Supporting Information

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SI Text

Cell Culture, Plasmids, and Reagents. PC3, and WPMY1 cells were obtained from the American Type Culture Collection. We maintained cell lines in RPMI 1640 media with 10% FCS, 25 units/ml penicillin and 25 μg/ml streptomycin. We stably transfected human prostate cancer PC3 cells with AR cDNA driven by human promoter 3.6 kb, as described (1), and named this cell line PC3-AR9. We selected neomycin resistant cells by incubation with 500 μg/ml G418. DHT was purchased from Sigma.

Light Microscopy Procedures. Tissue samples were fixed in 5% neutral buffered formalin, embedded in paraffin and cut into 5-μm-thick slide sections. After H&E or immunostaining, we first identified the desired area by light microscopy using a low power dry objective lens. We then placed a small drop of oil on the coverslip for operation of the oil immersion lens. High magnification and high resolution ($\times$1,000) images of interested area were obtained in the light microscope by using oil immersion objective lenses. We counted the percentage of the positive cells under oil immersion lens. Results were averaged from at least five different viewing areas.

RNA Extraction, RT-PCR, and Real-Time RT-PCR. We harvested tissues or cultured cells in TRIzol (Invitrogen) and extracted total RNA following the manufacturer's instructions. We reverse transcribed (RT) 5 μg total RNA into 20 μl cDNA by the SuperScript III kit (Invitrogen) with oligo(dT) primer. The 20 μl cDNA was then diluted by water into 200 μl. Two μl reverse transcribed cDNA were used for PCR and real-time quantitative PCR using the MyCycler thermal cycler (Bio-RAD) with Taq polymerase and on the iCycler IQ multicolor real-time PCR detection system, respectively. We designed primers by Beacon Designer 2 software and used the β-actin expression level as control to calculate the relative gene expression among different samples. We calculated threshold (CT) values by subtracting the control CT value from the corresponding β-actin CT at each time point. We confirmed the absence of nonspecific amplification products by agarose-gel electrophoresis.

Immunohistochemistry Staining. We fixed samples in 5% neutral buffered formalin and embedded in paraffin. We used the primary antibodies of the rabbit anti-Ki67 (Abcam), the rabbit anti-Tag (Santa Cruz), the rabbit anti-AR (C19) (Santa Cruz Biotechnology), anti-CK5 (Covance), anti-CK8 (Abcam), and anti-CD44 (Cell Signaling). The primary antibody was recognized by the biotinylated secondary antibody (Vector), and visualized by VECTASTAIN ABC peroxidase system and peroxidase substrate DAB kit (Vector). The positive staining signals were semiquantitated by Image J software.

BrdU Incorporation Assay. We purchased 5′-Bromo-2′-deoxyuridine (BrdU) from Sigma and dissolved it in double distilled water at 10 mg/ml. Starting at 24 h before sacrifice, we injected mice i.p. every 6 h with 10 μg BrdU per gram body weight. Following harvest, we embedded tissues in paraffin and labeled them with the BrdU Staining Kit (Zymed) following manufacturer's instructions.

TUNEL Assay. We purchased Fluorescein–Frag EL DNA Fragmentation Detection Kit (Calbiochem), labeled paraffin-embedded tissue sections following the manufacturer's instructions, and counted the labeled nuclei by using a standard fluorescein filter at 465–495 nm.

Laser-Capture Microdissection to Obtain Selected Prostate Cells. We embedded prostate tissues in O.C.T. on dry ice and stored at -80°C until cutting. We cut 5-μm sections onto plain uncoated glass slides and immediately performed H&E staining using HistoGene LCM Frozen Section Staining Kit (Arcturus) following the manufacturer's instructions. On the Pixcell II LCM system, we laser transferred the prostatic epithelium and stroma onto different caps. After microdissection, we merged caps in RNA extraction buffer of PicoPure RNA Isolation Kit (Arcturus) and isolated RNA following the manufacturer's instructions. We performed one round of RNA amplification using RiboAmp RNA Amplification Kit (Arcturus). The resultant cDNA was used to perform quantitative real-time PCR to determine the genes of interest.

Statistics. We presented the data as the mean $\pm$ standard deviation (SD). We made comparisons between groups using a two-sided Student's t test. Differences with P values $^{*}P < 0.05$, $^{**}P < 0.01$, were considered significant. We analyzed Survival curves by Kaplan–Meier analysis and log-rank tests.

Results and Discussion

Generation and Confirmation of pes-ARKO-TRAMP Mice That Lack AR Only in Prostate Epithelium. We first mated female flox/AR (C57BL/6) mice with TRAMP (FVB) mice (2) to generate flox/AR-TRAMP (C57BL/6 x TRAMP-FVB) mice. We then crossed these mice with Pb-Cre (C57BL/6) mice (3) to generate pes-ARKO-TRAMP (C57BL/6 x TRAMP-FVB) mice (Fig. S1*a*).

We genotyped all pups by PCR from tail snip DNA, as described (4). As shown in Fig. S1b, both WT-TRAMP and pes-ARKO-TRAMP pups showed T-ag bands, whereas only pes-ARKO-TRAMP mice had floxed AR (Fig. S1*b Middle*) and Pb-cre (Fig. S1*b Bottom*) bands. We analyzed mRNA level via PCR from anterior prostate (AP), dorsolateral prostate (DLP), ventral prostate (VP), and seminal vesicles (SV) with primers specific for deleted exon 1 and exon 3 of the AR and demonstrated that AR-exon 2 was excised in AP, DLP, and VP from pes-ARKO-TRAMP mice, but not from WT-TRAMP mice (Fig. S1*c*).

To monitor the AR knockout efficiency in pes-ARKO-TRAMP mice, we applied quantitative real-time RT-PCR to measure the expression of AR-exon 2 mRNA that was extracted from prostate epithelium via laser capture microdissection (LCM) (5). We showed AR mRNA was knocked down gradually to 90% in 16-wk-old pes-ARKO-TRAMP mice (data not shown). We confirmed loss of AR in the prostate epithelium, including luminal and basal cells, but not in stromal cells by immunohistochemical staining of AR expression in prostate from 16-wks-old pes-ARKO-TRAMP mice (data not shown).

Generation and Confirmation of ind-ARKO-TRAMP Mice with Knockdown of AR in Prostate. The ind-ARKO-TRAMP mice in which prostate AR could be knocked down in both epithelium and stroma were generated via mating female flox/AR-TRAMP (C57BL/6xTRAMP-FVB) mice with Mx-Cre (C57BL/6/FVB) mice (6) (Fig. S1*a*). i.p. injection of polyinosinic-polycytidylic acid (pI-pC) into ind-ARKO-TRAMP mice then induced the knockdown of AR in various tissues, including the prostate (6).

We verified the genotypes by PCR using tail snip DNA as templates. We found that both WT-TRAMP and ind-ARKO-TRAMP mice showed T-ag bands (Fig. S1*b Top*), whereas only ind-ARKO-TRAMP mice had floxed AR bands (Fig. S1*b Middle*) and Mx-Cre bands (Fig. S1*b Bottom*). We confirmed the knockdown of AR in ind-ARKO-TRAMP mice by detecting the mRNA with deletion of AR-exon 2 in different organs, such as AP, DLP, VP, seminal vesicle (SV) (Fig. S1*c*), liver, spleen, and testis (data not shown).

We monitored the knockdown efficiency in the ind-ARKO-TRAMP mice that were injected with pI-pC at 12 wk, and found AR mRNA was knocked down by 40–50% in prostate, 40% in testis, 20% in SV, and 80% in liver of both 16-wk-old and 20-wk-old mice (Fig. S2*a*). We used immunohistochemical staining of AR to confirm the knockdown of AR in both epithelium and stroma of 16-wk-old ind-ARKO-TRAMP mice (Fig. S2*b*). We measured AR mRNA from LCM-isolated epithelium or stroma of 16-wk-old ind-ARKO-TRAMP mice to confirm the loss of 60% AR mRNA in epithelium and 50% AR mRNA in stromal cells as compared with their pI-pC injected WT-TRAMP littermates (Fig. S2*c*).

1. Altuwaijri S, *et al.* (2007) Expression of human AR cDNA driven by its own promoter results in mild promotion, but not suppression, of growth in human prostate cancer PC-3 cells. Asian J Androl 9:181–188.
2. Hill RE, de Avila DM, Bertrand KP, Greenberg NM, Reeves JJ (2003) Immunization against luteinizing hormone-releasing hormone fusion proteins does not decrease prostate cancer in the transgenic adenocarcinoma mouse prostate model. *Exp Biol Med* 228:818–822.
3. Wu X, *et al*, (2001) Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation. *Mech Dev* 101:61–69.
4. Miyamoto H, *et al.* (2007) Promotion of bladder cancer development and progression by androgen receptor signals. *J Natl Cancer Inst* 99:558–568.
5. Schutze K, Lahr G (1998) Identification of expressed genes by laser-mediated manipulation of single cells. *Nat Biotechnol* 16:737–742.
6. Kuhn R, Schwenk F, Aguet M, Rajewsky K (1995) Inducible gene targeting in mice. *Science* 269:1427–1429.

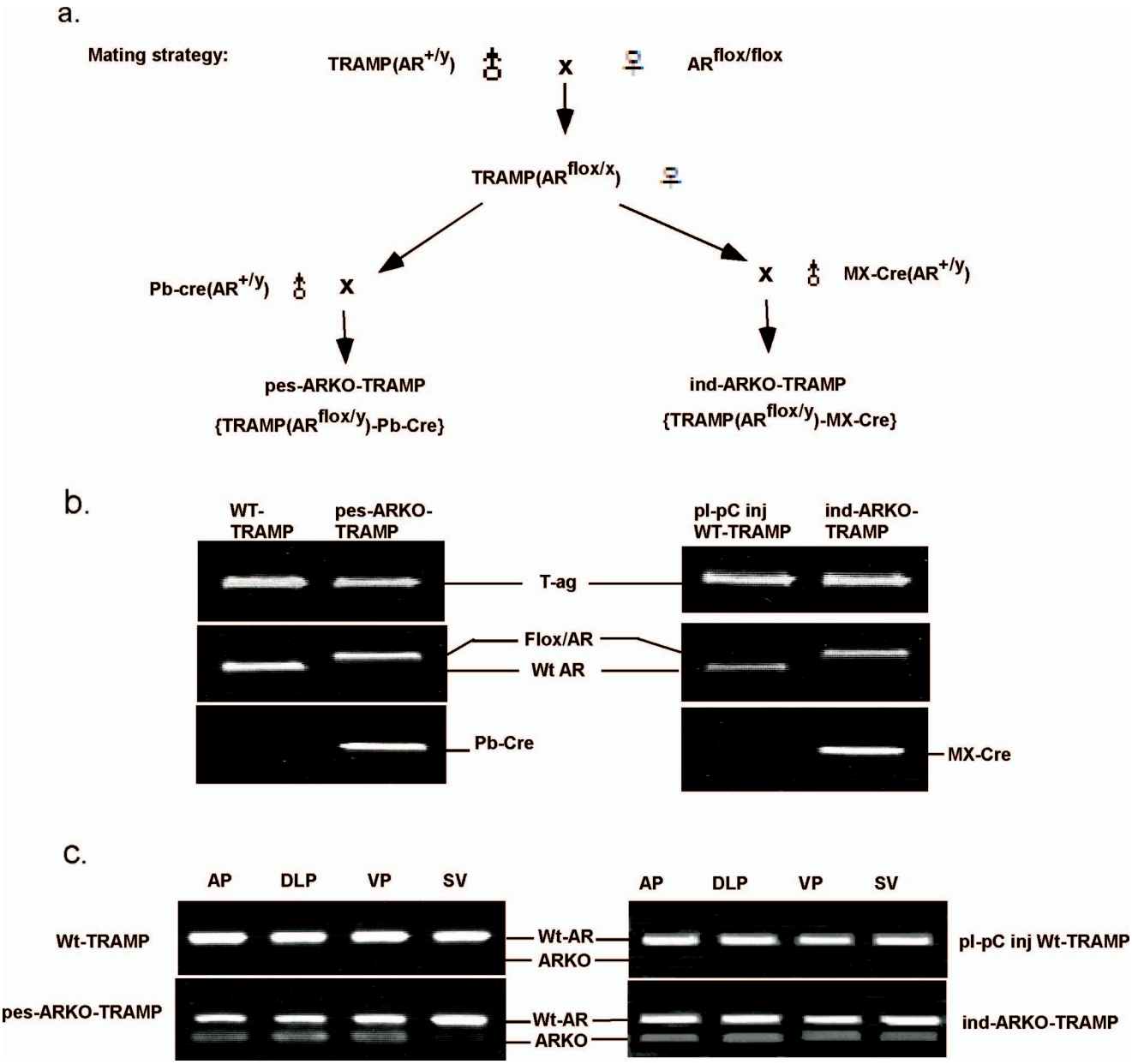

Fig. S1. Generation and confirmation of pes-ARKO-TRAMP mice and ind-ARKO-TRAMP mice. (*a*) Mating strategy of pes-ARKO-TRAMP (B6xTRAMP-FVB) and ind-ARKO-TRAMP mice (B6xTRAMP-FVB). (*b*) We used genotype screening of mice from tail snip DNA and T-ag (SV40) primer to identify TRAMP mice at 4 wks old (*Top*). We used primers ''2–3'' and ''select'' [Yeh S, *et al.* (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: An *in vivo* model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci USA* 99:13498–13503] that amplify AR-exon 2 and the surrounding intron region to identify the floxed AR allele in pes-ARKO-TRAMP mice and ind-ARKO-TRAMP mice (*Middle*). We used primers specific for Pb-Cre and Mx-Cre to identify Pb-Cre and Mx-Cre transgenic mice, respectively *(Bottom)*. (*c*) We confirmed AR knockout by detecting the exon 2 deletion in AR mRNA, using exon 1 and exon 3 primers, specific ARKO bands by RT-PCR amplifying AR mRNA from different organs. In pes-ARKO-TRAMP mice, we showed ARKO bands in dorsal lateral prostate (DLP), ventral prostate (VP) and anterior prostate (AP), but no significant band in seminal vesicles (SV) compared to Wt-TRAMP. In ind-ARKO-TRAMP mice, we found ARKO bands in DLP, VP, AP, and SV.

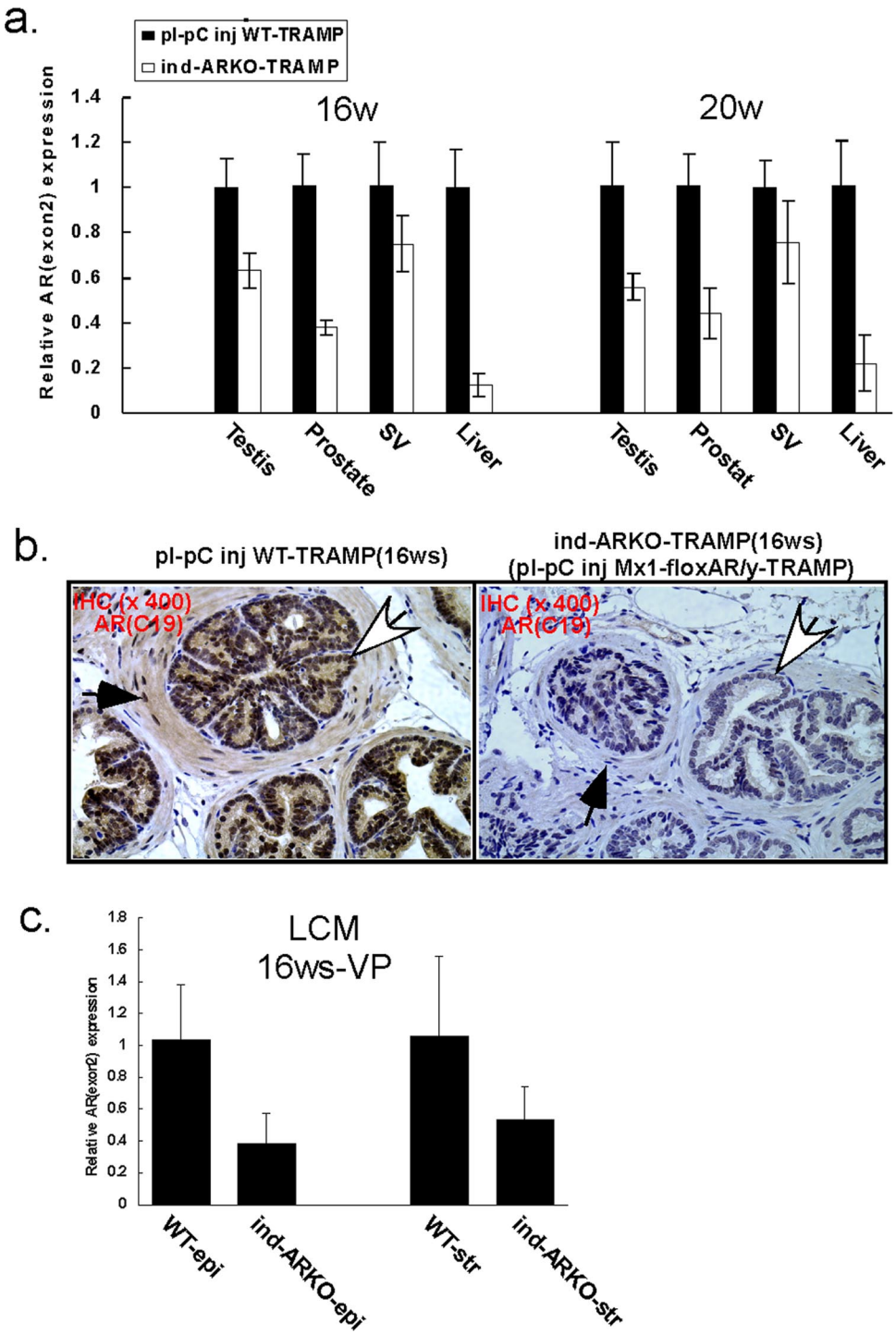

Fig. S2. (*a*) We performed pI-pC injection at the age of 12 wks. At the age of 4 wks and 8 wks following pI-pC injection (at 16-wk-old and 20-wk-old), we determined AR knockdown in different organs at various degrees by using Real-time RT-PCR to detect relative expression levels of AR-exon 2 mRNA. (*b*) IHC AR staining showed AR protein was partially lost in ventral prostate epithelium (white arrows) and stroma (black arrows) of ind-ARKO-TRAMP mice compared to WT-TRAMP mice at 16-wk-old. (*c*) We demonstrated that the AR was partially knocked down in both the epithelium (ind-ARKO-epi) and stroma (ind-ARKO-str), separated by LCM, in ventral prostate of ind-ARKO-TRAMP mice using real-time RT-PCR of AR-exon 2.