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Visualization and quantification of mouse prostate development by *in situ* hybridization

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Abstract

The purpose of this study was to validate a combined *in situ* hybridization (ISH)/immunohistochemistry (IHC) staining method for visualizing and quantifying mouse prostatic buds. To refine animal usage in prostate development studies, we also determined whether a comparable number of prostatic buds were formed in male and female mouse urogenital sinus (UGS) explants grown *in vitro* in the presence of androgen. We used IHC to label UGS epithelium and ISH to label prostatic buds with one of three different prostatic bud marking riboprobes: a previously identified prostatic bud marker, NK-3 transcription factor, locus 1 (*Nkx3-1*), and two newly identified prostatic bud markers, wingless-related MMTV integration site 10b (*Wnt10b*) and ectodysplasin-A receptor (*Edar*). We calculated total buds formed per UGS and the proportion marked by each mRNA after male UGS development *in vivo* and male and female UGS development *in vitro*. *Nkx3-1* was first to mark the prostate field during UGS development *in vivo* but all three mRNAs marked prostatic buds during later developmental stages. The mRNAs localized to different domains: *Nkx3-1* was present along about half the prostatic bud length while *Edar* and *Wnt10b* were restricted to distal bud tips. None of the mRNAs marked all buds formed *in vitro* and the proportion marked was developmental stage- and gender-dependent. *Nkx3-1* marked the highest proportion of prostatic buds during *in vitro* UGS development. Together, our results reveal that ISH staining of mouse UGS can be used to quantify prostatic bud number, *Nkx3-1* is currently the best suited riboprobe for this method, and female UGSs cannot be used interchangeably with male UGSs when conducting prostate development studies *in vitro*. We also found that *Nkx3-1*, *Edar*, and *Wnt10b* mark different prostatic bud regions and are likely to be useful in future studies of regional differences in prostatic bud gene expression.

Keywords

Prostate; UGS; Organ culture; LUT; Explant

1. Introduction

Mouse prostate development begins *in utero* from a transitory developmental sub-compartment of the lower urogenital tract known as the definitive urogenital sinus (UGS). In this manuscript the UGS is defined as the prostatic bud forming region of the male pelvic urethra and the equivalent region of the female pelvic urethra. In most placental mammals, prostate development begins shortly after initiation of fetal testicular androgen synthesis. Androgen receptor (AR) activation in UGS stroma induces prostatic bud formation in UGS

epithelium. In C57BL/6J male mice, prostatic bud initiation begins around embryonic day (E) 16.5 and is completed by about E18.5 (Lin et al., 2003). During postnatal development prostatic buds continue to elongate, undergo branching morphogenesis, canalize and undergo differentiation to form the mature prostate ductal network (Cunha et al., 1987; Lin et al., 2003; Sugimura et al., 1986).

Prostatic budding and branching morphogenesis can be activated *in vitro* by incubating the isolated male UGS, female UGS, or neonatal mouse prostate in organ culture media containing testosterone or its more potent metabolite, 5 α dihydrotestosterone (DHT) (Cunha, 1972). Androgens induce formation of budlike structures in both male and female UGS explants and given sufficient time, many of these budding structures will form primary and secondary branches in a pattern that mimics their development *in vivo* (Cunha et al., 1987; Price and Williams-Ashman, 1961). The organ culture model of prostate development can be used to: (1) rescue prostate development in mouse mutants predisposed to mortality during prostatic bud formation, (2) evaluate fetal prostate teratogenicity of chemicals that would otherwise disrupt pregnancy or harm the pregnant dam or fetus and (3) maintain consistent androgen levels during prostate development.

We and others have quantified prostate development by visualizing and counting the number of prostatic buds and branches formed *in vivo* and *in vitro* (Allgeier et al., 2010; Buresh et al., 2010; Lin et al., 2003; Timms, 2008). Several methods have been developed for this purpose, including light microscopy (Price, 1963), scanning electron microscopy (Lin et al., 2003), confocal microscopy (Buresh et al., 2010) and three dimensional serial reconstruction of UGS histological sections (Timms et al., 1994; Timms, 2008). All of these methods are capable of visualizing buds arising from the UGS and urethra and all have advantages and disadvantages. A common disadvantage is many of these methods require specialized equipment (scanning electron or confocal microscopy) or specialized computer programs (to conduct serial section reconstruction), which places them out of reach of some research laboratories.

On the other hand, many molecular biology laboratories are outfitted with a hybridization oven and a dissecting microscope and this equipment in conjunction with other basic research equipment is sufficient for conducting *in situ* hybridization (ISH). Prostatic buds can be visualized in the UGS by ISH staining with a riboprobe against a prostate selective mRNA, NK-3 transcription factor, locus 1 (*Nkx3-1*). In fact, this method has already been used to visualize prostatic buds and assess prostate development qualitatively (Bieberich et al., 1996; Chen, 2005; Gao et al., 2005; Ghosh et al., 2011; Prins et al., 2001, 2006; Tanaka et al., 2000; Thomsen et al., 2008; Wang et al., 2008). Our goal herein was to determine whether a comparable method can be used to assess prostate development quantitatively by counting ISH-stained prostatic buds.

Nkx3-1 is considered the earliest mRNA marker of mouse prostate identity and was reported to be present in mouse UGS epithelium beginning at E15.5, prior to prostatic bud initiation (Bhatia-Gaur et al., 1999). *Nkx3-1* appears to mark all prostatic buds. Importantly, however, *Nkx3-1* is not a prostate bud specific marker because it is also detected in closely apposed urethral and bulbourethral gland buds in males (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997) and in epithelial outgrowths of the female mouse UGS which do not typically develop into functional secretory structures (Allgeier et al., 2010). We recently identified wingless related MMTV integration site 10b (*Wnt10b*) (Abler et al., 2011a; Mehta et al., 2011) as a selective prostatic bud marker and in this study, characterize ectodysplasin-A receptor (*Edar*) as another newly identified prostatic bud marker. Neither *Wnt10b* nor *Edar* mark urethral gland buds. Whether they mark the same prostatic bud domains as *Nkx3-1* or the same proportion of buds as *Nkx3-1* had not been determined.

There were two goals of this study. The first was to use ISH to stain UGSs with *Nkx3-1*, *Edar*, or *Wnt10b* riboprobes and determine which riboprobe was best suited for visualizing and quantifying prostatic bud formation from the UGS during *in vivo* and *in vitro* development. Our second goal was to determine whether the number and mRNA expression of these markers was comparable between prostatic buds formed in female and male UGS explants cultured in the presence of androgen. To meet these goals, we compared the expression patterns and proportion of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* riboprobes after *in vivo* and *in vitro* prostate development. We determined that *Nkx3-1*, *Edar* and *Wnt10b* mRNAs mark distinct domains of all epithelial buds of the UGS *in vivo* but mark only a subset of buds formed *in vitro*. Furthermore, the proportion of prostatic buds marked by these mRNAs *in vitro* was developmental stage- and gender-dependent. Our experimental results inform a set of guidelines for using ISH to mark and quantify prostatic buds during mouse prostate development *in vitro* and *in vivo*. They also reveal previously unrecognized molecular differences in epithelial buds formed after *in vitro* versus *in vivo* UGS development

2. Materials and methods

2.1. Animals

C57B/6J mice were purchased from Jackson Labs (Bar Harbor, ME) and housed in polysulfone cages containing corn cob bedding. Mice were 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) 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. Female mice were paired overnight with males to obtain timed-pregnant dams. The morning of copulatory plug identification was considered 0.5 dpc. Dams were euthanized by CO₂ asphyxiation.

2.2. In situ hybridization (ISH)

Detailed protocols are available at www.gudmap.org and were described previously for vibratome-cut UGS sections (Abler et al. 2011b). The following modifications were used in this study to stain whole-mount UGS tissues: 0.5% Triton X-100 was added to all solutions and 1mg/mL collagenase (from *Clostridium histolyticum* #C9891, Sigma-Aldrich, St. Louis MO) was combined with 5 ug/mL proteinase K to enhance tissue permeability. Extensive washing with tris-buffered saline (TBS, 24 h, 25 °C) facilitated removal of un-bound antibody from whole-mount tissue. BM Purple was used as an alkaline phosphatase chromagen for detection of digoxigenin-labeled riboprobes. *Nkx3-1* (NCBI GeneID: 18095) and *Wnt10b* (NCBI GeneID: 22410) riboprobes were generated as described previously (Abler et al., 2011a; Mehta et al., 2011). The 649 base pair *Edar* (NCBI GeneID: 13608) probe corresponds to positions 2852 to 3499 of the NCBI reference sequence (RefSeq) NM_010100.3. The staining pattern for each hybridized riboprobe was assessed in at least three litter-independent fetuses. Male and female tissues were processed as a single experimental unit to allow for qualitative comparisons between sexes.

2.3. UGS organ culture

UGSs were dissected from male and female fetuses, placed on 0.4 µm Millicell-CM filters (Millipore, Bedford, MA) and cultured as described previously (Vezina et al. 2008) in media containing DHT (diluted from an ethanol stock solution to a final media concentration of 10 nM) and 0.1% dimethylsulfoxide (DMSO). UGSs were cultured for 7 days with replacement of media every 2 days.

2.4. Immunohistochemistry (IHC)

Immunofluorescent staining of ISH-stained vibratome-cut sections was conducted as described previously (Abler et al., 2011a). For whole-mount prostate tissues, ISH was conducted first and tissues were fixed at 4 °C for 24 h in 4% PFA. Tissues were washed at 25 °C for 15 min in TBS containing 0.5% Triton X and 0.1% Tween20 (TBSTx) and blocked at 25 °C for 1 h in TBSTx containing 1% Blocking Reagent (Roche Diagnostics, Indianapolis, IN), 5% normal goat sera, 1% bovine serum albumin fraction 5 (RGBTx) and 1% DMSO. Tissues were then incubated at 4 °C for 24 h in rabbit anti-CDH1 (#3195 Cell Signaling Technologies, Beverly, MA), diluted 1:750 in RGBTx. Tissues were washed at 4 °C for 24 h in TBS containing 0.5% Triton X, tissues were then incubated at 4 °C overnight in biotinylated goat anti-rabbit secondary antibody (BA1000 Vector Laboratories, Burlingame, CA) diluted 1:500 in RGBTx. Tissues were washed at 25 °C for 2 h in TBSTx and incubated in 1x avidin biotin complex (ABC) reagent (PK6100, Vectastain Elite ABC Kit, Vector Laboratories) according to manufacturer's instructions. Following incubation in ABC reagent tissues were rinsed at 25 °C for 1 hr with TBSTx and incubated in 3,3'-diaminobenzidine substrate (SK-4100, Vector Laboratories) according to manufacturer's instructions. Some tissues were imaged in whole mount. Other tissues were dehydrated into ethanol, cleared in XS-3 xylene substitute (Statlab, McKinney, TX) to preserve BM Purple and DAB color intensities, infiltrated with paraffin, cut into 5 µm sections, dewaxed in XS-3 and mounted with Permount (Fisher Scientific, Fair Lawn, NJ) prior to imaging.

2.5. Prostatic bud counting

The number of prostatic buds per UGS was computed as the average count from three persons, each of whom was blinded to the experimental conditions. Only buds within the UGS, as defined in the introduction, were counted (buds on the caudal portion of the urethra, which typically form urethral glands and not prostate gland buds, were excluded from analysis). Additionally, only bud stalks were counted, a bud with one or more branches was only counted as one bud. 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* = 3 litter independent samples per group.

3. Results

3.1. *Edar* mRNA selectively marks prostatic buds in developing mouse UGS

We and others previously described *Nkx3-1* and *Wnt10b* mRNA expression patterns in embryonic day (E) 17.5 and postnatal day (P) 0 UGS tissue sections (Abler et al., 2011a; Bhatia-Gaur et al., 1999). In conjunction with a recent ISH screen, we identified *Edar* as a prostatic bud marker. The *Edar* expression pattern has not been reported previously in prostate and our first goal was to visualize and describe its expression pattern in the UGS during prostatic bud initiation at E17.5. ISH was used to visualize *Edar* mRNA in E17.5 male and female mouse UGS sections. Tissue sections were then immunohistochemically stained with antibodies against anti-cadherin1 (CDH1) and smooth muscle actin alpha 2 (ACTA2) so that the *Edar* expression could be assessed and described in the context of other UGS tissue compartments (Fig. 1). *Edar* was detected specifically in prostatic bud tips (Fig. 1A: arrowheads) and therefore was not detected in buds where the distal tip was not present in the tissue section. *Edar* was not detected elsewhere in male UGS epithelium or stroma, or in any female UGS tissue compartment at E17.5 (Fig. 1B). Therefore, *Edar*, *Nkx3-1* and *Wnt10b* all appear to be selective mouse prostatic bud markers in E17.5 mouse UGS.

3.2. *Nkx3-1*, *Edar*, and *Wnt10b* are specific markers for mouse prostatic buds formed *in vivo*

We next assessed which selective prostatic bud marker would be best suited for marking prostate during fetal and neonatal development *in vivo*. The most useful marker would be visible in UGS epithelium during prostatic bud specification (E14.5–15.5, prior to the emergence of visible prostatic buds) and would exhibit selective expression in prostatic bud epithelium during bud initiation, elongation and branching morphogenesis. To determine which mRNA marker best fit these criteria, we compared *Nkx3-1*, *Edar* and *Wnt10b* mRNA expression patterns in whole mount E15.5, 16.5, 17.5 and 18.5 male UGSs and in postnatal day (P) 5 prostates. At E15.5, a stage when no prostatic buds are visible by scanning electron microscopic analysis of male UGS epithelium (Lin et al. 2003), *Nkx3-1*, *Edar* and *Wnt10b* were detected in UGS epithelium (Fig. 2A–C). *Nkx3-1* mRNA was restricted to fields where ventral, anterior and dorsolateral prostate will arise (Fig. 2A; inset) while *Edar* and *Wnt10b* were expressed in a diffuse pattern throughout UGS epithelium at this stage (Fig. 2B and C; inset). At E16.5, *Nkx3-1* and *Wnt10b* were detected in anterior prostatic buds and both were noticeably concentrated in UGS fields where dorsolateral and ventral prostatic buds are known to form about one day later (Fig. 2D and F; inset), *Edar* remained diffuse in UGS epithelium (Fig. 2E; inset). At E17.5 and E18.5, *Nkx3-1*, *Edar* and *Wnt10b* were detected in all anterior, ventral and dorsolateral prostatic buds (Fig. 2G–L).

In order to describe *Nkx3-1*, *Edar* and *Wnt10b* mRNA expression patterns in the context of UGS epithelial microanatomy at E18.5 and P5, ISH stained tissue specimens were co-stained by IHC with an anti-cadherin 1 (CDH1) antibody that marks all UGS epithelium. This co-staining procedure allowed us to determine whether each riboprobe marked all epithelial buds or only a subset and whether each marked the entire length of prostatic buds or only a fractional part. The ISH/IHC co-staining procedure also facilitated improved visualization of urethral gland buds. *Nkx3-1* mRNA was detected in urethral gland buds at E18.5 (Fig. 2J; open arrowheads). Diffuse *Edar* staining was observed in urethral mesenchyme at E18.5, but neither *Edar* nor *Wnt10b* were detected in urethral gland bud epithelium at this stage (Fig. 2K and L). The three mRNAs also exhibited regional expression pattern differences in prostatic buds at E18.5 and in prostatic buds undergoing branching morphogenesis at P5 (Fig. 2J–O). *Nkx3-1* was detected in at least half of the length of the main prostatic buds and in all branched tips (Fig. 2J and M; inset). *Edar* and *Wnt10b* were not detected in main prostatic buds but were instead localized to bud tips distal to primary and secondary branch points (Fig. 2K,L,N,O; inset). *Nkx3-1* was also detected in P5 urethral gland buds (Fig. 2M; open arrowheads) but neither *Edar* nor *Wnt10b* were detected in urethral gland buds at this stage (Fig. 2N and O). Together our results indicate that *Nkx3-1* is the most appropriate mRNA for marking the prostate field at E15.5, prior to bud initiation, and that *Nkx3-1*, *Edar* and *Wnt10b* are equally useful for marking prostatic buds after bud initiation and during branching morphogenesis (E17.5–P5). Our results therefore validate ISH for *Nkx3-1*, *Edar* and *Wnt10b* mRNAs as a tool for (1) distinguishing prostate from urethral gland buds and (2) marking unique domains of the proximodistal prostatic bud axis beginning after E18.5 and extending to at least P5.

3.3. *Nkx3-1*, *Edar* and *Wnt10b* mark the same quantity of prostatic buds formed *in vivo*

One of our overarching goals was to validate ISH as a method for visualizing and quantifying prostate development. To this end, we compared the number of anterior, ventral, dorsolateral and total prostatic buds marked by *Nkx3-1*, *Edar* and *Wnt10b* ISH in the E18.5 male UGS. There were no statistically significant differences among numbers of anterior, ventral, dorsolateral or total prostatic buds detected by each riboprobe (Fig. 3, $n = 3$). Furthermore, we did not identify any CDH1-positive buds in the UGS that were unmarked by *Nkx3-1*, *Edar* or *Wnt10b*. Care must be taken in quantifying *Nkx3-1* marked buds on or

after E17.5 because it marks prostatic and urethral gland buds. However, the distinct anatomical location of urethral gland buds, which are generally caudal to prostatic buds, allows the majority of urethral gland buds to be visually excluded from prostatic bud quantification. Together, these results suggest that all three mRNAs are equally useful for marking and quantifying prostatic buds formed during *in vivo* mouse prostate development.

3.4. *Nkx3-1*, *Edar* and *Wnt10b* mark different quantities of prostatic buds formed *in vitro*

Morphological similarities exist between prostatic buds and branches formed *in vivo* versus those formed *in vitro* (Doles et al., 2005). However, it is not known whether mRNA expression in prostatic buds and branches formed *in vivo* is recapitulated during *in vitro* prostate development. Our next goal was to assess the utility of ISH/IHC staining as a method for quantifying prostate development in cultured UGS explants. We wanted this method to be broadly applicable for *in vitro* studies of prostate development. Since some investigators focus such studies on early stages of prostate ductal development (bud formation) and others on later stages (branching morphogenesis), we assessed *Nkx3-1*, *Edar* and *Wnt10b* mRNA expression in UGS explants initiated at two different stages: E14.5 (before prostatic bud initiation) and E17.5 (after prostatic bud initiation). UGSs were incubated for 7 days in organ culture media containing 5 α -dihydrotestosterone (DHT, 10 nM). UGS explants were stained by ISH to visualize expression patterns of *Nkx3-1*, *Edar* and *Wnt10b* and then by IHC to visualize CDH1 in all bud and non-bud UGS epithelium (Figs. 4 and 5). This staining method allowed us to quantify mRNA marked epithelial buds, total epithelial buds, and the proportion of buds marked by each mRNA as a fraction of the total. Since *Nkx3-1*, *Edar* and *Wnt10b* mark different bud regions, to maintain consistency in quantification, mRNA marked buds with no branches were counted the same as buds with several mRNA marked branches. In other words, only the number of main ducts that exhibited mRNA staining in one or more regions of the proximodistal axis was counted. The anatomically distinct urethral gland buds were excluded from analysis. We determined that this method did not bias our ability to identify prostatic buds based on the fact that the computed total bud number (mRNA marked+mRNA unmarked) did not appreciably differ among age- and gender-matched *Nkx3-1*, *Edar* and *Wnt10b* stained UGS groups (results not shown, $n = 3$ per group).

Our first observation was surprising. Though *Nkx3-1*, *Edar* and *Wnt10b* appear to mark nearly all epithelial buds formed in the UGS during development *in vivo* (Fig. 2), these mRNAs marked only a subset (68% or fewer at E14.5 and 89% or fewer at E17.5) of buds formed *in vitro* (Figs. 4 and 5). Of particular note, *Nkx3-1* was detected in urethral gland buds after *in vivo* development but was either less abundant or not detected in urethral gland buds of males and females after *in vitro* development (Figs. 4 and 5; arrows). This result reveals a potential difference in the molecular composition of prostatic and urethral gland buds formed *in vitro* versus those formed *in vivo*.

The proportion of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* mRNAs was developmental stage- and gender-dependent. In E14.5 male UGS explants, *Nkx3-1* was found to mark a greater proportion of buds than *Edar* and *Wnt10b* (Fig. 4A–D). However, in E14.5 female UGS explants, *Nkx3-1* and *Edar* both marked a greater proportion of buds than *Wnt10b* (Fig. 4E–H). In E17.5 male UGS explants, there was no detectable difference in the proportion of buds marked by *Nkx3-1*, *Edar* and *Wnt10b* (Fig. 5A–D). However, in E17.5 female UGS explants, the proportion of *Nkx3-1* and *Edar* marked buds was greater than the proportion of *Wnt10b* marked buds (Fig. 5E–H). Together, our results indicate that *Nkx3-1* marks the greatest proportion of prostatic buds formed in male UGS explants, while *Nkx3-1* and *Edar* are equally efficient in marking prostatic buds formed in female UGS explants. Furthermore, our results reveal that age and gender of UGS explants at the time of

organ culture initiation influences the proportion of buds marked by *Nkx3-1*, *Edar* and *Wnt10b*.

3.5. The number of buds formed *in vitro* and the proportion marked by *Nkx3-1* depends on gender and developmental stage at which UGS organ culture was initiated

Mechanistic studies of prostate development are challenged by the fact that the mouse UGS is small and many UGS tissues must often be pooled to obtain sufficient tissue for downstream molecular and biochemical analyses. This problem is amplified in transgenic mouse studies, where dozens of litters are necessary to yield a sufficient number of genotype appropriate UGS tissues. Female UGSs are known to form prostatic buds in response to androgens (Cunha, 1972). As a method to reduce or refine animal usage for prostatic bud formation studies, female UGSs could conceivably be used (1) in combination with male UGS explants to increase experimental sample size and (2) in place of male UGSs to serve as control tissues naïve to high levels of *in vivo* androgen signaling. To determine whether such a use of female UGSs is appropriate, we compared prostatic bud formation in male and female UGS explants.

We first compared total UGS bud number between male *versus* female UGS explants incubated *in vitro* in the presence of androgens. Only main ducts were counted to eliminate differences in branching morphogenesis between age and gender. The anatomically distinct urethral gland buds were excluded from analysis. In order to achieve the highest statistical power for this analysis we pooled total UGS bud counts among age and gender matched *Nkx3-1*, *Edar* and *Wnt10b* stained groups. This sample pooling was feasible since total UGS bud counts did not detectably differ among age and gender matched ISH stained groups (results not shown). In E14.5 UGS explants, there was no detectable difference between the total number of buds formed in male and female tissues (Fig. 6A). However, E17.5 male UGS explants formed significantly more total buds than female UGS explants (Fig. 6B), an expected result since, at the time of organ culture initiation, prostatic buds were present in E17.5 males and absent in E17.5 females. Since *Nkx3-1* marked the greatest proportion of buds across gender and developmental stage, we next compared the proportion of *Nkx3-1* marked buds to the total buds formed in male *versus* female UGS explants incubated *in vitro* in the presence of androgens. UGS explants from E14.5 males formed a significantly greater proportion of *Nkx3-1* marked buds compared to those from E14.5 females (Fig. 6C). Yet, E17.5 male and female UGS explants formed the same proportion of *Nkx3-1* marked buds (Fig. 6D). Together, our results reveal that not all buds formed in UGS explants are the same and that composition of the prostatic bud population in UGS explants is influenced by UGS gender and developmental stage.

4. Discussion

A multitude of drugs, xenobiotics, transgenes, and gene deletions have been shown to impair, delay, reposition or accelerate prostate development by altering the number of prostatic buds formed *in vivo* and *in vitro*. Prostatic bud number therefore represents an important phenotypic endpoint for “quantifying” early stages of prostate development. We described a method that combines ISH and IHC staining to quantify two early prostate development endpoints in the same UGS: total prostatic bud number and proportional bud number marked by prostate-selective mRNAs. This method does not require expensive laboratory equipment and complements existing methods employed to quantify prostate development.

Using this newly validated method for counting prostatic buds, we revealed a noteworthy *in vivo versus in vitro* difference in molecular composition of prostatic buds. All prostatic buds formed *in vivo* were marked by *Nkx3-1*, *Edar*, and *Wnt10b*. Yet only a fraction of buds

formed *in vitro* were marked by the same prostate-selective markers. Possible explanations are that *in vitro* (1) culture conditions do not completely reproduce the needed battery of prostate morphogenetic signals that are present *in vivo* (2) UGS mesenchyme is impaired in its ability to instruct the appropriate prostate identity onto bud epithelium (3) prostatic bud epithelium is compromised in its ability to respond appropriately to UGS mesenchyme signals. Our results suggest that sole reliance on prostatic bud number as a metric for *in vitro* prostate development neglects a potentially relevant endpoint—whether or not buds express appropriate prostate marker mRNAs. The fractional number of prostatic buds marked by *Nkx3-1*, *Edar*, and *Wnt10b* mRNAs may also be relevant for assessing prostate development after genetic, pharmacological or toxicological manipulations *in vivo*. While we found that these prostate-selective mRNAs are present in all prostatic buds formed during *in vivo* prostate development in untreated wild type control male fetuses, whether the same is true for transgenic or chemically exposed male fetuses is not known.

Another important aspect of this study is that it establishes, with experimental evidence, that male and female UGS explants cannot be used interchangeably to assess androgen-dependent prostatic bud formation *in vitro*. Androgen-exposed male and female UGS explants varied in both the number of buds formed and the proportion of buds marked by prostate-selective mRNAs. These endpoints were further affected by the developmental stage of the male or female UGS used for organ culture. Though the total number of buds formed did not significantly differ between E14.5 male and female UGS explant cultures, prostatic buds formed in E14.5 male UGSs were more likely to express *Nkx3-1* than those formed in E14.5 female UGSs. On the other hand, while E17.5 male UGS explants formed significantly more buds than E17.5 female UGSs, there was not a significant male *versus* female difference in the *Nkx3-1*-marked bud proportion at this stage. Our results support the notion that some androgen-induced buds in E14.5 female UGS explants are developmentally delayed or deviate from a normal prostate gene expression program. The latter possibility is consistent with the fact that, during its peri-natal maturation, the female UGS loses its ability to initiate androgen-dependent prostatic bud formation (Cunha, 1975). Based on our results, the female UGS may begin losing its androgen responsiveness much earlier in development than previously appreciated, as early as E14.5, as evidenced by a failure to express appropriate prostate gene expression in all androgen-induced buds.

We identified *Wnt10b* and *Edar* as additional prostatic bud markers that can be used in conjunction with, or in some cases in place of, the more widely known *Nkx3-1* prostatic bud marker (Bhatia-Gaur et al., 1999). All three mRNAs have the benefit of marking prostatic buds over a relatively long prostate development window. Therefore, ISH staining of the UGS with *Nkx3-1*, *Edar*, or *Wnt10b* can be used to visualize and compare prostatic bud number among different experimental groups, even if experimental treatments reduce or delay prostate development.

ISH staining for *Nkx3-1* is particularly useful for visualizing the mouse prostate field prior to prostatic bud initiation. This method could be used to more clearly delineate the mechanism by which a particular experimental condition impairs prostatic bud formation. Absence of detectable *Nkx3-1* ISH staining in the UGS on or after the normal stage of bud initiation (>E16.5) may indicate that specification of the prostate field was delayed or impaired. Alternatively, the presence of *Nkx3-1* and absence of prostatic buds may indicate that the prostate field was specified appropriately but that buds failed to elongate. Distinguishing between these two possibilities refines the genes and signaling pathways likely to be involved in the prostatic bud impairment mechanism.

ISH staining for *Edar* and *Wnt10b* mRNAs can be used to distinguish prostatic from urethral gland buds. *Nkx3-1* was shown here and in previous studies (Abler et al., 2011a, Bhatia-

Gaur et al., 1999) to mark both prostatic and urethral gland buds. On the other hand, *Edar* and *Wnt10b* were detected in prostate buds and not in urethral gland buds. Visualization of *Nkx3-1*, *Edar*, and *Wnt10b* could be used separately or in conjunction to focus on one particular urogenital bud population or to visualize segmental boundaries in the developing mouse urethra.

An interesting future application for our results is that they can be used to conduct prostate cell lineage analyses and to enrich for highly-specific developing prostate cell populations for gene expression profiling and biochemical studies. It is currently possible to target genes for deletion using an *Nkx3-1-Cre* recombinase gene (Thomsen et al., 2008) and conduct lineage tracing of prostate bud epithelial cells using a tamoxifen inducible *Nkx3-1^{CreERT2}* with R26R-YFP or R26R-lacZ reporters (Wang et al., 2009). It had not been possible to compare the fate of cells at the tips of prostatic buds *versus* those in bud proximal regions, or to separate epithelial cells in prostatic bud tips from those in the proximal bud region. Yet, in this report we identified differential expression of *Nkx3-1*, *Edar*, and *Wnt10b* along the axis of prostatic buds. Early in development, all three mRNAs mark the entire length of prostatic buds. During prostate bud elongation and branching morphogenesis *in vivo* (E18.5 until at least P5) and *in vitro* (E17.5 plus at least 7 days in organ culture), *Edar* and *Wnt10b* uniquely mark prostatic bud tips. At the same developmental stages, *Nkx3-1* is present in prostatic bud tips and extends proximally towards the urethra. The specific prostatic bud domain marked by *Edar* and *Wnt10b* represents a unique prostatic bud cell subpopulation because these cells are at the leading edge of prostatic buds as they invade surrounding mesenchyme. It may be possible in future studies to use *Nkx3-1*, *Edar*, and *Wnt10b* expression as cell sorting tools to identify mRNAs and proteins that are distinctively expressed or activated in the invasive prostatic growth environment in prostatic bud tips.

Finally, the presence of *Edar* and *Wnt10b* in developing prostate ducts provides insight into the complex signaling pathways involved in prostatic bud specification, initiation and branching morphogenesis. These mRNAs are known members of the tumor necrosis factor (TNF) and WNT signaling pathways and indicate important roles for these signaling pathways in prostate development. Intriguingly, *Edar* and WNT signaling have been shown to interact to establish hair follicle patterning (Laurikkala et al., 2002; Zhang et al., 2009) whether the same interaction occurs during prostate development is currently unknown.

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Abbreviations

AR	androgen receptor
CDH1	Cadherin 1
DHT	5 α dihydrotestosterone
Edar	ectodysplasin-A receptor
E	embryonic day
Nkx3-1	NK-3 transcription factor locus 1
UGS	urogenital sinus
Wnt10b	wingless related MMTV integration site 10b

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Fig. 1.

Edar mRNA distribution in E17.5 male and female mouse UGS. (A and B) Near mid-sagittal sections (50 μ m) of embryonic day (E)17.5 male and female UGSs were stained by ISH to visualize mRNA expression of ectodysplasin-A receptor (*Edar*). Sections were then immunofluorescently stained with antibodies against smooth muscle actin alpha 2 (ACTA2, green) and cadherin 1 (CDH1, red). Results are representative of three litter-independent samples per group. Abbreviations: bl, bladder; ed, ejaculatory duct; lv, lower vagina; sv, seminal vesicle; uv, upper vagina. Solid arrowheads indicate prostatic buds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Developmental time course of *Nkx3-1*, *Edar*, and *Wnt10b* mRNA expression in whole-mount male fetal UGS and neonatal prostate. (A–O) ISH was used to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b* in embryonic day (E)15.5, 16.5, 17.5, and 18.5 male mouse UGS and postnatal day (P)5 mouse prostate. (J–O) To better visualize individual mRNA distribution, E18.5 UGS and P5 prostate were also immunohistochemically stained with an antibody against cadherin 1 (CDH1, red) to visualize all epithelium. (A–F) Insets are magnified images of whole mount tissues. (J–O) Insets are magnified images of 5 μ m stained tissue sections to reveal staining details within the proximal distal axis of prostatic buds and branches. Results are representative of three

litter-independent samples per group. Abbreviations: bl, bladder; d, distal; p, proximal; sv, seminal vesicle. Open arrowheads indicate representative urethral gland buds. All images are of the same magnification.

Fig. 3.

Nkx3-1, *Edar*, and *Wnt10b* mark all macroscopically visible prostatic buds after UGS development *in vivo*. E18.5 male UGS tissues were stained by ISH and IHC as described in Fig. 2 and the number of anterior, ventral, dorsolateral and total prostatic buds marked by each mRNA was determined. Results are reported as mean $\pm$ SE, $n = 3$ litter independent UGS tissues per group. One-way analysis of variance (ANOVA) was used to determine that there was no statistically significant difference ($p < 0.05$) in the mean prostatic bud number marked by *Nkx3-1*, *Edar* or *Wnt10b*.

Fig. 4.

Nkx3-1, *Edar*, and *Wnt10b* mark only a subset of prostatic buds after E14.5 UGS development *in vitro*. E14.5 (A–D) male and (E–H) female UGSs were grown for seven days in organ culture media containing vehicle (0.1% ethanol) or 5 α dihydrotestosterone (DHT, 10 nM). At the end of the incubation period, UGSs were stained by ISH to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b*. Tissues were then immunohistochemically stained with an antibody against cadherin 1 (CDH1, red) to visualize epithelium and mRNA-unmarked buds. Insets are magnified images of 5 μ m stained tissue sections to reveal staining details within representative mRNA marked and mRNA unmarked prostatic buds. Solid arrowheads indicate mRNA stained buds. Open arrowheads indicate buds without detectable mRNA staining. Arrows indicate urethral gland buds. Abbreviations: bl, bladder. Solid arrowheads indicate mRNA stained buds. Results are reported as mean $\pm$ SE, $n = 3$ litter independent UGS tissues per group. One-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post-hoc test was used to determine whether *Edar* or *Wnt10b* marked a different proportion of buds compared to *Nkx3-1*. Asterisks indicate a significant difference compared to *Nkx3-1* ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Nkx3-1, *Edar*, and *Wnt10b* mark only a subset of prostatic buds after E17.5 UGS development *in vitro*. E17.5 (A–D) male and (E–H) female UGSs were grown for seven days in organ culture media containing vehicle (0.1% ethanol) or 5 α dihydrotestosterone (DHT, 10 nM). At the end of the incubation period, UGSs were stained by ISH to visualize mRNA expression (purple) of *Nkx3-1*, *Edar*, and *Wnt10b*. Tissues were then stained by immunohistochemistry with an antibody against cadherin 1 (CDH1, red) to visualize epithelium and mRNA-unmarked buds. Solid arrowheads indicate mRNA stained buds. Open arrowheads indicate buds without detectable mRNA staining. Arrows indicate urethral gland buds. Insets are magnified images of 5 μ m stained tissue sections to reveal details of representative mRNA marked and mRNA unmarked prostatic buds. Though we detected epithelial buds marked by *Wnt10b* in some whole mount UGSs, we did not detect it in 5 μ m sections. Results are mean $\pm$ SE, $n = 3$ litter independent UGS tissues per group. One-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post-hoc test was used to determine whether *Edar* or *Wnt10b* marked a different proportion of buds compared to *Nkx3-1*. Asterisks indicate a significant difference compared to *Nkx3-1* ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

UGS gender and age at organ culture initiation influence the mean number of prostatic buds formed and the proportion marked by *Nkx3-1*. E14.5 and E17.5 male and female UGSs were stained by ISH and IHC as described in Fig. 5 and the number of *Nkx3-1* mRNA-marked and unmarked buds was determined for each UGS. Student's *t*-test was used to assess male *versus* female UGS differences in (A–B) the mean number of prostatic buds formed and (C–D) the proportion of buds marked by *Nkx3-1* after *in vitro* UGS development in the presence of 5 α dihydrotestosterone (DHT, 10 nM). Results are mean $\pm$ SE, $n = 3$ litter independent UGS tissues per group. Asterisks indicate a significant difference between male and female UGS ($p < 0.05$).