An Indispensable Role of Androgen Receptor in Wnt Responsive Cells During Prostate Development, Maturation, and Regeneration

YONGFENG HE,a,b ERIKA HOOKER,a,b EUN-JEONG YU,a,b HUIQING WU,c GERALD R. CUNHA,d ZIJIE SUN a,b

Key Words. Androgen receptor • Wnt signaling • β-Catenin • Prostate development

ABSTRACT

Androgen signaling is essential for prostate development, morphogenesis, and regeneration. Emerging evidence indicates that Wnt/L β-catenin signaling also contributes to prostate development specifically through regulation of cell fate determination. Prostatic Axin2-expressing cells are able to respond to Wnt signals and possess the progenitor properties to regenerate prostatic epithelium. Despite critical roles of both signaling pathways, the biological significance of androgen receptor (AR) in Axin2-expressing/Wnt-responsive cells remains largely unexplored. In this study, we investigated this important question using a series of newly generated mouse models. Deletion of Ar in embryonic Axin2-expressing cells impaired early prostate development in both ex vivo and tissue implantation experiments. When Ar expression was deleted in prostatic Axin2-expressing cells at pre-puberty stages, it results in smaller and underdeveloped prostates. A subpopulation of Axin2 expressing cells in prostate epithelium is resistant to castration and, following androgen supplementation, is capable to expand to prostatic luminal cells. Deletion of Ar in these Axin2-expressing cells reduces their regenerative ability. These lines of evidence demonstrate an indispensable role for the Ar in Wnt-responsive cells during the course of prostate development, morphogenesis, and regeneration, which also imply an underlying interaction between the androgen and Wnt signaling pathways in the mouse prostate.

SIGNIFICANCE STATEMENT

Androgen and Wnt signaling pathways are essential for prostate development and tumorigenesis. However, the molecular mechanisms underlying these pathways in regulating prostate development, maturation, and regeneration remains largely unexplored. This study developed a series of novel and biologically relevant mouse models to assess the biological role of Ar in Axin2-expressing/Wnt-responsive cells during prostate development and regeneration. Deletion of Ar in embryonic Axin2-expressing cells at pre-puberty stages, it results in smaller and underdeveloped prostates. A subpopulation of Axin2 expressing cells in prostate epithelium is resistant to castration and, following androgen supplementation, is capable to expand to prostatic luminal cells. Deletion of Ar in these Axin2-expressing cells reduces their regenerative ability. These lines of evidence demonstrate an indispensable role for the Ar in Wnt-responsive cells during the course of prostate development, morphogenesis, and regeneration, which also imply an underlying interaction between the androgen and Wnt signaling pathways in the mouse prostate.

INTRODUCTION

Androgen signaling is mediated through the androgen receptor (AR) and its ligands, testosterone and 5α-dihydrotestosterone (DHT) [1, 2]. The AR is a member of the nuclear hormone receptor superfamily [3], and plays a decisive role in prostate development, maturation, and tumorigenesis [1, 2]. Mouse prostatic development initiates at embryonic day 17.5 (E17.5) from the urogenital sinus (UGS) in response to rising levels of testicular androgens [4]. After birth, the unbranched buds and ducts in the ventral, dorsal, and lateral prostatic lobes grow peripherally to give rise to lobe-specific ductal branching patterns [4, 5]. Morphogenesis of the entire prostatic complex in mice is completed between postnatal days 15 and 30 (P15–30) [5]. Circulating androgens levels rise during puberty (P25–40), resulting in continued prostate growth and maturation [5, 6]. Prostatic regeneration through repeated cycles of androgen deprivation and replacement further demonstrate...
that androgen signaling is essential for prostatic regeneration and growth [4, 7].

Recent studies demonstrate that the canonical Wnt signaling pathway, whose effects are mainly mediated through β-catenin, plays a critical role in prostate development, morphogenesis, and organogenesis [8–11]. Activation of Wnt/β-catenin signaling in prostatic epithelium is necessary for the development of the prostate [9, 10, 12]. Deletion of the ctnnb1 gene in the mouse prostate at embryonic stages results in inhibition of prostatic development [10]. Moreover, Axin2, a well-established direct transcriptional target of β-catenin and a component of the destruction complex in the negative feedback loop of the canonical Wnt signaling pathways, is expressed in both embryonic and adult prostate [13, 14]. In embryonic stages, Axin2-expressing cells can be observed in both the urogenital sinus mesenchyme (UGM) and the urogenital sinus epithelium (UGE), while its expression is limited to the luminal epithelium in adult stages [9, 10, 19]. Recent studies have shown that Axin2-expressing cells have stem/progenitor cell properties in a variety of mouse tissues, including the prostate [15–19]. Moreover, deletion of β-catenin or expression of stabilized β-catenin in prostatic Axin2-expressing epithelial cells results in abnormal prostate development or oncogenic transformation, respectively [19]. In addition, the direct interaction between the AR and β-catenin has also been observed in prostate cancer cells [20–22].

Although emerging evidence has shown that both the androgen and Wnt signaling pathways play a critical role in prostate development and tumorigenesis, it is unclear how Wnt signaling pathways specifically control cell fate commitment and determination during the course of prostate development, maturation, and regeneration. Specifically, it is also unknown the role of androgen signaling in regulating prostatic Wnt responsive progenitor cells. In this study, we directly assess these important questions using relevant mouse models and other experimental approaches. Our results reveal that androgen signaling plays an essential role in Wnt/β-catenin mediated prostate cell fate commitment, implicating a molecular mechanism underlying the regulatory roles of androgen and Wnt signaling pathways in prostate development and regeneration.

**Materials and Methods**

Mouse Mating and Genotyping

Axin2CreERT2 and Rosa26RtmG mice were obtained from Jackson Laboratories, Bar Harbor, ME, the United States; stock 18867 and stock 7676 [18, 23]. ArloxY mice were obtained from Dr. Guido Verhoeven [24]. All animal procedures were performed based on animal care guidelines approved by the Institutional Animal Care and Use Committee at Beckman Research Institute and City of Hope, and Stanford University.

Mouse Procedures

Castration of adult male mice was performed as described previously [25]. For androgen supplementation, testosterone pellets (12.5 mg, Innovative Research of America, Sarasota, FL, the United States) were placed subcutaneously in castrated mice. To elicit genetic recombination mice received a single intraperitoneal injection of 4 mg/25 g body weight tamoxifen (TM) (Sigma-Aldrich, St. Louis, the United States) suspended in corn oil, corresponding to a total dose of 1 mg for prepubescent mice (injected at P14) and 4 mg of TM for adult mice (injected at P84). To label Axin2-expressing cells in embryos, pregnant mothers received a single intraperitoneal injection at E13.5, totaling 1 mg/25g body weight.

**Histology and Immunostaining**

Prostatic tissues were fixed in 10% neutral-buffered formalin, and processed into paraffin. Five-micron serial sections were cut and processed from xylene to water through a decreasing ethanol gradient. For histology, H&E staining was performed as reported earlier [26, 27]. For immunohistochemistry, slides were treated by boiling in 0.01 M Citrate buffer (pH 6.0) for antigen retrieval, incubated in 0.3% H2O2 for 15 minutes, blocked in 5% normal goat serum for 30 minutes, and incubated with primary antibodies diluted in 1% normal goat serum at 4°C overnight. Slides were incubated with biotinylated secondary antibodies for 1 hour then with horseradish peroxidase streptavidin (SA-5004, Vector Laboratories, Burlingame, CA, the United States) for 30 minutes and visualized by DAB kit (SK-4100, Vector Laboratories). Slides were counterstained with 5% (w/v) Harris Hematoxylin, and coverslips were mounted with Permount Mounting Medium (SP15–500, Fisher Scientific, Hampton, NH, the United States).

For immunofluorescent staining and detection of mTmG signals, mouse tissues were fixed in 4% PFA at 4°C overnight, cryoprotected in 30% sucrose at 4°C overnight, and embedded in OCT (Tissue-Tek, Torrance, CA, USA). Five-micron sections were obtained using a Leica cryostat, and slides were washed three times with phosphate-buffered saline (PBS). For detection of membrane-bound Tomato (mT) and membrane-bound green fluorescent protein (mGFP) signals, slides were directly mounted with VECTASHEILD Mounting Medium with DAPI (H-1200, Vector Laboratories). For immunofluorescence staining, slides were treated for antigen retrieval as described above. Tissues were then blocked in 5% normal goat serum for 30 minutes, and incubated with primary antibodies diluted in 1% normal goat serum at 4°C overnight. Slides were washed in PBS then incubated with fluorescent-conjugated secondary antibodies for 1 hour, and then mounted as described above.

**Ex Vivo Culture and Manipulation**

Female mice were mated overnight. The following day was considered as E0.5 if a vaginal plug was detected. The UGS were dissected at E14.5 and placed on 0.4 µM Millicell-CM filters (MilliporeSigma, Burlington, MA, the United States) in 6-well tissue culture plates. Culture media consisted of DMEM-F12 (1:1) media, supplemented with nonessential amino acids, 1% insulin-transferrin-selectin, l-glutamine, Penicillin/Streptomycin, and 1 × 10−8 M DHT. 2 µM of (Z)-4-Hydroxytamoxifen was added to the media to activate Axin2CreER. Media was changed every 2 days, and the UGS explants were cultured for 7 days and then processed for histological and immunofluorescent analyses.

**Antibodies**

The following primary antibodies were used: anti-GFP (Rabbit, 1:1,000, A11122, Molecular Probes, Eugene, OR, the United States), anti-GFP (mouse, 1:300, 2955, Cell Signaling, Danvers, MA, the United States), anti-GFP (Chicken, 1:2,000, ab13970, Abcam, Cambridge, MA, the United States), anti-KS (rabbit,
1:3,000, PRB-160P, Covance), anti-K8 (mouse, 1:2,500, MMS-162P, Covance, Brea, CA, the United States), anti-p63 (mouse, 1:200, sc-8431, Santa Cruz, Biotechnology, Santa Cruz, CA, the United States), anti-Ki67 (mouse, 1:1,000, NCL-kil67, Novocasta

In this study, we also examine the colocalization of Ar and GFP expression within the UGM at E15.5 (Fig. 2A4, 2A5), in the closely adjoining UGE (Fig. 2B1, 2B2, 2B3), and in newly formed prostatic buds (arrow heads, Fig. 2B5) at E17.5. The expansion of Ar and GFP double positive cells from the UGM into both the UGM and UGE between E15.5 and E17.5 suggests an important role for Wnt/β-catenin signaling in androgen-induced UGE formation and prostatic bud initiation. Since Axin2-expressing epithelial cells have been shown to possess progenitor cell properties [19], we further evaluated the Ar and GFP double-positive cells in both E15.5 and 17.5 tissues using triple immunofluorescence approaches. At E15.5, the majority of Ar and GFP double positive cells are localized in the UGM (Fig. 2C4), but a small minority also appears in the UGE (Fig. 2C4). Double positive epithelial cells were immunoreactive to antibodies against E-cadherin (Arrowheads, Fig. 2C4, 2C5), Ck8 (Fig. 2D4, 2D5), p63 (Fig. 2E4, 2E5), and in some cases also to Ki67 (Fig. 2F4, 2F5). At E17.5, Ar and GFP positive UGM cells are mainly localized in the areas adjacent to the UGE and in the newly formed prostatic buds (Fig. 2G4, 2G5), which appear immunoresponsive to the both Ck8 and p63 antibodies (Arrowheads, Fig. 2H4, 2H5, 2I4, 2I5) as well as Ki67 antibody (Arrowheads, Fig. 2J4, 2J5). Our observation of endogenous Ar expression in Axin2 positive cells in the UGS is interesting, and it suggests a biological role of androgen signaling in Wnt/β-catenin-mediated prostatic differentiation and development.

Deletion of Ar Expression in Axin2 Positive Cells Impairs Embryonic Prostate Development

Currently, it is still unclear how Wnt signaling regulates epithelial cell fate commitment and determination during the course of prostate development and organogenesis. To directly assess the role of the Ar in prostatic Wnt-responsive cells during prostate development, maturation, and regeneration, we generated R26RtmGfl/Y;Arfloxed/Y:Axin2creRT2/−;ROsmTmGfl/Y (R26RtmGfl/Y;Arfloxed/Y;Axin2creRT2/−) compound mice, in which TM-induced Cre activity results in conditional deletion of the Ar and induced expression of GFP in Axin2-expressing cells (Fig. 3A). We did not observe abnormalities in embryonic prostatic development in the above fetuses (data not shown). We then isolated UGS tissues from E14.5 male R26RtmGfl/Y;Arfloxed/Y;Axin2creRT2/− and

Using immunohistochemical approaches, we assessed Ar expression, and observed uniform nuclear staining of Ar protein in both UGM and UGE areas at E15.5 and E17.5 (Fig. 1D1–1D4). We further evaluated the cellular identity of Ar positive cells using co-immunofluorescence (co-IF) analyses. As shown in Figure 1E2 and 1F2, E-cadherin expression appears within UGE areas of both E15.5 and E17.5 mice (Fig. 1E, 1F). At E17.5 E-cadherin staining was observed in epithelial cells of the newly formed prostatic buds (arrow heads, Fig. 1F2–1F4) coexpressing Ar (Fig. 1F3, 1F4). Further analyses show that a subset of these Ar-expressing epithelial cells is also immunoreactive for Ck8, p63, and Ki67 (Supporting Information Fig. S2). Intriguingly, a portion of E-cadherin-positive epithelial cells in the UGSs at E15.5 and E17.5 were also membrane-bound GFP positive, indicating their Wnt-responsive cellular identities (compare Fig. 1C3, 1C4, 1F3, 1F4). Taken together, this data suggests an interaction between androgen and Wnt/β-catenin signaling pathways during the course of embryonic prostatic development. To explore this idea, we directly examined the colocalization of Ar and GFP expression within the UGM at E15.5 (Fig. 2A4, 2A5), in the closely adjoining UGE (Fig. 2B1–2B4), and in newly formed prostatic buds (arrow heads, Fig. 2B5) at E17.5. The expansion of Ar and GFP double positive cells from the UGM into both the UGM and UGE between E15.5 and E17.5 suggests an important role for Wnt/β-catenin signaling in androgen-induced UGE formation and prostatic bud initiation.
control $R_{26}^{m{TmG}}$:Axin2CreERT2/+ fetuses, and cultured them with 2 μM of (Z)-4-Hydroxytamoxifen (Fig. 3B). After 7 days of ex vivo culture, sizes of UGS tissues from $R_{26}^{m{TmG}}$:Arf/Y:Axin2CreERT2/+ mice appeared smaller than ones from $R_{26}^{m{TmG}}$:Axin2CreERT2/+ controls ($n = 4$), respectively. The representative images are shown in Figure 3C1, 3C2, 3D1, 3D2. Histologically, we observed glandular structures in the center of UGS of $R_{26}^{m{TmG}}$:Axin2CreERT2/+ controls, which is very similar to normal prostatic budding formation in UGS in vivo, but not in $R_{26}^{m{TmG}}$:Arf/Y:Axin2CreERT2/+ controls (Fig. 3E1–3E4). Analysis of E-cadherin expression in frozen sections revealed more prostatic budding in cultured UGS tissues of $R_{26}^{m{TmG}}$:Axin2CreERT2/+ controls than of $R_{26}^{m{TmG}}$:Arf/Y:Axin2CreERT2/+ fetuses (Fig. 3F1–3F4). This provides the first line of evidence that demonstrates the decisive role of Ar in Axin2 expressing UGE cells in regulating prostatic bud formation and early prostatic development. We then performed co-IF approaches to assess Ar expression in mouse UGE cells. As shown in Figure 3G1, 3G2, and 3I, almost all of E-cadherin-positive UGE cells are Ar positive in $R_{26}^{m{TmG}}$:Axin2CreERT2/+ controls. In contrast, only a few E-cadherin and Ar positive cells were observed in UGE from $R_{26}^{m{TmG}}$:Arf/Y:Axin2CreERT2/+ fetuses (Fig. 3G3, 3G4, 3I). Accordingly, there is a significant reduction of Ki67 positive cells in UGE of $R_{26}^{m{TmG}}$:Arf/Y:Axin2CreERT2/+ in comparison with UGE cells of $R_{26}^{m{TmG}}$:Axin2CreERT2/+ controls (Fig. 3H1–3H4, 3I, Supporting Information Table S2).
Figure 2. Expression of Ar in Axin2-expressing cells during early development of mouse prostate. (A, B): Ar (red) and GFP (green) expression in male urogenital sinus (UGS) isolated at E15.5 and E17.5. (C–F): Colocalization of Ar (red) and GFP (green) with E-cad, CK8, p63, or Ki67 (blue) in UGS tissue isolated at E15.5. (G–J): Colocalization of Ar (red) and GFP (green) with E-cad, CK8, p63, or Ki67 (blue) in UGS tissue isolated at E17.5. Abbreviations: GFP, green fluorescent protein; UGE, urogenital sinus epithelium; UGM, urogenital sinus mesenchyme.
Figure 3. Ar is required in Axin2-expressing cells during prostatic bud formation of ex vivo cultured UGS. (A): Illustration of floxed alleles present in R26R<sup>WT</sup>:Ar<sup>F</sup>/Y<sup>1</sup>:Axin2<sup>CreERT2</sup>/1 mice. (B): Diagram depicting experimental timeline for ex vivo culture of UGS tissues. (C): Gross images of ex vivo cultured UGS tissues isolated from R26R<sup>WT</sup>:Axin2<sup>CreERT2</sup>/1 or R26R<sup>WT</sup>:Ar<sup>F</sup>/Y<sup>1</sup>:Axin2<sup>CreERT2</sup>/1 mice. (D): E-cadherin expression and localization on sections of ex vivo cultured UGS tissues. (E): H&E stained sections of ex vivo cultured UGS tissues. (F): Ar or E-cadherin (brown) expression pattern in sections of UGS tissues following 7 days of ex vivo culture. (G, H): Colocalization of E-cadherin (green) with Ar or Ki67 (red) in UGS cultured for 7 days ex vivo. (I): Percentage of Ar-expressing cells or Ki67-expressing cells present in urogenital epithelium. **, p < .01. Abbreviation: UGS, urogenital sinus.
together, our results show that deletion of Ar expression in Axin2 positive cells at an embryonic stage reduces prostatic epithelial cell proliferation and impairs prostatic gland formation.

To further assess the role of Ar in Axin2 positive cells during the course of prostatic development, we took another approach by implanting UGS tissues isolated from both R26RmTmG/1:Arf/Y:Axin2CreERT2/1 and R26RmTmG/1:Axin2CreERT2/1 fetuses under kidney capsule (Fig. 4A). After 8 weeks of implantation, UGS grafts isolated from R26RmTmG/1:Arf/Y:Axin2CreERT2/1 were smaller in size and weighed less than ones from R26RmTmG/1:Axin2CreERT2/1 controls, (see the representative images in Fig. 4B1, 4B2, 4C). In co-IF experiments, we observed much more AR positive prostatic epithelial cells in the grafts of R26RmTmG/1:Axin2CreERT2/1 controls (Fig. 4E1, 4E2) than those of R26RmTmG/1:Arf/Y:Axin2CreERT2/1 mice (Fig. 4E3, 4E4). As a consequence, Ar deleted grafts also showed less Ki67 expressing cells than controls (Fig. 4F1, 4F2, 4F4, Supporting Information Table S3). Intriguingly, we observed smaller and abnormal acini with flattened epithelial cells in prostatic glands of R26RmTmG/1:Arf/Y:Axin2CreERT2/1 grafts (Arrows, Fig. 4H1–4H4), suggesting an impaired prostate development phenotype. We only observed a minor subset of the

Figure 4. Deletion of Ar in Axin2 expressing cells during embryonic stage impairs prostate gland formation in renal capsule of SCID mice. (A): Schematic demonstrating experimental timeline including activation of Axin2CreER, urogenital sinus (UGS) collection, renal capsule transplantation and analysis. (B): Gross images of xenografts from UGS tissue isolated from R26RmTmG/1:Axin2CreERT2/1 or R26RmTmG/1:Axin2CreERT2/1 mice. (C): Graphical representation of xenograft weight from grown from mice of each genotype. **, p < .01. (D): Quantification of Ki67 positive cells in the epithelial cells from xenografts of each genotype. ***, p < .01. (E, F): Colocalization of E-cadherin (green) with Ar or Ki67 (red) in R26RmTmG/1:Axin2CreERT2/1 and R26RmTmG/1:Arf/Y:Axin2CreERT2/1 xenografts. (G, H): Representative H&E staining of tissue sections from xenografts of each genotype.
Figure 5. Ar is required in prepubescent Axin2-expressing cells for prostate development. (A): Illustration depicting experimental timeline of Axin2CreERT2 activation (TM injection) and tissue analysis. (B): Gross images of prostates isolated at P56 from R26RmTmG/1:Axin2CreERT2/1 or R26RmTmG/1:Arf/Y:Axin2CreERT2/1 mice. (C): Graphical representation of the ratio of prostate wet weight/body weight. **, p < .01. (D): Quantification of Ki67 positive cells per 1,000 epithelial cells (E-cadherin positive) from prostate tissues of each genotype. *, p < .05. (E, F): Colocalization of E-cadherin (green) with Ar or Ki67 (red) in prostates isolated from R26RmTmG/1:Axin2CreERT2/1 or R26RmTmG/1:Arf/Y:Axin2CreERT2/1 mice. (G, H): H&E staining of tissue sections from prostates of each genotype. Abbreviations: AP, anterior prostate; D/LP, dorsolateral prostate; VP, ventral prostate; TM, tamoxifen.
Figure 6. Ar is required for the regenerative potential of castration-resistant Axin2-expressing cells. (A): Diagram of experimental design describing timelines for castration, activation of Axin2CreERT2 (TM injection), androgen supplementation (regeneration) and analysis. (B–E): mTmG analysis of both castrated and regenerated prostate tissues from mice of different genotypes. (F): Graphical quantification of the ratio of mG positive cells per total cells in different lobes of both castrated and regenerated prostates. **, p < .01. (G): Colocalization of GFP (green) and Ar (red) in frozen sections of both castrated and regenerated prostate tissues from R26RmTmG+;Axin2CreERT2+ and R26RmTmG+;Arf/Y:Axin2CreERT2+ mice. Abbreviations: AP, anterior prostate; D/LP, dorsolateral prostate; VP, ventral prostate; TM, tamoxifen.
GFP-positive epithelial cells appeared flattened. These flattened cells are also immunoreactive to Ck8 but not Ck5 antibody (Supporting Information Fig. S3), suggesting they are of luminal and not basal origin. These data are consistent with the results of ex vivo UGS cultures and implicate conditional deletion of Ar expression in Axin2-expressing epithelial cells impairs embryonic prostate development.

**Deletion of Ar Expression in Prepubescent Axin2-Expressing Cells Negatively Regulates Prostate Maturation and Morphogenesis**

Morphogenesis of the entire prostatic complex in mice is completed between postnatal days 15 and 30 (P15–30) and is tightly regulated by the androgen signaling pathway [4, 5]. Additionally, it has been shown that during this period Axin2-expressing cells expand to form the majority of luminal compartment in the mature prostate [19]. Thus, we next examined the effect of Ar deletion in prepubescent Axin2-expressing cells during expansion of the luminal lineages at puberty. We injected TM into both R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ and R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice at postnatal day 14, P14 (Fig. 5A). Analysis of those mice after 6 weeks, at P56, showed smaller and underdeveloped prostates in mutant R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice in comparison to R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ controls (Fig. 5B1, 5B3). Specifically, anterior prostate and dorsolateral prostate of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ lobes are much smaller in size than those in controls (Fig. 5B2 vs. 5B4; blue and pink arrows). The prostate weights of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ were also less than those of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ controls (Fig. 5C). Ar expression was significantly reduced in prostatic glands of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice in comparison to those of controls (Fig. 5E1–5E4). Less Ki67 positive epithelial cells appear in prostatic tissues isolated from R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice than those from R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ controls (Fig. 5D, 5F1–5F4, Supporting Information Table S4). As expected, normal prostatic glandular architecture was present in R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ control mice (Fig. 5G1–5G6). In contrast, pathological changes featured with scattered foci of marked glandular atrophy were observed in prostatic lobes of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice (arrows, Fig. 5H1–5H6). IHC analyses showed most of the flattened cells are immune-reactive to Ck8, but not Ar and Ck5 antibody (data not shown). These lines of evidence demonstrate that conditional deletion of Ar expression in prepubescent Axin2-expressing cells impairs prostate maturation and morphogenesis.

**The Regenerative Ability of Axin2 Expression Cells Depends on Ar-Mediated Signaling Events**

Axin2 expressing prostatic epithelial cells have been shown to possess luminal cell properties and have ability to generate prostatic luminal cells [19]. To explore the role of the Ar in Axin2 expressing cells, we assessed the ability of castration-resistant Axin2-expressing cells in prostate regeneration. Both R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ and R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice were castrated at 8 weeks, administered TM at 12 weeks, and then analyzed at 13 weeks of age (Fig. 6A). Histological analyses showed regressed glands in all prostatic lobes of castrated mice in both genotypes (data not shown). Membrane GFP (mGFP) positive, Axin2-expressing cells, were present in all prostatic lobes of both R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ and R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice (Fig. 6B1–6B3, 6D1–6D3). We observed that most mGFP positive Axin2-expressing cells are immunoreactive to Ck8 antibody as we reported earlier [19]. Following androgen supplementation we tracked the fate of these castration-resistant Axin2 expressing cells through androgen-mediated prostatic regeneration (Fig. 6A). After regeneration, a significant increase of mGFP-positive luminal cells was observed in prostatic lobes in R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice (Fig. 6B1–6B3, 6D1–6D3, 6E1–6E3, 6F, Supporting Information Table S5). Interestingly, we noticed that the majority of mGFP positive cells in regenerated prostatic tissues of R26RmTmG+/A\(y^{+}\)/Axin2-CreERT2+/+ mice showed Ar positive staining, indicating that the requirement of Ar in Axin2-expressing cell mediated regeneration (Arrowheads, Fig. 6D4). Thus, these lines of evidence demonstrate that deletion of Ar expression in Axin2 expressing cells significantly reduces the regenerative ability of those Wnt/β-catenin-responsive cells.

**DISCUSSION**

Mouse prostate develops from the UGS at embryonic day 16.5–17.5. During this short period, the cells in epithelial layer outgrow into surrounding mesenchyme to form prostatic buds [4]. The process of prostatic bud formation requires interaction between the UGE and the UGM. It has been demonstrated that androgen signaling is essential for prostate early development. Androgen actions upon UGS are mediated solely through the AR. Mutation of the Ar in Tfm mice results in complete absence of prostate development, demonstrating the indispensable role of the Ar in prostate development [30]. Canonical Wnt signaling pathways that are mainly mediated through β-catenin play a critical role in prostate development. Deletion of β-catenin in the UGE impairs prostatic bud formation in vivo and ex vivo [9, 10, 12]. Expression of Axin2, a direct downstream target of Wnt signaling, occurs at E15.5 and was induced by the presence of androgen within the UGE [10]. Emerging evidence has shown that Axin2-expressing cells function as Wnt responsive cells that possess progenitor properties during the course of prostate development and regeneration [19]. In this study, we directly addressed the biological significance of the AR in Axin2-expressing cells in regulating prostatic cell fate commitment and differentiation during prostate development, maturation, and regeneration.

Earlier studies have demonstrated Ar expression in the UGM during prostatic buds formation as early as E17 [29, 31]. Using both IHC and IF approaches, we detected extensive Ar expression in both UGE and UGM at or before E15.5, before bud formation. Our observation is consistent with the recent study that demonstrated Ar in the UGE at E14 [32]. Nuclear staining of Ar in both the UGE and UGM further indicates androgen-mediated nuclear translocation of Ar in UGS tissues. Intriguingly, we observed coexpression of Ar and Axin2 in UGE starting at E15.5, suggesting a possible crosstalk between...
androgen/Ar and wnt/β-catenin signaling pathways during early prostate development. In addition, both Axin2 activated GFP expression and Ar expression were coexpressed in newly formed prostatic buds at E17.5. These data provide the first line of evidence demonstrating that both androgen and Wnt signaling pathways are active in prostatic bud formation. To directly assess the role of Ar in Axin2-expressing cells, we generated R26RmTmG/::Arf/Y::Axin2CreERT2/+ mice, which exhibited conditional deletion of Ar and simultaneous expression of GFP in Wnt responsive cells. We did not observe notable defects in prostatic development in the above mice when TM was administered at E13.5. However, deletion of Ar expression in Axin2-expressing cells elicited impaired prostatic bud formation in both ex vivo culture and kidney capsule transplantation experiments. Using co-IF approaches, we further demonstrated a link between the deletion of Ar expression in Axin2-expressing cells and the above defect phenotypes. Several factors may contribute to the discrepancy that we observed in vivo versus ex vivo and implantation experiments. For example, the dosage of TM used to inject R26RmTmG/::Arf/Y::Axin2CreERT2/+ female mice is relatively lower than the one that was used in the ex vivo culture approaches. Thus, we observed much greater recombination effects from the later than the former. Moreover, additional factors may exist in vivo and be able to compensate the effect of Ar deletion in a physiological context.

Mouse prostatic ductal branching morphogenesis starts through the interaction between prostatic epithelial buds and mesenchymal pads [33], which is completed between postnatal days 15 and 30 (P15–30) [25]. During puberty between P25 and 40, circulating androgens levels raise, which further promotes prostate growth and maturation [6, 34]. It has been shown that the prepubescent Axin2-expressing epithelial cells have prostatic progenitor capabilities [19]. Thus, we injected TM at P14 to selectively delete Ar expression in prepubescent Axin2-expressing cells. Both the size and weight of prostates isolated from R26RmTmG/::Arf/Y::Axin2CreERT2/+ and Arf/Y::Axin2-CreERT2/+ only mice were significantly reduced as compared to their normal counterparts. The lack or reduced expression of both the Ar and Kd67 in underdeveloped prostate tissues further implicates an important role of Ar in Axin2-expressing cells in prostate maturation and morphogenesis during the period of pre-puberty. The function of the Ar in prostatic luminal cells has been assessed previously with different promoter-driven Cre strains [35–37]. Disruption of AR signaling in prostatic luminal cells appears to damage normal cellular barriers, increase apoptosis, and impair cellular maturity, which further induces inflammation and cell proliferation [35–37]. In this study, we observed prostatic atrophy and reduced cell proliferation when Ar expression was deleted in R26RmTmG/::Arf/Y::Axin2CreERT2/+ mice. Specifically, flattened epithelial cells appeared in prostate glands of the above mice. IHC analyses showed that these cells arise from Ar-native Axin2-expressing cells and possess luminal characteristics, such as CK8 expression. While we did not observe any overt effect on the secretory functions of these cells, further study is needed to characterize the biological significance of these flattened luminal cells. Given the fact that prostatic luminal Axin2 expressing cells possess prostatic stem/progenitor properties, our data are consistent with the previous studies, and suggest that Ar expression in prepubescent Axin2-expressing cells is important for mouse prostate maturation and morphogenesis.

Prostatic stem/progenitor cells in adult mice are able to survive androgen withdrawal and to regenerate upon androgen supplementation [38]. It has been shown that a sub-population of Axin2-expressing cells are resistant to castration and able to regenerate to prostatic luminal cells upon androgen administration [19]. In this study, we performed a series of experiments to determine the role of Ar in Axin2-expressing cells during the course of prostate regeneration. We observed the existence of castration-resistant Axin2-expressing cells in all four prostatic lobes of both R26RmTmG/::Arf/Y::Axin2CreERT2/+ and R26RmTmG/::Axin2CreERT2/+ controls. In the presence of androgens, those cells are able to expand and regenerate prostatic luminal cells in R26RmTmG/::Axin2CreERT2/+ controls, demonstrating their regenerative ability as prostatic progenitor epithelial cells. However, deletion of Ar expression in Axin2 expressing cells reduced and eliminated the regenerative ability in R26RmTmG/::Arf/Y::Axin2CreERT2/+ mice. These data suggest an indispensable role of the Ar in Axin2-expressing cells in response to androgen mediated prostate regeneration. In this study, we have noted that the both mGFP expression and Ar deletion do not always simultaneously occur in TM-induced Axin2-expressing cells of the regenerative prostate tissues in R26RmTmG/::Arf/Y::Axin2CreERT2/+ mice. Although we do not know the precise reasons for the mosaic Ar expression in the experiments, it is likely resulted by incomplete Cre-mediated recombination. Further studies are needed to further investigate the cellular identity of the Ar-positive Axin2-expressing cells in R26RmTmG/::Arf/Y::Axin2CreERT2/+ mice. In addition, high dosages of TM or other systems may be used to fully delete the Ar in Axin2-expressing cells to better determine the effect of Ar in prostate regeneration.

Prostatic Axin2-expressing cells respond to Wnt/β-catenin signaling, possess progenitor properties, and are able to expand the luminal epithelial cell lineage during prostatic development, maturation, and regeneration. They can also survive androgen withdrawal and regenerate prostatic luminal epithelial cells following androgen replacement. As detailed in this study, we provide several lines of experimental evidence demonstrating an indispensable role of Ar in the cellular events regulated through Axin2-expressing cells. In addition, the results also suggest a new molecular mechanism for androgen signaling to regulate Wnt/β-catenin mediated prostate cell fate commitment and differentiation.

**CONCLUSION**

In this study, we demonstrated that deletion of Ar in embryonic Axin2-expressing cells impaired early prostate development in both ex vivo and tissue implantation experiments. We also shown that when Ar expression was deleted in prostatic Axin2-expressing cells at mouse pre-puberty, it results in smaller and underdeveloped prostates. Moreover, we observed that a subpopulation of Axin2-expressing prostatic epithelial cells is resistant to castration and, then following androgen supplementation, is capable to expand to prostatic...
luminal cells. Deletion of Ar in these Axin2-expressing cells reduces their regenerative ability. Our data indicate an indispensable role for the Ar in Wnt-responsive cells during the course of prostate development, morphogenesis, and regeneration.

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REFERENCES

1 Balk SP. Androgen receptor as a target in androgen-independent prostate cancer. Urology 2002;60:132–138; discussion 138–139.

2 Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002;20:3001–3015.

3 Zhou ZX, Wong CJ, Sar M. The androgen receptor: An overview. Recent Prog Horm Res 1994;49:249–274.

4 Cunha GR, Donjacour AA, Cooke PS et al. The endocrinology and developmental biology of the prostate. Endocr Rev 1987;8:338–362.

5 Sugimura Y, Cunha GR, Donjacour AA. Morphogenesis of ductal networks in the mouse prostate. Biol Reprod 1986;34:961–971.

6 Hayashi N, Sugimura Y, Kawamura J et al. Morphological and functional heterogeneity in the rat prostatic gland. Biol Reprod 1991;45:308–321.

7 Cunha GR, Donjacour AA, Sugimura Y. Stromal-epithelial interactions and heterogeneity of proliferative activity within the prostate. Biochem Cell Biol 1986;64:608–614.

8 Prins GS, Putz O. Molecular signaling pathways that regulate prostate gland development. Differentiation 2008;76:641–659.

9 Francis JC, Thomsen MK, Taketo MM et al. beta-catenin is required for prostate development and cooperates with Pten loss to drive invasive carcinoma. PLoS Genet 2013;9:e1003180.

10 Simons BW, Hurley PJ, Huang Z et al. Wnt signaling though beta-catenin is required for prostate lineage specification. Dev Biol 2012;371:246–255.

11 Ontiveros CS, Salm SH, Wilson EL. Axin2 expression identifies progenitor cells in the murine prostate. Prostate 2008;68:1263–1272.

12 Mehta V, Schmitz CT, Keil KP et al. Beta-catenin (CTNNB1) induces Bmp expression in androgen-independent prostate cancer. Urology 2012;76:641–659.

13 Zhou Z, Wong CJ, Sar M. The androgen receptor: An overview. Recent Prog Horm Res 1994;49:249–274.

14 Jho EH, Zhang T, Domon C et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002;22:1172–1183.

15 Bowman AN, van Amerongen R, Palmer TD et al. Lineage tracing with Axin2 reveals distinct developmental and adult populations of Wnt/beta-catenin-responsive neural stem cells. Proc Natl Acad Sci USA 2013;110:7324–7329.

16 Ardehali R, Ali SR, Inlay MA et al. Prospective isolation of human embryonic stem cell-derived cardiovascular progenitors that integrate into human fetal heart tissue. Proc Natl Acad Sci USA 2013;110:3405–3410.

17 Blauwkamp TA, Nigam S, Ardehali R et al. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. Nat Commun 2012;3:1070.

18 van Amerongen R, Bowman AN, Nusse R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell 2012;11:387–400.

19 Lee SH, Johnson DT, Luong R et al. Wnt/beta-catenin-responsive cells in prostatic development and regeneration. STEM CELLS Transl Med 2015;3:3356–3367.

20 Mulholland DJ, Cheng H, Reid K et al. The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. J Biol Chem 2002;277:17933–17943.

21 Traichi CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60:4709–4713.

22 Yang F, Li X, Sharma M et al. Linking beta-catenin to androgen-signaling pathway. J Biol Chem 2002;277:11336–11344.

23 Muzumdar MD, Tasic B, Miyamichi K et al. A global double-fluorescent Cre reporter mouse. Genesis 2007;45:593–605.

24 De Gendt K, Swinnen JV, Saunders PT et al. A sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in mice. Proc Natl Acad Sci USA 2004;101:1327–1332.

25 Sugimura Y, Cunha GR, Bigsby RM. Androgenic induction of DNA synthesis in prostatic glands induced in the urothelium of testicular feminized (Tfm/Y) mice. J Steroid Biochem 1981;14:1317–1324.

26 Cooke PS, Young P, Cunha GR. Androgen receptor expression in developing male reproductive organs. Endocrinology 1991;128:2867–2873.

27 Keil KP, Abler LL, Laporta J et al. Androgen receptor DNA methylation regulates the timing and androgen sensitivity of mouse prostatic ductal development. Dev Biol 2014;396:237–245.

28 Thomson AA, Timms BG, Barton L et al. The role of smooth muscle in regulating prostatic induction. Development 2002;129:1905–1912.

29 Staeck A, Donjacour AA, Brody J et al. Mouse urogenital development: A practical approach. Differentiation 2003;71:402–413.

30 Simanainen U, Allan CM, Lim P et al. Disruption of prostate epithelial androgen receptor impedes prostate lobe-specific growth and function. Endocrinology 2007;148:2264–2272.

31 Wu CT, Altuwaijri S, Rickle WA et al. Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. Proc Natl Acad Sci USA 2007;104:12679–12684.

32 Zhang B, Kwon OJ, Henry G et al. Non-cell-autonomous regulation of prostate epithelial homeostasis by androgen receptor. Mol Cell 2016;63:976–989.

33 English HF, Santen RJ, Isaacs JT. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate 1987;11:229–242.

AUTHOR CONTRIBUTIONS

Y.H.: conception and design, collection and assembly of data, and manuscript writing; E.H. and G.R.C.: data analysis and interpretation and manuscript writing; E.-J.Y. and H.W.: data analysis and interpretation; Z.S.: conception and design, collection and assembly of data, manuscript writing, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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