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Beta-catenin and estrogen signaling collaborate to drive cyclin D1 expression in developing mouse prostate

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ABSTRACT

Androgen, beta-catenin (CTNNB1), and estrogen pathways stimulate proliferative growth of developing mouse prostate but how these pathways interact is not fully understood. We previously found that androgens induce CTNNB1 signaling in mouse urogenital sinus (UGS) epithelium from which prostatic ductal epithelium derives. Others have shown that low estradiol concentrations induce UGS epithelial proliferative growth. Here, we found that CTNNB1 signaling overlaps cyclin D1 (CCND1) expression in prostatic buds and we used a genetic approach to test whether CTNNB1 signaling induces CCND1 expression. We observed an unexpected sexually dimorphic response to hyperactive CCNTB1 signaling: in male mouse UGS it increased *Ccnd1* mRNA abundance without increasing its protein abundance but in female UGS it increased *Ccnd1* mRNA and protein abundance, suggesting a potential role for estrogens in stabilizing CCND1 protein. Treating wild type male UGS explants with androgen and either 17β-estradiol or a proteasome inhibitor increased CCND1 protein and KI67 labeling in prostatic bud epithelium. Together, our results are consistent with an epithelial proliferative growth mechanism linking CTNNB1-driven *Ccnd1* transcription and estrogen-mediated CCND1 protein stabilization.

1. Introduction

Coordinated cell proliferation guides formation of normal tissue structure in the fetus and maintains tissue homeostasis in the adult. In endocrine-responsive tissues such as the prostate, hormones control cell proliferation across nearly all stages of life. Understanding how hormones interact with each other as well as cell cycle factors is paramount to understanding endocrine organ development and homeostasis.

This study examines the interaction between hormones and proliferative growth signaling pathways in developing mouse prostate. Mammalian prostate derives from the urogenital sinus (UGS) a transient fetal structure positioned between the bladder and urethra. The mouse UGS is an intriguing organ from which to investigate interactions between sex hormones and cell proliferation because both male and female UGS respond to androgens and estrogens. Androgen receptors are detectable in the UGS of both sexes and when activated, drive mouse prostate development in both sexes (Takeda and Chang, 1991; Takeda et al., 1986). Estrogen receptors are also expressed by male and female UGS (Omoto et al., 2005). While neither 17β-estradiol nor estrogen receptor α and β are required for normal prostatic bud formation, their activation can augment androgen-induced prostate proliferative growth (Allgeier et al., 2008; Bianco et al., 2006; McPherson et al., 2001; Prins et al., 2001; Timms et al., 2005; vom Saal et al., 1997; Welshons et al., 2003). Pinpointing downstream targets of androgen and estrogen signaling pathways will help to elucidate how these pathways interact to drive prostate proliferative growth.

To elucidate mechanisms of androgen signaling in developing prostate, we previously compared expression of hundreds of mRNAs across male and female mouse UGS (Abler et al., 2011a; Georgas et al., 2015) and gudmap.org. We found that CTNNB1-responsive transcripts are detected in male but not female UGS epithelium and that functional androgen receptors are required for CTNNB1 target gene expression (Mehta et al., 2011). Subsequent studies offered evidence of a CTNNB1 requirement in specifying UGS epithelium into prostate epithelium and for prostatic bud formation (Francis et al., 2013; Mehta et al., 2013; Simons et al., 2012). Though these studies link androgens to CTNNB1

Abbreviations: CCND1, (cyclin D1); CTNNB1, (beta-catenin); UGS, (urogenital sinus

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signaling and prostate development, how CTNNB1 guides proliferative growth processes in the UGS and prostate has yet to be elucidated.

Cyclin D1 (CCND1) interacts with cyclin-dependent kinases and the retinoblastoma protein to promote G1 to S phase progression of the cell cycle (Baldin et al., 1993) and has been identified as a direct transcriptional target of CTNNB1 signaling in multiple organs (Kikuchi, 2000; Lin et al., 2000; Tetsu and McCormick, 1999). CTNNB1 signaling stimulates CCND1 and cellular proliferation in the stomach (Soutto et al., 2015), uterus, breast (Lin et al., 2000; Liu et al., 2014), and colon (Tetsu and McCormick, 1999). Whether CTNNB1 activates CCND1 and proliferative growth in prostatic development has not been examined.

This study's objective was to spatially map CCND1 in the developing mouse prostate and examine its regulation by androgen, estrogen, and CTNNB1 signaling. CCND1 was noticeably more abundant in male compared to female UGS and within males, was regionally abundant in prostatic bud tips where proliferating cells are concentrated and CTNNB1 signaling is enriched. Yet, while genetic activation of CTNNB1 signaling induced *Ccnd1* mRNA expression in male and female UGS, it only increased CCND1 protein in females. Using UGS explant cultures, we determined that a low (100 pM) concentration of 17β-estradiol combined with androgen increased CCND1 protein expression more than androgen alone. We also found that combining androgen with proteasome inhibition, a known function of estrogen signaling (Zhou et al., 2014), mimicked the actions of estrogen by increasing the number of CCND1 protein-positive prostatic bud epithelial cells. Together, our results are consistent with a mechanism by which androgens activate CTNNB1 signaling in UGS epithelium to stimulate *Ccnd1* transcription and estrogens stabilize CCND1 protein levels to augment proliferative growth. This mechanism may underlie previous observations of estrogen-mediated increases in prostatic growth and development.

2. Materials and methods

2.1. Animals

C57BL/6 J mice (Stock #000664) and $Shh^{tm2(cre/ERT2)Cjt}/J$ mice (Stock #005623) (Harfe et al., 2004) were purchased from Jackson Laboratory (Bar Harbor, ME). $Ctnnb1^{tm1Mmt/tm1Mmt}$ mice (Harada et al., 1999) were from Makoto Mark Taketo (Kyoto University, Japan). Mice were housed in polysulfone cages containing corn cob bedding and maintained on a 12 h light and dark cycle at 25 ± 5 °C and 20–50% relative humidity. Feed (Diet 2019 for males and Diet 7002 for pregnant females, Harlan Teklad, Madison, WI, USA) and water were available ad libitum. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. To obtain timed-pregnant dams, females were paired overnight with males and the next morning was considered 0 days post coitus (dpc). Mice harboring the dominant stable *Ctnnb1* gain-of-function (iGOF) allele in UGS epithelium ($Shh^{creERT2/+}$; $Ctnnb1^{tm1Mmt/tm1Mmt}$) and genotypic control mice ($Shh^{+/+}$; $Ctnnb1^{tm1Mmt/tm1Mmt}$) were generated as described previously (Mehta et al., 2013). Dams were euthanized by CO_2 asphyxiation for embryo collection.

2.2. In situ hybridization (ISH)

UGSs were fixed overnight in 4% paraformaldehyde (PFA), dehydrated into methanol and stored at −20 °C. On the day of sectioning, UGSs were rehydrated into phosphate buffered saline and cut with a vibrating microtome into 50 μm (sagittal) sections as described previously (Abler et al., 2011a, 2011b; Keil et al., 2012a, 2012b). Protocols for polymerase chain reaction-based riboprobe synthesis and ISH staining are at www.gudmap.org. The staining pattern for each hybridized riboprobe was assessed in at least two UGS sections / mouse fetus and at least three litter-independent fetuses. Replicate male and female tissue sections were processed as a single experimental unit to allow for qualitative comparisons among replicates and between males and females. Riboprobes for *Axin2* and *Wif1* were described previously (Keil et al., 2012b; Mehta et al., 2011). The 551 base pair *Ccnd1* (National Center for Biotechnology Information [NCBI] GeneID: 12443) riboprobe corresponds to positions 2813 to 3363 of the NCBI reference sequence NM_007631.2. The primers used for polymerase chain reaction amplification were 5′-TGG GAC CAC ATG GGA CAG-3′ and 5′-CGA TGT TAA TAC GAC TCA CTA TAG GGA CCG GAG ACT CAG AGC AAA TC-3′. A synthetic T7 RNA polymerase recognition sequence was incorporated into the reverse primer (underlined).

2.3. Immunohistochemistry (IHC)

Immunofluorescent staining of paraffin sections was performed as described previously (Abler et al., 2011a; Mehta et al., 2011). Antibodies include: rabbit anti-CCND1 (Abcam #ab16663 Cambridge MA), mouse anti-CTNNB1 (BD Transduction labs # 610153, San Jose, CA), rabbit anti-KI67 (Abcam #ab15580), Alexafluor 488-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch #115-547-003, West Grove, PA), Alexafluor 594-conjugated goat anti-mouse IgG (Jackson ImmunoResearch #111-516-045). Tissue sections were also stained with 4′,6-diamidino-2-phenylindole, dilactate (DAPI) to label cell nuclei.

2.4. Organ culture

14 dpc wild-type male and female UGSs were grown for 4 days on 0.4-μm Millicell-CM filters (Millipore, Billerica, MA) as described previously (Vezina et al., 2008). Media were supplemented with 5α-dihydrotestosterone (DHT; 10 nM, Sigma, St. Louis MO), 17-β estradiol (10 nM or 100 pM, Sigma Aldrich), and the proteasome inhibitor Z-Leu-Leu-Leu-al (10 μM, Sigma). Media and supplements were changed every 2 d.

2.5. Statistical analyses

The percentage of CCND1 and KI67 positive epithelial cells was determined and averaged across 3–5 representative 200X magnified fields from at least 3 mice per group. $CTNNB1^{iGOF}$ cell islands were identified by the presence of detectable cytosolic and nuclear CTNNB1 staining. Statistical analysis was conducted using R version 2.13.1. Student's *t*-test and analysis of variance (ANOVA) were conducted on untransformed data that passed Bartlett's test for homogeneity of variance and appeared to be normally distributed. Tukey's Honest Significant Difference (HSD) test was used for post-hoc analysis, and p values of less than 0.05 were considered significant. All results are reported as mean ± SE, $n \geq 3$ litter independent mice per group.

3. Results and discussion

3.1. CTNNB1-responsive mRNA expression in prostatic bud tips overlaps CCND1 and KI67 protein expression

Our first objective was to visualize and spatially map CCND1 protein distribution in 18 dpc wild type male and female mouse UGS. In male UGS, CCND1 immunolabled cells were concentrated in prostatic bud tips and were rare in non-budding epithelium and mesenchyme (Fig. 1A-B). By contrast, CCND1 immunolabeled cells were rare across the entire female UGS (Fig. 1A-B), indicating androgens potentially support CCND1 expression in male UGSs. CCND1 drives the G1 to S cell cycle transition and we observed many CCND1 and KI67 co-labeled male UGS epithelial cells (Fig. 1C).