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Authors

He, Yongfeng
Hooker, Erika
Yu, Eun-Jeong
[et al.](#)

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

Yongfeng He^{a,c}, Erika Hooker^{a,c}, Eun-Jeong Yu^{a,c}, Huiqing Wu^b, Gerald R. Cunha^d, and Zijie Sun^{a,c}

^aDepartment of Cancer Biology, City of Hope, Duarte, CA 91010-3000

^bDepartment of Pathology, Beckman Research Institute and Cancer Center, City of Hope, Duarte, CA 91010-3000

^cDepartment of Urology, Stanford University School of Medicine, Stanford, CA 94305

^dDepartment of Urology, School of Medicine, University of California San Francisco, San Francisco, CA 94143

Abstract

Androgen signaling is essential for prostate development, morphogenesis, and regeneration. Emerging evidence indicates that Wnt/ 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.

Correspondence: Zijie Sun, Ph.D, MD, Beckman Research Institute and Cancer Center, Beckman Bldg, Room 2311, 1500 E. Duarte Rd., Duarte, 1500 Duarte Road, Duarte, CA 91010 0300, Tel: (626) 218-0955, zjsun@coh.org.

Author contributions: Yongfeng He: Conception and design, collection and assembly of data, and manuscript writing

Erika Hooker: Data analysis and interpretation and manuscript writing

Eun-Jeong Yu: Data analysis and interpretation

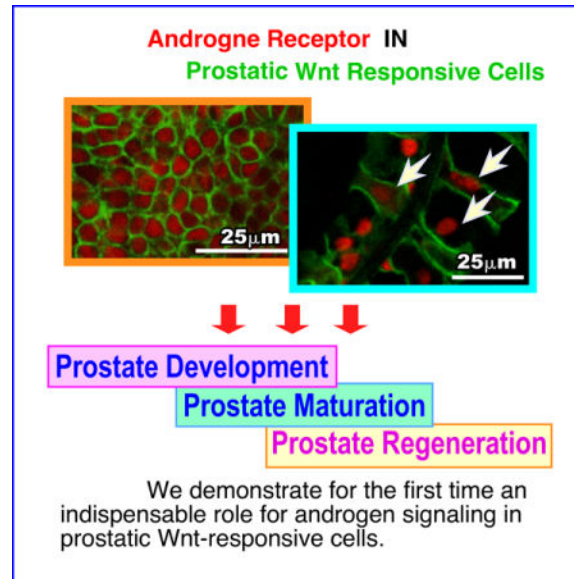
Huiqing Wu: Data analysis and interpretation

Gerald R. Cunha: Data analysis and interpretation and manuscript writing

Zijie Sun: Conception and design, collection and assembly of data, manuscript writing, and final approval of manuscript

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Graphical abstract



Keywords

Androgen receptor; Wnt signaling; β -catenin; Prostate development

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

Axin2^{CreERT2} and *Rosa*^{mTmG} mice were obtained from Jackson Laboratories; stock 18867 and stock 7676) [18, 23]. *Ar*^{fllox/Y} 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.

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) were placed subcutaneously in castrated mice. To elicit genetic recombination mice received a single intraperitoneal injection of 4 mg/25 g body weight tamoxifen (Sigma) suspended in corn oil, corresponding to a total dose of 1 mg for prepubescent mice (injected at P14) and 4 mg of tamoxifen for adult mice (injected at P84). To label *Axin2*-expressing cells in embryos, pregnant mothers received a single intraperitoneal injection at E13.5, totaling 1mg/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, hematoxylin-eosin staining was performed as reported earlier [26, 27]. For immunohistochemistry, slides were treated by boiling in 0.01M

Citrate buffer (pH 6.0) for antigen retrieval, incubated in 0.3% H₂O₂ for 15 min, blocked in 5% normal goat serum for 30 min, 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) 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).

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). Five micron sections were obtained using a Leica cryostat, and slides were washed three times with PBS. For detection of membrane-bound Tomato (mT) and membrane-bound green fluorescent protein (mGFP) signals, slides were directly mounted with VECTASHIELD 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 min, 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 urogenital sinuses (UGS) were dissected at E14.5 and placed on 0.4 μM Millicell-CM filters (Millipore) in 6-well tissue culture plates. Culture media consisted of DMEM-F12 (1:1) media, supplemented with nonessential amino acids, 1% insulin-transferrin-selectin (ITS), L-glutamine, Penicillin/Streptomycin, and 1×10⁻⁸ M dihydrotestosterone (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), anti-GFP (mouse, 1:300, 2955, Cell Signaling), anti-GFP (Chicken, 1:2000, ab13970, Abcam), anti-K5 (rabbit, 1:3000, PRB-160P, Covance), anti-K8 (mouse, 1:2500, MMS-162P, Covance), anti-p63 (mouse, 1:200, sc-8431, Santa Cruz), anti-Ki67 (mouse, 1:1000, NCL-ki67, Novacastra), anti-E-cadherin (mouse, 1:300, c20820, BD Transduction Laboratories), anti-androgen receptor (rabbit, 1:500, sc-816, Santa Cruz). Biotinylated anti-rabbit or anti-mouse secondary antibodies (BA-1000 or BA-9200, Vector Laboratories) were used for immunohistochemistry experiments. For immunofluorescence studies AlexaFluor-conjugated anti-rabbit, anti-mouse or anti-chicken antibodies (Molecular Probes) were used.

Microscope image acquisition

Images of H&E and immunohistochemistry were acquired on an Axio Lab. A1 microscope using 10x and 40x Zeiss A-Plan objectives with a Canon EOS 1000D camera and using Axiovision software (Carl Zeiss). Images of immunofluorescent staining and mTmG signals

and were acquired on a Nikon ECLIPSE E800 epi-fluorescence microscope using 20x and 40x Nikon Plan Fluor objectives with an QImaging RETIGA EXi camera and using QCapture software (QImaging). Epithelial and mesenchymal cell numbers were counted manually using 40x photomicrographs as described in Supplementary Tables S1-S5. Statistical analyses were performed using 2-tailed Student's *t* test or 2-way ANOVA.

RESULTS

Distinctive co-localization of the AR and Axin 2 in the urogenital sinus of embryonic mice

Expression of *Axin2* has been observed in mouse UGE [10]. Using *Axin2*^{CreERT2/+};*Rosa*^{mTmG/+} mice [18] (Fig. 1A), we assessed Axin2 expression at embryonic stages during the course of early urogenital development in mice (Fig. 1B). Prostatic buds emerge from mouse UGE at E16.5 to E17.5 [4]. To evaluate Axin2 expression, we injected tamoxifen (TM) at E13.5 and examined induced membrane-bound green fluorescent protein (mGFP) expression at E15.5 and 17.5 (Fig. 1C). We observed TM-induced GFP expression at E15.5 mainly in urogenital sinus mesenchyme (UGM) in close apposition to the adjoining UGE (Fig. 1C1-2). At E17.5, Axin2 expressing cells were detected in both UGM and UGE (Fig. 1C3, Table S1), especially, in the newly formed prostatic epithelial buds (arrow heads, Fig. 1C4). Since Axin2 has been well-established as a downstream target of Wnt/ β -catenin signaling pathways [13, 14], the above observation implicates the activation of Wnt/ β -catenin signaling in both prostatic epithelium and mesenchyme at prostate embryonic development. In this study, we also examine the expression of Lef-1 and CyclinD1, two bona fide Wnt/ β -catenin downstream targets in Axin2-expressing cells. We observed the co-expression of mGFP with Lef-1 or CyclinD1 in Axin2-expressing cells, which provides an additional line of evidence for the responsiveness of Axin2-expressing cells to Wnt/ β -catenin signaling (Supplemental Figure 1).

Expression of Ar protein has been observed as early as at E13 in mouse UGM prior to prostatic bud formation [28, 29], suggesting that Ar is likely to be expressed in Axin2-expressing Wnt-responsive cells that are found in the UGM. 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. 1D1-4). We further evaluated the cellular identity of Ar positive cells using co-immunofluorescence (co-IF) analyses. As shown in Figure 1E2 and F2, E-cadherin expression appears within UGE areas of both E15.5 and E17.5 mice (Fig. 1E-F). At E17.5 E-cadherin staining was observed in epithelial cells of the newly formed prostatic buds (arrow heads, Fig. 1F2-4) co-expressing Ar (Fig. 1F3 and 1F4). Further analyses show that a subset of these Ar-expressing epithelial cells is also immunoreactive for Ck8, p63, and Ki67 (Supplemental Figure 2). Intriguingly, a portion of E-cadherin-positive epithelial cells in the UGS at E15.5 and E17.5 were also membrane-bound GFP positive, indicating their Wnt-responsive cellular identities (compare Fig. 1C3-4, and 1F3-4). 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 co-localization of Ar and GFP expression within the UGM at E15.5 (Fig. 2A4-5), in the closely adjoining UGE (Fig. 2B1-4), 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 UGE and UGM between E15.5-17.5 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. 2C4-5), Ck8 (Fig. 2D4-5), p63 (Fig. 2E4-5), and in some cases also to Ki67 (Fig. 2F4-5). 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. 2G4-5), which appear immunoresponsive to the both Ck8 and p63 antibodies (Arrowheads, Fig. 2H4-5, 2I4-5) as well as Ki67 antibody (Arrowheads, Fig. 2J4-5). 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 $R26R^{mTmG/+}; Ar^{floxed/Y}; Axin2^{CreERT2/+}$ ($R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$) 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 $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ and control $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ fetuses, and cultured them with 2 μ M of (Z)-4-Hydroxytamoxifen (Fig. 3B). After seven days of *ex vivo* culture, sizes of UGS tissues from $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ mice appeared smaller than ones from $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ controls (n=4), respectively. The representative images are shown in Figure 3C1-2 and 3D1-2. Histologically, we observed glandular structures in the center of UGS of $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ controls, which is very similar to normal prostatic budding formation in UGS *in vivo*, but not in $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ fetuses (Fig. 3E1-4). Analysis of E-cadherin expression in frozen sections revealed more prostatic budding in cultured UGS tissues of $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ controls than of $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ fetuses (Fig. 3F1-4). 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 Fig. 3G1-2 and 3I, almost all of E-cadherin-positive UGE cells are Ar positive in $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ controls. In contrast, only a few E-cadherin and Ar positive cells were observed in UGE from $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ fetuses (Fig. 3G3-4, and 3I). Accordingly, there is a significant reduction of Ki67 positive cells in UGE of $R26R^{mTmG/+}; Ar^{f/Y}; Axin2^{CreERT2/+}$ in comparison with UGE cells of $R26R^{mTmG/+}; Axin2^{CreERT2/+}$ controls (Fig. 3H1-4 and 3I, Table S2). Taken 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 $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ and $R26R^{mTmG/+};Axin2^{CreERT2/+}$ fetuses under kidney capsule (Fig. 4A). After 8 weeks of implantation, UGS grafts isolated from $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ were smaller in size and weighed less than ones from $R26R^{mTmG/+};Axin2^{CreERT2/+}$ controls, (see the representative images in Fig. 4B1-B2 and 4C). In co-IF experiments, we observed much more AR positive prostatic epithelial cells in the grafts of $R26R^{mTmG/+};Axin2^{CreERT2/+}$ controls (Fig. 4E1-2) than those of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice (Fig. 4E3-4). As a consequence, Ar deleted grafts also showed less Ki67 expressing cells than controls (Fig. 4F1-4, Table S3). Intriguingly, we observed smaller and abnormal acini with flattened epithelial cells in prostatic glands of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ grafts (Arrows, Fig. 4H1-4), suggesting an impaired prostate development phenotype. We only observed a minor subset of the GFP-positive epithelial cells appeared flattened. These flattened cells are also immunoreactive to Ck8 but not Ck5 antibody (Supplemental Figure 3), 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 to 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 $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ and $R26R^{mTmG/+};Axin2^{CreERT2/+}$ mice at postnatal day 14, P14 (Fig. 5A). Analysis of those mice after six weeks, at P56, showed smaller and underdeveloped prostates in mutant $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice in comparison to $R26R^{mTmG/+};Axin2^{CreERT2/+}$ controls (Fig. 5B1 and 5B3). Specifically, anterior prostate (AP) and dorsolateral prostate (D/LP) of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ lobes are much smaller in size than those in controls (Fig. 5B2 versus B4, blue and pink arrowheads). The prostate weights of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice are also less than those of $R26R^{mTmG/+};Axin2^{CreERT2/+}$ controls (Fig. 5C). Ar expression was significantly reduced in prostatic glands of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice in comparison to those of controls (Fig. 5E1-4). Less Ki67 positive epithelial cells appear in prostatic tissues isolated from $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice than those from $R26R^{mTmG/+};Axin2^{CreERT2/+}$ controls (Fig. 5D, and F1-F4, Table S4). As expected, normal prostatic glandular architecture was present in $R26R^{mTmG/+};Axin2^{CreERT2/+}$ control mice (Fig. 5G1-6). In contrast, pathological changes featured with scattered foci of marked glandular attenuation was observed in prostatic lobes of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice (arrows, Fig.

5H1-6). 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 relies 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 $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ and $R26R^{mTmG/+};Axin2^{CreERT2/+}$ 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 $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ and $R26R^{mTmG/+};Axin2^{CreERT2/+}$ mice (Fig. 6B1-3 and D1-3). 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 $R26R^{mTmG/+};Axin2^{CreERT2/+}$ mice (Fig. 6B1-3, 6C1-3, and 6F). In contrast, only slight expansion of mGFP-positive cells revealed in prostatic tissues of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice (Fig. 6D1-3, E1-3, and 6F, Table S5). Interestingly, we noticed that the majority of mGFP positive cells in regenerated prostatic tissues of $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice showed Ar positive staining, indicating the requirement of Ar in Axin2 expressing cell mediated regeneration (Arrowheads, Fig. 6G4). 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 a 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 co-expression 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 co-expressed 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 *R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}* 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 administrated 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 *R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}* 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 P25-40, 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 *R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}* and *Ar^{f/Y};Axin2^{CreERT2/+}* only mice were significantly reduced as compared to their normal counterparts. The lack or reduced expression of both the Ar and Ki67 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 *R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}* 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 *R26^{mTmG/+}:Ar^{f/Y}:Axin2^{CreERT2/+}* mice and *R26^{mTmG/+}:Axin2^{CreERT2/+}* controls. In the presence of androgens, those cells are able to expand and regenerate prostatic luminal cells in *R26^{mTmG/+}:Axin2^{CreERT2/+}* 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 *R26^{mTmG/+}:Ar^{f/Y}:Axin2^{CreERT2/+}* 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 *R26^{mTmG/+}:Ar^{f/Y}:Axin2^{CreERT2/+}* 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 *R26^{mTmG/+}:Ar^{f/Y}:Axin2^{CreERT2/+}* 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.

CONCLUSIONS

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANT 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. In this study, we developed a series of novel and biologically relevant mouse models to assess the biological role of Ar in Wnt responsive cells at different stages during mouse prostate development. Using these mouse models, we demonstrated for the first time that Ar expression is indispensable in Axin2-expressing cells during prostate development and regeneration. Deletion of Ar in Axin2-expressing cells at embryonic or pre-pubescent stage impairs prostate early development and results in underdeveloped prostate, respectively. When Ar was deleted in castration-resistant Axin2-expressing cells, the regenerative ability of Axin2-expressing cells was decreased significantly.

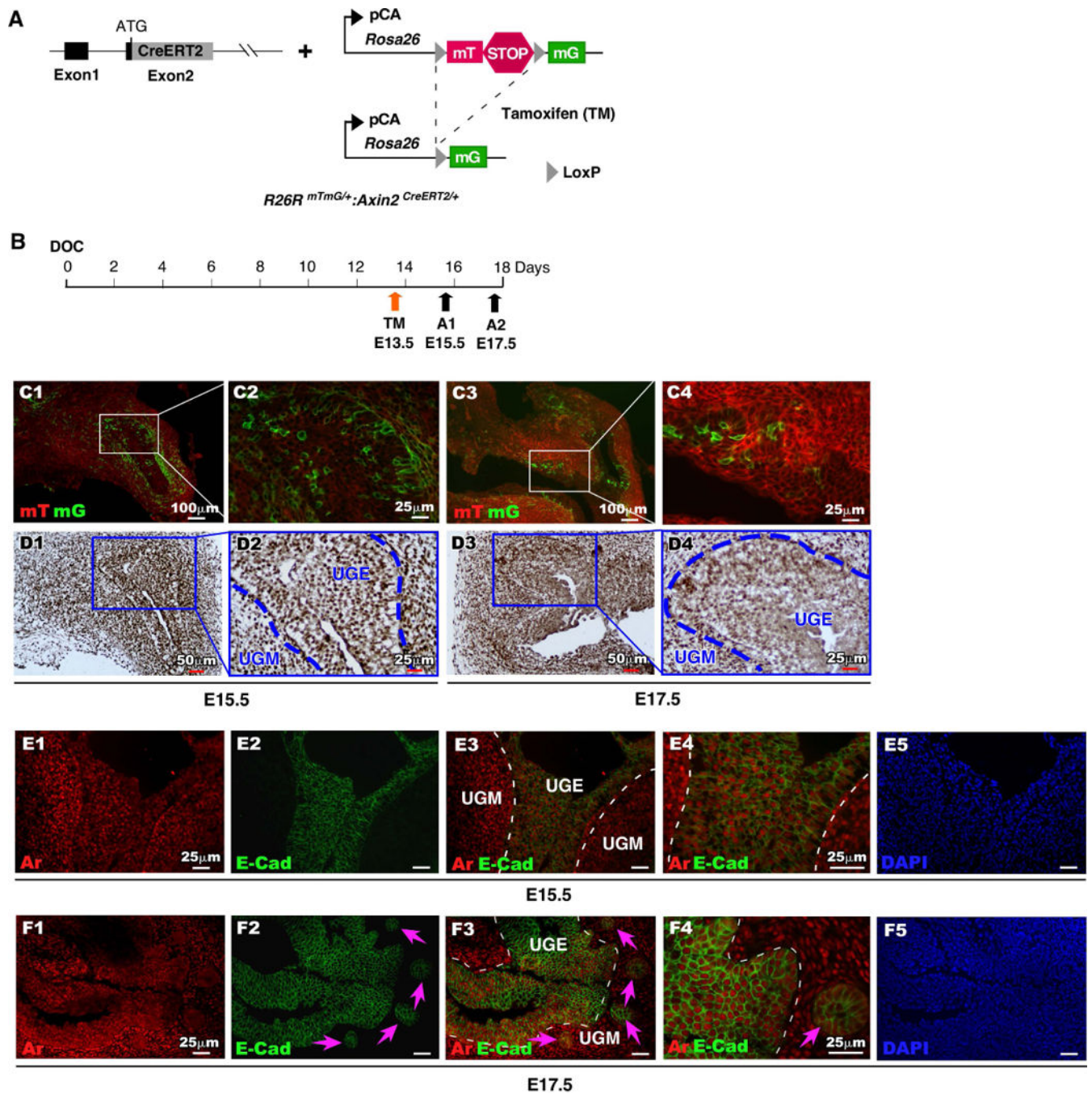


Figure 1. Temporal analysis of Axin2 and Ar expression during early development of mouse prostate

(A): Illustration of transgenic alleles present in $R26R^{mTmG/+};Axin2^{CreERT2/+}$ mice. (B): Schematic depicting experimental timeline for labeling and analyzing UGS tissues during embryonic stage. (C): mTomato (red) or mGFP (green) expression in frozen tissue sections of male UGS from E15.5 and E17.5. (D): Ar expression (brown) in male UGS tissues from both E15.5 and E17.5. (E-F): Localization of Ar (red) and E-cadherin (green) in male UGS tissues isolated at E15.5 and E17.5.

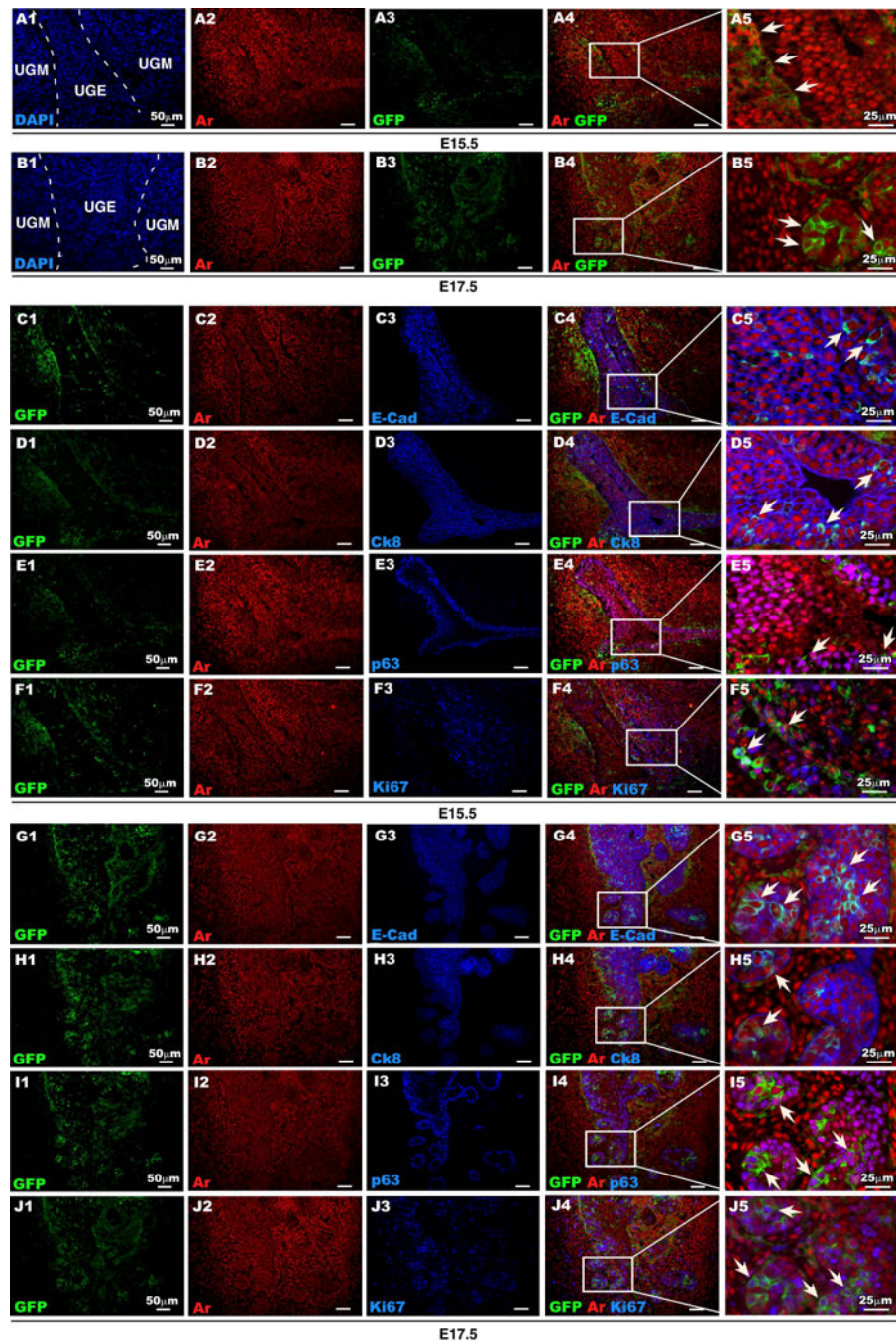


Figure 2. Expression of Ar in Axin2-expressing cells during early development of mouse prostate (A-B): Ar (red) and GFP (green) expression in male UGS isolated at E15.5 and E17.5. **(C-F):** Colocalization of Ar (red) and GFP (green) with E-cad, CK8, p63 or Ki67 (blue) in UGS tissue isolated at E15.5. **(G-J):** Colocalization of Ar (red) and GFP (green) with E-cad, CK8, p63 or Ki67 (blue) in UGS tissue isolated at E17.5.

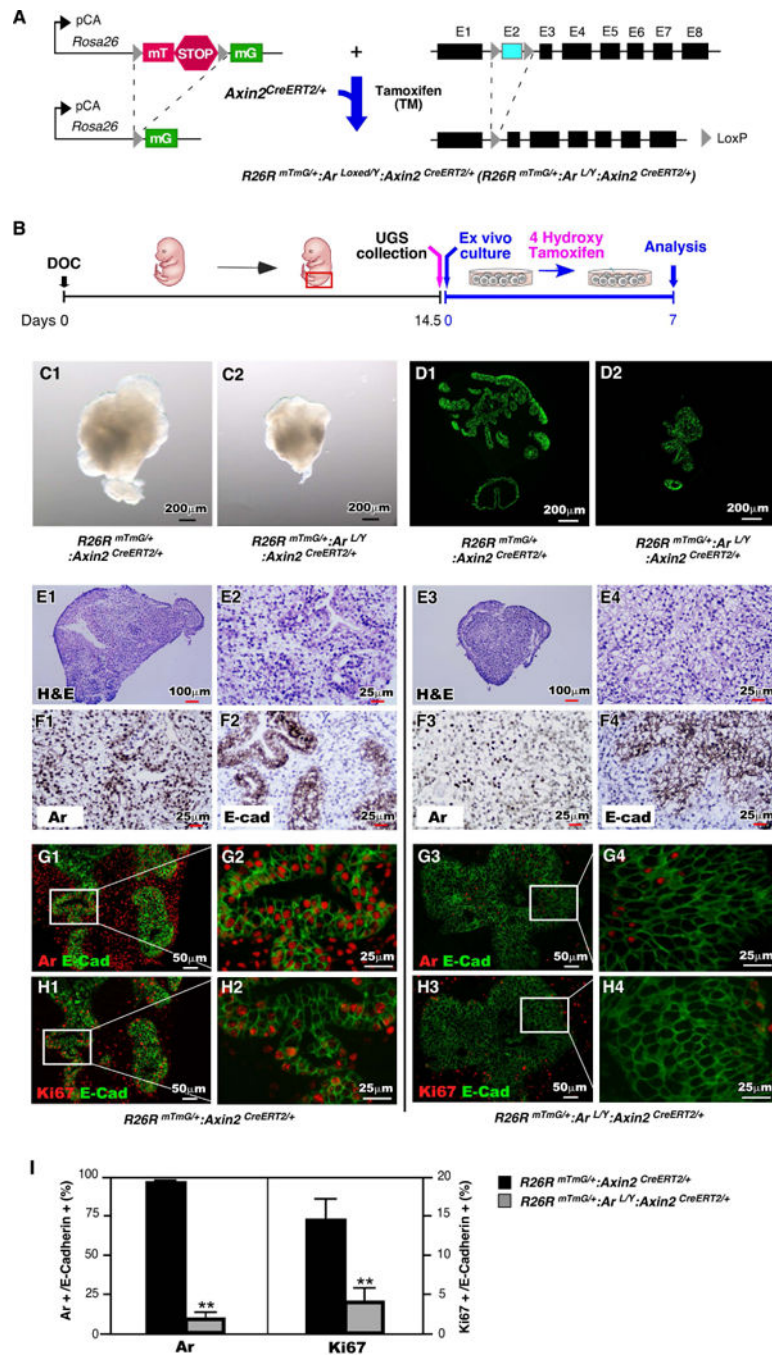


Figure 3. Ar is required in Axin2-expressing cells during prostatic bud formation of ex vivo cultured UGS

(A): Illustration of floxed alleles present in *R26R^{mTmG/+};Ar^{f/Y+};Axin2^{CreERT2/+}* 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^{mTmG/+};Axin2^{CreERT2/+}* or *R26R^{mTmG/+};Ar^{f/Y+};Axin2^{CreERT2/+}* 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<0.01.

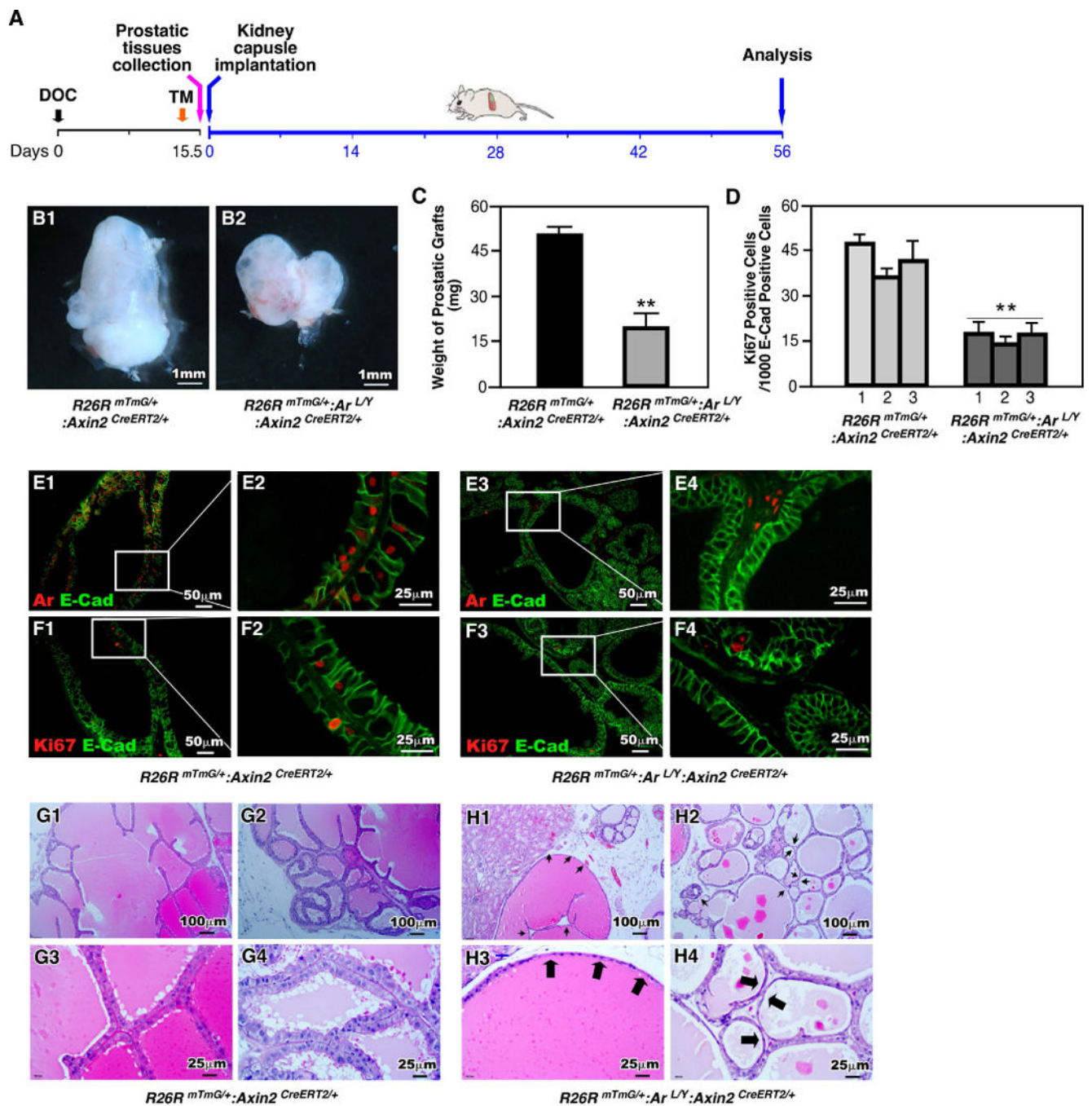


Figure 4. Deletion of Ar in Axin2 expressing cells during embryonic stage impairs prostate gland formation in renal capsule of SCID mice

(A): Schematic demonstrating experimental timeline including activation of Axin2^{CreER}, UGS collection, renal capsule transplantation and analysis. (B): Gross images of xenografts from UGS tissue isolated from *R26R^{mTmG/+};
:Axin2^{CreERT2/+}* or *R26R^{mTmG/+};
:Ar^{LY};
:Axin2^{CreERT2/+}* mice. (C): Graphical representation of xenograft weight from grown from mice of each genotype. ** P<0.01. (D): Quantification of Ki67 positive cells in the epithelial cells from xenografts of each genotype. ** P<0.01. (E-F):

Colocalization of E-cadherin (green) with Ar or Ki67 (red) in *R26^{fl};^{mTmG/+};^{CreERT2/+}Axin2^{CreERT2/+}* and *R26^{fl};^{mTmG/+};^{Ar^{f/Y};^{CreERT2/+}Axin2^{CreERT2/+}}* xenografts. **(G-H)**: Representative H&E staining of tissue sections from xenografts of each genotype.

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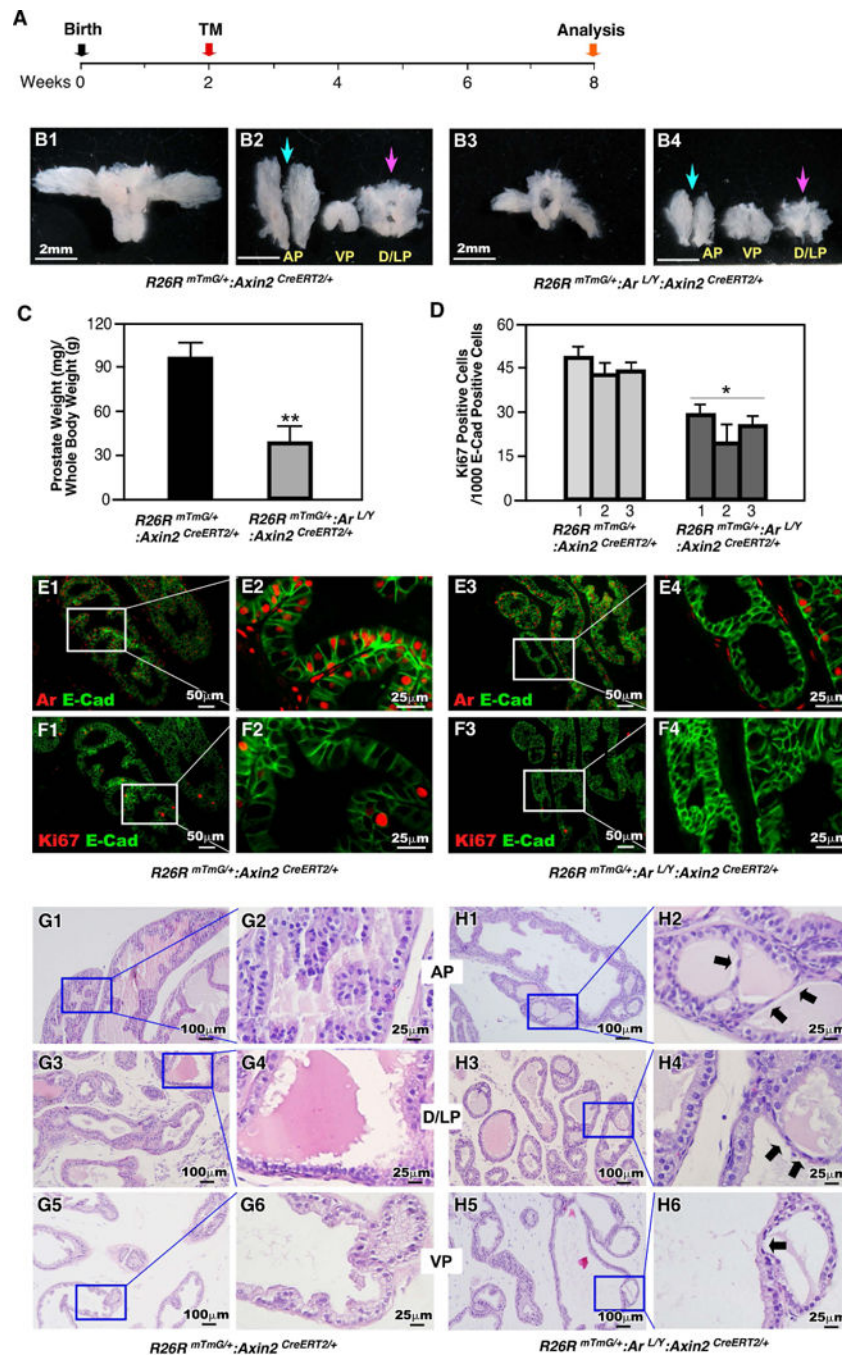


Figure 5. Ar is required in prepubescent Axin2-expressing cells for prostate development (A): Illustration depicting experimental timeline of Axin2^{CreER} activation (TM injection) and tissue analysis. (B): Gross images of prostates isolated at P56 from $R26R^{mTmG/+};Axin2^{CreERT2/+}$ or $R26R^{mTmG/+};Ar^{LY};Axin2^{CreERT2/+}$ mice. (C): Graphical representation of the ratio of prostate wet weight/body weight. ** P<0.01. (D): Quantification of Ki67 positive cells per 1000 epithelial cells (E-cadherin positive) from prostate tissues of each genotype. * P<0.05. (E-F): Colocalization of E-cadherin (green) with Ar or Ki67 (red) in prostates isolated from $R26R^{mTmG/+};Axin2^{CreERT2/+}$ or

R26^{mTmG/+};Arl^{f/Y};Axin2^{CreERT2/+} mice. **(G-H)**: H&E staining of tissue sections from prostates of each genotype.

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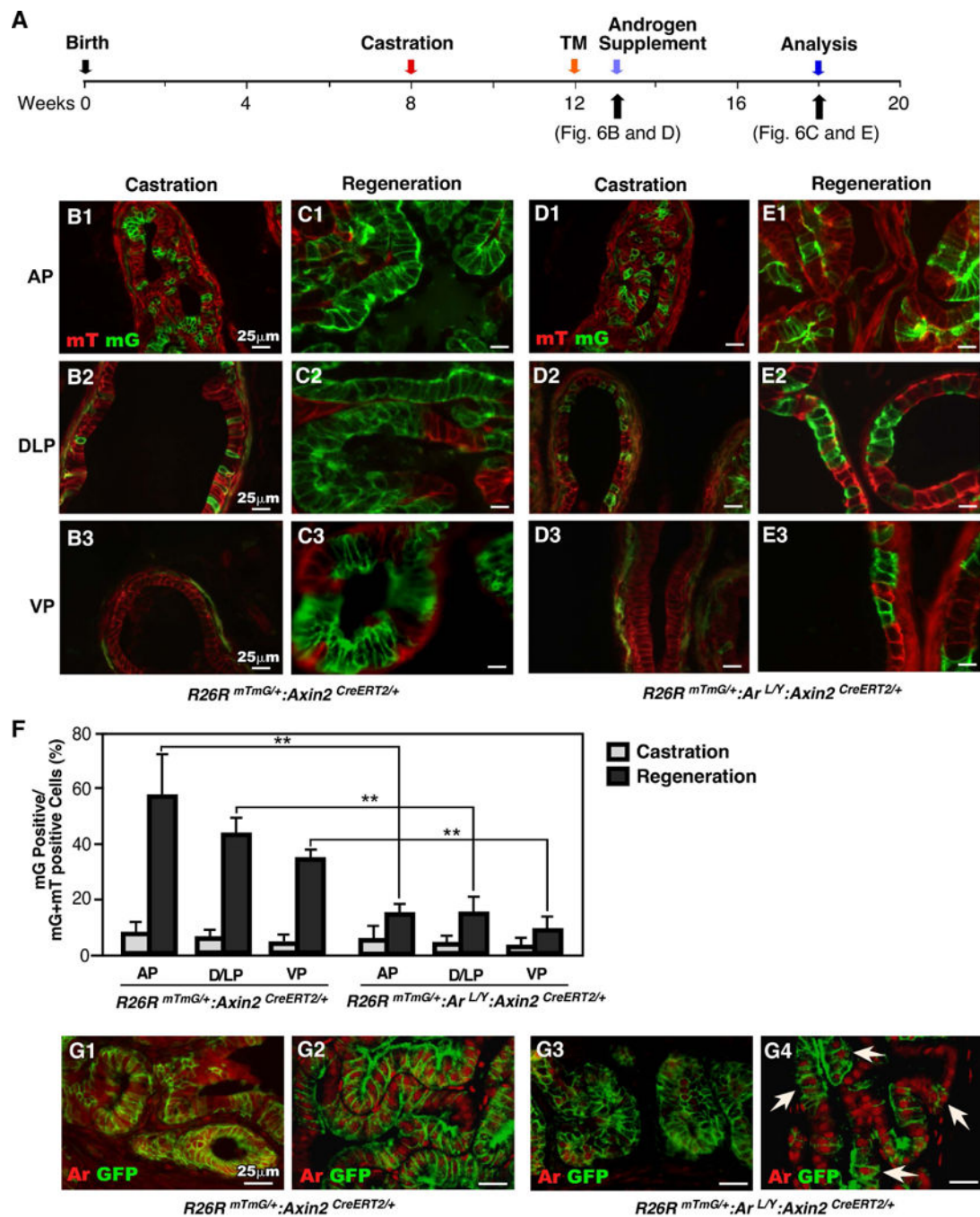


Figure 6. Ar is required for the regenerative potential of castration-resistant Axin2-expressing cells

(A): Diagram of experimental design describing timelines for castration, activation of Axin2^{CreER} (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<0.01. (G) Colocalization of

GFP (green) and Ar (red) in frozen sections of both castrated and regenerated prostate tissues from $R26R^{mTmG/+};Axin2^{CreERT2/+}$ and $R26R^{mTmG/+};Ar^{f/Y};Axin2^{CreERT2/+}$ mice.

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