Supplementary Data

Confirming decalcification of tibiae

Discarded EDTA (1 mL) was used to assess the radioactivity chelating from the bone using a liquid scintillation counter (Beckman Coulter LS-6500). Decalcification was measured using a calcium oxalate precipitation chemical test; 2 mL of citrate phosphate buffer (0.20 M citric acid, 0.16 M dibasic potassium phosphate, pH 3.2-3.6) was added to 1 mL of discarded EDTA followed by the addition of 5 ml of 5% ammonium oxalate solution. After a 30 min incubation, if no precipitate was seen then the result was considered negative. Two negative tests in a row signified a sample had completed decalcification.

Processing tibiae for embedment and sectioning

After decalcification was complete, tibiae were rinsed 3 times in PBS for 5 min each, and then in distilled water 3 times for 5 min each. The tibiae were processed for embedment using an automated tissue processing unit (Leica ASP300S). that carried out dehydration, clearance, and wax infiltration of samples. The following steps were performed in the sequence listed for 45 min each: 70% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 100% ethanol, 100% ethanol, xylene, xylene, paraffin, and paraffin.

The processed tissue was embedded in paraffin ring blocks using an embedding console (Leica Biosystems HistoCore Arcadia). Bones were oriented to be cut in a transverse manner. Tibiae of approximately 16 mm in length were cut transversely into four 4-mm segments. The segments were then arranged in the block so that the proximal end of each section would cut at the same time. The segments of the tibia were embedded from proximal to distal with the cutting orientation consistent for each bone segment. The 5-μm transverse sections were cut using a rotary microtome (Reichert HistoSTAT). Sections were dried at room temperature overnight. Serial sectioning of individual limbs was performed until at least 100 human breast cancer cells were identified in a tibia.

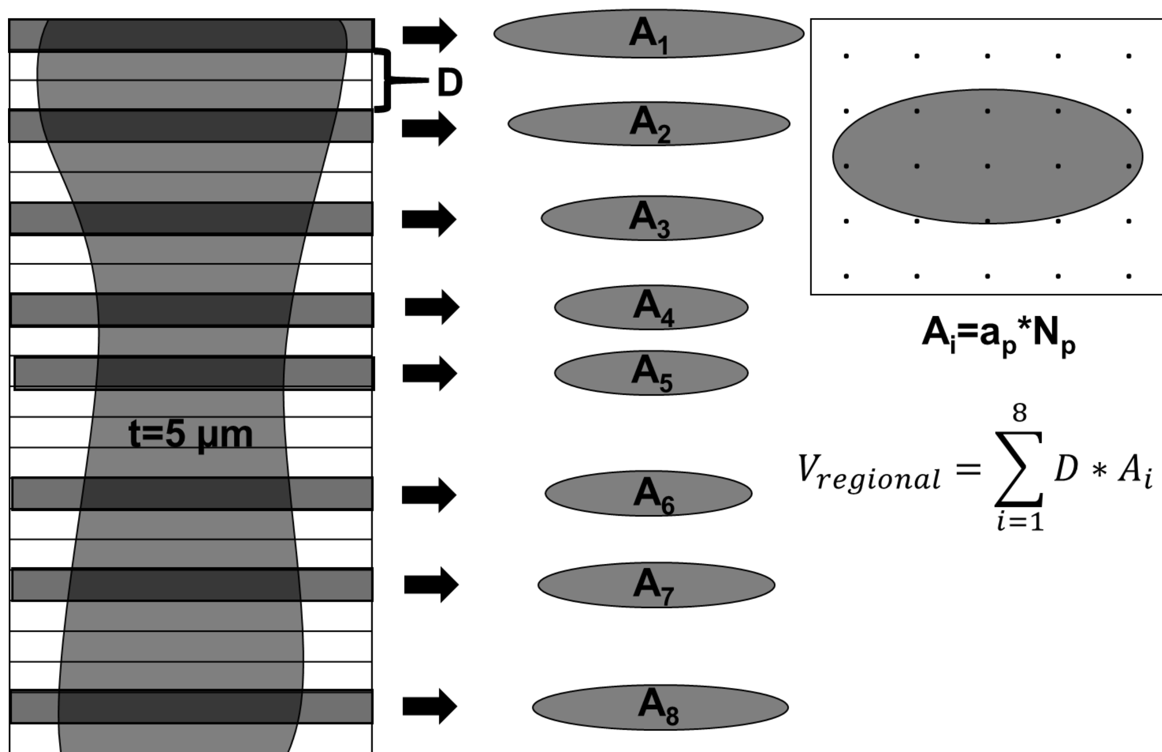
Histological screening of tissue sections

Tissue sections were first confirmed to contain inoculated human breast cancer cells before analyses of biological effects. Dried sections were deparaffinized in the heated compartment of a Leica ST5010 Autostainer XL for 1 h at 60°C. The subsequent deparaffinization (2 washes with 100% xylene for 5 min, 2 washes with 100% ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 5 min, 2 washes with water for 5 min) was automatically carried out by the same instrument. Sections were cover-slipped and

imaged with a Nikon A1R Confocal Laser Microscope. A 488 nm Argon laser was used to excite the CellTracker[TM] Green fluorophore.

Additional confirmation of human breast cancer cells was done with hematoxylin and eosin staining using adjacent sections. The process was automated with the aforementioned Autostainer XL (5 min 60°C in oven, 2 washes with xylene, 2 washes with 100% ethanol, 1 wash with 95% ethanol, one with 70% ethanol, one wash with water, staining with hematoxylin for 5 min, one wash with water, one wash with 1% hydrochloric acid in 70% ethanol (Leica), one wash with water, staining with bluing solution for 30 s, one wash with water, one wash with 95% alcohol, staining with eosin for 1 min, one wash with 70% ethanol, two washes with 95% ethanol, two washes with 100% ethanol, two washes with xylene).

Stereological determination of tumor location and cell density



Supplementary Figure S1. Estimating regional tumor volume within transverse sections. 5 µm thick transverse bone sections containing tumor were cut. 8 sections within a region were sampled (dark gray). These sections had a distance, D, between them. Section areas, A_i, were measured by placing a uniformly spaced point grid over the two-dimensional sectioning containing the tumor. Each point represented a known area, a_p. A_i was the product of a_p multiplied by the number of grid points falling within the tumor. The regional tumor volume, $V_{regional}$, was the summation of the tumor areas, A_i, multiplied by the distance between them, D.

Supplemental Table S1. Tumor dimensions of the human breast cancer cells inoculated into tibiae

Cell Type	MDA-MB-231*	MCF-7*
Cross-sectional tumor diameter (μm)	250 ± 32	203 ± 29
Distance from cross-sectional tumor center to bone surface (μm)	84 ± 11	100 ± 24
Number of tumor cells per cross section	103 ± 17**	54 ± 13
Cell density (cells/μm^3)	$2.5 \times 10^{-4} \pm 3.5 \times 10^{-5}$**	$1.6 \times 10^{-4} \pm 1.9 \times 10^{-5}$
Packing density (percentage of tumor volume)	28 ± 3.8	36 ± 4.4

*Values provided are mean and standard error of the mean
** Statistically significant $p<0.05$

Radiosensitivity of Inoculated Human Breast Cancer Cells

Preparation for Cell Irradiation. MCF-7-luc-F5 and MDA-MB-231-luc-D3H1 cells, grown in the L-15 medium, were prepared for alpha particle irradiation as described in Neti *et al*. (1). Briefly, 1.5-μm thick Mylar®-bottomed dishes were precoated with FNC solution (AthenaES™) containing fibronectin and collagen, overlaid with 2 mL of growth medium, and incubated at 37 ºC. After 30 min, the medium was aspirated, and the cells were seeded. MDA-MB-231 cells were seeded at a density of 5.0×10^5 cells/dish while MCF-7 cells were seeded at a density of 7.0×10^5 cells/dish. Irradiations were carried out, as described below, 1-3 days following cell seeding.

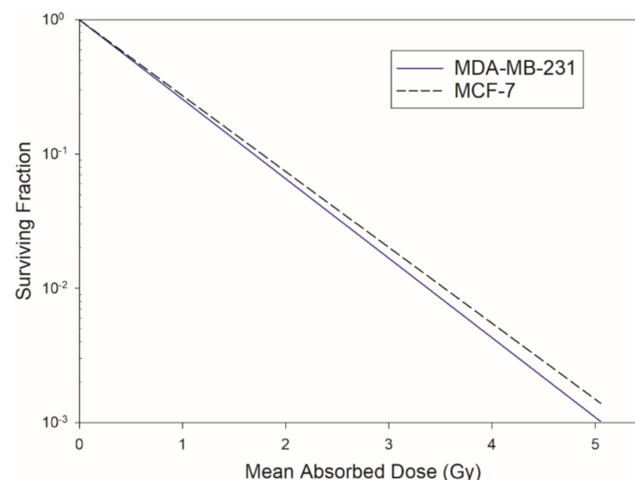
Alpha particle irradiation. The alpha particle irradiator was the one used by Neti *et al*. (1). Cells were irradiated at 37 ºC at a mean absorbed dose rate of 8.0 cGy/min. The ^{241}Am alpha particle source is located underneath the Mylar®-bottomed dish, and the alpha particles pass through the Mylar® growing surface perpendicularly at an average energy of 2.9 MeV when striking the cells. A photographic shutter is placed on the

source window aiding accurate delivery of the prescribed absorbed dose. Control cells were placed on a shutter with no radioactive source underneath. Mean absorbed doses used were 0, 0.9, 1.7, 2.5, 3.4, 4.2, and 5.0 Gy with one dish per dose.

Clonogenic cell survival. As described in Neti et al. (1), Cell survival was evaluated by quantifying colony formation following alpha particle irradiation. Immediately after irradiation cells were harvested by trypsinization and suspended in 2.5 mL of L-15 medium. The cells were syringed through a 21-gauge needle 5 times to disrupt clumps. A Z-Series Coulter Counter (Beckman Coulter) determined the concentration of cells. Cell suspensions were serially diluted and seeded into T-25 flasks in triplicate. Colonies were given 1-2 weeks to form, washed 3 times with saline, fixed with 90% ethanol, and stained with 1% crystal violet. Colonies consisting of at least 50 cells were counted and the surviving fraction relative to controls determined. Experiments were repeated 2-3 times and the surviving fractions calculated. The data were fitted with the linear quadratic model using SigmaPlot v14.

$$S = e^{-\alpha D - \beta D^2}$$

where *S* is the fraction of cells surviving absorbed dose *D*. The α and β are the linear and quadratic parameters. The raw data are reported in Rajon *et al*. (2); the curve fits are presented here for clarity. The α parameter for the MDA-MB-231 survival curve was 1.4 ± 0.26 Gy^{-1} while the corresponding parameter for MCF-7 cells was 1.3 ± 0.50 Gy^{-1}. The β parameter for both cell lines' survival curves was essentially 0. The corresponding D_{37} values for these cell lines were 0.73 and 0.77 Gy, respectively (2).



Supplementary Figure S2. Clonogenic survival of alpha-particle irradiated MDA-MB-231 (solid line) and MCF-7 (dashed line) human breast cancer cells. Curves represent least squares fits to the data for surviving fraction as a function of mean absorbed dose from 2-3 experiments. The explicit data points are given in Rajon *et al*. (2).

Supplementary Table S2. Statistical significance within treatment groups and timepoints for percentage of cells with one or more γ-H2AX foci

Inoculated Cell Line, Region, and Timepoint	Test	Significance	Notes
MDA-MB-231 Irradiated 1 day	ANOVA post hoc Tukey Test	p=0.026 600 kBq/kg vs control	
MDA-MB-231 Irradiated 3 days	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, 50 kBq/kg vs control	
MDA-MB-231 Bystander 1 day	ANOVA	p=0.058	No post hoc test run
MDA-MB-231 Bystander 3 days	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, 50 kBq/kg vs control	
MCF-7 Irradiated 1 day	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, 600 kBq/kg vs 50 kBq/kg	
MCF-7 Irradiated 3 days	ANOVA post hoc Tukey Test	p<0.001, 600 kBq/kg vs control p=0.004 600 kBq/kg vs 50 kBq/kg	
MCF-7 Bystander 1 day	ANOVA post hoc Tukey Test	p<0.001, 600 kBq/kg vs control p=0.003 600 kBq/kg vs 50 kBq/kg	
MCF-7 Bystander 3 days	Kruskal Wallis Ranks Dunn’s Method Pairwise	p=0.052, 600 kBq/kg vs control p=0.03 600 kBq/kg vs 50 kBq/kg	Failed normality test
Osteocytes MDA-MB-231 1 day	Kruskal Wallis Ranks Dunn’s Method Pairwise	p<0.05, 600 kBq/kg vs control	Failed normality test
Osteocytes MDA-MB-231 3 days	Kruskal Wallis Ranks Dunn’s Method Pairwise	p<0.05, 600 kBq/kg vs control	Failed normality test
Osteocytes MCF-7 1 day	Kruskal Wallis Ranks Dunn’s Method Pairwise	p<0.05, 600 kBq/kg vs control	Failed normality test
Osteocytes MCF-7 3 days	Kruskal Wallis Ranks Dunn’s Method Pairwise	p<0.05, 600 kBq/kg vs control	Failed equal variance test

Supplementary Table S3. Statistical significance within treatment groups and timepoints for percentage of bystander cancer cells with 0, 1-2, 3-5 and 5+ γ-H2AX foci

Inoculated Cell Line, Timepoint, Foci Number	Test	Significance	Notes
MDA-MB-231 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.045 50 kBq/kg vs control	
MDA-MB-231 1 day 1-2 foci	ANOVA	p=0.198	No post hoc test run
MDA-MB-231 1 day 3-5 foci	ANOVA post hoc Tukey Test	p=0.067 600 kBq/kg vs control, p=0.052 50 kBq/kg vs control	
MDA-MB-231 1 day 5+ foci	ANOVA	p=0.097	No post hoc test run
MDA-MB-231 3 days 0 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, 50 kBq/kg vs control	
MDA-MB-231 3 days 1-2 foci	ANOVA post hoc Tukey Test	p=0.003 600 kBq/kg vs control, p=0.008 50 kBq/kg vs control	
MDA-MB-231 3 days 3-5 foci	ANOVA post hoc Tukey Test	p=0.009 600 kBq/kg vs control	
MDA-MB-231 3 days 5+ foci	ANOVA	p=0.887	No post hoc test run
MCF-7 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.003 600 kBq/kg vs control, p=0.006 600 kBq/kg vs 50 kBq/kg	
MCF-7 1 day 1-2 foci	ANOVA	p=0.089	No post hoc test run
MCF-7 1 day 3-5 foci	ANOVA post hoc Tukey Test	p=0.005 600 kBq/kg vs control, p=0.03 600 kBq/kg vs 50 kBq/kg	
MCF-7 1 day 5+ foci	ANOVA	p=0.077	No post hoc test run
MCF-7 3 days 0 foci	Kruskal Wallis Ranks Dunn’s Method Pairwise	p=0.019 600 kBq/kg vs control, p=0.047 600 kBq/kg vs 50 kBq/kg	Failed normality test
MCF-7 3 days 1-2 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, p=0.005 600 kBq/kg vs 50 kBq/kg	
MCF-7 3 days 3-5 foci	ANOVA post hoc Tukey Test	p=0.056	No post hoc test run
MCF-7 3 days 5+ foci	ANOVA post hoc Tukey Test	p=0.174	No post hoc test run

Supplementary Table S4. Statistical significance within treatment groups and timepoints for percentage of irradiated cancer cells with 0, 1-2, 3-5 and 5+ γ-H2AX foci

Inoculated Cell Line, Timepoint, Foci Number	Test	Significance	Notes
MDA-MB-231 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.033 600 kBq/kg vs control	
MDA-MB-231 1 day 1-2 foci	ANOVA	p=0.316	No post hoc test run
MDA-MB-231 1 day 3-5 foci	Kruskal Wallis Ranks	p=0.185	Failed normality test No post hoc test run
MDA-MB-231 1 day 5+ foci	ANOVA	p=0.168	No post hoc test run
MDA-MB-231 3 days 0 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, 50 kBq/kg vs control	
MDA-MB-231 3 days 1-2 foci	ANOVA post hoc Tukey Test	p=0.036 600 kBq/kg vs control, p=0.004 50 kBq/kg vs control	
MDA-MB-231 3 days 3-5 foci	ANOVA post hoc Tukey Test	p=0.017 600 kBq/kg vs control, p=0.018 50 kBq/kg vs control	
MDA-MB-231 3 days 5+ foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.021 600 kBq/kg vs control	Failed normality test
MCF-7 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.009 600 kBq/kg vs control, p=0.013 600 kBq/kg vs 50 kBq/kg	
MCF-7 1 day 1-2 foci	ANOVA post hoc Tukey Test	p=0.009 50 kBq/kg vs control	
MCF-7 1 day 3-5 foci	ANOVA	p=0.128	No post hoc test run
MCF-7 1 day 5+ foci	ANOVA post hoc Tukey Test	p<0.04 600 kBq/kg vs control, p=0.007 600 kBq/kg vs 50 kBq/kg	
MCF-7 3 days 0 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, p=0.004 600 kBq/kg vs 50 kBq/kg	
MCF-7 3 days 1-2 foci	ANOVA post hoc Tukey Test	p=0.018 50 kBq/kg vs control	
MCF-7 3 days 3-5 foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.006 600 kBq/kg vs control	Failed normality test
MCF-7 3 days 5+ foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.005 600 kBq/kg vs control	Failed equal variance test

Supplementary Table S5. Statistical significance within treatment groups and timepoints for percentage of osteocyte cells with 0, 1-2, 3-5 and 5+ γ-H2AX foci

Inoculated Cell Line, Timepoint, Foci Number	Test	Significance	Notes
MDA-MB-231 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.033 600 kBq/kg vs control	
MDA-MB-231 1 day 1-2 foci	ANOVA	p=0.316	No post hoc test run
MDA-MB-231 1 day 3-5 foci	Kruskal Wallis Ranks	p=0.185	Failed normality test No post hoc test run
MDA-MB-231 1 day 5+ foci	ANOVA	p=0.168	No post hoc test run
MDA-MB-231 3 days 0 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, p=0.002 50 kBq/kg vs control	
MDA-MB-231 3 days 1-2 foci	ANOVA post hoc Tukey Test	p=0.036 600 kBq/kg vs control, p=0.004 50 kBq/kg vs control	
MDA-MB-231 3 days 3-5 foci	ANOVA post hoc Tukey Test	p=0.017 600 kBq/kg vs control, p=0.018 50 kBq/kg vs control	
MDA-MB-231 3 days 5+ foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.021 600 kBq/kg vs control	Failed normality test
MCF-7 1 day 0 foci	ANOVA post hoc Tukey Test	p=0.003 600 kBq/kg vs control, p=0.006 600 kBq/kg vs 50 kBq/kg	
MCF-7 1 day 1-2 foci	ANOVA post hoc Tukey Test	p=0.009 50 kBq/kg vs control	
MCF-7 1 day 3-5 foci	ANOVA	p=0.128	No post hoc test run
MCF-7 1 day 5+ foci	ANOVA post hoc Tukey Test	p=0.04 600 kBq/kg vs control, p=0.007 600 kBq/kg vs 50 kBq/kg	
MCF-7 3 days 0 foci	ANOVA post hoc Tukey Test	p<0.001 600 kBq/kg vs control, p=0.004 600 kBq/kg vs 50 kBq/kg	
MCF-7 3 days 1-2 foci	ANOVA post hoc Tukey Test	p=0.018 50 kBq/kg vs control	
MCF-7 3 days 3-5 foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.006 600kBq/kg vs control	Failed normality test
MCF-7 3 days 5+ foci	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.005 600kBq/kg vs control	Failed equal variance test

Supplementary Table S6. Statistical significance within treatment groups and timepoints for percentage of cells that are TUNEL$^+$

Inoculated Cell Line, Region, and Timepoint	Test	Significance	Notes
MDA-MB-231 Irradiated 1 day	ANOVA post hoc Tukey Test	p=0.046, 600 kBq/kg vs control p=0.006 50 kBq/kg vs control	
MDA-MB-231 Irradiated 3 days	Kruskal Wallis Ranks	p=0.064	Failed normality test No post hoc test run
MDA-MB-231 Bystander 1 day	ANOVA post hoc Tukey Test	p=0.009, 50 kBq/kg vs control	
MDA-MB-231 Bystander 3 days	ANOVA post hoc Tukey Test	p=0.011 600 kBq/kg vs control	
MCF-7 Irradiated 1 day	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.009 600 kBq/kg vs control	Failed normality test
MCF-7 Irradiated 3 days	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.007 600 kBq/kg vs control p=0.022 50 kBq/kg vs control	Failed normality test
MCF-7 Bystander 1 day	Kruskal Wallis Ranks	p=0.124	Failed normality test No post hoc test run
MCF-7 Bystander 3 days	Kruskal Wallis Ranks	p=0.331	Failed normality test No post hoc tests run
Osteocytes MDA-MB-231 1 day	ANOVA post hoc Tukey Test	p=0.088	No post hoc test run
Osteocytes MDA-MB-231 3 days	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.008 600 kBq/kg vs control, p=0.027 50 kBq/kg vs control	Failed normality test
Osteocytes MCF-7 1 day	Kruskal Wallis Ranks Dunn's Method Pairwise	p=0.006 600 kBq/kg vs control	Failed normality test
Osteocytes MCF-7-231 3 days	Kruskal Wallis Ranks	p=0.056	No post hoc test run

References

1. Neti PV, de Toledo SM, Perumal V, Azzam EI, Howell RW. A multi-port low-fluence alpha-particle irradiator: fabrication, testing and benchmark radiobiological studies. *Radiation research* 2004;**161**(6):732-8.
2. Rajon DAC, B.S.; Leung, C.N.; Bäck, T.A.; Fritton, J.C.; Azzam, E.I.; Howell, R.W. Modeling Direct and Bystander Effects that cause Growth Delay of Breast Cancer Xenografts in Bone Marrow of Mice Treated with Radium-223. 2021;**submitted**.