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FGFR1-WNT-TGF- β signaling in prostate cancer mouse models recapitulates human reactive stroma

Julienne L. Carstens^{1,3}, Payam Shahi^{1,5}, Susan Van Tsang², Billie Smith³, Chad J. Creighton³, Yiqun Zhang³, Amber Seamans³, Mamatha Seethammagari⁴, Indira Vedula¹, Jonathan M. Levitt¹, Michael M. Ittmann^{2,3,6}, David R. Rowley^{1,3}, and David M. Spencer^{1,3,4}

¹Department of Pathology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

²Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

³Dan L Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

Abstract

The reactive stroma surrounding tumor lesions performs critical roles ranging from supporting tumor cell proliferation to inducing tumorigenesis and metastasis. Therefore, it is critical to understand the cellular components and signaling control mechanisms that underlay the etiology of reactive stroma. Previous studies have individually implicated fibroblast growth factor receptor 1 (FGFR1) and canonical WNT/ β -catenin signaling in prostate cancer progression and the initiation and maintenance of a reactive stroma; however, both pathways are frequently found co-activated in cancer tissue. Using autochthonous transgenic mouse models for inducible FGFR1 (JOCK1) and prostate-specific and ubiquitously expressed inducible β -catenin (Pro-Cat and Ubi-Cat, respectively) and bigenic crosses between these lines (Pro-Cat $\times$ JOCK1 and Ubi-Cat $\times$ JOCK1), we describe WNT-induced synergistic acceleration of FGFR1-driven adenocarcinoma, associated with a pronounced fibroblastic reactive stroma activation surrounding prostatic intraepithelial neoplasia (mPIN) lesions found both in situ and reconstitution assays. Both mouse and human reactive stroma exhibited increased transforming growth factor-beta (TGF- β) signaling adjacent to pathologic lesions likely contributing to invasion. Furthermore, elevated stromal TGF- β signaling was associated with higher Gleason scores in archived human biopsies, mirroring murine patterns. Our findings establish the importance of the FGFR1-WNT-TGF- β signaling axes as driving forces behind reactive stroma in aggressive prostate adenocarcinomas, deepening their relevance as therapeutic targets.

Keywords

Prostate Cancer; Reactive Stroma; FGFR1; WNT; TGF- β

Corresponding Authors: David M. Spencer and David R. Rowley, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA, 713-798-6220, dspencer@bcm.edu and drowley@bcm.edu.

⁴Current address:

Bellicum Pharmaceuticals, Houston, TX, USA

⁵Department of Anatomy, University of California, San Francisco, CA, USA

⁶Michael E. DeBakey Dept. of Veterans Affairs Medical Center, Houston, Texas 77030

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INTRODUCTION

Cancer growth and metastasis require proliferation, cell migration, and stromal remodeling, functions also critical during organogenesis and wound repair. The fibroblast growth factors (FGFs) and their receptors (FGFRs) play pivotal roles in development, wound healing and tumorigenesis (1). FGFR1 is a receptor tyrosine kinase that signals through the RAF/MEK/MAPK-ERK1/2 kinase cascade and PI3K/AKT axis, both of which are well described oncogenic pathways, shown to promote androgen-independence in prostate cancer (2,3). WNT signaling is vital for embryogenesis, homeostasis of adult tissues, and wound repair, as well as being associated with malignancy (4–11). In the absence of WNT ligands, the GSK-3 β -containing “destruction complex” phosphorylates β -catenin, targeting it for proteasomal degradation (4). Canonical WNT signaling involves WNT ligands binding to co-receptors Frizzled and lipoprotein receptor-related protein (LRP)-5/6, facilitating disruption of the destruction complex, allowing β -catenin to translocate to the nucleus.

There are many examples of FGF and WNT signaling cooperating in development and tumorigenesis (12–14). In mammary tissue, FGFR1 rapidly accelerates *Wnt-1*-induced carcinomas (15), while FGFR1 targets, MAPK/pERK, are known to modulate WNT signaling and colorectal tumorigenicity (16). WNT signaling also cooperates with activated K-RAS and is upregulated in FGFR1-driven prostate cancer (10,17). Additionally, recent queries of two independent databases revealed a modulation of either FGF and/or canonical Wnt signaling members in 81% and 92% of completely characterized prostate adenocarcinoma cases, respectively. Modulation of both pathways within the same case occurred 38% and 72% of the cases, respectively (18). Furthermore, tumor-associated reactive stroma is similar to hyper-proliferative stroma found during wound repair. Appropriately, mechanical injury modulates the FGF and WNT, as well as TGF- β , signaling pathways (19–24). Finally, the expression of FGF, WNT and TGF- β signaling primes bone metastatic niches. These intricate developmental and wound-repair interactions support the synergistic cooperation of these signaling pathways in carcinogenesis that includes a poorly understood, complementary stromal component.

We hypothesized that FGFR1 and canonical Wnt signaling, within the same epithelia cells (intracellular), should accelerate tumor progression, given previously described data (discussed below). However, it was unclear whether (intra / intercellular) paracrine signals derived from associated stromal tissue would further accelerate tumor progression, producing a second potential target for therapeutic intervention. In order to study epithelial FGFR1 signaling, we previously generated the synthetic ligand-inducible FGFR1 transgenic mouse model, JOCK1, via use of the prostate epithelium-specific, composite probasin promoter, ARR₂PB (25). Addition of a lipid-permeable chemical inducer of dimerization (CID), AP20187, causes the intracellular signaling domains of FGFR1 to oligomerize, inducing downstream signaling (26,27). We previously demonstrated that prolonged induction (~ 60 weeks) of FGFR1 results in a step-wise progression to invasive prostate cancer with distant metastasis (17). Furthermore, we recently demonstrated that induced aggregation of the WNT co-receptor LRP5, is sufficient to induce β -catenin stabilization, similar to canonical WNT signaling (28). We subsequently developed two novel transgenic mouse models wherein WNT signaling can be specifically activated in the prostate epithelium, yielding “Pro-Cat” mice (*Prostatic Activator of β -catenin*; ARR₂PB promoter) or ubiquitously, in “Ubi-Cat” mice (*Ubiquitous Activator of β -catenin*; H-2K^b promoter) (28). Interestingly, Pro-Cat mice do not progress beyond prostatic hyperplasia; however, after a year of induction, by which time murine prostatic epithelia can undergo natural wounding (29), two out of six Ubi-Cat mice developed adenocarcinoma (28). Furthermore, WNT signaling derived from Ubi-Cat adult stroma enhanced prostate tissue reconstitutions, further supporting an additional role for stromal WNT signaling in tumorigenesis (28).

In this report, we delineate synergism between FGF and WNT pathways across different sub-tissue layers in prostate carcinogenesis by crossing Pro-Cat and Ubi-Cat lines onto the JOCK1 background. We observe that WNT signaling accelerates FGFR1-driven adenocarcinoma, through both intracellular crosstalk (Pro-Cat $\times$ JOCK1) and intra/intercellular crosstalk (Ubi-Cat $\times$ JOCK1) by significantly accelerating adenocarcinoma initiation, while intra/intercellular crosstalk accelerates tumor progression. We also observed that WNT signaling drives an inflammatory, fibroblastic reactive stroma in both endogenous tumor and reconstitutions assays, in contrast to the primarily myofibroblastic reactive stroma seen in human prostate cancer. Finally, we have identified increased stromal TGF- β signaling in both human and murine tumors as a potential mechanism for accelerated tumorigenesis.

MATERIALS AND METHODS

Animals and Treatment

Mice were housed in a pathogen-free facility following approved IACUC protocols. Generation and genotyping of transgenic mice have been previously described (27,28). Mice were treated biweekly starting at 6 weeks of age by i.p. injections of AP20187 (Ariad Pharmaceuticals) at 2 mg/kg in drug diluent (16.7% propanediol, 22.5% PEG400, 1.25% TWEEN 80). 8-week-old nu/nu mice were procured from Charles River Laboratories. See Table 2 for N – values.

Human Tissue Microarrays

Tissue microarrays have been described previously (32). Radical prostatectomy tissues were collected with informed consent, as approved by the Baylor College of Medicine Institutional Review Board, by the Prostate Cancer Specialized Program of Research Excellence (SPORC). All patients underwent surgery for clinically localized prostate cancer and received no prior treatment.

Histology and Immunohistochemistry

Tissues were fixed in either 10% buffered formalin (JOCK1, Ubi-Cat, and Ubi-Cat $\times$ JOCK1) or 4% paraformaldehyde (Pro-Cat and Pro-Cat $\times$ JOCK1) overnight, embedded in paraffin, and 5- μ m sections stained with hematoxylin and eosin or Sigma-Aldrich Accustain Trichrome Stain system.

Immunohistochemistry (IHC) analysis of 5- μ m sections was performed as follows for each antibody using standard light microscopy. *SMA*: no antigen retrieval; 1:400 overnight, 4°C mouse monoclonal smooth muscle alpha actin (Sigma A2547); Secondary - BioCare's MM AP Polymer 30 minutes at RT; Vector red substrate for 20 minutes at RT. *Vimentin*: 20 minutes heated EDTA buffer pH 8.0 antigen retrieval; 1:50 overnight, 4°C goat polyclonal vimentin (sc-7557); Secondary - BioCare's goat HRP detection kit 15 minutes at RT; Substrate - DAB, enhanced by BioCare's DAB Sparkle. *Tenascin C*: heated EDTA buffer, pH 8.0 antigen retrieval; 1:10 overnight, 4°C rabbit monoclonal tenascin C (ab108930); Secondary - 1:500 Anti-rabbit, 1 hour at RT; Vector red substrate, 30 minutes at RT. *Phospho-Smad2*: heated Citrate Buffer, pH 6.0 (Diagnostic BioSystems), 20 minutes antigen retrieval; 1:100 overnight, 4°C, pSmad2 (Millipore, AB3849); Secondary- Rabbit on Rodent (BioCare Medical), for 30 minutes at RT; Substrate - DAB, enhanced by BioCare's DAB Sparkle. *Ki-67*: heated Citrate Buffer, pH 6.0 (Diagnostic BioSystems), 20 minutes antigen retrieval; 1:100 overnight, 4°C, anti-Ki-67 (SP6 Neomarkers RM-9106-SO); Secondary- Rabbit on Rodent (BioCare Medical), for 30 minutes at RT; Substrate - DAB, enhanced by BioCare's DAB Sparkle.

Prostate Reconstitution

Embryonic stroma: prostates from 7–13-week untreated JOCK1, Pro-Cat × JOCK1 or Ubi-Cat × JOCK1 mice were isolated and sorted for basal progenitors (LSC), as previously described (30). 100,000 LSC cells were mixed with 100,000 rat urogenital sinus mesenchyme (UGSM) in 100µl Matrigel® and transplanted subcutaneously into nu/nu mice and treated ± CID for 8 weeks or 30 weeks (JOCK1 grafts). Adult stroma: prostates from adult untreated wild type, JOCK1, or Ubi-Cat mice were isolated, and sorted for two populations: LSC (Lin⁻, Sca1⁺, CD49f⁺) and stroma (Lin⁻, Sca1⁺, CD49f⁻). 100,000 cells were mixed from each population from different genotypes in 100µl Matrigel®, transplanted subcutaneously into nu/nu mice and treated ± CID (16 weeks).

Laser Capture Microdissection and Whole Genome Arrays

Frozen samples were collected in OCT medium and stored at -80°C. mPIN lesions and adjacent reactive stroma were captured from 5-µm sections on the Pixcell Laser Capture machine. Total RNA was isolated using the CapSure® Macro LCM caps (Applied Biosystems LCM0211) and amplified using Arcturus™ RiboAmp® PLUS (Applied Biosystems KIT0501). Amplified RNA was labeled using the Invitrogen cDNA labeling system (L1015-06) and hybridized to the Agilent Whole Mouse Genome Oligo (4×44) Microarray (G4122) chip for analysis.

Statistical Analyses

Pathological Grading—Histological analysis was performed by a board-certified pathologist (MI) in a blinded fashion. The penetrance of each histological phenotype throughout the prostate (whole anterior, dorsal/lateral and ventral lobes) was graded on a scale of 0–3 (0 none; 1 25%; 2 ~ 50%; 3 75%). Scores for each phenotype were averaged across all mice in a particular group, giving the mean group score. Group phenotype scores were then weighted summed ((2 × PIN score) + (3 × Adenocarcinoma score) + (4 × Sarcomatoid Carcinoma score) + (5 × Sarcoma score)) to derive the total pathological score for each group (plotted with SEM). Tissues were also scored for reactive stroma penetrance (disorganized stroma, characterized by fibroblasts, neovasculature and inflammation) on a scale of 0–3 (0 none; 1 25%; 2 ~ 50%; 3 75%). Finally, the score for each mouse was averaged to get the mean score for the group (reported with SEM). See Table 2 for the number of mice in each group. Statistical significance was determined by Two-way ANOVA with Bonferroni multiple comparisons within the interaction effect. Two-tailed post-hoc power analysis for an α of 0.05 was also performed.

To determine the ratio of p-Smad2 in reactive stroma adjacent to increasing epithelia grades, 20 random high power (60× optic) fields of each mouse group and 387 normal, 272 PIN and 343 tumor fields of patient samples were examined. Positive cells were defined as any stromal cells (outside the basement membrane: includes fibroblasts, muscle cells, and endothelial cells; excludes immune cells and epithelial cells) with clear nuclear staining (brown IHC over a faint blue counterstain). The total number of p-Smad2⁺ stromal nuclei were divided by the total number of stromal cells per field, and these values were averaged to determine the mean of each group with significance determined by Two-way ANOVA with Bonferroni multiple comparisons. Significance of specific human subsets was determined by a t-test or Mann-Whitney Rank sum test, as needed.

Percent Ki-67⁺ stromal cells were quantified by Inform software analysis of ten random 10× micrographs imaged by a Nuance camera. Percentages for each micrograph were calculated and averaged to give a mean for each mouse. The mean score for each mouse was averaged to give the mean score for the group (reported value).

Prostate Reconstitution Assays—Groups were compared for statistical significance with one-tailed student's t-tests.

Gene Expression Analysis—Expression arrays were processed and loess-normalized using BioConductor, and heat maps were generated using JavaTreeView (47). Array data have been deposited in the Gene Expression Omnibus (GEO), accession GSE41344.

RESULTS

WNT Signaling Accelerates FGFR1-Driven Prostate Adenocarcinoma

Both murine and human prostate tumorigenesis advances through well-characterized pathological “hallmarks”: epithelial hyperplasia, mPIN, adenocarcinoma, and transitional sarcomatoid. Transitional sarcomatoid lesions have been recently reclassified as sarcomatoid carcinoma and is considered a histological manifestation of EMT (Figure S3) (31). Our previous work demonstrated JOCK1 mice steadily progress through these hallmarks, similar to human prostate cancer, and reaches an adenocarcinoma state by 40 weeks of CID treatment (Figure 1)(17). To determine whether canonical WNT signaling accelerates JOCK1 progression, thereby modeling more aggressive human cancer, we crossed Pro-Cat (inducible WNT signaling in prostate epithelium) and Ubi-Cat mice (ubiquitous expression of inducible WNT signaling) with JOCK1 animals (Figure S1 and S2). Both strains were treated with CID biweekly for 12, 24, 40 or 60 weeks. Tissues were collected and analyzed for the presence and penetrance of progression hallmarks. Seminal vesicle occlusion correlated with prostate transformation (Figure S5); all other tissues were histologically normal (data not shown). Pro-Cat mice did not progress beyond minor hyperplasia. When combined with FGFR1 signaling, all Pro-Cat × JOCK1 mice developed adenocarcinoma significantly faster than monogenic JOCK1 mice (24 weeks vs. 40 weeks). However, tumors in Pro-Cat × JOCK1 mice did not progress until 60 weeks of treatment, similar to JOCK1, consistent with an age-dependent and self-limiting progression threshold or “brake”. Similar to Pro-Cat animals, most Ubi-Cat mice did not progress beyond hyperplasia; however, after one year of CID treatment, two out of six mice developed adenocarcinoma (28), also suggesting an age-dependent, self-limiting step. When crossed to JOCK1, all Ubi-Cat × JOCK1 mice displayed the same significant acceleration in initiation as Pro-Cat × JOCK1 mice; however Ubi-Cat × JOCK1 mice continued to progress, resulting in tissues containing 50% sarcomatoid carcinoma by 40 weeks, 20 weeks earlier than JOCK1 or Pro-Cat × JOCK1 mice. Prostate tumors phenocopied the JOCK1 histopathology with heterogeneous, invasive hyperchromatic epithelial cells and loss of the basal cell layer, as previously described. These data suggest, the addition of WNT signaling changed the rate of progression: intracellular crosstalk (Pro-Cat × JOCK1) accelerated FGFR1-driven adenocarcinoma initiation, while intra- and inter-cellular crosstalk (Ubi-Cat × JOCK1) accelerated both progression and initiation.

Transgenic Progenitors Reconstitute mPIN and Adenocarcinoma

We next investigated the ability of basal progenitor cells, known as LSC (Lin⁻, Sca1⁺, CD49f⁺), to reconstitute prostate pathology seen in the endogenous tumors. LSC cells from JOCK1, Pro-Cat × JOCK1 and Ubi-Cat × JOCK1 mice were isolated, transplanted subcutaneously along with inductive rat urogenital sinus mesenchyme in Matrigel® into recipient mice, and treated ± CID for 8 weeks (28). Consistent with the autochthonous tumor progression, all transgenic LSC grafts displayed hyperplasia within 8 weeks. Interestingly, JOCK1 grafts also displayed mPIN, like the double-transgenic lines (Figure 2A), indicating loss of a tumor-suppressing function, typically distinguishing WNT-accelerated FGFR1-driven pathology. To test whether JOCK1 grafts also achieved accelerated adenocarcinoma, we extended CID treatment and observed reconstitution of adenocarcinoma-like lesions by

30 weeks, ten weeks earlier than autochthonous JOCK1 tumors (Figure 2B). This accelerated tumorigenesis could be attributable to the use of pro-inductive embryonic stroma, which, when combined with oncogenic initiation (FGFR1), recapitulated the pronounced, tumor-associated reactive stroma observed in double-transgenic mice. Thus, in the presence of inductive stroma, reconstituted grafts, containing FGFR1 and FGFR1/WNT signaling-activated progenitor cells, can progress to similar levels of pathology as in autochthonous transgenic tissues.

WNT Drives an Inflammatory, Fibroblastic Reactive Stroma

To further characterize the reactive stroma, we performed immunohistochemical analysis of several reactive stroma markers in histologically similar tumors from JOCK1 (*i.e.*, following 40 weeks of CID treatment) and Pro-Cat × JOCK1 and Ubi-Cat × JOCK1 (24 weeks of treatment). The reactive stroma was very collagen-rich (Figure 3A and S4; Trichrome, blue staining) with Ubi-Cat × JOCK1 being the most dense. The smooth muscle α -actin (SMA) layer surrounding the acini in normal stroma is greatly reduced in reactive stroma. This correlates with an overall decrease in SMA; however, JOCK1 tissues still displayed focal areas of dense SMA staining (pictured). We also observed dense expression of the fibroblastic marker, vimentin, in all reactive stroma (Figure 3A and S4). Furthermore, focal expression of the reactive stroma marker, Tenascin C, closely correlated with invasive lesions, as previously observed in human prostate cancer (32). Finally, overall proliferation across genotypes (Figure 3A; Ki-67) correlated with rate of progression. Interestingly, the reactive stroma of Ubi-Cat × JOCK1 tumors revealed considerably more Ki-67⁺ cells in the stroma. Thus, β -catenin stabilization-accelerated FGFR1-driven prostate cancer, producing a heterogeneous, proliferative reactive stroma that is predominantly fibroblastic, as opposed to a myofibroblastic phenotype reported in human prostate cancer.

To further understand the relationship between the reactive stroma and cancer progression, we quantified the stromal pathological score across each group (Figure 3B). Development of reactive stroma was CID-dependent and consistently arose in conjunction with mPIN, prior to adenocarcinoma. This observation suggests the presence of reactive stroma could be driving prostate cancer development, as previously suggested in humans (32). Finally, there was significantly more reactive stroma in the double-transgenic mice over time with the most pronounced difference seen in the Ubi-Cat × JOCK1 animals (Figure 3B). Interestingly, in the two Ubi-Cat mice that developed CID-dependent tumors, reactive stroma was observed only at focal sites of adenocarcinoma and was not observed in normal or mPIN tissue, suggesting canonical WNT signaling in autochthonous epithelium and stroma (without oncogenic stimuli) is not sufficient to drive a reactive stroma. Taken together, these data indicate that WNT signaling drives stromal expansion and when accompanied by appropriate oncogenic signaling (*e.g.*, FGFR1), correlates with aggressive prostate cancer.

Stromal WNT Signaling Induction Induces a Fibroblastic Reactive Stroma with Lymphocyte Infiltration

Although we observed cooperative effects of FGFR1 and WNT signaling, limitations of autochthonous tumor models and the lack of an all-inclusive stromal promoter precluded a full assessment of whether WNT signaling in the stroma alone is sufficient to synergize with FGFR1 signaling in the epithelium. To address this, we utilized the ubiquitous iLRP5 expression in Ubi-Cat mice for prostate reconstitution assays. LSC cells and stromal cells (Lin⁻, Sca1⁺, CD49f⁻) from adult wild type (WT), JOCK1, or Ubi-Cat mice were isolated. Cell populations from different genotypes were mixed 1:1 in Matrigel® and transplanted subcutaneously into athymic mice. Ubi-Cat stroma mixed with WT LSC cells in the absence of CID had very poor engraftment (in reference to very small acini size and number and not

“take” rate), and only small, single-cell layer acini (28). However, in the presence of canonical WNT signaling (+ CID), acini were more numerous and developed, though their overall size was small. Interestingly, WNT-induced stroma was thicker with spindle cell-like morphology and contained vascular (Figure 4A). Likewise, in the absence of CID, JOCK1 LSC and WT stroma had poor engraftment. The induction of FGFR1 signaling in the epithelium was sufficient to improve engraftment, without aberrant pathology, as was seen when combined with embryonic stroma (Figure 2). Without CID, Ubi-Cat stroma and JOCK1 LSC grafts had similarly poor engraftment rates and small acini (Figure 4A and B). However, in the presence of WNT signaling, acini contained proliferative lesions and were significantly larger than CID-treated WT LSC + Ubi-Cat stroma (Figure S6). As shown in Figure 4A, a reactive host response by histological parameters is apparent in CID-treated JOCK1 LSC + Ubi-Cat stroma grafts. Meanwhile, control grafts (*i.e.*, minus CID or CID-treated WT LSC + Ubi-Cat stroma) exhibited very little reactive stroma (Figure 4A). These experiments demonstrate that WNT signaling alone in the stroma is associated with a reactive stroma phenotype in synergy with FGFR1-induced proliferation and pre-malignant transformation.

Aggressive Adenocarcinoma Correlates with Modulated Reactive Stroma and TGF- β Signaling

To elucidate the mechanism behind the WNT-accelerated FGFR1 adenocarcinoma, we performed laser capture microdissection (LCM) to separate pre-cancerous (hyperplasia and mPIN) epithelia and adjacent reactive stroma cells. We utilized frozen samples from the same timepoints as above and performed gene expression profiling (Figure 5A). Comparing the more aggressive Pro-Cat $\times$ JOCK1 pre-cancerous tissue to JOCK1, we discovered modulation of the FGF axis receptors (*i.e.*, *FGFR1*, 2, 3, and 4) and ligands (*i.e.*, *FGFs* 3, 7 and 8) in both the epithelium and stroma, demonstrating a feed-forward loop that could lead to accelerated CID-independence, previously described in JOCK1-driven tumors (17, 26, 27). Furthermore, we observed an upregulation of various metastasis-associated genes (*Snail2*, *Twist1*, and *R-Ras*) in the epithelium of both bigenic lines versus JOCK1 (Figure S7). Interestingly, we also observed a significant upregulation of TGF- β ligands in the epithelium of double-transgenic compared to JOCK1 mice and a downregulation of TGF- β receptors in both the epithelium and stroma (Figure 5B). This is consistent with the recent discovery that loss of TGF- β RII in a subset of stromal cells (but not all) promotes a more aggressive disease (33). Although Pro-Cat $\times$ JOCK1 and Ubi-Cat $\times$ JOCK1 tissues were compared, no notable differences were observed at this particular timepoint of “accelerated initiation”; however, later timepoints in disease progression might reveal gene expression changes that could distinguish an “accelerated progression” signature.

To explore which cells were responding to the increased TGF- β ligands, we examined nuclear localization of phosphorylated-Smad2 (p-Smad2) as a read-out for TGF- β receptor activation in JOCK1, Pro-Cat $\times$ JOCK1, Ubi-Cat $\times$ JOCK1 tissues and in a human tissue array, consisting of normal, mPIN and tumor samples from matched donors. Approximately 90% of normal prostate epithelium darkly stained for p-Smad2. In light of the established anti-proliferative role of TGF- β , this supports a tightly controlled signaling mechanism to maintain epithelial homeostasis (Figure 5C). Interestingly, mPIN lesions showed high expression of nuclear p-Smad2, while hyperplastic areas showed p-Smad2 heterogeneity, suggesting that a change in homeostatic signaling permits neoplasia. Furthermore, most of the nuclei in normal stroma faintly to modestly stain for nuclear p-Smad2, while reactive stroma stained more intensely and heterogeneously. To investigate the association and potential role of this heterogeneity, we analyzed the percentage of nuclear p-Smad2⁺ stromal cells adjacent to various types of epithelium. Overall, the expression of tumor-associated stromal p-Smad2 correlated with increased disease aggressiveness in the mouse models

(Figure 5D). This suggests that the canonical WNT-driven partial loss of TGF- β receptors and increase in ligands identified by LCM could initiate the reactive stroma, which collaborates with neoplastic epithelium to drive accelerated adenocarcinogenesis in double-transgenic mice. Moreover, the higher overall TGF- β signaling observed in Ubi-Cat $\times$ JOCK1 stroma suggests that slight changes in TGF- β signaling in an already primed pre-neoplastic background elicits more rapid progression.

When the patient data was split according to pathological scores, we discovered that Gleason 7 relative to Gleason 5–6 tumors and stage T2/T3a relative to T3b or N1 tumors revealed a strong trend ($p = 0.073$ and $p = 0.112$, respectively) towards higher TGF- β signaling in the stroma immediately adjacent to the tumors; however, the relatively limited cohort size ($N = 49$) may have tempered statistical significance. Interestingly, tumor-associated stroma containing greater than 60% nuclear pSmad2⁺ cells is significantly associated with more aggressive tumors than stroma that was less than 60% positive ($p = 0.003$) (Figure 5D). Finally, four patients had recorded biochemical recurrence with a trend in higher tumor-associated TGF- β ; however, the small number of patients did not lend statistical significance. Together, these data demonstrate both bigenic mouse lines model several key attributes of human prostate cancer progression: (1) pathological progression, (2) expression of many important gene signatures, and (3) alterations in stromal TGF- β signaling. This last point is particularly relevant, as patients with higher stromal TGF- β signaling present with more aggressive disease, which is paralleled in the more aggressive Ubi-Cat $\times$ JOCK1 compared to Pro-Cat $\times$ JOCK1 or monogenic JOCK1 tumors.

Novel WNT Signaling Signature in Human Grade 3

While the role of FGFR1 in human prostate carcinogenesis is well established, a regulatory role of canonical WNT signaling during therapy resistance in response to treatment-induced wound healing is only beginning to be unraveled, and WNT signaling in the pre-treated stroma has not been investigated. To this end, we used the ontogeny analysis tool in DAVID Bioinformatics Resources 6.7 to interrogate the microarray data collected by Dakhova and colleagues, who compared normal human stroma to reactive stroma grade 3 (RSG3, characterized by at least a 1:1 ratio between reactive stroma and cancer epithelium; 34). We discovered a distinct WNT signature, consisting of the upregulation (1.2-fold and $p < 0.001$) of 15 genes implicated in a WNT signaling cascade (Figure S8A) (35,36). Even though the WNT signature observed in the CAT $\times$ JOCK transgenic animals contains different misregulated genes (Figure S8B), it nonetheless validates the importance of stromal WNT signaling in progression of human carcinogenesis. Furthermore, this WNT signature reveals the modulation of several WNT ligands (*Wnt7a*, *Wnt3* and *Wnt6*) in Pro-Cat $\times$ JOCK1 and a dramatic upregulation of *Wnt3* in Ubi-Cat $\times$ JOCK1, indicating that induced FGF and canonical WNT signaling could induce the production of more WNT ligands, as seen in development, that could act on the stroma needed for cancer initiation (Figure S6)(37).

DISCUSSION

Tumor-associated epithelial cells are surrounded by supporting cells and matrices, together termed “stroma”. The stroma contains vasculature, smooth muscle, nerves, fibroblasts, and immune cells (31). Following wounding, this diversity further expands in response to various stimuli (*e.g.*, TGF- β , FGF and WNT signaling) to facilitate fibroblast migration, myofibroblast differentiation, epithelial cell proliferation, and homing of inflammatory cells to close the breach and eliminate pathogens (31). These same innate functions are capable of responding to basement membrane breaches caused by neoplastic growth. However, growth stimuli further exacerbate tumorigenicity, eliciting a classic “chicken-or-egg” dilemma, as to

which tissue-type evolves first and promotes/recruits the other. This report supports tumor-promoting mutations in the epithelial cells occur first, which subsequently induce reactive stroma growth. In turn, reactive stroma is then required to co-evolve with a dysplastic epithelium to drive malignant transformation to adenocarcinoma.

We and others have previously shown the transforming ability of chronic FGFR1 stimulation when expressed in prostate epithelia cells (17,38). Using the Pro-Cat $\times$ JOCK1 and Ubi-Cat $\times$ JOCK1 mice, we show that canonical WNT signaling accelerates FGFR1-driven adenocarcinoma, due largely to an accelerated conversion to a fibroblastic reactive stroma. While the mechanisms whereby WNT accelerates this wound-like reactive stroma are unknown, migratory and metastatic roles for the WNT pathway have been reported. For example, FGF and canonical WNT signaling are highly expressed and result in the osteoblastic phenotype of AR- bone metastasis (39). Furthermore, anti-cancer therapeutics promote canonical WNT signaling in prostate cancer stroma via WNT16B, leading to carcinoma progression and therapeutic resistance (24). As a correlate with the role of WNT signaling, we have identified the decrease of *TGF- β RII* and an increase in TGF- β ligands in these bigenic tumors as the focus of future study.

Others have shown the decrease in TGF- β RII and concomitant increase in overall signaling is found in over 60% of human prostate cancers (40) and is sufficient to induce malignant transformation in prostate reconstitutions and mouse models (41–44). Using known prostate-associated oncogenes, we serendipitously observed a similar increase in TGF- β signaling, which correlates strongly with the co-evolving neoplasia (mPIN) and reactive stroma, as a likely mechanism for progression from mPIN to adenocarcinoma. Likewise, in a human tissue array we observed the same pattern of Smad2 activation. Furthermore, tumors with greater than 60% stromal TGF- β signaling significantly correlated with higher Gleason grades, resembling the signaling pattern of the more aggressive Ubi-Cat $\times$ JOCK1 mice. These findings agree with the “two-step computational model”, whereby TGF- β -nonresponsive stromal cells are required for the first (epithelial) transformation step and TGF- β -responsive cells are needed for the second (invasion) step of prostate cancer progression (45). Interestingly, this model demonstrated that canonical WNT signaling was a vital paracrine factor secreted by the TGF- β RII-deficient stromal cells as a mechanism for epithelial transformation. Although this model does not rule out other explanations, this bipartite TGF- β model could further explain the higher levels of nuclear p-Smad2 staining seen in Ubi-Cat $\times$ JOCK1 animals, as enforced transgenic WNT signaling in the stromal compartment could circumvent the initial requirement of TGF- β -nonresponsive cells to secrete *Wnt3a* for the proliferation step, allowing TGF- β -responsive (p-Smad2⁺) cells to efficiently promote epithelial invasion and accelerated progression.

The observed epithelial phenotype of our double-transgenic mice correlates with progression hallmarks identified in patients; even to the extent that sarcomatoid carcinomas are now accepted as histological manifestations of EMT (17, 31, 46). The tumor-associated stroma, however, reveals both similarities and distinct differences between mouse and human diseased tissue. The reactive stroma seen in these mice “closely resembles the stroma of [the] aggressive human prostate cancer subgroup (RSG3)” (31). Furthermore, these models display a fibroblastic reactive stroma (i.e., high-level collagen and vimentin staining, a decrease in smooth muscle α -actin, and focal areas of tenascin c) all of which indicate a fibroblastic reactive stroma. In humans, these fibroblasts, more commonly referred to as CAFs (cancer-associated fibroblasts), make up approximately 50% of reactive stroma in Gleason 3 foci and are progressively replaced by myofibroblasts in Gleason 4–5 foci (32). In more advanced foci, myofibroblasts are the majority and share both fibroblastic (i.e., tenascin c and vimentin) and smooth muscle markers (i.e., smooth muscle α -actin). This difference correlates with the dissimilarities seen in the normal stroma of mice and men.

Normal murine stroma is sparse, with only a thin layer of smooth muscle surrounding the acini, whereas human stroma consists of dense areas of smooth muscle filling the space surrounding the glands. Therefore, it is easy to conceptualize that the characterization of the tumor-associated fibroblasts found in each species will correlate with the characteristics of the normal stroma. In contrast, the predominant myofibroblasts may be derived from CAFs or from another stem/progenitor cell types. Therefore, the lack of myofibroblasts in murine prostate cancer may not be due to the starting material, but a difference in progression and/or development. Despite this difference, these murine models clearly model CAFs, infiltration of immune cells, and upregulation of key signaling pathways, like those initiated by TGF- β , FGFs, and WNTs, which are all present in human cancers.

In conclusion, we have demonstrated the ability of canonical WNT signaling to accelerate FGFR1-driven carcinogenesis, by promoting a fibroblastic reactive stroma, characterized by increased TGF- β heterogeneity. As reactive stroma has now been shown to be predictive of prostate cancer-specific death (47), this work provides a powerful new model to test and validate the importance of reactive stroma in human prostate cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Progression of tumor phenotypic hallmarks over time in five mouse models

(A) Graphical representation of tumor progression kinetics. (See Table 2 for N-value of mice per group exhibiting the reported phenotype)

(B) Ventral lobe H&E. Bar = 100 μ m. Column 1: Control (30 weeks). Transgenic lines have minor background hyperplasia that is consistent throughout observed timepoints. Column 2: 12 weeks CID-treated. Ubi-Cat animals exhibit a thickened stroma, whereas Pro-Cat animals have minor hyperplasia and slightly thickened stroma. JOCK1, Ubi-Cat × JOCK1 and Pro-Cat × JOCK1 animals have marked hyperplasia and focal areas of mPIN. Columns 3: 24 weeks CID-treated. Pro-Cat animals have areas of hyperplasia while Ubi-Cat animals have abnormal proliferation and thickened stroma. Ubi-Cat × JOCK1 and Pro-Cat × JOCK1

animals have marked hyperplasia, high-grade mPIN and adenocarcinoma; significantly faster than JOCK1 which did not obtain this level of progression until 40 weeks of treatment. Column 4: 40 weeks CID-treated. JOCK1 and Pro-Cat $\times$ JOCK1 have adenocarcinoma while Ubi-Cat $\times$ JOCK1 mice have advanced adenocarcinoma with sarcomatoid carcinomas. Column 5: Ventral prostate at 40w CID. Bar = 500 μ m. (C) Weighted Pathological Score for each JOCK1, Pro-Cat $\times$ JOCK1 and Ubi-Cat $\times$ JOCK1 time point $\pm$ CID. P-values determine by two-way ANOVA. Post-hoc power analysis (α = 0.05, Directional) for CID vs. No CID and 24w CID Double transgenic vs. JOCK1 was greater than 0.995; 40w CID Pro-Cat $\times$ JOCK1 vs. JOCK1 and Ubi-Cat $\times$ JOCK1 vs. JOCK1 was 0.70 and 0.99, respectively.

Figure 2. Reconstituted prostates develop hyperplasia, mPIN, and adenocarcinoma

(A) H&E analysis of basal progenitor cells (LSC) mixed with UGSM prostate grafts.

Activation of CID-inducible transgenes for 8 weeks induced hyperplastic acini and mPIN lesion. Bar = 100 μm (N = 4).

(B) Activation of JOCK1 grafts for 30 weeks resulted in adenocarcinoma, 10 weeks earlier than autochthonous JOCK1 tumors (N = 12).

Figure 3. FGFR1 and WNT induce a fibroblastic reactive stroma

(A) Adenocarcinoma lesions of JOCK1 40 weeks CID; Pro-Cat $\times$ JOCK1 and Ubi-Cat $\times$ JOCK1 24 weeks CID. Bar = 200 μ m. N = 3. (Row 1) Trichrome. (Row 2) Smooth Muscle α -actin (SMA; muscle and myofibroblast marker). (Row 3) Vimentin (fibroblast marker). (Row 4) Tenascin C (reactive stroma marker). (Row 5) Ki-67 (proliferation marker); percent Ki-67⁺ stromal cells in bottom left-hand corner. The induced reactive stroma is collagen and vimentin-rich with focal areas of tenascin C staining accompanied by a loss of smooth muscle, hallmarks of a fibroblastic reactive stroma.

(B) Inset H&E representative micrographs of reactive stroma. Bar = 200 μ m. Inset bar = 10 μ m. Each lobe was scored for the presence and penetrance of reactive stroma on a scale of

0–3 (0 none; 1 ~ 25%; 2 ~ 50%; 3 ~ 75%), mean score for each group is plotted $\pm$ SEM. Reactive stroma is a CID-dependent stromal disorganization, characterized by fibroblasts, neovasculature and inflammation. Penetrance of reactive stroma significantly increases over time in both bigenic mice, with the Ubi-Cat $\times$ JOCK1 increase being more pronounced. Significance determined by Two-way ANOVA. (See Table 2 for N-value in each group; denominator).

Figure 4. WNT activation in the stroma alone is sufficient to induce fibroblastic reactive stroma
 (A) H&E analysis of WT or JOCK1 basal progenitor cells (LSC) mixed adult Ubi-Cat stromal cells grafts. Activation of stromal WNT for 16 weeks increased acini formation and a thickened fibroblastic reactive stroma, compared to no CID. Activation of FGFR1 in the epithelium and WNT in the stroma also increased acini number, size and hyperplasia with a fibroblastic reactive stroma. Bar = 100 μ m
 (B) Average acini area for each transplant type. N = 3. Significance determined by t-test.

Figure 5. FGFR1 and WNT promote TGF-β signaling

(A) Selected genes (centered on centroid mean of groups) from epithelium/mPIN and reactive stroma LCM. (a) and (d) JOCK1 CID vs. No CID; (b) and (e) Pro-Cat × JOCK CID vs. JOCK1 CID; (c) and (g) Ubi-Cat × JOCK1 CID vs. JOCK1 CID. The expression patterns of the promoters correlate with the similar patterns between Pro-Cat × JOCK1 and Ubi-Cat × JOCK1 epithelium and the less similar patterns between Pro-Cat × JOCK1 and Ubi-Cat × JOCK1 stroma. There is a marked downregulation of TGF-β receptors and an upregulation of TGF-β ligands in both the epithelium and stroma of Pro-Cat × JOCK1 and Ubi-Cat × JOCK1 mice.

(B) qRT-PCR validation for TGF-β ligands and receptors.

(C) pSmad2 IHC. Bar = 20 μ m. (a) Normal murine epithelium; (b) mPIN; (c) Normal human epithelium; (d) PIN; (e) Normal murine stroma; (f) Mouse reactive stroma, negative nuclei (black arrow) immediately adjacent to positive nuclei (white arrow head); (g) Normal human stroma; (h) Human reactive stroma.

(D) Mean percent pSmad2⁺ stromal nuclei adjacent to tumor epithelium (N = 20 for each murine group, N > 350 for each human; plotted + StDev). No significant difference between JOCK1 and Pro-Cat $\times$ JOCK1. Significant increase in Ubi-Cat $\times$ JOCK1 in pSmad2⁺ stromal cells. Correlative pattern in higher (7 vs. <7) Gleason Score patient tumors. Two-way ANOVA *p < 0.05; ** p < 0.01

Table 1**Similarities and Differences between Mouse and Human Prostatic Stroma**

Similarities	Differences
<ul style="list-style-type: none"> Both stain strongly for fibroblastic markers vimentin, collagen and tenascin c Both reactive stroma exhibit immune infiltration, nerve innervation and neovasculature Both have similar key signaling molecules <ul style="list-style-type: none"> ○ FGF ○ WNT ○ EGF ○ TGF-β Both exhibit a partial loss of TGF-βRII but an overall increase in TGF-β signaling as a driving force behind neoplasia and tumorigenesis 	<ul style="list-style-type: none"> Humans have dense normal stroma, while Mice have sparse stroma Human reactive stroma is predominately myofibroblastic (stain strongly for SMA), Mice predominately fibroblastic

List of similarities and differences between human and murine prostatic stroma under normal and reactive conditions. Information provided is collected from this work and the current understanding of the literature.

Table 2
Mice Per Group Exhibiting Reported Histological Phenotype at 50% Tissue Penetration

	JOCK1	Pro-Cat	Pro-Cat × JOCK1	Ubi-Cat	Ubi-Cat × JOCK1
12 weeks	5/5 *	3/3	3/3 *	3/3	6/7 *
24 weeks	5/5 *	7/7	5/6 *	3/3	3/4 *
40 weeks	4/9 *	4/4	4/6 *	3/3	3/3 *
60 weeks	9/14 *	6/6	4/6 *	2/6	NA

Data represent number of mice exhibiting reported phenotype (i.e. adenocarcinoma) with at least 50% (a score of 2) tissue penetration out of the number of mice in each group (treatment time with CID in each genotype).

(*) CID vs. No CID post-hoc power analysis greater than 0.995 ($\alpha = 0.05$, Directional); Pro- Cat and Ubi-Cat mice were not significantly different than the null.