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TITLE: Defining a Role for the Oncogene Beta-Catenin in Prostate Epithelial Growth and Invasion

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  We are investigating the molecular basis of prostate development in order to understand the pathways which control prostate epithelial differentiation, proliferation, and invasion. In the last year, we were able to develop and validate a new system for interrogation of gene function in prostate development. Using this model, we demonstrated an absolute requirement for canonical Wnt signaling in prostate lineage specification and bud formation.					
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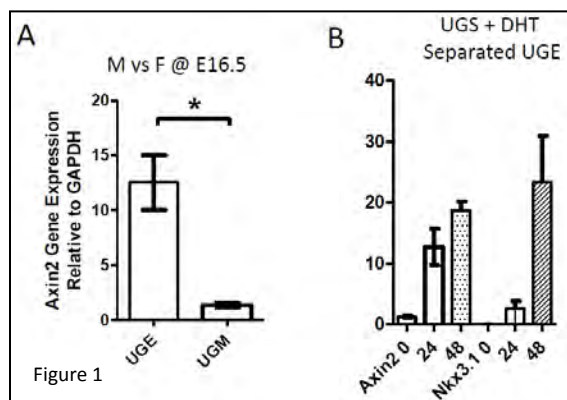
## Introduction

Developmental pathways which control proliferation, differentiation, and invasion in embryogenesis are frequently found to be directing similar programs, although in an unregulated fashion, during carcinogenesis. Wnt signaling is required for branching morphogenesis in a variety of organ systems, and our previous publications have suggested a role for Wnt in prostate development. Understanding the normal function of Wnt signaling in early prostate development may shed light on its function in cancer. Through the work funded by this grant, we have determined that canonical Wnt signaling is required for the earliest stages of prostate lineage commitment and prostate bud induction.

## Body

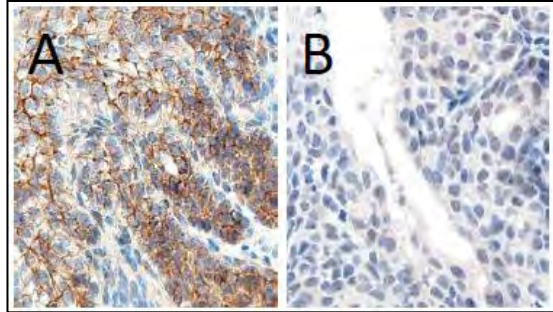
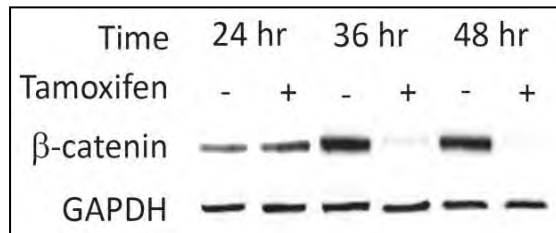
Our work began with two major challenges. First, although not included in our original Statement of Work, we sought to determine the timing and location of androgen dependent Wnt signaling in the developing UGS. Then we sought to refine and validate our system for conditional gene knockout in the developing prostate. We determined the best approach would be to use a drug-inducible Cre driver (ERT-Cre, tamoxifen-inducible Cre) to control gene knockout. These mice were obtained from Jackson Labs and crossed to homozygous floxed-Ctnnb1 (beta-catenin) mice and homozygous floxed-APC mice to generate homozygous ERT-Cre x Ctnnb1<sup>fl/fl</sup> mice and homozygous ERT-Cre x APC<sup>fl/fl</sup>. These colonies were expanded to generate several breeding pairs. This completed Stage 1 from the approved Statement of Work.

**Canonical Wnt signaling is present in the UGE and precedes prostatic differentiation.** In order to localize canonical Wnt signaling in the UGS, we compared *Axin2* expression, an indicator of canonical Wnt signaling, between the mesenchymal and epithelial compartments of the UGS at E16.5 by



quantitative real-time PCR. At this time point, the male UGS has been exposed to androgens, but prostate buds have not formed. There was a significant increase in *Axin2* expression only in the epithelium when comparing male UGSs to female UGSs (Fig1A). Furthermore, when androgen naïve UGSs are cultured in vitro in the presence of androgen, *Axin2* expression is induced in the UGE at 24 hours, which precedes significant prostatic differentiation, as evidenced by *Nkx3.1* expression at 48 hours (Fig1B).

**Our model is capable of rapid and efficient knockout of beta-catenin.** When the breeding colony was established, the mice were mated in 12-hour overnight matings to produce timed pregnant embryos for analysis. We first sought to validate our method by determining the time course and efficiency of gene knockout using this inducible system. By harvesting the embryonic urogenital sinus (UGS), from ERT-Cre x Ctnnb1 flox/flox embryos and treating these tissues in vitro with tamoxifen, we were able to determine that beta-catenin expression is greatly reduced by 36 hours after treatment and essentially complete by 48 hours (Fig 2). This confirmed that our system was capable of rapidly and completely



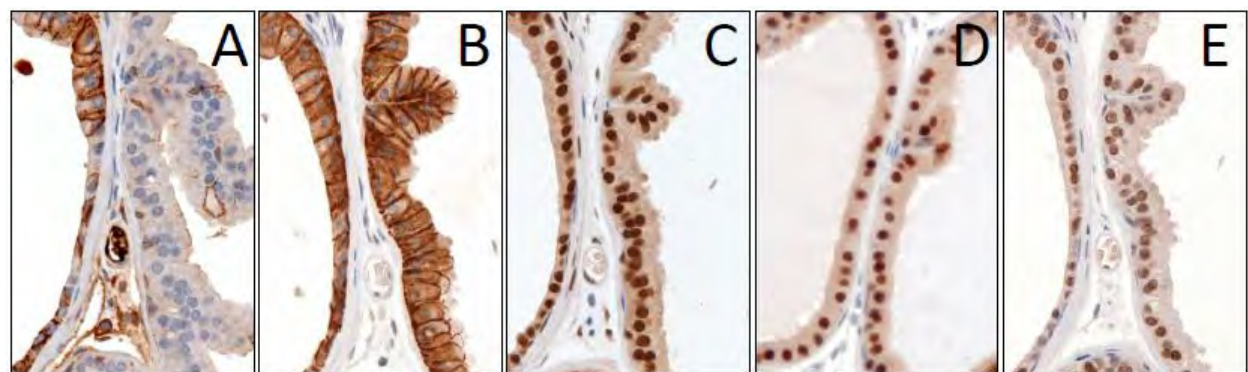
**Figure 2:** Western blot analysis of E16.5 day UGS treated with 2  $\mu$ M Tamoxifen or vehicle and harvested at the indicated time points. Beta-catenin IHC of control (A) and treated (B) UGSs.

deleting a target gene, which would allow us to determine the temporal requirement for beta-catenin in prostate development. Having generated the required breeder pairs and validated our system, we were able to collect the tissues outlined in the Statement of Work Stage 2.

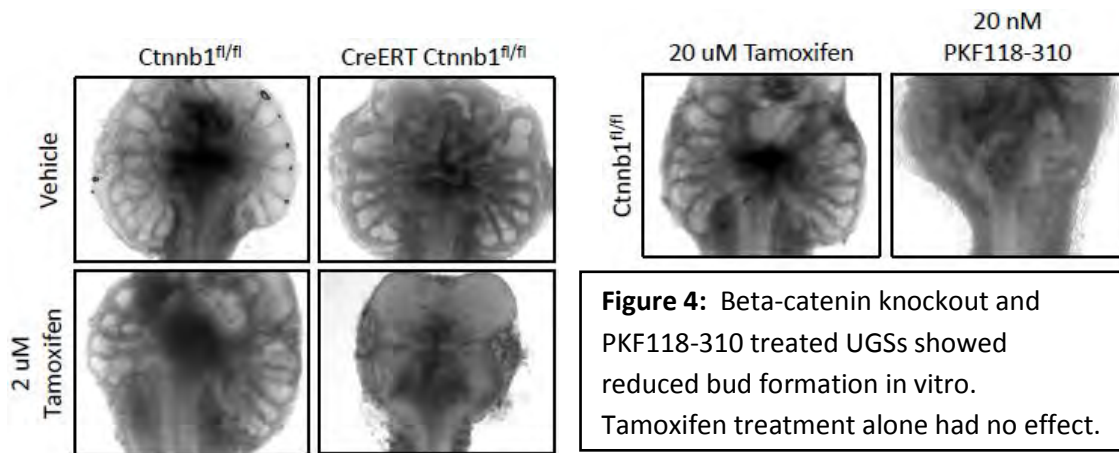
#### **Loss of beta-catenin does not affect differentiation or viability of mature luminal prostate epithelial cells.**

Because beta-catenin has many functions in the cells, we needed to determine if deletion of beta-catenin we sought to exclude the possibility that Wnt/beta-catenin signaling was essential for survival of committed prostate epithelial cells, or that loss of beta-catenin would interfere with E-cadherin localization and cell-cell adhesion. To accomplish this, we generated Probasin-Cre *Ctnnb1<sup>fl/fl</sup>* mice and examined their prostates. Probasin driven Cre expression is initiated well after the prostate morphogenesis, so this should have no effect on prostate differentiation. Because Probasin-Cre shows

mosaic expression in the prostate, we were able to compare beta-catenin deleted and replete cells in the same gland. Both cell types showed normal histology and expression of E-cadherin, AR, Nkx3.1 and Hoxb13 (Fig. 3 A-E). These data confirm that non-Wnt signaling roles for beta-catenin were not responsible for the failure of beta-catenin null UGSs to develop.

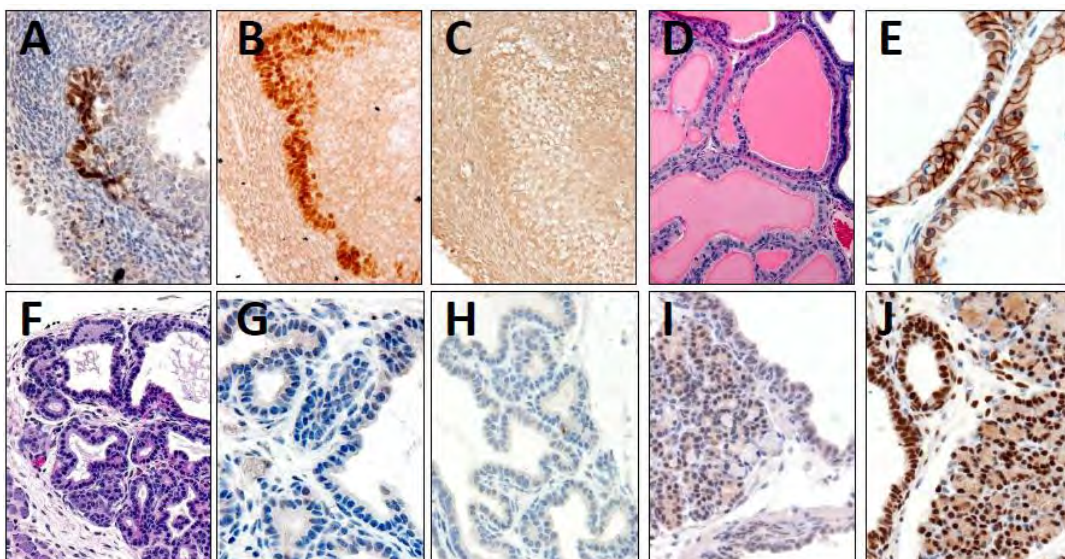


**Figure 3:** Probasin-Cre x *Ctnnb1<sup>fl/fl</sup>* adult prostate showing mosaic loss of beta-catenin (A). Consecutive sections show normal E-cadherin (B), AR (C), Nkx3.1 (D), and Hoxb13 (E) expression in cells with and without beta-catenin.



**Wnt disruption prevents prostate bud formation.** After harvesting E15.5 embryos, we compared the effects of genetic knockout using our inducible system to small molecule inhibition of Wnt signaling in wildtype embryos. Genetic knockout resulted in near complete inhibition of prostate bud outgrowth (Fig 4). Treatment of wildtype UGSs with the small molecule inhibitor PKF118-310 resulted in nearly identical inhibition of bud outgrowth (Fig 4). Tamoxifen treatment of wildtype embryos showed no inhibition of bud growth. The slightly higher rate of bud growth in drug treated UGSs is presumed to be due to incomplete inhibition of the Wnt pathway. Activation of Wnt pathway by knocking out APC had no effect on prostate bud initiation and elongation. This completed SOW Stage 3.1 and 3.2.

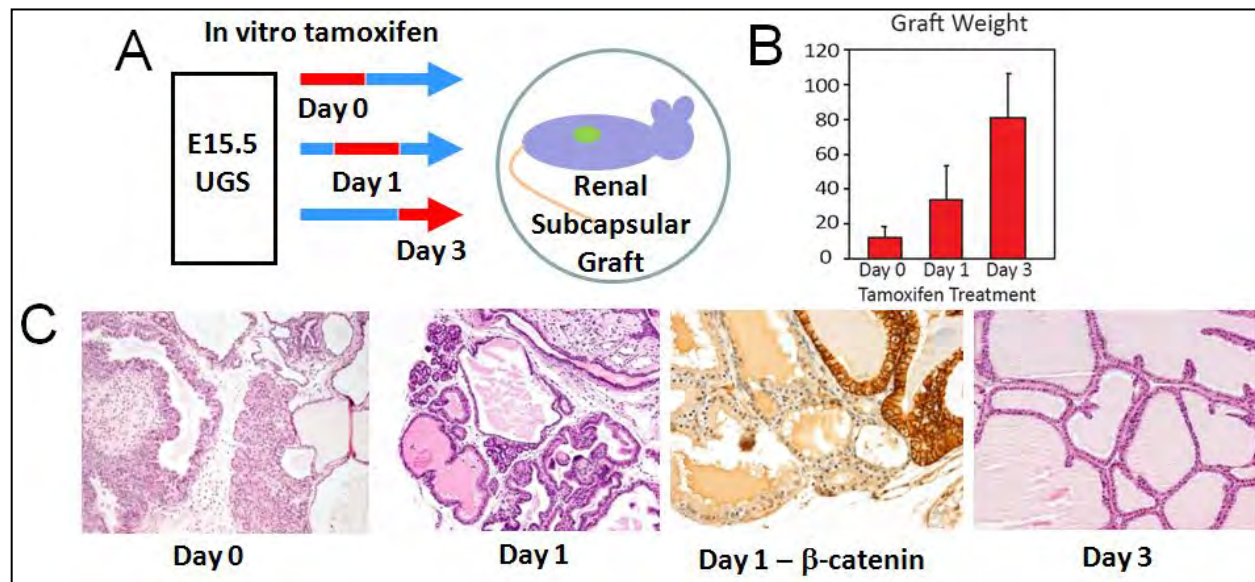
**Beta-catenin null UGS fails to adopt prostatic identity in organ culture and in vivo.** Nkx3.1 is a transcription factor that robustly marks prostatic epithelium and is the earliest known marker of prostatic differentiation. In vivo, Nkx3.1 expression can be seen in the urogenital sinus epithelium at the sites of bud formation. In beta-catenin null UGSs, buds fail to appear and Nkx3.1 is not expressed despite androgen exposure in culture (Fig 5C). This suggests a complete failure of prostatic differentiation. We can conclude from this that canonical Wnt signaling is required for the initial stages of prostate lineage specification in the UGS.





**Figure 5 (previous page):** Nkx3.1 expression in early prostate buds of (A) E17.5 wildtype UGS, (B) *In vitro* organ culture, and (C) beta-catenin deleted UGSs. Subcapsular renal grafts after *in vitro* culture with vehicle (D, E) show normal prostate histology (D) and b-catenin expression (E), but Tamoxifen treated UGSs fail to form prostate tissue (F), express beta-catenin (G) or Nkx3.1 (H). Hoxb13 expression is limited to small foci of cells with periurethral gland differentiation (I). All grafted tissue retains AR expression (J).

**Wnt signaling is only required for a brief window during prostate lineage specification.** To determine how long canonical Wnt signaling is required for prostate development, we collected UGSs at E15.5 and allowed them to develop for 0, 24, or 72 hours before deleting beta-catenin by treating them with tamoxifen for 24 hours, as outlined in our Statement of Work Stage 3.3. The prostates were then grafted under the kidney capsule of a host mouse and allowed to mature before harvesting them for analysis (Fig 6). UGSs treated with tamoxifen at the onset of organ culture (Day 0) showed no identifiable prostate tissue by histology or immunohistochemistry for Nkx3.1 after 6 weeks of growth (Fig 5H). Delaying beta-catenin knockout for one day resulted in grafts that were triple the weight of day 0 and which contained a small amount of prostate tissue. Prostate glands developed which expressed no beta-catenin, suggesting that canonical Wnt signaling is only required for the first 24-48 hours after androgen exposure. After this critical stage of lineage commitment and bud initiation, prostate development can continue without Wnt signaling. UGSs treated with tamoxifen after 3 days of androgen exposure developed normally, despite the absence of beta-catenin (Fig 6).



**Figure 6:** A graphic representing the time course deletion experiment (A). Graft weights of 5 UGSs treated with tamoxifen 0, 1, or 3 days after androgen treatment and grown for 6 weeks under the renal capsule (B). H&E stains and beta-catenin immunohistochemistry of Day 0, 1, and 3 grafts (C).

**Wnt signaling and androgen receptor function.** To determine if increased or decreased Wnt signaling can affect androgen receptor function, we harvested E15.5 UGSs from Esr1-Cre x beta-catenin<sup>fl/fl</sup> and Esr1-Cre APC<sup>fl/fl</sup> embryos and treated them with 2 uM tamoxifen and zero, low, normal, or high androgen to determine if high androgen could compensate for beta-catenin knockout, and if upregulated Wnt signaling (APC knockout) would allow bud formation with low or no androgen. This experiment was outlined in the Statement of Work Stage 3.4. We saw no differences between these groups and the control (no tamoxifen) groups with respect to bud number and length.

### **Key Research Accomplishments**

- Development and validation of a novel system to interrogate gene function in prostate development
- Demonstration that Wnt signaling is required for prostate lineage specification and prostate budding
- Identification of a 24-48 hour period of initial prostate lineage specification where canonical Wnt signaling is absolutely required, after which Wnt is dispensable

### **Key Training Accomplishments**

Courses Taken:

- New Approaches to Cancer Prevention and Therapy
- Developmental Biology
- Great Experiments in Biology
- Mouse Phenotyping
- Introduction to Effective Instruction
- Designing Experiments and Writing a Research Paper
- Cancer Biology: From Cause to Cure

Journal Clubs:

- Brady Urology Journal Club, weekly attendance and two presentations
- Pathobiology Journal Club, weekly attendance
- Cancer Biology Seminar Series, weekly attendance

Teaching Experience:

- Lecturer, Toxicologic Pathology, Johns Hopkins School of Public Health 2010 & 2011
- Lecturer, Comparative Pathobiology, Johns Hopkins University School of Medicine 2010 & 2011
- Faculty, Workshop on Phenotyping Mouse Models of Lung Disease, The Jackson Laboratory, September 2009, 2010, & 2011



- Lab instructor, Pathology for Graduate Students, Johns Hopkins University School of Medicine, 2009 & 2010

#### Mentoring:

- Oversight of laboratory rotations for one medical student, two undergraduate student, and two veterinary student

#### **Reportable Outcomes**

##### Abstracts/Posters/Presentations:

- 06/07/2010 Grand Rounds, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland
- 04/10/2010 Multi-Institutional Prostate Cancer Meeting, Fort Lauderdale, Florida
- 04/08/2010 12<sup>th</sup> Annual Pathology Young Investigator's Day Poster Session, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland
  - Award: 1<sup>st</sup> place, basic research
- 02/06/2010 5<sup>th</sup> Annual Prostate Research Day, Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland
  - Award: John L. Willey First Place Poster Award
- 10/30/2010 American College of Veterinary Pathologists Annual Meeting, Baltimore, Maryland
  - Award: Young Investigator Award, Experimental Disease
- 11/14/2010 Society of Basic Urologic Research Annual Meeting, Atlanta, Georgia
  - Travel award and podium presentation
- 03/09/2011 PCRP IMPaCT Conference, Orlando, Florida

#### **Conclusion**

During the award period, we have been able to complete the research and training goals as outlined for this period in our approved Statement of Work. In doing so, we have developed a novel system for determining gene function in the developing prostate, and have identified a critical role for canonical Wnt signaling and the oncogene beta-catenin in prostate epithelial identity specification and bud induction. We have presented in poster form at several meetings, and have prepared a manuscript which we expect to submit for publication this month.