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Development of an ErbB-overexpressing A-431 Optical Reporting Tumor Xenograft Model to Assess Targeted Photodynamic Therapy Regimens

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Abstract

To better assess the efficacy of erbB-targeted therapies, it would help to have optical reporting human tumor xenograft models that abundantly express erbB receptors. A-431 cells have frequently been used in erbB1-targeting studies, but a well-characterized optical reporting version of the cell line has not been readily available. In this study, optical reporting A-431 clones were developed that express both a fluorescent protein reporter (green, GFP; or red, RFP) and a bioluminescent reporter, firefly luciferase. Reporter genes were transduced into cells using commercial lentiviral vectors, and clonal selection was carried out using a series of procedures. A number of clones were isolated for further characterization. A GFP/luciferase clone, A-431/D4, and an RFP/luciferase clone, A-431/G4, were obtained that exhibit erbB1 expression levels and tumor growth kinetics similar to the parental cells. To demonstrate the utility of the optical reporting clones, A-431/G4 tumors were grown subcutaneously in nude mice and treated with vascular-targeted photodynamic therapy (PDT), which targets the angiogenic consequences of erbB signaling. The A-431/G4 tumor model permitted highly sensitive longitudinal monitoring of PDT treatment response using optical imaging. A-431/D4 and A-431/G4 optical reporting tumor models should also prove useful for assessing therapies that directly target the erbB1 receptor.

INTRODUCTION

The two erbB family tyrosine kinase growth factor receptors, epidermal growth factor receptor (EGFR; also known as erbB1 or HER1) and HER2 (also known as erbB2), have been shown to play an important role in cancer therapy. The overexpression of these receptors in many types of cancers is associated with aggressive disease and poor patient prognoses (1–3). Monoclonal antibodies (MAbs) against these receptors can block receptor ligand binding, inhibit receptor kinase activity, and bring about receptor internalization and down-regulation. Moreover, they can disrupt and arrest cell cycle progression and may also induce antibody-dependent cellular cytotoxicity (3–5). A number of anti-erbB receptor MAbs have been approved for cancer therapy or are in clinical trials including nimotuzumab, cetuximab (Erbix), panitumumab (ABX-EGF or Vectibix), and trastuzumab (Herceptin) (6–11). Low-molecular-weight drug alternatives like gefitinib (Iressa, ZD1839)

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SUPPORTING MATERIALS

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Figure S2 can be found at DOI: xxxx-xxxxxx.s2.

Figure S3 can be found at DOI: xxxx-xxxxxx.s3.

and erlotinib (Tarceva, OSI-774) have also proven clinically effective for blocking activation of the EGFR tyrosine kinase (12–14).

Ongoing research aimed at improving the efficacy of anti-erbB therapeutics often includes the use of A-431 cells, as indicated by hundreds of peer-reviewed publications. A-431 cells were originally established from an epidermoid carcinoma and are a commonly used model for high EGFR expression (15,16). In addition, A-431 cells are markedly sensitive to mitogenic stimuli and have no functional p53, a potent tumor suppressor gene (17,18). All research involving A-431 cells would benefit tremendously from an effective and inexpensive imaging modality that is capable of detecting small numbers of cells in vitro and in vivo. Optical imaging of fluorescent and bioluminescent reporters is a powerful tool that fits this bill. It allows for noninvasive and highly sensitive monitoring of biological processes, which in turn enables facile and efficient preclinical evaluation of new therapies (19). Optical imaging reporter technology could also hold promise for clinical applications where tumors are specifically tagged using tumor targeting vectors (20,21). The approach requires the introduction of fluorescent protein and/or luciferase reporter genes into cells and an appropriate optical imaging system. We cover all aspects involved in developing an optical reporting A-431 tumor model including 1.) gene transduction using commercially available lentiviral vectors, 2.) clonal selection, 3.) phenotypic validation of selected clones by comparison to the parental A-431 cell line (EGFR immunofluorescence staining, tumor growth kinetics, and response to therapy), and 4.) in vitro and in vivo imaging using a home-built optical imager.

A-431 cells expressing a fluorescent protein and luciferase serve as an optical reporting tumor model for real-time in vivo evaluation of EGFR-targeted cancer therapies. As a first demonstration of the utility of the model, we have used it to assess the efficacy of a vascular-targeted photodynamic therapy (PDT) treatment regimen (19,22), since EGFR signaling has been tied to tumor angiogenesis (23–25). Eventually, we plan to use this tumor model to investigate PDT regimens that directly target the EGFR with anti-EGFR photosensitizer immunoconjugates (26,27) or that combine PDT with a therapeutic anti-EGFR MAb, an adjuvant approach which has already been shown to enhance chemotherapy and radiotherapy (28).

MATERIALS AND METHODS

Cell line and lentiviral vectors

A-431 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C in ATCC-formulated Dulbecco's Modified Eagle's Medium containing 4 mM L-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum. Cell medium was supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin.

Optical reporter vectors were obtained from commercial sources and came as ~200 µl aliquots of transduction-ready lentiviral particles containing ~10⁷ transducing units/ml. A-431 cells were first transduced with either GFP or TurboRFP (29) (Evrogen JSC; Moscow, Russia) optical reporter genes using the lentiviral vectors Lenti-Green or Lenti-Red from Biogenova Corp. (Frederick, MD). Selected A-431 clones expressing high levels of GFP or TurboRFP were then transduced with the gene of a non-secreted form of firefly luciferase using the lentiviral vector Cignal Lenti Positive Control (CLS-PCL) from SABiosciences Corp. (Frederick, MD). All reporter genes are driven by the CMV promoter (30,31) for high-level constitutive expression. The Lenti-Red and CLS-PCL vectors also contain blasticidin and puromycin resistance genes, respectively, for selection purposes.

Lentiviral transductions and selection of clones

A-431 cells were plated in 96-well plates at 2.5×10^3 , 5×10^3 , 10^4 , and 2×10^4 cells per well. After incubation of the cells for 24 hours, the culture medium was replaced with a Lenti-Green or Lenti-Red lentiviral particle solution diluted with fresh culture media to obtain multiplicity of infection (MOI) ratios ranging from 2.5 to 100 virus particles per cell. To enhance transduction efficiency, the lentiviral particle media mixture was supplemented with polybrene (hexadimethrine bromide; Sigma-Aldrich, Allentown PA) at a final concentration of 8 $\mu\text{g/ml}$. Cells were incubated 24 to 48 hours with the lentiviral mixtures, and then the mixtures were removed and replaced with fresh medium. After another incubation period of 24 to 96 hours, cells were observed by fluorescence microscopy to verify the initiation of fluorescent protein expression. Cells were then removed from the wells by trypsinization and transferred to 24-well plates for expansion. Following two more passages and further expansion into T75 flasks, stock aliquots of cells were frozen back in a 10% dimethyl sulfoxide (DMSO)/90% fetal bovine serum cryopreservative solution for liquid nitrogen storage.

For selection of bright fluorescent protein expressing A-431 clones, 20 to 25 60-mm culture dishes were plated with 100 cells per dish of either the Lenti-Green or Lenti-Red transduced A-431 cultures. Approximately 1.5 to 2 weeks after plating, individual colonies containing ~100 cells or more had formed on the plates. Highly fluorescent colonies were distinguished from non-fluorescent or poorly fluorescent colonies by fluorescence microscopy, and the locations of the brightest colonies were marked for selection. Isolation of marked colonies was accomplished by washing the plates with Dulbecco's phosphate buffered saline without calcium or magnesium (PBS), removing most of the PBS while leaving a thin wet film to ensure the cells did not dry out, and quickly scraping away unwanted cells around the marked fluorescent colony using a sterile cell scraper. A small drop ($\leq 30 \mu\text{l}$) of trypsin/ethylenediaminetetraacetic acid (EDTA) solution was then applied to the isolated colony (the drop stayed in place over the colony due to surface tension). Following trypsinization, the colony of cells was transferred to a 96-well plate, further expanded into a 24-well plate, and finally expanded into T75 flasks. In our hands, this clonal isolation technique was easier to perform and more reliable than using traditional cloning cylinders. The selection process ultimately yielded five GFP clones and three RFP clones.

One GFP clone and one RFP clone were chosen for subsequent transduction with the luciferase lentiviral vector, CLS-PCL. The initial steps of the transduction procedure using the CLS-PCL vector were similar to those described above for the Lenti-Green and Lenti-Red vectors. Cells were plated at 2.5×10^3 cells per well, and MOIs were roughly 50 to 70. However, after removal of the lentivirus followed by a 24-hour incubation period to allow the cells sufficient time to begin expressing the luciferase (*luc*) and puromycin resistance transgenes, the cells were subsequently cultured in the presence of 0.5 $\mu\text{g/ml}$ puromycin over the next ~2.5 weeks. During this period the CLS-PCL transduced cultures were expanded into T75 flasks, and stock aliquots of cells were frozen back. For both the GFP and RFP clone cultures that had been transduced with the CLS-PCL vector and treated with puromycin, six 60-mm culture dishes were plated with 100 cells per dish. This allowed isolation of five GFP/*luc* and five RFP/*luc* puromycin-resistant clones using the previously described clonal isolation procedure, with the exception that isolated clones could not actually be observed by bioluminescence under the microscope (our microscope set-up was not sensitive enough to image bioluminescence). Instead, colonies were randomly selected off the plates and expanded into T75 flasks, and these cultures were then used to prepare cell suspensions. The cell densities of the suspensions were determined using a hemacytometer, and cell pellets containing 3.5×10^6 cells/pellet were prepared by centrifugation (8 minutes at 125 g). After removal of the supernatants, the pellets were resuspended in conical tubes with culture medium containing 150 $\mu\text{g/ml}$ luciferin (D-luciferin potassium salt; Gold

Biotechnology, Inc.; St. Louis, MO) and then placed in a tube holder for immediate imaging on a home-built imager (described below) to assess luciferase activity. Image exposure times were 25 seconds. Two high-expressing GFP/luc clones, designated A-431/D1 and A-431/D4, and one high-expressing RFP/luc clone, designated A-431/G4, were identified using this procedure.

Animals and tumor models

Female athymic NCr-nu/nu mice, 4 to 5 weeks old, were purchased through the Animal Production Program of the National Cancer Institute at Frederick (Frederick, MD) and housed in microisolator cages in a laminar flow unit (Tecniplast USA, Exton, PA) under controlled ambient light. For tumor inoculations, imaging, PDT, and tumor measurements, mice were anesthetized by intraperitoneal (i.p.) injection with ketamine:xylazine (40:6 mg/ml) prepared in a saline solution. Depending on animal size and the procedure being performed, doses ranged from 60:9 to 120:18 mg per kg body weight, which allowed mice to remain anesthetized for 0.5 to 1.5 hours. In the infrequent event that an animal began to awake prematurely during a procedure, a maintenance dosage of ketamine only (40 mg/ml, doses typically 40 to 120 mg per kg body weight) was given to prolong the anesthesia period. All animal experiments were approved by Dartmouth College's Institutional Animal Care and Use Committee.

Cultures of optical reporting A-431/D1, D4, and G4 clones and A-431 parental cells that had reached 75 to 90% confluency were harvested by trypsinization off of culture flasks and prepared as suspensions in complete culture medium. Cell densities were determined using a hemacytometer. Cells were pelleted (8 minutes at 125 g), supernatant was removed, and pellets were resuspended with complete culture media to obtain tumor inoculate suspensions at the desired cell densities. For tumor growth studies and development of ex vivo cell cultures from established tumors, inoculates of $\sim 3.3 \times 10^6$ cells were prepared in 100 μ l aliquots in insulin syringes (0.5 cc, 28g 1/2" needle), and one inoculate per mouse was injected subcutaneously in the left pectoral region. For optical imaging sensitivity studies, inoculates ranging from 10^4 to 10^6 cells were prepared in 50 μ l aliquots, and 4 to 5 inoculates per mouse were injected intradermally at various sites on the ventral surface of the animals. Mice were then immediately imaged.

Three-dimensional tumor sizes were measured at least weekly with a digital caliper, and tumor volumes were calculated using the formula $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. Animals were euthanized before tumor volume exceeded 1500 mm³.

Fluorescent protein and EGFR immunofluorescence microscopy

To examine whether the optical reporting A-431 clones continued to stably express fluorescent proteins and the EGFR after growth as tumors in nude mice, mice bearing A-431/D1, D4, and G4 tumors (tumor volumes >150 mm³) were sacrificed, and their tumors were immediately removed and processed to develop ex vivo cell cultures. Excised tumors were minced into small pieces (1–3 mm in size), washed two times with PBS, and pelleted. The supernatant was decanted, and the minced tumor tissue preparations were resuspended and incubated with an excess of 0.25% trypsin/EDTA at 4°C. After 24 hours, the excess trypsin/EDTA was decanted, and the minced tumor preparations were incubated for 30 minutes at 37°C. The trypsinization reaction was then stopped by the addition of complete culture medium, and the minced tumor preparations were pressed through a cell dissociation sieve (tissue grinder kit; Aldrich, Milwaukee, WI). The resulting coarse cell suspensions were transferred to T75 flasks for culturing. After a week, non-adherent material was removed, and cells that had attached to the flasks were passaged and expanded. To eliminate fibroblasts and other stromal cells that persisted in the cultures, cultures were treated with

puromycin (up to 5 $\mu\text{g/ml}$) for 1 to 3 days. After sufficient expansion, stock aliquots of the ex vivo cultures were frozen for liquid nitrogen storage.

For microscopy of fluorescent protein expression, $\sim 1.5 \times 10^5$ cells per well of the ex vivo cultures were plated on sterilized coverslips (No.1, 22 mm square; Corning Inc. Life Sciences, Lowell, MA) in 6-well plates and grown for 2 to 3 days. Media was removed from the wells, and the cells were washed with PBS. Coverslips were then mounted with the cell side of the coverslips oriented upward and covered with a thin droplet layer of PBS for live cell imaging. A reflected fluorescence microscope (model BX51 equipped with a BX-RFA fluorescence illuminator and filter sets U-MWIB2 and U-MWIG2 for GFP and RFP fluorescence, respectively; Olympus America Inc., Melville, NY) with a filtered arc lamp fluorescence illuminator (X-cite 120; EXFO America, Richardson, TX) was used for imaging. Images were acquired as 8-bit RGB TIFF files at 100x magnification using a high resolution CCD color camera (Q-Color3; Olympus America, Inc.) controlled by QCapture 2.52 image acquisition software (Quantitative Imaging Corp., Surrey, BC, Canada). Room lights were kept off, and exposure times were 1 second.

For immunofluorescence microscopy of the EGFR, cells were grown on coverslips as previously described, rinsed with PBS, and then fixed in ice-cold methanol:acetone (1:1) at -20°C for 10 minutes. After air drying, the coverslips were covered with 1% bovine serum albumin (BSA) in PBS blocking buffer and incubated for 30 minutes at 37°C . Blocking buffer was then removed, and coverslips were covered with a thin layer of 10 $\mu\text{g/ml}$ Eribitux (ImClone Systems Inc., Branchburg, NJ) primary antibody in blocking buffer ($\sim 150 \mu\text{l}$ per coverslip) and incubated at 37°C in a humidified atmosphere for 1 hour. The primary antibody was then removed, and three 5-minute washes in PBS were performed. For controls, some coverslips were incubated with blocking buffer only. Coverslips were then incubated with 15 $\mu\text{g/ml}$ fluorescein labeled anti-human IgG secondary antibody (Vector Laboratories, Inc., Burlingame, CA) in blocking buffer using a procedure similar to that described for the primary antibody incubation. After removal of secondary antibody and washing with PBS, coverslips were inverted and mounted onto slides with a drop of mounting media (VECTASHIELD, Vector Laboratories, Inc.). Mounted coverslips were imaged by fluorescence microscopy using acquisition methods as previously described for GFP imaging. Image exposure times were 0.2 seconds.

Fluorescence and bioluminescence small animal imager

A home-built small animal imager was constructed for imaging fluorescence and bioluminescence and was housed in a darkroom (see Fig. 1A). The imaging system consisted of a highly sensitive 12-bit cooled (-11°C) CCD camera (SensicamQE; The Cooke Corporation, Romulus, MI) equipped with a manual zoom lens (2/3" 12.5–75mm f1.2 6X model M6Z 1212, Computar; CBC (America) Corp., Commack, NY). It was configured to have a working distance of approximately one meter between the zoom lens and the anti-reflection coated glass window platen imaging stage (10 $\times$ 12 inches; Edmund Optics Inc., Barrington, NJ). Specimens usually were first positioned on a separate glass plate, which was then placed on the window platen stage for imaging. Two different light sources, a 670 nm diode laser (model HPD7401, High Power Devices, Inc., North Brunswick, NJ) for photosensitizer fluorescence imaging and a filtered white light source (model M1000, Stocker Yale, Salem, NH) for fluorescent protein imaging, were positioned on opposite sides of the camera at a sufficient distance to keep direct reflection images of the light sources out of the imaging field of view. The 670 nm diode laser light was delivered through a multimode fiber optic (1mm diameter, 3 m length, 0.37 numerical aperture UV-Vis-NIR silica core/silica clad patch cable fiber; Multimode Fiber Optics, Inc., Hackettstown, NJ), and the beam was expanded using a 3.1 mm focal length micro aspheric lens (0.68 numerical aperture, multilayer broadband coating; Geltech/LightPath Technologies, Inc.,

Orlando, FL). The filtered white light was delivered through a liquid light guide (5mm core diameter, 6 foot length, 0.55 numerical aperture; Edmund Optics Inc.), and the beam was expanded using a 15 mm focal length plano-convex lens (15 mm diameter, MgF₂ coating; Edmund Optics Inc.). To achieve the best light intensity uniformity across the expanded beam, the fiber optic and liquid light guide were looped with curvatures at or near their specified minimum bend radii. Stray reflected light from the fluorescence excitation sources was minimized by partially enclosing the imager with black foam board (in Fig. 1A, the foam board was removed to allow clearer viewing of the imager).

Fluorescence emission filters were mounted on a filter holder placed directly over the zoom lens, and excitation filters for the filtered white light source were mounted within the light source housing in front of the liquid light guide coupler. 685 nm long pass (Omega Optical, Inc., Brattleboro, VT), 520±20 nm bandpass (520DF40; Omega Optical, Inc.), and 610±38 nm bandpass (HQ610/75m; Chroma Technology Corp., Rockingham, VT) emission filters were used for imaging photosensitizer, GFP, and RFP fluorescence, respectively. 470±20 nm and 540±20 nm bandpass filters (470DF40x and HQ540/40x, Chroma Technology Corp.) were used for GFP and RFP excitation, respectively. For bioluminescence imaging, emission filters were removed from the camera lens, light sources were turned off, and all light leaks in the dark room were covered with black masking tape or felt. Mice were pre-imaged prior to administration of luciferin to obtain a baseline dark image. After i.p. injection of luciferin (10 µl per g body weight of a freshly prepared 15 mg/ml solution dissolved in PBS), mice were continuously imaged every 5 to 15 minutes up to ~1 hour post-injection. A white light reference image was also acquired using the white light source without any excitation filter mounted. This allowed the anatomic location of the bioluminescence signal to be visualized in a superimposed composite image consisting of a color-coded bioluminescence image overlaid on a gray scale reference image. To minimize glare when acquiring reference or photosensitizer fluorescence images, a linear polarizer film (Edmund Optics Inc.) was mounted above the beam expanding lens, and another polarizer film in a cross polarization orientation was mounted above the camera lens.

Software (CamWare v1.00; PCO AG, Kelheim, Germany) provided by the camera manufacturer was used to control the camera, and images were saved as 16-bit TIFF files (image pixel values were preserved as the original 12-bit camera values). Camera settings were generally set to analog gain normal, low light mode off, 1×1 binning, and with a region of interest (ROI) of 1376×1040 pixels (i.e., the full sensor imaging area and maximum resolution was utilized). The lens was typically set with the zoom halfway between the 50 and 75 mm markings on the lens barrel, which was optimal for whole body imaging of mice (imaging of larger specimens up to the size of a rat was possible with smaller zoom settings). The lens f-stop was set at 2, and the focus was near the 3.5 mark but varied slightly depending on the imaging mode. Image files were processed and analyzed with the image processing program, ImageJ (32).

Photosensitizer

Pyropheophorbide-a (PPa) was purchased from Frontier Scientific, Inc. (Logan, UT) and was provided to a contract peptide synthesis facility (Biopeptide Co., Inc., San Diego, CA) for synthesis of a short linear polyethylene glycol (PEG) PPa-peptide photosensitizer conjugate, sPPp (sequence: N-ε-(PPa-Asp)-Lys-PEG(11-mer)-PEG(11-mer)-amide) using standard Fmoc solid-phase peptide synthesis methods (see Supporting Materials Fig. S1 for the chemical structure of sPPp). The purity of the conjugate was determined to be >95% by HPLC analysis, and the molecular weight (1,977 g/mole) was verified by electrospray ionization mass spectrometry (see Supporting Materials Fig. S2 for HPLC and mass spectrometry data). The sPPp photosensitizer conjugate was acetylated by reaction with excess acetic anhydride (~2.5 ml of ~7 mg/ml sPPp in 50 mM NaHCO₃ buffer was rapidly

mixed with ~4 ml of DMSO and 0.1 ml of acetic anhydride, and the reaction was allowed to proceed for 1 hour at room temperature). The resulting acetylated-sPPp (Ac-sPPp) preparation was diluted with ~2 volumes of distilled/de-ionized water (DDI-H₂O) and then dialyzed against 4 liters of DDI-H₂O using a 1-kDa molecular weight cut-off dialysis membrane tubing (Spectra/Por 7; Spectrum Laboratories Inc., Rancho Dominguez, CA) with two changes of dialysate. Lyophilization of the purified Ac-sPPp allowed recovery of the product as a dry solid. The Ac-sPPp preparation was found to be at least 90% pure by HPLC analysis, and the molecular weight (2,019 g/mole) was verified by electrospray ionization mass spectrometry (see Supporting Materials Fig. S3 for HPLC and mass spectrometry data). All reaction, purification, and processing steps were performed in the dark or under low-light conditions.

For PDT studies, the dried Ac-sPPp was directly dissolved in PBS, and the concentration of the stock solution was determined by absorbance spectrometry. The Ac-sPPp stock was diluted 1 to 100 into DMSO, and an absorbance spectrum was taken using a Cary 50 Bio UV-visible absorbance spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA). An extinction coefficient of 47,500 M⁻¹cm⁻¹ at 668 nm was used to calculate the concentration of the Ac-sPPp solution, based on the assumption that the extinction coefficient of Ac-sPPp in DMSO is similar to that of the PPa methyl ester parent compound in its monomeric, disaggregated state (33).

PDT treatment

Approximately 10 to 15 days after tumor inoculation, tumors grew to a size suitable for PDT experiments. Mice with tumors ~150 mm³ in size were administered 60 nmoles of Ac-sPPp in PBS per 20 g of body weight (equivalently ~1.6 mg PPa content per kg body weight) via a lateral tail vein bolus injection. Sixteen hours later, mice were irradiated at their tumor sites with 100 J/cm² of 670 nm light at an incident fluence rate of ~50 mW/cm² (irradiations took ~33 minutes). After 1.5 to 3 weeks, PDT-treated mice frequently exhibited tumor regrowth. Some of the mice with recurring tumors were administered another dose of Ac-sPPp, and PDT treatment was repeated. The photodynamic treatment parameters (i.e., photosensitizer and light dosages and timing of the light dose) used here were based on in vivo dosing studies as well as photosensitizer biodistribution and pharmacokinetic studies employing whole body fluorescence imaging techniques. The full details of these studies are beyond the scope of this paper and will be reported elsewhere.

An illuminator was constructed to perform PDT irradiations (see Fig. 1B). It consisted of a high-powered 670 nm laser diode coupled to a beam expander mounted above a small glass plate platform upon which Scotch-taped aluminum foil blocking screens were positioned. The animal was anesthetized and then immobilized with tape on a black felt-covered platform with its ventral side up and its tumor centered directly below the beam expander and blocking screen platform. The components of the fiber optic delivery cable and the beam expander for the PDT illuminator were identical to the analogous components described above for the small animal imager. For PDT, the fiber optic connecting the 670 nm laser to the imager was swapped to re-direct the laser through a different fiber optic routed to the PDT illuminator. The incident fluence rate at the plane of the tumor was adjusted to ~50 mW/cm², and the shape of the beam area was adjusted to cover the tumor as well as a ~2 mm border around the tumor perimeter.

RESULTS AND DISCUSSION

Optical setups

Small animal imager—The home-built small animal imager used in this study (see Fig. 1A) was designed for fluorescence, chemiluminescence, and bioluminescence imaging of a wide variety of specimens including small animals, electrophoretic gels, blots, and tissue culture plates. In fluorescence imaging mode, the imager's one-meter working distance allowed excitation light to strike the image plane at an angle that was close to normal, which minimized problems associated with non-uniform illumination of the specimen (the intensity of the excitation light varied less than ~10% over the maximal imageable area). This working distance also helped to ensure optimal filtering of the light reaching the camera, since light must pass through interference filters at near normal incidence for best filter performance. While it is generally considered more optimal to position the specimen as close as possible to the camera for imaging very weak optical signals and/or small features, in bioluminescence imaging mode as few as ~100 A-431/G4 cells (in 96-well plates) could be detected at a 1-meter working distance using a 10-minute exposure. Ultimately, camera sensor noise limited bioluminescence imaging sensitivity when attempting exposures much longer than 10 minutes, whereas imperfect filtering was the main factor limiting fluorescence imaging sensitivity. Side-reflector mirrors placed on either side of the specimen and oriented at 45° to the window platen stage have subsequently been integrated into the setup to obtain left and right side views in addition to the main front surface view of the specimen (not shown). However, images acquired with side-reflectors required the f-stop to be adjusted to higher values (>2) in order to keep all views in focus, which in turn necessitated longer exposures.

PDT illuminator—Figure 1B shows the illuminator setup that was used to irradiate animals for PDT. The illuminator design allowed easy adjustment of fluence rate as well as size and shape of the illumination area. Before placing an animal under the illuminator, fluence rate was adjusted by raising or lowering the beam expander to adjust its distance to the tumor plane. The laser was then switched to aiming beam mode, and the mouse was inserted under the illuminator to adjust beam area and shape. This was accomplished by placing 1 to 3 small blocking screens on a glass platform positioned between the beam expander optic and the mouse. Screens were oriented and configured to shape the beam as necessary. Beam adjustments usually took less than 5 minutes, and no PDT effects resulted from the procedure since it was done under the laser's low intensity aiming beam.

Lentiviral transduction with optical reporting genes and selection of clones

A variety of optical reporters were investigated to examine their potential advantages and disadvantages. A-431 cells were first transduced with a commercial GFP or RFP lentiviral vector, and then one bright GFP and one bright RFP clone were chosen for transduction with a commercial firefly luciferase lentiviral vector. Sequential transductions with separate fluorescent protein and luciferase lentiviruses were chosen over a single transduction or single transfection procedure using more sophisticated fluorescent protein-luciferase fusion reporter constructs because it has been noted that reporters are often not as active when expressed as fusions (34). Moreover, fusion reporter constructs were not readily available as commercial off-the-shelf products at the time this study was initiated. Another potential advantage of sequential transductions is that it may be of use to have two differently labeled bioluminescent clones (e.g., GFP versus RFP) for highly sensitive simultaneous in vivo tracking. One clone could serve as a control and the other could be used to study various biological manipulations (e.g., knockout or down-regulation of a gene-product to examine its influence on an experimental treatment).

As judged by fluorescence microscopy, MOIs of 25 to 100 were most effective for the GFP and RFP lentiviral transductions, and there did not appear to be any major improvements in fluorescent protein expression as MOIs were increased above 50. Based on these qualitative observations, MOIs of 50 to 70 were used for the luciferase lentiviral transductions.

GFP- and RFP-transduced cultures were first expanded prior to selecting individual clones. After expansion, roughly 2.5–6% of the cells were brightly expressing GFP, and only ~1% of the cells were brightly expressing RFP. While the GFP lentiviral vector contained no antibiotic resistance selection gene, the RFP lentiviral vector contained a blasticidin resistance gene. Although it was determined that A-431 parental cells rapidly died when exposed to as little as 2.5 µg/ml blasticidin, blasticidin selection of RFP-transduced cultures failed to yield any highly expressing RFP cells. This may have been due to the fact that there were so few bright RFP-positive cells to start with, and expression of the blasticidin resistance gene may not have correlated well with RFP expression. Consequently, GFP and RFP clones were isolated off of sparsely seeded tissue culture plates using fluorescence microscopy to identify the brightest colonies. This isolation procedure yielded five different GFP clones and three different RFP clones.

Only one GFP and one RFP clone were chosen for further transduction with the luciferase lentiviral vector. The choice of these particular GFP and RFP clones was based on their similar proliferation and expansion rates as compared to the parental A-431 cells under identical culture conditions. Because the luciferase lentiviral vector also contained a puromycin resistance gene, one day after transduction cells were grown under the selection pressure of 0.5 µg/ml puromycin to enrich for cells expressing high levels of the luciferase and puromycin resistance transgenes. Once the transduced cultures had been expanded into T75 flasks, they were considered free of puromycin-susceptible cells, and the culture medium was no longer supplemented with puromycin. At this stage, five GFP/luc and five RFP/luc puromycin-resistant clones were obtained by random selection off of sparsely seeded plates using clonal isolation procedures similar to those used to select GFP⁺ and RFP⁺ clones. Our microscope set-up was not sensitive enough to image bioluminescence, so the puromycin-resistant clones were instead expanded into T75 flasks and then prepared as resuspended cell pellets for bioluminescence imaging using our home-built imager. Figure 2A shows a composite bioluminescence image comparing the parental GFP/luc transduction culture with the various isolated GFP/luc clones (similar results were observed with the RFP/luc clones and are not shown). Interestingly, there was a wide range of luciferase activity amongst the different clones, and one of the clones, D5, exhibited no measurable bioluminescence. Antibiotic selection is clearly not entirely reliable since puromycin resistance does not necessarily correlate directly with luciferase activity. However, this is understandable considering that the optical reporting and antibiotic selection genes are driven by different promoters. Using the cell pellet bioluminescence as a guide, two bright GFP/luc clones, D1 and D4, and one bright RFP/luc clone, G4, were chosen for further characterization.

Characterization of selected optical reporting clones

To determine whether the selected A-431 clones continued to stably express optical reporting genes as well as high levels of the EGFR when grown as tumors in nude mice, mice bearing A-431/D1, D4, and G4 tumors were sacrificed, and their tumors were removed and processed to develop ex vivo cell cultures. The ex vivo cell cultures were expanded into T75 flasks and treated with puromycin for up to three days to remove any non-tumor cells. The cultures were then used to grow cells on coverslips for fluorescent protein microscopy and EGFR immunofluorescence microscopy.

Maintenance of stable optical reporting gene expression—Figure 2B shows that all cells from the A-431/D4 and A-431/G4 ex vivo cultures continued to express high levels of GFP and RFP, respectively. GFP expression for the D1 clone was similar to D4 and is not shown. To allow for better visualization, image pixel values were remapped (for RFP images, pixel values 7–60 were remapped to 0–255, and for GFP images, pixel values 7–240 were remapped to 0–255; image exposure times were one second for both RFP and GFP images). Therefore, although it is not evident in the presented images, the GFP fluorescence of the D1 and D4 cells was significantly brighter than the RFP fluorescence of the G4 cells. This most likely explains why the minimum number of D1 or D4 cells that could be detected in vivo by GFP imaging was slightly less than the minimum number of G4 cells that could be detected by RFP imaging (see the following section, **Imaging sensitivity of the optical reporting clones**). For both the GFP and RFP clones, fluorescent protein localization is fairly evenly distributed throughout the cellular space. However, the G4 clone appears to exhibit a greater cell-to-cell variation in fluorescent protein brightness than the D1 and D4 clones. Morphologically, all of the optical reporting clone cells were very similar to parental A-431 cells, with the exception that the D4 clone appeared to have a somewhat greater fraction of larger-sized cells.

Because our microscope setup was not sensitive enough to do bioluminescence microscopy, we did not conduct comparable studies to verify that all cells derived from ex vivo tumor cultures also continued to express high levels of luciferase. In any case, such experiments seemed unnecessary considering that the lentiviral vectors used for introducing the fluorescent protein and luciferase transgenes into the cells were very similar in their constructions, and all optical reporting gene constructs were under the control of a CMV promoter. Consequently, it appears safe to assume that the observed maintenance of stable fluorescent protein expression by the various optical reporting A-431 clones after growth as tumor xenografts also extends to the luciferase reporter gene.

Maintenance of EGFR expression—Figure 3 shows that cells from the A-431/D4 and A-431/G4 ex vivo cultures continued to express very high levels the EGFR. Moreover, their EGFR immunofluorescence staining was very similar to the typical EGFR staining pattern observed for parental A-431 cells (16) (EGFR immunofluorescence staining of the D1 clone was similar and is not shown). A-431 cells express approximately 2.6×10^6 EGFR per cell, which is a salient characteristic of the cell line. The very high EGFR expression level of A-431 cells makes them ideal for testing anti-EGFR targeting strategies.

Tumor growth characteristics—To examine the tumor growth characteristics of the various selected optical reporting clones, tumors were inoculated subcutaneously in the left pectoral region and regularly measured. Unexpectedly, initial efforts to grow tumors from the D1 and D4 clones were disappointing, since tumor growth rates were markedly slower compared to tumors grown from parental A-431 cells (data not shown). However, sizeable D1 and D4 tumors eventually developed in all mice, and it was possible to harvest the tumors and develop ex vivo cultures. The ex vivo D1 and D4 cultures were then used to inoculate tumors in mice, and these tumors developed more readily and grew at rates more comparable to parental A-431 tumors. In contrast, the G4 clone never exhibited unusually slow tumor growth, and it was unnecessary to develop an ex vivo culture of this clone to achieve optimal tumor growth.

Tumor growth curves for the various optical reporting A-431 clones and the parental A-431 cell line are shown in Figure 4 (data shown in Figs. 4C and 4D are for tumors grown from the ex vivo D4 and D1 cultures, respectively). To obtain average fits of the tumor growth curves, an exponential tumor growth characteristic was assumed (35) for tumor sizes in the range of approximately 20 to 500 mm³. This allowed the estimation of mean tumor doubling

times (td). The td values were all roughly the same for parental A-431, G4, and D4 tumors (3.4 – 4.2 days), but for D1 tumors td was somewhat longer (6.6 days). In addition, unlike parental A-431, G4, and D4 tumors, D1 tumors ulcerated and began to exhibit extensive necrosis early on when tumors had only reached 300 to 450 mm³, which ultimately resulted in erratic, slow tumor growth. At this time it is unclear why the D1 tumors grew so poorly, but it is possible that they were implanted too superficially and grew intradermally rather than subcutaneously (generally speaking, tumors growing deeper under the skin do not ulcerate or ulcerate at much larger sizes than tumors growing more superficially or intradermally). Consequently, we have decided not to use the D1 clone in subsequent studies until this issue can be resolved. Although parental A-431, G4, and D4 tumors grew more regularly than the D1 tumors, they also began to show signs of ulceration and scabbing when tumors reached roughly 450 to 800 mm³. Once ulceration set in, the rate of tumor growth typically slowed somewhat. Ulceration in the A-431 tumor model has been noted by others (36) and appears to be a normal tumor progression phenomenon of the model.

Imaging sensitivity of the optical reporting clones

For optical imaging sensitivity studies, inoculates ranging from 10⁴ up to 10⁶ cells were prepared in 50 µl volume aliquots, and 4 to 5 inoculates per mouse were injected intradermally at various sites on the ventral surface. Immediately following inoculation, mice were imaged by fluorescent protein imaging and then by bioluminescence imaging.

Using our home-built imager in fluorescent protein imaging mode it was possible to detect inoculates as small as 10⁵ cells for the GFP-expressing D1 and D4 clones and as small as 3×10⁵ cells for the RFP-expressing G4 clone (data not shown). On the other hand, under a fluorescence microscope, individual cells of the optical reporting clones could clearly be distinguished from parental A-431 cells. The disparity in detection sensitivity between fluorescence protein microscopy imaging (a detection limit of 1 cell) and whole-animal fluorescent protein imaging (detection limits around 10⁵ to 3×10⁵ cells) requires some explanation. First, it must be realized that the sensitivity of whole-animal fluorescent protein imaging with our home-built imager is limited somewhat because the coupling of an interference emission filter with a monochrome camera is not optimal for distinguishing endogenous tissue fluorescence from exogenous fluorescent protein signals. In this respect, replacing the monochrome camera with a color camera or incorporating a tunable RGB filter (e.g., a tunable liquid crystal filter) would potentially allow better spectral distinction of fluorescent protein signals. Second, interference filters cannot completely block all of the excitation light reflected off of the specimen, and consequently, the excitation light background in the images increases as image exposures are increased in an attempt to detect weak fluorescence signals. This is compounded by the fact that unlike microscopy, in a whole-body imaging setting the contribution of the excitation light to the image background is not uniform because tissue thickness varies across an animal's body, and the optical properties of different tissues vary significantly. Third, in vivo imaging even at superficial intradermal depths results in significant light scattering and absorption that can substantially diffuse and muffle fluorescent protein signals, especially in a whole-body imaging setting. By comparison, scattering and absorption is usually much less of an issue in fluorescence microscopy imaging.

In contrast to whole-body fluorescent protein imaging, a much smaller inoculate size of 10⁴ cells could easily be detected for all of the optical reporting clones (D1, D4, and G4) using our home-built imager in bioluminescence imaging mode. Figure 5A shows a representative bioluminescence image of a mouse implanted at multiple sites with G4 cells, and figure 5B shows a plot of the integrated bioluminescence signal versus G4 inoculate size. The relationship between bioluminescence signal and inoculate size is roughly linear in the range 10⁴ to 3×10⁵ cells, but slightly deviates from linearity above 3×10⁵ cells. This general trend

was also observed with the other optical reporting clones, D1 and D4 (data not shown). Possible explanations for the deviation from linearity above 3×10^5 cells are: (1) oxygen delivery to freshly implanted tumor inoculates may have been limited since no vasculature had yet been established to directly supply the tumor cells; therefore, for larger inoculate sizes, there may have been less oxygen per tumor cell available to sustain the luciferase reaction; (2) for similar reasons, there also may have been less luciferin per tumor cell for larger inoculate sizes. In fact, it is somewhat surprising that *in vivo* bioluminescence was detectable immediately after tumor inoculation. Obviously diffusion by itself without any establishment of tumor neovasculature permitted sufficient delivery of oxygen and luciferin to tumor cells.

While we have not specifically attempted to image optical reporting tumor inoculates smaller than 10^4 cells *in vivo*, it should be feasible since the bioluminescence signal-to-noise was still very good at 10^4 cells, and image exposure times can be further increased. *In vitro*, we have detected as few as ~ 100 A-431/G4 cells per well in 96-well plates using the home-built imager in bioluminescence imaging mode with 10-minute exposures. The bioluminescence signal increased linearly with cell number in the range 0 to $\sim 25,000$ cells per well and began to plateau above $\sim 30,000$ cells per well, presumably due to the cells approaching confluency in the 96-well plates (data not shown).

Based on these imaging sensitivity experiments, it is clear that luciferase bioluminescence imaging is far more sensitive than fluorescent protein imaging when performing macroscopic *in vivo* imaging, although fluorescent protein imaging is certainly exquisitely sensitive in a microscopic imaging setting. A distinct advantage of bioluminescence imaging is that the background noise of the image is easily determined by taking a control dark image, i.e., an image of the animal prior to administering luciferin. However, in fluorescent protein imaging, the background is not as easily distinguishable due to endogenous tissue fluorescence and imperfect filtering of the backscattered excitation light, both of which vary across the body of the animal and also depend on the exact placement and orientation of the animal in the imaging field. Another major advantage of bioluminescence imaging is that the activating agent luciferin is delivered throughout the body via *i.p.* injection, whereas in fluorescent protein imaging, tissue penetration of the activating excitation light is fairly limited. We observed the consequences of these imaging modality differences when we attempted to implant A-431/G4 cells deep under the skin of a mouse and accidentally injected some of the inoculate into the peritoneal cavity. We found that small disseminated A-431/G4 tumor nodules developed in the peritoneal cavity, which could easily be seen by bioluminescence imaging but could not be seen as well or at all by RFP imaging (data not shown). This distinct depth advantage of bioluminescence imaging over fluorescent protein imaging has also been noted by others (37).

Monitoring tumor treatment with bioluminescence imaging

We have previously shown that a novel vascular-targeting photosensitizer, Ac-sPPp, could completely eradicate A-431 tumors in a nude mouse model when PDT was given as a repeated regimen, but A-431 tumors nearly always grew back if PDT was only administered once (unpublished data presented at the 10th NSTI Nanotech Conference, June 2008 and the 15th International Congress on Photobiology, June 2009). To better visualize A-431 tumor recurrence following initial PDT and then subsequent tumor eradication following repeated PDT, we used the A-431/G4 optical reporting tumor model to monitor tumor response. Figure 6 shows a representative longitudinal bioluminescence imaging data set of an A-431/G4 tumor-bearing mouse treated with repeated vascular-targeted Ac-sPPp PDT. Five days after the first PDT treatment there was a dramatic reduction in tumor bioluminescence but a small focus of weak bioluminescence still remained. The small focus of surviving tumor was on the order of 10^4 cells or less, based on earlier sensitivity studies (see Fig. 5). This was too

small to discern as living tumor tissue by the unaided eye. Furthermore, in contrast to bioluminescence imaging, fluorescent protein imaging could not definitively demonstrate the presence of this small surviving tumor focus since treatment-induced tissue damage and tumor scabbing was highly RFP-fluorescent and had not cleared or sloughed away by five days post-PDT (data not shown). Twelve days after initial PDT, bioluminescence imaging showed that the small tumor focus had begun to grow back. By 20 days, the recurrent tumor reached $\sim 50 \text{ mm}^3$ in size and was once again highly bioluminescent. At this point, PDT was repeated, and bioluminescence imaging 3 days later indicated that all surviving tumor had been destroyed. Another bioluminescence image was taken 18 days after the second PDT treatment verifying the tumor had been completely eradicated. The mouse was monitored for another 140 days, and no tumor recurrence was observed. Later when the mouse was rechallenged with tumor inoculate, it was able to grow a tumor $>800 \text{ mm}^3$, which strongly suggests that the observed tumor cure was attributable to repeated vascular-targeted PDT and not a result of spontaneous tumor regression due to leaky immunity developing in the nude mouse.

The utility of optical reporting tumor models in PDT research has been previously demonstrated by others (19,38,39). We have now developed an A-431 optical reporting tumor model that is particularly useful for evaluating erbB1-targeted therapies. The optical reporting A-431 cells are exceptionally sensitive for detecting and imaging very small numbers of tumor cells in both in vitro assays and in vivo treatment studies. Although the cells express both fluorescent proteins and luciferase, in a whole-body imaging setting they are most sensitively imaged with bioluminescence imaging. Moreover, we observed that bioluminescence imaging allows a quicker and more definitive assessment of tumor response following therapy compared to fluorescent protein imaging. The main reason for this is that firefly luciferase bioluminescence is a reliable and rapid indicator for viable, metabolically-active tumor tissue because oxygen and ATP, two key molecules involved in active metabolic processes, are required for the firefly luciferase bioluminescence reaction. In contrast, PDT-damaged tumor tissue and scabbing can still exhibit significant fluorescent protein contrast until the dead tissue has cleared or sloughed. Chen and colleagues reported the use of a GFP-expressing tumor model to evaluate tumor response to PDT and were able to observe significant changes in tumor GFP brightness after treatment. However, they acknowledged GFP is not the best indicator of tumor viability because sufficient time is required for it to be fully degraded (38).

The present study shows that vascular-targeted PDT is an effective approach for treating the erbB1-overexpressing A-431 tumor model. Vascular-targeted PDT does not directly target erbB1. However, it does effectively treat the tumor vasculature, which is a consequence of the angiogenesis-promoting signaling cascades downstream from erbB receptor stimulation. As noted by many others, the success of PDT is notably improved when used as a repeated regimen. In fact, in our A-431 tumor model, we have observed that repeated vascular PDT treatment is almost always required to achieve complete tumor eradication. The reason vascular PDT is more effective when repeated appears to stem from the fact that tumor vasculature is known to undergo pathologically related disturbances in perfusion efficiency, which can temporarily result in the escape of isolated tumor regions from the therapeutic effects of vascular PDT (19).

In future studies, the optical reporting A-431 tumor model should prove an invaluable tool to assess new erbB1-targeted therapeutics and to develop more effective erbB1-targeted treatment protocols. To this end, we aim to use this model to study the efficacy of new Ac-sPPp immunoconjugates that we have been developing for erbB receptor-targeted PDT. We also plan to use the model to investigate combination therapies involving Ac-sPPp vascular-targeted PDT coupled with anti-erbB MAb adjuvant therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Small animal imager and PDT illuminator. A, full-length view of the small animal imager with a close-up view showing details of the illumination optics, camera, and fluorescence emission filter mounted on the camera zoom lens. B, PDT illuminator with mouse being aligned for treatment. Expanded laser light beam passes down through a blocking screen that shapes the beam spot area to illuminate only the tumor site. See text for further details.

Figure 2. Bioluminescence imaging and fluorescence microscopy of optical reporting A-431 clones. A, bioluminescence images of resuspended cell pellets prepared from the A-431-GFP/luc parental culture and various selected GFP/luc clones, D1–D5. Cell pellets were imaged immediately after resuspension with culture medium containing 150 μ g/ml luciferin. B, fluorescence microscopy of RFP/luc and GFP/luc expressing A-431 clones grown on glass coverslips. Optical reporting clone cells were from ex vivo cultures developed from tumors grown in mice. A-431 parental cells, which do not express fluorescent proteins, are also shown as controls. Images were taken at 100X magnification, and scale bars are 50 μ m.

Figure 3.

Immunofluorescence microscopy of EGFR expression comparing optical reporting clones to the A-431 parental cell line. Optical reporting clone cells were from ex vivo cultures developed from tumors grown in mice. A control image (A-431 parental, no primary) shows that no significant nonspecific background fluorescence is observed when the primary anti-EGFR C225 antibody is omitted from the labeling procedure. Images were taken at 100X magnification, and scale bars are 50 μm .

Figure 4.

Tumor growth curves comparing optical reporting clones to the A-431 parental cell line. A, A-431 parental tumors; B, A-431/G4 tumors; C, A-431/D4 tumors; and D, A-431/D1 tumors. Growth curves were aligned with tumor volume $\sim 150 \text{ mm}^3$ at $t=0$ (indicated by the dashed vertical lines). Data were aligned this way because PDT was typically administered when tumors reached 150 mm^3 (only data for untreated tumors is shown here). Averaged fit curves were calculated assuming exponential growth for tumor sizes in the range of approximately 20 to 500 mm^3 . For the D1 and D4 growth curves, the data are for tumors grown from ex vivo cultured cells derived from previously established tumors. See text for additional details.

Figure 5.

In vivo imaging sensitivity of an optical reporting clone. A, bioluminescence image of a mouse implanted intradermally at multiple sites with A-431/G4 cells (5-minute exposure taken ~30 minutes after i.p. injection of luciferin). Number of cells implanted per site ranged from 10^4 cells (dimkest spot) to 10^6 cells (brightest spot). Bioluminescence imaging was performed immediately after implanting cells. Note: Image shows the log of the bioluminescence pixel values (color scale) to allow clearer viewing of values ranging over several orders of magnitude. B, plot of bioluminescence signal versus number of A-431/G4 cells implanted. Each data point represents the signal average of 3 to 4 separate implant sites, with standard deviations indicated by error bars.

Figure 6. Monitoring PDT efficacy with bioluminescence imaging. A mouse bearing an A-431/G4 tumor grown subcutaneously in the left pectoral region was treated with two rounds of PDT using the vascular targeting photosensitizer Ac-sPPp, and tumor luciferase activity was monitored at various time points before and after treatment. The tumor was $\sim 150 \text{ mm}^3$ prior to treatment. For each round of PDT, the mouse was injected via the tail vein with 60 nmoles PPa content of Ac-sPPp, and then at 16 hours post injection the tumor site was irradiated with 100 J/cm^2 of 670nm light delivered at $\sim 50 \text{ mW/cm}^2$. The first PDT was given on day 1, and the second PDT was given on day 22. Bioluminescence images were taken roughly 30 minutes after i.p. injection of luciferin, and depending on the bioluminescence intensity, image exposures were either 25 s or 300 s (indicated for each image). Note: Images show the log of the bioluminescence pixel values (color scale) to allow clearer viewing of values ranging over several orders of magnitude, and bioluminescence signals were thresholded so only pixels with values greater than 7 are seen.