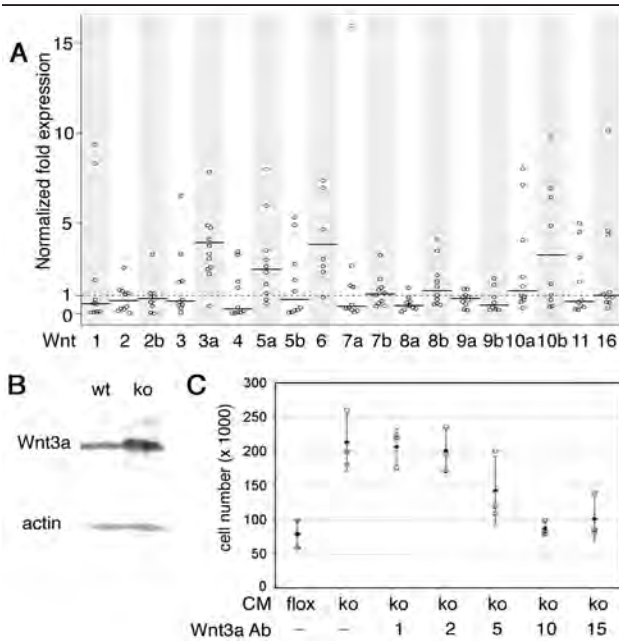
#### Wnt3a mediates increased tumorigenicity of prostate tumors by *Tgfr2*<sup>fspKO</sup> mouse prostate stroma

In light of the observed Twist expression and recent publications indicating the importance of Wnt signaling

mice was four-fold greater than that from *Tgfr2*<sup>flxE2/flxE2</sup> mice in the seven-month progression model, as determined by quantitating phosphorylated-histone H3 expression (Figure 3A). The mouse dorsolateral prostate (mDLP) antibody was used to localize secretions found in differentiated prostatic epithelium, was present in the *Tgfr2*<sup>flxE2/flxE2</sup> tissues but was focally absent in *Tgfr2*<sup>fspKO</sup> tissues (Figure 3B) (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*<sup>flxE2/flxE2</sup> tissues, as expected (Figure 3C) (Kurita et al., 2004). In contrast, p63 positive cells were rare and scattered in the *Tgfr2*<sup>fspKO</sup> prostate rescues, supportive of the progression of adenocarcinoma and indicating a disruption of the



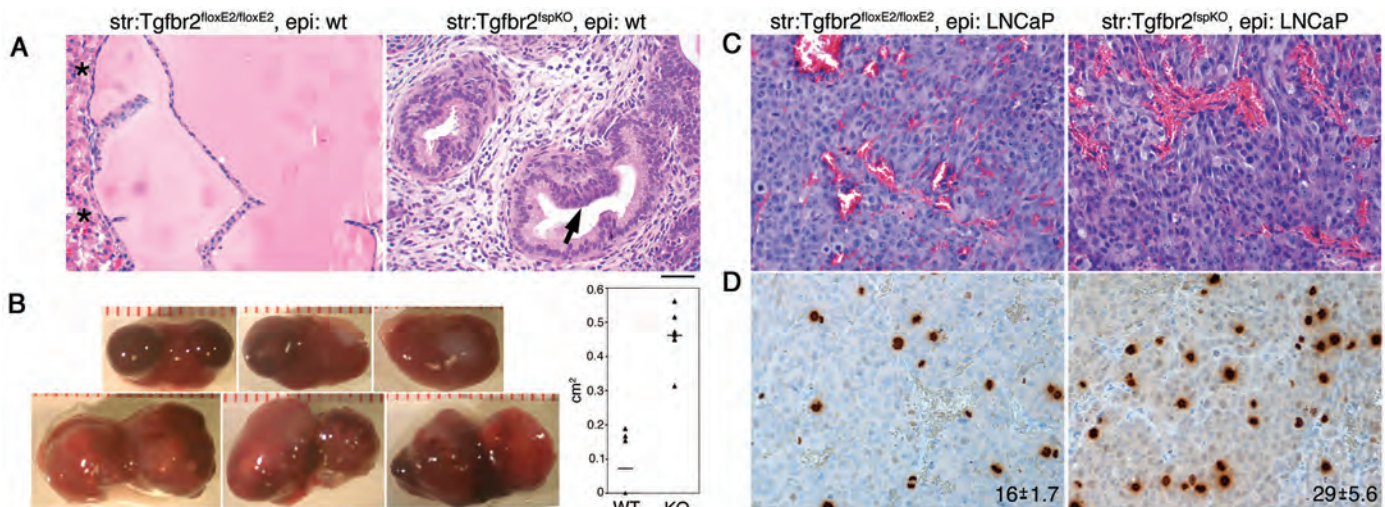
**Figure 3. *Tgfr2*<sup>fspKO</sup> prostates develop adenocarcinoma in seven months.** A. Phosphorylated-histone H3 staining of the mitotic cells suggested higher proliferation rate in the *Tgfr2*<sup>fspKO</sup> prostates rescues than the normal *Tgfr2*<sup>flxE2/flxE2</sup> prostates rescues. The mean ± standard deviation of positive staining is indicated in each panel ( $P < 0.01$ ,  $n = 6$  for both test and control). B. Immunohistochemistry for dorsolateral prostate (DLP) was detectable control *Tgfr2*<sup>flxE2/flxE2</sup> prostates, but often lost in the *Tgfr2*<sup>fspKO</sup> tissue rescues. C. Immunohistochemistry for p63 revealed disorganized staining pattern in the prostates of *Tgfr2*<sup>fspKO</sup> compared to *Tgfr2*<sup>flxE2/flxE2</sup>. D. Immunohistochemistry for Twist expression was positive in the adenocarcinoma *Tgfr2*<sup>fspKO</sup> tissues, but not expressed in the *Tgfr2*<sup>flxE2/flxE2</sup> prostates. The sections were nuclear counterstained with hematoxylin (blue). Scale bar represents 50 μm.



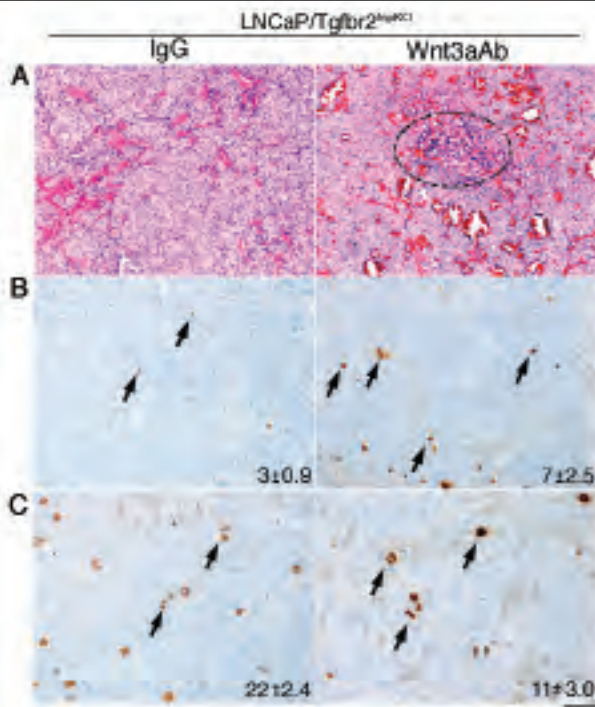
**Figure 4.  $Tgfr2^{fspKO}$  prostatic stromal cells have elevated Wnt3a expression.** **A.** The screening of 19 Wnt isoforms by real-time PCR revealed specific Wnt isoforms to have greater mRNA expression by cultured  $Tgfr2^{fspKO}$  prostatic stromal cells relative to control,  $Tgfr2^{floxE2/floxE2}$  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. **C.** Cell counting was used to measure LNCaP cell proliferation following incubation with  $Tgfr2^{floxE2/floxE2}$  or  $Tgfr2^{fspKO}$  prostatic stromal conditioned media. The addition of Wnt3a neutralizing antibody inhibited LNCaP cell proliferation in a dose dependent manner. The graphs indicate mean  $\pm$  standard deviation ( $P < 0.01$ ,  $n = 12$ ).

in prostate cancer progression, we screened for the expression of nineteen Wnt ligand isoforms as putative paracrine mediators of the loss of TGF- $\beta$  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 4A). 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 at higher levels 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 4B). The Wnt3a neutralizing antibody reduced the proliferative effect of  $Tgfr2^{fspKO}$  conditioned medium in a dose dependent manner. LNCaP cell proliferation was decreased to comparable levels as cells grown in  $Tgfr2^{floxE2/floxE2}$  conditioned medium in the presence of 10 ng/ml Wnt3a neutralizing antibody.

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



**Figure 5.  $Tgfr2^{fspKO}$  prostatic stromal cells increase tumorigenicity of prostate epithelial cells.** **A.** Tissue recombinant of  $Tgfr2^{floxE2/floxE2}$  or  $Tgfr2^{fspKO}$  mouse prostatic stromal cells with wild type mouse prostatic epithelial organoids recapitulated the histology of the respective intact mice. **B.** The gross representations of the LNCaP/ $Tgfr2^{fspKO}$  tumors in renal xenografts were larger than control, LNCaP/ $Tgfr2^{floxE2/floxE2}$  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/ $Tgfr2^{floxE2/floxE2}$  and LNCaP/ $Tgfr2^{fspKO}$  recombinant tumors histology showed little difference. **D.** Immunohistochemistry for phosphorylated-histone H3, indicated the mitotic index of LNCaP/ $Tgfr2^{fspKO}$  tumors to be greater than LNCaP/ $Tgfr2^{floxE2/floxE2}$  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  $\mu$ m for panels A, C, and D.



**Figure 6. Wnt3a neutralizing antibody inhibits tumorigenic progression of LNCaP/Tgfr2<sup>fspKO</sup> 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 100  $\mu$ m for panel A and 50  $\mu$ m for panels B and C.

C57/Bl6 male mice for eight weeks, we found that the Tgfr2<sup>flloxE2/flloxE2</sup> stroma-associated prostatic grafts were comparable to intact prostates of wild type mice. The Tgfr2<sup>fspKO</sup> stroma-associated prostatic grafts developed PIN lesions, recapitulating the intact Tgfr2<sup>fspKO</sup> 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<sup>fspKO</sup> compared to tumors of LNCaP/Tgfr2<sup>flloxE2/flloxE2</sup> 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<sup>fspKO</sup> tissue recombinants compared to LNCaP/Tgfr2<sup>flloxE2/flloxE2</sup> 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<sup>fspKO</sup> 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 associated with mice 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-histone3 immuno-localization. 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- $\beta$  signaling in the prostatic stromal compartments is associated with canonical Wnt signaling

in the adjacent epithelia to support tumor initiation and further tumor progression. Blocking Wnt3a activity in tumors deficient in stromal TGF- $\beta$  signaling was effective in reducing tumor growth.

#### T $\beta$ RII expression is lost in stromal cells of human prostate adenocarcinomas

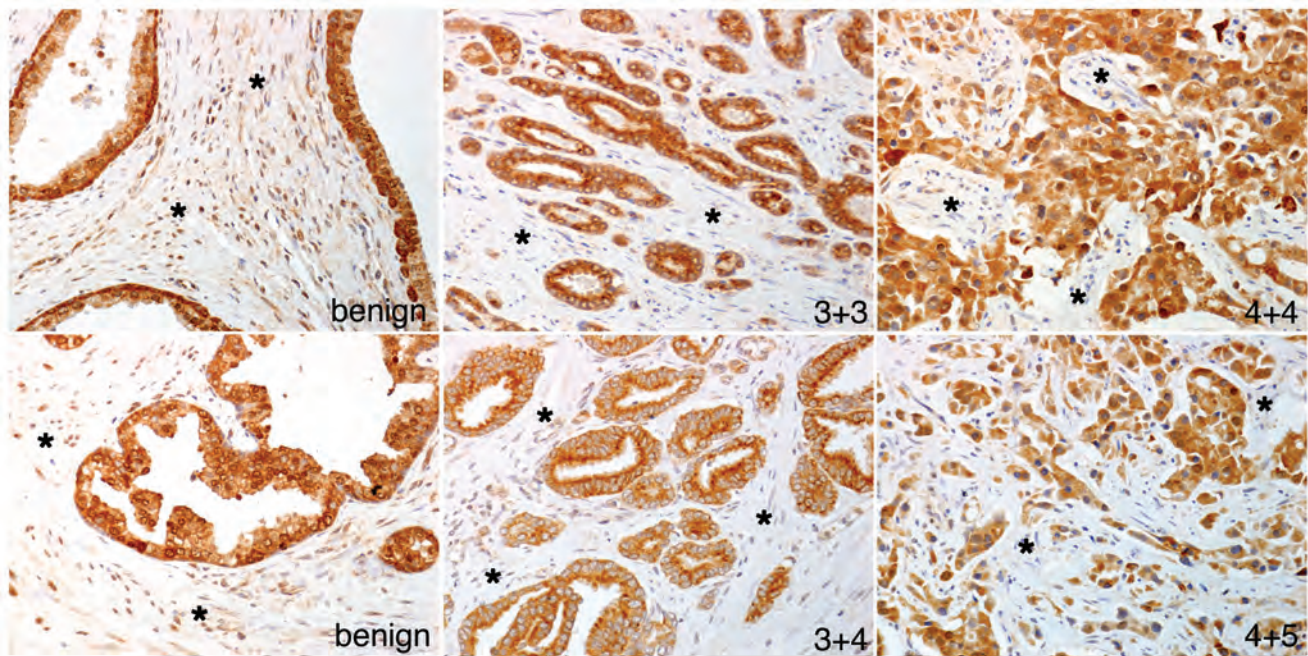
Finally, to explore the role of stromal TGF- $\beta$  signaling in human prostate cancer progression, we localized T $\beta$ RII expression by immunohistochemistry of 140 benign and malignant prostate tissues. Prostate samples from patients who underwent radical prostatectomy were obtained from Vanderbilt 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 positive and negative in the respective Tgfr2<sup>flloxE2/flloxE2</sup> and Tgfr2<sup>fspKO</sup> prostates in Fig 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- $\beta$  expression and a specific Gleason score. As the proportions of stromal TGF- $\beta$  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. Multivariant



analysis suggested the odds of positive stromal T $\beta$ 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).

## KEY RESEARCH ACCOMPLISHMENTS

- We provide in vivo data supporting stromal TGF- $\beta$  signaling can regulate prostate cancer androgen responsiveness in a tissue recombination model.
- We showed that Wnt5a is expressed by the prostatic stroma following androgen ablation in a short window.
- The activation of Wnt5a is regulated by TGF $\beta$  responsivity of the prostatic stroma
- Specific inhibition of the Frizzled receptor 2 in the epithelial compartment can result in regression of prostates that were otherwise refractile to androgen ablation.
- Wnt3a is downregulated by TGF- $\beta$  stromal signaling.
- The neutralization of Wnt3a can reduce the tumorigenesis induced by the loss of TGF- $\beta$  signaling in the stroma.
- The loss of TGF- $\beta$  receptor II expression is observed in human prostate adenocarcinoma associated stromal cells.



Histopathology of Specimen	Total	Stromal T $\beta$ RII (+)	
		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%
<b>Gleason 6-10</b>	<b>107</b>	<b>33</b>	<b>30.8%</b>

**Figure 7.** Immunohistochemistry for TGF- $\beta$  type II receptor (T $\beta$ RII) expression is not detectable in stromal cells of human prostate adenocarcinomas. The pathologic grade of the representative immunohistochemistry images are indicated as benign or Gleason score. Note T $\beta$ RII was consistently expressed in epithelial cells, but often lost in stromal cells of neoplastic tissues. Scale bar represents 50  $\mu$ m. The table indicates the distribution of tissue pathology with positive histochemical T $\beta$ RII staining in the stromal compartment.

## REPORTABLE OUTCOMES

### Research

#### Manuscripts

Veronica R. Placencio, Ali-Reza Sharif-Afshar, Xiaohong Li, Hongxia Huang, Consolate Uwamariya, Simon W. Hayward, Neil A. Bhowmick (2008) TGF- $\beta$  Responsiveness of the Stroma Dictates Prostatic Sensitivity to Androgen Ablation. *Cancer Res.* 68:4709-4718.

Xiaohong Li, Yongqing Wang, Ali-Reza Sharif-Afshar, Consolate Uwamariya, Andrew Yi, Kenichiro Ishii, Simon W. Hayward, Robert J. Matusik, Neil A. Bhowmick (2008) Urothelial transdifferentiation to prostate epithelia is mediated by paracrine TGF- $\beta$  signaling. *Differentiation*. In press.

Xiaohong Li, Veronica Placencio, Juan M. Iturregui, Consolate Uwamariya, Ali-Reza Afshar-Sherif, Tatsuki Koyama, Simon W. Hayward, Neil A. Bhowmick (2008) Loss of TGF- $\beta$  type II receptor expression in the prostatic stroma is associated with tumor progression. *Oncogene*. In press.

### Awards received based on work supported by this grant

Veronica Placencio (Graduate student supported grant) received Travel Awards to Society For Basic Urologic Research Meeting in 2005 to Miami, FL and 2006 to Phoenix AZ.

1RO1 CA108646    Bhowmick (PI)    8/01/04 – 7/31/09  
 NIH/NCI  
 TGF $\beta$  Signals in Prostate Stromal-Epithelial Interactions  
 The goal of this project is to specifically identify the TGF $\beta$ -mediated signals in the stroma that mediate prostate androgen responsiveness.  
 Role: Principal Investigator

PC073536    Jackson (PI)    4/01/08 – 3/31/10  
 DOD USMRMC Prostate Cancer Research Program, Postdoctoral Training Grant  
 TGF- $\beta$  Signaling in Stromal Differentiation and Prostate Cancer Progression  
 This project identifies the role of TGF- $\beta$  in the differentiation in the prostate and bone microenvironment in the context of tumor growth.  
 Role: Mentor

F31 GM079879    Placencio (PI)    6/01/07 – 5/31/11  
 NIH/NRSA Pre-doctoral training grant  
 TGF-beta and Androgen Signaling During Prostate Regression  
 The project identifies the mechanism the prostate stroma regulates prostate regression following androgen ablation. Paracrine signaling pathways involving TGF- $\beta$ , Wnt, and androgen signaling are studied.  
 Role: Mentor

U54 CA126505    Matrisian (PI)    10/1/06 – 09/30/11  
 Paracrine TGF- $\beta$  signaling in tumor initiation and progression  
 Our project in this program project is to examine the role of downstream TGF- $\beta$  signals in prostate epithelial neoplastic progression using xenografting and mathematical models.  
 Role: Project Co-director (Project 2)

### Products

CDNA construct, cell lines, and animal models developed

- Development of a in vivo tissue recombination model that incorporates large T antigen expressing prostatic epithelia with stromal cells deficient in TGF $\beta$  responsivity.
- Transgenic mouse line termed F $\beta$ KO/TOPGal that enables the study of canonical Wnt signaling in the context of stromal cells deficient for TGF $\beta$  responsivity.

## CONCLUSION

Surgery and androgen ablation therapy remains the major treatment for prostate cancer. However, within a year of treatment >80% of prostate cancer becomes androgen independent as a result of documented mutations in AR (50%) and unknown factors (50%). In this study, we showed that the Wnt3a neutralizing antibody inhibited LNCaP cell proliferation promoted by Tgfr2<sup>fspKO</sup> prostatic stromal cells. The in vivo experiment revealed neutralizing Wnt3a mediated more 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. Like other targeted monoclonal antibody-based therapies in the clinic, the Wnt3a neutralizing antibody was well tolerated by the host mice. Thus such Wnt antagonists may prove effective for prostate cancer patients, specifically those with undetectable stromal TβRII expression. We conclude that disruption of TGF-β signaling in the prostatic stromal cells up regulates the expression of Wnt3a to promote tumorigenesis in a paracrine manner.

New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Based on the studies described, androgen ablation therapy can cause paracrine Wnt signaling by the stromal compartment. Wnt ligand expression is apparently a mechanism for maintaining prostatic tissues in the absence of androgens. Thus, the administration of androgen ablation therapy to subjects with hormone refractile prostatic epithelia would not only be ineffective, rather have counter-indications for further aggressiveness of the cancer. Future prostate cancer therapies would most likely benefit by not only antagonizing the traditional androgen signaling pathway, but acting on Wnt signaling as well. This would allow therapies to target both the epithelial and stromal compartments as well as androgen dependent and independent tumor cells. Understanding paracrine interactions of TGF-β, androgen, and Wnt signaling in regulating prostate regression fosters the advancement of therapeutic options.

The progression of the grafted prostate tissue rescues from Tgfr2<sup>fspKO</sup> 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 Tgfr2<sup>fspKO</sup> 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 Tgfr2<sup>fspKO</sup> 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 Tgfr2<sup>fspKO</sup> 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 Tgfr2<sup>floxE2/floxE2</sup> and Tgfr2<sup>fspKO</sup> 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 TβRII 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).

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