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A Dose- and Time-Controllable Syngeneic Animal Model of Breast Cancer Microcalcification

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Abstract

Introduction—The development of novel diagnostic agents for the detection of breast cancer microcalcifications requires a reliable animal model. Based on previous work from our group, we hypothesized that a single systemic injection of recombinant bone morphogenetic protein-2 (rBMP-2) could be used to create such a model.

Methods—The cDNA encoding mature human BMP-2 was expressed in BL21(DE3) bacteria, purified to homogeneity, and re-folded as a dimer. Bioactivity was confirmed using a C2C12 alkaline phosphatase assay. rBMP-2 was radiolabeled with ^{99m}Tc and its biodistribution and clearance were quantified after both intravenous (IV) and intraperitoneal (IP) injection. Fischer 344 rats bearing syngeneic R3230 breast tumors received a single intraperitoneal injection of rBMP-2 at a specified dose. Tumor microcalcification was quantified over time using micro single-photon emission computed tomography (SPECT) and micro computed tomography (CT).

Results—rBMP-2 could be expressed in *E. coli* at high levels, isolated at >95% purity, and refolded to a bioactive dimer. Beta-phase half-life was 30.5 min after IV administration and 47.6 min after IP administration. Renal excretion was the primary mode of clearance. A single IP injection of ≥ 50 µg rBMP-2 when tumors were not yet palpable resulted in dose-dependent microcalcification in 8

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COMPETING INTERESTS

Fangbing Liu: None.

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AUTHOR CONTRIBUTIONS

Fangbing Liu: Preparation of all reagents. Conducted all experiments. Wrote first draft of manuscript. Edited final draft of manuscript.
Preeti Misra: ^{99m}Tc-radiolabeling and purification of BMP-2. *In vivo* biodistribution and clearance experiments. Reviewed final draft of manuscript.

Elaine Lunsford: Micro CT and micro SPECT experiments and quantitative analysis of data. Reviewed final draft of manuscript.

Joanne T. Vannah: Micro CT and micro SPECT experiments and quantitative analysis of data. Reviewed final draft of manuscript.

Yuxia Liu: Micro CT and micro SPECT experiments and quantitative analysis of data. Reviewed final draft of manuscript.

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of 8 R3230 tumors. No calcification was found in control tumors, or in normal tissues and organs of animals injected with rBMP-2. Tumor calcification increased progressively between weeks 2 to 4 post-rBMP-2 injection.

Conclusions—A single IP injection of rBMP-2 in rats bearing a syngeneic breast cancer will produce dose-dependent and time-dependent microcalcifications. This animal model lays the foundation for the development of novel diagnostic radiotracers for breast cancer.

Keywords

Breast cancer; microcalcifications; BMP-2; animal models; micro SPECT/CT; micro CT

INTRODUCTION

Presently, breast cancer screening of the general population relies on x-ray mammography. However, x-ray mammography has relatively poor sensitivity, specificity, and positive predictive value (PPV), and is particularly difficult in younger women with dense breasts. The PPV depends on a number of factors, including age, with recent values for screening women ages 40 to 49, 50 to 59, 60 to 69, and over 70 of approximately 22%, 35%, 45%, and 50%, respectively (see [1]). In 30% to 50% of women eventually diagnosed with breast cancer, microcalcification was the key diagnostic feature seen on the mammogram [2]. Because the physical principle underlying x-ray mammography is the linear attenuation coefficient, though, it cannot distinguish among different chemical forms of calcium crystals. Therefore, only crude metrics, such as clustering and crystal size [3] are available to the mammographer to distinguish between benign and malignant calcifications.

Recent advances in breast cancer screening for high-risk populations include magnetic resonance imaging with dynamic contrast-enhancement (DCE-MRI; see, for example, [4]) and positron emission mammography (PEM; [5–7]). Although both methods have demonstrated improved sensitivity over mammography, neither can detect microcalcifications. Yet, microcalcifications can ultimately be found in 88% of non-palpable tumors [8] and a high percentage of interval breast cancers [9]. These reports suggest that microcalcification may actually correlate with breast cancer aggressiveness.

Since MRI provides superb anatomical imaging of the breast, and positron emission imaging systems are already available for functional breast imaging, a major focus of our laboratory and others is the development of novel diagnostic agents to highlight malignant calcifications with high sensitivity and specificity [10–13]. Much of these efforts are focused on the ability of certain bisphosphonates to distinguish between the two major chemical forms of calcium crystals in the breast. Birefringent and colorless type I crystals (calcium oxalate) are found more frequently in benign ductal cysts [2]. Non-birefringent and basophilic type II crystals (calcium hydroxyapatite; HA) are most often seen in proliferative lesions and are associated with malignant cells [14].

The key technology needed to develop positron emission tomography (PET) or PEM radiotracers for improved breast cancer diagnosis is a reliable, reproducible, small animal model that produces spontaneous breast cancer microcalcifications. In 2008, our group published the first such model, which required adenoviral expression of bone morphogenetic protein-2 (BMP-2) under BL2+ conditions [15]. Based on the known biological effects of BMP-2 as an osteo-inductive agent [16], and the results from our prior animal model that suggested BMP-2 could act systemically on breast tumors [15], we hypothesized that it may be possible to produce an animal model of breast cancer microcalcification using only a single injection of recombinant BMP-2 (rBMP-2).

MATERIALS AND METHODS

Expression of rBMP-2 in *E. coli*

The mature human BMP-2 coding sequence was purchased from the American Type Culture Collection (ATCC, Rockville, MD) as a lambda gt10 phage and amplified by the polymerase chain reaction (PCR) using the following primers:

5' primer: 5' GGATAACCATGGCACAAGCCAAACACAAACAGCGGA 3'

3' primer: 5' GGAAACGGATTCCTTAGCGACACCCACAACCCTCCAC 3'

NcoI and BamHI restriction endonuclease recognition sites are shown underlined within the sequences. A start codon (ATG) was contained within the NcoI site and a stop codon (TAA) was introduced immediately prior to the BamHI site. The NcoI and BamHI digested fragment was cloned into pET-15b (Novagen, San Diego, CA) as shown in Figure 1A, and the coding sequence verified by DNA sequencing.

Expression vectors were transformed into competent cells BL21(DE3) bacteria and overnight cultures of single colonies were inoculated with 5 ml of Luria-Bertani broth supplemented with 100 µg/ml ampicillin and grown at 37°C for 4.5 h. Cultures were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Induced cultures were centrifuged at 5000 × g for 20 min and the pellets were suspended in sample buffer or stored at -20°C. Lysates and purified proteins were separated on 4 to 20% gradient gels and stained with Coomassie Brilliant Blue R250 (EMD Chemicals, Gibbstown, NJ). The colony with the highest level of rBMP-2 expression was chosen for subsequent purification.

Purification and Refolding of rBMP-2

Purification and refolding were performed as described previously [17] with slight modifications. Briefly, cells were induced as described above, then the cell pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0) and frozen at -80°C until use. The frozen pellet was resuspended in 20 mM Tris-HCl (pH 8.5), 0.5 mM EDTA, and 2% (v/v) Triton X-100. After vigorous vortexing, the suspension was ultrasonicated for 2 min on ice and centrifuged at 26,000 × g for 30 min at 4°C. This last step was then repeated to yield washed inclusion bodies.

The washed inclusion bodies were solubilized with 6 M guanidinium hydrochloride (Gnd-HCl) and soluble rBMP-2 was refolded for at least 72 h at RT in the presence of 6 M Gnd-HCl, 0.75 M 2-(Cyclohexylamino)ethanesulfonic acid (CHES) buffer, pH 8.5 and 3 mM glutathione (2:1 ratio of reduced:oxidized) with constant stirring. The renaturation mixture was extensively dialyzed against 4 M urea in 20 mM Tris-HCl (pH 8.0) and passed through a 0.22 µm filter (Minisart, Sartorius, Göttingen, Germany). 10 mg of total protein was applied to a 5-ml HiTrap heparin-sepharose column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and equilibrated with 5 column volumes (CV) of dialysis buffer consisting of 4 M urea and 20 mM Tris, pH 8.0. The column was washed again with 5 CV of dialysis buffer, and the monomeric and dimeric forms of rBMP-2 were eluted using 350 mM and 500 mM of NaCl, respectively. The eluted dimer fraction was concentrated to 1 mg/ml, and urea was removed, by repetitive ultrafiltration using a 5,000 MWCO spin cartridge (Vivaspin, Vivascience, Germany) and 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES) buffer, pH 5.0. For long-term storage, rBMP-2 was freeze-dried in 50 mM MES (pH 5.0) without loss of biological activity.

Measurement of rBMP-2 Biological Activity

C2C12 cells from the ATCC were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine at 37°C in a humidified atmosphere with 5% CO₂. The alkaline phosphatase (ALP) activity assay has been described in detail previously [18]. Briefly, 3×10^4 cells per well were plated in 96-well plates (Nunc, Roskilde, Denmark) and cultured in 2% FBS, 1% L-glutamine and rBMP-2 at the concentration specified. rBMP-2 from a commercial source (R&D Systems, Minneapolis, MN) was used as a positive control. After 72 h, cells were washed with phosphate buffered saline (PBS), pH 7.4 and alkaline phosphatase activity of cell lysates was measured on a Versamax (Molecular Devices, Sunnyvale, CA) absorbance plate reader using pNPP (Sigma-Aldrich) as the substrate.

Radiolabeling of rBMP-2

The N-hydroxysuccinimide (NHS) ester of [^{99m}Tc-MAS₃] was prepared with high radiochemical purity (>99%) and high specific activity ($\approx 6,675$ Ci/mmol) in dimethyl sulfoxide (DMSO) using solid-phase pre-loading technique within 20 min as described in detail previously [19]. For radiolabeling, 1 nmol (12.5 µg) of rBMP-2 as a dried powder was resuspended in 50 µL of DMSO. Of note, rBMP-2 resuspended in DMSO retained its bioactivity after dilution into aqueous buffer (data not shown). Conjugation was performed by the addition of 500 µL of [^{99m}Tc-MAS₃]-NHS (4 mCi, 0.36 nmol) to rBMP-2 in DMSO, followed by 20 µmol of triethylamine from a 4 M stock, and constant stirring at RT for 1 h. [^{99m}Tc-MAS₃]-rBMP-2 was purified to homogeneity by gel-filtration chromatography using a 8×300 mm, 60 Å Diol (Catalog #DL06S053008WT; YMC, Kyoto, Japan) gel-filtration column and PBS, pH 7.4 as mobile phase. Purified [^{99m}Tc-MAS₃]-rBMP-2 was concentrated to 1 mCi/ml using a 3,000 MWCO Vivaspinn cartridge prior to injection.

Syngeneic Rat Model of Breast Cancer Microcalcification

Animals were used under the supervision of an approved institutional protocol. Female Fischer 344 rats were from Taconic Farms (Hudson, NY). At the time of injection, rats averaged 7 to 9 weeks of age and weighed $130 \text{ g} \pm 20 \text{ g}$. For tumor cell inoculation, rats were anesthetized with 2% isoflurane/balance O₂. R3230 cells adapted to cell culture [15] were grown in DMEM supplemented with 10% FBS and antibiotics (penicillin [100 U/ml] and streptomycin [100 mg/ml]). After trypsinization, $\approx 2 \times 10^7$ cells in 0.3 mL DMEM were injected subcutaneously into the fat pad. Bioactive rBMP-2 (10 µg, 50 µg, and 100 µg) or vehicle control (PBS) was administered as a single intraperitoneal (IP) injection into R3230 tumor-bearing rats (n = 4 animals per group) at 4 days post-tumor cell inoculation. Resected tumors were fixed in formalin, processed for histology, and stained with hematoxylin and eosin (H&E) or by the method of von Kossa [20].

Biodistribution and Clearance of [^{99m}Tc-MAS₃]-rBMP-2

100 µCi [^{99m}Tc-MAS₃]-rBMP-2 was injected IV or IP into groups of four R3230-bearing Fischer 344 rats. Blood samples were collected at 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h. Rats were sacrificed at 4 h post-injection, total body radioactivity was measured in a dose calibrator (Capintec, Ramsey, NJ), then tumor, organs, and tissues were dissected, rinsed briefly with saline, and weighed. Radioactivity was quantified using a model 1470 Wallac Wizard (Perkin Elmer, Wellesley, MA) ten-detector gamma counter.

Multi-Modality Imaging of Breast Cancer Microcalcification

Tumor growth and tumor calcification were assessed weekly using micro SPECT/CT. 1 mCi of ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) in 0.2 ml saline was injected intravenously 4 h before imaging. Animals were anesthetized with 2% isoflurane/balance O₂. Imaging was

performed on a NanoSPECT/CT (Bioscan, Washington, DC) scanner equipped with an 8W x-ray source running at 65 kV (123 mA), and a 48 μm pitch CMOS-CCD x-ray detector. Continuous helical micro CT scanning was employed with the following parameters: 1 sec exposure, 240 angles, 1.3 magnification, 37 mm pitch (1 field-of-view), and a 512×256 pixel frame size (192 μm pixels). Images were reconstructed as 170×170 pixel transverse matrices with varying axial length and slice thickness of 0.4 mm (isotropic voxel size 0.4 mm) using filtered-back projection (SheppLogan filtering). Helical micro SPECT was performed using a four-headed gamma camera outfitted with multi-pinhole collimators having 2.5 mm diameter pinholes (36 total). Images were acquired over 360° in 48 projections of 50 sec each using a 256×256 frame size (1.0 mm pixels). The micro-SPECT images were reconstructed as 86×86 pixel transverse matrices with varying axial length and slice thickness of 0.8 mm (isotropic voxel size 0.8 mm). Quantitation of tumor volume, percent calcification, and $^{99\text{m}}\text{Tc}$ -MDP uptake was performed using InVivoScope software (Bioscan).

Statistical Analyses

All experiments were repeated at least twice. A Student's t-test was used to examine the differences between the experimental groups.

RESULTS

Recombinant Protein Expression, Refolding, Purification, and Bioactivity of BMP2

The 13 kDa mature fragment (amino acids 283 to 396) of rBMP-2 (Figure 1A) was initially expressed as an insoluble protein in inclusion bodies, but could be refolded to a bioactive dimer and purified to homogeneity using heparin-sepharose chromatography (Figure 1B). As judged by non-reducing gels (Figure 1B), our purification protocol resulted in >90% dimer and >95% overall protein purity. Refolded dimeric protein maintained bioactivity, as assayed by its ability to induce alkaline phosphatase in C2C12 cells (Figure 1C).

Biodistribution and Clearance of rBMP-2

rBMP-2 was radiolabeled with $^{99\text{m}}\text{Tc}$ and purified to $\geq 95\%$ to achieve a final specific activity of 1,987 Ci/mmol (data not shown). After IV or IP injection, [$^{99\text{m}}\text{Tc}$ -MAS₃]-rBMP-2 exhibited a beta-phase elimination half-life in blood of 30.5 min and 47.6 min, respectively (data not shown). Clearance from the body was primarily through excretion into urine. At 4 h post-injection, 44% of the IV injected dose and 70% of the IP injected dose remained in the body.

Distribution in major organs and tissues is shown in Figure 2. After IV injection, the only statistically significant uptake above background was seen in kidney and stomach. After IP injection, spleen, liver, stomach, and kidney exhibited high uptake, consistent with absorption of [$^{99\text{m}}\text{Tc}$ -MAS₃]-rBMP-2 into bowel lymphatics. Tumor uptake of systemically administered BMP-2 did not differ from background (Figure 2).

Time- and Dose-Controllable Microcalcification of Breast Cancer after a Single IP Dose of rBMP-2

R3230 cells inoculated into the mammary pad of syngeneic Fischer 344 rats develop reproducible, histology-confirmed breast tumors over the course of 4 weeks, with exponential growth during weeks 2 to 4 after inoculation (Figure 3). There were no statistically significant differences in tumor volume over time in animals injected IP with 0, 10, 50, or 100 μg rBMP-2 (Figure 3).

Because of prolonged blood half-life and body retention, IP injection was chosen over IV injection to test the effect of rBMP-2 on breast cancer microcalcification. A single IP injection of ≥ 50 μg rBMP-2 resulted in dose- and time-dependent calcification of all breast tumors (n

= 4 animals per group; 8 animals total) by 4 weeks post-injection (Figure 3), with a statistically significant difference between 50 µg and 100 µg doses seen by both CT ($p = 0.008$) and SPECT ($p = 0.002$). Minimal calcification also developed in 1 of 4 rats administered the 10 µg dose.

In the absence of rBMP-2, tumor calcification was not detectable by CT. Breast tumor microcalcifications were confirmed by both micro CT and ^{99m}Tc -MDP micro SPECT imaging (Figures 3 and 4A), although a false-positive ^{99m}Tc -MDP signal was occasionally seen in large necrotic tumors, likely due to altered perfusion. Interestingly, full body scanning from nose to tail did not reveal any microcalcification in normal tissues or organs (Figure 4A and data not shown) suggesting that R3230 cells were uniquely capable of inducing microcalcification after stimulation with rBMP-2. Histological analysis confirmed the presence of malignant cells and tissue microcalcification (Figure 4B).

DISCUSSION

Breast cancer microcalcification is prevalent and clinically important. Until now, though, there hasn't been a syngeneic small animal model of the process that could be used to develop improved diagnostic agents. In particular, our interest and that of many groups is in the development of positron emission tomography (PET) radiotracers for use in conjunction with MRI. In this scenario, PET imaging would complement anatomic (MRI) and functional (DCE) information to improve sensitivity and diagnostic accuracy. Our group has already described near-infrared fluorescent [11–13,21] and ^{99m}Tc -labeled [10] pamidronate derivatives with ≥ 8 -fold specificity for the malignancy-associated calcium salt HA over the other endogenous calcium salts calcium oxalate, calcium carbonate, calcium phosphate, and calcium pyrophosphate. We also confirmed that BMP-2 induced calcification in R3230 tumors was primarily HA [15]. The availability of the animal model we describe in this study will greatly expedite PET agent development since microcalcifications can be induced with only a single IP injection, and their abundance and timing controlled precisely.

It is unknown at present whether the biological effect we see with rBMP-2 is from the dimeric ($\approx 90\%$ of total) or monomeric ($\approx 10\%$ of total) form. Although the dimeric form is felt to be the most bioactive [22,23], trace levels of monomer are always present in the rBMP-2 preparation. Regardless, the ability to produce large quantities of rBMP-2, freeze it, and create the model with only a single IP injection eliminates the major problems with our previously described animal model [15]. In particular, it eliminates the need to generate large quantities of adenovirus, to transduce R3230 cells under BL2+ conditions, and to grow tumors under BL2+ conditions.

This study highlights the fact that there appears to be something special about R3230 tumors or their microenvironment, such that they are poised to produce microcalcifications after only a single stimulus. Our results are consistent with a study showing significant differences in gene expression between single and continuous application of BMP-2 to MCF-7 cells *in vitro* [24]. Our results are also consistent with a recent study suggesting that an excess of BMP-2 relative to matrix Gla protein (MGP) results in trans-differentiation of smooth muscle cells to osteochondrogenic precursors, leading to vasculature calcification [25]. An unanswered question in our animal model, now the subject of ongoing studies, is whether tumor microcalcification is arising from alterations in malignant epithelial cells or normal mesenchymal cells in the tumor stroma.

Another unanswered question is whether similar bioactivity of rBMP-2 would be seen in humans. Recently, recombinant BMP-2 has been used in large clinical trials to assess its efficacy in spinal fusion [26]. By performing pre- and post-trial mammograms, such trials may offer an opportunity to determine whether BMP-2 induces microcalcification in otherwise

occult breast cancers. If so, a single pre-mammogram BMP-2 injection could be used as an adjunct to improve sensitivity of the test. Of course, such use of BMP-2 also needs to be considered in the context of studies suggesting that BMP-2 expression in breast cancer can lead to increased invasion, proliferation, and a more aggressive phenotype [27–30].

CONCLUSIONS

We have developed a straightforward syngeneic animal model of breast cancer microcalcification that requires only a single injection of recombinant BMP-2, and which permits precise adjustment of the level of calcification over time.

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ABBREVIATIONS

%ID	Percent injected dose
%ID/g	Percent injected dose per gram of tissue
aa	Amine acids
ALP	Alkaline phosphatase
BMP-2	Bone morphogenetic protein-2
CT	Computed tomography
CV	Column volumes
DCE	Dynamic contrast-enhanced
DMEM	Dulbecco's modified Eagle's medium
DMSI	Dimethyl sulfoxide
FBS	Fetal bovine serum
Gnd-HCl	Guanidinium hydrochloride
HA	Hydroxyapatite
H&E	Hematoxylin and eosin
IP	Intraperitoneal
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IV	Intravenous
MDP	Methylene diphosphonate
MIP	Maximal intensity projection
MRI	Magnetic resonance imaging
NHS	N-hydroxysuccinimide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEM	Positron emission mammography
PET	Positron emission tomography

PPV	Positive predictive value
rBMP-2	Recombinant bone morphogenetic protein-2
SPECT	Single-photon emission computed tomography

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Figure 1. Expression, Purification, and Bioactivity of Recombinant BMP-2

A. Crystal structure of amino acids (aa) 283 to 396 of human BMP-2 at 2.7 Å resolution [31]. Lysine residues available for conjugation to [^{99m}Tc -MAS $_3$]-NHS are indicated. The bacterial expression construct used for recombinant protein expression is also shown.

B. Purification of rBMP-2 from bacterial lysates. Reduced (lanes 1–4) and non-reduced (lane 5) Coomassie blue-stained reduced SDS-PAGE gel. Lane 1: crude extract of bacteria expressing pET-15b/rBMP-2 and induced with 1 mM IPTG at 37°C for 4 h; 2: crude extract of bacteria expressing the empty vector pET-15b and induced with 1 mM IPTG at 37°C for 4 h; 3: 200 ng of rBMP-2 after heparin column purification; 4, 400 ng of rBMP-2 after heparin column purification. 5 = 1 µg of rBMP-2 after heparin column purification. Molecular weight markers (in kDa) are shown at left. M = monomer. D = dimer.

C. Alkaline phosphatase activity of C2C12 cells stimulated for 72° with increasing concentrations of rBMP-2.

Figure 2. Biodistribution of [^{99m}Tc -MAS₃]-rBMP-2 in Tumor-Bearing Rats at 4 h Post-Injection
Shown are the percent injected dose per gram (%ID/g) for the major tissues and organs of R3230 tumor-bearing Fischer 344 rats after intravenous (top) or intraperitoneal (bottom) injection of [^{99m}Tc -MAS₃]-rBMP-2.

Figure 3. Dose- and Time-Dependence of Breast Tumor Growth and Microcalcification
 Shown are tumor volume (mean $\pm$ SEM; top), tumor calcification (as a percentage of total volume; mean $\pm$ SEM; middle), and uptake of ^{99m}Tc-methylene diphosphonate (Tc-99m-MDP; mean $\pm$ SEM; bottom) for groups of n = 4 rats receiving a single injection of rBMP-2, at the dose indicated, 4 d after tumor inoculation.

Figure 4. Macroscopic and Microscopic Imaging of R3230 Syngeneic Breast Cancers in the Presence and Absence of rBMP-2

A. Micro SPECT/CT imaging of tumor-bearing rats in the absence (top; Control) and presence of rBMP-2 (bottom). Tumors (arrowheads) were grown for a total of 4 weeks, with a single injection of rBMP-2 given to the experimental group 4 d after tumor inoculation. Shown are the maximal intensity projection (MIP) micro CT images (left), micro SPECT images (middle),

and micro SPECT/CT fusion images (right). CT and SPECT images have identical acquisition parameters and normalizations. Images are representative of $n = 4$ animals per group.

B. Histological analysis of R3230 tumors. Shown are H&E staining (top) and von Kossa staining (bottom) of the tumors shown in Figure 3A. Magnification = 400X.