AEG-1 promoter-mediated imaging of prostate cancer

Akrita Bhatnagar,1 Yuchuan Wang,1 Ronnie C. Mease,1 Matthew Gabrielson,1 Polina Sysa,1 Il Minn,1 Gilbert Green,1 Brian Simmons,2 Kathleen Gabrielson,2 Siddik Sarkar,3 Paul B. Fisher,3,4,5 Martin G. Pomper1

1Russell H. Morgan Department of Radiology and Radiological Science; 2Department of Molecular and Comparative Pathobiology, Johns Hopkins Medical Institutions, Baltimore, MD 21287; 3Department of Human and Molecular Genetics, 4VCU Institute of Molecular Medicine, 5VCU Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, 23298

Running Title: AEG-Prom for imaging prostate cancer

Key Words: molecular-genetic imaging, bioluminescence, SPECT, metastasis, nanoparticle, PC3

Financial Support: Prostate Cancer Foundation (MGP, PBF, George Sgouros), CA151838 (MGP), Patrick C. Walsh Foundation (MGP) and the National Foundation for Cancer Research (PBF)

Corresponding Author: Martin G. Pomper, M.D., Ph.D.
Johns Hopkins Medical School
1550 Orleans St., 492 CRB II
Baltimore, MD 21287
Ph: 410-955-2789
Fax: 410-817-0990
Email: mpomper@jhmi.edu

Conflict of Interest: none

Word Count: 143 (abstract), 4,999 (text), 980 (figure legends); Figures: 5; Tables: 0
ABSTRACT

We describe a new imaging method for detecting prostate cancer, whether localized or disseminated and metastatic to soft tissues and bone. The method relies on the use of imaging reporter genes under the control of the promoter of AEG-1 (MTDH), which is selectively active only in malignant cells. Through systemic, nanoparticle-based delivery of the imaging construct, lesions can be identified through bioluminescence imaging and single photon emission-computed tomography in the PC3-ML murine model of prostate cancer at high sensitivity. This approach is applicable for the detection of prostate cancer metastases, including bone lesions for which there is no current reliable agent for non-invasive clinical imaging. Further, the approach compares favorably to accepted and emerging clinical standards, including positron emission tomography with $[^{18}\text{F}]$fluorodeoxyglucose and $[^{18}\text{F}]$sodium fluoride. Our results offer a preclinical proof of concept that rationalizes clinical evaluation in patients with advanced prostate cancer.
INTRODUCTION

A transcription-based imaging, therapeutic or theranostic system can be considered for clinical translation if it meets certain criteria such as high tumor specificity, broad application and minimal toxicity (1, 2). The first two criteria can be met through the choice of a strong and tumor-specific promoter. For example, cancer-specific gene therapy with the osteocalcin promoter, delivered through intra-lesional administration of an adeno-viral vector, caused apoptosis in a subset of patients with prostate cancer (PCa) (2, 3). We have shown that cancer-specific imaging could be accomplished in experimental models of human melanoma and breast cancer by systemic delivery of imaging reporters under the transcriptional control of the progression elevated gene-3 promoter (PEG-Prom) (1, 4). Here we describe a nanoparticle-based, molecular-genetic imaging system employing the astrocyte elevated gene-1 promoter (AEG-Prom) (5) for detecting metastases due to PCa, including to bone, for which there is no reliable clinical imaging agent.

AEG-1 was first identified using subtraction hybridization as an up-regulated gene in primary human fetal astrocytes infected with HIV-1 (6, 7). Subsequent studies identified AEG-1 as a metastasis-associated gene in the mouse, called metadherin (MTDH) (8), and as a lysine-rich CEACAM1 co-isolated gene in the rat, called LYRIC (9). Recent studies in multiple cancers confirm a significant role for AEG-1 as an oncogene (10) implicated in cancer development and progression in many organ sites (11). Based on the diverse roles of AEG-1 in tumor progression, including transformation, growth regulation, cell survival, prevention of apoptosis, cell
migration and invasion, metastasis, angiogenesis, and resistance to chemotherapy (12), this
gene may provide a viable target for developing therapies for diverse cancers. Expression of
*AEG-1* involves transcriptional regulation through defined sites in its promoter (5). A minimal
promoter region of *AEG-1* was identified by its association with oncogenic Ha-ras-induced
transformation (5). *AEG-1* is a downstream target of the *Ha-ras* and *c-myc* oncogenes,
accounting in part for its tumor-specific expression. We have previously shown that AEG-Prom
is activated by the binding of the transcription factors c-Myc and its partner Max to the two E-
box elements of the promoter in Ha-ras-transformed rodent and immortalized transformed
astrocyte cell lines (5). AEG-1 interacts with PLZF, the transcriptional repressor that regulates
the expression of the genes involved in cell growth and apoptosis (13).

Although molecular-genetic imaging with AEG-Prom should be generally applicable to a variety
of malignancies, our initial study performed here was in part to demonstrate the utility of this
system in a relevant and challenging application, namely, for molecular imaging of PCa. We
also focus on PCa because positron emission tomography (PET) with [*18*F]fluorodeoxyglucose
(FDG), which is the current clinical standard for a wide variety of malignancies, does not work
particularly well for this disease (14). Although a variety of new molecular imaging agents for
PET with computed tomography (PET/CT) of PCa are emerging, such as [*18*F]NaF (NaF) (15, 16),
[*11*C]- and [*18*F]choline (17-19), [*18*F]FDHT (20), anti-[*18*F]FACBC (21) and [*18*F]DCFBC (22), some
are limited to detecting bone lesions (NaF), have significant overlap with normal prostate tissue
(the cholines), or have not yet been extensively tested in the clinic. To maintain relevance to
clinical translation, we used a linear polyethyleneimine (l-PEI) nanoparticle to deliver the
construct systemically. Nanoparticles comprised of l-PEI are being used in a variety of ongoing clinical trials (23-25). We describe AEG-Prom-mediated imaging in tumors derived from PC3-ML cells, a human androgen-independent invasive and metastatic model of PCa (26-28). We show that imaging with AEG-Prom delineates lesions from PCa as well as or with higher sensitivity than FDG- or NaF-PET/CT in this model system.

MATERIALS AND METHODS

Cloning of plasmid constructs. pPEG-Luc and pAEG-Luc were generated as described previously (4, 29). The firefly luciferase-encoding gene in pAEG-Luc was replaced by the HSV1-tk-encoding sequence from pORF-HSVtk plasmid (InvivoGen, San Diego, CA) to generate pAEG-HSV1tk. Details of cloning by restriction enzyme digestion and other conditions are available upon request. The plasmid DNA was purified with the EndoFree Plasmid Kit (Qiagen, Valencia, CA). Endotoxin level was ensured as < 2.5 endotoxin units per mg of plasmid DNA.

Cell lines. PC3-ML-Luc (stable transfectants) and PC3-ML were provided by Dr. Mauricio Reginato (Drexel University, Philadelphia, PA). These were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro, Manassas, VA) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antimycotic solution (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C, 5% CO2. PrEC (normal prostate epithelium) cells were provided by Dr. John T. Isaacs (Johns Hopkins School of Medicine, Baltimore, MD). Those were grown in keratinocyte serum-free medium (total [Ca2+] is 75 ± 2 μmol/L) supplemented with bovine pituitary extract and recombinant epidermal growth factor (Invitrogen Life Technologies, Grand Island, NY).
Transient transfection and luciferase assay. The following PCa cell lines: PC3, PC3-ML, LNCaP, DU145, ARCaP-E, ARCaP-M, RWPE-1 (primary cells immortalized with HPV-18) and PrEC (primary cells) were plated in 6-well plates (BD Biosciences, Bedford, MA, USA) at $180 \times 10^3 - 200 \times 10^3$ cells. Cells were transfected using in-vitro jetPRIME® (Polyplus-transfection, Illkirch, France) according to the manufacturer’s instructions. The indicated cells were transfected with Luc under the experimental promoters AEG-Prom, PEG-Prom, and a promoter-less empty vector (control) as a pDNA-PEI polyplex. Luminescence was normalized for transfection efficiency by co-transfection with a vector expressing renilla luciferase, pGL4.74[hRluc/TK] (Promega, Madison, WI). After 48 h of transfection, the expression level of the Luc reporter was measured by the Dual Luciferase Reporter Assay kit (Promega). Luminescence was normalized for cell number (by µg total protein) using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

Construction of mutant AEG-Prom. The mEbox1 and mEbox2 sites were mutated in the wild-type pAEG-Luc plasmid to generate the pAEG-mEbox1&2-Luc plasmid. The consensus E-box sequences, CACGTG, for mEbox1 and mEbox2, were mutated into AGAGTG and AGATTG, respectively, using the QuikChange Lightening Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The sequences of the forward (F) and reverse (R) primers used for mutagenesis were F: 5’ CCCGCCCCGCCCCAGAGTGACGCCCA and R: 5’ GGACGACCGTGGGTCAATCTGGCGCC. The mutated E-box sequences and the luciferase sequence were confirmed by sequencing (Macrogen USA, Cambridge, MA). PC3-ML cells were
transiently transfected with the wild-type and mutated plasmid for the subsequent luciferase assay, as described above.

**Western blot analysis.** The plated cells were harvested and lysed using cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA) supplemented with 1 mM PMSF (Sigma-Aldrich) with Protease cocktail inhibitor, Phosphatase inhibitor, (Roche, Indianapolis, IN). The whole cellular proteins were separated using 10% SDS-PAGE. For western blotting the primary antibodies used were rabbit monoclonal anti-c-Myc (1: 1000, Cell Signaling Technology, Inc.) and mouse monoclonal anti-β-actin (1: 2000, Sigma-Aldrich). The secondary antibodies used were HRP-conjugated polyclonal goat anti-mouse IgG (1:1000; Dako, Carpinteria, CA) and polyclonal swine anti-rabbit IgG (1:3000; Dako).

**Generation of an in vivo experimental model of metastatic PCa.** Protocols involving the use of animals were approved by the Johns Hopkins Animal Care and Use Committee. Four-to-six-week old male NOG (NOD/Shi-scid/IL-2Rγnull) mice were purchased from the Sidney Kimmel Comprehensive Cancer Center’s Animal Resources Core (Johns Hopkins School of Medicine). PC3-ML and PC3-ML-Luc cells were expanded over three to five passages. Cells were harvested and diluted in sterile Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} (Invitrogen Life Technologies). For intravenous injection, mice were administered 1 x 10^6 PC3-ML cells in 100 μL of sterile Dulbecco's PBS via the tail vein. To ensure hematogenous dissemination, including to the bone, the cells were injected into the left ventricle of the heart (27, 28). For this intra-cardiac model mice were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and inoculated into
the left ventricle with $5 \times 10^4$ PC3-ML-Luc enriched or PC3-ML cells in a total volume of 100 $\mu$L of sterile Dulbecco's PBS using a 26$^{3/4}$ gauge needle. To image the PC3-ML-Luc cells with BLI, mice were injected intraperitoneally (IP) with 100 $\mu$L of 25 mg/mL of d-luciferin solution (Caliper LifeSciences, Hopkinton, MA), and BLI was performed 20 min after the intra-cardiac injection to detect the distribution of cells. Mice were imaged weekly. Images were acquired on an IVIS Spectrum small animal imaging system (Caliper Life Sciences, Alameda, CA) and results were analyzed using Living Image software (Caliper Life Sciences). A group of age-matched healthy NOG mice served as a negative control for the PCa metastasis model.

**Enrichment of PC3-ML-Luc cells.** The PC3-ML-Luc cells were further selected for bone-homing tendency. Mice bearing PC3-ML-Luc tumors developed through the intra-cardiac injection method were monitored for tumor formation by BLI. After five weeks, following euthanasia the femur and tibia of the regions demonstrating clear signal were aseptically dissected. The tumor cells were established in culture by mincing the epiphysis and flushing the bone marrow with 1X PBS (Invitrogen Life Technologies) as described previously (26). The subpopulations of cells selected using a Transwell migration chamber with an 8 $\mu$m pore size (BD Falcon, San Jose, California) were tested and confirmed for Luc expression as described previously (30), but using 1 mM of d-luciferin, potassium salt (Gold Biotechnology, St. Louis, MO). The radionuclide imaging experiments were performed with the enriched PC3-ML-Luc cell lines.

**Systemic delivery of plasmid constructs.** Low molecular weight l-PEI-based cationic polymer, in vivo-jetPEI® (Polyplus Transfection), was used for gene delivery. The DNA-PEI polyplex was
formed according to the manufacturer’s instructions. For systemic delivery 40 µg of DNA and 4.8 µL of 150 mM in vivo-jetPEI® was diluted in endotoxin-free 5% (wt/vol) glucose separately. The glucose solutions of DNA and I-PEI polymer were then mixed together to give an N:P ratio (the number of nitrogen residues of vivo-jetPEI® per number of phosphate groups of DNA) of 6:1 in a total volume of 400 µL. The DNA-PEI polyplex was injected IV as two 200 µL injections with a 5 min interval.

**Bioluminescence imaging.** *In vivo* BLI was conducted at 24 and 48 h after the systemic delivery of reporter genes. Mice were imaged with the IVIS Spectrum. For each imaging session mice were injected IP with 150 mg/kg of d-luciferin, potassium salt under anesthesia using a 2.0% isoflurane/oxygen mixture. *Ex vivo* BLI was conducted within 10 min of necropsy. Living Image 2.5 and Living Image 3.1 software were used for image acquisition and analysis.

**SPECT-CT imaging and data analysis.** At 48 h after injection of pAEG-HSV1tk/PEI polyplex, animals were injected IV with 37.0 MBq (1.0 mCi) of \[^{125}\text{I}\]FIAU. At 18-20 h after radiotracer injection, imaging data were acquired with the X-SPECT small-animal SPECT-CT system (Gamma Medica Ideas, Northridge, CA) using the low-energy single pinhole collimator (1.0 mm aperture). Focused lung and liver imaging were acquired with a radius of rotation of 3.35 cm and whole-body imaging was undertaken with a radius of rotation of 7.00 cm. Mice were imaged in 64 projections at 45 sec of acquisition per projection. SPECT images were co-registered with the corresponding 512-slice CT images. Tomographic image datasets were reconstructed with the 2D ordered subsets-expectation maximum (OS-EM) algorithm. AMIDE (31) and PMOD (v3.3,
PMOD Technologies Ltd, Zurich, Switzerland) software were used for image quantification and analysis.

**FDG- and NaF-PET/CT imaging and analysis.** 9.25 MBq (0.25 mCi) of each imaging agent was injected via the tail vein. Animals were placed on a heating pad and were allowed mobility during the 1 h radiotracer uptake period. The animals were then subjected to isoflurane anesthesia. Whole-body images were acquired with the eXplore Vista small animal PET scanner (GE Healthcare, Milwaukee, WI) using the 250-700 keV energy window. Acquisition time was 30 min (two bed positions, 15 min per bed position). Mice were fasted for 6 - 12 h before receiving FDG to minimize radiotracer accumulation in non-tumor tissues. FDG and NaF imaging was done between four and five weeks after injection of PC3-ML-Luc cells. PET images were co-registered with the corresponding 512-slice CT images. Tomographic image datasets were reconstructed with the 3D OS-EM algorithm with three iterations and 12 subsets and were analyzed with AMIDE software (31).

**Histological analysis.** After BLI data acquisition at 48 h after pAEG-Luc-PEI delivery, each organ demonstrating expression of Luc was collected and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin blocks. Serial paraffin longitudinal sections were stained with goat anti-luciferase polyclonal antibody (Promega) or rabbit anti-Myc polyclonal antibody (Epitomics, Burlingame, CA). Horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-goat antibody was used as a secondary antibody. HRP activity was detected with 3, 3′-diaminobenzidine (DAB) substrate chromogen (EnVision™+ Kit, Dako, Carpinteria, CA).
Consecutive sections of each tissue sample were stained with hematoxylin and eosin (H & E) and were photographed with a Zeiss photomicroscope III.

**Quantitative real-time PCR.** After imaging experiments, animals were euthanized and their lung and liver tissue were harvested and snap frozen. Total DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer’s instructions. 100 ng of purified total DNA from each animal was used as a template. Quantitative real-time PCR was performed in triplicate per template using the inventoried Taqman® Gene Expression Assays (Cat. #4331182, Life Technologies, Grand Island, NY) with the FAM dye labeled primer set for Luc. Reaction conditions were set as 50°C for 2 min, 95°C for 10 min and 50 cycles of 95°C for 15 sec, 60°C for 1 min followed by the disassociation step of 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec in a Bio-Rad iQ™5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA). Data were analyzed by the absolute quantification method using a standard curve by iQ5 v2.0 software (Bio-Rad). Quantified data was normalized relative to the amplification of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) DNA.

**Radiographic and gross visualization of bone lesions.** A Faxitron MX20 Specimen X-ray system (Faxitron Corp., Tuscon, AZ) with digital exposures of 25 kV, 17 sec was used. Films were obtained on Kodak Portal Pack Oncology X-ray film (25.4 x 30.5 cm) for 22 kV, 15 sec. For gross pathology, bone tissues were fixed in 10% neutral buffered formalin and were decalcified for 2 h in Decal® (Decal Chemical Corp., Suffern, NY) and cut in thin slices.
**Statistical Analysis.** For BLI error bars in graphical data represent mean ± standard deviation (SD). *P*-values < 0.05 were considered to be statistically significant.

**RESULTS**

**Comparison of cancer specificity of AEG-Prom and PEG-Prom by bioluminescence imaging (BLI) in PCa.** To examine the cancer-specific activity of AEG-Prom we constructed two plasmids, pAEG-Luc, expressing firefly luciferase, and pAEG-HSV1*tk*, expressing the herpes simplex virus type I thymidine kinase ([Supplemental Fig. S1](#)). AEG-Prom drives the expression of the imaging reporter genes firefly luciferase (Luc) and HSV1-*tk*, which enable BLI and radionuclide based-techniques, respectively. Given the high sensitivity and ease of BLI, our initial studies used this modality for proof of concept. The HSV1-*tk* reporter gene was used, as before (1), to provide a method that has a clear path to clinical translation. The PEG-Prom construct, namely, pPEG-Luc, was generated previously (1), and was used as a comparison for some of the current studies.

Using BLI we tested the cancer specificity of AEG-Prom and PEG-Prom in different PCa cell lines, including HPV-18 transformed normal immortal prostate epithelial cells (RWPE-1), PC3, PC3-ML, LNCaP, DU-145, AR-CaP-E (metastatic resistant epithelial clone), AR-CaP-M (metastatic prone mesenchymal clone), and in the non-malignant counterpart cells of prostate epithelium. Robust expression from AEG-Prom and PEG-Prom was observed only in the malignant cell lines, whereas promoter activity was negligible in the normal prostate epithelial cells (PrEC) ([Fig. 1A](#)). To elucidate further the role of c-Myc in the activation of AEG-Prom, we engineered an AEG-
Prom containing mutations in the two E-box elements, to which the c-Myc transcription factor was hypothesized to bind in PCa cells, to produce pAEG-mEbox1&2-Luc, similar to the one reported in Lee et al. (5) (Fig. 1B). The mutant pAEG construct, pAEG-mEbox1&2-Luc, consists of AGAGTG and AGATTG in lieu of the consensus CACGTG in the Ebox1 and 2 regions of the promoter, respectively. Those constructs were transiently transfected into PC3-ML cells, and the promoter activities were compared with those of the wild-type AEG-Prom construct, pAEG-Luc. As shown in Fig. 1B, the pAEG-mEbox1&2-Luc is still active in the PC3-ML cell line, although there was a three-fold reduction in the extent of activation of the AEG-Prom construct. Furthermore, we note that the AEG-Prom activity increases by six-fold as it goes from a no-\textit{c-myc} state in the AR-CaP-E cells to a substantial \textit{c-myc} state in the AR-CaP-M clone (Fig. 1A) (Supplemental Fig. S2 shows c-Myc levels). These results indicate that AEG-Prom activity is regulated primarily, but not exclusively, by the c-Myc transcription factor in these cancer cells.

We then tested and compared the specificity of AEG-Prom and PEG-Prom \textit{in vivo} in a relevant experimental model of PCa. To develop this model we used two human PCa sub-lines selected from initial metastases of the parental human PC3 cells that targeted the murine lumbar vertebrae, hence ML (metastasis lumbar). We used PC3-ML cells and the luciferase-tagged version of the PC3-ML cells, namely, PC3-ML-Luc (26-28), which were injected either intravenously (IV) or directly into the left ventricle of the heart to ensure widespread dissemination – including to bone. BLI confirmed the presence of widespread metastases to liver, kidney, lung and bone after IV injection of PC3-ML-Luc cells (Supplemental Fig. S3). We
assumed a similar time course for the development of metastases from the PC3-ML cells that did not express Luc so that we could use them in conjunction with the AEG-Prom-driven system to identify metastatic lesions by BLI. Mice received an IV dose of pAEG-Luc-PEI and pPEG-Luc-PEI polyplexes (Fig. 2 and Supplemental Fig. S4). Twenty-four and 48 h after plasmid DNA delivery, BLI revealed AEG-Prom- and PEG-Prom-driven gene expression above background only in the model demonstrating metastasis (Fig. 2B, D) and not in the healthy control group (Fig. 2A, C).

Histological analysis of the photon-emitting regions within lung for the animals treated with pAEG-Luc or pPEG-Luc showed the presence of tumor and the correlative Luc expression in the cancer models, but not in the controls (Fig. 2E, F, I, J). In the lungs Luc expression was detected by immunohistochemistry (IHC) from uniformly scattered tumor cells with some forming large, nodular aggregates. Lesions infiltrate capillaries, interstitium, septae and larger blood vessels (Fig. 2F, J). The kidneys also demonstrated multiple metastases. Tumor replaced all normal tissue except individual glomeruli (indicated by “G” in Fig. 2G, K). Tumor cells in the liver formed multifocal nodules that in some cases demonstrated adjacent necrosis. Necrotic centers (indicated by “N” in Fig. 2H, L) correlated with a lack of Luc expression. We have also shown that expression of c-myc correlates with AEG-Prom-driven Luc expression within tumor (Fig. 2E, F, G). Similar expression of the c-myc or the Luc genes was not evident in the healthy, control mice.

BLI signal intensity was significantly higher in the PCa group compared to controls within lung at
both the 24 and 48 h time points (after administration of pAEG-Luc and pPEG-Luc) ($P < 0.0001$; **Fig. 2M**). Moreover, at the 48 h time point we observed an approximately two-fold higher $\text{Luc}$ gene expression from the AEG-Prom group as compared to the PEG-Prom group (**Fig. 2M**). To compare the transfection efficiency between the lungs in pAEG-Luc and pPEG-Luc treated PCa-1-3 (**Fig. 2M** and **Supplemental Fig. S5**), we quantified the amount of plasmid DNA delivered to each of the lung tissues. We performed quantitative real-time PCR with a primer set designed to amplify a region of the Luc-encoding gene in the pAEG-Luc and pPEG-Luc plasmids (**Supplemental Fig. S5**). We used total DNA extracted from the lungs as a template. The difference in transfection efficiency in the PCa lungs between the pAEG-Luc and the pPEG-Luc group was not significant. That confirms that Luc expression from the pAEG-luc treated PCa models was due to the higher tumor-specific activity of AEG-Prom rather than higher transfection efficiency to malignant tissues. A possible reason for elevated expression using AEG-Prom *in vivo* includes that a human gene may demonstrate more productive interaction with the human proteins of the Ras-signaling pathway such as c-Myc (PEG-Prom is of rat origin and expression is not dependent on these signaling pathways) (4). Alternatively, AEG-Prom expression might be elevated further *in vivo* as a consequence of epithelial to mesenchymal transition (EMT). Further experimentation will be required to determine if that hypothesis is correct.

To enable reliable formation of metastasis to bone, a tissue prominently involved in human metastatic PCa, we injected PC3-ML-Luc and PC3-ML cells through an intra-cardiac route (**Supplemental Fig. S3A**). Once timing for the development of metastases was determined for
the luciferase-expressing cells, we then studied metastases due to PC3-ML cells using the pAEG-Luc-PEI polypex. At 48 h after plasmid delivery we observed AEG-Prom-mediated expression of Luc from the PC3-ML models, as shown for PCa-4 and PCa-2 and not from controls (Figs. 3 and 4, respectively). For PCa-4, when imaged seven weeks after cell injection, BLI was able to detect cancer cells in the left tibia (Fig. 3B), as confirmed by histological analysis (Fig. 3C). The BLI signal intensity, from deep within the bone, was weak in vivo, likely due to attenuation by living tissues (32).

Ex vivo BLI of PCa-2, when imaged five weeks after cell injection, showed the presence of tumor in the lungs, liver, adrenals and kidneys, as also confirmed by gross pathology, histological analysis and Luc IHC (Fig. 4C, D and Supplemental Fig. S6). To study the transfection efficiencies of systemically delivered construct within lung and liver (Fig. 4E and Supplemental Figs. S6 and S7), we quantified the amount of plasmid DNA delivered to each of these tissues. We performed quantitative real-time PCR with a primer set designed to amplify a region of the Luc-encoding gene in the pAEG-Luc plasmid. We used total DNA extracted from the lung and liver as a template. The differences in transfection efficiency between areas of high tumor burden vs. those of low tumor burden within liver in same animal, as well as between areas of high tumor burden within liver vs. normal liver, were significant at $P < 0.0005$ and $P = 0.0078$, respectively. Lower transfection efficiency in diseased vs. normal liver was likely due to lower delivery of plasmid to the diseased tissue, as demonstrated previously for PEG-Prom and lung replete with metastases (1). PCR was also performed in tissues from control animals after pAEG-Luc delivery. Differences in transfection efficiency within lungs and liver between the
control group and the PCa group were not significant. That confirms that higher visualized Luc expression from the PCa models is due to the tumor-specific activity of AEG-Prom rather than higher transfection efficiency to malignant tissues.

**Radionuclide imaging of cancer via AEG-Prom.** BLI is limited to pre-clinical studies due to the dependence of signal on tissue depth, the need for administration of exogenous D-luciferin substrate at relatively high concentration for light emission, rapid consumption of D-luciferin leading to unstable signal, and low anatomic resolution (1). Accordingly we cloned pAEG-HSV1tk ([Supplemental Fig. S1B](#)), which can be detected by the radionuclide-based techniques of PET or single photon emission computed tomography (SPECT), upon administration of a suitably radiolabeled nucleoside analog (33). We examined the SPECT-CT imaging capabilities of pAEG-HSV1tk for detection of bone and soft tissue metastases in the PC3-ML model. Approximately five weeks after receiving an intra-cardiac administration of PC3-ML-Luc cells, the PCa group and the corresponding controls received pAEG-HSV1tk–PEI polyplex. Forty-eight hours after plasmid delivery, mice received the known HSV1-TK substrate, 2'-fluoro-2'-deoxy-β-d-5-[^125]Iiodouracil-arabinofuranoside ([^125]FIAU) (29), and were imaged at 18-20 h after injection of radiotracer. Fig. 5 shows a representative example, PCa-3, for which we were able to detect the presence of multiple metastatic lesions with the pAEG-HSV1tk system. We then compared the sensitivity of the AEG-Prom imaging system to the current clinical PET-based methods for detecting soft tissue (FDG-PET) and bony (NaF-PET) metastatic lesions due to PCa. Fig. 5A, B show representative examples, PCa-3, and a healthy control, Ctrl-1, imaged with each method. Because NaF is a bone-seeking agent, there is substantial uptake within the normal
skeleton (34), which may obscure lesions within bone (**Fig. 5A**). Moreover, on NaF bone scan skeletal metastases are seen indirectly, depending on the reaction of bone to the lesion, while the AEG-Prom polyplex images tumor directly. The NaF-PET/CT study for PCa-3 appears similar to that for Ctrl-1. NaF-PET/CT failed to identify the metastases within the tibia and axial skeleton. The same mouse also underwent FDG-PET/CT, which was only able to identify a lesion in the left scapula (L1, **Fig. 5B**).

BLI performed *ex vivo* and gross pathology of lesions within the right humerus, dorsal thoracic wall, ribs, sternum and the heart confirmed that tumor was the source of signal seen on the living images (**Fig. 5C** – **E** and **Supplemental Fig. S8**, respectively). We were able to identify a 3 mm tumor nodule on the heart (L2, **Supplemental Fig. S8B**), a 5 mm lesion in the dorsal thoracic wall adjacent to the mid-spine (L3, **Fig. 5D**) and a 1 mm lesion in the ventral midline of the sternum (L4, **Fig. 5D**). Furthermore, the macroscopic picture confirmed metastases in the bone marrow within the proximal tibia (L5, **Fig. 5E**, red dotted circles). An *ex vivo* plain film image of the pelvis of PCa-3 did not delineate bone lesions clearly (**Supplemental Fig. S8C**), suggesting advanced changes in skeletal morphology might be needed for detection with conventional imaging modalities.

**DISCUSSION**

Our goal was to develop a systemically deliverable construct for molecular-genetic imaging of metastatic lesions within both soft tissue and bone in a relevant model of PCa. Others have developed *in vivo* molecular-genetic imaging agents for PCa using adenoviral mediated,
prostate-specific regulatory elements. Native androgen-dependent promoters/enhancers derived from prostate-specific antigen (PSA) (35), probasin (36), human glandular kallikrein2 (37) and the prostate-specific membrane antigen (PSMA) (38) have been used to drive transgene expression, but in a tissue-restricted, rather than a tumor-specific manner (39). Additionally, promoters such as PSES (PSA promoter/enhancer) have been improved by incorporating the TSTA system, a two-step transcriptional amplification mechanism using the Gal4-VP16 fusion protein to enhance the transcriptional activity of weak PSES (40).

In comparison to the above mentioned prostate-specific promoters, the tumor-specific promoters of PEG-3 and AEG-1 have certain features that might render them more specific and selective while at the same time instill them with greater utility, namely to use them in a variety of cancers beyond PCa. PEG-Prom and AEG-Prom: [1] maintain universal cancer specificity regardless of the tissue of origin; [2] do not require amplification to achieve high sensitivity; and, [3] are systemically delivered using a non-viral delivery vehicle. We note that the expression levels of both the AEG-Prom and PEG-Prom increase in the mesenchymal clone of the ARCaP cell line compared with the epithelial clone of the same cell line. (Fig. 1A), indicating a possible level of involvement in EMT.

To recapitulate the clinical characteristics of PCa metastasis, we implemented a bone metastatic model of PCa, which occurs spontaneously after the IV injection of PCa cells without orthotopic injection directly to bone. In animals showing tibial lesions using BLI of PC3-ML-Luc cells, subsequent SPECT/CT was able to detect these lesions in all animals tested (Fig. 5B).
perhaps the example closest to ours, Wu et al. previously utilized the PSES-TSTA bioluminescent vector to identify tibial bone marrow metastases that could not be detected by FDG- or NaF-PET/CT (38). In addition to using a tissue-specific promoter, that study also differed from ours in that an orthotopic tibial PCa model was used, an adenoviral vector served as the delivery vehicle and TSTA amplification was employed to boost the promoter activity by several orders of magnitude as compared to the parental PSES vector (40).

By using a biodegradable polymer, in vivo-jetPEI®, we tried to avoid certain problems that may arise when employing viral vectors, such as immune-mediated toxicity, inflammation and liver tropism (41). We checked the ability of the non-viral delivery vehicle to provide widespread, systemic dissemination of plasmid by conducting quantitative PCR on sections of lung and liver and compared the transfection efficiency between controls and animals affected with PCa (Fig. 4). Group differences in gene delivery between lung and liver were insignificant. This study also confirmed our earlier results that nanoparticle delivery is most efficient to well-vascularized tissues (1). Liver tissue sections with a high tumor burden had significantly lower ($P < 0.005$) delivery of plasmid DNA, possibly due to the reduced vasculature of this tissue, which was also likely under high hydrostatic pressure and was adjacent to necrotic areas.

Although imaging was mediated through activation of AEG-Prom, delivery was in part mediated through the enhanced permeability and retention (EPR) effect, interaction of positively charged DNA-PEI polyplex with the cell membrane followed by endocystosis, release from endosomes and entry into the nucleus (42).
The methods used in this report are intended to enable rapid clinical translation. Accordingly, for systemic delivery we used a non-viral delivery vehicle, which has seen clinical use (l-PEI) (ClinicalTrials.gov #NCT01435720), and have employed an imaging reporter gene/probe pair (HSV1-tk/FIAU) that has previously been used in patients (33). Nevertheless there are several hurdles that must be overcome, arguably the most significant of which is the delivery of the nanoparticles to the malignant tissue. Several excellent reviews on that topic have recently been published (43-45), with recognized obstacles including tumor heterogeneity, elevated interstitial fluid pressure, shifting properties of the microenvironment and the difficulty of translating optimized conditions for animal systems to the clinic (46, 47). Strategies for enhancing tumor delivery include surface functionalization by affinity agents, particularly agonists that promote internalization (48), and surface coating with polyethylene glycol to increase circulation times. Other aspects requiring optimization include the reporter gene/probe pair, assuring that the gene is non-immunogenic and that the probe has pharmacokinetics suitable for detection using widely available imaging modalities (49).

AEG-Prom enables a sensitive method for molecular-genetic imaging of PCa in vivo. From mutational analysis of AEG-Prom we have shown that its activation relies significantly on c-Myc binding to the two E-box elements as discussed above. As Ras-mediated c-Myc signal transduction is a pathway present in nearly all malignancies yet is absent in normal tissue (50), we anticipate that AEG-Prom will enable imaging of a wide variety of cancers directly and specifically.
ACKNOWLEDGEMENTS

We appreciate funding from the A. David Mazzone Research Awards Program and technical support from S. Nimmagadda and X. Guo (Johns Hopkins University).

REFERENCES


FIGURE LEGENDS

Figure 1. AEG-Prom and PEG-Prom are active in PCa cell lines but remain silent in normal prostate epithelial cells. (A) Human PCa cell lines DU-145, PC3, PC3-ML, LNCaP, ARCaP-E and ARCaP-M and the normal counterparts, immortalized prostate epithelial cells (RWPE-1) and prostatic epithelial cells (PrEC), were tested for the promoter activities of pPEG-Luc and pAEG-
Luc using a dual luciferase assay. The indicated cells were transfected with Luc under control of the experimental promoters AEG-Prom, PEG-Prom, and a promoter-less empty vector (control) as a pDNA-PEI polyplex. (B) PC3-ML cells transfected with each pAEG-Luc and mutant pAEG-mEbox1&2-Luc plasmids. Luminescence was normalized for transfection efficiency (by co-transfection with the pGL4.74[hRluc/TK] vector, which expresses renilla luciferase) and for cell number (by µg total protein). Column heights signify mean ± standard deviation (SD) for three independent experiments.

Figure 2. Comparison of AEG-Prom and PEG-Prom activity in an experimental model of metastatic human PCa (PC3-ML). Top panels (A-D): (A, C) Representative healthy control mice, Ctrl-1 and Ctrl-2 (n = 4). (B, D) Representative tumor models, PCa-1 and PCa-2 (n = 4), developed by IV administration of PC3-ML cells. (A, B) BLI at 48 h after delivery of the AEG-Prom-driven firefly luciferase construct (pAEG-Luc-PEI) in Ctrl-1 and PCa-1, respectively. (C, D) BLI at 48 h after delivery of the PEG-Prom-driven firefly luciferase construct (pPEG-Luc-PEI) in Ctrl-2 and PCa-2, respectively. Each mouse was imaged from four orientations (D, dorsal; V, ventral; L, left side; R, right side) with a scale in photons/sec/cm²/steradian. Pseudocolor images from the four groups were adjusted to the same threshold. Bottom panels (E-L): Histological analysis of the photon-emitting regions in pAEG-Luc-PEI- and pPEG-Luc-PEI-treated mice that received IV PC3-ML cells (PCa-1 and PCa-2) and controls (Ctrl-1 and Ctrl-2). Microscopic lesions can be visualized with hematoxylin and eosin (H & E) (left) and immunohistochemistry of Luc and Myc expression (dark brown stain, right) in tumors in the lung (F, J), kidney (G, K), and liver (H, L) but not in the lungs from Ctrl-1 or Ctrl-2 (E, I) or the
necrotic liver regions (D, H – as indicated by the letter N). G, glomerulus; T, tumor; N, necrosis.

(M) Total BLI photon flux emanating from the lung in the Ctrl and PC3-ML groups at 24 h and 48 h after injection of pAEG-Luc-PEI and pPEG-Luc-PEI polyplexes. Data points obtained from individual animals are displayed in Supplemental Fig. S4. The difference between photon emission from PC3-ML and Ctrl groups was significant (*P < 0.0001).

Figure 3. AEG-Prom-driven Luc detects tibial lesion in a model of human PCa metastatic to bone (PC3-ML). 5 x 10^4 cells were inoculated into the left cardiac ventricle to achieve hematogenous spread, providing skeletal metastasis. BLI experiments were conducted at seven weeks after inoculation with tumor cells. (A, B) BLI at 48 h after delivery of pAEG-Luc-PEI in a representative healthy control, Ctrl-1 (A), and the PC3-ML model, PCa-4 (B). (C) Histological analysis confirmed tumor metastasis (T) next to the bone marrow (BM) in the left tibia, but not in the right tibia of PCa-4.

Figure 4. Luc expression in human PCa (PC3-ML) models is due to cancer-specific AEG-Prom activity rather than to differences in transfection efficiency between normal and malignant tissue. (A, B) BLI showing Luc expression in a representative healthy control Ctrl-1 (A) (n = 4) and a PCa model, PCa-2 (B) (n = 4). The images were acquired at 48 h after the intravenous delivery of pAEG-Luc-PEI polyplex. (C-D) Ex vivo BLI, gross pathology and whole body images (with red boxes) reveal the source of signal. The dissected organs were imaged within five min following euthanasia. H & E staining confirmed the extensive metastasis in (C) lung, (D) liver,
the right adrenal and the right renal cortex (Supplemental Fig. S6). Luciferase IHC of consecutive sections showed the correlative luciferase expression (brown stain). (E)

Comparison of Luc plasmid delivery to high tumor burden and low tumor burden areas in liver and lungs of the PCa group (n = 3, PCa-1-3) and liver and lung sections of control (n = 3, Ctrl-1-3). The absolute amount of pAEG-Luc in lung and liver tissues of each animal was quantified by quantitative real-time PCR. The differences in transfection efficiency between areas of high tumor burden vs. those of low tumor burden within liver in same animal (*P < 0.0005), as well as between areas of high tumor burden within liver vs. normal liver (**P = 0.0078), were significant. No significant difference was observed in transfection efficiency in the lungs and liver tissue between the control group and the PCa group. Error bars represent mean ± standard deviation (SD).

Figure 5. AEG-Prom-based SPECT/CT imaging detects distant metastasis not identified by NaF- or FDG-PET/CT. The experimental metastases model, PCa-3 (n = 5), was developed by intra-cardiac injection of 5 x 10⁴ PC3-ML-Luc cells. SPECT/CT images for PCa-3 and a representative healthy control Ctrl-1 were obtained at 18 - 20 h after [¹²⁵I]FIAU injection, which was 66 - 68 h after IV administration of pAEG-HSV1tk-PEI polyplex. (A, B) NaF-PET/CT, AEG-Prom SPECT/CT and FDG-PET imaging in Ctrl-1 (A) and the PC3-ML-Luc model, PCa-3 (B). To enhance the dynamic range of the image display, SPECT signals from the gastrointestinal tract (site of FIAU metabolism) were manually segmented and excluded from the AEG-Prom image in panel B. (D, E) Gross pathology of the metastatic nodules that were located on the basis of the SPECT/CT images. (C, D) Ex vivo BLI of the dissected organs, imaged within 20 min of euthanasia. (C-E) In
PCa-3, pAEG-HSV1tk identified lesions across the right shoulder (L1), dorsal thoracic wall adjacent to mid spine (L3, black dotted circle) and the ventral midline of the sternum (L4, white dotted circle), and within the knee joints next to the bone marrow (L5, red dotted circle), as confirmed by gross pathology. A possible L1 lesion was detected by FDG/PET (B). Color bar represents percentage injected dose per gram of tissue, (%ID/g).
Figure 1

A

Normalized Emission Ratio/μg Total Protein

- pPEG-Luc
- pAEG-Luc
- Empty

B

Normalized Emission Ratio/μg Total Protein

- PC3-ML

pAEG-Luc
pAEG-mEbox1&2-Luc
Quantification of the cancer-specific activity of AEG-Prom and PEG-Prom based on BLI of the control and PCa groups:

<table>
<thead>
<tr>
<th>Control</th>
<th>pAEG (24h)</th>
<th>Ctrl</th>
<th>pPEG (24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl-1</td>
<td>3.0x10^5</td>
<td>Ctrl-2</td>
<td>6.0x10^5</td>
</tr>
<tr>
<td>PC3-ML</td>
<td>6.0x10^5</td>
<td>PC3-ML</td>
<td>9.0x10^5</td>
</tr>
<tr>
<td>PCa-1</td>
<td>1.2x10^6</td>
<td>PCa-2</td>
<td>1.5x10^6</td>
</tr>
</tbody>
</table>

Note: * denotes statistical significance.
Figure 4

A

Control
Ctrl-1

PC3-ML
PCa-2

B

Ex vivo BLI
Gross Pathology
H & E
IHC (Anti-Luc)

C

D

E

(Normalized ng Luc pDNA)/(100ng gDNA)

PCa Liver
High Tumor Burden
PCa Liver
Low Tumor Burden
PCa Lungs Tumor
Ctrl Liver

Ex vivo BLI
Gross Pathology
H & E
IHC (Anti-Luc)
Figure 5

A

Control
Ctrl-1

B

PC3-ML-Luc
PCa-3

C

D

E

NaF-PET
AEG-Prom SPECT/CT
FDG-PET

Heart

L1, L2, L3, L4

L1, L2, L3, L4

L1, L2, L3, L4

L5

L5
AEG-1 promoter-mediated imaging of prostate cancer

Akrita Bhatnagar, Yuchuan Wang, Ronnie C. Mease, et al.

Cancer Res  Published OnlineFirst August 21, 2014.