AR Variant AR\textsuperscript{v567es} Induces Carcinogenesis in a Novel Transgenic Mouse Model of Prostate Cancer\textsuperscript{1,2}

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Abstract
Androgen deprivation therapy remains the primary treatment modality for patients with metastatic prostate cancer but is uniformly marked by progression to castration-resistant prostate cancer (CRPC) after a period of regression. Continued activation of androgen receptor (AR) signaling is attributed as one of the most important mechanisms underlying failure of therapy. Recently, the discovery of constitutively active AR splice variants (AR-Vs) adds more credence to this idea. Expression of AR-Vs in metastases portends a rapid progression of the tumor. However, the precise role of the AR-Vs in CRPC still remains unknown. AR\textsuperscript{v567es} is one of the two AR variants frequently found in human CRPC xenografts and metastases. Herein, we developed a probasin (Pb) promoter–driven AR\textsuperscript{v567es} transgenic mouse, Pb-AR\textsuperscript{v567es}, to evaluate the role of AR\textsuperscript{v567es} in both autonomous prostate growth and progression to CRPC. We found that expression of AR\textsuperscript{v567es} in the prostate results in epithelial hyperplasia by 16 weeks and invasive adenocarcinoma is evident by 1 year of age. The underlying genetic cellular events involved a cell cycle–related transcriptome and differential expression of a spectrum of genes that are critical for tumor initiation and progression. These findings indicate that AR\textsuperscript{v567es} could induce tumorigenesis de novo and signifies the critical role of AR-Vs in CRPC. Thus, the Pb-AR\textsuperscript{v567es} mouse could provide a novel model in which the role of AR variants in prostate cancer progression can be examined.

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Introduction
Patients with castration-resistant prostate cancer (CRPC) have an average survival time of 16 to 18 months from identification of recurrence [1–3]. Despite intensive investigation, treatment resistance of CRPC remains a significant clinical challenge, as the underlying mechanisms are still not fully understood. Persistence of intratumoral androgens [3–6], activation of alternate signaling pathways that could enhance androgen receptor (AR) activity in the presence of lower levels of androgen [7–11], and overexpression of AR that could promote sensitivity to low levels of ligand are all proposed mechanisms for the development of CRPC. Recently, the discovery of constitutively active AR splice variants (AR-Vs) adds credence to the idea that continued signaling through the AR plays a strong role in the development of CRPC [12–15].

The AR-Vs have different structures with each lacking portions of the ligand-binding domain [16–21]. Functionally, these truncated

Abbreviations: AR, androgen receptor; AR-FL, AR full length; AR-Vs, androgen receptor splice variants; AR\textsuperscript{v567es}, AR variant with exons 5, 6, and 7 skipped; CRPC, castration-resistant prostate cancer; EMT, epithelial to mesenchymal transition; GSEA, gene set enrichment analysis; GU, genitourinary; Pb, probasin; PIN, prostatic intraepithelial neoplasia; RQ, relative quantification; Tg, transgenic; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; Ube2c, murine E2 ubiquitin-conjugating enzyme; Wt, wild type

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\textsuperscript{2}This article refers to supplementary materials, which are designated by Tables W1 and W2 and Figures W1 to W3 and are available online at www.neoplasia.com.

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AR variants are constitutively active, ligand-independent transcription factors that can support androgen-independent expression of AR target genes. The potential role of AR-Vs in driving prostate cancer progression is supported by several independent clinical correlation studies [17,18,22] and provides a conceptually simple explanation for the development of resistance to prostate cancer therapies that target the ligand-binding domain. However, the mechanisms by which the splice variants could mediate progression of prostate cancer following castration still need to be elucidated.

ARv567es, in which exons 5, 6, and 7 are deleted, is one of the two AR variants frequently found in human CRPC xenografts and human metastases [12,20,22,23]. Expression in metastases portends a rapid progression of the tumor [22]. Currently, the limited studies on ARv567es are confined to expression testing with xenografts and functional exploration in in vitro cell lines [12,20,22,23]. The precise role of the ARv567es in normal prostate growth and CRPC is unknown.

**Materials and Methods**

**Generation of a Transgenic Mouse Model Expressing Human AR Variant, ARv567es, in Prostate Epithelium**

Mice were bred and housed under specific pathogen-free conditions in the University of Washington animal facility in accordance with institutional guidelines. To create the Pb-ARv567es transgenic (Tg) mouse used in this study, we replaced the SV40T fragment from the A2R2PB-SV40T expression cassette [24] with the cDNA fragment encoding ARv567es. The construction of cDNA encoding ARv567es has been previously described [20,24]. The entire Pb-ARv567es expression cassette was gel isolated following digestion with Sphi and was microinjected into fertilized C57BL/6 embryos (performed at the University of Washington Comparative Medicine Transgenic Core Facility).

**Genotyping**

Tg progeny were identified by polymerase chain reaction (PCR) analysis of DNA extracted from tail biopsies of 3-week-old mice. Tail tips were lysed (25 mM NaOH and 2 mM EDTA) and boiled for 30 minutes; after cooling, the mixture was placed in neutralization buffer (40 mM Trizma base). Integration of a single intact ARv567es transgene copy was confirmed by genomic PCR with limited template dilution using AccuPrime Taq high fidelity DNA polymerase (Life Technologies, Grand Island, NY). A forward primer specific for rabbit β-globin intron (5′-CCGGGCCCCTCTGCTAACCA-3′), and a reverse primer specific for ARv567es (5′-TCCCTCGTCCGAGGTGCTGG-3′).

**Castration Studies**

Castration studies were performed on 16-week-old mice and 49- to 52-week-old mice. Pb-ARv567es and wild-type (Wt) littermates were either castrated or had sham surgery performed. Mice were sacrificed 3 weeks post-surgery. At the time of sacrifice, the prostate and genitourinary (GU) organs as well as liver, lung, and kidneys were harvested and weighed. Half of the prostate was then placed in formalin and the other half in RNAlater. All other organs collected were placed only in formalin.

**Polymerase Chain Reaction**

Total RNA was obtained from prostates using TRIzol (Life Technologies). RNA was converted to cDNA using SuperScript First-Strand Synthesis System according to the manufacturer’s protocol with random primers (Life Technologies). Relative quantitative reverse transcription-PCR (qRT-PCR) was then performed using a ViiA 7 Real-Time PCR system (Life Technologies) and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). PCR data were analyzed using ViiA 7 Software (Life Technologies). Target mRNA levels were normalized against Rpl13a levels. ΔΔCT method was used to compare the relative expression, and relative quantification (RQ) value was achieved. The distribution of RQ of individuals in the Tg and Wt groups was displayed as mean ± 1 SEM. TaqMan probe was designed to target the unique ARv567es exon 4 and exon 8 junction: 6FAM-CCTTGGCGCTGTTGGAGA-MGBNFQ. The following primer pairs were used:

<table>
<thead>
<tr>
<th>Forward PCR Primer</th>
<th>Reverse PCR Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARv567es</td>
<td>CTT TGC TCT CTA GCC TCA ATG AA</td>
</tr>
<tr>
<td>Forward: CCT TGC TCT CTA GCC TCA ATG AA</td>
<td>Reverse: GGT GAT TAG CAG GTC AAA AGT GAA CT</td>
</tr>
<tr>
<td>Murine E2 ubiquitin-conjugating enzyme (Ube2c)</td>
<td>GGG AGA GTT TGT ACC TCA GGT</td>
</tr>
<tr>
<td>Forward: GGG AGA GTT TGT ACC TCA GGT</td>
<td>Reverse: GGG CAC CAG TAA CAA TGG AG</td>
</tr>
<tr>
<td>Fkbpl</td>
<td>GAT GGT GTG ACT GAT GGT GG</td>
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<tr>
<td>Forward: GAT GGT GTG ACT GAT GGT GG</td>
<td>Reverse: GGC AAA TGG CTT CTT TCT GT</td>
</tr>
<tr>
<td>Fgfbp1</td>
<td>GGC AAA TGG CTT CTT TCT GT</td>
</tr>
<tr>
<td>Forward: GGC AAA TGG CTT CTT TCT GT</td>
<td>Reverse: CCT AGG CAT CAA CAT TCC TG</td>
</tr>
<tr>
<td>Nkx3.1</td>
<td>Rev</td>
</tr>
<tr>
<td>Forward: GCA GGC TAT TGC TCA TCA CA</td>
<td>Reverse: GGT GAT TAG CAG GTC AAA AGT GAA CT</td>
</tr>
<tr>
<td>Rpl13a</td>
<td>AGG GCC TAT GCC CCA CCA</td>
</tr>
<tr>
<td>Forward: AGG GCC TAT GCC CCA CCA</td>
<td>Reverse: TCT GAT TAA CCA CCA CCT GGT CTC TGG</td>
</tr>
<tr>
<td>Gsd2</td>
<td>GCC ATG AAC GCC TGC CTA</td>
</tr>
<tr>
<td>Forward: GCC ATG AAC GCC TGC CTA</td>
<td>Reverse: GTC ATC AAC TCA</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>GGC AAA TGG CTT CTT TCT GT</td>
</tr>
<tr>
<td>Wnt11</td>
<td>GGT GGT GTG ACT GAT GGT GG</td>
</tr>
<tr>
<td>Forward: GAT GGT GTG ACT GAT GGT GG</td>
<td>Reverse: ACT TCT GAC CTG CAG CTC TA</td>
</tr>
<tr>
<td>Lbp</td>
<td>GAG GGG TGA TGC TCA TTA AA</td>
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<tr>
<td>Forward: GAG GGG TGA TGC TCA TTA AA</td>
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</tr>
<tr>
<td>Chodl</td>
<td>GAG GGG TGA TGC TCA TTA AA</td>
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<tr>
<td>Forward: GAG GGG TGA TGC TCA TTA AA</td>
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</tr>
</tbody>
</table>

**Immunohistochemistry**

Prostates were fixed with formalin and then paraffin embedded. Ten-micrometer-thick sections were made for hematoxylin and eosin (H&E) staining as well as immunohistochemistry (IHC). We used the Elite Vectastain ABC kits for IHC (Vector Laboratories, Inc, Burlingame, CA). For better antigen retrieval, slides were placed in a 0.01 M citrate buffer for 25 minutes and then blocked with 5% serum in phosphate-buffered saline (PBS). For mouse antibodies, we used an additional blocking step (avidin blocking solution followed by a biotin blocking solution; Vector Laboratories, Inc). When staining for non-nuclear proteins, hematoxylin was used as a counterstain. Primary and secondary antibodies were diluted in 5% serum/PBS. The human-specific AR N-terminal antibody AR441 (Santa Cruz Biotechnology, Santa Cruz, CA) and the murine-specific C-terminal antibody EP670Y (Epitomics, Burlingame, CA) were used for detecting ARv567es or endogenous mouse AR, respectively. Other primary antibodies used were Ki-67 (Thermo Scientific, Fremont, CA), Ube2c (Boston Biochem, Cambridge, MA), FoxA1, Twist, Vimentin (Abcam, Cambridge, MA), and E-cadherin (BD Biosciences, San Jose, CA).
DNA Fragmentation Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

The presence of apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), using the DEAEEND Colorimetric Apoptosis Detection System (Promega, Madison, WI) as per the manufacturer’s instructions. Briefly, paraffin-embedded slides were fixed in 4% paraformaldehyde in PBS for 15 minutes after pretreatment. After washes, tissues were permeabilized in proteinase K for 30 minutes and then labeled in TdT reaction mix for 60 minutes at 37°C. Reaction was stopped by immersion in 2× saline-sodium citrate buffer (SCC) for 15 minutes, and then streptavidin-HRP was added for 30 minutes followed by DAB stain and hematoxylin counterstain.

Statistical Analyses

Weight data are displayed as mean ± 1 SD. Real-time PCR data are displayed as mean ± 1 SEM. When two groups were compared, two-tailed Student’s t test was used (GraphPad Prism, version 5.0d; GraphPad Software, La Jolla, CA). A P value of .05 or less was considered significant.

Results

To evaluate the role of the constitutively active AR splice variant ARv567es in the autonomous state, we developed a probasin (Pb) promoter–driven ARv567es Tg mouse, Pb-ARv567es. We targeted the ARv567es coding sequence to the prostate epithelium of C57BL/6 mouse under the drive of the minimal rat Pb promoter (-426 to +28 bp) [24], which we designated as the Pb-ARv567es mouse. Integration of a single intact ARv567es transgene copy was confirmed by genomic PCR with limited template dilution (Figure W1). Real-time RT-PCR using a TaqMan probe targeted to the exon 4/8 junction of the variant demonstrated the profuse presence of ARv567es transcripts in Pb-ARv567es mice compared to Wt littermates (Figure 1A). Protein expression of ARv567es in the prostate epithelium was further confirmed by immunohistochemistry staining with the human-specific AR N-terminal antibody AR441, which was not detected in the Wt littermates (Figure 1B) and no antibody control (Figure W3A). ARv567es protein expression was detected in all four prostate lobes with a tendency of higher expression in lateral and ventral lobes.

To investigate whether ARv567es functions in normal prostate growth, and whether androgen could affect its function, we performed castration and sham surgery on Pb-ARv567es and Wt mice at 16 weeks of age. Three weeks post-surgery, the prostate and GU organs were harvested and weighed. As shown in Figure 1, C and D, and Table W1 in both the castrated and sham Pb-ARv567es mice, the prostate and GU organs showed significantly higher relative weights compared with those of Wt littermates (P < .05). Accordingly, the proliferation rate of Pb-ARv567es mouse prostate increased with a...
Figure 2. AR^{v567es} induced prostatic hyperplasia, PIN, and adenocarcinoma (×400). (A) Foci hyperplasia lesions (arrows) were evident in Pb-AR^{v567es} mice at 16 to 20 weeks of age, demonstrated by increased numbers of cells with normal or enlarged size in hyperproliferated epithelium. Comparatively normal prostatic epithelium could be seen in adjacent gland (arrowheads) showing normal single layer structure. (B) PIN in Pb-AR^{v567es} mice at 30 to 40 weeks of age (arrows) showed overlapping of cells, elongation of nuclei, and prominence of nucleoli. (C) Prostate histology in old mice, 50 to 60 weeks. Wt group demonstrated normal-looking prostate with single layer of glandular epithelium, normal nuclear and nuclear-to-cytoplasm ratio and low proliferation, and amorphic secretions containing numerous atrophic epithelium. Castrated Wt mice showed atrophic cells and epithelium but retained organized structure. Pb-AR^{v567es} mice showed low-grade PIN in anterior prostate (AP) and dorsal prostate (DP) and high-grade PIN in lateral prostate (LP) and ventral prostate (VP), which are shown as overlapping of epithelial cells forming multiple layer of epithelium, enlargement of nuclei and high nuclear-to-cytoplasm ratio, and hyperchromatic nuclei with prominent nucleoli. Ventral prostate showed more advanced progression to well-differentiated adenocarcinoma, which is shown as cellular changes corresponding to dysplasia, increased quantity of small glands, and lymphocyte infiltration in stroma. Castrated Pb-AR^{v567es} mice showed typical well-differentiated adenocarcinoma appearance, which is shown as increased quantity of small glands, thickened stroma with infiltrated tumor cells, and round, dysplastic, and hyperchromatic nuclei with very little cytoplasm. Lymphocyte infiltration is very common.
significantly higher Ki-67 index compared with Wt littermates \((P < 0.05)\) (Figures 1E and W3B). These data show that while AR\(^{v567es}\) does not maintain the normal differentiated growth of the prostate, AR\(^{v567es}\) does increase the proliferative rate of the prostate epithelium as seen by the increased weight of the GU tract and prostate post-castration in Pb-AR\(^{v567es}\) versus Wt mice. The effect of AR\(^{v567es}\) on apoptosis was also determined by TUNEL assay (Figure W3C). Comparable appearances of apoptotic cells in Tg mice and Wt littermates indicated an insignificant role of AR\(^{v567es}\) on apoptosis protection.

Using qPCR and immunohistochemistry staining, we confirmed the continued expression and nuclear localization of AR\(^{v567es}\) after castration (Figure W2, A and B), which signified the constitutive activity of AR\(^{v567es}\) in the absence of ligand binding. We also examined the endogenous mouse AR levels by IHC using a mouse-specific C-terminal antibody, EP670Y, and showed that the expression of mouse AR was significantly decreased after castration with only hints of staining in the cytoplasm (Figures W2C).

To further characterize the features of the Pb-AR\(^{v567es}\) mouse, the prostate histology was serially evaluated from 16 weeks of age up to 1 year (Table W2). Consistent with the prostate weight outcomes, foci of hyperplasia could be seen in young adults (16 weeks; Figure 2A), with a gradual progression to prostatic intraepithelial neoplasia (PIN) lesions by 40 weeks (Figure 2B). To investigate differences between intact and castrate mouse histology in older Pb-AR\(^{v567es}\) mice, castration and sham operations were also performed on 50-week-old mice. Prostate histology was examined 3 weeks post-castration. Well-differentiated adenocarcinoma could be seen in the sham-operated mice (Figure 2C), which was characterized as increased quantity of small

![Figure 3. Invasive adenocarcinoma in the ventral prostate of Pb-AR\(^{v567es}\) mice. (A and B) Low power (×100) showing well-differentiated adenocarcinoma in ventral prostates. Widespread and essentially diffuse PIN containing glands mixed with small glands (arrowheads) and tumor cell infiltrations (*) in the surrounding fibromuscular stroma or looser connective tissue with desmoplastic response. (C and D) Intermediate power (×200) showing unequivocal invasive small acinar adenocarcinoma, extensively extending into stroma and periprostatic loose connective tissue (arrowheads). (E) Focus on a gland with complete epithelial cells infiltrating into surrounding periprostatic connective tissue (*) and also forming small acini (arrowheads). (F) High power (×400) showing a distinct sheet of tumor cell invasion into stroma (arrow) and a typical well-differentiated gland formed (arrowhead). (G–I) High magnification (×400). Immunohistochemistry of epithelial cell marker FoxA1 showing unequivocal invasive small acinar adenocarcinoma (arrowheads), extensively extending into stroma and surrounding periprostatic loose connective tissue. In addition to invasion into stroma and periprostatic tissue, overgrowing of epithelium causes cell shedding into the lumen (*). (G) A cluster of tumor cells appearing in the blood vessel (arrow) signifying the possibility of metastasis through the bloodstream. (J) FoxA1 staining in age-matched Wt littermate (arrowhead). IHC with AR441 antibody showed the expression of AR\(^{v567es}\) in glands (K) and stroma (L) in the invasive tumor.](image-url)
glands, stromal thickening with desmoplastic response, hyperchromatic round nuclei with increased mitosis, and frequent inflammation. More advanced progression to invasive adenocarcinoma is evident in the castrated group (Figure 3, A–F), which displayed a ventral prostate preference. The characterization of invasive adenocarcinoma includes pronounced extension of smaller, well-formed acini into surrounding stroma and periprostatic loose connective tissue with significant desmoplastic response and inflammation [25].

To characterize the invasive adenocarcinoma, we performed IHC against FOXA1 as well as epithelial to mesenchymal transition (EMT) markers. FOXA1 staining confirmed epithelial tumor cell infiltration into the periprostatic stroma and connective tissues where they formed acini glands (Figure 3, G–I). In one view (Figure 3G), tumor clumps can be seen in an interstitial blood vessel, which may signify the possibility of metastasis through the blood stream. AR441 staining (Figure 3, K and L) showed constitutive expression of variant ARv567es in the invasive tumor area. EMT markers were also examined. Loss of E-cadherin and higher expression of Twist and Vimentin (Figure 4A) strongly suggested that an EMT was one of the most important mechanisms underlying ARv567es-induced invasive phenotype development [26–28]. Except randomly localized hyperplasia, none of the Wt littermates showed PIN or adenocarcinoma in all the age groups (Table W2). These data strongly indicated that ARv567es can induce prostate cancer to develop in a de novo way, and it has the potential to enhance the progression of androgen-dependent disease to CRPC.

Recently, Hu et al. [29] characterized the distinct expression patterns of gene sets induced by AR full length (AR-FL) and AR-Vs and found that AR-Vs induced a top gene set enriched with cell cycle genes, compared with the biosynthesis, metabolism, and secretion
Figure 5. Mouse whole genome expression oligonucleotide microarray analyses showing the differentially regulated transcriptome in \( \text{Pb-AR}^{\text{567es}} \) prostate. (A) Oncology-related gene categories that were significantly downregulated in \( \text{Pb-AR}^{\text{567es}} \) mice by microarray GSEA (false discovery rate < 25%). (B) Oncology signatures significantly upregulated in \( \text{Pb-AR}^{\text{567es}} \) mice by microarray GSEA (false discovery rate < 25%). (C) qPCR verification of SINGH_KRAS_DEPENDENCY_SIGNATURE up-regulation by showing higher expression of Kras and Fgfbp1. (D) qPCR verification of HINATA_NFKB_IMMUN-INF signature up-regulation by showing higher expression of Nfkb1 and Ccl2. (E) qPCR verification of HINATA_NFKB_IMMUN-INF signature up-regulation including \( \beta \)-catenin gene, Wnt11, Lbp, and Chodl.
gene sets induced by AR-FL [29]. Of the cell cycle genes increased by AR-Vs, Ube2c is the most outstanding and its expression is significantly correlated with AR-Vs but not AR-FL [29]. To investigate whether ARv567es could also induce a similar transcriptome in the Pb-ARv567es Tg mouse, we ran real-time PCR on prostates from intact and castrated Pb-ARv567es mice; Tg prostates from castrated mice showed increased expression of mitotic genes similar to those seen in CRPC patients, including Ube2c (Figure 4C). Upregulated Ube2c protein levels were further confirmed by immunohistochemistry compared with Wt control (Figure 4B). Other genes of interest that were upregulated included the canonical AR-regulated gene Fkbp5, the Nkx3.1 homeobox gene, and the proliferation marker Ki-67 (Figure 4C), consistent with the IHC data (Figure 1E). This gene expression pattern demonstrated that the AR variant in mouse prostate functions in a way closely mimicking that in human cancer. Furthermore, our Pb-ARv567es mouse demonstrates that the distinct AR variant transcriptome is a common and direct molecular consequence when AR-Vs occur and further suggests that activation of this transcriptome increases prostate epithelial cell progression by enhancing passage through the cell cycle.

To identify potential changes in RNA transcripts underlying ARv567es-mediated tumorigenesis, we further examined the transcriptional reprogramming of Tg mice prostates using mouse whole genome expression oligonucleotide microarray analyses. Gene set enrichment analysis (GSEA; Figure 5, A and B) showed differentially regulated gene sets that included inflammatory-related cytokines, transcriptional factors, and tumorigenesis-associated factors. We further verified some genes in the upregulated oncology signatures including Kras (Figure 5C), nuclear factor kappa B (NF-kB) (Figure 5D), and β-catenin/Wnt pathway (Figure 5E) related markers. These data suggest that ARv567es induces a reprogramming of cellular transcriptional activity and upregulates the expression of a spectrum of genes that are critical for tumor initiation and progression.

Discussion
Two constitutively active AR-Vs have been described in clinical disease, AR-V7 and ARv567es. AR-V7 is reported in most prostate tissues both benign and malignant, but ARv567es has only been seen in malignant prostate glands [12,20,22,23]. Although ARv567es is not the most frequently expressed AR-V, we chose to use ARv567es in our Tg mouse because this variant is the most restricted to prostate malignancy.

The most impressive finding in the present study was that ARv567es could induce prostate cancer autonomously from de novo as well as contribute to progression to CRPC following castration, findings that have not been previously reported. These findings are of great value in light of the current differing hypotheses concerning the role of AR-Vs in prostate cancer progression, especially CRPC: one hypothesis regards the occurrence of variants as a phenomenon, as only the by-product of CRPC, whereas the other hypothesis attributes to AR-Vs a role in CRPC, in which AR-Vs precipitate the progression and contribute to the devastating outcome of CRPC. In our data, we showed that ARv567es could induce cancer development de novo as well as induce progression to CRPC.

A mitotic transcriptome has been associated with CRPC in human studies [12,17,19,30]. Importantly, recent data showed the AR-Vs are associated with a more mitotic transcriptome than AR-FL [22,29]. Consistent with these findings, our data also showed upregulated mitotic genes including Ki67 and Ube2c. Ube2c is an anaphase-promoting complex/cyclosome–specific E2 ubiquitin-conjugating enzyme that has a critical role in anaphase-promoting complex/cyclosome–dependent M-phase cell cycle progression by inactivating the M-phase checkpoint. Ube2c has been shown to be a prominent oncogene and is found overexpressed in various types of solid tumors including late-stage prostate cancer [30,31]. In addition to in vitro studies, these data from our Tg mouse further support that an AR-V–activated mitotic transcriptome plays a critical role in CRPC.

In addition to the transcriptome shift to mitotic genes that augments proliferation and cell growth, panels of tumorigenesis-related gene signatures were differentially regulated. In the Tg prostates, significant up-regulation of five oncogenic signatures that have been associated with prostate cancer initiation, progression, and metastasis was detected by GSEA: K-RAS, NF-kB, STK33, Gli1, and β-catenin. K-RAS has been associated with prostate cancer progression, specifically a role in EMT and metastatic progression [32]. Further, in cells that are K-RAS dependent, STK33 promotes cancer cell viability [33,34]. The inflammatory transcription factor NF-kB plays an early role in cellular transformation as well as therapy resistance and metastatic progression [35,36]. Overexpression of β-catenin contributes to invasion of prostate cancer [37], whereas Gli1, which binds directly to the AR, contributes to the development of CRPC [38]. Examination of the cross talk of ARv567es with these oncologic pathways would be a highly necessary task in future studies.

Given the evidence of the autonomous process of tumorigenesis induced by ARv567es as well as its role in the development of CRPC, this novel Tg mouse will be an excellent model for more in-depth studies of the role of AR variant in prostate cancer development and progression. Future studies crossing the Pb-ARv567es with current CRPC mouse models would provide unique CRPC models in which to examine the direct role of AR variant in CRPC development, progression, and therapy resistance.

Acknowledgments
We thank the Comparative Animal Department at the University of Washington for their assistance in developing the Pb-ARv567es mouse and the Histopathology Laboratory at the University of Washington for sectioning and H&E staining of mouse tissues. We thank Robert Matusik for his insights on the prostate pathology of the Tg mice.

References


Figure W1. (A) Diagrammatic representation of the Tg construct. The human AR splice variant AR\textsuperscript{667es} coding sequence was subcloned between AR2Pb promoter and the human growth hormone polyadenylation signal. Exon 8 (E8) is short and unique in spliced AR\textsuperscript{667es} with a stop codon after the first 29 nucleotides, which results in a reading frame shift. (B) RT-PCR genotyping identified one Pb-AR\textsuperscript{667es} founder (No. 187) with the expression of AR\textsuperscript{667es} from a panel of 10 founders. (C) Representative qPCR identifying homozygous and heterozygous offspring in the positively genotyped Pb-AR\textsuperscript{667es} mice. Mouse Nos. 729, 733, 734, 736, 737, 744, 745, and 747 are heterozygous, and the others are homozygous.

Table W1. Weight Data of Body, GU Organs, and Prostate Expressed as Mean ± SD (g).

<table>
<thead>
<tr>
<th></th>
<th>Body</th>
<th>GU</th>
<th>Prostate</th>
<th>Relative GU</th>
<th>Relative Prostate</th>
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<tr>
<td>Wt-sham (n = 6)</td>
<td>31.16 ± 1.32</td>
<td>0.46 ± 0.072</td>
<td>0.13 ± 0.016</td>
<td>0.0146 ± 0.0019</td>
<td>0.0041 ± 0.0005</td>
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<tr>
<td>Wt-castration (n = 8)</td>
<td>28.39 ± 0.88</td>
<td>0.11 ± 0.018</td>
<td>0.05 ± 0.008</td>
<td>0.0039 ± 0.0006</td>
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<tr>
<td>Tg-sham (n = 7)</td>
<td>36.80 ± 3.23</td>
<td>0.64 ± 0.002</td>
<td>0.17 ± 0.023</td>
<td>0.0173 ± 0.0020</td>
<td>0.0047 ± 0.0005</td>
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<tr>
<td>Tg-castration (n = 7)</td>
<td>33.16 ± 2.82</td>
<td>0.22 ± 0.096</td>
<td>0.07 ± 0.013</td>
<td>0.0065 ± 0.0024</td>
<td>0.0022 ± 0.0004</td>
</tr>
</tbody>
</table>

Pb-AR\textsuperscript{667es} Tg mice and Wt littermates received castration or sham operation at the age of 16 weeks and were harvested 3 weeks post-surgery. Relative weight is GU or prostate weight normalized by body weight.
Figure W2. Constitutive expression pattern of AR<sup>v567es</sup> in the mouse model Pb-AR<sup>v567es</sup> mice. (A) mRNA level of AR<sup>v567es</sup> showed lower but no significant changes in Pb-AR<sup>v567es</sup> mice after castration. (B) The nuclear expression of AR<sup>v567es</sup> protein remained in castrated Pb-AR<sup>v567es</sup> prostate. (C) IHC of endogenous mouse AR protein showed significant decrease after castration both in Pb-AR<sup>v567es</sup> mice and Wt littermates.

Table W2. Pathology Distribution of Pb-AR<sup>v567es</sup> and Wt Prostate.

<table>
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<tr>
<th>Age (Weeks)</th>
<th>Pathology</th>
<th>Intact Pb-AR&lt;sup&gt;v567es&lt;/sup&gt;</th>
<th>Castrated Pb-AR&lt;sup&gt;v567es&lt;/sup&gt;</th>
<th>Intact Wt</th>
<th>Castrated Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-20</td>
<td>Hyperplasia</td>
<td>7/7 (100%)</td>
<td>7/7 (100%)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>30-40</td>
<td>Hyperplasia</td>
<td>2/5 (40%)</td>
<td>N/A</td>
<td>1/6 (16.7%)</td>
<td>N/A</td>
</tr>
<tr>
<td>50-60</td>
<td>Hyperplasia</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6 (33.4%)</td>
<td>2/6 (33.4%)</td>
</tr>
<tr>
<td></td>
<td>High-grade PIN</td>
<td>2/6 (33.3%)</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>4/6 (66.7%)</td>
<td>6/6 (100%)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Localized</td>
<td>4/6 (66.7%)</td>
<td>4/6 (66.7%)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Invasive</td>
<td>0/6</td>
<td>2/6 (33.3%)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

N/A, castration is not available in mice at 30 to 40 weeks of age.
Figure W3. (A) No antibody control for AR441 staining on Pb-ARv567es mice of 16 to 20 weeks of age. HRP-anti-mouse IgG secondary antibody was used without adding AR441 antibody (×400). (B) Representative Ki-67 IHC pictures in young mice of 16 to 20 weeks of age (×400). (C) Representative TUNEL pictures in young mice of 16 to 20 weeks of age (×400).