Loss of stromal androgen receptor leads to suppressed prostate tumourigenesis via modulation of pro-inflammatory cytokines/chemokines

Kuo-Pao Lai1, Shinichi Yamashita1, Chiung-Kuei Huang1, Shuyuan Yeh1, Chawnshang Chang1,2*

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INTRODUCTION

Emerging evidence has shown that the tumour microenvironments play crucial roles in the prostate cancer (PCa) development/progression (Ammirante et al, 2010; Erez et al, 2010; Santos et al, 2009; Trimboli et al, 2009). The tumour stromal cells can foster the oncogenic epithelial cell growth through secretion of paracrine growth factors and providing a more favorable microenvironment for tumour growth. One line of evidence used tissue recombination to show that when initiated benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 cells (immortalized by SV40-T antigen) were combined with normal fibroblasts or carcinoma associated fibroblasts (CAFs), large tumour growth only occurred in BPH-1 with CAFs recombinants. The results indicated that the tumourigenesis of BPH-1 cells required signalling support from CAFs, but not from normal fibroblasts (Cunha et al, 2002, 2003; Hayward et al, 2001). To determine the stromal androgen receptor (AR) roles in the elicitation of hormonal-mediated carcinogenesis, tissue recombinants from wild-type (WT) or testicular feminized (Tfm) urogenital sinus mesenchyme (UGM) were mixed with BPH-1 cells following estradiol 17-beta (E2) + testosterone (T) treatment. The BPH-1 cells could undergo tumourigenesis and develop tumours only in the presence of functional...
mesenchymal AR (Cunha et al., 2002, 2003, 2004; Ricke et al., 2006). Furthermore, tissue recombinant result from PC-3 (prostate epithelium) and AR siRNA knockdown in WPMY-1 cells (prostate stroma) revealed that stromal AR is essential to support PC-3 cell growth, leading to larger tumour development (Niu et al., 2008a, b). Taken together, the previous studies using tissue recombination suggested that stromal AR could promote PCa development/progression. However, how stromal AR influences oncogenic epithelium cell growth, especially in the real physiological condition with intact immune systems in mice, is still unresolved.

The phosphatase and tensin homologue deleted from chromosome 10 (PTEN) or mutated in multiple advanced cancers (MMAC1) gene was originally identified as a tumour suppressor gene located at chromosome 10q23 (Myers et al., 1997). The PTEN gene is one of the most frequently mutated or deleted genes in various human cancers including lymphoid, breast, endometrial, glioblastoma and prostate (Bose et al., 1998; Wang et al., 2003; Webber et al., 1999). PTEN is a multifunctional phosphatase to dephosphorylate the substrate of phosphatidylinositol (3,4,5)-triphosphate (PIP3; Maehama & Dixon, 1998).

By dephosphorylating the D3 position of PIP3, PTEN negatively regulates the PI3K pathway and triggers its downstream Akt activation. The lipid phosphatase activity and Akt activity were associated with tumour suppression (Goberdhan & Wilson, 2003; Li et al., 1997). PTEN mutations or deletions have been implicated in the PCa development (Gray et al., 1998; Whang et al., 1996), being present in 30% of primary prostate tumours (Dahia, 2000) and around 63% of metastatic human prostate cancers (MMAC1) gene was originally identified as a tumour suppressor gene located at chromosome 10q23 (Myers et al., 1997). The PTEN gene is one of the most frequently mutated or deleted genes in various human cancers including lymphoid, breast, endometrial, glioblastoma and prostate (Bose et al., 1998; Wang et al., 2003; Webber et al., 1999). PTEN is a multifunctional phosphatase to dephosphorylate the substrate of phosphatidylinositol (3,4,5)-triphosphate (PIP3; Maehama & Dixon, 1998). By dephosphorylating the D3 position of PIP3, PTEN negatively regulates the PI3K pathway and triggers its downstream Akt activation. The lipid phosphatase activity and Akt activity were associated with tumour suppression (Goberdhan & Wilson, 2003; Li et al., 1997). PTEN mutations or deletions have been implicated in the PCa development (Gray et al., 1998; Whang et al., 1996), being present in 30% of primary prostate tumours (Dahia, 2000) and around 63% of metastatic human prostate tissue samples (Suzuki et al., 1998; Trotman et al., 2006, 2003).

To approach the PTEN pathophysiological roles in PCa development, several PTEN mutant mouse lines have been established and characterized (Backman et al., 2004; Cully et al., 2006; Di Cristofoano et al., 2001; Podsypanina et al., 1999; Trotman et al., 2003; Wang et al., 2003). Heterozygous PTEN mutant mice (Pten+/−) were reported to show prostatic intraepithelial neoplasia (PIN) lesions in the adult mice, however, PIN lesions never proceed to PCs or metastatic tumours, suggesting that loss of one allele of the PTEN gene is not sufficient to cause the onset of PCs (Chen et al., 2006; Park et al., 2002). The biallelic mutation of the PTEN gene or the 2nd hit of loss-of-function in tumour suppressor genes could promote advanced PCs progression (Abate-Shen et al., 2003; Chen et al., 2006; Kim et al., 2002; Wang et al., 2003). Histopathological phenotypes revealed that invasive adenocarcinoma was frequently observed in epithelial PIN null mice older than 12 weeks (Wang et al., 2003). Other PTEN specific KO mice were concurrently established by breeding mouse mammary tumour virus-long terminal repeat promoter cre mice with floxed Pten mice, resulting in aberrant prostate morphology that exhibited high grade-PIN (HG-PIN) with frequent invasion into adjacent stromal compartments (Backman et al., 2004).

Since prostate stromal AR has a potential contribution to the PCa development, we characterized this stromal fibromuscular ARKO (dARKO) mouse model with PTEN haplo-insufficiency. Here, we identified that stromal fibromuscular AR could regulate PIN lesion formation, epithelium proliferation and the tumour-promoting microenvironment in Pten+/− mice. Finally, targeting stromal fibromuscular AR via the AR degradation using ASC-99® could yield the therapeutic effects through modulation of the stromal–epithelial interaction and alteration of the tumour-promoting microenvironments.

RESULTS

Generation and characterization of dARKO/Pten+/− mice lacking AR in cancer associated stromal cells

To dissect the stromal AR roles in prostate tumourgenesis, we generated a new mouse model (named as dARKO/Pten+/−) that has selective AR deletion in stromal cells (both fibroblasts and smooth muscle cells; Lai et al., 2012) via mating mice carrying FSP1-cre (Blowersick et al., 2004) and transgelin cre (Tgln-cre; Li et al., 1996) with floxed AR mice (Yeh et al., 2002), then bred the resulting FSP1-cre/Tgln-cre/ARlox+/− mice with Pten+/− mice (see detailed mating strategy in Fig 1A). The tail genotyping results demonstrated the new dARKO/Pten+/− mice carried the transgenes, FSP1-cre, Tgln-cre, floxed AR allele and had PTEN heterozygosity (Fig 1B).

Immunohistochemistry (IHC) staining of dorsolateral prostate (DLPs) lobes from Wt-AR/Pten+/−, Wt-AR/Pten+/− and dARKO/Pten+/− mice using an antibody against the AR C-terminal showed partial AR deletion, which was restricted to the stromal fibromuscular cells, but this deletion was not seen in the adjacent epithelium (Fig 1C).

Consistent with a previous report (Podsypanina et al., 1999), we found that in the Wt-AR/Pten+/− mice, the anterior-prostates (APs) and DLPs had larger size compared to those found in Wt-AR/Pten+/− mice, indicating the development of PIN lesions (Chen et al., 2006; Park et al., 2002; Podsypanina et al., 1999). We then examined the effects of loss of stromal fibromuscular AR on PIN lesion development judged by prostate size. As shown in Fig 1D, we found the prostate size displayed significant reduction (APs and DLPs) in dARKO/Pten+/− mice, suggesting that stromal fibromuscular AR might play positive roles to promote PIN lesion development.

Furthermore, the serum testosterone levels were found to be similar between dARKO/Pten+/− mice and their Wt littermates, indicating that altered phenotypes (described in the next sections) found in the dARKO/Pten+/− mice were not due to the change of circulating testosterone (Fig 1E).

Collectively, results from Fig 1A–E showed that we successfully established the stromal dARKO mice with PTEN deficiency and also observed the reduced prostate size in dARKO/Pten+/− mice.

Loss of stromal fibromuscular AR diminished PIN lesion development

We then applied H&E staining to examine PIN lesions in 9-month-old Wt-AR/Pten+/− mice and found multicellular layered or cribriform structures of low-grade/high-grade PIN (LC/HG-PIN) prostate glands (Fig 2A arrows) as compared to a single layer of epithelial glandular structure in Wt-AR/Pten+/− mice. This Pten+/− mouse is a germline mutation and the PIN lesion was concurrently
observed in the proximal and distal regions of prostate ducts (Supporting Information Fig S1A and B arrows), which was distinct from using the cre-loxP system to knockout PTEN alleles in the prostate epithelium. Comparing two prostate epithelial specific cre mediated PTEN disruptions (PSA-cre and probasin-cre), the PSA-cre driven PTEN deletion caused hyperplasia was restricted to the distal luminal epithelial cells (Korsten et al, 2009). However, the probasin-cre mediated PTEN deletion caused tumour development arising from basal/stem cells in the proximal regions (Wang et al, 2006). Therefore, the phenotypes we observed from Pten\(^{+/−}\) mice were not due to the differential effects of Pten gene loss in luminal or basal/stem epithelial cells.

Importantly, we found DLPs from dARKO/Pten\(^{+/−}\) mice displayed diminished PIN formation as compared to those found in the Wt-AR/Pten\(^{+/−}\) mice (Fig 2A and Supporting Information Fig S1). The LG-/HG-PIN lesion quantification results (Fig 2B) further confirmed that the loss of stromal fibromuscular AR not only reduced the PIN initiation, but also retarded the LG-PIN progression.

Loss of PTEN leads to activation of phospho-Akt (p-Akt), which can further trigger the downstream signals such as mammalian target of rapamycin (mTOR), forkhead box 01A (FOXO1A) and phosphorylated S6 Ribosomal Protein (p-S6RP) to influence PCA cell survival and cell cycle control (Trotman et al, 2006, 2003). Therefore, we applied p-Akt (at Ser 473) IHC staining and found the staining signal was significantly reduced in the dARKO/Pten\(^{+/−}\) mice as compared to that found in Wt-AR/Pten\(^{+/−}\) mice (Fig 2C arrows), implicating reduction of PIN formation. As expected, we also found similar results in p-S6RP (at Ser 235/236) IHC staining in the dARKO/Pten\(^{+/−}\) mice (Fig 2D arrows).

Together, results from Fig 1D and Fig 2A–D clearly demonstrated that stromal fibromuscular AR played positive roles in promoting PIN lesion development. This positive role of stromal AR is in contrast to our earlier report showing negative roles of AR in basal/intermediate epithelial cells to suppress PCA progression/invasion and indicates a differential AR role depending on the cell type (Niu et al, 2008a, 2008b, 2010).
Figure 2. Loss of stromal fibromuscular AR leads to diminished PIN lesion development. \( N = 6-7 \) mice per group.

A. Histological examination of 9-month-old Wt-AR/Pten\(^{+/+}\), Wt-AR/Pten\(^{+-}\) and dARKO/Pten\(^{+-}\) mouse DLPs subjected to H&E staining. The PIN lesions are presented as multi-layered epithelium and typical cribriform structures pointed out by arrows. The asterisk marks the reactive stroma surrounding the PIN lesions and arrowheads indicate infiltrated immune cells located in the stromal compartments. Scale Bars = 200\( \mu \)m (100×) and 50\( \mu \)m (400×).

B. Quantification of LG-/HG-PIN in Wt-AR/Pten\(^{+/+}\), Wt-AR/Pten\(^{-/-}\) and dARKO/Pten\(^{-/-}\) mouse DLPs at 9-month-old (\( N = 5 \) mice for each group). Data are presented as mean ± SEM. \(^*\)p < 0.05, \(^{**}\)p < 0.001.

C. IHC staining against p-Akt at Ser473. Arrows indicate p-Akt positive epithelium. Scale Bars = 200\( \mu \)m (100×) and 50\( \mu \)m (400×).

D. IHC staining of p-S6RP (Ser235/236). Arrows indicate p-S6RP positive epithelium. Scale Bars = 200\( \mu \)m (100×) and 50\( \mu \)m (400×).
Stromal fibromuscular AR plays a positive role in promoting PIN development and modulates the tumour-promoting microenvironment

In order to dissect the possible mechanisms of how stromal fibromuscular AR could play a positive role in PIN development, we first examined its influences on the adjacent epithelial cell proliferation. The BrdU staining (for proliferation index) of epithelial cells was decreased in dARKO/Pten+/− mice as compared to that found in the Wt-AR/Pten+/− mice (Fig 3A arrows and quantitation in right panel), suggesting that epithelial cell proliferation within the PIN lesions still relied on stromal AR mediated signals.

Next, we characterized several features of the tumour-promoting microenvironments such as extracellular matrix (ECM) remodelling, angiogenesis and immune cell infiltration. We applied Mason’s Trichrome staining to evaluate the ECM remodelling of collagen deposition, which has been suggested to play crucial roles in the tumour initiation and progression (Santos et al, 2009; Trimbo et al, 2009). We also found loss of stromal fibromuscular AR resulted in decreased collagen fiber accumulation in the dARKO/Pten+/− mice (Fig 3B asterisks and quantitation in right panel). Moreover, we used IHC staining with an antibody against CD34 (endothelial cell marker to monitor angiogenic status) to confirm that neovasculature formation was substantially decreased in the dARKO/Pten+/− mice (Fig 3C arrows and quantitation in right panel).

Chronic inflammation and/or recurrent infection have been suggested to be involved in the etiology of PCa development (Roseman et al, 1984). Early reports also showed that infiltrating immune cells frequently accompanied the PIN lesions, which suggested immune cells might contribute to the development of PIN and PCa (De Marzo et al, 1999; Putzi & De Marzo, 2000). As expected, using IHC staining with antibodies against CD3 (T-cell marker) and F4/80 (macrophage marker), we found infiltrating T-cells/macrophages were significantly reduced in the dARKO/Pten+/− mice (Fig 3A arrows and quantitation in right panel). Furthermore, we analysed the tumour microenvironments by detection of collagen deposition (Fig 4E), neovasculature formation (Fig 4F; upper panels) and macrophage infiltration (Fig 4F; lower panels) and consistently each result showed a reduction in the Pten+/−, Rag2+/− mice compared to Pten+/−, Rag2+/− mice.

Collectively, after removal of T-/B-cells in Pten+/− mice, we observed less PIN lesion accompanied by less tumour-promoting microenvironmental changes, suggesting that T and/or B-cells mediated inflammation may also contribute to the PIN development.

Abolishment of T-/B-cells in Pten+/− mice displays less PIN lesion development

According to the previous results in Figs 2 and 3, we found loss of stromal fibromuscular AR results in decreased PIN lesion and attenuation of tumour-promoting microenvironment. Then, we were interested to know whether infiltrating lymphocytes play any significant roles to promote or suppress PIN development, therefore, we established the Pten+/− mice with Rag2 gene deletion. In the Pten+/−, Rag2−/− mice, there are no mature T-/B-cells because Rag2 is essential for V(D)J rearrangement (Shinkai et al, 1992). Data on the generation of these mutant mice are provided in Fig 4A. We then characterized the PIN lesion in 7–8-month-old Pten−/−, Rag2−/− and Pten−/−, Rag2−/− mice and found Pten+/−, Rag2−/− mice displayed PIN lesion reduction compared to Pten−/−, Rag2−/− mice (Fig 4B). To further confirm the PIN lesion, we used p-AKT (at Ser 473) IHC staining (Fig 4C) as well as Western blotting (WB) from mouse DLPs (Fig 4D) and found similar results correlating with H&E staining. These results suggested that depletion of T-/B-cells infiltration and their mediated inflammatory response results in decreased PIN lesion formation.

Furthermore, we analysed the tumour microenvironments of these mutant mice (Fig 4). To explore the roles of immune cells mediated inflammation, which may contribute to the PIN development during prostate tumourigenesis (de Visser et al, 2006; Karin & Greten, 2005), we examined whether AR in cancer-associated fibroblasts (CAFs) was able to control pro-inflammatory cytokines/chemokines production to affect the immune cells recruitment. We used primary culture of prostate stromal cells (PrSCs) from DLPs of Pten−/+ and Pten−/− mice and used lentivirus carrying ARsiRNA (ARsi) to knockdown AR compared to ARscramble (ARscr) controls. As shown in Fig 5A, we successfully isolated the PrSCs from Pten−/+ and Pten−/− mouse prostates and established the AR siRNA knockdown stromal cells. The results showed that p-AKT (at Ser 473) expression was only elevated in the PrSCs isolated from Pten−/+ mice, but not from Pten−/− controls (Fig 5A).

Next, the conditioned media (CM) obtained from four groups of PrSCs isolated from Pten−/+ DLPs was prepared: Pten−/+ ARscr+DHT treatment, Pten−/+ ARscr+EtOH, Pten−/+ ARscr+DHT and Pten−/+ ARsi+DHT. The CM from each group was subjected to pro-inflammatory cytokine array analysis (Fig 5B) and we found Pten−/+ PrSCs displayed significant up-regulation of several cytokines/chemokines: such as interferon gamma-induced protein (IP-10); keratinocyte-derived chemokine (KC); thymus and activation regulated chemokine (TARC); tissue inhibitor of metalloproteinase 1 (TIMP-1); monocyte chemotactic protein-5 (MCP-5); macrophage inflammatory protein-1α (MIP-1α); macrophage inflammatory protein-1β (MIP-1β); regulated upon activation, normal T-cell expressed and secreted (RANTES); stromal cell-derived factor-1 (SDF-1); and interleukin-10 (IL-10), compared...
Figure 3.
to Pten\(^{+/+}\) controls (Fig 5B). Interestingly, we found the levels of MIP-1\(^{\alpha}\), MIP-1\(^{\beta}\), MIP-2 and IL-10 were substantially reduced in Pten\(^{+/–}\) ARsi + DHT group compared to Pten\(^{+/+}\) ARscr + DHT, which was confirmed by dot-quantification (Fig 5C) and real-time PCR analysis (Fig 5D). The similar result was also seen in the Pten\(^{+/–}\) PrSCs isolated from APs (Supporting Information Fig S2).

We then studied the mechanisms by which AR regulates the expression of MIP-1\(^{\beta}\), which was altered significantly in Pten\(^{+/–}\) ARsi PrSCs (Fig 5). First, we surveyed the mouse MIP-1\(^{\beta}\) 5 kb promoter region to identify the potential androgen responsive elements (AREs) by transcriptional element search system (TESS, U of Pennsylvania, Philadelphia, PA). Based on the survey results, we found the potential AREs (half-palindromic motif) mainly located within the 2 kb promoter region. Therefore, we performed the ChIP assay to examine whether AR can directly bind to the MIP-1\(^{\beta}\) promoter region in mouse PrSCs. The partial mouse MIP-1\(^{\beta}\) promoter region and the primer sets used to amplify AR-bound DNA are provided (Supporting Information Fig S3A). The results showed that AR failed to bind to the immunoprecipitated promoter regions of MIP-1\(^{\beta}\) (Supporting Information Fig S3B, upper and middle panels), however, AR can bind to the keratinocyte growth factor promoter (Heitzer & DeFranco, 2006) that served as positive control (Supporting Information Fig S3B, lower panel). These results were further corroborated with mouse MIP-1\(^{\beta}\) promoter luciferase assay. We constructed the mouse MIP-1\(^{\beta}\) promoter (-1,989 to +68) into pGL3-basic vector and determined whether AR can activate the MIP-1\(^{\beta}\) promoter luciferase assay. We showed diminished PIN lesion formation, most markedly in Pten\(^{+/–}\) mice. After 2 months of injection, we harvested the prostate tissues and characterized the histology. The DLPs from ASC-J9 treated Pten\(^{+/–}\) mice showed diminished PIN lesion formation, most markedly in HG-PIN as compared to vehicle treated Pten\(^{+/–}\) mice (Fig 6B). The LG-/HG-PIN lesion quantification is also provided (Fig 6B, right panel). The PIN lesions were further examined by staining of p-Akt (Ser473) and the results were similar to the H&E staining data (Fig 6C). To ensure ASC-J9\(^{R}\) can degrade AR in the Pten\(^{+/–}\) mice, we employed AR IHC and prostate tissues WB to assess the degradation of AR on MIP-1\(^{\beta}\) is unlikely through direct promoter binding. Instead, the cooperation with NF-kappaB to activate MIP-1\(^{\beta}\) promoter may play more important role for the AR-mediated regulation. Although it is also possible that AR may bind to the enhancer region of MIP-1\(^{\beta}\) or modulate the co-occupied transcriptional factors to regulate MIP-1\(^{\beta}\) expression, which needs further investigation.

Together, results from Fig 5A–D and Supporting Information Figs S3 and S4 suggested that stromal AR in CAFs was able to regulate pro-inflammatory cytokines/chemokines expression such as MIP-1\(^{\alpha}\), MIP-1\(^{\beta}\), MIP-2 and IL-10, to affect immune cell recruitment and modulate inflammatory responses in Pten\(^{+/–}\) prostate. For the MIP-1\(^{\beta}\) gene regulation, AR may cooperate with NF-KappaB to activate MIP-1\(^{\beta}\) promoter after IL-1\(^{\beta}\) stimulation, instead of directly binding to MIP-1\(^{\beta}\) promoter.

ASC-J9\(^{R}\) suppresses PIN lesion development, in part, via stromal AR degradation

To further confirm the findings in Figs 2 and 3 showing that stromal AR can regulate PIN development and the tumour-promoting microenvironmet, we used the AR degradation enhancer, ASC-J9\(^{R}\), which was developed to interrupt the interaction of AR and its coregulator ARA55 in stromal cells and ARA70 in luminal epithelial cells. This disruption might then lead to the degradation of AR and suppress AR-mediated cell growth (Lai et al., manuscript submitted). The potential therapeutic effects of ASC-J9\(^{R}\) have been demonstrated in vitro in several AR related diseases such as bladder cancer, liver cancer and spinal and bulbar muscular atrophy (SBMA) a motor-neuron disease (Miyamoto et al, 2007; Wu et al, 2010; Yang et al, 2007).

First, we used primary cultured of stromal cells from Pten\(^{+/–}\) mice and treated with vehicle or ASC-J9\(^{R}\) to show AR protein could be degraded (Fig 6A). To examine the in vitro effects of ASC-J9\(^{R}\), we injected DMSO-vehicle or ASC-J9\(^{R}\) every other day starting with 6-month-old Pten\(^{+/–}\) mice. After 2 months of injection, we harvested the prostate tissues and characterized the histology. The DLPs from ASC-J9\(^{R}\) treated Pten\(^{+/–}\) mice showed diminished PIN lesion formation, most markedly in HG-PIN as compared to vehicle treated Pten\(^{+/–}\) mice (Fig 6B). The LG-/HG-PIN lesion quantification is also provided (Fig 6B, right panel). The PIN lesions were further examined by staining of p-Akt (Ser473) and the results were similar to the H&E staining data (Fig 6C). To ensure ASC-J9\(^{R}\) can degrade AR in the Pten\(^{+/–}\) mice, we employed AR IHC and prostate tissues WB to
Figure 4. Depletion of T-/B-cells attenuates PIN lesion formation in Pten−/− mice.
A. Genotyping of Pten+/+, Rag2+/+; Pten−/−, Rag2+/+ and Pten+/+, Rag2−/− mice by using Pten and Rag2 primer sets. Tail genomic DNA was isolated and the PCR genotyping was performed to identify Wt or KO of Pten and Rag2 genes.
B. H&E staining of 7–8-month-old Pten+/+, Rag2+/+ and Pten−/−, Rag2−/− mice. The PIN lesions are pointed-out by arrows and arrowheads indicate infiltrated immune cells located in the stromal compartments. The asterisk marks the reactive stroma. N = 4–5 mice per group. Scale Bars = 200 μm (100×) and 50 μm (400×).
C. IHC staining of p-Akt at Ser473. Arrows indicate p-Akt positive epithelium. N = 4–5 mice per group. Scale Bars = 200 μm (100×) and 50 μm (400×).
D. Proteins were extracted from DLPs of three genotypes of mice and p-Akt/Akt immunoblotting assays were used to determine the expression levels. The data are presented from one Pten+/+, Rag2+/+ mouse and three individual Pten−/−, Rag2+/+ or Pten+/+, Rag2−/− mice. N = 4–5 mice per group. Scale Bars = 200 μm (100×) and 50 μm (400×).
E. Pten−/−, Rag2+/+ and Pten−/−, Rag2−/− mouse DLPs were subjected to Masson’s Trichrome staining. The blue staining indicates collagen fibers (asterisk). N = 4–5 mice per group. Scale Bars = 200 μm (100×) and 50 μm (400×).
F. CD31 and F4/80 IHC stainings were applied to detect endothelium and macrophages in the upper and lower panel, respectively. Arrows indicate CD31+ or F4/80 positive cells. N = 4–5 mice per group. Scale Bars = 50 μm (400×).
confirm ASC-J9\textsuperscript{K} effects (Fig 6D–E). Note that ASC-J9\textsuperscript{K} can also degrade luminal epithelial AR protein, therefore, we were able to observe decreased epithelial AR amounts in the ASC-J9\textsuperscript{K} treated mouse prostate (Fig 6D).

Collectively, we demonstrated that ASC-J9\textsuperscript{K} could reduce PIN lesion development in Pten\textsuperscript{+/-} mice, at least in part, due to the stromal AR degradation.

ASC-J9\textsuperscript{K} impedes PIN lesion development and attenuates the tumour microenvironment in Pten\textsuperscript{+/-} mice

We further utilized the BrdU incorporation assay to identify proliferating epithelial cells and the results indicated that Pten\textsuperscript{+/-} mice with ASC-J9\textsuperscript{K} treatment had markedly reduced proliferation signals within the PIN areas (Fig 7A; left panel and quantitation in right panel). Moreover, tumour microenviron-
Figure 6.
mental characterization was conducted using staining with Mason’s Trichrome, CD31 IHC and CD3 IHC (Fig 7B–D; left panels and quantitation in right panels) to show ASC-J9 treated cells also suppressed these changes.

Together, the phenotypic characterization results from Fig 6B–C and Fig 7A–D are consistent with data from mice lacking stromal fibromuscular AR (Figs 2 and 3) and demonstrated that injection of ASC-J9 into Pten+/− mice could effectively suppress PIN development with attenuation of the tumour microenvironment remodelling partially through stromal AR degradation.

**DISCUSSION**

Reciprocal signalling between tumour cells and adjacent stromal cells plays pivotal roles in orchestrating the oncogenic epithelial cellular and molecular mechanisms. The cross-talk between tumours and various stromal cells in the PCAs are still not fully understood. We developed the stromal fibromuscular ARKO mice to examine PCa initiation in Pten+/− mice. Our experimental results with these compound mutant mice highlight the crucial roles of stromal fibromuscular AR on the regulation of adjacent epithelial lesion development through a paracrine manner and re-organization of a favourable microenvironment. In this study, we also observed a reduction in collagen deposition, neovascularature formation and immune cells infiltration in this dARKO/Pten+/− mouse as the features of tumour-promoting microenvironments (Fig 8). Although we still did not have the direct evidence to support that these microenvironmental changes are exclusively due to the stromal AR deletion, it is likely that these changes are attributed to the intermixing effects of loss of stromal AR and reduction of PIN lesion in dARKO/Pten+/− mice. The oncogenic epithelium within the PIN lesion was also able to secrete growth factors and cytokines/chemokines to create a more favorable microenvironment including the ECM remodelling, angiogenesis and immune cell infiltration.

Emerging evidence of risk factors such as infectious agents and environmental factors have been proposed to precede the development of PIN lesions (De Marzo et al, 1999; De Marzo et al, 2007). Epidemiological studies also suggested that chronic inflammation and/or recurrent infection might contribute to prostate carcinogenesis (Griewank et al, 2010; Karin & Greten, 2005; Karin et al, 2006). Proliferative inflammatory atrophy (PIA) lesions of the glandular epithelial foci could be a connection between prostatitis and PIN or PCa (De Marzo et al, 2007; Palapattu et al, 2005). The inflammatory lesions in the aging prostate are frequently associated with atrophic epithelium and some fractions of epithelium undergo active proliferation, which may develop into PIN or PCa. In the molecular aspects, several genetic alteration such as glutathione S-transferase pi 1 (GSTP1), NKF3.1, p27 and PTEN have been regarded as the markers of PIN and PCa (Wagenlehner et al, 2007). Collectively, this epidemiological and genetic evidence might suggest a causative role of inflammation in the complicated process of prostate carcinogenesis, implicating the important contribution of immune cells in the prostate pathogenesis.

Regarding the functions of tumour associated stromal cells, tumour associated fibroblasts (TAFs) are distinguishable from normal fibroblasts and a subset of TAFs has been characterized as myofibroblasts based on the expression of smooth muscle α-actin (SMAα; Santos et al, 2009). TAFs in skin were demonstrated to be able to produce pro-inflammatory cytokines to foster epithelium growth, but the functions of TAFs in PCa are unclear (Erez et al, 2010). In addition to TAFs, B-cells residing in the skin were able to ‘educate’ normal fibroblasts to evolve into TAFs, which can produce various pro-inflammatory cytokines in order to evoke tumour initiation (Erez et al, 2010). Furthermore, the sensitivity to castration in PCa can be modulated through infiltrated B-cells derived lymphotoxic-epithelium engagement in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (Ammirante et al, 2010). Overall, inflammatory responses from either infiltrated immune cells or TAFs may contribute, in part, to the PCa development. In this report, we demonstrated that AR in CAFs could modulate cytokines/chemokines production to affect the immune cells recruitment. Although we have not identified whether T-cells or B-cells are more important in mediating this tumour-promoting inflammatory response in Pten+/− mice, further studies by crossing T-cell or B-cell deficient mice with Pten+/− mice may provide direct answers to address this question.

**Figure 6.** ASC-J9® suppresses PIN lesion development via AR degradation.

A. Primary cultures of PrSCs from Pten+/− mice were prepared. The PrSCs were cultured in 10% CD-FBS medium for 2 days and treated with E1OH, 10 nM DHT, 10 μM ASC-J9®, or DHT + ASC-J9® for 24 h. The protein extracts were harvested and subjected to WB analysis using antibodies against Vimentin (Vim), SMA, AR and GAPDH. The stromal cells were pooled from 5 to 6 Pten+/− mice at 22–24 weeks old. The quantification data of AR amounts are shown and normalized with GAPDH loading controls (right panel).

B. ASC-J9® displays chemopreventive effects in Pten+/− mouse model. ASC-J9® was administrated to 6-month-old Pten+/− mice and the DLPs were harvested after 2 months treatment. Histological examination of 8-month-old Pten+/− DMSO, Pten+/− DMSO and Pten+/− ASC-J9® treated mouse DLPs were subjected to H&E staining. The PIN lesions are pointed out by arrows. Quantification results are shown in the right panel. N = 4–5 mice per group. Scale Bars = 200 μm (100 ×) and 50 μm (400 ×).

C. p-Akt (Ser473) IHC staining in Pten+/− DMSO, Pten+/− DMSO and Pten+/− ASC-J9® treated mouse DLPs were performed. Arrows indicate p-Akt positive staining in the PIN lesion. N = 4–5 mice per group. Scale Bars = 200 μm (100 ×) and 50 μm (400 ×).

D. DLPs from the three groups of mice were subjected to AR IHC staining. Arrows indicate the AR immunoreactive cells and arrowheads show weaker intensity in ASC-J9® treated group. N = 4–5 mice per group. Scale Bars = 200 μm (100 ×) and 50 μm (400 ×).

E. The DLPs tissue protein extracts were isolated from the three groups of mice and AR immunoblotting assay was used to determine AR expression. The data are presented from three individual Pten+/− DMSO and Pten+/− ASC-J9® treated mice.
Research Article

Stromal AR regulates PIN in Pten null mice

Figure 7.

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As noted, tissue recombination experiments conducted in immunodeficient mice, which lack functional T- and B-cell mediated immune responses. The elicited T-/B-cells inflammation has been associated with the tumour progression through pro-inflammatory cytokine production and recruitment of macrophages (Balkwill et al, 2005; Coussens & Werb, 2002; Karin et al, 2006). Furthermore, subrenal capsule implementation of tissue recombinants provide enriched nutrition and ample oxygen supply for recombinants growth, however, this does not mimic the real in vivo prostate microenvironment. The ECM composition, basement membrane components and matrix/metalloproteinase factors are distinct between renal capsules and prostate. Therefore, by generating conditional stromal fibromuscular ARKO mice with the cre-loxP system, we can breed with other transgenic/KO mice such as TRAMP, Nkx3.1 KO, p53 KO and e-Myc transgenic mice to further understand stromal AR roles in vivo with intact immune systems.

Interestingly, a previous report has suggested that human prostate tumour samples with higher mesenchymal stem cells (MSCs) marker CD90 expression might contain the tumour-promoting potentials of CAFs (Zhao & Peehl, 2009). To characterize the MSCs population in the Pten+/−/PrSCs and explore the potential AR regulation, we performed QPCR analysis to examine MSC markers (CD29, CD44 and CD90). The results showed that PrSCs isolated from Pten+/− mice expressed these three MSC markers, with higher expression in CD90 (Supporting Information Fig S5D). By using genetic knockout (WT vs. ARKO bone marrow-MSCs, BM-MSCs) or AR siRNA knockdown approach in Pten+/− PrSCs to disrupt AR expression (Supporting Information Fig S5A), we did not see ablation of AR could reverse these MSC marker expressions (Supporting Information Fig S5B–D). However, we could observe that loss of AR in BM-MSCs could enhance self-renewal by CFU-F assay (Supporting Information Fig S5E). Therefore, we reasoned that knockdown of stromal AR might increase MSCs population, which has the potential to promote PCA development, however, this may not happen in the dARKO-Pten+/− mice because dARKO-Pten+/− mice showed reduced PIN lesion.

Finally, the AR degradation enhancer, ASC-J9®, has been extensively examined in several types of cells such as motor neuronal cells, bladder cancer cells, hepatocarcinoma cells and macrophages in wound tissues (Lai et al, 2012). ASC-J9® was purchased from Sigma–Aldrich and used according to the manufacturer’s instructions (Invitrogen). The Masson’s Trichrome staining kit was purchased from Taconic (Germantown, NY). Animal procedures were conducted in accordance with the protocols approved by the University of Rochester Committee on Animal Resources.

The primary cultured prostate stromal cells were prepared by cutting the prostates into small pieces (1 mm3) and further digested into single cells by using 1% collagenase (Roche). The primary cultured PrSCs were grown in DMEM/F12 (1:1) with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% insulin–transferrin–selenium (ITS, Gibco) and the mixed populations were collected for characterization. ASC-J9® (5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one) was a gift from AndroScience (San-Diego, CA) and was generated as described previously (Wu et al, 2010; Yang et al, 2007). ASC-J9® (dissolved in DMSO and diluted with corn oil) was i.p. injected into mice at a dose of 75 mg/kg every other day until the end of each study. Control mice received DMSO in corn oil only.

**Histological and immunohistochemical analysis**

Four percent of paraformaldehyde fixed prostates from individual genotypes of mice were subjected to paraffin embedding and sectioning (5 μm). To determine the phenotypic characterization of dARKO/Pten+/− mice, tissue sections were sequentially stained with H&E and IHC for characterization. For the BrdU incorporation assay, the BrdU injection protocol was described previously (Wu et al, 2007) and BrdU immunostaining was performed following the manufacturer’s instructions (InVitrogen). The Masson’s Trichrome staining kit was purchased from Sigma–Aldrich and used according to the manufacturer’s instructions. For IHC staining, the antibodies against AR (C-19, Santa Cruz Biotechnology), Pten (138G6, Cell Signaling), p-Akt at Ser473 (D9E, Cell Signaling), p-S6RP at Ser235/236 (91B2, 2595, Cell Signaling) were used. The DLPs from three treated groups of mice were subjected to histological characterization by BrdU staining (left panel) and the quantification results are provided in the right panel. The blue colors indicate collagen fibers defined by asterisks and quantification results are shown in the right panel.

**Figure 7. ASC-J9® impedes PIN lesion development and alleviates tumour-promoting microenvironment in Pten+/− mice. N = 5–6 mice per group.**

A. The DLPs from three treated groups of mice were subjected to histological characterization by BrdU staining (left panel) and the quantification results are provided in the right panel.

B. Pten+/− DMSO, Pten+/− DMSO and Pten+/− ASC-J9® treated mouse DLPs were subjected to Masson’s Trichrome staining to measure deposited collagen fibers. The blue colors indicate collagen fibers defined by asterisks and quantification results are shown in the right panel.

C. CD31 IHC staining. Arrows indicate CD31+ endothelial cells in the stromal compartment and quantification results are shown in the right panel.

D. CD3 IHC staining. Arrows indicate CD3+ T-cells and quantification results are shown in the right panel.
Stromal AR regulates PIN in Pten null mice

The paper explained

PROBLEM:
The interaction of epithelium and stroma plays a crucial role to determine the rodent and human prostate development. Although the tissue recombination has demonstrated that mesenchymal/stromal AR is indispensable for the rodent prostate organogenesis, the stromal AR function in the prostate cancer (PCa) development is still unclear especially under the real physiological condition such as in immune intact mice compared to tissue recombinants implanted into SCID mice. Here, we elucidated the importance of stromal AR in prostate tumourigenesis in PTEN deficient mice and dissected the mechanisms of cross-talk between stromal AR and adjacent oncogenic epithelium for prostatic intraepithelial neoplasia (PIN) development.

RESULTS:
Using a cre-loxP based conditional knockout system, we established a compound mutant mouse model, which has deleted AR in fibroblasts and smooth muscle cells with PTEN haploinsufficiency. By characterizing the phenotypes, we described that stromal AR is essential to control the epithelium proliferation and is able to modulate the surrounding tumour-promoting microenvironments. Those tumour-promoting microenvironmental features include the immune cell infiltration, angiogenesis and extracellular matrix (ECM) remodelling, which may contribute to the promotion of tumour initiation in Pten<sup>−/−</sup> mice. The data may only reflect PIN observed in the peripheral zone of human prostate.

IMPACT:
We have highlighted the critical role of stromal AR in the prostate tumourigenesis in mice with PTEN deletion, which frequently occurs in the human PCa patients. These valuable preclinical findings might not only advance our knowledge of the pathophysiological role of stromal AR, but also help us to develop a new therapeutic agent such as we used here, AR degradation enhancer, ASC-J9<sup>®</sup>. The successful clinical trials of this compound would be beneficial for patients who suffer from early stage PCa.

Figure 8. Stromal fibromuscular AR regulates PIN lesion through secretion of paracrine factors and alteration of surrounding microenvironments. A proposed model for the roles of CAFs AR to mediate tumour-promoting microenvironment and cross-talk between different cell types in the PIN areas of Pten<sup>−/−</sup> mice, illustrating the functional roles of AR in the ECM remodelling, angiogenesis and tumour-promoting inflammatory response.
Cell Signaling), vimentin (LN-6, Sigma–Aldrich), SMA (1A4, Sigma–Aldrich), CD3 (Sigma–Aldrich), F4/80 (A3-1, Abcam) and anti-CD31 (Abcam) were used.

Serum testosterone detection
Mice of the indicated genotypes were killed at 9 months old, blood was drawn by cardiac puncture and immediately assayed for serum testosterone concentration by an EIA kit following the manufacturer's instructions (Diagnostic Systems Laboratories).

Genotyping, RNA extraction, RT-PCR and real-time quantitative PCR analysis
Genomic DNA from mouse tails was purified by phenol/chloroform extraction and used as templates for PCR reactions to detect transgenes. Total RNA was prepared from cells with Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis was carried out by reverse transcriptase PCR (RT-PCR) with Superscript RNase H-reverse transcriptase (Invitrogen). Expression levels of AR, MIP-1α, MIP-1β, MIP-2 and IL-10 were determined by quantitative real-time PCR using iCycler real-time PCR amplifier (Bio-Rad Laboratories). The relative copy numbers of GAPDH were quantified by real-time PCR using iCycler real-time PCR amplifier (Bio-Rad Laboratories). The relative copy numbers of GAPDH were quantified by real-time PCR using iCycler real-time PCR amplifier (Bio-Rad Laboratories). The relative copy numbers of GAPDH were quantified by real-time PCR using iCycler real-time PCR amplifier (Bio-Rad Laboratories). The relative copy numbers of GAPDH were quantified by real-time PCR using iCycler real-time PCR amplifier (Bio-Rad Laboratories).

Statistics
The data are presented as mean± SEM. The student t-test was employed to compare two groups and one way ANOVA was used to compare more than two groups. p-Values of "p<0.05; **p<0.01; and ***p<0.001 were considered statistically significant.

For more detailed Materials and Methods see the Supporting Information Table.

Pro-inflammatory cytokine array analysis
Primary cultures of prostate stromal cells from Pten−/− mouse prostates were prepared and lentivirus carrying ARscr or ARsiRNA was used as control or to knockdown AR, respectively. The CM from each treated group was collected and subjected to cytokines analysis. The proinflammatory cytokine array was obtained from R&D systems and performed according to the manufacturer’s instructions.

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Supporting Information is available at EMBO Molecular Medicine online.

Conflict of interest statement: ASC-J9® was patented by the University of Rochester, the University of North Carolina, and AndroScience Corp., and then licensed to AndroScience Corp. Both the University of Rochester and C. Chang own royalties and equity in AndroScience Corp. The other authors declare that they have no conflict of interest.

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