Non-cell-autonomous regulation of prostate epithelial homeostasis by androgen receptor

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Summary

Prostate inflammation has been suggested as an etiology for benign prostatic hyperplasia (BPH). We show that decreased expression of the androgen receptor (AR) in luminal cells of human BPH specimens correlates with a higher degree of regional prostatic inflammation. However, the cause-and-effect relationship between the two events remains unclear. We investigated specifically whether attenuating AR activity in prostate luminal cells induces inflammation. Disrupting luminal cell AR signaling in mouse models promotes cytokine production cell-autonomously, impairs epithelial barrier function, and induces immune cell infiltration, which further augments local production of cytokines and chemokines including IL-1 and Ccl2. This inflammatory microenvironment promotes AR-independent prostatic epithelial proliferation, which can be abolished by ablating IL-1 signaling or depleting its major cellular source, the macrophages. This
study demonstrates that disrupting luminal AR signaling promotes prostate inflammation, which may serve as a mechanism for resistance to androgen-targeted therapy for BPH.

**eTOC**

Zhang et al demonstrate that decreased AR signaling in mouse prostate luminal cells upregulates cytokines and chemokines cell-autonomously and impairs epithelial barrier function. These lead to an increased immune cell infiltration into the prostate. IL-1 generated by macrophages promotes AR-independent epithelial proliferation by effecting prostate stromal cells.

**Keywords**

Androgen receptor; epithelial homeostasis; benign prostatic hyperplasia; inflammation; microenvironment; macrophage; interleukin-1

**Introduction**

Benign prostatic hyperplasia (BPH) is a progressive condition in aging men that is characterized by the enlargement of the periurethral region of the prostate gland due to nonmalignant proliferation in both the epithelial and stromal compartments (Bushman, 2009; Isaacs, 2008). The underlying etiology remains unclear. Androgen receptor-mediated signaling has been shown to play a role in BPH progression (Izumi et al., 2013), and androgen targeted therapies such as finasteride, a 5-α-reductase inhibitor (5ARI), are clinically employed for the treatment of BPH (McConnell et al., 2003).

Paradoxically, male ageing is associated with a substantial increase in the incidence of BPH, but progressive and continuous declines in serum testosterone levels (Harman et al., 2001). Androgen negatively modulates immune and inflammatory responses (Schmidt et al., 2006) while prostate inflammation has been suspected as an etiological factor for BPH (Bushman, 2009). Therefore, it has been hypothesized that declining androgen levels increases the incidence of BPH by promoting prostate inflammation. This hypothesis is supported by several lines of evidence. For example, higher testosterone levels are associated with a decreased risk of BPH (Kristal et al., 2008). In addition, anti-androgen therapies for BPH
and prostate cancer induce immune cell infiltration and increased production of pro-
inflammatory cytokines (Maggio et al., 2005; Maggio et al., 2006; Sorrentino et al., 2011).
Finally, androgen supplementation suppresses metabolic syndrome-associated prostate
inflammation in a rabbit model (Vignozzi et al., 2012b). These observations suggest that
androgin signaling is necessary to suppress the development of prostatic inflammation.

Various mechanisms have been proposed to account for how androgen-mediated signaling
suppresses prostate inflammation. A prevailing theory is that a decreased ratio of androgen
to estrogen disrupts the balance between androgen-mediated immunosuppressive action and
estrogen-mediated proinflammatory signaling, thereby leading to prostate inflammation
(Cutolo et al., 2002). A second mechanism suggests a direct impact of AR signaling on the
production of inflammatory cytokines by prostate stromal or epithelial cells (Vignozzi et al.,
2012a). Finally, AR has also been shown to impinge on prostatic micro-environmental
homeostasis by regulating prostate glandular architecture (Meng et al., 2011). However, the
contributions of individual mechanisms to prostate inflammation have not been validated in vivo
due to various technical limitations. Androgen deprivation studies cannot distinguish
the contribution of androgen action in different cell populations. In studies using Ar
knockout mouse models, Ar was often disrupted in all epithelial cells at the pre-pubertal
stage (Simanainen et al., 2007; Wu et al., 2007). Therefore, it is difficult to distinguish
whether the resulting phenotypes reflect the role of AR in epithelial development or
homeostasis, and whether the altered biology is caused directly by loss of intracellular AR
action or indirectly through non-cell-autonomous mechanisms. In this study, we employed
an improved mouse model to specifically disrupt AR expression in a fraction of adult murine
prostate luminal epithelial cells and investigated whether and how decreased AR signaling in
prostate luminal cells induces prostate inflammation.

Results

AR expression is decreased in luminal cells of human BPH specimens and is inversely
correlated with the degree of regional prostatic inflammation

Prostate volume is an objective determinant of BPH progression. Previous studies suggested
that inflammation was increased with prostate volume based on subjective pathological
review (Nickel et al., 2007). We performed flow cytometry to quantitate the percentage of
CD45+ cells in 35 patient samples across a broad range of prostate volumes. The specimens
were transition zone samples from 5ARI-treament naïve patients who underwent open
simple prostatectomy for obstructive symptoms due to large volume BPH (Figure S1A).
Given the difficulties in acquiring rapid autopsy tissues from age-matched men with
congruent clinical information, the best age-matched, small to medium volume transition
zone samples available came from patients being treated by transurethral resection of the
prostate (TURP) and from patients undergoing robotic assisted prostatectomy for low
Gleason grade, peripheral zone localized tumors (Strand et al., 2015). As shown by
representative FACS plots in Figure 1A, we observed a significantly higher percentage of
CD45+ leukocytes in larger volume prostates. Figure 1B shows that the percentage of
leukocytes correlated with prostate volume (R² = 0.23, p = 0.004, n=35).
We next sought to investigate whether the AR expression level is altered during BPH progression. Briefly, prostate basal cells (CD45−/CD31−/EpCAM+/CD49fhigh/CD26low), luminal cells (CD45−/CD31−/EpCAM+/CD49flow/CD26high), and stromal cells (CD45−/CD31−/EpCAM−/CD49flow/CD26−) were FACS-isolated as reported previously (Goldstein et al., 2011) and efficient isolation was validated by qPCR analysis of lineage marker expression (Figure S1B). Luminal epithelial AR ($R^2 = 0.23$, $p = 0.03$) and PSA ($R^2 = 0.25$, $p = 0.02$) transcript levels are inversely correlated with prostate volume ($n=21$, Figure 1C), but basal and stromal AR transcript levels are not significantly altered (Figure S1C). The AR primers recognize both the full length AR and the alternatively spliced AR isoforms. Finally, using a tissue microarray established at the Baylor College of Medicine, we further showed that AR is expressed at a lower level significantly in BPH tissues of larger prostates (> 30 cc) than in those of smaller prostates (< 30 cc) (Figure 1D), although there was no significant correlation between the AR expression scores and prostate volumes.

Coimmunostaining of AR and CD45 was performed to determine whether a physical association existed between AR-low glands and inflammation. Regardless of prostate volume, we noted that inflamed glands were also deficient in luminal epithelial AR levels although adjacent non-inflamed glands displayed normal AR levels (Figure 1E and 1F). To quantitate the phenotypic correlation between inflammation and AR expression, we used ImageJ to segment inflammatory cells and AR-positive cells across a range of inflamed and non-inflamed regions as represented in Figure 1E and 1F. After analyzing over 150 images across 21 patient samples, we found that regional inflammation was inversely correlated with AR positivity ($R^2 = 0.32$, $p < 0.0001$, Figure 1G). Interestingly, Ki67+ proliferating epithelial cells were frequently observed in the AR-low specimens collected from both the University of Texas Southwestern Medical Center and Baylor College of Medicine (Figure S1D and S1E). Collectively, these results demonstrate that luminal cell-specific AR expression is decreased during BPH progression and is inversely correlated with the degree of regional prostatic inflammation.

**Temporal induction of Ar knockout in adult murine prostate luminal epithelial cells**

The cause-and-effect relationship between inflammation and decreased AR expression in the luminal cells remains unclear. We sought to use mouse models to specifically investigate whether decreased Ar expression in the luminal cells is capable of inducing prostate inflammation. We generated K8-CreERT2;Arfl/Y mice (hereafter referred to as the K8-Ar mice) by breeding an Ar conditional line (De Gendt et al., 2004) with a K8-CreERT2 transgenic mouse line that harbors the tamoxifen-responding CreERT2 transgene driven by the promoter of the luminal cell marker cytokeratin 8 (K8) (Zhang et al., 2012). As shown in Figure 2A, 8-week-old K8-Ar mice were treated with tamoxifen transiently to induce Ar knockout in prostate luminal cells. The dosage of tamoxifen (9mg/40g/day for 4 days) for effective Ar disruption was determined by titration (Figure S2A). Littermate Arfl/Y mice (hereafter referred to as the control) were treated likewise and used as controls. There is no significant difference in prostate morphology, weight (Figure 2B), and glandular structures (Figure S2B) even 10 weeks after Ar deletion. Previous studies showed that prostate epithelial-specific Ar deletion at the prepubertal stage driven by a probasin promoter (PB-Cre:Arfl/Y, hereafter referred to as the PB-Ar model) led to an expansion of intermediate
cells that are dual positive for both K8 and the basal cell marker cytokeratin 5 (K5) (Niu et al., 2011). This did not occur in our model (Figure S2C), suggesting that the emergence of K5⁺K8⁺ intermediate cells in the PB-Ar model may reflect impaired epithelial maturation due to prepubertal disruption of Ar signaling.

Coimmunostaining of Ar and K8 showed that Ar is detectable in 97.8% of luminal cells in the control group (Figure 2C). In contrast, only 27.9% of luminal cells expressed Ar in the dorsolateral prostate lobes (DLP) of K8-Ar mice 2 weeks after tamoxifen treatment. QRT-PCR analysis showed that three typical Ar target genes Nkx3.1, Pbsn and Psp94 were substantially downregulated (Figure 2D), corroborating successful Ar deletion. In contrast, Ar deletion was very inefficient in the anterior (AP) and ventral (VP) lobes (Figure 2C). Therefore, we focused on DLPs in the following studies unless otherwise noted.

Interestingly, the percentage of Ar⁺ luminal cells in K8-Ar mice increased gradually (27.87% at 2 weeks vs. 53.4% at 10 weeks post tamoxifen treatment), albeit remaining significantly less than that in the control mice (Figure 2E). These results show that disrupting Ar does not lead to acute death of all luminal epithelial cells, but Ar-null luminal cells contribute less to the maintenance of the luminal cell lineage than Ar-intact luminal cells.

**Ar deletion in luminal cells augments apoptosis cell-autonomously and promotes cell proliferation non-cell-autonomously**

We investigated why Ar-null cells were less competent in maintaining epithelial homeostasis. Coimmunostaining of BrdU and K8 showed that the proliferation index of luminal cells increased by 3.65 and 2.40 fold at 2 wks and 10 wks post Ar deletion, respectively (Figure 3A; 3B; 3C and 3D). This increased proliferation is consistent with the previous reports using the PB-Ar model (Simanainen et al., 2007; Wu et al., 2007), which was considered as a direct result of loss of intracellular Ar transcriptional activity since Ar positively regulates luminal cell differentiation. However, we found that the increased BrdU incorporation took place in both the Ar⁻ and Ar⁺ cells (pie charts, Figure 3C and 3D). The percentages of the newly-generated Ar⁺ luminal cells (BrdU⁺Ar⁺K8⁺) correlated with those of the Ar⁺ luminal cells (Ar⁺K8⁺) at both 2 weeks (Figure 3E) and 10 weeks (Figure 3F) after induced Ar deletion. These results suggest that the Ar⁻ and Ar⁺ luminal cells proliferated at similar rates. Collectively, our study implies that disrupting Ar activity in luminal cells leads to the formation of extracellular signaling that in turn promotes the proliferation of both Ar-null and Ar-intact cells.

Coimmunostaining of BrdU and K5 showed that the proliferative index of K5⁺ basal cells was also increased by 3.75 and 2.65 fold at 2 and 10 wks post Ar deletion, respectively (Figure 3G). Immunostaining of the other basal cell marker P63 confirmed the increased basal cell numbers (Figure S3A). These results imply that the extracellular signaling induced by AR deletion in luminal cells is capable of promoting proliferation of both luminal and basal cells.

Immunostaining of the cleaved caspase 3 (CC3) also revealed that the apoptotic index of K8⁺ luminal cells was increased by 2.96 and 2.17 fold at 2 and 10 weeks after induced Ar deletion in luminal cells augments apoptosis cell-autonomously and promotes cell proliferation non-cell-autonomously
deletion, respectively (Figure 3H, p<0.005). We were unable to determine the Ar status of the CC3+ cells directly because antibody host species are incompatible. These CC3+ cells are very likely Ar-null because the Ar-null and Ar-intact cells proliferated at the same rate (Figure 3E and 3F) but the percentage of Ar-null cells decreased during ageing (Figure 2E). In addition, the percentage of Ar-null luminal cells decreased by 1/3 from 72% at 2 weeks to 47% at 10 weeks after Ar deletion (Figure 2E), whereas the percentage of CC3+ cells was also reduced by 1/3 (from 1.39% to 0.89%) at the same time (Figure 3H). These results support that Ar is necessary for the optimal survival of the luminal cells, and explain why Ar-null luminal cells are less competent than Ar-intact cells in contributing to epithelial homeostasis during ageing (Figure 2E).

To determine whether Ar deletion induced senescence, we performed qRT-PCR analysis of senescence-associated cell cycle regulators, Western blot analysis of phosphor-H2AX, and the senescence-associated β-galactosidase assay. Our results showed no sign of cellular senescence after Ar deletion (Figure S3B; S3C and S3D).

Ar deletion in prostate luminal cells promotes production of inflammatory cytokines and chemokines and immune cell infiltration into the prostate

To identify the extracellular signaling that promotes epithelial proliferation, we performed RNA sequencing of FACS-isolated luminal cells from tamoxifen treated control and K8-Ar mice. We identified 1483 and 313 genes that were upregulated and downregulated, respectively, by at least 1.4 fold in the K8-Ar group. Gene ontology analysis reveals that groups related to immune and inflammatory responses were upregulated in luminal cells of the K8-Ar group (Figure 4A). This is consistent with previous reports showing repression of cytokines by androgen in various types of cells (Lin et al., 2013; Vignozzi et al., 2012a).

Figure 4B shows that Nfκb2, Tgfb3, and many cytokines and chemokines were all upregulated in K8-Ar luminal cells. Interestingly, IκBs were also upregulated, indicating a negative feedback response to increased inflammatory signaling.

We then examined whether increased production of inflammatory factors affected lineage composition in prostatic microenvironment by FACS analysis. Two weeks after tamoxifen treatment, the percentage of CD45+ leukocytes in the prostate tissues of K8-Ar mice was approximately 1.62 fold of that in the control mice. The percentages of T cells, macrophages and neutrophils in the prostates were all increased significantly (Figure 4C and Figure S4A). The extent of leukocyte infiltration correlated to the percentage of Ar-null luminal cells. For example, leukocyte infiltration was not significantly augmented in anterior and ventral prostates where Ar deletion was very inefficient (Figure S4B). In addition, leukocyte infiltration in dorsolateral lobes declined at 10 weeks after tamoxifen treatment (Figure S4C), which is also consistent with the decreasing percentage of Ar-null luminal cells during this period of time (Figure 2E). Leukocyte infiltration was not reported in previous studies using the PB-Ar model (Simanainen et al., 2007; Wu et al., 2007). We generated PB-Ar mice and confirmed leukocyte infiltration in 10-wk-old mice (Figure S4D), but the increase in epithelial proliferation at this age did not reach statistical significance (Figure S4E). Interestingly, it is difficult or impossible to notice these phenotypes, especially the increased epithelial proliferation, in other mouse models in which Ar activity is suppressed in both...
epithelial and stromal cells such as castration and enzalutamide treatment (Figure S4F and S4G). This is because suppressing Ar signaling in stromal cells inhibited epithelial proliferation; hence there is no control available to enable the detection of potential increase in epithelial proliferation induced by loss of Ar signaling in the luminal cells.

We reasoned that the increased immune cell infiltration may further affect prostatic local concentration of cytokines and chemokines. A pathway-focused PCR array was performed to determine the expression of 84 cytokines, chemokines and their receptors in total prostate tissues at 2 weeks post-tamoxifen treatment. Table S1 shows that, among those genes that expressed at an easily detectable level (> 0.001 of that of Gapdh), 15 were upregulated and 8 were down-regulated by at least 1.5 fold in the Ar KO groups. Chemokines such as Ccl17 have been shown previously to play a role in prostatic infiltration of T cells in human specimens upon androgen deprivation therapy (Sorrentino et al., 2011), while Ccl2 and Ccl4 have been shown to promote recruitment of neutrophils and macrophages (Fang et al., 2013; Qian et al., 2011). An important cytokine Il-6 that is not included in the PCR array was assayed separately and shown not altered in total prostate tissues (Figure S4H). A cytokine antibody array confirmed that many of these cytokines and chemokines, including IL-1α, IL-1β, Ccl2, and Cxcl10 etc., were also upregulated at the protein level (Table S1 and Figure S4I).

Among the upregulated genes, Il1α, Il1β, Tnfa, Ccl2, and Cxcl10 are expressed at relatively higher levels (> 0.01 of that of Gapdh), and have been shown previously to regulate prostate epithelial proliferation and apoptosis. We further determined the cellular source of these cytokines through qRT-PCR analysis using FACS-sorted different cell lineages. As expected, Ar and its target genes were significantly downregulated in the luminal cells isolated from tamoxifen-treated K8-Ar mice (Figure S4J). Figure 4D; 4E and 4F show that Tnfa, Il1α and Il1β are mainly expressed by leukocytes. Tnfa and Il1β were upregulated in the leukocytes of K8-Ar mice. The IL-1 receptor antagonist Il1rn was also upregulated in the K8-Ar group (Table S1), reflecting a feedback response to the increased IL-1 signaling. Ccl2 is mainly expressed by prostate stromal cells (Figure 4G). Cxcl10 is expressed by prostate basal, stromal cells, and leukocytes and its expression was upregulated in these cells in the K8-Ar group (Figure 4H). Finally, analyses shown in Figure 4D to Figure 4I also confirm the RNA-seq result (Figure 4A) that cytokines and chemokines including Il1s, Ccl2, and Il6 were upregulated in the Ar null luminal cells. Collectively, these results show that although many cytokines and chemokines are upregulated in the Ar null luminal cells, the increased expression of these cytokines and chemokines in the prostate tissues of K8-Ar mice is mainly due to the infiltrated leukocytes, especially neutrophils and macrophages as they are the major sources of these inflammatory factors.

**IL-1 is both essential and sufficient for Ar deletion-induced epithelial proliferation**

QRT-PCR analyses show that Il1α and Il1β are both significantly upregulated in human BPH specimens (Figure 5A), as has been reported previously (Giri and Ittmann, 2000). IL-1α upregulated CCL2 and CXCL10 significantly in the primary human prostate stromal cells (Figure 5B). We reasoned that IL-1 plays a critical role in modulating the expression of cytokines and chemokines by prostate stromal cells and promoting epithelial proliferation.
We investigated whether increased Il1α expression is capable of promoting prostate epithelial proliferation in vivo using a prostate regeneration assay. In this assay, dissociated adult mouse prostate epithelial cells are mixed with urogenital sinus mesenchymal cells (UGSM) and transplanted under the renal capsules of immune-deficient male host SCID/Beige mice (Xin et al., 2003). UGSM cells are able to promote epithelial cell proliferation and regeneration of prostate tissues. Increased epithelial proliferation will result in more robust regeneration in this assay.

UGSM cells were infected with a lentivirus expressing functionally active Il1α (Figure S5A and S5B) and control lentivirus separately. Figure 5C schematically illustrates the experimental design. To test the impact of IL-1α on epithelial cell proliferation, we performed prostate regeneration assays by combining epithelial cells from C57Bl/6 mice with Il1α-expressing UGSM cells and control UGSM cells separately. In addition, we also took advantage of prostate epithelial cells from Il1r1 null mice to determine whether IL-1α directly promotes epithelial cell proliferation, or alternatively stimulates stromal cells to secrete growth factors that in turn promote epithelial proliferation. Figure 5D shows that Il1r1 null and WT prostate epithelial cells regenerated prostate tissues at similar potency when stimulated with the same type of UGSM cells. In contrast, both Il1r1 null and WT prostate epithelial cells regenerated more robustly when combined with Il1α-expressing UGSM cells than with WT UGSM cells. Coimmunostaining of BrdU and E-cadherin shows that the proliferative indices of Il1r1 null and WT epithelial cells in the Il1α-expressing UGSM group are 1.41 fold and 1.73 fold of those in the WT UGSM group, respectively (Figure 5E). Of note, increased Il1α expression is not sufficient to induce hyperplastic epithelial growth (Figure S5C). Similar results were obtained when Il1α was expressed in prostate epithelial cells instead of UGSM cells (data not shown). Collectively, these studies demonstrate that increased expression of IL-1α is sufficient to promote prostate epithelial cell proliferation, and that IL-1α affects prostate stromal cells, which in turn promotes prostate epithelial cell proliferation. This is in agreement with previous studies showing that IL-1α stimulates production of IGF and FGF7 by prostate stromal cells, both of which are capable of promoting epithelial proliferation (Giri and Ittmann, 2000; Jerde and Bushman, 2009).

To determine whether IL-1 is essential for the increased epithelial proliferation induced by loss-of-function of Ar, we bred the Il1r1 null mouse strain with the K8-Ar model and generated a cohort of K8-Ar-Illr1KO and control K8-Ar mice. The experimental procedure is the same as shown in Figure 2A. Immunostaining shows that Ar was deleted at similar frequencies in the K8-Ar and K8-Ar-Illr1KO mice upon tamoxifen treatment (Figure 6A). Figure 6B shows that although the number of CD45+ leukocytes in the prostates of Illr1 null mice was similar to that in wild type mouse (ArWT Illr1WT versus ArWT Illr1KO), the infiltration of CD45+ leukocytes induced by Ar deletion was abolished in the K8-Ar-Illr1KO mice as compared to the K8-AR mice (ArKO Illr1WT versus K8-AR KO Illr1KO). In addition, although Illr1 knockout did not affect epithelial cell proliferation under the physiological condition (ArWT Illr1WT versus ArWT Illr1KO, Figure 6C), Ar deletion-induced epithelial proliferation was completely abolished in the K8-Ar-Illr1KO mice (ArKO Illr1WT versus ArKO Illr1KO, Figure 6C). These results demonstrate that IL-1 plays an essential role in...
immune cell infiltration and epithelial proliferation induced by loss of Ar signaling in luminal cells.

Macrophages are the major sources of ILIs in the prostate tissues (Figure S6A). The macrophage marker CD68 is expressed at a significantly higher level in human BPH specimens than in control normal specimens (Figure 6D), indicating an increased density of macrophages in human BPH samples. We sought to determine whether depleting macrophages using the monoclonal antibody against Csf-1R would also abolish the increased epithelial proliferation in tamoxifen treated K8-Ar mice. The anti-Csf-1R antibody treatment suppressed prostatic infiltration of macrophages induced by Ar deletion (Figure 6E), and abolished the increased epithelial proliferation induced by Ar deletion (Figure 6F). We also investigated the role of T cells using the Rag2−/− mice that are devoid of mature T and B cells. Tamoxifen treatment induced Ar deletion at comparable frequencies in the K8-CreER<sup>TL2</sup>:Ar<sup>fl/Y</sup>;Rag2−/− triple transgenic mice (hereafter referred to as K8-Ar-Rag2) and the control K8-Ar mice (Figure S6B). Figure 6G confirms the absence of mature T cells in the prostate tissues of K8-Ar-Rag2 mice. Ar deletion was still able to slightly induce epithelial proliferation in K8-Ar-Rag2 mice as compared to the control Rag2−/− mice, but the increase was much less compared to that in the K8-Ar mice (Fig. 6H). Of note, neither difference is statistically significant. Collectively, these results indicate that infiltrated T cells may also mediate signaling that promotes epithelial proliferation, but the infiltrated macrophages play the most essential role initiating the signaling that promotes epithelial proliferation.

**Loss of Ar activity impairs the integrity of prostate epithelial cell tight junctions**

We reasoned that the increased cytokine expression induced by Ar loss in luminal cells promotes leukocyte infiltration into the prostate. But how do these luminal cell-expressed cytokines reach peri-glandiular spaces to initiate an immune-inflammatory cascade? Nuclear receptors including AR have been reported to regulate the expression of the tight junction component Claudins in various epithelial cells (Ichikawa-Tomikawa et al., 2011; Meng et al., 2005; Meng et al., 2011). We hypothesized that Ar deletion impairs prostate epithelial cell tight junctions so that the luminal cell-expressed cytokines can leak into peri-glandular spaces and induce immune cell infiltration. We investigated whether Ar affects expression of several claudins (Cldn1, 3, 4, 7, 8) that are highly expressed in prostate tissues (Meng et al., 2011). Individual prostate cell lineages were FACS-isolated from K8-Ar and control mice. Quantitative RT-PCR analysis showed that Cldn3, Cldn4, and Cldn8 were all significantly down-regulated in the prostate luminal cells of K8-Ar mice (Figure 7A). Expression of Cldn7 is not affected while Cldn1 is mainly expressed in the basal cells. Western blot analysis confirmed that CLDN4, but not CLDN3, was also downregulated at the protein level (Figure 7B). QRT-PCR analysis further confirmed that CLDN3, CLDN4, and CLDN8 were also downregulated in FACS-isolated human prostate luminal epithelial cells from large volume prostates compared with those from small volume prostates (Figure 7C). A whole genome Ar ChIP-Seq analysis performed using total prostate tissues of 10-week-old C57Bl/6 mice showed that Ar binds at the promoter regions of Cldn3, 4, and 8 (Figure 7D), supporting a direct transcriptional regulation. We further performed transmission electron microscopic analysis to examine whether disrupting Ar impairs the structure of epithelial tight junctions. Tight junctions in the tamoxifen-treated control mice were detected as close
contacts between two neighboring plasma membranes (blue arrows, Figure 7E). Intercellular spaces were even and the lateral plasma membranes were straight (yellow arrows, Figure 7E). However, prostate tissues of tamoxifen-treated K8-Ar mice displayed dilatations of tight junctions (red arrows, Figure 7E). The intercellular spaces were unevenly widened and lateral plasma membranes between adjacent cells were often much more convoluted (circle, Figure 7E). Collectively, these results demonstrate that loss of epithelial Ar function impairs the integrity of epithelial cell tight junctions.

**Discussion**

We demonstrate that decreased Ar signaling in luminal epithelial cells causes cell-autonomous upregulation of cytokines and chemokines and impairs epithelial tight junctions by downregulating claudins. As a result, cytokines and chemokines reach peri-glandular spaces where they impact stromal cells and resident immune cells to promote immune cell infiltration, resulting in higher prostatic local production of cytokines and chemokines. IL-1 is capable of promoting AR-independent epithelial proliferation by effecting stromal cells.

**A vicious cycle of prostate inflammation**

Although decreased AR expression in the luminal cells is correlated with a higher degree of prostate inflammation, the cause-and effect relationship between the events remains unclear. We demonstrate directly in vivo that attenuation of luminal epithelial AR signaling is capable of inducing prostate inflammation. On the other hand, inflammation has also been shown to suppress expression of AR and critical enzymes for testosterone metabolism (Debelec-Butuner et al., 2014; Khalili et al., 2010; Shinohara et al., 2013; Simons et al., 2014; Vignozzi et al., 2012a). Together, these results suggest a mutual induction between prostate inflammation and reduced luminal AR expression and imply the existence of a vicious cycle of prostate inflammation enforced by declining epithelial AR signaling. Such a self-enforcing feedback loop could be critical in maintaining a sustained chronic inflammation during BPH progression.

We showed that Ar deletion in luminal cells upregulated expression of many cytokines and chemokines. These cytokines are unlikely direct Ar targets as there is no Ar binding site in these genes based on our whole genome Ar ChIP-Seq analysis (data not shown). Instead, it is more likely that Ar regulates the expression of these cytokines indirectly by interfering with other signaling pathways such as STATs and NFκb etc. Besides AR-regulated production of cytokines from luminal epithelial cells, other mechanisms may also contribute to the initiation of aseptic prostatic inflammation. For example, antibodies against prostate secreted antigens are often identified in BPH and prostate cancer patients, hence an autoimmune response towards prostate secretory proteins has been suspected to initiate aseptic prostate inflammation (Lokant and Naz, 2014). Our result that attenuation of AR signaling impairs the integrity of prostate epithelial cell tight junctions may also explain how prostatic secreted proteins reach peri-glandular spaces to elicit autoimmune responses. Finally, circulating PSA levels have been shown to positively correlate with prostate volume in BPH patients (Nickel, 2003), but we showed that PSA is downregulated in luminal cells.
in large volume prostate specimens. These two seemingly contradictory observations can also be reconciled by the impaired epithelial barrier function during BPH progression.

A remaining outstanding question is how AR is downregulated in luminal cells of BPH specimens. One potential mechanism is the reduced receptor stability due to declining serum androgen level during ageing. Since serum testosterone level does not always correlate with that within the prostate tissues, we attempted to investigate the correlation between age and prostatic testosterone levels in available archived human prostate specimens collected from 1995 to 2004 at Baylor College of Medicine using liquid chromatography triple quadruple mass spectrometry. Unfortunately, we were unable to detect testosterone in most specimens probably because they were too ancient. The other mechanism for AR downregulation is epigenetic silencing of AR or genes involved in testosterone metabolism. Future studies will aim to address these mechanisms.

**Anti-inflammatory drugs may improve therapeutic efficacy of androgen targeted therapies**

AR signaling exists in both prostate epithelial and stromal cells. Our studies using the models of castration, enzalutamide treatment and the K8-Ar or PB-Ar mice suggested that stromal AR signaling is critical for epithelial cell proliferation (Figure S4F and S4G) while epithelial AR signaling impacts cell survival more than proliferation (Figure 3C; 3D; 3H and Figure S4E). These explain why androgen-targeted therapies are effective for treating both BPH and prostate cancer. However, the MTOPS study shows that 5-α-reductase inhibitor therapy only leads to a 34% reduction of risk for clinical progression of LUTS/BPH (McConnell et al., 2003), suggesting the existence of unrecognized mechanisms that promote disease progression. 5ARI decreased the risk of low-grade prostate cancer but at the same time increased the risk of high-grade cancer (Thompson et al., 2003). In addition, although prostate cancers respond to androgen targeted therapies, they often recur and become castration-resistant. Many underlying molecular mechanisms have been reported previously. We reason that the inflammatory microenvironment induced by AR inhibition in luminal cells may contribute partially to the resistance and recurrence. Our study predicts that anti-inflammatory drugs such as the IL-1α inhibitor anakinra may boost therapeutic efficacies of anti-androgen therapies for these diseases.

**Impaired epithelial integrity is a common mechanism for dysregulated epithelial growth**

Breaching epithelial integrity has been shown to induce pathological epithelial proliferation in various organs (Pasparakis, 2012). Interestingly, similar to our study, tissue inflammation was often proven as an underlying molecular mechanism for epithelial proliferation. For example, Demehri et al. showed that disrupting Notch signaling in rodent skin impaired skin-barrier integrity, which led to severe inflammation that in turn drove epidermal hyperplasia (Demehri et al., 2009). Alternatively, inflammation can also be initiated by bacterial infection. For example, ablating NF-κB signaling in skin and intestinal epithelia disrupt epithelial barriers and lead to inflammation triggered by improper penetration of commensal bacteria (Pasparakis, 2012). In the current study, we showed that decreasing AR signaling impairs prostatic epithelial integrity and leads to aseptic inflammation. As bacterial infection is one of the proven etiological factors for prostate inflammation, it is intuitive to propose that declining androgen signaling may also mitigate the development of
bacterial infection-mediated inflammation. In summary, our study adds an additional example from an unconventional barrier tissue to highlight the impact of epithelial barrier function on tissue homeostasis.

Limitations of the K8-Ar mouse model

The K8-Ar model enables us to disrupt Ar specifically in a fraction of adult mouse prostate luminal cells. This made it possible for us to discover the non-cell-autonomous mechanism through which Ar regulates epithelial homeostasis. Despite the unique feature, there are several limitations in the model. First, we used the genetic approach to disrupt Ar in luminal epithelial cells abruptly, which unlikely happens the same way in humans. Other ways to suppress Ar signaling include castration and enzalutamide treatment. But our study shows that in BPH specimens only AR expression in luminal cells was decreased while those in basal and stromal cells were not. Therefore, castration and enzalutamide treatment do not faithfully recapitulate the situation in BPH either. In contrast, we specifically disrupted AR in a small fraction of luminal cells in the K8-Ar model, which is not a perfect but still is the best available way to recapitulate the situation in BPH. Second, AR knockout epithelial cells underwent apoptosis at a higher rate than wild type cells. They were gradually replaced by AR wild type cells, which led to the attenuation of leukocyte infiltration and epithelial proliferation. Therefore, we were not able to observe a sustained and deteriorating BPH-like phenotype in this model. But Wu et al did report an increase in size of ventral prostate lobes in old mice using the PB-Ar model (Wu et al., 2007). Finally, BPH is a disease of non-malignant growth of both stromal and epithelial compartment, whereas we only observed major differences in epithelial cell proliferation. This is probably because mouse prostates have much fewer stromal components than human prostates, or the signaling for stromal expansion is absent in our experimental setting. However, despite the differences between human and mouse prostates, we have confirmed in human specimens all major molecular signaling that we discovered in the K8-Ar model. To date, there is no perfect mouse model for BPH. Therefore, we believe that different models may be utilized to address specific questions.

Experimental Procedures

Patient specimens

Human prostate specimens used in this study were obtained at the University of Texas Southwestern Medical Center and at the Baylor College of Medicine. Institutional Review Board approval was obtained for medical record review to collect retrospective clinical and pathological data on each patient at both institutions.

Mice

All animals used in this study received humane care in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication, 1996 edition, and the protocol was approved by the Institutional Animal Care Committee of Baylor College of Medicine.
Statistical analyses

All experiments were performed using 3–8 mice in independent experiments. Data are presented as mean ± SEM. Student’s t test was used to determine significance between groups. Linear regression was used to determine correlation between groups. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance; more details are found in the figure legends.

Please refer to the Supplemental Materials for more details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AR expression is lower in luminal cells in inflamed human prostate tissues.
AR in luminal cells regulates epithelial barrier function and cytokine production.
Disrupting luminal AR signaling induces AR-independent epithelial proliferation.
IL-1 generated by macrophages regulates AR-independent epithelial proliferation.
Figure 1. Focally inflamed prostate glands display low AR expression and are increased with prostate volume in BPH

(A) Representative FACS plots of small and large volume human prostate fractionated into leukocytes, epithelial cells, and stromal cells. (B) Positive correlation between percentage of leukocytes by FACS and prostate volume. (C) Inversed correlations between expressions of AR and PSA in FACS-isolated luminal cells and prostate volume. Gene expression level is normalized to that of RPL27 and fold change is calculated by normalizing expression level to the lowest value sample, which makes the lowest value 1. (D) Bar graph shows comparison of AR expression level in BPH tissues from small (<30cc) and large prostates (>30cc). AR expression level was blindly determined via pathological review based on an AR staining score (0–9) that is defined by multiplying staining intensity score (0–3) with staining extent score (0–3) in luminal epithelial cells. Images on right represent weak, medium, and strong staining, respectively. *: p<0.05. (E–F) Representative images of non-inflamed (E) and inflamed (F) epithelial glands quantitated by an ImageJ macro. Scale bars = 50μm. (G) Quantitative analysis of ImageJ-transformed images shows that AR-positive cells are inversely correlated with CD45-positive cells. See also Figure S1.
Figure 2. Disrupting Ar in adult murine prostate luminal cells using K8-CreER<sup>T2;Ar<sup>fl/Y</sup> model (A) Schematic illustration of experimental procedure. Tmx: tamoxifen. (B) Representative images of prostate tissues from K8-CreER<sup>T2;Ar<sup>fl/Y</sup> (AR<sup>KO</sup>) and Ar<sup>fl/Y</sup> (AR<sup>WT</sup>) mice. Bars = 2mm. Bar graphs show means ± SEM of prostate weight. N=5 per group. (C) Immunostaining of cytokeratin 8 (K8) and AR in anterior (AP), dorsolateral (DLP) and ventral (VP) prostate lobes of AR<sup>KO</sup> and AR<sup>WT</sup> mice at 2 wks after Tmx treatment. Bars = 25μm. Bar graphs show means ± SEM of percentage of Ar<sup>+</sup> cells in K8<sup>+</sup> luminal cells. N=3 per group. (D) QRT-PCR analysis of three Ar target genes in prostates of AR<sup>KO</sup> and AR<sup>WT</sup> mice 2 wks after Tmx treatment. Data represent means ± SEM. N=3 per group. (E) Immunostaining of K8 and AR in DLP of AR<sup>KO</sup> and AR<sup>WT</sup> mice. Bar graphs show means ± SEM. N=3 per group. Bars = 25μm. *: p<0.05; **: p<0.01; ***, p<0.005. See also Figure S2.
Figure 3. Ar deletion in luminal cells induces Ar-independent epithelial proliferation

(A–B) Coimmunostaining of Ar, cytokeratin 8 (K8) and BrdU in prostates of K8-CreER\textsuperscript{T2},Ar\textsuperscript{fl/Y} (AR\textsuperscript{KO}) and Ar\textsuperscript{fl/Y} (AR\textsuperscript{WT}) mice at 2wks (A) and 10 wks (B) after tamoxifen (Tmx) treatment. (C–D) Bar graphs show means ± SEM of percentages of AR\textsuperscript{+} cells and BrdU\textsuperscript{+} cells in luminal cells. Pie charts show percentages of AR\textsuperscript{+} and AR\textsuperscript{−} cells in BrdU\textsuperscript{+} cells. N=5 per group. (E–F) Linear regression analyses reveal a significant correlation between percentages of AR\textsuperscript{+} cells and newly-formed AR\textsuperscript{+} cells in K8-CreER\textsuperscript{T2},Ar\textsuperscript{fl/Y} mice at both 2 weeks (E) and 10 weeks (F) after tamoxifen-induced AR deletion. (G) Coimmunostaining of cytokeratin 5 (K5) and BrdU. Bar graphs show means ± SEM from 5 mice per group. (H) Coimmunostaining of cleaved caspase 3 (CC3) and cytokeratin 8 (K8). Bar graphs show means ± SEM of percentage of CC3\textsuperscript{+} cells from 5 mice per group. All immunostaining were performed using dorsolateral prostates. Quantification is based on results collected from randomly-taken images that cover >70% area of DLP. Bars = 25μm. *: p<0.05; **: p<0.01; ***: p<0.005. See also Figure S3.
Figure 4. Ar deletion results in an inflammatory microenvironment in prostates

(A) Gene Ontology pathways that are significantly enriched (-log10 p-value) in luminal cells of tamoxifen-treated K8-Ar versus those of tamoxifen-treated control mice. P values were by one-sided Fisher’s exact test.

(B) Heatmap of inflammation-related genes upregulated in FACS-isolated luminal cells of tamoxifen-treated K8-Ar mice.

(C) Bar graphs show means ± SEM of percentages of leukocytes and major immune cell lineages in dorsolateral prostates of K8-CreER<sup>T2</sup>; Ar<sup>KO</sup> (AR<sup>KO</sup>) and Ar<sup>WT</sup> (AR<sup>WT</sup>) mice 2 wks after tamoxifen treatment. N=5 per group.

(D–I) QRT-PCR analyses of gene expression in different prostate cell lineages of AR<sup>KO</sup> and AR<sup>WT</sup> mice at 2 weeks after tamoxifen treatment. Bar graphs show means ± SEM from 3 independent experiments. *: p<0.05; **: p<0.01; ***: p<0.005. See also Figure S4 and Table S1.
Figure 5. IL-1α promotes prostate epithelial proliferation

(A) Bar graphs show means ± SEM of expression of *IL1α* and *IL1β* in normal human prostate (N=11) and BPH tissues (N=35) by qRT-PCR. (B) Bar graphs show means ± SEM of expression of *CCL2* and *CXCL10* by human primary prostate stromal cells in response to IL-1α treatment from 3 independent experiments. (C) Schematic illustration of experimental design. (D) Transillumination images of regenerated prostate tissues. Bar graphs show means ± SEM of weight of regenerated prostates. Bars = 5mm. (E) Coimmunostaining of BrdU and E-Cadherin in regenerated prostate tissues. Bar graph shows means ± SEM Bars = 50μm.*: p<0.05; **: p<0.01; ***, p<0.005. See also Figure S5.
Figure 6. Luminal proliferation induced by Ar deletion is abolished by ablating Il-1 signaling or depleting macrophages

(A) Coimmunostaining of K8 and Ar in K8-Ar (AR^{KO} II1r1^{WT}) and K8-Ar-II1r1^{KO} (AR^{KO} II1r1^{KO}) mice at 2 wks after Tmx treatment. Bar graph shows means ± SEM of percentage of AR^{+} cells in K8^{+} luminal cells. N=5

(B) Bar graph shows means ± SEM of CD45^{+} % in prostates. N=5 per group.

(C) Coimmunostaining of K8 and BrdU 2 wks after Tmx treatment. Bar graph shows means ± SEM of percentage of proliferating luminal cells. N=5 per group.

(D) Bar graph shows means ± SEM of expression of CD68 in normal human prostate (N=11) and BPH tissues (N=35) by qRT-PCR.

(E) Bar graph shows means ± SEM of percentage of F4/80^{+} macrophages in prostates of K8-Ar mice treated with tamoxifen and anti-CSF-1 antibody or control IgG2a antibody. N=3 per group.

(F) Coimmunostaining of K8 and BrdU in K8-Ar mice 2 wks after treatment with tamoxifen and anti-CSF-1 antibody or control IgG2a antibody. Bar graph shows means ± SEM of percentage of proliferating luminal cells. N=5 per group.

(G) Bar graph shows means ± SEM of percentage of T cells in prostates of K8-Ar-Rag2 (AR^{KO} Rag2^{KO}) and K8-Ar (AR^{KO} Rag2^{WT}) mice 2 wks after tamoxifen treatment. N=3 per group.

(H) Coimmunostaining of K8 and BrdU in AR^{KO} Rag2^{KO} and AR^{KO} Rag2^{WT} mice 2 wks after tamoxifen treatment. Bar graph shows means ± SEM of percentage of proliferating luminal cells. N=3 per group.
immunostaining were performed using dorsolateral prostates. Bars = 25μm. *: p<0.05; **: p<0.01; ***: p<0.005. See also Figure S6.
Figure 7. Loss of AR function impairs epithelial cell tight junctions

(A) QRT-PCR analyses of Cldns in FACS-isolated prostate cell lineages from K8-Ar (AR<sup>KO</sup>) and AR<sup>WT</sup> mice at 2 wks after tamoxifen treatment. Bar graphs show means ± SEM from 3 independent experiments. *: p<0.05; **: p<0.01. (B) Western blot analyses of CLDN3 and CLDN4 in prostate lysates of AR<sup>KO</sup> and AR<sup>WT</sup> mice at 2 wks after tamoxifen treatment. Each lane represents an independent specimen. NRAEV: normalized relative arbitrary expression value by β-actin. (C) QRT-PCR analyses of CLDNs in FACS-isolated luminal cells from large and small volume prostates. *: p<0.05; **: p<0.01. (D) UCSC genome browser tracks of Cldn gene loci with ChIP-Seq data for Ar binding. (E) Ultrastructure of prostate epithelium in AR<sup>KO</sup> and AR<sup>WT</sup> mice at 2 weeks after tamoxifen treatment by electron microscopy. Blue and yellow arrows point to tight junction and intercellular spaces in specimens of AR<sup>WT</sup> mice, respectively. Red arrow and circled area show dilatation of tight junction and convoluted lateral plasma membranes between adjacent cells in AR<sup>KO</sup> mice, respectively. L: lumen. Black bars=100nm; red bars=200nm.