EZC-Prostate Models Offer High Sensitivity and Specificity for Noninvasive Imaging of Prostate Cancer Progression and Androgen Receptor Action

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Abstract

In vivo imaging advances have greatly expanded the use of animal cancer models. Herein, we describe two new models that permit prostate imaging \textit{ex vivo}, \textit{in vivo}, and \textit{in utero}. Further, we show the use of these models for detecting small metastasis and testing reagents that modulate the androgen receptor (AR) axis. A luciferase reporter gene was directed to the prostate epithelium using three composite promoters called human kallikrein 2 (hk2)-E3/P, PSA-E2/P, and ARB-P, derived from hk2, PSA, and rat probasin regulatory elements, to generate the EZC1, EZC2, and EZC3-prostate mice, respectively. EZC2 and EZC3-prostate display robust expression in the prostate with only minimal detectable expression in other organs, including testes and epididymis. Luciferase expression was detected as early as embryonic day 13 (E13) in the urogenital track. To image prostate cancer progression, lines of EZC mice were bred with prostate cancer models TRAMP and JOC1, and imaged longitudinally. When crossed with prostate cancer models, EZC3 facilitated detection of metastatic lesions although total prostate luciferase expression was static or reduced due to weakening of AR-regulated promoters. Castration reduced luciferase expression by 90% and 97% in EZC2 and EZC3 mice, respectively, and use of GnRH antagonist also led to extensive inhibition of reporter activity. The EZC-prostate model permits prostate imaging \textit{in vivo} and should be useful for imaging prostate development, growth, metastasis, and response to treatment noninvasively and longitudinally. These models also provide powerful new reagents for developing improved drugs that inhibit the AR axis. (Cancer Res 2006; 66(12): 6199-209)

Introduction

Animal models that reflect the natural history of human disease and/or human pathobiology can help elucidate the molecular basis of disease pathogenesis and accelerate the pace of drug development. There is now an increasing repertoire of animal models for numerous distinct types of cancer, such as prostate cancer (reviewed in refs. 1, 2). Because autochthonous models develop progressive disease over time, advances in molecular and optical imaging now permit imaging of early tumor growth or metastasis. These new noninvasive techniques can complement results obtained by direct methods at the study end point, thereby hastening the pace of research while reducing the number of animals required (reviewed in ref. 3).

Approaches to noninvasive imaging differ by a number of key variables, such as spatial resolution, depth, imaging times, sensitivity, type of probe, cost, and others (reviewed in ref. 4). The highest spatial resolution is obtained with magnetic resonance imaging and computed tomography that can get close to 25 mm with long (>15 minutes) scan times. Molecular imaging using positron emission tomography (PET) and techniques based on detection of γ-rays produced by radiolabeled probes can provide resolution near 1 to 2 mm, but these methods require expensive equipment and relatively slow throughput. A new three-dimensional ultrasound microimaging technology has been developed with very good spatial resolution at low cost that has been used to image spontaneous models of prostate cancer (5). Alternatively, advances in charged coupled devices (CCD) have made optical imaging quite feasible for both fluorescence and especially bioluminescence imaging (BLI), where typically the oxidation of d-luciferin $[\text{D-}(-\text{)}-2-(6\text{-hydroxy-2'-benzothiazolyl})\text{thiazone-4-carboxylic acid}]$ is observed following the Mg-ATP- and O$_2$-dependent catalysis by firefly luciferase (6). The main advantages of BLI is sensitivity: Background is virtually absent in mammals imaged in light-tight specimen chambers attached to cryogenically cooled ($\leq-105^\circ$C) CCD cameras. Another advantage of BLI is speed, as multiple animals can be scanned simultaneously in seconds to minutes in a single image acquisition with minimal postprocessing. Drawbacks include the partial opacity of highly vascularized tissue and melanin-pigmented skin, light scattering from tissue membranes and hair of most visible light, and planar two-dimensional images lacking depth information; however, newer "red-shifted" reporter proteins (i.e., producing light $>600$ nm) and other technologies may overcome these obstacles (7–10). Despite these limitations, imaging of as few as 100 tumor cells in the peritoneal cavity has been shown (10).

We previously reported development and characterization of a mouse model originally named EZC-prostate based on prostate epithelial-directed firefly (\textit{Photinus pyralis}) luciferase and enhanced green fluorescent protein (EGFP) expression (11). The majority of expression was directed by prostate, with minor confounding signals coming from the testes and intestines. Longitudinal measurements showed strong androgen receptor (AR) responsiveness of luciferase reporter driven by the composite human kallikrein 2 (hk2) promoter, hk2-E3/P (12). In the present study, we describe two new prostate-directed expression systems that were used to build new luciferase reporter models, called EZC2-prostate and EZC3-prostate, based on the composite probasin promoter, ARB-PB (13), and a composite prostate-specific antigen promoter, PSA-E2/P (14), respectively. These new models direct
even more exquisite prostate specificity than the original model, allowing for detection of distant soft-tissue metastasis when bred unto the TRAMP prostate cancer background (15, 16). Early detection of distant metastasis in living mice should greatly reduce the number of animals required in some studies. Moreover, localization of metastasis ex vivo should facilitate histologic analysis. The use of AR-responsive reporter models also allows remote appraisal of AR activity during development and prostatic tumor progression. Although AR levels are maintained or even elevated in the majority of cells within human prostate metastasis (17), AR levels typically drop in a subset of cells or poorly differentiated tumors, especially those displaying neuroendocrine markers (18–20). This observation has been used to suggest that AR signaling maintains the differentiated state of prostate epithelial cells, but loss of this signaling axis in the presence of other genetic and epigenetic events can drive dedifferentiation or reprogramming of cells in both human and animal models (reviewed in ref. 21). Consistent with this hypothesis, we observed a sharp decrease in reporter activity during cancer progression in bigenic models on both the TRAMP background and the JOCK1 model (22). Moreover, distant metastasis in bigenic EZC3-prostate x TRAMP (i.e., EZC-TRAMP) mice have low-level (but detectable) reporter signal, consistent with their prostatic origin. 

Although palliative therapies can extend life span by several years, virtually all patients treated with surgical or medical androgen deprivation, combined with AR blockade, eventually succumb to an aggressive hormone-refractory prostate cancer (reviewed by ref. 23). Targets of androgen action include blockade of GnRH/LH-RH signaling (e.g., leuprolide, Abarelix), blockade of 5α-reductase that converts testosterone to the more potent androgen, 5α-dihydrotestosterone (e.g., finasteride, dutasteride), nonsteroidal antiandrogens that act as AR antagonist (e.g., flutamide, bicalutamide), and selective AR modulators that likely prevent tissue-specific coactivator function (24, 25). To test the use of the EZC system in predicting response to hormone withdrawal therapy, we show that the GnRH antagonist, PPI-258, can almost completely eliminate prostate-directed reporter activity to levels comparable with that of castration (up to 3 orders of magnitude).

These new genetically engineered mouse models should accelerate the pace of drug development and our understanding of the role of AR signaling during prostate cancer progression. 

**Materials and Methods**

**Constructs.** pARR-PB-KBPA-Luc and pPSA-E2/P-KBPA-Luc were composed of the ~500 bp composite ARR-PB promoter originally from pSK/ARR-PB (13) or the ~2.9 kb PSA-E2/P promoter (12), respectively, cloned upstream of the KCR intron in KBPA (26), which is, in turn, upstream of the modified firefly (*Photinus sp.*) luciferase gene from pGL3-Basic (Promega, Madison, WI) and a polyadenylate acid site from bovine growth hormone. Briefly, IRES-EGFP was released from plasmid phK2-E3/P-IRES-EGFP (11) by EcoRI digestion and religation to give phK2-E3/P-KBPA-Luc. To get pARR-PB-KBPA-Luc, the XbaI/SpeI ARR-PB fragment from pAdox/ARR-PB-EFPP (27) was blunt ligated into the NotI/BamHI sites of phK2-E3/P-KBPA-Luc. Similarly, the NotI/XbaI (blunt) PSA-E2/P fragment from pSH1/PSA-E2/P-SEAP (27) was ligated into NotI/BamHI (blunt)-digested phK2-E3/P-KBPA-Luc to produce pPSA-E2/P-KBPA-Luc.

**Generation and screening of transgenic mice.** The minimal eukaryotic-derived fragments were released from plasmids pARR-PB-KBPA-Luc and pPSA-E2/P-KBPA-Luc with BsiHI and gel-purified with Gene Clean kit (Bio101, Vista, CA). The EZC2-prostate (ARR-PB-luc) and EZC3-prostate (PSA-E2/P-luc) transgenic mice were generated by microinjection of purified fragments into the male pronuclei of fertilized FVB oocytes by the Transgenic Core Lab at Baylor College of Medicine. EZC-prostate mice based on plasmid hK2-E3/P-Luc-IRESGFP were previously described (11). Mice were maintained in the Transgenic Mouse Facility, a pathogen-free environment, in compliance with Baylor College of Medicine policy. Mouse tail DNA was extracted using the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) and screened by PCR using primers for the firefly luciferase genes *Luc-p1*, 5'–ACCCAGATTGGGAAGACGCCAAAAC-3' and *Luc-p2*, 5'TGCAGATAATCCGAATGGTTGCG-3'. DNA quality control primers for mouse casein: forward, 5'-GATGTTGCTCCGCGG-TAAAGGT-3', and reverse, 5'-AGAAACGAGGTATGGTGGAGG-3'.

**Luciferase assays.** To assay tissue-derived luciferase activity, animals were euthanized and dissected. Tissue specimens from the ventral, dorsolateral, and anterior prostate lobes were microdissected and stored temporarily on ice before homogenization in lysis buffer. Other tissue specimens, including testes, epididymis, seminal vesicle, penis, as well as brain, salivary glands, thymus, lung, heart, stomach, cecum, small intestine, large intestine, liver, adrenal gland, kidney, pancreas, and spleen were also analyzed to determine the tissue specificity of the EZC, EZC2, and EZC3 promoters. Tissues were homogenized with a PRO250 homogenizer (Pro Scientific, Inc., Oxford, CT) in 300 μl luciferase lysis buffer (Promega) containing 1/100 diluted protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Specimens were centrifuged at 8000 rpm for 5 minutes and placed temporarily on ice. Luciferase activity of the cell lysates was measured with a TD 20/20 luminometer (Turner Designs, Inc., Mt. View, CA) and the protein concentration was determined using the detergent-compatible protein assay system (Bio-Rad, Hercules, CA) in a Beckman DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA). The luminescence results are reported as relative light units per milligram of protein.

**Histologic and immunohistochemical examination.** The microdissected prostate parts and other tissues indicated above were fixed overnight in 10% formaldehyde and transferred to 1:1 formaldehyde/ethanol for 1 hour followed by transfer to 70% ethanol until processing. Tissues were processed in a series of increasing ethanol concentrations and embedded in paraffin wax. Five-micrometer sections were cut and stained with H&E. Immunostaining for firefly luciferase was also done. Briefly, sections were deparaffinized in xylene, rehydrated in decreasing ethanol from 100% to 80%, microwaved for 10 minutes in 10 mmol/L citrate buffer (pH 6.0) at 95°C to 99°C for antigen retrieval, and finally endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Non-specific binding was abolished with 10% Power Blocker (BioGenex, San Ramon, CA) for 10 minutes. Tissue sections were incubated overnight at 4°C with a 1:2,000 dilution of 10 mg/mL biotin-conjugated goat anti–firefly luciferase antibody (Abcam, Hartford, CT). After four PBS washes with 0.1% Tween 20 for 2 hours, sections were incubated with horseradish peroxidase–conjugated streptavidin using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) for 45 minutes at room temperature. Peroxidase activity was revealed with 3,3’-diaminobenzidine (DAB) tetrahydrochlorurate using a DAB kit (Vector Laboratories) according to the protocol provided. Finally, sections were washed in distilled water for terminating the reaction, counterstained with 1% methyl green, dehydrated, and mounted.

**Imaging and quantification of bioluminescence data.** Mice were anesthetized with a mixture of 1.5% isoflurane/air using an Inhalation Anesthesia System (VetEquip, Inc., Pleasant Hill, CA). n-Luciferin (Xenogen, Alameda, CA) was i.p. injected at 40 mg/kg mouse body weight (unless otherwise specified). Ten minutes after n-luciferin injection, mice were imaged with an IVIS Imaging System (Xenogen) with continuous 1% to 2% isoflurane exposure. Imaging variables were maintained for comparative analysis. Gray scale–reflected images and pseudocolored images reflecting bioluminescence were superimposed and analyzed using the Living Image software (Xenogen). A region of interest (ROI) was manually selected over relevant regions of signal intensity. The area of the ROI was kept constant within experiments and the intensity was recorded as total photon counts per second per cm² within a ROI. In some experiments, after imaging living mice, animals were euthanized and organs of interest were removed, arranged on black, bioluminescence-free paper, and *ex vivo* imaged within 30 minutes.
Castration of mice and administration of reagents. To study the effect of androgen ablation on EZC2-Prostate-driven firefly luciferase expression, 20-week-old male mice, were anesthetized with Rodent Combo Anesthetic III (37.6 mg/mL ketamine, 1.92 mg/mL xylazine, and 0.38 mg/mL acepromazine) at 240 mg/kg mouse weight, and cleaned around the scrotum with 70% ethanol. A 1-cm incision was made at the tip of the scrotum. The testes lying in the sacs can be seen by placing pressure on the lower abdomen. A 5-mm incision was made into each sac, and the testes, epididymis, vas deferens, and spermatic blood vessels were pulled out. A single ligature was placed around the spermatic blood vessels and vas deferens. The testes and epididymis were removed by severing the blood vessels and vas deferens distal to the ligature. The remaining vas deferens was pushed back into the sac and the incision was sutured. The castrated mice were put on a heating pad until recovery and were given injectable Buprenex (buprenorphine hydrochloride; Reckitt Benckiser Healthcare UK Ltd., England, United Kingdom). Sham-operated, age-matched males were used as controls. The orchiectomized mice were imaged biweekly until day 16 postcastration when animals were given testosterone pellets (10 mg/21-day release-Innovative Research of America, Sarasota, FL) for experimental groups and placebo for control groups. The mouse was shaved on dorsal side and cleaned with 70% ethanol. A 5-mm incision was made in the center of the back, the pellet was placed under the skin with forceps and the incision was clipped with surgical staples. The mice were measured biweekly and after 2 weeks animals were euthanized and imaged ex vivo as above. Mice treated with GnRH antagonist, PPI-258 (Praecis Pharma, Waltham, MA), were treated with a single s.c. 50 mg/kg dose (5 mg/mL 0.9% saline) of PPI-258-CMC, suspended in a time-release depot, designed to last for at least 4 weeks. For treatment of EZC-JOCK mice with chemical inducer of dimerization (CID), AP20187 was dissolved in 16.7% propanol, 22.5% PEG400, 1.25% Tween 80, and injected i.p. biweekly at 2 mg/kg. Alternatively, carrier alone was injected.

Statistical analysis. Statistical significance was determined between indicated groups primarily using a nonpaired, two-sided Student’s t test (Microsoft Excel 2004).

Results

Development of EZC-prostate models. We previously reported that in vivo chemiluminescent imaging of the prostate was possible (11). Although reporter expression driven by the composite hK2-based promoter, hK2-E3/P, was primarily (>98%) specific for prostate tissue, low-level androgen-independent expression in the cecum and lower intestines of some animals produced a disproportionate confounding signal during living imaging. Therefore, we have now developed two models that expressed luciferase under the transcriptional regulation of the alternative prostate-epithelial-targeted promoters, namely ARR2PB, based on the rat probasin-targeted promoter (13) and PSA-E2/P, based on the prostate-specific antigen promoter (12).

We were able to select new founder lines using both ARR2PB (one of three) and PSA-E2/P (two of four) that showed highly robust and prostate-specific expression, and have named them EZC2-prostate (EZC2) and EZC3-prostate (EZC3), respectively. Both new lines showed extremely high luminescence in the lower abdomen of living mice, requiring only seconds for imaging (Fig. 1A). Dissection of these lines and ex vivo imaging of tissues confirmed that the major luminescence was derived from the relatively small (~20 mg) ventral prostate lobes. Moreover, luciferase expression was almost undetectable in the intestines and all internal organs of both new lines (Fig. 1B). Additional relatively weak signals in the extremities of EZC3 mice are distant from the prostate and likely target tissues of prostate cancer metastasis in mice, such as draining lymph nodes, lungs, and liver. Thus, EZC2- and EZC3-prostate models reflect the highest overall signal intensity and tissue specificity ever seen in a prostate-directed bioluminescence model.

Tissue-specific and temporal regulation of reporter expression. To quantitate the tissue specificity of these new lines, we conducted sensitive luciferase assays on a broad panel of tissues from 12-week-old mice from each line (Fig. 2A-C). This analysis further confirmed that the major reporter signal from each line was derived from the ventral prostate with minor signals coming from various other sources. For EZC2, minor relative signals (>0.1%) were detected in the epididymis (2.1%), dorsolateral prostate (0.7%), and penis (0.4%; Fig. 2B). For EZC3, minor signals (>0.1%) were detected in dorsolateral prostate (1.6%), testes (1.1%), and anterior prostate (0.2%). In contrast, for the original EZC-prostate (renamed EZC1), minor signals (≥0.5%) came from a larger number of tissues, including dorsolateral prostate (7.8%), cecum (6.1%), anterior prostate (3.2%), large intestines (2.0%), testes (1.4%), spleen (1.4%), small intestines (1.1%), penis (0.5%), and

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thymus (0.4%; Fig. 2C). Thus, careful tissue analysis confirmed the robust expression and prostate specificity of EZC2 and EZC3.

An advantage of live imaging is the ability to undertake longitudinal analysis of reporter activity. In accordance with the large increase in testosterone production and prostate size during puberty, all three distinct EZC-prostate lines showed an increase in AR-driven reporter activity in living mice after 4 weeks of age (Fig. 3A and B). Moreover, in EZC2 mice, the amount of luciferase per milligram prostate tissue showed a very large (~100-fold) increase from 4 to 8 weeks (Fig. 3C). A somewhat more conservative increase was observed during puberty in EZC3-prostates (Supplementary Fig. S1), perhaps reflecting greater sensitivity of the PSA-E2/P promoter to lower testosterone levels. In EZC1 mice, the biggest temporal increase in reporter signal occurred earlier, between 3 and 4 weeks, although off-target reporter expression in their intestines may have partially confounded pubertal changes in AR activity during prostate development (data not shown). Thus, in all three of the EZC series of prostate models, AR activity in the prostate could be measured as a surrogate marker for prostate growth and development.

To determine whether the EZC system was active in utero, gravid, nontransgenic female mice bearing embryonic day 10 (E10) to E19 EZC2 embryos were administered β-luciferin. Although the signal intensity was weak relative to adult transgenic mice, by 10 minutes after substrate injection, fetal signals were readily detected after E14 in some mice (Fig. 4A). Although some intermouse variability was seen, likely due to positioning of embryos and stochastic efficiency of substrate delivery, in general the average signal intensity peaked at E17 and dropped steadily until birth (data not shown). Removal of the uterine horn 15 or 19 days post coitum confirmed that all of the detectable signals came from transgenic embryos although the absolute levels of luciferase activity varied per embryo (Fig. 4B). Also, we were unable to find a significant correlation between signal intensity and gender or fetus position in the uterus (Fig. 4C). The weakest signals, however, did originate from the smallest embryos. Luminescence was localized to the urogenital tract of both male and female embryos, as well as from other tissues, including the penis, vagina, upper and lower jaws (and other head regions), and the upper limbs (Fig. 4D). In contrast, as in adults, there did not seem to be reporter gene expression in the internal organs, including the liver, lungs, and intestines.

The signals we detected at the ends of the upper and lower jaws and the upper limbs (likely from skeletal muscle) at E15 and E19 were essentially undetectable by neonatal day 1 or 2 (Fig. 4F). Similarly, the urogenital tract–derived signal was difficult to detect after day 3, commensurate with the predicted drop in circulating androgens. Interestingly, reporter activity in the lower abdomen continued to increase in intensity during early development despite near undetectable signaling from the urogenital sinus (Fig. 4F). Dissection and ex vivo imaging showed that this weak signal came primarily from the intestines, and leveled off following puberty. Thus, these very sensitive androgen-responsive promoters can drive a spatiotemporal program of expression during development that may differ from adults, likely reflecting changes in both AR levels and coactivators.

**Imaging prostate cancer progression and metastasis with EZC-prostate models.** One of the primary goals of generating organ-specific reporter mice is the ability to noninvasively image changes in the gland during normal development or pathologic states, such as neoplasia. To test whether the EZC-prostate model could be used to measure prostate hyperplasia and cancer progression, EZC1-, EZC2-, and EZC3-prostate mice were bred with two distinct animal models, the recently described inducible prostate intraepithelial neoplasia model, JOCK1 (22), and the well-characterized TRAMP model (15, 20). In the JOCK-1 model, administration of a lipid-permeable dimerizing drug leads to cross-linking and activation of a prostate-targeted isoform of FGFR-1 that carries cytoplasmic tyrosine kinase domains linked to tandem dimerizer drug-binding domains but lacks any extracellular ligand-binding capacity. Signaling ensues within minutes of dimerizer delivery and proliferation is detectable within 24 hours. Hyperplasia is evident within 2 weeks of biweekly dimerizer administration; by 6 to 8 weeks, the ducts are filled with dysplastic cells, and by 24 weeks high-grade prostate intraepithelial neoplasia is widespread. Further, within 40 weeks of treatment, adenocarcinoma is reproducibly seen.3 Despite a large increase in cellular content, we did not detect a concomitant increase in reporter activity in biogenic mice treated with dimerizer drug for up to 40 weeks (Fig. 5A and B). Histologic analysis showed a reduction

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in luciferase expression per cell following FGFR1-mediated hyperplasia and dysplasia (Supplementary Fig. S2). Ex vivo chemiluminescent imaging of 60-week-old EZC2-JOCK prostates (from above) also shows that AR activity is diminished per cell during FGFR1-stimulated progression, which increases total prostate volume, leading to an overall ~5-fold decrease in reporter activity ex vivo (Fig. 5C).

In the TRAMP model, histologic well-differentiated adenocarcinoma can be detected by 12 weeks of age that progresses to poorly differentiated adenocarcinoma with metastasis over the next 12 to 18 weeks (20). Although skin and hair pigment from the C57BL/6 background lower reporter activity by at least 10-fold, chemiluminescence was readily detected in the lower abdomen of living mice in all three models (Figs. 6 and 7; Supplementary Fig. S3). Despite reproducible tumor progression, we were unable to detect significant concomitant increases in reporter activity in living mice (Fig. 6A, B and 7; Supplementary Fig. S3), consistent with a loss of promoter activity as a function of tumor progression. Higher reporter variability in the EZC1-TRAMP model (i.e., hK2-E3/P-luc × TRAMP) over time versus EZC2-TRAMP is apparently due to unpredictable tumor-mediated displacements of the faintly glowing intestines in the EZC1 model, which luminesce as a function of surface proximity (Fig. 6A and C). The decrease in reporter activity in the prostates of tumor-bearing mice may be due to a number of factors, including reduced blood flow and hypoxia in necrotic tissue, decreases in AR expression during dedifferentiation, and possible changes in coactivator function. Interestingly, reporter activity can still be seen in the less involved anterior prostate even when other prostate lobes become fused within a large tumor mass (Fig. 6C).

**Imaging metastasis with the EZC-prostate models.** In a minority of EZC1-TRAMP and EZC2-TRAMP mice (10-20%), distant site metastasis is visible at necropsy (Fig. 6D). Although reporter activity in the ventral prostate of normal EZC1-prostate mice is ~20-fold greater per milligram of tissue than in the intestines (Fig. 2), reporter activity in distant metastasis in EZC1-TRAMP

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**Figure 3.** Kinetics of luciferase expression in EZC-prostate mice. A, male EZC2-prostate mice were anesthetized and chemiluminescence was imaged 10 minutes after i.p. injection with D-luciferin (40 mg/kg) at the ages shown. Three representative mice and one nontransgenic mouse are shown. B, reporter activity at various ages for all three EZC-prostate lines. Points, mean photon counts (n = 3) summed over the entire chemiluminescent lower half of each mouse; bars, SD. *, P < 0.05, EZC3 versus EZC1; #, P < 0.05, EZC2 versus EZC1; ^, P < 0.05, EZC3 (8 versus 4 weeks). Kinetics of tissue-specific reporter expression in EZC2-prostate mice. Luciferase activity was quantitated in each tissue extract using a luminometer. Columns, mean luciferase activity (relative luciferase units per milligram of total protein); bars, SD.
mice is actually weaker than that of intestinal tissue. However, lowering the pseudocolor threshold reveals reporter activity in the easily visible hypochromic metastatic tumors, consistent with prostate epithelial origin, and this bioluminescence follows the outline of the gross metastasis (Fig. 6E). Imaging tissues ex vivo can also reveal metastasis within tissues, such as in the enlarged spleen of this animal despite the absence of metastatic nodules on the surface. Therefore, although imaging of prostate metastasis in living mice may be challenging when AR-regulated promoters are used to express luciferase activity, prostate metastasis in distant organs can be easily detected ex vivo.

As opposed to EZC1 and, to a lesser extent, EZC2 mice, the near absence of detectable extraprostatic reporter activity in the abdominal region of EZC3 mice led to the prediction that distant metastasis should be easily detectable in the EZC3-TRAMP model. Similar to EZC1- and EZC2-TRAMP, reporter activity from the lower abdomen of EZC3-TRAMP dropped with age in 40% (4 of 10) of animals imaged fortnightly until euthanasia [due to age (up to 32 weeks), distress, or prohibitive tumor size], consistent with prostate cancer progression (Fig. 7A and Supplementary Fig. S3A, C, E, and F). Moreover, in 50% of mice analyzed until euthanasia, we witnessed a large increase in disseminated reporter activity in living mice (Fig. 7A and Supplementary Fig. S3A, B, C, E, and F). Upon autopsy, we observed reporter activity in numerous tissues, including pancreas (10 of 10) para-aortic (prostate draining) and mesenteric lymph nodes (6 of 10), kidneys (3 of 10), adrenal glands (2 of 10), liver (2 of 10), spleen (2 of 10), and salivary glands (1 of 10). Histologic analysis confirmed that putative tumors imaged in living mice were present in suspect organs (Fig. 7C-F). Thus, the EZC3-prostate model can be used to identify prostate metastasis in living mice and to localize metastasis to specific organ sites ex vivo.

**In vivo imaging of AR activity.** The three EZC-prostate models were based on highly AR-responsive promoters, leading to the prediction that they should permit remote determination of AR activity in living mice. To test this hypothesis, we compared in vivo reporter activity between castrated and intact mice. Circulating DHT levels in intact mice varied widely (from 50 to 3,000 pg/mL),
but dropped below detection (<5 pg/mL) in all castrated mice (data not shown). Although there was some variability in signal intensity in intact mice, likely due to these large variations in circulating androgen levels in cohabitating male animals, the luminescence of castrate mice were quite reproducible and dropped rapidly (3-4 days) to ~10% of total intact levels in living EZC2-prostate mice (Fig. 8A and C). Ectopic delivery of testosterone (10 mg/pellet/21 days) led to recovery of intact reporter levels over a 3-week period. The lengthened time required for reporter rebound versus suppression was likely due to the additional time needed for restoring prostate cellularity. Addition of testosterone pellets to intact mice led to only a slight increase in average reporter levels (Fig. 8A), likely reflecting intact homeostatic regulation of circulating testosterone levels in intact mice. Thus, EZC-prostate mice permit high-throughput appraisal of AR activity in mouse prostates, which could be used to facilitate the characterization and development of drugs that target the AR axis.

As a proof-of-principle of this novel assay for AR activity, we treated EZC2-prostate mice with a 20-day depot (50 mg/kg total) of the gonadotropin-releasing hormone antagonist, PPI-258. Similar to castration, PPI-258 administration led to a relatively rapid drop in total (lower abdomen) reporter activity to ~10% of untreated mice (Fig. 8B and C). As above, addition of testosterone during GnRH blockade led to reporter rebound, consistent with the mechanism for GnRH antagonist function, which targets luteinizing hormone production in the pituitary gland. Thus, the EZC2-prostate model should provide a convenient model for testing some classes of new drugs targeting the AR axis.

Because residual androgen-independent signaling in some tissues could, in principle, limit the total drop in reporter activity, we analyzed tissues \textit{ex vivo} in intact and castrated mice (Fig. 8D). Surprisingly, the drop in reporter activity from prostate tissue following castration was >1,000-fold, much larger than the measured change in intact mice when chemiluminescence over the entire lower abdomen was integrated. As described above, weak reporter activity was also detected in intestines, which was not subject to the effects of castration. Thus, narrowing the region-of-interest for measuring reporter activity during analysis to just over the prostate should further improve the sensitivity of the EZC2-prostate model for remote detection of AR activity.

In EZC3-prostate mice, castrate levels of total lower abdomen reporter activity in living mice were ~3% of intact levels (Fig. 9A and B), suggesting that this model may provide even greater AR sensitivity than EZ2 mice. Interestingly, the drop in reporter activity from prostate tissue following castration was not as great as in EZC2-prostate mice (~65-fold versus ~2,500-fold), but the near
absence of intestinal reporter activity apparently more than compensates for residual prostate-localized signal (Fig. 9C). Peripheral signals in feet and jaw are well removed from prostate-derived signals. Thus, the EZC3-prostate model should be ideal for testing the efficacy of some classes of AR-targeted drugs.

Discussion

A number of traditional and novel imaging modalities have now been applied to small-animal studies (reviewed in ref. 28). Many of these applications are driven by technology improvements with miniaturization of probes and detectors appropriate for small animals. This sequence contrasts bioluminescent imaging, as its applications in animal research have preceded clinical applications in which the significant absorption and scattering of visible light by most vascularized tissues has limited applications to superficial lesions. Despite possible limited translational applications, BLI has proven quite practical for tumor, signaling, and other applications in small animals, particularly mice.

Other methodologies are also unlikely to have the throughput of optical imaging, as in micro-PET, micro–computed tomography, micro–single-photon emission computed tomography, micro–magnetic resonance imaging, and ultrasound, animals are scanned and imaged individually by skilled personnel and large computer files are required to reconstruct and quantify images in three dimensions; however, continuous improvements in computer power will obviate this concern. Similarly, however, it is likely that reengineering of optical imaging stations to accommodate multi-angle imaging can greatly improve linearity and dimensionality, coincident with engineering of luciferase and fluorescent reporters with favorable red-shifted emission profiles that better penetrate mammalian tissue. Therefore, despite the allure of optical imaging, the ultimate choice of imaging technologies is application and budget driven.

Although inevitable improvements in imaging will further expand the use of the EZC-prostate models described here, there are already several current applications of these prostate reporter mice. First, we were able to successfully compare three distinct (12–14), robust prostate-specific promoters in an unbiased in vivo setting. Due to the strength of all three AR-responsive composite promoters used in this study, we were able to easily image mice in 10 to 30 seconds. Weaker tissue-specific promoters can require additional manipulations, such as two-step transcriptional amplification (29), to amplify reporter activity to convenient levels for imaging; however, prostate-directed luciferase activity based on two-step transcriptional amplification technology seems to have much lower tissue specificity than the EZC-prostate models (30). In the composite promoters used for the EZC models, multimerization of AREs was largely responsible for magnifying the transcriptional rates while maintaining extremely high (>99%) tissue specificity; however, each line displayed unique characteristics. This comparison indicated that the composite PSA-E2/P and ARR2PB promoters were probably somewhat (~2-fold) more potent and prostate specific than hK2-E3/P, whereas hK2-E3/P
seemed to be less susceptible to position-effect variegation (i.e., qualitatively similar expression patterns in six of six founders). All promoters were highly AR dependent with dramatic drops in prostate reporter expression up to 2 or 3 orders of magnitude in the EZC3-prostate (PSA) and EZC2-prostate (ARR2PB) models, respectively. At the same time, low-level AR-independent expression in the intestines of EZC1-prostate (hK2) and EZC2-prostate mice make the EZC3-prostate model likely better for appraising AR activity. Although qualitatively similar data is likely to occur in gene therapy vectors (12, 27), adjacent promoter elements, such as those found in viral vectors, can modulate tissue specificity (31).

We were also able to detect relatively small metastasis in TRAMP mice when bred onto the EZC3-prostate background, facilitated by the extremely low background expression outside of the urogenital tract. Low-level signals from the intestines of EZC1- and EZC2-prostate mice make detection of distant metastasis more challenging in these models as stochastic movements of the gastrointestinal tract to surface proximal and distal positions can reduce signal-to-noise ratio. Although all three promoters may be useful for prostate epithelial-specific expression of most transgenes, the extreme sensitivity of BLI can cause confounding signals from even minute levels of luciferase that are orders of magnitude lower than in the prostate, especially if the nontargeted expression emanates from surface-proximal or relatively large tissues. Thus, the unusually high prostate specificity of the EZC3-prostate model makes it ideal for identifying early metastasis in genetically engineered mice predisposed to prostate cancer.

Background bioluminescence is much less of a factor when applying BLI to the tracking of adoptively transferred luciferase-transfected cells. In numerous reports, tumor cell growth and metastasis were accurately measured and identified with BLI, although in vivo imaging was less sensitive than ex vivo imaging as expected (32). Further, response of tumor cells to traditional therapies or immunotherapy has been accurately monitored in vivo as well as the tracking of leukocytes to neoplastic tissue (33). Despite the extra challenges of transgenic models, a number of reports indicate their broad use (reviewed in ref. 8).

Due to both the exquisite AR sensitivity of both the three composite promoters used in this study and the involution of the prostate that occurs following medical or chemical castration, the EZC-prostate models are also useful for monitoring AR activity in the prostate in living mice. As proof-of-principle, we were able to show a drop in reporter activity up to 2 orders of magnitude following castration in living EZC2- and EZC3-prostate mice. Similar results were achieved when GnRH antagonist, PPI-258, was added to EZC2-prostate mice. This model should permit comparisons of not only distinct reagents but also of dosing schedules and routes of injection. Of course, there are always concerns about the applicability of animal studies to humans, but due to high conservation of the AR axis, these mice should be appropriate for preclinical evaluation of other classes of AR-blocking compounds. The high magnitude of the inhibition following castration was somewhat surprising as adrenal-derived androgens comprise up to 5% of circulating testosterone and have been shown to activate the...
AR (reviewed in ref. 25). Nevertheless, the combination of prostate involution and promoter inhibition following castration or GnRH antagonist administration almost completely inhibited prostate-derived luciferase in all three models. It will be interesting to evaluate other classes of drugs, such as 5α-reductase inhibitors, nonsteroidal antiandrogens, and selective AR modulators using these mice. Similarly, these models should be useful for elucidating the potential roles of numerous transcriptional coactivators associated with AR signaling in normal or prostate cancer tissue (34, 35).

The recent development of genetically engineered mouse modeling prostate cancer has stimulated our understanding of disease progression and has been a platform for treatment development. Further developments in imaging technologies will synergize with that work. These new EZC-prostate models should not only expand the use of transgenic prostate cancer mouse models, but will also be useful for drug and treatment development and a better understanding of the role(s) of the AR axis during prostate development and disease progression.

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Figure 9. Effects of castration on EZC3-prostate mice. A and B, in vivo imaging of luciferase activity in representative intact (A) or castrate (B, 4 weeks following castration) EZC3-prostate mice. C, ex vivo imaging of liver, intestines (cecum and colon only), prostates, and spleen of intact and castrate (4 weeks) mice. The average ± SD of photon flux (ps/cm²/sr) is shown to the right. Note that prostates from intact mice were measured separately to avoid reflected light. *, P < 0.05.

References

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