Noncanonical Wnt signaling mediates androgen-dependent tumor growth in a mouse model of prostate cancer

Sayuri Takahashi, Tomoyuki Watanabe, Maiko Okada, Kazuki Inoue, Takashi Ueda, Ichiro Takada, Tetsuro Watabe, Yoko Yamamoto, Toru Fukuda, Takashi Nakamura, Chihiro Akimoto, Tetsuya Fujimura, Maiko Hoshino, Yuuki Imaia, Daniel Metzger, Kohei Miyazono, Yasuhiro Minamig, Pierre Chambon, Tadaichi Kitamura, Takahiro Matsumoto, and Shigeaki Kato

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Prostate cancer development is associated with hyperactive androgen signaling. However, the molecular link between androgen receptor (AR) function and humoral factors remains elusive. A prostate cancer mouse model was generated by selectively mutating the AR threonine 877 into alanine in prostatic epithelial cells through Cre-ERT2-mediated targeted somatic mutagenesis. Such AR point mutant mice (AR<sup>T877A</sup>) developed hypertrophic prostates with responses to both an androgen antagonist and estrogen, although no prostatic tumor was seen. In prostate cancer model transgenic mice, the onset of prostatic tumorigenesis as well as tumor growth was significantly potentiated by introduction of the AR T877A mutation into the prostate. Genetic screening of mice identified Wnt-5a as an activator. Enhanced Wnt-5a expression was detected in the malignant prostate tumors of patients, whereas in benign prostatic hyperplasia such aberrant up-regulation was not obvious. These findings suggest that a noncanonical Wnt signal stimulates development of prostatic tumors with AR hyperfunction.

The androgen receptor (AR) is pivotal in prostate cancer development, because androgen depletion or androgen antagonism is clinically successful in attenuating prostate cancer growth in its initial stages. However, most patients treated with antiandrogen therapies eventually transition into a “hormone-refractory” state (2, 7), which is usually aggressive and frequently lethal. Up-regulation of AR target genes also occurs during the transitional stage (2, 7). Rising serum levels of prostate-specific antigen (PSA) accompany the development of the hormone-refractory state (8). Thus, enhancement of AR-mediated androgen signaling appears to portend the onset of tumor progression, although its molecular basis remains largely elusive.

AR is a member of the nuclear steroid receptor gene superfamily, and acts as a hormone-dependent transcription factor (9). Activation of AR by ligand binding transcriptionally controls expression of the target genes (10, 11). Like the other members, AR is functionally and structurally divided into five domains, A–E. The most conserved, the C domain, in the middle of the receptor protein encodes the DNA binding domain, whereas the C-terminal E domain encompasses the hormone/ligand binding domain (LBD). The N-terminal A/B domains and the LBD bear the transcription activation function and transcriptional coactivators or coregulator complexes for ligand-dependent transcriptional regulation (12). As tumors transition, they generate AR mutations leading to altered responses to steroid hormones and synthetic ligands, including resistance to androgen antagonists (13, 14). Most point mutations found in prostate tumors of hormone-refractory patients have been mapped in the AR LBD (15, 16). As the hydrophobic pocket in the LBD specifically recognizes and captures androgens and AR ligands, the mutated amino acid residues in the LBD, particularly in the motifs constituting the pocket, are presumed to impair specificity in hormone/ligand recognition. Indeed, in vivo cell cultures, such human (h)AR point mutants display acquired responsiveness to other steroid hormones and androgen antagonists (17, 18). The point mutation threonine 877 to alanine (T877A) in the LBD is one of the most common mutations in prostate tumors (15, 16). Significantly, the hAR point mutants found in hormone-refractory patients appear hyperactive in terms of transactivation, because the mutants are hypersensitive to endogenous non–androgenic steroid hormones as well as to clinically used AR synthetic antagonists. In addition to the aberrant hyperfunction of the AR mutants, cellular signals from the tumor cells and surrounding nontumor cells also contribute to prostate tumor progression (19). Growth factors secreted by the stroma and prostate epithelium act in concert with signaling through the activated AR to stimulate prostate cancer cell growth in vitro (20). However, in animal models, the roles of these humoral factors remain to be investigated. Several mouse prostate cancer models have been established (21), and spontaneous prostatic tumorigenesis is seen in one of the transgenic murine prostate cancer model (TRAMP) lines expressing a T-antigen gene (22). However, androgen dependency in prostatic tumor development and onset could not be recapitulated in such mouse models (21).

To determine whether aberrantly potentiated AR signaling stimulates prostate cancer growth in vivo, we have generated an experimental prostate cancer model in mice by selectively mutating AR T877 to A in prostatic epithelial cells of adult mice, through Cre-ERT2-mediated spatiotemporally controlled targeted somatic mutagenesis (23). Such AR point mutant mice (AR<sup>T877A</sup>) exhibited hypertrophic prostates but failed to develop prostate tumors. In orchidectomized AR mutants, hydroxylutamide (HF), a clinically used androgen antagonist, stimulated prostate growth. By introducing the T877A mutation into epithelial cells of the prostate of adult TRAMP transgenic mice, the onset of tumor formation was shortened and tumor growth was stimulated.


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1To whom correspondence should be addressed. E-mail: uskato@mail.ecc.u-tokyo.ac.jp.

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netic screening of tumor growth factors in these mice led to the identification of a noncanonical Wnt ligand (Wnt-5a) that accelerated tumor growth. Importantly, enhanced Wnt-5a expression was also present in tumor cells of prostate cancer patients but not in prostatic cells from patients with benign prostatic hyperplasia. These findings demonstrate that the genetic mutation T877A in the AR promotes prostatic tumor growth by stimulating a noncanonical Wnt signaling pathway.

Results

Introduction of the Human T877A AR Mutation into the Mouse AR Gene Locus. To create conditional mutants (T877A) located in the AR LBD, we replaced the mouse genomic DNA segment encompassing AR exons 6–8 with a DNA segment containing the corresponding human coding sequence flanked by three loxP sites, followed by a similar DNA segment encoding the T877A mutation (Fig. 1A). The knocked-in and floxed (L2) mouse line (ARloxfllox) (24, 25), in which the Neo cassette was deleted by Cre-mediated excision in embryonic stem cells, had no overt abnormalities and grew normally (Fig. S1A). The prostate was phenotypically normal, showing considerable budding and differentiation into columnar glandular epithelium. The size of the prostate, however, was smaller than that of the wild-type mouse at 10 wk of age (Fig. S1 C and D). When evaluated at 20 wk, the growth of the ventral prostate (VP) was delayed in comparison with the anterior (AP) and dorsal (DP) prostates and the seminal vesicle (SV). ARloxfllox males were fertile but their reproductive activity was reduced. However, the smaller prostates were structurally intact. Because growth of the prostate is dependent on AR-mediated androgen signaling (24–26), it is likely that the prostatic phenotype of the ARloxfllox results from hypofunction of the AR protein. The ARloxfllox showed normal levels of AR mRNA but reduced AR protein levels (Fig. S2 C and D). Therefore, the genetic manipulation may have affected stabilization of the AR protein. Serum testosterone levels were up-regulated, owing to decreased negative feedback on androgen biosynthesis (Fig. S1B). Thus, although androgen signaling in ARloxfllox mice appears reduced but not significantly impaired, we concluded that this floxed mouse line was appropriate for further study.

Selective Introduction of the T877A AR Mutation into Prosthetic Epithelial Cells of Adult Mice. We then used the KLKB1-Cre-ERT2 mouse line (23) and a T877A mouse line to generate a prostatic epithelium-specific, T877A knock-in. First, prostate-specific excision by Cre was confirmed using a testes mouse line in which the β-galactosidase gene was induced by Cre-mediated excision (27). Cre enzymatic activity (the Cre-ERT2 system) requires activation of an estrogen receptor point mutant which is sensitive only to an ERbinding, tamoxifen (TAM), but not to endogenous estrogens (23). Following treatment with TAM for 5 d, clear staining of β-galactosidase was seen in the prostates (Fig. S2A) but not in the other tissues tested. We then crossed the ARloxfllox mice with the KLKB1-Cre-ERT2 mouse line to replace protein expression of the wild-type hAR LBD by the point-mutated (T877A) hAR LBD through Cre-mediated excision of the loxP sites (Fig. 1A), generating a KLKB1-Cre-ERT2, ARloxfllox mouse line. Then, these mice at the age of 16 wk were treated with TAM for 5 d to generate ARloxflloxloxmice, in which AR was selectively expressed in prostatic epithelial cells. These mice exhibited no overt abnormalities and grew normally. Similar to ARloxfllox mice, the ARloxflloxlox mice were fertile but had impaired reproductive activity. The T877A mutation was detectable in the genomic sequences of prostates from 15 out of 20 mice (Fig. S2B). The expression levels of the AR transcript and protein in the ARloxflloxlox mice were indistinguishable from those of ARloxfllox mice (Fig. S2 C and D). From these observations, we concluded that the Cre-ERT2 system was successful in selectively and inducibly expressing the AR T877A mutant in the mouse prostate.

Fig. 1. Selective introduction of the AR T877A mutation into prostatic epithelial cells of adult mice. (A) The targeting strategy for insertion of the ART877A mutation is illustrated. The diagram shows the wild-type AR genomic locus, targeting vector, floxed ARloxfllox, and loxP sites. Targeted ES cells were injected into embryonic stem cells of the KLKB1-Cre-ERT2 mice. We isolated colonies of mice that were inducible by tamoxifen (TAM) treatment. (B) Ventral prostates (VP) and total prostates (TP) of ARloxfllox mice (n = 10) and ARloxfllox mice (n = 10) were weighed at 16, 24, and 52 wk of age. The ventral prostate lobes of ARloxfllox mice were about 1.5 times heavier than those of the control mice (*P < 0.05, **P < 0.01 by one-way ANOVA). The anterior and dorsal prostates showed similar results. Error bars represent the SD. (C) Prostate lobes of 16- and 52-wk-old ARloxfllox mice and ARloxfllox mice under a dark-field dissection microscope. ARloxfllox mutation promoted prostate lobe growth. (D) Hematoxylin and eosin-stained ventral prostates of 24-wk-old ARloxfllox mice and ARloxfllox mice.
analysis of the \( \text{AR}^{\text{pe-T877A/Y}} \) mice at 52 wk (Fig. 1 C and D) or later. Because endogenous testosterone levels were significantly up-regulated (Fig. S1B), the mice were castrated (24, 27). Prostate development in castrated \( \text{AR}^{\text{pe-T877A/Y}} \) mice was severely retarded at 24 wk of age. VP (Fig. 2 A and B) and total prostate (TP) (Fig. 2 A) were restored by 5α-dihydrotestosterone (DHT) treatment for 3 wk. This DHT response was seen in both castrated \( \text{AR}^{\text{lox/Y}} \) and \( \text{AR}^{\text{pe-T877A/Y}} \) mice. These observations suggested that prostatic development of these lines was dependent on endogenous androgen. However, the TR77A point mutation was not potent enough to trigger tumorigenesis.

**Growth of the Prostate in \( \text{AR}^{\text{pe-T877A/Y}} \) Mice Is Stimulated by both an Androgen Antagonist and Estrogen.** At the initial stages of prostate cancer, while the cells retain hormone responsiveness, patients are treated with synthetic androgen antagonists to attenuate androgen-induced tumor development. However, after several years of treatment, the hormone-dependent state is frequently lost and new genetic mutations appear in the AR exons encoding the LBD (15, 16, 28). We therefore asked whether \( \text{AR}^{\text{pe-T877A/Y}} \) mice develop a response similar to that seen in hormone-refractory prostate cancer patients. A 3-wk treatment with HF, a clinically used AR antagonist, stimulated prostate growth in \( \text{AR}^{\text{pe-T877A/Y}} \) mice but not in \( \text{AR}^{\text{lox/Y}} \) mice (Fig. 2 A and B). Likewise, high doses (160 µg/d for 3 wk) of exogenous 17β-estradiol (E2) clearly stimulated prostatic growth, whereas another AR antagonist, bicalutamide (BIC; Casodex), elicited no stimulatory effect (Fig. 2 C). Thus, it appeared likely that \( \text{AR}^{\text{pe-T877A/Y}} \) mice recapitulate the abnormal responses seen in hormone-refractory prostate cancer patients.

**Accelerated Tumorigenesis Induced by the T877A AR Mutation in an Experimental Mouse Model of Prostate Cancer.** Because \( \text{AR}^{\text{pe-T877A/Y}} \) mice failed to develop detectable prostate tumors even after 1 yr, we tested the impact of the T877A AR mutation on prostate tumor growth in a known experimental prostate cancer model (TRAMP). This mouse line spontaneously develops prostate cancer in which the tumor resembles that seen in humans (22). TRAMP mice were crossed with KLKB1-Cre-ERT2; \( \text{AR}^{\text{lox/Y}} \) mice to generate a TRAMP; KLKB1-Cre-ERT2; \( \text{AR}^{\text{lox/Y}} \) mouse line. At 16 wk of age, these mice were treated with TAM for 5 d to introduce the TRAMP mutation into the prostate. This mouse line (TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \)) exhibited an earlier onset of prostate tumor growth than did the TRAMP-\( \text{AR}^{\text{lox/Y}} \) mice (Fig. 3 A and C). The rapid growth of the prostate tumor led to the death of all of the TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice at around 36 wk of age. In contrast, TRAMP-\( \text{AR}^{\text{lox/Y}} \) mice survived past 48 wk (Fig. 3 A). Consistently, the expression levels of several known AR target genes (Probasin, Psp94, Fkbp51, Sfk37) (29–31) were higher in prostate tumors from TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) than those of TRAMP-\( \text{AR}^{\text{lox/Y}} \) (Fig. 3 D). However, no remarkable alteration in the expression levels of the tumor-related genes was detected (Fig. S3). Histologically, the prostate tumor phenotype of TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice at 19 wk was indistinguishable from that of TRAMP-\( \text{AR}^{\text{lox/Y}} \) mice (Fig. 3 B).

To test whether both the early onset and the rapid growth of the prostatic tumors in TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) were tumor-autonomous and/or androgen-dependent, we transplanted the tumors into the ventral prostate lobe of nude mice (Fig. S4 A). The prostatic tumors continued to grow. At 8 wk posttransplantation, the tumors derived from TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice were three times larger than those from TRAMP-\( \text{AR}^{\text{lox/Y}} \) mice (Fig. S4 B–D). When these mice were castrated, such tumor growth was more attenuated from TRAMP-\( \text{AR}^{\text{lox/Y}} \) mice than from TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice (Fig. S5 A and B), suggesting that tumor growth is dependent on activated AR function. From these findings, we presumed that the tumor progression in TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice can be attributed to AR hyperfunction induced by endogenous hormones. Moreover, we assumed that a certain factor(s) expressed in the prostate potentiated the growth of prostatic tumors harboring the T877A mutation.

**Noncanonical Wnt Ligand Acts as an Activator in Experimental Prostate Cancer.** We reasoned that the function of the AR mutant in tumor growth was potentiated by cross-talk with prostatic growth factors and/or cytokine signaling pathways. Several major candidate factors (Fgf10, Stat3, Tab2, Wnt-5a), already documented to modulate prostate growth in vivo and in vitro, were examined by crossing the TRAMP; KLKB1-Cre-ERT2; \( \text{AR}^{\text{lox/Y}} \) mouse line with deficient in the candidate factors (32–37). The offspring at 16 wk of age were then treated with TAM to introduce the AR\(^{\text{pe-T877A/Y}}\) mutation. Of the factors tested, haploinsufficiency of Wnt-5a (33) remarkably abrogated the early onset and early lethality of the prostate tumors in TRAMP-\( \text{AR}^{\text{pe-T877A/Y}} \) mice, and conferred prostatic growth similar to that observed in the TRAMP-\( \text{AR}^{\text{lox/Y}} \) control line (Fig. 4 A). Thus, Wnt-5a appeared to be an activator of AR-mediated prostate cancer growth. Wnt-5a is a ligand involved in the noncanonical Wnt signaling pathway that also coregulates the transactivation function of PPARγ (33). During studies of Wnt-5a action associating with AR hyperfunction, LNCaP cells were found to endogenously express Wnt-5a (Fig. 4 B and Fig. S6). When Wnt-5a was knocked down (Fig. 4 B and Fig. S6), transactivation function of liganded AR was clearly reduced.

![Fig. 2. Aberrant prostate growth responses to hormonal and antagonist signals in AR\(^{\text{pe-T877A/Y}}\) mice. (A) AR\(^{\text{pe-T877A/Y}}\) mice and AR\(^{\text{lox/Y}}\) mice at 24 wk of age were divided into eight groups (n = 5 per group) and treated with hormones (DHT, HF, E2, or BIC) for 3 wk after castration or a sham operation. Prostates were processed, weighed, and stained with hematoxylin and eosin. (B) Typical ventral prostates of castrated AR\(^{\text{pe-T877A/Y}}\) mice after hormonal treatment. (C) Hematoxylin and eosin staining of ventral prostates of AR\(^{\text{pe-T877A/Y}}\) mice treated with the indicated hormones. The prostatic tubule structure in AR\(^{\text{pe-T877A/Y}}\) mice was maintained by treatment with either HF or E2.](http://www.pnas.org/cgi/doi/10.1073/pnas.1014850108)
lowered (Fig. 4C). Consistently, androgen-dependent proliferation of LNCaP cells was attenuated by this Wnt-5a knockdown (Fig. 4D and Fig. S7).

**Aberrant Wnt-5a Expression in Human Prostate Tumors.** To address the impact of Wnt-5a on human prostate cancer, we examined the expression of Wnt-5a in human prostate cancers. In benign prostatic hyperplasia, Wnt-5a expression levels were normal by immunohistochemical analysis (Fig. 5B). In contrast, malignant prostate tumors (Dataset S1) demonstrated aberrantly up-regulated Wnt-5a expression compared with that in the surrounding normal tissues (Fig. 5A and B). Stromal–epithelial interactions and the bioactive factors produced by these interactions have proven to be important in cancer progression (19, 20). Here we examined whether Wnt-5a in carcinoma cells is acting in an autocrine fashion. By an immunofluorescence analysis, expressions of Wnt-5a and AR proteins were seen in both carcinoma and stromal cell types (Fig. 5C). However, colocalized expression of these two factors was predominantly detected in the carcinoma cells (Fig. 5C). Thus, it is likely that the Wnt-5a effect in carcinoma cells occurs primarily in an autocrine fashion. These findings suggest that Wnt-5a can serve as an activator of AR-mediated androgen signaling for prostate growth under certain pathological conditions in the human prostate, as seen in mice.

**Discussion**

Mouse models of prostate cancer have been developed to improve understanding of the molecular basis of prostate carcinogenesis and explore treatment regimens for prostate cancer patients (21). Some model limitations are inherent because prostatic structure and major areas/cells of tumors are not identical in human patients and mouse models. Nonetheless, the impact of oncogenic and antimetastatic genes in prostatic tumors has been tested by mouse genetic approaches (21). Among such candidate factors AR is of prime interest, because it is involved in all stages of prostate cancer (16, 38). Androgen actions are indispensable for prostate development and function, and appear stimulatory for tumorigenesis, evidently acting through AR. Moreover, AR is considered a key factor in prostate cancer even after transition to the androgen-independent state (16, 38). Whatever the function of AR in androgen-dependent and -independent prostate cancer, mouse models to assess AR function have been invaluable owing to the innate roles of AR in prostate development (24, 25). To avoid this difficulty, we have introduced a modified Cre-loxP system to selectively introduce a point mutation into the AR LBD in the prostates of adult mice (ARflox/Y mice). This mutation (T877A) is often seen in prostatic tumors of androgen-independent patients (17, 18).

Neoplasia was undetectable in the prostates of the ARflox/Y mice even after 1 y, indicating that a single mutation rendering AR hyperactive is not sufficient to trigger prostatic tumorigenesis. The onset of prostatic tumors bearing hyperfunctioning AR thus appears to require at least one additional factor in mice, as already predicted in the literature (21, 28). In our initial pilot experiment, we sought to compare the impact of the T877A mutation on prostate tumorigenesis between Ptenfl/Y mice and the transplanted prostate tumors from the TRAMP-ARflox/Y line consistently enhanced tumor development in TRAMP mice and, more importantly, androgen dependency became evident. Thus, the TRAMP-ARfllox/Y line constitutes a unique mouse model of androgen-dependent prostate cancer, and will be useful for exploration of the molecular basis underlying the transition from androgen dependency to the refractory state.

**TRAMP-ARfllox/Y** mice demonstrated that the T877A mutation significantly shortened the onset of tumor development and accelerated tumor growth. The average lifespan of the TRAMP-ARfllox/Y line was greater than 48 wk, whereas that of TRAMP-ARfllox/Y mice was less than 36 wk. TRAMP-ARfllox/Y mice succumbed to tumors that were equivalent in size to their total body weight. Furthermore, the transplanted prostate tumors from the TRAMP-ARfllox/Y mice grew three times as fast as the control line in nude mice. These findings suggest that a prostatic factor potentiates the effect of the AR hyperfunction that drives prostatic tumor progression.

Using a microarray analysis and quantitative PCR validation (39) of TRAMP-ARfllox/Y mice, we observed the expected alterations in tumor genes such as TERT and Cdk2 (40, 41) and the tumor suppressor genes NKX3.1, ATBF1, and p21 (42, 43) (Fig. S3). However, in this analysis, we were unable to identify new candidate genes accounting for tumor growth at this stage. Therefore, we opted for a genetic screening strategy that crossed
mice deficient in regulators of cell proliferation. Wnt-5a was identified as an activator of early onset and rapid growth of prostate tumors in TRAMP-AR<sup>T877A/Y</sup> mice. We also found aberrantly up-regulated expression of Wnt-5a in human prostate tumors. Wnt-5a is a noncanonical Wnt ligand involved in cell proliferation and differentiation, particularly during embryonic development (34, 44, 45).

It is conceivable that ectopic Wnt-5a expression potentiates cancer cell proliferation (46, 47), because Wnt-5a protein is aberrantly up-regulated in tumors compared with the normal tissues of prostate cancer patients. In accordance with this observation, Wnt-5a was able to coregulate the transactivation function of TRAMP-AR<sup>T877A/Y</sup> mice and Wnt-5a<sup>T877A/Y</sup>, TRAMP-AR<sup>T877A/Y</sup> mice were also analyzed as controls. Note that even at 28 wk of age, Wnt-5a<sup>T877A/Y</sup> and Wnt-5a<sup>T877A/Y</sup> mice did not develop significant prostate tumors (<p>0.05, <p>0.01 with one-way ANOVA). (B) Efficiency of the indicated siRNAs toward human Wnt-5a in LNCaP cells. Western blot with α-Wnt-5a (R&D Systems) or α-actin (Santa Cruz Biotechnology) was performed 48 h after the transfection of siRNAs. (C and D) The attenuation of AR-mediated androgen signaling in LNCaP cells by Wnt-5a knockdown. LNCaP cells transfected with siRNAs were subjected to transfection for a reporter luciferase assay (C) or a cell proliferation assay (D). AR ligands were incubated with the cells for 12 h (C) or 6 d (D). Each point represents the mean ± SD of three separate experiments (p<0.05, **p<0.01 with two-way ANOVA).

Materials and Methods

Generation of the Mouse Model. The genomic AR DNA fragment was isolated from a TT2 embryonic stem (ES) cell genomic library. The targeting vector was created by inserting human cDNA of wild-type AR exons 6–8 with two loxP sites and human cDNA of AR<sup>T877A</sup> exons 6–8 together with a positive Neo selective marker (Neo<sup>+</sup>) (Fig. 1A). A thymidine kinase gene (HSV-tk) cassette was attached to the 5' end of the targeting vector for negative selection. The targeted TT2 ES clones were selected after positive-negative selection with Southern analysis, and then aggregated with single eight-cell embryos from CD-1 mice (32, 48). The floxed AR mice (AR<sup>flox/Y</sup>) were crossed with a hybrid C57BL/6 and CBA genetic background, were backcrossed for four generations into a C57BL/6J background. These mice were mated with KLKB1-Cre-ERT<sup>2</sup> transgenic mice that express the tamoxifen-dependent Cre<sup>ER</sup><sup>2</sup> recombinase under the control of the human PSA promoter, allowing us to induce AR deficiency in the prostate stroma and epithelium in a tamoxifen-dependent manner. The AR-deficient mice were then mated with AR<sup>T877A/Y</sup> mice to generate AR<sup>T877A</sup> and AR<sup>T877A</sup> mice. Mice were divided into nine groups (n = 5 per group). Prostates of mice from each group were dissected and weighed every 2 wk. Wnt-5a<sup>T877A/Y</sup>, TRAMP-AR<sup>T877A/Y</sup> or Wnt-5a<sup>T877A/Y</sup> mice were analyzed as controls. Note that even at 28 wk of age, Wnt-5a<sup>T877A/Y</sup> and Wnt-5a<sup>T877A/Y</sup> mice did not develop significant prostate tumors (<p>0.05, <p>0.01 with one-way ANOVA).

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to introduce the AR T877A mutation selectively into epithelial cells of fully differentiated prostates of adult mice (23). Tamoxifen administration (daily injection (1 mg/d) for 5 to 6-wk-old mice) was performed as described (23). To generate Wnt-5a+/TRAMP-ARflOxy mice, we first mated KLK8 Cre:ER<sup>+</sup> AR<sup>flOxy</sup> mice with TRAMP transgenic [C57BL/6-Tg (TRAMP) 8247Nlg (the Jackson Laboratory) mice. Then we interbred TRAMP-AR<sup>T877A/Y</sup> mice with mice heterozygous for Wnt-5a deficiency (33). All mice were maintained according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

**Analysis of Prostate Morphology.** Mice were fully anesthetized and ventral, anterior, and dorsal prostate lobes were removed, weighed, and examined under a dark-field dissection microscope. Then, prostate tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were stained with H&E or Masson's trichrome, miniced in 10% volume of physiological saline. A volume (0.05 mL) of minced tissue was injected into a ventral prostate lobe of nude mice (BALB/C, Scl-nu). Prostates were dissected and weighed 8 wk after transplantation.

**Statistical Analysis.** For all graphs, data are represented as means ± SD. Statistical differences were determined using one-way ANOVA or two-way ANOVA followed by post hoc comparison with Fisher's protected least significant difference test or Student's t test. Statistical significance is displayed as *P < 0.05 or **P < 0.01.

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**Castroversion and Hormone Replacement.** Twenty-four-week-old mice were castrated or sham-operated, and slow-releasing pellets of DHT (160 μg/d), HF (160 μg/d), E2 (160 μg/d), or Casodex (BIC) (4.1 μg/d) (Innovative Research of America, Schaumburg, IL) were implanted s.c. in the scapular region behind the neck (27). After 3 wk, ventral prostates were resected, weighed, and examined.

**Transplantation of Prostate Cancer.** When prostate tumors in TRAMP-AR<sup>T877A/Y</sup> mice and TRAMP-AR<sup>flOxy</sup> mice reached ~5,000 mg, tumors were resected and minced in a 10% volume of physiological saline. A volume (0.05 mL) of minced tissue was injected into a ventral prostate lobe of nude mice (BALB/C, Scl-nu). Prostates were dissected and weighed 8 wk after transplantation.
Supporting Information

Takahashi et al. 10.1073/pnas.1014850108

SI Materials and Methods

Immunohistochemistry. Sections were subjected to boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (1). The cooled sections were incubated in 1% H2O2 for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4 °C. Sections were then incubated with anti-Wnt-5a (Imgenex) or anti-AR (Santa Cruz Biotechnology) in 3% BSA overnight at 4 °C. Negative controls were incubated in 3% BSA without primary antibody. The sections were then incubated with secondary antibodies for 2 h at room temperature, washed with PBS, and incubated with an avidin–bixin complex for 1 h (Vector Laboratories). Signals were visualized with 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma), and then slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

For immunofluorescence double staining (2, 3), the sections were treated as above and first stained with anti-Wnt-5a followed by signal amplification with TSA Plus Fluorescein Systems (PerkinElmer). After biotin blocking, the sections were then stained with anti-AR, and signal was amplified with TSA Plus Cy3 Fluorescence Systems (PerkinElmer). Confocal microscopy analysis was carried out on a Zeiss LSM 510 confocal laser scanning system.

siRNA. Knockdown was induced using siRNA duplex targeting human Wnt-5a. The transfection was performed using Lipofectamine 2000 (Invitrogen) with an siRNA concentration of 300–600 nM for ~8 × 105 cells per 10 cm-dish. Forty-eight hours after the transfection, these cells were subjected to assays. For a cell proliferation assay, 24 h after the transfection, cells (~2 × 106 cells/mL) were plated on 12-well plates (Corning).

ON-TARGETplus SMARTpool Human WNT5A (siWnt5a) was purchased from Thermo Scientific (Dharmacon) following the manufacturer’s instructions. Experimental samples were treated as above and then slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

real-time RT-PCR. For real-time RT-PCR, ventral prostate lobes were homogenized in ISOGEN (Nippon Gene) and total RNA was extracted according to the manufacturer’s instructions. Oligo-dT-primed cDNA was synthesized from 1 μg of prostate RNA by using SuperScript reverse transcriptase (Gibco-BRL) in a 20-μL reaction volume, 1 μL of which was then diluted serially. Primer sets for PCR were as follows: Wnt-5a forward, 5′-GGCCATATTTTCTCCTTTCG-3′; Wnt-5a reverse, 5′-CAGAGAGGTGTGCTCCTATAAA-3′; GAPDH forward, 5′-ACCACATTCATGCACTAC-3′; GAPDH reverse, 5′-TCCACACCTGTGTGCTGTA-3′. Real-time quantitative RT-PCR was performed using SYBR Premix Ex TaqII (Takara) with a Thermal Dice Cycler (Takara) according to the manufacturer’s instructions. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. To correct for variations in both RNA recovery and the efficiency of reverse transcription, GAPDH cDNA was amplified and quantified in each cDNA preparation (4, 5).

Cell Proliferation Assay. LNCaP cells transfected with siRNA in RPMI 1640 with 10% charcoal-stripped FBS and antibiotics were plated on 12-well culture plates (Corning) at ~2 × 105 cell/mL (day 0) and cultured with 5α-dihydrotestosterone (DHT), hydroxyflutamide (HF), or bicalutamide (BIC) for 6 d. In the absence of the ligands, 100% EtOH was added as a control. Cell numbers were counted using a trypan blue exclusion test at each point as shown in Fig. 4D. All values are means ± SD from at least three independent experiments.

Plasmids. Expression vectors for full-length human AR were inserted into a pcDNA3 vector (Invitrogen). The ARE-tk-Luc reporter vectors were constructed as described (6).

Reporter Assays. LNCaP cells (all 70–80% confluent) were transfected with plasmids using Superfect Reagent (QIAGEN) on 12-well plates. DHT (10−6 M), BIC (10−7 M), and HF (10−7 M) were added 12 h after transfection and then cells were incubated for 12 h. Luciferase activities were determined using the Dual Luciferase Assay System (Promega). As a reference plasmid to normalize transfection efficiency, 2 ng pRL-CMV plasmid (Promega) was cotransfected in all experiments. For RNAi, a two-step transfection was performed with Lipofectamine 2000 (Invitrogen) and Superfect Reagent (QIAGEN) following the manufacturer’s recommendation.

Fig. S1. (A) The growth curves of AR\textsuperscript{flox/Y} and AR\textsuperscript{pe-T877A/Y} mice were normal and comparable to that of wild-type (AR\textsuperscript{WT/Y}) mice. (B) Endocrine markers of mice at 20 wk of age. Serum testosterone levels were up-regulated in AR\textsuperscript{flox/Y} and AR\textsuperscript{pe-T877A/Y} mice. LH, luteinizing hormone. (C) Ventral prostate (VP), anterior prostate (AP), dorsal prostate (DP), and seminal vesicle (SV) of AR\textsuperscript{flox/Y} mice at 10 wk of age were remarkably more hypoplastic than those of AR\textsuperscript{WT/Y} mice. (D) Decreased wet weights in VP, AP, and DP of AR\textsuperscript{flox/Y} mice.
Fig. S2. (A) LacZ staining of the ventral prostates of KLKB1-Cre-ER<sup>T2</sup>; CAG-CAT-Z mice. KLKB1-Cre-ER<sup>T2</sup> mice were crossed with CAG-CAT-Z mice. Tamoxifen (TAM) was administered to the offspring at 6 wk of age followed by resection of the ventral prostate. LacZ expression, resulting from Cre-mediated excision, induced by TAM, was seen exclusively in the prostate epithelial cells. (B) Sequence analysis of cDNA extracted from the ventral prostates of AR<sup>T877A/Y</sup> mice. Sequences from exon 6 to exon 8 of the AR gene locus were analyzed, and the point mutation of T to A at codon 877 was confirmed. (C) No significant alterations were detected in AR mRNA levels in the prostates of AR<sup>flox/Y</sup> and AR<sup>T877A/Y</sup> mice by semiquantitative RT-PCR. (D) Reduced expression level of AR protein in the prostates of AR<sup>flox/Y</sup> and AR<sup>T877A/Y</sup> mice. The indicated proteins were visualized by Western blotting with α-AR or α-β-actin antibody. (E) Immunohistochemical staining demonstrating increased levels of cleaved caspase-3 and BrdU incorporation in prostatic cells. The number of BrdU-positive cells increased more in AR<sup>T877A/Y</sup> mice than in AR<sup>flox/Y</sup> mice following DHT treatment for 3 d. The sections were counterstained with hematoxylin. (F) Hematoxylin and eosin staining of prostate lobes from 24-wk-old transgenic murine prostate cancer model (TRAMP)-AR<sup>T877A/Y</sup> mice and 36-wk-old TRAMP-AR<sup>flox/Y</sup> mice revealed no histological difference.
Fig. S3. Alterations in gene expression of several major prostate cancer oncogenes (A) and tumor suppressor genes (B). Quantitative RT-PCR analysis of tumor-related genes was performed in the mice, and the results of representative five independent experiments are shown.

Fig. S4. Prostate tumors from TRAMP-AR<sup>pe-T877A</sup>/Y and TRAMP-AR<sup>flox/Y</sup> mice were transplanted into the ventral prostate lobes of nude mice at 8 wk of age. (A) Schematic representation of the experimental procedure for transplantation of prostate tumors. (B) Photographs of representative nude mice (Upper) and tumors (Lower). (C) Transplanted prostate weights are shown (**P < 0.01 with one-way ANOVA). (D) Hematoxylin and eosin staining of transplanted tumors.
**Fig. S5.** (A) The effect of castration in nude mice on the growth of transplanted tumors from TRAMP-AR<sup>pe-T877A/Y</sup> mice (*P < 0.05 with one-way ANOVA). Black columns indicate prostate tumors of TRAMP-AR<sup>pe-T877A/Y</sup> mice, and white columns indicate prostate tumors of TRAMP-AR<sup>flox/Y</sup> mice. (B) Photographs of representative nude mice (Upper) and tumors (Lower).

**Fig. S6.** Efficiencies of the indicated siRNA toward human Wnt-5a in LNCaP cells were analyzed by quantitative RT-PCR with the primer sets for human Wnt-5a.

**Fig. S7.** No significant alterations of cell proliferation were detected in BIC-treated LNCaP cells. LNCaP cells transfected with siRNAs were subjected to a cell proliferation assay. BIC was incubated with the cells for 6 d. Each point represents the mean ± SD of three separate experiments.
Dataset S1. Human prostate specimens

Dataset S1
Pathological diagnosis was based on World Health Organization histological grading and Gleason grade.