**Carcinogenesis** vol.33 no.7 pp.1391–1398, 2012
doi:0.1093/carcin/bgs153
*Advance Access publication April 25, 2012*

# Androgen hormone action in prostatic carcinogenesis: stromal androgen receptors mediate prostate cancer progression, malignant transformation and metastasis

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**It has been postulated that prostatic carcinogenesis is androgen dependent and that androgens mediate their effects primarily through epithelial cells; however, definitive proof of androgen hormone action in prostate cancer (PRCA) progression is lacking. Here we demonstrate through genetic loss of function experiments that PRCA progression is androgen dependent and that androgen dependency occurs via prostatic stromal androgen receptors (AR) but not epithelial AR. Utilizing tissue recombination models of prostatic carcinogenesis, loss of AR function was evaluated by surgical castration or genetic deletion. Loss of AR function prevented prostatic carcinogenesis, malignant transformation and metastasis. Tissue-specific evaluation of androgen hormone action demonstrated that epithelial AR was not necessary for PRCA progression, whereas stromal AR was essential for PRCA progression, malignant transformation and metastasis. Stromal AR was not necessary for prostatic maintenance, suggesting that the lack of cancer progression due to stromal AR deletion was not related to altered prostatic homeostasis. Gene expression analysis identified numerous androgen-regulated stromal factors. Four candidate stromal AR-regulated genes were secreted growth factors: fibroblast growth factors-2, -7, -10 and hepatocyte growth factor which were significantly affected by androgens and antiandrogens in stromal cells grown *in vitro*. These data support the concept that androgens are necessary for PRCA progression and that the androgen-regulated stromal microenvironment is essential to carcinogenesis, malignant transformation and metastasis and may serve as a potential target in the prevention of PRCA.**

## Introduction

Prostate cancer (PRCA) is estimated to be detected in over 217 730 men in USA alone in 2010 (1). Many of these patients will undergo treatment to halt or slow the disease. Many more will be diagnosed with advanced PRCA for which there is no cure. Moreover, this year >32 050 men are projected to die from this disease. Goals of early detection, surgery, radiation therapy and hormone therapy are to decrease morbidity and mortality and increase life span. To achieve these goals, a better understanding of hormone action in the prostate must be achieved.

**Abbreviations:** AR, androgen receptors; ARKO, androgen receptor knockout; $E_2$, Estradiol-17β; FGF, fibroblast growth factor; hPrE, human prostatic epithelia; mPrE, mouse prostate epithelia; PRCA, prostate cancer; QPCR, quantitative reverse transcription–polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; shAR, AR small hairpin RNA; Str, stromal cells; T, testosterone; TGF, transforming growth factor.

Sex steroids are known to play an important role in prostatic carcinogenesis. In men and dogs, ratios of serum androgens:estrogens decrease with age (2,3). Estradiol-17β ($E_2$) in combination with testosterone (T) induces PRCA in mice, rats and human cells (4–9). These models support the hypothesis that the hormonal milieu induces PRCA initiation and/or progression. Determining how steroid hormones, especially androgens, stimulate carcinogenesis of the prostate will help elucidate fundamental mechanisms involved in this disease, lead to better therapeutics and may lead to increased life expectancy.

The prostate is a primary target organ for androgens. As such, androgens regulate prostatic development, growth and function. Androgenic effects are mediated via androgen receptors (AR) expressed in both epithelium and stroma of the prostate. Developmental biology studies have demonstrated that prostatic development and growth are mediated via signaling through stromal AR but not epithelial AR (10). Synthesis of epithelial androgen-dependent secretory proteins is thought to be dependent upon epithelial AR (11). The respective roles of AR signaling within epithelial versus stromal tissue layers in prostate growth and development have been elucidated by the analysis of tissue recombinants composed of AR-positive wild-type and AR-negative mouse epithelium and stroma. Similarly, PRCA is dependent upon androgens and removal of androgens increases cell death and decreases cellular proliferation. Hence, androgen deprivation therapy is the standard of care for men with advanced PRCA. The role of androgens in earlier stages of PRCA progression is not known but is assumed to be important. Furthermore, the role of epithelial versus stromal AR signaling in prostatic carcinogenesis remains unclear.

Currently, there is a lack of information on how human prostatic epithelial cells progress from benign to malignant states under the influence of androgens. This is due, in part, to the lack of models that evaluate early stages of human carcinogenesis that progress to metastasis. Models used for the current study do not incorporate highly mutagenic agents, overexpression of oncogenes or deletion of tumor suppressors to induce carcinogenesis or malignant transformation. Instead, circulating steroid hormones are used to elicit carcinogenesis of the benign prostatic tissues. To better understand androgen hormone action and stromal–epithelial interactions in prostatic carcinogenesis, we have utilized tissue recombination models of carcinogenesis where loss of AR function is achieved within prostatic epithelial and stromal elements. The objectives of these studies were to determine if prostatic carcinogenesis was dependent upon androgens and AR signaling. Additionally, we wanted to determine if stromal and/or epithelial AR was necessary for PRCA progression. To date, it is assumed that AR signaling during PRCA progression is mediated via epithelial AR (12–14). Determination of the androgen action at the tissue compartment level (epithelial versus stromal) during PRCA progression will help elucidate the role of androgens in PRCA progression. Moreover, understanding androgen hormone action may lead to better therapies, which may selectively target tissue-specific ARs of the prostate.

## Materials and methods

### *Animals*

All animals in these experiments were approved by University of Wisconsin and University of Rochester Medical Center's University Committee on Animal Resources and housed in the University's animal facilities with food and drinking water *ad libitum* under controlled conditions (12 h light, 12 h dark, 20°C ± 2°C).

Surgical insertion of T + $E_2$ slow-releasing implants and castration of male mice were performed as described previously (6,8). Castration was verified by histological analysis of testes and by verifying the decrease in size of seminal vesicles and prostates of treated animals. In all cases, castrated animals had

clear and small seminal vesicles and prostates, whereas in intact hosts, seminal vesicles and prostates were large and full of secretory fluid. Androgen receptor knockout (ARKO) mice, known as testicular feminization mice, were obtained from Jackson Laboratories (Bar Harbor, ME). Tails or forelimbs were collected from day 16 mouse embryos. DNA was isolated using the Maxwell 16 instrument and mouse tail DNA purification kit (Promega, Madison, WI). Genotyping was performed according to a protocol from Jackson Labs (http://jaxmice.jax.org/strain/001809.html).

*Tissue and cell collection and propagation*

Stromal cells (Str) were derived from mouse urogenital sinus mesenchyme collected on gestational day 16.5 as described previously (6,15,16). Urogenital sinus mesenchyme was minced into small pieces using two 28-gauge needles and allowed to attach and grow in media for 28 days. Cells were then trypsinized, counted and used in subsequent experiments. Human benign prostatic epithelial cells, termed human prostatic epithelia (hPrE)$^{BPH\text{-}1}$ (17), were used from passages 12–15 for all experiments. RWPE-1 cells were purchased from ATCC (Manassas, VA). Stroma from wild-type animals (Str$^{WT}$) were used for androgen-regulated gene expression experiments and were grown to confluency in T25 flasks in Dulbecco's modified Eagle's medium media with 5% charcoal-stripped fetal bovine serum with ×1 antibiotic/antimycotic with or without 12 nM T with or without 10 μM hydroxyl-flutamide. RNA was isolated from Str$^{WT}$ cells and evaluated at the University of Rochester Medical Center's Functional Genomics Core for gene expression. Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) was used to analyze gene expression.

*Reverse transcription–polymerase chain reaction and quantitative reverse transcription–polymerase chain reactions*

Reverse transcription–polymerase chain reaction (RT–PCR) and quantitative reverse transcription–polymerase chain reaction (QPCR) were performed as described previously (18,19). Briefly, RT–PCR conditions consisted of 3 min at 95°C and then 45 cycles of 10 s at 95°C, 30 s at 58°C and 30 s at 72°C. AR transcripts of ∼168 and 171 bp were observed in human and mouse RNAs. No cross-reactivity was observed between mouse and human primers. QPCR conditions consisted of 3 min at 95°C and then 40 cycles of 10 s at 95°C and 30 s at 58°C. Relative expression of genes were normalized to two housekeeping genes, β-glucuronidase (*GUS*) and β-2-microglobulin (*B2M*), using the ΔΔCT method within the Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA). All QPCR and RT–PCR primers were purchased from IDT (Iowa City, IA) (Supplementary Table I is available at Carcinogenesis Online).

*Preparation and processing of grafts, cell isolation and regrafting experiments*

Tissue recombinants, cell isolation and regrafting experiments were as described previously (6) with the following changes: 250 000 Str$^{WT}$ cells were collected from urogenital sinus mesenchyme of ARKO mice Str$^{ARKO}$ and mixed with 100 000 hPrE wild-type empty vector (hPrE$^{BPH\text{-}1}$) or the RWPE-1 human prostate epithelial cell line (hPrE$^{RWPE\text{-}1}$) or mouse prostate epithelia (mPrE) suspended in 30 μl rat tail type I collagen as described previously (6,8,15). These tissue recombinants were then grafted beneath the renal capsule of adult male athymic mice from the NCI (Frederick, MD) with or without subcutaneous slow releasing T (25 mg) and $E_2$ (2.5 mg) implants. Tissue recombinants containing mPrE from double heterozygous *Pten* and *Trp53* (mPrE$^{pten\pm p53\pm}$) mice were used as described previously (10,15,20) without the addition of exogenous hormones. Double heterozygous *Pten* and *Trp53* mice were generously provided by Dr Allan Balmain (University of California San Francisco, San Francisco, CA). *Pten* and *Trp53* genotyping was performed as described previously (15). Regrafting of purified epithelia (100 000 cells) or tissues (10 mg) was performed as we have described previously (6,8,15). Regrafting of Str$^{ARKO}$ + hPrE$^{BPH\text{-}1}$ tissue recombinants was achieved by growing 10 mg of tissue under the renal capsule of new male untreated hosts for an additional 6 months.

*Immunohistochemistry*

Immunohistochemical procedures including Ki67 labeling indices were performed as described previously (6,8,19,21). The following changes were made: freshly cut (<2 week old) tissue sections were used for detection of SV40 T-antigen (1:500, SCBT, Santa Cruz, CA) and AR (1:50, ABR, Golden, CO). Non-specific binding sites were blocked with a 2.5% (v:v) dilution of appropriate normal horse serum in phosphate-buffered saline (Vector Labs, Temecula, CA).

*AR small hairpin RNA construction and expression*

Use of AR small hairpin RNA (shAR) construct to effectively reduce AR protein has been described previously (18). Construction of shAR occurred by using the pSUPER.retro.puro retroviral vector (OligoEngine, Seattle, WA). The shAR oligonucleotide was subcloned into the vector BglII–HindIII site to generate pSUPER.retro-AR-small hairpin RNA. The retroviral construct was then transfected into Phoenix Ampho cells [SD 3443 (ATCC)] and viral supernatant harvested 48 h after transfection. The tissue culture medium containing virus was passed through a 0.45 μm filter and supplemented with 4 μg/ml hexadimethrine bromide (Sigma, H9268). Cells were then cultured in the viral supernatant for at least 6 h and allowed to recover for 24 h in fresh medium. Infected cells were then selected with 3 μg/ml puromycin for 48 h. BPH-1 cells were infected with either control empty vector (hPrE$^{BPH\text{-}1}$) or shAR vector (hPrE$^{BPH\text{-}1shAR}$) and subsequently grafted under the renal capsule of male athymic mice.

*Cancer incidence in tissue recombinant grafts*

Paraffin blocks containing grafts were sectioned and mounted on microscope slides. Three serial sections were placed on each slide representing a total of 18 μm of tissue thickness. Every 10th (for benign tissues) and 25th (for cancerous tissue) slide was stained with hematoxylin and eosin and examined histopathologically. Samples were scored for the presence or absence of malignant histopathology, benign architecture and squamous metaplasia. Malignancy was defined by both cytologic and architectural criteria and later confirmed by transplanting purified epithelial cells derived from the harvested grafts into untreated nude hosts as described previously (6,8,22).

*Statistics*

Data were analyzed by general linear models analysis of variance (Prizm, La Jolla, CA). When the *F*-test was significant ($P < 0.05$), differences among means were evaluated by Student-Newman-Keuls post-test comparison or *t*-tests when appropriate.

## Results

*Androgen deprivation prevents PRCA progression*

To determine if PRCA progression was dependent upon androgenic support, we evaluated two methods, surgical castration and genetic deletion of AR. Tissue recombinants composed of wild-type mouse stroma (Str$^{WT}$) plus mPrE from pten and p53 double heterozygous mice (mPrE$^{pten\pm p53\pm}$) (15) grown in intact hosts ($n = 6$) underwent carcinogenesis as evidenced by tumor formation and histopathological analysis (Figure 1). However, when Str$^{WT}$ + mPrE$^{pten\pm p53\pm}$ tissue recombinants ($n = 6$) were grown in castrate male mouse hosts, only small non-invasive grafts were observed. Tissue recombinants grown in castrate hosts were histologically benign with no invasion or nuclear atypia and were similar to Str$^{WT}$ + mPrE$^{WT}$ untreated control tissue recombinants (data not shown). Str$^{WT}$ + mPrE$^{pten\pm p53\pm}$ grafts grown in castrate hosts were significantly ($P < 0.001$) smaller (13.6 versus 177.6 mg) and had significantly ($P < 0.05$) fewer proliferating cells (3.8 versus 6.5; Figure 1B.).

To determine if genetic loss of AR function in stroma and epithelia was necessary for PRCA progression, we utilized tissue recombinants grown in T + $E_2$-treated hosts as described previously (6,8). Genetic loss of AR function was achieved via stable expression of small hairpin RNA toward AR (shAR) in hPrE termed here as hPrE$^{BPH\text{-}1shAR}$ or via use of AR knockout Str (Str$^{ARKO}$). Tissue recombinants composed of Str$^{ARKO}$ + hPrE$^{BPH\text{-}1shAR}$ ($n = 6$) grown in T + $E_2$-treated hosts did not develop into cancer unlike control hormone-treated Str$^{WT}$ + hPrE$^{BPH\text{-}1}$ tissue recombinants ($n = 7$), which developed into large invasive tumors that metastasized to the lymph node. Genetic loss of AR in both stroma and epithelial compartments led to significantly ($P < 0.01$) smaller tissue mass (13.5 versus 218.0 mg) and significantly ($P < 0.001$) lower proliferation (4.8 versus 24.4; Figure 1D). These data demonstrate that mouse and human PRCA progression can be prevented by surgical and genetic methods. Validation of tissue- and species-specific loss of AR expression in epithelial cells and Str was determined by RT–PCR and immunohistochemistry (Supplementary Figure 1 is available at Carcinogenesis Online). AR was expressed in both stroma and epithelia of all tissue recombinants (data not shown).

*Tissue-specific loss of AR function in prostatic carcinogenesis*

Genetic deletion of AR in stroma and epithelia prevented human PRCA progression; therefore, to evaluate the contribution of stromal versus epithelial AR in carcinogenesis, tissue-specific AR deletion was evaluated. To determine if AR tissue specificity was important

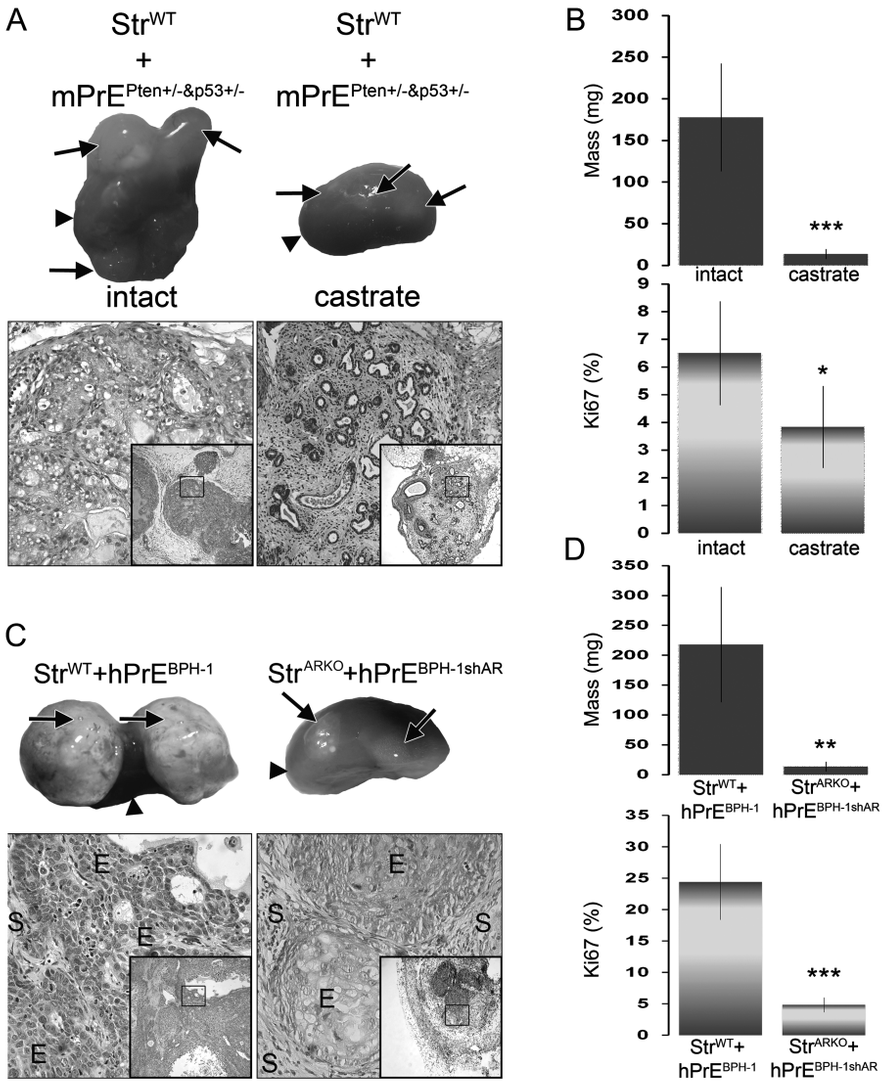


**Fig. 1.** Androgens and AR are necessary for mouse and human PRCA progression. (**A**) Tissue recombinants composed of mouse embryonic prostatic stroma (Str) + adult mPrE from double heterozygous pten and p53 mice ($mPrE^{pten\pm p53\pm}$) develop large tumors after 2 months of growth under the kidney capsule of adult intact male athymic mice (left panel arrows). Hematoxylin and eosin (H&E) analyses of Str + $mPrE^{pten\pm p53\pm}$ tissue recombinants grown in intact hosts demonstrated that high-grade prostatic intraepithelial neoplasia (PIN) developed, whereas tissue recombinants grown in castrated hosts developed into small non-invasive grafts with histologically benign prostate tissue (right panels, insets are at low magnification). (**B**) Castration of male hosts led to significantly ($P < 0.001$) smaller Str + $mPrE^{pten\pm p53\pm}$ tissue recombinants as well as decreased ($P < 0.05$) labeling indices for proliferation marker Ki67. (**C**) Analysis of tissue recombinants that genetically lack AR in stroma and epithelia do not develop into PRCA. Tissue recombinants composed of Str plus vector control $hPrE^{BPH\text{-}1}$ develop into large bulky malignant tumors after 4 months of growth under the kidney capsule of adult T- and estradiol-treated male athymic mouse hosts (left panel arrows). Tissue recombinants (arrows) composed of Str from ARKO mice plus $hPrE^{BPH\text{-}1}$ that stably expressed small hairpin RNA for AR (shAR) develop into small non-invasive prostate grafts (right panel). H&E analyses of $Str^{ARKO}$ + $hPrE^{BPH\text{-}1shAR}$ tissue recombinants grown in hormone-treated mice demonstrated benign histopathology with no invasion (lower right panel), unlike $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants where invasive cancerous histopathology was exhibited (lower left panel). (**D**) Genetic loss of stromal and epithelial AR led to significantly ($P < 0.01$) smaller tissue recombinants as well as significantly ($P < 0.001$) decreased labeling indices for proliferation marker Ki67 when compared with wild-type, AR-positive tissue recombinants. E, prostate epithelia; S, prostate stroma; arrowhead, kidney.

in prostatic carcinogenesis, tissue recombinants composed of $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ ($n = 7$) and $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ ($n = 8$) were used (Figure 2). Genetic loss of epithelial AR function did not appreciably affect PRCA progression as evidenced by the ability of $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ tissue recombinants to develop into large invasive cancers. These cancers did not differ in size (192.8 versus 171.2 mg) or histopathology compared with control $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants when grown in hormone-treated hosts. Similarly, proliferation (Ki67 labeling indices) was not different (24.8 versus 26.0) between these two groups. Contrasting this were the results of genetic loss of stromal AR function during cancer progression, where $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants developed into benign non-invasive prostate tissue. Furthermore, genetic loss of stromal AR led to significantly ($P < 0.001$) smaller grafts (26.4 versus 171.2 mg) that proliferated less (2.2 versus 4.4; $P < 0.001$).

To determine if stromal AR was necessary in PRCA progression in other models, we utilized the $Str^{WT}$ + $mPrE^{pten\pm p53\pm}$ and $Str^{WT}$ + $hPrE^{RWPE\text{-}1}$ PRCA models. Loss of stromal AR function was achieved using $Str^{ARKO}$ to generate $Str^{ARKO}$ + $mPrE^{pten\pm p53\pm}$ and $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ tissue recombinants. Results were similar to those observed in $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants, as demonstrated by histologically benign prostate tissue and significantly ($P < 0.001$) smaller grafts: 16.9 versus 303.8 and 4.4 versus 23.4, respectively (Figure 2D). As determined by RT–PCR and immunohistochemistry,

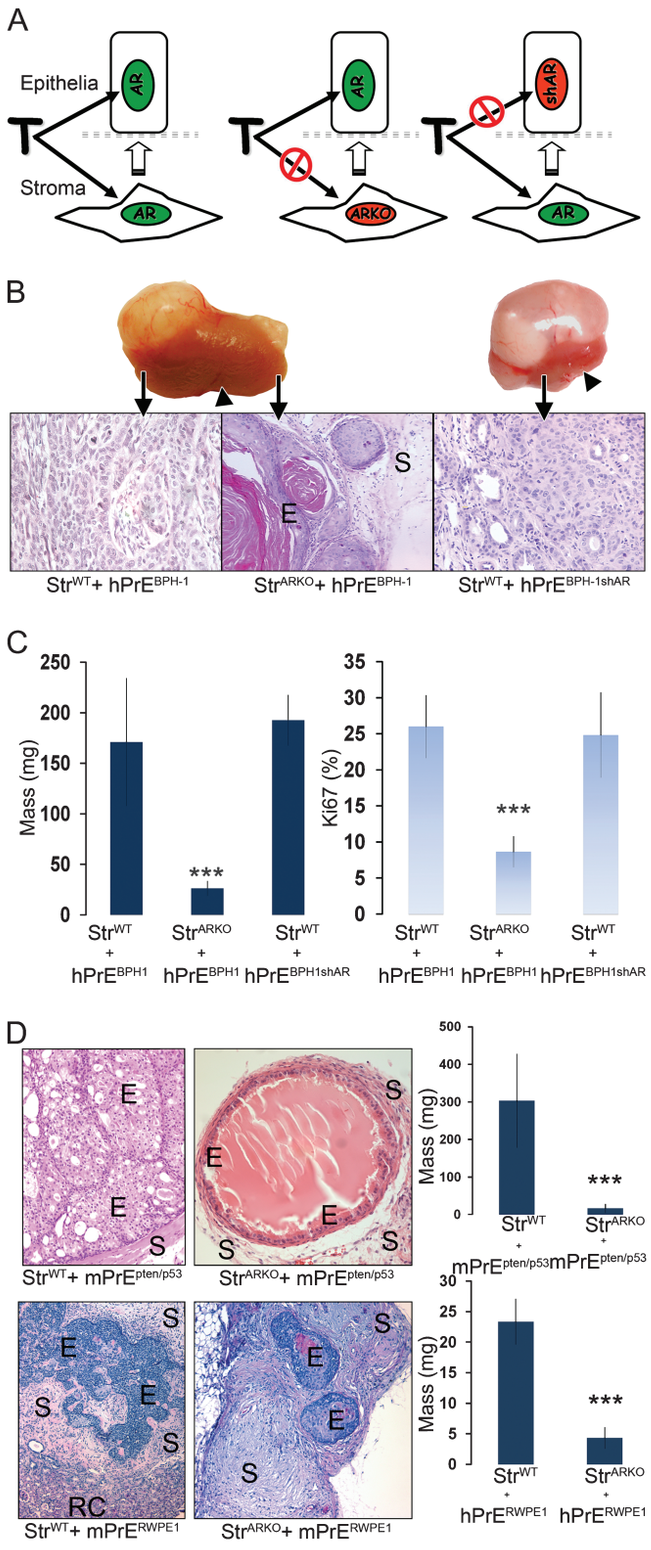


**Fig. 2.** Stromal AR but not epithelial AR is necessary for PRCA progression. (**A**) Schematic representation of androgen hormone action using genetically altered tissue recombinants induced to undergo carcinogenesis. Genetic loss of stromal AR function was achieved by use of ARKO Str, termed $Str^{ARKO}$, or stable expression of small hairpin RNA for AR in hPrE termed $hPrE^{BPH\text{-}1shAR}$. Three possible tissue recombination experiments were performed: AR present in both prostatic stroma and prostatic epithelia (left panel), AR present only in the epithelia but not in the stroma (middle panel) or AR present in the stroma but not in the epithelia (right panel). (**B**) Whole mounts and hematoxylin and eosin (H&E) staining of tissue-specific AR deletion. Wild-type (WT) littermate mouse stroma ($Str^{WT}$) plus empty vector control WT hPrE ($hPrE^{BPH\text{-}1}$) tissue recombinants grown in T + $E_2$-treated hosts to induce carcinogenesis (upper left panel). Tissue recombinants were noticeably smaller when the stroma lacked AR ($Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$; upper middle panel) compared with $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants, whereas loss of epithelial AR function ($Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$) led to the development of large invasive tumors (upper right). Histopathological analysis of tissue recombinants using H&E staining demonstrated that loss of stromal AR, but not epithelial AR, led to benign prostatic architecture. Note that epithelia (E) do not invade into surrounding stroma (S, middle panel). Genetic loss of AR function in both stroma and epithelia led to benign grafts (data not shown). (**C**) Determination of mass and proliferation (Ki67 labeling index) of tissue recombinants that lacked tissue-specific AR. Loss of stromal AR, found in $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants, led to significantly ($P < 0.001$) smaller tissue mass and proliferation. (**D**) To determine the necessity of stromal AR in other models of prostatic carcinogenesis, $Str^{WT}$ + mouse $PrE^{pten\pm p53\pm}$ and $Str^{WT}$ + $hPrE^{RWPE\text{-}1}$ + (T + $E_2$) models of prostatic carcinogenesis were utilized (see Materials and methods for details). Tissue recombinants composed of $Str^{WT}$ + $mPrE^{pten\pm p53\pm}$ and $Str^{WT}$ + $hPrE^{RWPE\text{-}1}$ + (T + $E_2$) developed into cancer, whereas $Str^{ARKO}$ + $mPrE^{pten\pm p53\pm}$ and $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ + (T + $E_2$) developed small, benign and non-invasive tissue. Histological analysis of $Str^{ARKO}$ + $mPrE^{pten\pm P53\pm}$ and $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ tissue recombinants demonstrated benign histology with no pathologic evidence of invasion or cancer. Loss of stromal AR, found in $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ and $Str^{ARKO}$ + $mPrE^{pten\pm p53\pm}$ tissue recombinants led to significantly ($P < 0.001$) smaller tissue mass. E, prostate epithelia; S, prostate stroma; arrowhead, kidney.

AR was detected in tissue recombinants accordingly. That is, AR was found in $Str^{WT}$ and hPrE, but not in $Str^{ARKO}$ and $hPrE^{BPH\text{-}1shAR}$ (Supplementary Figure 2 is available at Carcinogenesis Online). Collectively, these data support the concept that stromal AR, but not epithelial AR, is necessary for PRCA progression in human and mouse model systems.

*Determination of malignant transformation*

To determine if genetic loss of AR function affected the ability of hPrE to undergo malignant transformation, we isolated hPrE from $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ ($n = 5$), $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ ($n = 5$) and $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ ($n = 5$) tissue recombinants and transplanted them into athymic intact male mouse hosts as described previously (6,22). Purified hPrE free from stroma that were isolated from $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ or $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ tissue recombinants developed into large cancerous tissues when grown without stroma or hormones into new untreated male hosts. Tumor mass was not different (144.0 versus 147.0 mg) between groups (Figure 3). In contrast, epithelial cells isolated from tissue recombinants devoid of stromal AR (i.e. $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$) formed significantly ($P < 0.01$) smaller masses (6.3 versus 147.0 mg) and did not undergo malignant transformation when transplanted without stroma or hormones into new untreated intact male hosts. In tumors derived from purified epithelial cells isolated from $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ and $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$, metastasis was observed in 2/5 and 3/5 animals, respectively, whereas grafts derived from $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ did not metastasize (Supplementary Table II is available at Carcinogenesis Online). Proliferation (Ki67 labeling indices) of hPrE tumors derived from $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants was significantly ($P < 0.001$) less than hPrE tumors derived from $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ or $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ tissue recombinants (2.6 versus 25.8 and 29.4; Figure 3C). To determine if loss of stromal AR function in PRCA progression needed extended periods of time to develop into cancer, $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissue recombinants were grown in new hosts for an additional 6 months. Ultimately, $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ grew into large tumors and $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ tissues remained small and histologically benign. Collectively, these data support the concept that stromal, but not epithelial, AR is necessary for carcinogenesis, malignant transformation and metastasis of prostatic epithelia.

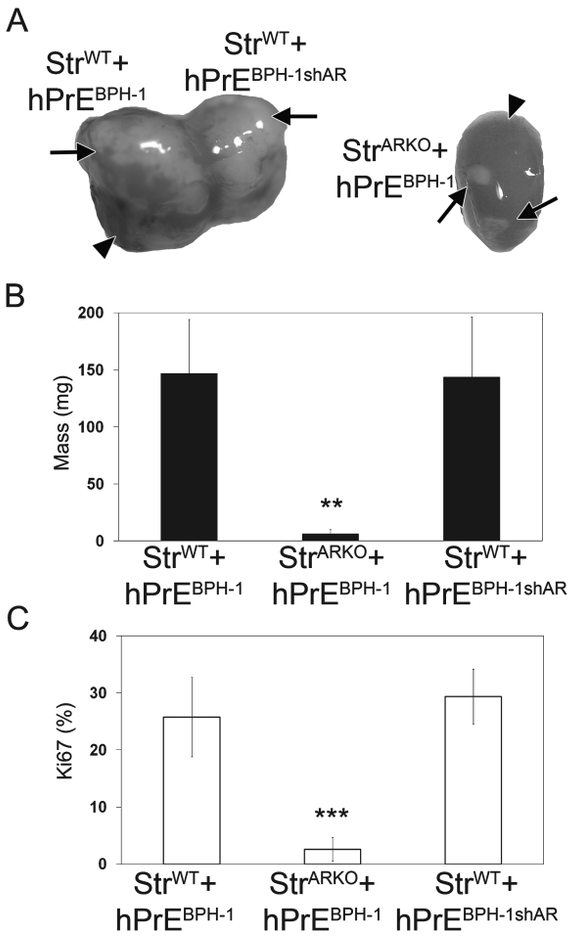


**Fig. 3.** Stromal AR but not epithelial AR is necessary for malignant transformation of hPrE. (**A**) Tissue recombinants (arrows) consisting of $Str^{WT}$ + $hPrE^{BPH\text{-}1}$, $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ or $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ were grown in hormone-treated animals (see text for details). hPrE were purified using G418 selection from respective tissue recombinants. hPrE cells (100 000 cells), free from mouse cells, were grafted into a new intact male host without stroma or hormones. Purified hPrE from $Str^{WT}$ + $hPrE^{BPH\text{-}1shAR}$ tissue recombinants (right side of kidney) underwent malignant transformation as evidenced by the development of large bulky tumors not different from hPrE isolated from $Str^{WT}$ + $hPrE^{BPH\text{-}1}$ (left side of kidney) when grown in new male hosts without stroma or hormones. Isolation of hPrE from tissue recombinants that lacked stromal AR ($Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$) developed into small, non-invasive tissue (right panel) when grown in new host. (**B**) Mass of isolated hPrE in (**A**) grown in male mouse hosts for 2 months without hormones or stroma. Note that hPrE isolated from tissue recombinants grown with ($Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$) were significantly ($P < 0.01$) smaller in mass and (**C**) contained significantly ($P < 0.001$) fewer Ki67-positive cells. Arrowheads, kidney.

*Stromal AR is not required for prostate maintenance*

The lack of carcinogenesis observed in $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$, $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ and $Str^{ARKO}$ + $mPrE^{pten\pm p53\pm}$ tissue recombinants may be explained by at least two possibilities. First, stromal AR may be necessary for prostatic homeostasis as described previously (16). Accordingly, loss of stromal AR may lead to a lack of prostatic homeostasis and hence prostate identity and prostatic carcinogenesis may not ensue. Alternatively, paracrine-acting growth factors regulated by stromal AR may be necessary to induce PRCA progression. Therefore, to investigate these possibilities, we evaluated growth of human and murine wild-type prostate epithelium ($hPrE^{BPH\text{-}1}$, $hPrE^{RWPE\text{-}1}$ and $mPrE^{WT}$) combined with $Str^{ARKO}$ in untreated intact male hosts. As expected, all untreated wild-type tissue recombinants ($Str^{WT}$ + $hPrE^{BPH\text{-}1}$, $Str^{WT}$ + $hPrE^{RWPE\text{-}1}$ and $Str^{WT}$ + $mPrE^{WT}$) developed into benign prostatic tissues similar to those described previously (6,11,20) (data not shown). Similarly, loss of stromal AR function, as observed in $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$, $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ and $Str^{ARKO}$ + $mPrE^{WT}$ tissue recombinants, led to prostate tissue development not different from their wild-type counterparts. No differences in mass were observed in $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$ (15.5 versus 11.9 mg) and $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ (12.9 versus 10.0 mg) tissue recombinants. Tissue recombinants composed of $Str^{ARKO}$ + $hPrE^{BPH\text{-}1}$, $Str^{ARKO}$ + $hPrE^{RWPE\text{-}1}$ or $Str^{ARKO}$ + $mPrE^{WT}$ lacked expression of stromal AR as demonstrated by species-specific RT–PCR or immunohistochemistry (Figure 4).

*Identification of candidate androgen-regulated genes in prostatic stroma*

In three different prostatic carcinogenesis models, stromal AR was necessary for PRCA development *in vivo*, and lack of stromal AR did not lead to decreased prostate homeostasis. We hypothesized that stromal-derived AR-regulated growth factors may be involved in carcinogenesis and may be expressed in stroma used in these models. To investigate this hypothesis and gain insight into which growth factors may play a role in prostate epithelial cell proliferation and progression, we evaluated the effects of T and estradiol on $Str^{WT}$ RNA using Affymetrix 430 2.0 gene expression arrays. These results were deposited at http://www.ncbi.nlm.nih.gov/geo/. A subset of these genes were validated by QPCR (Supplementary Table III is available at Carcinogenesis Online). We also evaluated androgen-regulated gene expression of secreted factors by QPCR. Compared with untreated (Unt) cells, T-treated $Str^{WT}$ ($n = 4$) expressed significantly ($P < 0.05$) higher levels of fibroblast growth factor (FGF)-2 (2.1-fold), FGF-7 (1.6-fold) and FGF-10 (2.6-fold), transforming growth factor (TGF)-β (1.8-fold) and hepatocyte growth factor (2.2-fold) (Figure 5). Of the androgen-induced genes, only TGF-β was not significantly decreased by the anti-androgen hydroxy-flutamide. These androgen-regulated paracrine factors have been shown to be important in prostate biology and therefore may represent potential stromal AR-regulated genes, i.e. putative andromedins, involved in prostatic carcinogenesis.

## Discussion

For decades, it has been speculated that androgens working via the AR are necessary for the development of PRCA. In fact, it is commonly stated that eunuchs do not get PRCA. Although this may be intuitive, formal investigation has not clearly proven this point. Experimentally, surgical, pharmacologic and genetic loss of AR function has reduced cancer growth but has not prevented cancer progression (18,23–25). Hence, definitive proof of the necessity of AR/AR signaling in PRCA is missing. Here we demonstrate that androgen deprivation in the form of surgical castration or complete genetic loss of AR function prevents the manifestation of PRCA in three human or mouse models of carcinogenesis. The concept that PRCA can be prevented at an early stage is enticing because currently there is no cure for PRCA. Additionally, PRCA progression takes decades to manifest and thus multiple therapies could be used over time to prevent the progression of cancer. Mechanistic data supporting the prevention of PRCA progression using androgen deprivation methodologies are lacking, thus gaining insight into androgen hormone action during PRCA progression is important to understand. The proof of principle provided herein that human PRCA progression can be prevented at an early stage may pave the way for new therapies or methods in the treatment of PRCA.

It is well established that androgens, AR signaling and stromal–epithelial interactions are essential for prostate function. The prostate contains ARs in stromal and epithelial tissue compartments. Therefore, it is likely that androgens elicit effects in the prostate via both stromal and epithelial AR signaling throughout life and disease progression. In cancer, androgens are thought to mediate their effects primarily via AR signaling in epithelial (i.e. carcinoma) cells. This may be in part because both primary prostate tumors and metastases

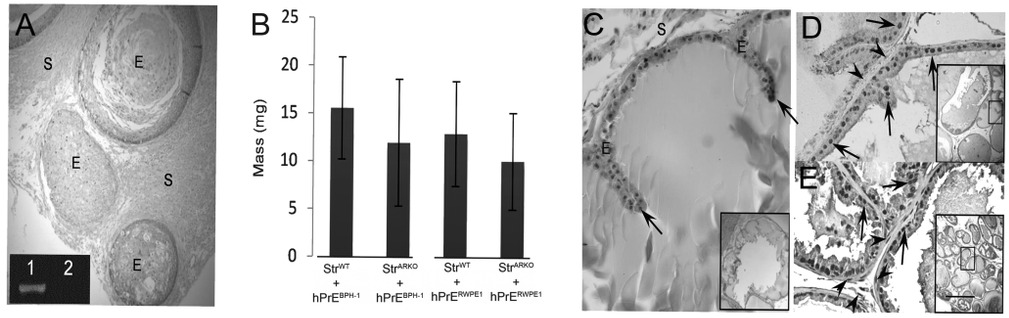


**Fig. 4.** Prostatic stromal AR is not necessary for maintenance of adult prostate phenotype. (**A**) Hematoxylin and eosin (H&E) analysis of tissue recombinants composed of $Str^{ARKO}$ + $hPrE^{BPH-1}$, grown in untreated intact male hosts, which developed into benign prostatic tissue, were not different from $Str^{WT}$ + $hPrE^{BPH-1}$ controls (data not shown). Note prostate epithelia (E) do not invade into surrounding stroma (S). Similar results were observed in $Str^{ARKO}$ + $hPrE^{RWPE-1}$ and $Str^{WT}$ + $hPrE^{RWPE-1}$ (data not shown). Species-specific AR RT–PCR was performed on $Str^{ARKO}$ + $hPrE^{BPH-1}$ tissue recombinants. Lane 1 AR primers were specific for human and lane 2 primers were specific for mouse AR (similar results were observed for $Str^{WT}$ + $hPrE^{RWPE-1}$). Note continued expression of human epithelial AR messenger RNA (mRNA) but not mouse stromal AR mRNA. (**B**) Mass of tissue recombinants composed of $Str^{WT}$ + $hPrE^{BPH-1}$, $Str^{ARKO}$ + $hPrE^{BPH-1}$, $Str^{WT}$ + $hPrE^{RWPE-1}$ and $Str^{ARKO}$ + $hPrE^{RWPE-1}$ grown in untreated intact male hosts. Note that mass of tissue recombinants lacking stromal AR were not significantly different than wild-type tissue recombinants. (**C**) H&E analysis of tissue recombinants composed of $Str^{ARKO}$ + $mPrE^{WT}$. Tissue recombinants developed non-invasive, histologically benign tissue similar to control untreated $Str^{WT}$ + $mPrE^{WT}$ tissue recombinants (data not shown). Note that the $mPrE^{WT}$ differentiated into normal prostatic tissue with glandular infoldings (arrows). (**D**) Tissue recombinants composed of $Str^{ARKO}$ + $mPrE^{WT}$ express AR (brown nuclear staining) in epithelial cells (arrows) but not within Str (arrowheads). (**E**) AR was localized to nuclei within epithelia (arrows) and stroma (arrowheads) of control $Str^{WT}$ + $mPrE^{WT}$ tissue recombinants. Insets are at low magnification.

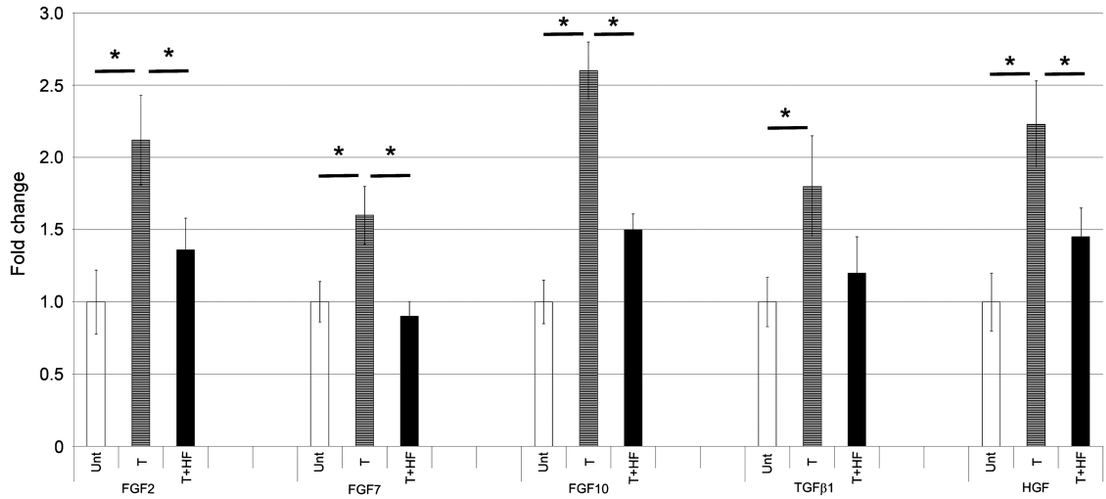


**Fig. 5.** Identification of candidate stromal-derived androgen-regulated factors. To determine if T affected transcriptional regulation of candidate factors, Str were grown *in vitro* with media containing 5% charcoal-stripped fetal bovine serum, with or without T (12 nM). Confluent Str were grown in respective media (72 h) and QPCR gene expression analysis of *FGF-2*, *-7* and *-10*, *TGF-β* and hepatocyte growth factor (*HGF*) was performed. Addition of T significantly ($P < 0.05$) stimulated Str gene expression of *FGF-2*, *-7*, *-10*, *TGF-β1* and *HGF*. Addition of the anti-androgen, hydroxy-flutamide (HF), significantly ($P < 0.05$) decreased *FGF-2*, *-7*, *-10* and *HGF* but not *TGF-β1*. Data are illustrated as fold change.

contain numerous carcinoma cells and fewer Str. However, prostatic carcinogenesis is a dynamic process, which takes many years to manifest. Lesions start in an environment of relatively high ratio of stromal:epithelial cells that changes during progression to a low ratio of stromal:epithelial cells in high-grade PRCAs. Moreover, epithelial AR expression generally increases in PRCA progression (12,26,27), whereas stromal AR generally decreases with progression (27,28). Collectively, this leads to an environment where stromal AR signaling and hence stromal AR regulation decreases with PRCA progression.

To determine the role of epithelial versus stromal AR in PRCA progression, we evaluated genetic loss of AR function in epithelia and stroma in tissue recombinants that undergo androgen-dependent hormonal carcinogenesis (6,15). The $Str^{WT}$ + $hPrE^{BPH-1}$ model progresses from benign, non-tumorigenic prostate tissue into malignant PRCA that metastasizes (6). These human tissue recombinants give rise to prostate tissue that is androgen dependent and contain genomic changes as well as progression markers that are consistent with PRCA progression in men (6,8,22,29). Loss of epithelial AR within tissue recombinants ($Str^{WT}$ + $hPrE^{BPH-1shAR}$) stimulated to undergo carcinogenesis led to invasive cancers after 3 months of growth, which was similar to tissue recombinants with AR-positive stroma and epithelia (i.e. $Str^{WT}$ + $hPrE^{BPH-1}$; Figure 2B). This suggested that epithelial AR was not necessary for cancer progression and malignant transformation. These findings are in agreement with previous data that demonstrated prostatic epithelial AR was not necessary for mouse prostatic carcinogenesis (18). Contrasting these results were experiments where tissue-specific loss of stromal AR function was evaluated. Loss of stromal AR function did not result in PRCA but instead led to the development of benign prostatic tissue growth (Figure 2B). Furthermore, hPrE isolated from $Str^{ARKO}$ + $hPrE^{BPH-1}$ tissue recombinants did not undergo malignant transformation or metastasis because these cells did not form cancers or metastasis when grown in new untreated male hosts (Figure 3A). Interestingly, although human epithelial cells were in contact with stroma and were exposed to hormones, the mere

loss of stromal, but not epithelial, AR apparently altered the tissue microenvironment sufficiently to prevent malignancy. An alternative explanation of the necessity of stromal AR in carcinogenesis is that stromal AR is an accelerator for carcinogenesis. To evaluate this possibility, we regrafted $Str^{ARKO}$ + $hPrE^{BPH-1}$ tissue recombinants into new male hosts for an additional 6 months. No cancer or metastasis was observed in these samples (Supplementary Table II is available at Carcinogenesis Online). This evidence further supports the concept that stromal AR signaling is necessary for carcinogenesis.

The concept of AR necessity in carcinogenesis may provide insight to how hormonally regulated organs such as the prostate rely on the stroma to progress from benign to malignant states and thus therapeutic targeting of intermediate events may be possible in the future. The loss of stromal AR in these studies is consistent with previous results where genetic loss of stromal AR function in experimental metastasis significantly reduced metastatic tumor size and proliferation, yet metastasis still occurred (18,30). An important distinction here is that a change in 'state' (i.e. from benign to malignant transformation or metastasis) was not observed. The idea that stromal AR regulates human PRCA progression was confirmed using other models of prostatic carcinogenesis including mPrE from *Pten* and *Trp53* double heterozygous mice (15) (Figure 2D) and the benign prostatic human epithelial cell line RWPE-1 ($hPrE^{RWPE-1}$) (31). These experiments demonstrated that stromal AR signaling was necessary for early stages of PRCA progression and malignant transformation in these models. Collectively, these data provide new mechanistic insight into androgen action as well as provide evidence that stroma control PRCA progression at early stages. Therefore, these data provide evidence of a stromal-specific target for future therapies in which to combat PRCA progression. The means of therapeutically preventing PRCA progression has been demonstrated in the Prostate Cancer Prevention Trial and Reduction by Dutasteride of Prostate Cancer Events (REDUCE) clinical trials. Although it is likely that reduction in androgen production would affect AR signaling in both stromal and epithelial tissue layers, our genetic proof of principle reported here suggests that decreased stromal AR signaling would be a potential mechanism of action for preventing PRCA progression in these trials. Hence, development of therapeutics that target the stroma may be particularly useful in the prevention of PRCA progression.

We have demonstrated previously that stromal AR is necessary for prostate development (10). Without stromal AR, prostatic tissues develop into vaginal-like tissues during organogenesis. As surmised, loss of stromal AR in these experiments may lead to prostate regression or interrupted prostate homeostasis preventing prostate development and hence PRCA. To determine if the lack of PRCA progression observed in $Str^{ARKO}$ + $hPrE^{BPH-1}$, $Str^{ARKO}$ + $hPrE^{RWPE-1}$ or $Str^{ARKO}$ + $mPrE^{Pten\pm p53\pm}$ tissue recombinants grown in untreated hosts was associated with the loss of prostatic maintenance, we evaluated genetic loss of stromal AR function in prostate homeostasis. Stromal AR was not necessary for maintenance of mature prostatic tissue phenotype in $Str^{ARKO}$ + $hPrE^{BPH-1}$, $Str^{ARKO}$ + $hPrE^{RWPE-1}$ or $Str^{ARKO}$ + $mPrE^{WT}$ tissue recombinants. These data suggest that stromal AR is not a significant prostatic maintenance factor in adult prostatic tissues unlike what is observed in prostate development, where loss of stromal AR function leads to phenotypically female tissues. These data are also consistent with the concept that stromal AR is a necessary contributor to PRCA progression.

To determine stromal androgen-regulated genes that may be involved in PRCA progression, we evaluated gene expression. A large number of genes were upregulated with the addition of hormones including various paracrine-acting growth factors known to be involved in the prostate/PRCA. Interestingly, members of the FGF, TGF-β and hepatocyte growth factor families were androgen regulated within Str but TGF-β expression was not significantly influenced with the addition of the anti-androgen, hydroxy-flutamide. Thus, androgen-regulated FGF and hepatocyte growth factor family members may be necessary for PRCA progression. In this regard, the FGF signaling pathway has recently been implicated in carcinogenesis in the mouse (32–34). Therefore, androgens working via stromal AR may elicit increased FGF expression or its bioactivity, which in turn may stimulate PRCA progression and malignant transformation. Collectively, these data provide evidence that androgens working primarily through stromal AR, but not epithelial AR, promote stromal growth factor expression, which may facilitate PRCA progression (Supplementary Figure 3 is available at Carcinogenesis Online). Future studies will evaluate the gain and loss of function of these stromal AR-regulated factors in PRCA progression.

The experiments performed here demonstrated that PRCA progression can be prevented. Furthermore, it was demonstrated that stromal AR but not epithelial AR was the key androgen signaling event in hormone-induced carcinogenesis of human and mPrE. Although others (34–36) have demonstrated that stromal factors can induce carcinogenesis, here we demonstrated that a single stromal factor, AR, is necessary for carcinogenesis. Additionally, the standard of care for PRCA is androgen ablation therapy, which indiscriminately targets both Str and epithelial cells via their ARs at different time points in progression. Thus, knowledge of androgen hormone action may provide the key to prevention of PRCA progression, particularly at early stages of the disease. The concept of targeting the tumor stromal microenvironment is attractive since it is genetically stable relative to the epithelial/carcinoma cell during carcinogenesis. Therefore, therapies targeting the stroma could be more effective because the Str may respond consistently to the administered therapeutic. Future studies will evaluate the efficacy of therapies that target stromal factors in the prevention of PRCA progression.

## Supplementary material

Supplementary Tables I–III and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

## Funding

National Institutes of Health (grant number CA123199 to W.A.R.).

## Acknowledgements

We would like to thank Pam Weller, Don Hom and Mei Cao for their technical assistance.

*Conflict of Interest Statement:* None declared.

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*Received November 9, 2011; revised April 13, 2012; accepted April 19, 2012*