Transgenic Mouse Model for Rapid Pharmacodynamic Evaluation of Antiandrogens

Katharine Ellwood-Yen,[1] John Wongvipat,[2] and Charles Sawyers[1,2]

[1]Departments of Medicine, Urology, Molecular, and Medical Pharmacology, Jonsson Comprehensive Cancer Center and [2]Howard Hughes Medical Institute, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California

Abstract

Persistent androgen receptor signaling has been implicated as a critical factor in prostate cancer progression even at the hormone-refractory stage and provides strong rationale for developing novel androgen receptor antagonists. Traditional models for *in vivo* evaluation of antiandrogens are cumbersome because they rely on physiologic end points, such as the size of androgen-dependent tissues. Here, we describe a transgenic mouse (ARR2 Pb-Lux) that expresses luciferase specifically in the prostate in an androgen-dependent fashion. This signal is reduced by castration or by treatment with bicalutamide and can be quantified through noninvasive bioluminescent imaging. ARR2 Pb-Lux mice provide a novel method for rapid pharmacodynamic evaluation of novel pharmacologic compounds designed to inhibit androgen receptor signaling. (Cancer Res 2006; 66(21): 10513-6)

Introduction

Treatment of advanced prostate cancer is focused on drugs that interfere with androgen receptor function either through lowering androgen levels or through competitive antagonism at the ligand-binding site of the androgen receptor. Although effective initially, nearly all patients eventually relapse, despite continued treatment with these drugs, with hormone-refractory prostate cancer, the lethal stage of the disease. Several lines of evidence implicate altered androgen receptor function as the major cause of hormone-refractory disease through androgen receptor gene amplification, overexpression, or mutation or through ligand-independent androgen receptor activation by various kinase signaling pathways (1–4).

Compounds in an antiandrogen drug development program typically proceed through a series of *in vitro*, cell-based, and *in vivo* screens to inform selection of an appropriate clinical candidate. Whereas *in vitro* and cell-based assays are relatively straightforward, *in vivo* methods to assess antiandrogen activity are complex and cumbersome. For example, a typical readout is the wet weight of the ventral, lateral, and dorsal lobes of the prostate in rats treated with the compound of interest >14 days (5, 6). Although the development and maintenance of these glands is exquisitely androgen dependent and accurately reflects physiologic androgen receptor function, this assay presents numerous experimental challenges that preclude evaluation of large numbers of compounds, such as the treatment time, dynamic range of the prostate weight, and number of animals required to obtain conclusive information. To circumvent these shortcomings, we developed a reporter mouse (ARR2 Pb-Lux) with androgen-dependent luciferase expression specifically in the prostate. Androgen receptor activity can be measured noninvasively in these mice through bioluminescent imaging over a 70-fold dynamic range. We show the use of ARR2 Pb-Lux mice for evaluation of antiandrogens by treatment with bicalutamide, a nonsteroidal androgen receptor antagonist currently in clinical use. These mice offer a quantitative method for rapid, noninvasive evaluation of the pharmacodynamic properties of androgen receptor inhibitors and could complement current prostate cancer models as a useful drug development tool in the search for compounds effective in hormone-refractory prostate cancer.

Materials and Methods

Construct design for prostate-specific luciferase activity. The following gene fragments were subcloned into the Bluescript (KS+) backbone (Stratagene). The polyadenylic acid tail of the insulin receptor gene (*PAI*) was subcloned into the *Bam*HI/*Not*I site of the Bluescript KS+ multiple cloning site (MCS). The 5′ flanking ARR_2Pb promoter region (−286/+28) of the rat probasin gene fused to an additional androgen response region (7) was subcloned into the *Kpn*I/*Eco*RV restriction sites located in the MCS. The firefly luciferase cDNA was isolated from the pSP-luc+ cloning vector (Promega) using *Nhe*I and *Eco*RV, and the resulting fragment was filled in using the large fragment Klenow polymerase. Following gel purification, the blunt-ended product was subcloned into the *Eco*RV site of the Bluescript KS+ ARR_2Pb-PAI backbone, thus generating the ARR_2Pb-Lux-PAI transgenic construct. The completed construct was sequenced and tested for promoter inducibility by androgen in LNCaP cells by transient transfection before microinjection into FVB ova.

Generation of transgenic ARR_2Pb-Lux-PAI mice. The ARR_2Pb-Lux-PAI construct was linearized with *Kpn*I/*Not*I, microinjected into fertilized FVB ova, and transplanted into a pseudopregnant female (Transgenic Mouse Facility, University of California at Irvine, Irvine, CA). Transgenic founders were screened by PCR using genomic DNA isolated from tail snips. The 5′ primer was specific to the ARR_2Pb promoter (5′-ARR2Pb-CAATGTCTGTGTACAACTGCCAACTGGGATGC), and the 3′ primer was located at the end of the luciferase cDNA (5′-TTACACGGCGATCT-TTCCGCCCTTCTTGGCCTTTATGAGGATCTCTC). A PCR product of 1,847 bp was generated from the ARR_2Pb-Lux-PAI mice, and six founder lines were obtained (designated 1.3, 1.11, 3.4, 4.2, 10.10, and 99.1). Breedings were carried out and four ARR_2Pb-Lux-PAI founders obtained germ-line transmission. These mice were bred and the offspring were aged to determine if luciferase activity could be detected specifically in the prostate of transgene-positive male mice. Prostates were isolated "en block" from transgenic and wild-type (WT) mice at 2 to 12 weeks as well as at 6, 9, 12, and 16 months. Superficial and deep H&E sections were examined on the same tissue to document the presence/absence of an abnormal phenotype based on transgene expression (i.e., hyperplasia mPIN, microinvasion, and invasive adenocarcinoma).

Luciferase imaging of ARR_2Pb-Lux-PAI mice. Luciferin substrate (100 μL) was injected i.p. at a concentration of 15 mg/mL, and 5 minutes later, mice were anesthetized using isoflurane (Abbott Laboratories). *In vivo*

imaging using a cooled charged-coupled device ISIS camera was done, and data were analyzed using Living Image 2.20 software.

Mouse dissections, tissue isolation, serum collection, and castration. Urogenital organs were isolated and prostates were microdissected in a Petri dish containing 10 mL of cold PBS ($1\times$ PBS; Life Technologies) under a dissecting microscope. Adipose tissue surrounding the mouse prostates was cleared using forceps. One half was used to obtain protein, whereas the other half was fixed in 10% phosphate-buffered formalin for histology (Fisher Scientific). The liver, testes, bone from the spine, brain, kidneys, and lungs were also isolated for both histologic examination as well as protein analysis to check for nonspecific expression of luciferase as described (8). Serum testosterone levels were measured in the mice by an outside vendor, IDEXX Preclinical Research Services, who used RIAs to measure the levels. Serum was isolated via an orbital sinus venipuncture. For castration experiments, mice were anesthetized using isoflurane and castration was done as described (8).

Luciferase assays of ARR$_2$Pb-Lux-PAI mice. Tissue was microdissected as stated above, and individual organs were lysed in $1\times$ passive lysis buffer (Promega) plus protease inhibitors (Calbiochem) using a PowerGen 125 tissue homogenizer from Fisher Scientific. Following lysis, luciferase assays were done using the Luciferase Assay System from Promega.

Results and Discussion

Prostate-specific, androgen-dependent luciferase expression in ARR2 Pb-Lux mice. To generate mice with androgen receptor–dependent expression of luciferase, we used the well-characterized, androgen-dependent ARR$_2$Pb promoter (7). The linearized transgenic construct (Fig. 1*A*) was microinjected into FVB ovum, and the resulting six transgenic founders were analyzed for transgene expression using Xenogen IVIS imaging system. Of the six founders, all showed prostate-specific luciferase expression but only two passed the transgene through the germ line to F1 progeny. On further characterization of both founder lines, one consistently showed higher transgene expression than the other and was therefore used to establish a larger colony (Fig. 1*B*, *Tg-2*). The luciferase expression observed in these mice clearly originated from the prostate based on independent analysis of the urogenital tissues obtained from these mice at the time of sacrifice. Microdissection of the urogenital tissue into the bladder, seminal vesicles, anterior, dorsolateral, and ventral prostate lobes showed luciferase expression in the ventral and dorsal-lateral prostatic lobes as visualized by luciferase assays conducted on tissue lysates (Fig. 1*C* and *D*). The prostates of these mice appeared normal as assessed by standard histology, indicating that transgene expression does cause pathologic changes (data not shown).

To examine the androgen dependence of the transgene, mice were castrated and reimaged using bioluminescence. After castration, luciferase expression was nearly completely attenuated by 3 days (Fig. 2*A* and *B*). The very low level of expression observed in Fig. 2*D* and *F* after castration is most likely due to residual adrenal androgens (as in humans) and/or stimulation by
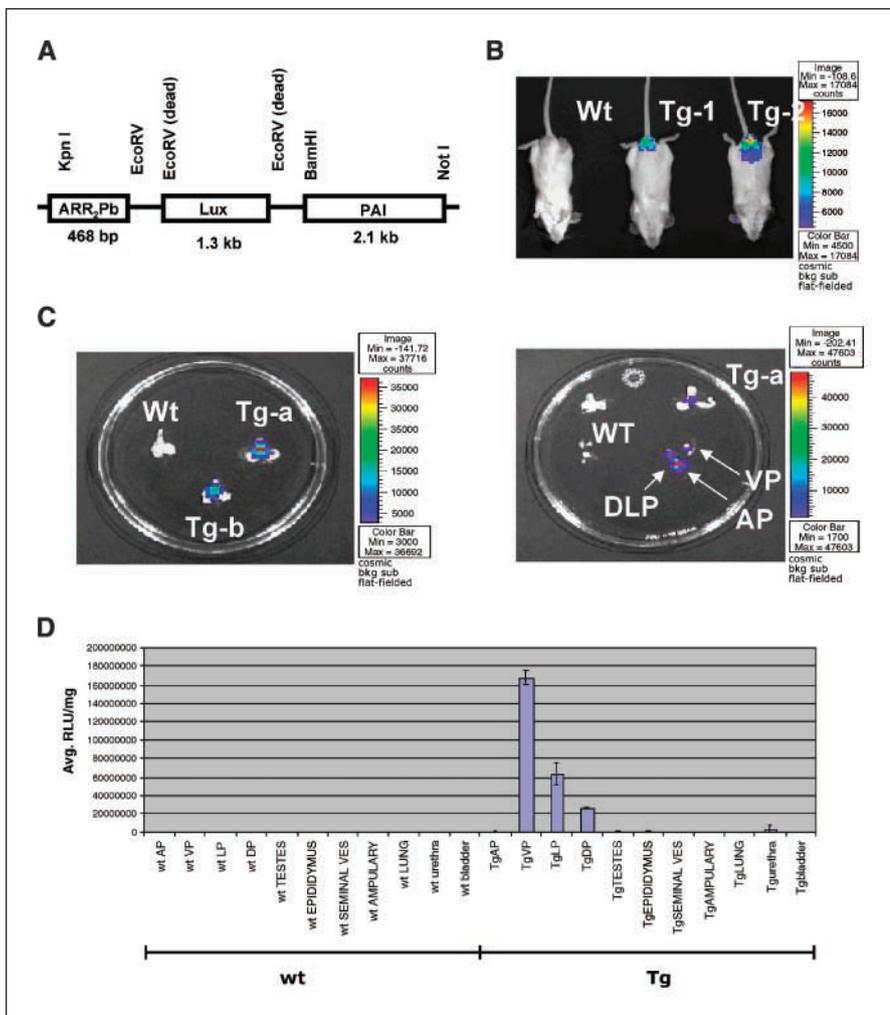

Figure 1. Generation of the ARR$_2$Pb-Lux mouse. *A,* the transgenic construct used to microinject FVB ovum contains the prostate-specific ARR$_2$Pb promoter driving expression of the firefly luciferase gene. *B, in vivo* imaging of WT and two transgenic mice (Tg-1 and Tg-2 at 6 weeks of age) shows prostate-specific luciferase expression. *C, ex vivo* image of the urogenital tissue following microdissection of two transgenic mice (Tg-a and Tg-b) and the WT control. The four prostate lobes were dissected away from the total urogenital tissue (bladder and seminal vesicles) and imaged. *AP,* anterior prostate; *VP,* ventral prostate; *DLP,* dorsal lateral prostate. *D,* luciferase assays done on lysates from organs microdissected from the urogenital tract of both WT and transgenic animals.
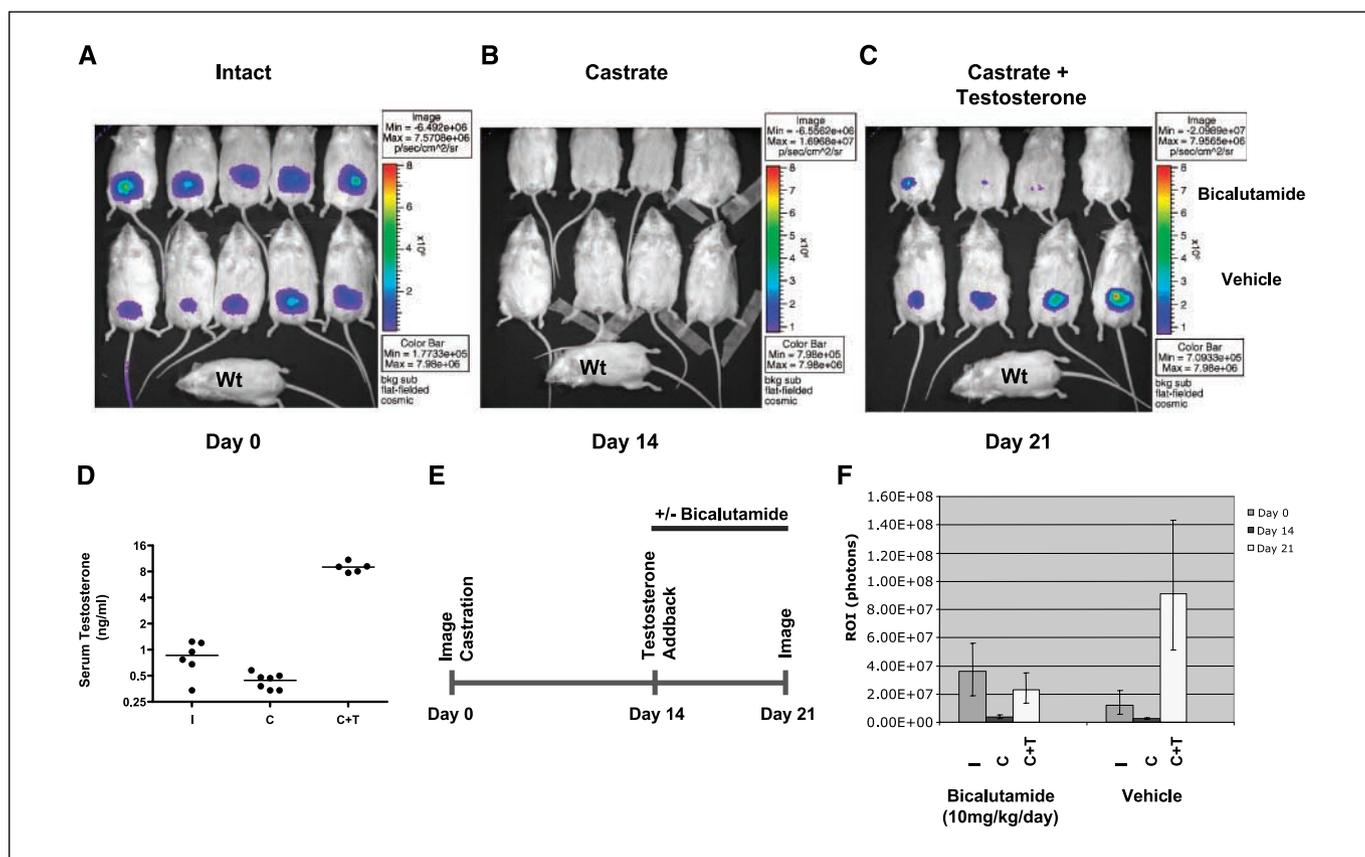
Figure 2. Castration and bicalutamide are both capable of inhibiting androgen receptor signaling *in vivo*. *A,* prostate-specific luciferase activity is seen in the mouse prostate at the start of the experiment (day 0) before castration. WT is a nontransgenic mouse control. *B,* castration abolishes the luciferase signal in all animals. At 2 weeks after castration (day 14), before the addition of testosterone, no luciferase expression is observed. *C,* mice were reimaged 1 week (day 21) following treatment with either bicalutamide or vehicle in the presence of testosterone. Bicalutamide treatment reduces luciferase signal in the prostate (*top*) when compared with vehicle-treated mice (*bottom*). *D,* serum testosterone levels vary from 0.3 to 1.2 ng/mL in intact (*I*) transgenic mice. Castrated (*C*) mice have serum testosterone levels ranging from 0.34 to 0.58 ng/mL in each mouse. After implantation of time-release testosterone pellets (2.5 mg/kg/d) to castrated animals (*C+T*), serum levels were between 7.7 and 10.9 ng/mL. *Lines,* mark the average testosterone level in each group. *E,* the figure outlines the experimental design. On day 0, animals were imaged and then castrated. On day 14, animals were imaged followed by implantation of a 90-day release testosterone pellet (2.5 mg/kg/d) and treated with either vehicle or bicalutamide (10 mg/kg/d). On day 21, all animals were reimaged. *F,* quantification of the luciferase signal seen in (*A*) to (*C*) shows a 4.2-fold reduction in the luciferase signal when the mice were treated with bicalutamide rather than vehicle for 1 week.

glucocorticoids as suggested by Zhang et al. (7). After surgical implantation of time-release exogenous testosterone pellets (2.5 mg/kg/d), luciferase expression was restored to levels even higher than baseline, consistent with the increased levels of serum testosterone following testosterone addback (compare the four mice in Fig. 2*C, bottom* with Fig. 2*A, D,* and *F*).

Inhibition of luciferase expression by bicalutamide. To determine if ARR2 Pb-Lux mice might be used to assess pharmacologic inhibitors of the androgen receptor, we evaluated the effect of bicalutamide, an antiandrogen commonly used in clinical treatment of prostate cancer (9), on luciferase expression. In preliminary studies, we noted substantial differences in the baseline serum testosterone levels of intact mice (0.3-1.2 ng/mL), whereas levels in transgenic mice following castration were consistently within a narrower range (0.34 and 0.58 ng/mL; Fig. 2*D*). Because of concerns that this variability in baseline testosterone levels might affect the assessment of a competitive androgen receptor antagonist, we sought to standardize the assay by castrating ARR2 Pb-Lux mice and then surgically implanting a sustained release testosterone pellet (2.5 mg/kg/d) in each mouse 14 days after castration (Fig. 2*E*). As expected, these mice had more consistent baseline testosterone levels (7.7-10.9 ng/mL; Fig. 2*D*),

and luciferase expression was restored after castration (see panel of four mice in Fig. 2*B* and *C, bottom*; quantification in Fig. 2*F*). On treatment with bicalutamide (10 mg/kg/d by oral gavage), luciferase expression was consistently inhibited in all mice, with a 4.2-fold drop in the entire cohort (Fig. 2*B, C,* and *F*). This magnitude of reporter gene suppression is physiologically significant because histologic evaluation of the treated mice showed involution and other pathologic changes comparable with castrated mice and in stark contrast to fully restored glandular architecture of vehicle-treated mice (Fig. 3*A-D*). Notably, bicalutamide was effective despite the fact that testosterone levels following androgen addback were significantly higher than those in noncastrated animals (Fig. 2*D* and *F*). We have not explored the activity of bicalutamide in castrate mice implanted with lower dose androgen pellets that might replicate the serum testosterone levels found in intact mice while eliminating the wide variability.

Although the primary goal of this work was to develop a reporter mouse for noninvasive measurement of androgen receptor activity, ARR2 Pb-Lux mice may also have use in tracking prostate cancer progression when crossed to appropriate genetically engineered prostate cancer models. Indeed, we have crossed ARR2 Pb-Lux to Hi-Myc transgenic mice (7), generated bigenic animals, and showed
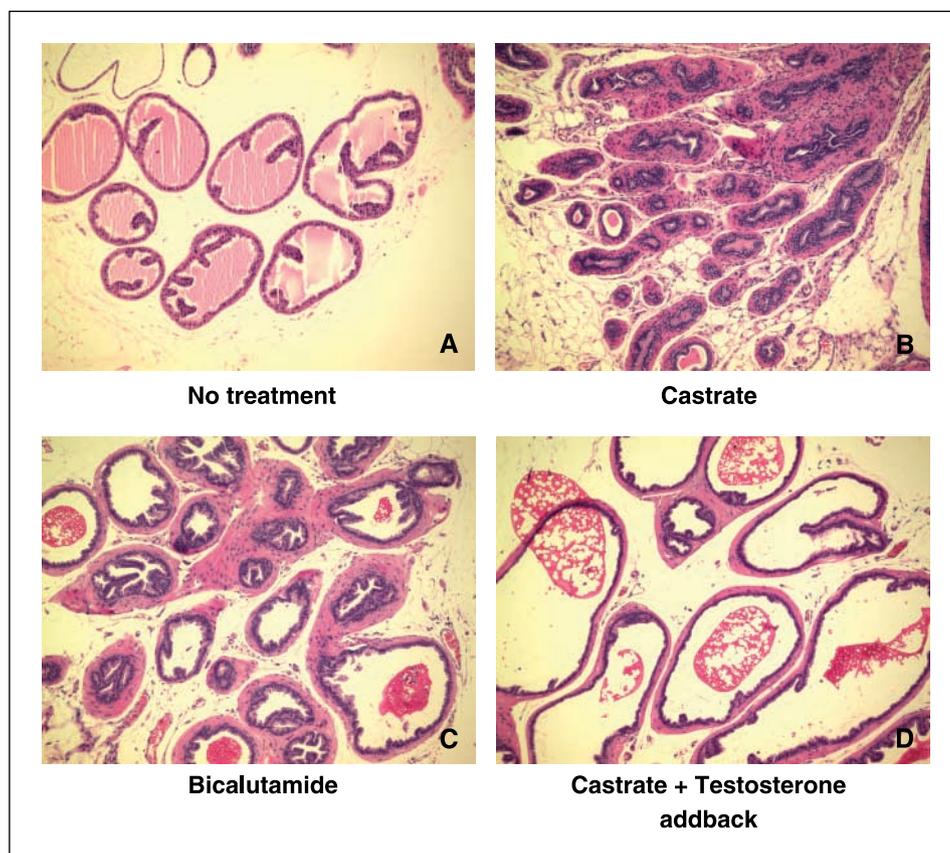
Figure 3. Histologic effect of bicalutamide treatment of the ARR2 Pb-Lux mouse prostate. *A,* H&E staining of the dorsal-lateral lobe of an intact nontreated transgenic mouse shows normal prostate architecture. *B,* dorsal-lateral prostate of a transgenic mouse 14 days after castration shows characteristic features of castration, including involution, atrophy, and fibrosis of the gland. *C,* dorsal-lateral lobes after bicalutamide treatment for 14 days show histologic features similar to the castrated mouse in (*B*). *D,* dorsal-lateral lobes of a mouse treated with castration and testosterone addback (2.5 mg/kg/d) for 14 days but not bicalutamide seem similar to the nontreated mouse in (*A*).

sustained expression of both transgenes in mice up to 16 months of age. Preliminary experiments fail to reveal a consistent quantitative change in luciferase expression as these mice develop prostatic epithelial neoplasia or invasive adenocarcinoma, suggesting that this model may not be useful for measuring local disease progression. Further studies using metastatic models are required to assess use in tracking disease outside the prostate gland.

Recently, other transgenic mouse models have been described that also express luciferase in the prostate. One differs from ARR$_2$Pb-Lux in that it relies on the sPSA prostate-specific promoter (10). Unlike the ARR$_2$Pb-Lux described here, sPSA-Lux mice show background luciferase expression in the bone marrow and the mouse caput epididymis (10). A very recent report describes another ARR$_2$Pb-Lux mouse (EZC3) with characteristics similar to that reported here; however, the sensitivity of luciferase expression in these mice to androgen receptor antagonists has not been evaluated (11). In summary, we describe a new transgenic mouse model with prostate-specific, androgen-dependent luciferase expression that may serve as a useful intermediate tool for rapid *in vivo* evaluation of candidate antiandrogens in drug development programs.

Acknowledgments

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