The systemic delivery of an oncolytic adenovirus expressing decorin inhibits bone metastasis in a mouse model of human prostate cancer

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In an effort to develop a new therapy for prostate cancer (PCa) bone metastases, we have created Ad.dcn, a recombinant oncolytic adenovirus carrying the human decorin gene. Infection of PC-3 and DU-145, the human prostate tumor cells, with Ad.dcn or a non-replicating adenovirus Ad(E1-).dcn resulted in decorin expression; Ad.dcn produced high viral titers and cytotoxicity in human prostate tumor cells. Adenoviral-mediated decorin expression inhibited Met, the Wnt/β-catenin signaling axis, vascular endothelial growth factor A, reduced mitochondrial DNA levels and inhibited tumor cell migration. To examine the antitumor response of Ad.dcn, PC-3-luc cells were inoculated in the left heart ventricle to establish bone metastases in nude mice. Ad.dcn, in conjunction with control replicating and non-replicating vectors were injected via tail vein. The real-time monitoring of mice, once a week, by bioluminescence imaging and X-ray radiography showed that Ad.dcn produced significant inhibition of skeletal metastases. Analyses of the mice at the terminal time point indicated a significant reduction in the tumor burden, osteoclast number, serum tartrate-resistant acid phosphatase 5b levels, osteocalcin levels, hypercalcemia, inhibition of cancer cachexia and an increase in the animal survival. Based on these studies, we believe that Ad.dcn can be developed as a potential new therapy for PCa bone metastasis.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men in the United States.1 Following initial standard treatments and subsequent androgen-deprivation therapy, many patients develop castration-resistant prostate cancer. During its advanced stage, PCa often metastasizes to bone, producing bone pain and spinal cord compression, and resulting in high morbidity and mortality.2 Existing therapies for the advanced PCa and bone metastases are only palliative in nature.3–8 Two currently available bone-protecting modalities, denosumab, an antibody against the receptor activator of nuclear factor kappa-B ligand, and bisphosphonates are quite effective in bone metastasis.16,17 Towards that end, we have now created Ad.dcn, an oncolytic adenovirus carrying the decorin gene. Decorin is a small leucine-rich proteoglycan, and low levels of decorin are generally considered to be a poor prognostic marker of PCa.18,19 Decorin is an attractive candidate as decorin protein can target and suppress multiple tyrosine kinase receptors, including Met, the Wnt/β-catenin signaling pathway, and effectively inhibit the angiogenic network.18,20–23 of all which are known to promote PCa tumorigenesis and bone metastases.24–27 Interestingly, decorin can also target the tumor-bone microenvironment and inhibit osteoclastogenesis, and promote the osteoblastogenesis.28,29 The systemic delivery of decorin protein, or local delivery of adenoviral vectors expressing decorin, can inhibit tumor growth.30–32 However, the systemic application of oncolytic adenoviruses expressing decorin for targeting bone metastases has not been previously investigated. In this report, we describe the construction of Ad.dcn and in vitro studies evaluating its replication potential and its ability to produce functional decorin in prostate tumor cells. We further describe the effect of systemic administration of Ad.dcn to inhibit PCa bone metastases and tumor-induced bone destructions in a mouse model. Based on our results described here, we believe that Ad.dcn can be potentially...
developed as an antitumor agent for robust targeting of PCa bone metastases.

RESULTS

Construction of Ad.dcn, Ad.dcn replication, viral-induced cytotoxicity and decorin production in the prostate tumor cell lines

Ad.dcn, a recombinant oncolytic adenovirus containing the decorin gene, and Ad(E1-).dcn, a non-replicating adenovirus containing the decorin gene, were created as described in the Materials and Methods. The schematic diagrams of Ad.dcn, Ad(E1-).dcn, Ad.luc (an oncolytic adenovirus carrying luciferase 2 gene) and Ad(E1-).luc (a non-replicating adenovirus carrying luciferase 2 gene) are shown in Figure 1a. The replication potential and the viral-induced cytotoxicity of the Ad.dcn and Ad(E1-).dcn, along with Ad.luc, Ad(E1-).luc and Ad(E1-).null were determined in two human prostate tumor cell lines, PC-3 and DU-145, and in a mouse prostate tumor cell line TRAMP-C2. Viral titers of Ad.dcn and Ad.luc were ~2000-times higher than those of replication-deficient Ad(E1-).null, Ad(E1-).dcn and Ad(E1-).luc in PC-3 cells and DU-145 cells (Figure 1b). Ad.dcn and Ad.luc produced a similar dose-dependent cytotoxicity in PC-3 cells (Figure 1c) and in DU-145 cells (Figure 1d). In TRAMP-C2 cells, minimum viral replication

Figure 1. Schematic diagrams of adenoviral vectors, viral replication, viral-induced cytotoxicity and protein expression in prostate tumor cell lines. (a) Schematic diagram of adenoviral constructs of Ad.dcn, Ad(E1-).dcn, Ad.luc and Ad(E1-).luc. Ad.dcn and Ad.luc have two small deletions, 01/07 (amino acids 4–25, and amino acids 111–123) in the E1A region, have deletion in E3 but contains ADP (adenoviral death protein). Ad(E1-).dcn, and Ad(E1-).luc are E1 and E3 minus. The maps are not drawn to scale. CMVp: CMV promoter, SV40pA: SV40 polyA, ITR: inverted terminal repeats. (b) Adenoviral replication in prostate tumor cells. Tumor cells were infected with 2.5 × 10⁴ VPs per cell for either 3 or 48 h. The viral burst sizes were obtained in HEK293 cells, and the ratios of the burst sizes of 48 and 3 h samples were calculated and are shown (c, d) Adenoviral-induced cytotoxicity in PC-3 and DU-145 cells. Cells were exposed to various doses of the viral vectors (in the range of 0.32 × 10²–1.25 × 10⁶ VPs/cell) for 7 days, and the viral-induced cytotoxicity were measured by staining the cells with the sulforhodamine B (e, f) Adenoviral-mediated decorin expression in prostate tumor cells. Tumor cells were exposed to various adenoviral vectors (2.5 × 10⁴ VPs per cell) for 48 h (first 24 h in the medium containing serum, and the second 24 h in the media without serum). The cell lysates and the media were subjected to western blot analyses for decorin (e), the cell lysates were subjected to western blot analyses for actin (e) and the media were subjected to ELISA for decorin expression (f) as described in Material and Methods.
Infection of the prostate tumor cell lines with Ad.dcn or Ad(E1-).dcn produced decorin protein, which was detected in both the cell lysates, and in the extracellular media (Figure 1e). The amounts of decorin protein released in the media from Ad.dcn and Ad(E1-).dcn-infected cells were similar (in the range of 1–4μg ml\(^{-1}\)) (Figure 1f). These results suggest that Ad.dcn can replicate and produce cytokoty in human prostate tumor cells, and that both Ad.dcn and Ad(E1-).dcn produce decorin protein in prostate tumor cells.

Adenoviral-expressed decorin reduces Met, β-catenin and vascular endothelial growth factor A expression, and migration of human prostate tumor cells
To examine if the decorin protein produced by the recombinant adenoviral vectors is functionally active, PC-3 cells were infected with Ad(E1-).dcn, and analyzed for multiple known target genes, MET, CTNNB1 (catenin (cadherin-associated protein) beta 1), and vascular endothelial growth factor A (VEGFA), by quantitative PCR (qPCR). The results indicated that Ad(E1-).dcn infection resulted in a significant downregulation of MET (P<0.01), CTNNB1 (P<0.001) and VEGFA (P<0.01) at the mRNA level (Figure 2a) following authentication of robust decorin mRNA expression. The Ad(E1-).dcn infection also resulted in significant reductions of Met, β-catenin and VEGFA protein expression (P<0.001) (Figures 2b and c). Ad(E1-).dcn infection of PC-3 cells also reduced mtDNA levels (P<0.01) (Figure 2d), indicating the induction of decorin-induced mitochondrial autophagy. Moreover, decorin containing conditioned media (about 3μg ml\(^{-1}\)) inhibited cell migration in a transwell migration assay (P<0.01) (Figure 2e). In a wound-healing assay, the decorin containing media also showed significant reductions in the wound areas filled at 16h (P<0.01), and at 24h (P<0.001) (Figure 2f). These results indicate that adenoviral-mediated decorin production is functionally and biologically active.

Systemic administration of Ad.dcn inhibits the progression of established PCa bone metastases
Next, the antitumor effects of Ad.dcn in a PCa bone metastasis model were examined. PC-3-luc cells were inoculated into the left heart ventricle of male nude mice. The mice were subjected to the healing assay, the decorin containing media also showed significant reductions during the earlier time points,17,33 the BLI of Ad.luc and Ad(E1-).luc were also effective (P<0.001 vs buffer), and produced better responses than Ad.luc (P<0.01 Ad.luc vs buffer; P<0.05 Ad.dcn vs Ad.luc) or Ad(E1-).dcn (P<0.05 Ad(E1-).dcn vs buffer; P<0.01 Ad.dcn vs Ad(E1-).dcn). Moreover, on day 60, only the Ad.dcn treatment resulted in significant inhibition of tumor size (P<0.05) (Figure 4c), and produced a significant number of skeletal tumor-free mice (P<0.05) (Figure 4d). There was no significant inhibition of tumor growth by Ad(E1-).luc treatment. These results suggest that Ad.dcn, Ad.luc and Ad(E1-).dcn treatments all resulted in the inhibition of bone metastases as examined by the BLI and X-ray radiography, and among the three vectors, Ad.dcn is the most effective in inhibiting skeletal metastases.

Systemic administration of Ad.dcn reduces tumor burden, inhibits bone destruction and increases animal survival
The tumor burden at the terminal time point (day 62) was examined by histomorphometric analyses. The representative hematoxylin and eosin staining ofibia and femur median sagittal sections from each group are shown in Figure 5a. The tumor areas were outlined with a yellow line (Figure 5a) and quantified. The buffer group had a large tumor burden, and the treatment of mice with Ad.dcn resulted in a significant reduction in tumor burden (P<0.05) (Figure 5b) and produced a significant tumor-free incidence (P<0.05) (Figure 5c). Analysis of serum decorin protein levels (on day 62) indicated that both the Ad.dcn and Ad(E1-).dcn-treated groups contained decorin, whereas no decorin protein was detected in the other treatment groups (Figure 5d).

The osteolytic bone destruction in the distal femur was further examined by synchrotron micro computed tomography. The reconstructed slices near the growth plate region and 1.45 mm below the growth plate showed extensive trabecular and cortical bone destruction in the buffer and Ad(E1-).luc-treated groups (Figure 5e, top two panels). The 3D renderings of the bone volume also showed lesions in the buffer and Ad(E1-).luc groups (Figure 5e, bottom panel). However, normal slices and bone architecture were observed in the bones examined from Ad.dcn, Ad(E1-).dcn and Ad.luc-treated groups (Figure 5e).

To further evaluate the effects of adenoviral vectors on bone resorption/formation, several relevant biomarkers were examined. These included tartrate-resistant acid phosphatase-positive osteoclasts in the bone/tumor interface and serum tartrate-resistant acid phosphatase 5b (TRACP 5b) levels as indicators of osteolytic bone destruction, serum osteocalcin as a marker of bone turn over, and serum calcium levels as an indicator of bone destruction. Representative bone samples stained for tartrate-resistant acid phosphatase-positive multinucleated osteoclasts along the bone/tumor interface are shown in Figure 6a, with arrows pointing to the multinucleated mature osteoclasts. Among all the treatment groups, only Ad.dcn treatment resulted in a significant reduction in the osteoclast number, compared with the buffer group (P<0.05) (Figure 6b). Treatment with Ad.dcn, Ad.luc, or Ad(E1-).dcn resulted in significant reductions in the TRACP 5b levels (Ad.dcn vs buffer P<0.001; Ad(E1-).dcn vs buffer P<0.01; Ad.luc vs buffer P<0.01) (Figure 6c). Ad.dcn, Ad(E1-).dcn and Ad. luc treatments also resulted in the reduction of serum osteocalcin levels; Ad.dcn and Ad.luc treatments were relatively more effective.

No significant inhibition of fold-increases was observed by the Ad(E1-).luc treatment.
Bone metastases were further examined using radiographic measurements taken weekly from day 21 onwards. The representative X-ray images on day 21, day 42 and day 60 from each group are shown in Figure 4a. Osteolytic lesions on the skeletal tumors are marked by yellow arrows. The skeletal tumor sizes were measured in both hind limbs of each mouse, over the course of the study. As shown in Figure 4b, Ad.dcn, Ad(E1-).dcn and Ad. luc treatments resulted in the inhibition of tumor progression. However, the Ad.dcn group was the most effective (P<0.001 vs buffer), and produced better responses than Ad.luc (P<0.01 Ad.luc vs buffer; P<0.05 Ad.dcn vs Ad.luc) or Ad(E1-).dcn (P<0.05 Ad(E1-).dcn vs buffer; P<0.01 Ad.dcn vs Ad(E1-).dcn). Moreover, on day 60, only the Ad.dcn treatment resulted in significant inhibition of tumor size (P<0.05) (Figure 4c), and produced a significant number of skeletal tumor-free mice (P<0.05) (Figure 4d). There was no significant inhibition of tumor growth by Ad(E1-).luc treatment. These results suggest that Ad.dcn, Ad.luc and Ad(E1-).dcn treatments all resulted in the inhibition of bone metastases as examined by the BLI and X-ray radiography, and among the three vectors, Ad.dcn is the most effective in inhibiting skeletal metastases.

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(P < 0.001) than Ad(E1-).dcn (P < 0.001) (Figure 6d). Ad.dcn treatment was effective in inhibiting hypercalcemia (P < 0.001), although both Ad.luc (P < 0.01) and Ad(E1-).dcn (P < 0.05) treatments were also highly effective in reducing calcium levels (Figure 6e). Ad(E1-).luc treatment had no significant effect in the assays conducted above (osteoclast number, TRACP 5b, osteocalcin and calcium levels) (Figures 6b–e).

Animal body weight was monitored weekly, as an indicator of cancer cachexia. In the buffer group, the mice began to lose body weight from day 51 onwards. By day 62, there was a significant reduction in the body weight of mice in the buffer group compared with the normal mice (P < 0.01) and the Ad.dcn treatment group (P < 0.05) (Figure 6f); only the Ad.dcn treatment group produced a significant survival advantage over the buffer group.
The major finding of our study is that the systemic delivery of Ad.dcn, an oncolytic adenovirus expressing decorin, is effective in inhibiting skeletal metastases and the bone destruction in a mouse model of human PCa. In multiple assays, the Ad.dcn was more potent than both Ad(E1-).dcn, a non-replicating adenovirus expressing decorin, and Ad.luc, a control oncolytic adenovirus (Table 1). Thus, adenoviral replication, coupled with concomitant decorin production in Ad.dcn, is critical in producing strong antitumor responses and a significant number of tumor-free mice, and inhibiting bone destruction, resulting in an increase in the animal survival.

Based on our findings, we propose the following model of Ad.dcn-mediated inhibition of bone metastases. Upon intravenous delivery of Ad.dcn in skeletal tumor-bearing mice, the virus is taken up by the skeletal tumors producing viral replication and tumor-destruction. Ad.dcn-infected tumor cells produce decorin, which is released into the tumor microenvironment, and targets group (as measured by the loss of 10% body weight) \( P < 0.05 \) (data not shown).

**DISCUSSION**

The major finding of our study is that the systemic delivery of Ad.dcn, an oncolytic adenovirus expressing decorin, is effective in inhibiting skeletal metastases and the bone destruction in a mouse model of human PCa. In multiple assays, the Ad.dcn was more potent than both Ad(E1-).dcn, a non-replicating adenovirus expressing decorin, and Ad.luc, a control oncolytic adenovirus (Table 1). Thus, adenoviral replication, coupled with concomitant decorin production in Ad.dcn, is critical in producing strong antitumor responses and a significant number of tumor-free mice, and inhibiting bone destruction, resulting in an increase in the animal survival.

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multiple tumor and stromal components, and interrupts the vicious cycle between tumor cells and the surrounding stromal cells, resulting in the inhibition of tumor growth and tumor-induced bone destruction. In support of this model, we have shown that the infection of prostate tumor cells with Ad.dcn resulted in viral replication, tumor cell killing and decorin production, which is released into the extracellular compartment. The prostate tumor cells ectopically expressing decorin responded in an autocrine manner, resulting in the downregulation of several tumor/bone metastases promoting decorin targets. These targets included MET, which encodes for a tyrosine kinase receptor that is often activated in PCA; CTNNB1, which encodes for β-catenin, the aberrant expression of β-catenin is associated with the prostate tumor growth and metastases; and VEGFA, the protein product of which is known to promote angiogenesis. The Ad.dcn-mediated decorin released in tumor microenvironment will also target the osteoclasts and the osteoblasts and inhibit osteoclast activity and promote the osteoblastogenesis. Ad.dcn-mediated decorin production was shown to inhibit the prostate tumor cell migration, a step that is necessary to establish the skeletal metastases. Interestingly, decorin expression in prostate tumor cells also resulted in decreased mtDNA content, a marker for mitochondrial turnover and degradation, consistent with the earlier studies in which soluble decorin was shown to evoke mitochondrial autophagy (mitophagy) in breast carcinoma cells via a novel mitophagic effector, mitostatin. Ad.dcn-induced autophagy of the prostate tumor cells in vivo could potentially stimulate the cell-mediated immune responses against the tumor cells and enhance the antitumor-responses. The ability of Ad(E1-).dcn to inhibit bone metastases, albeit weaker than Ad.dcn, is consistent with the proposed model, and are in agreement with the previous studies in which decorin was shown to inhibit the tumor growth of colon and breast carcinoma.

Although our in vitro experiments and the in vivo studies, describing the antitumor responses of Ad.dcn, corroborate with our proposed model, we realize that some of the steps need to be investigated further in vivo in a bone metastasis model. It will be also interesting to examine if the vector-mediated decorin expression can also enhance the intratumoral adenoviral spread, and hence its oncolytic potential, as previously suggested. As the human adenoviruses replicate poorly in mouse prostate...
tumor cells studied here, we need to identify an immune-competent syngeneic mouse bone metastases tumor model to examine the antitumor responses of Ad.dcn, and to examine additional immunotherapy strategies to further enhance the antitumor responses of Ad.dcn. Given that Ad.dcn can directly kill cancer cells and can simultaneously target multiple tumor-promoting signaling pathways, we believe that Ad.dcn can be potentially developed for the treatment of PCa skeletal metastases.

MATERIALS AND METHODS

Cell lines and adenoviruses

Human prostate tumor cell lines, PC-3 and DU-145, and a mouse prostate tumor cell line TRAMP-C2 were obtained from ATCC (Manassas, VA). PC-3-luc cell line was kindly provided by Kenneth Pienta (University of Michigan, Ann Arbor, MI, USA). All prostate tumor cell lines were maintained in RPMI-1640 media containing 10% fetal calf serum. To create Ad.dcn, the decorin gene was cloned in a shuttle vector and subjected to homologous recombination with adenoviral genomic DNA derived from adenoviral mutant dl01/07, using published methods. To create Ad(E1-).dcn, an AdEasy system was used for homologous recombination. Ad(E1-).null (the non-replicating adenovirus without any foreign transgene), Ad(E1-).luc (the non-replicating adenovirus containing firefly luciferase 2 gene) and Ad.luc (the conditionally replicating adenovirus containing firefly luciferase 2 gene) have been previously described. All adenoviral vectors were amplified in HEK293 cells (ATCC), and purified as described earlier. Adenoviral replication and cytotoxicity assays

Tumor cells were exposed to viral vectors (2.5 x 10⁸ VPs per cell) for 3 or 48 h, and the viral burst sizes in HEK293 cells as a indicators of viral replication were measured using Adeno-X Rapid Titer Kit (Clontech, Mountain view, CA, USA) as described previously. For cytotoxicity assays, cells were exposed to various concentrations of viral vectors for 7 days, and cell survival was examined using the sulforhodamine B staining method as described earlier. Adenoviral-mediated decorin expression

Cells were plated in six-well dishes (5 x 10⁶ cells per well). The following day, cells were infected with viral vectors (2.5 x 10⁸ VPs per cell). After 24 h,
the media was changed to serum-free media, and the incubations continued for another 24 h. The media and the cell lysates were subjected to western blot analyses using published methods except that the blots were probed with antibodies against human decorin (R&D systems, Minneapolis, MN, USA). Decorin levels in the media were examined by enzyme-linked immunosorbent assay using mouse anti-human decorin (R&D systems) and biotinylated mouse anti-human decorin (R&D systems), using a previously described method.

Gene expression analysis of canonical decorin target genes following infection with Ad(E1-).dcn
PC-3 cells were infected with either Ad(E1-).null or Ad(E1-).dcn (2.5 × 10^3 VPs per cell). To perform quantitative RT-PCR (qPCR) of decorin and decorin-regulated genes, MET, CTNNB1, and VEGFA, RNA were harvested and cDNA libraries generated after 48 h post infection. All samples were subjected to a DNase I digestion to eliminate potentially contaminating genomic DNA and viral plasmids prior to cDNA synthesis and qPCR analysis. ACTB served as the endogenous housekeeping gene. Fold changes were determined by the comparative ΔΔCt method.

Mitochondrial DNA (mtDNA) analysis following infection with Ad(E1-).dcn
PC-3 cells were infected with Ad(E1-).null or Ad(E1-).dcn (2.5 × 10^3 VPs per cell) for 48 h. Genomic DNA (gDNA) and mtDNA were isolated and interrogated via qPCR, as done previously, with mitochondrial encoded NADH dehydrogenase I serving as a mtDNA marker, and lipoprotein lipase (LPL) for gDNA analysis and normalization. Reported fold changes were determined by the comparative ΔΔCt method.

Transwell migration and wound-healing assays
PC-3 cells were infected with 2.5 × 10^4 VPs per cell of Ad.luc or Ad.dcn for 6 h. Cells were washed and incubations continued in serum-free media for 24 h.
Table 1. Summary of antitumor response and bone destruction assays

|          | BLI* | X-ray   | H&E  | OC TRACP 5b | ON Ca++ | Body weight | Survival |
|----------|------|---------|------|-------------|---------|-------------|----------|
|          | Progression | Fold change | Tumor size | Tumor free | Tumor size | Tumor free |          |
| Ad.dcn   | ***   | **      | NS    | NS          | NS      | NS          | NS       |
| Ad(E1-).dcn | *** | **      | NS    | NS          | **      | *           | NS       |
| Ad.luc   | ND    | NS      | NS    | NS          | NS      | NS          | NS       |
| Ad(E1-).luc | ND  | NS      | NS    | NS          | NS      | NS          | NS       |

Abbreviations: BLI, bioluminescence imaging; H&E, hematoxylin and eosin; *OC, osteoclast; *ON, osteocalcin; ND, not done; NS, not significant. * represents P < 0.05. ** represents P < 0.01. *** represents P < 0.001; all P-values were compared with the buffer group. * were analyzed by using a one-way ANOVA followed by Bonferroni post-tests. ** were analyzed statistically by using a two-way repeated-measure ANOVA followed by Bonferroni post-tests. *** were analyzed by log-rank (Mantel–Cox) test. a were analyzed by Fisher exact test.

24 h. The conditioned media was collected and subjected to ultracentrifugation to remove adeno viral particles. For transwell migration assay, 5.0 × 10^4 PC-3 cells were plated into each transwell with conditioned media in the top chamber. After 16 h, cells that migrated to the lower surface of the filter were stained using manufacturer’s protocol (Fisher Scientific, Pittsburgh, PA, USA). The cells per field of view were counted using > 100 magnification (2.54 mm² field area). Scratched wound-healing assays were carried out on nearly confluent PC-3 cells grown in six-well plates as described.47 Conditioned media from mock, Ad.luc or Ad.dcn-infected PC-3 cells were added, and at 0, 16 and 24 h, live cell images were taken with a Nikon DS-Fi1 camera. Gap distances between the two margins of the wounds were measured using Nikon image software and the percentages of wound area filled determined.

Animal studies
All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at NorthShore University HealthSystem.

Bone metastasis model and BLI
PC-3-luc cells (2.0 × 10^5 per mouse) were injected into the left heart ventricle of 4-week-old male nude mice (Nu/Nu) (Charles River laboratories, Wilmington, MA, USA). On day 9, mice were subjected to BLI in the dorsal and ventral positions using Xenogen IVIS Spectrum imaging equipment (Caliper life sciences, Hopkinton, MA, USA). The signal intensity was quantified within regions of interest in both left and right hind limbs as previously described.48 Mice that had fluid in the range of 2.0 × 10^2 – 1.0 × 10^3 photons per second were divided into various groups, with statistically indistinguishable BLI signals among each group. BLI was conducted weekly for the duration of the study.

X-ray radiography imaging
Mice were subjected to X-ray radiography on day 21, and weekly thereafter until day 60, using Faxitron (Faxitron X-ray Corporation, Wheeling, IL, USA) as previously described.45 Bone lesions were quantified in the femur and tibia of both hind limbs using Image J software (NIH, Bethesda, MD, USA) as described earlier.49 Bone histology and histomorphometric analysis
On day 62, mice were euthanized, and hind limbs were harvested, processed and stained with hematoxylin and eosin as previously described.48 Tumor burden per tibia/femur was quantified on hematoxylin and eosin-stained sections as previously described.48 Multinucleated tartrate-resistant acid phosphatase-positive osteoclasts at the bone-tumor interface were stained and counted as described earlier.48

Synchrotron micro computed tomography
Synchrotron micro computed tomography was performed using beamline 2-BM of the Advanced Photon Source at Argonne National Laboratory (Lemont, IL, USA) using the micro computed tomography instrument as previously described.57 X-ray photons of 22 keV were used, and the isotropic volume element (voxel) size in the reconstructions was 1.45 μm. 3D images of bone sections spanning 3.48 mm near/below the growth plate regions were constructed using MATLAB R2011a (The MathWorks, Inc, Natick, MA, USA).

Quantification of serum TRACP 5b, osteocalcin and calcium levels
At the terminal time point, blood was collected via cardiac puncture. The sera were obtained by centrifuging blood at 10 000 rpm for 5 min. Serum concentrations of TRACP 5b were measured by the MouseTRAP kit (Immunodiagnosticsystems, Phoenix, AZ, USA) as described.16 Osteocalcin levels were measured by BTI Mouse Osteocalcin EIA Kit (Biomedical Technologies, Ward Hill, MA, USA) as described.16 Calcium concentrations were measured using the Quantichrom calcium assay kit (BioAssay Systems, Hayward, CA, USA) as described.16

Statistical analysis
Data were presented as mean ± s.e.m. and statistically analyzed using GraphPad Prism software version 5 (GraphPad software, San Diego, CA, USA). Longitudinal data were analyzed using a two-way repeated-measure ANOVA followed by Bonferroni post hoc tests for the data obtained over the time course. For multiple group analyses, one-way ANOVA was performed. Student’s t-tests were performed to compare two sets of data. A Fisher exact test was used for the tumor incidence data in the X-ray and histomorphometric analyses. A log-rank (Mantel–Cox) test was performed to compare the survival distributions. Differences were considered significant at P < 0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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