Radiation therapy modulates tumor physical characteristics to reduce intratumoral pressure and enhance intratumoral drug delivery and retention

Running Title
Radiation reduces intratumoral pressure

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**Abstract**

**Purpose**: High intratumoral pressure, caused by tumor cell-to-cell interactions, interstitial fluid pressure, and surrounding stromal composition, plays a substantial role in resistance to intratumoral drug delivery and distribution. Since radiation therapy (XRT) is commonly administered in conjunction with different intratumoral drugs, it is important to assess how radiation can reduce pressure locally and help intratumoral drug administration and retention.

**Material and Methods**: 344SQ-Parental or 344SQ-anti-PD1-Resistant lung adenocarcinoma cells were established in 129Sv/Ev mice and irradiated with either 1Gy x 2, 5Gy x 3, 8Gy x 3, 12Gy x 3, or 20Gy
1. Intratumoral pressure was measured every 3-4 days post XRT. Contrast dye was injected into the tumors 3- and 6-days post XRT and imaged to measure drug retention.

**Results:** In the 344SQ-P model, low-dose radiation (1Gy x 2) created an early window of reduced intratumoral pressure 1-3 days post XRT compared to untreated control. High-dose stereotactic radiation (12Gy x 3) reduced intratumoral pressure 3-12 days post XRT, while 20Gy x 1 showed a delayed pressure reduction on day 12. Intermediate doses of radiation did not significantly impact intratumoral pressure. In the more aggressive 344SQ-R model, low-dose radiation reduced pressure 1-5 days post XRT, while 12Gy x 3 reduced pressure 1-3 days post XRT. Moreover, both 1Gy x 2 and 12Gy x 3 significantly improved drug retention 3 days post XRT; however, there was no significance detected 6 days after XRT. Lastly, histopathologic evaluation showed that 1Gy x 2 reduced collagen deposition within the tumor, while 12Gy x 3 led to more necrotic core and higher ECM formation in the tumor periphery.

**Conclusions:** Optimized low-dose XRT as well as higher stereotactic XRT regimen led to reduction in intratumoral pressure and increased drug retention. The findings from this work can be readily translated to the clinic in order to enhance intratumoral injections of various anti-cancer agents.
Introduction

Medical advances over the past years have elucidated and refined the concept of using immunotherapy in solid tumors. There is a growing number of clinical trials using immunotherapy in conjunction with chemotherapy and radiotherapy (XRT), emphasizing the concept that combining immunotherapy with other treatment modalities may be critical to achieve robust antitumor responses (1,2). It is particularly critical to shed light on radiation-based combinations, as XRT may prime antitumor immunity and reduce intratumoral pressure, the latter of which is the primary focus of this study (3-6). The rationale and importance of reducing intratumoral/intraoncotic pressure by XRT stems from the need to enhance intratumoral drug delivery and retention. Currently, 24 out of 130 clinical studies investigating immune modulating therapies involve intratumoral route of administration (7-11). The benefits of doing so include
avoiding off-target toxicity, using a lower and less toxic drug dose, and priming local T-cells for immune response (12,13).

Developments in image-guided local drug injection techniques are fast growing. However, elevated intratumoral pressure presents a barrier to this, as the pressure differential between the center and outer regions of solid tumors can cause hypoxia, increase metastatic potential, and compromise successful intratumoral drug delivery (14,15). Though the mechanisms are not entirely understood, elevated tumor interstitial or intratumoral pressure can be attributed to blood-vessel leakiness, lymph vessel abnormalities, interstitial fibrosis, modified interstitial matrix, and tumor cells proliferating within a confined space (15).

To address this, we propose the use of XRT to reduce the pressure-induced convection force that opposes the diffusion of therapeutic agents injected intratumorally. Cancer cells with their inhibitory stroma produce cytokines, such as TGF-β that further contribute to abnormal vasculature and thus elevate intratumoral pressure (15). We have recently shown that low-dose radiotherapy can in fact reduce TGF-β levels locally and modulate the tumor microenvironment (TME) (16). Beyond reducing intratumoral pressure, XRT has also been shown to re-oxygenate certain portions of irradiated tumors and increase \( pO_2 \) levels, hence increasing tumor treatability by overcoming hypoxia (17).

While certain physical and cellular attributes contributing to intratumoral pressure are well understood, there remain many aspects that warrant further research, especially in context of XRT (18,19). In this paper, we seek to optimize the radiation dose and fractionation schedule to find the best window for intratumoral drug delivery. To our knowledge, this is the first study that comprehensively explores how to use radiation to decrease intratumoral pressure, thereby increasing the success of intratumoral drug delivery into solid tumors.
Materials and Methods

Mice and tumor establishment

All animal protocols were reviewed and approved by the IACUC committee of XXXX. 344SQ-parental (344SQ-P) or 344SQ-anti-PD1-resistant (344SQ-R) lung adenocarcinoma cells were subcutaneously implanted on day 0 in the hind legs of 8 to 12 weeks old 129Sv/Ev mice, at a dose of 5x10^5 cells for parental and 0.5x10^5 for resistant. When tumors reached 7-8 mm in diameter, they were locally irradiated using a Cesium source. Radiation doses tested were 1Gy x 2, 5Gy x 3, 8Gy x 3, 12Gy x 3, and 20Gy x 1. The different fractions were scheduled such that all treatments were completed on Day 10 after tumor inoculation. Intratumoral pressure was recorded using a pressure transducer (Compass CT) at various time points (days 11, 13, 18, 22, 27, 33), and measurements were graphed accordingly. Tumor growth was also recorded twice per week using digital calipers and mice were euthanized when tumor diameter reached 14 mm in diameter.

In vivo evaluation of percutaneous intratumoral delivery and retention

344SQ-P tumors were established in 129Sv/Ev mice. XRT was delivered on day 7 to experimental groups as follows: Untreated control, 1Gy x 2, 5Gy x 3, 8Gy x 3, 12Gy x 3, and 20Gy x 1. Evaluation for intratumoral drug delivery deposition and retention were evaluated in the following manner. A 25-gauge needle was advanced into the tumor under ultrasound visualization (Siemens Acuson); ultrasound imaging guidance was utilized to ensure accurate positioning of the needle within the lesion. Next, 100 uL of an iodinated contrast agent (Visipaque 320) was delivered via the 25-gauge needle into the tumor under live fluoroscopic imaging (Siemens Artis-Q). To standardize the injection rate, the injections were performed using a syringe pump (Harvard Apparatus) at a rate of 5cc per minute. The Siemens Artis Q C-Arm was run at 7.5 frames per second to monitor the injections. The procedures were performed by an
interventional radiologist with 7 years of experience with preclinical and clinical intratumoral injection procedures. Animals were then immediately scanned with microCT imaging with 100 micron resolution (Bruker SkyScan). The volumetric images were then analyzed using a 3D image analysis software program (MIM Maestro) by a radiologist with 10 years of volumetric imaging analysis. The tumor volume as well as the volume of distribution of the injected contrast agent were calculated. The percent contrast agent retention was calculated by dividing contrast volume by tumor volume, multiplied by 100 to obtain % tumor fill.

**Tumors histopathologic evaluation**

344SQ-P tumor cells were subcutaneously injected in 129Sv/Ev mice on day 0. When tumors reached 7-8 mm in diameter, low-dose XRT (1Gy x 2) was given on days 7 and 8, while high-dose XRT (12Gy x 3) was given on days 7, 8, and 9. Low-dose tumors were harvested on day 10 and high-dose tumors were harvested on day 13. Mice were euthanized and the tumors were dissected and fixed with 10% neutral buffered formalin solution. Formalin fixed tumors were cut in half through the middle on the larger diameter plan, then the largest cut surfaces were obtained, processed, and embedded into paraffin blocks. From paraffin blocks, 4-µm thick sections of tumor tissues were cut and mounted on glass slides, and then stained with hematoxylin and eosin (H&E) and Masson’s trichrome stains following the methods in Histotechnology book: A Self-Instructional Text 2nd Edition (May 1, 1997), by Freida L. Carson, ISBN-13: 978-0891894117). Stained slides were first examined with Olympus BX41 microscope and then the slides were scanned with Aperio Scanscope AT2. For quantification of extracellular collagen stained with Masson’s trichrome method, we used the Aperio image analysis algorithms.

**Results**
Optimized radiation dosing reduces intratumoral pressure in the 344SQ-Parental lung adenocarcinoma model

To test the impact of radiation on intratumoral pressure, 0.5 x 10^6 344SQ-P lung adenocarcinoma cells were inoculated into the right hind legs of 129Sv/Ev mice on day 0. Additionally, the primary tumor growth measurements were monitored throughout the 35 day observation period (Fig. 1A). When compared to the control, all experimental groups showed increased anti-tumor responses (Ctrl vs 1Gy x 2: *P* < 0.0001, Ctrl vs 5Gy x 3: *P* < 0.0001, Ctrl vs 8Gy x 3: *P* < 0.0001, Ctrl vs 12Gy x 3: *P* < 0.0001, Ctrl vs 20Gy x 1: *P* < 0.0001). Furthermore, 12Gy x 3 controlled tumor growth better than 8Gy x 3 (*P* < 0.0001). Tumors that were irradiated with 1Gy x 2 and 12Gy x 3 responded similarly and were not significantly different (*P* = 0.0827). Optimized doses of radiation including both low-dose (1Gy x 2) and high-dose XRT (12Gy x 3 and 20Gy x 1), reduced intratumoral pressure levels during the measurement period (1 to 12 days post XRT), whereas the intermediate doses of radiation (5Gy x 3 and 8Gy x 3) did not show any significant reduction in intratumoral pressure (Fig. 1B). A multiple unpaired t-test was conducted to compare the mean intratumoral pressure from different doses of radiation against the control at each time point. Low-dose XRT showed an early window of low intratumoral pressure 1- and 3-days post XRT. On day 1, there was a significant drop in pressure from 104.8 mmHg in the control group to 67 mmHg in the 1Gy x 2 group (*P* = 0.0543). On day 3, the group that received two fractions of 1Gy had a mean intratumoral pressure of 69.6 mmHg versus 111.4 mmHg in the control (*P* = 0.0591). The tumors that were irradiated with three fractions of 12Gy showed a delayed window of reduced intratumoral pressure 3-, 8-, and 12-days post XRT (*P* = 0.0105, *P* = 0.040, and *P* = 0.013 respectively). On the contrary, tumors irradiated with a single fraction of 20Gy showed reduced pressure at 1- and 12-days post XRT (*P* = 0.0457, *P* = 0.009, respectively).

In order to check if there was any correlation between tumor size and intratumoral pressure at the first and last measurements taken for each group, we calculated the slope of the linear regression and
reported accordingly (Supplementary Fig. 1A). In general, there was an upward trend for control as well as radiation-treated groups with increase in tumor size.

**Optimized radiation dosing reduces intratumoral pressure at varying timepoints in the 344SQ-Resistant lung adenocarcinoma model**

To further examine the impact of radiation on intratumoral pressure, the same experimental design was used from figure 1, except that a different cancer cell line was used. Instead of using the 344SQ-Parental lung adenocarcinoma cell line, the more aggressive 344SQ-Resistant cancer cell line was injected. The primary tumor growth measurements were monitored over the 40 days observation period (Fig. 2A). All experimental groups controlled tumor growth better than the control group (Ctrl vs 1Gy x 2: $P = 0.0081$, Ctrl vs 5Gy x 3: $P < 0.0001$, Ctrl vs 8Gy x 3: $P < 0.0001$, Ctrl vs 12Gy x 3: $P < 0.0001$, Ctrl vs 20Gy x 1: $P < 0.0001$). There was no statistical difference in tumor growth between the group that received 8Gy x 3 and 12Gy x 3 ($P = 0.1566$). Additionally, 12Gy x 3 hampered tumor growth better than 1Gy x 2 ($P = 0.0001$). All doses of radiation produced a different window of reduced intratumoral pressure (Fig. 2B) with 1Gy x 2 producing the most statistical significance. Similar to the previous experiment using the 344SQ-P model, low-dose XRT created an early window of decreased intratumoral pressure 1-, 3-, and 5-days after the last fraction of XRT ($P = 0.0175$, $P = 0.0209$, $P = 0.006$). The 5Gy x 3 and 8Gy x 3 experimental groups only reduced pressure one day post XRT ($P = 0.0038$, $P = 0.0017$). 12Gy x 3 reduced intratumoral pressure one day post XRT ($P = 0.0203$) and had a strong trend of decreasing pressure on day 3 ($P = 0.073$). Contrary to the previous groups, 20Gy x 1 only had a late impact on intratumoral pressure 13 days post XRT ($P = 0.047$).

To check if there was any correlation between tumor size and intratumoral pressure at the first and last measurement points for each group, we calculated the slope of the linear regression and reported
accordingly (Supplementary Fig. 1B). As the tumor size increased the pressure decreased in the control group, showing that 344SQ-R model tends to become less stiff by size increase, a characteristic of highly metastatic/soft tumors. An opposite trend was noticed in the irradiated groups with the exception of 20Gy x 1 which favors high necrosis.

**Optimized radiation dosing (1Gy x 2 and 12Gy x 3) enhances tumor fill and drug retention at different timepoints**

In order to examine the effects of reduced intratumoral pressure on drug delivery and retention, the irradiated mice with 344SQ-P tumors were imaged 3- and 6-days post XRT (Fig. 3A, B). Three days post XRT (Fig. 3C), the mice that received two fractions of 1Gy radiation had a significant increase in tumor fill of intratumorally injected iodinated contrast dye with a mean of 14.07% vs. control 2.29% ($P = 0.008$). Mice that were irradiated with three fractions of 12Gy also showed a significant increase in tumor fill of 11.45% compared to control ($P = 0.005$). There was no significant increase in percent tumor fill detected 6 days post XRT (Fig. 3D); however, there was a strong increasing trend for the group that received one fraction of 20Gy vs. control (21.89% vs. 8.43%, $P = 0.058$).

**Low-dose radiation (1Gy x 2) downregulates the extracellular matrix (ECM) while high-dose radiation (12Gy x 3) upregulates peripheral ECM in 344SQ-P tumors**

To fully understand how intratumoral pressure is reduced, immunohistochemistry was performed on three groups: control, 1Gy x 2, and 12Gy x 3. Masson’s trichrome staining was used to visualize collagen and ECM (blue), nuclei (dark brown spots), muscle (red), and cytoplasm (pink). Afterwards, the histochemical images were analyzed using the Aperio Color Deconvolution Algorithm to measure the percentage of ECM present in the samples (Fig. 4A). The positive areas were shaded yellow to red.
depending on the intensity of the collagen stain and the negative areas were shaded blue (Fig. 4B). Based on the values obtained from the algorithm, two fractions of 1Gy XRT significantly lowered the percentage of ECM present \( (P = 0.037) \). Contrarily, three fractions of 12Gy XRT significantly increased the percentage of ECM present \( (P = 0.05) \). However, it is possible that high-dose radiation reduced the intratumoral pressure from direct killing of tumor cells.

**Discussion**

Intratumoral pressure is an essential obstacle that must be addressed and overcome in order to improve intratumoral drug delivery and retention. Solving this issue would allow intratumoral drugs to become more homogenously distributed throughout the tumor and in turn enhance anti-tumor responses (20). The increased pressure present in cancer cells, when compared to normal tissue cells, can be explained by interstitial fluid pressure (IFP), solid stress (SS), stiffness, and microarchitecture (21). The interstitial fluid space is primarily held together by SS, which is directly correlated with multiple factors, including extracellular matrix (ECM), which is comprised of collagen and hyaluronan, and cancer associated fibroblasts (CAFs). Therefore, an increase in ECM results in an overall increase in intratumoral pressure. Additionally, the cross-linking of ECM is the primary cause of matrix stiffening as well as changes in the matrix architecture (21). The ECM not only increases the intratumoral pressure but also promotes tumor growth, cancer cell migration, as well as resistance to apoptosis (22). Since radiation can be administered in conjunction with different intratumoral drugs used to treat cancer (oncolytic viruses, NLRP3 agonists, STING agonists, antibodies, nanoparticles, etc.), it is important to assess how radiation can reduce intratumoral pressure and help intratumoral drug delivery as well as retention (23).

The direct delivery of anti-cancer therapies into tumors, particularly immunostimulatory agents, is a flourishing strategy to overcome resistance mechanisms for systemically administered
immunotherapies and associated toxicities (24-28). In practice, however, it is clear that physical properties of tumors also impose challenges to intratumoral delivery and deposition, even when the therapeutic agent is directly injected into the tumor (25,28). Adjuvant interventions that can modulate the tumor’s physical properties (stiffness, fibrosis status, composition of the inhibitory stroma and extracellular matrix) to render the lesion more amenable to intratumoral injection have the potential to substantially augment the efficacy of these therapies.

In order to assess how different doses of radiation (1Gy x 2, 5Gy x 3, 8Gy x 3, 12Gy x 3, 20Gy x 1) would affect intratumoral pressure in the 344SQ-P murine model, we measured intratumoral pressure 1-, 3-, 8-, and 12-days after the last fraction of XRT using a compass CT pressure transducer. Additionally, tumor growth was also recorded to see the efficacy of the different radiation doses. We found that all groups significantly hampered tumor growth with 1Gy x 2, 12Gy x 3 and 20Gy x 1 being the most significant. Moreover, 12Gy x 3 controlled tumor growth better than 8Gy x 3. 12Gy x 3 and 1Gy x 2 had similar effects on tumor control. Low-dose XRT created an early window of low intratumoral pressure 1- to 3-days post XRT. A recent study showed that low-dose XRT modulates the tumor stroma and downregulates CAFs (29). This causes the stroma to become more permeable and allows effector immune cells (mainly NK and T cells) to infiltrate the TME. Once the NK and T cells infiltrate the stroma, immune-mediated killing begins. Additionally, transforming growth factor-beta (TGF-β) is reduced with low-dose XRT (16). While reduction of intratumoral pressure with low-dose XRT was attributed to modulation of the stroma and immune-mediated killing, high-dose XRT, on the other hand, physically killed tumor cells. With more necrosis and space between tumor cells, intratumoral pressure dropped. Three fractions of 12Gy produced a sustained window of low intratumoral pressure 3-, 8-, and 12-days post XRT. The effects of a single fraction of 20Gy were different in that it dropped pressure in the early (day 1) and late (day 12) timepoints post XRT.
To further examine the impact of different radiation regimens on intratumoral pressure, we followed the same experimental design as the first experiment; however, instead of the 344SQ-Parental cell line, we established tumors with an aggressive α-PD-1 resistant cell line, 344SQ-R (Kras mutated, p53 deficient) (30). Similar to the previous experiment, all doses of radiation significantly controlled tumor growth, but there were differences among these experimental groups. In 344SQ-R, three fractions of 8Gy controlled tumors similarly to three fractions 12Gy, whereas in 344SQ-P 12Gy proved to control tumor growth better than 8Gy. Another notable difference was that two fractions of 1Gy underperformed the efficacy of three fractions of 12Gy. This difference could be due to a more resilient inhibitory stroma associated with the 344SQ-R model, leading to reduced CD4+ and CD8+ tumor infiltrating lymphocytes (30). Low-dose XRT once again created an early window of decreased intratumoral pressure 1-, 3-, and 5-days after the last fraction of XRT in 344SQ-R, while three fractions of 12Gy only reduced intratumoral pressure 1 day post XRT (as opposed to the parental model with reduced pressure on days 3 to 12). Three fractions of 5Gy and 8Gy also only reduced pressure one day post XRT.

Extensive imaging was conducted to measure percent tumor fill after different doses of XRT 3 and 6 days post treatment. First, 100μL of iodinated contrast dye (Visipaque 320) was injected into the tumor with a syringe pump (Harvard Apparatus) at a rate of 5 cc per minute. Concurrently, live imaging was conducted using the Siemens Artis Q C-Arm. Mice were then transferred to the Bruker SkyScan 1276 for high resolution microCT imaging. Percent tumor fill was calculated from the images produced using the formula: (Contrast volume / Tumor volume) x 100. When intratumoral pressure was too high, it was possible to see the osmotic/oncotic pressure causing the contrast dye to re-enter the capillaries. Two fractions of 1Gy showed a significant increase in tumor fill 3 days post XRT, confirming the early window of low intratumoral pressure in the previous experiments. As pressure lowered, the contrast dye was able to be more homogenously distributed around the TME. Moreover, two fractions of 1Gy showed no significant increase in percent tumor fill 6-days post XRT. This is in line with the first set of experiments where intratumoral pressure was only reduced 1- to 3-days post 1Gy x 2. Percent tumor fill
of the 12Gy group also followed the pressure readings from figure 1B. Tumor fill was elevated 3- and 6-
days post 12Gy x 3; however, it was not statistically significant 6 days post XRT.

To investigate the mechanisms behind the increased tumor fill with two fractions of 1Gy and
three fractions of 12Gy, histopathologic evaluation was conducted using Masson’s trichrome to stain for
ECM, which consists mostly of collagen and hyaluronic acid (HA). Recent studies have shown that
several drugs that target collagen and/or hyaluronic acid reduce IFP. Examples include Collagenase,
PEGPH20 (targets HA), Losartan (targets Collagen and HA), and Bevacizumab (targets VEGFR) (31-34).
When VEGFR is inhibited, blood vessels are normalized, and blood flow is improved (34). Initial
analysis of the trichrome-stained slides showed a significant downregulation of ECM two days post low-
dose XRT and an upregulation of ECM four days post high-dose XRT. The reduced ECM with low-dose
XRT was as expected. As collagen is reduced in the interstitial space, SS is decreased, and pressure is
relieved from the tumor, enabling drugs to distribute intratumorally. Contrarily, The ECM was
upregulated with three fractions of 12Gy, most of which was observed to be deposited along the tumor
periphery, while the core presented with necrosis upon pathological evaluation. One explanation of
improved tumor fill with 12Gy x 3 is that the upregulated peripheral ECM traps the intratumoral drugs
inside and promotes retention. Overall, both doses of XRT (1Gy x 2 and 12Gy x 3) improved percent
tumor fill; however, the underlying mechanisms are different.

Conclusions

In conclusion, these novel findings with optimized radiotherapy doses will facilitate the delivery of
intratumoral drugs and enhance their retention to maximize tumor killing. This is directly applicable to
clinical settings where drugs have to be injected locally to avoid systemic toxicity and turn
immunologically ‘cold’ tumors into ‘hot’.
Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References


16. XXXX.


27. XXXX

28. XXXX

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30. XXXX


**Figure Legends**

![Figure 1](image_url)

**Figure 1**

**A**

Primary tumor growth

**B**

344150-2 intracolonic pressure
Fig. 1. Optimized doses of radiation reduced intratumoral pressure in the 344SQ-P model. (A) Tumors were monitored throughout the measurement period to ensure that radiation started once the tumors reached 7-8 mm. Measurements were also used to confirm that tumors did not grow past 15 mm in diameter. (B) Intratumoral pressure was measured 1-, 3-, 8-, and 12-days after the last fraction of XRT and multiple t tests were conducted to analyze the difference in intratumoral pressure at each time point. $P \leq 0.05$ was considered significant. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Fig. 2. Different doses of radiation reduce intratumoral pressure at varying timepoints in the 344SQ-R model. (A) The primary tumor was measured beginning on day 7 to ensure that tumors were ready to be irradiated, then measured twice per week to confirm that tumor did not surpass 15 mm in diameter. (B) Intratumoral pressure was measured 1-, 3-, 5-, 9-, and 13-days post XRT and multiple t tests were used to analyze the difference in intratumoral pressure at each time point. $P \leq 0.05$ was considered significant. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. 
Fig. 3. Optimized radiation dosing increases tumor fill and drug retention at different time frames in the 344SQ-P model. (A) Workflow for acquiring data and images regarding tumor fill 3- and 6-days post XRT. (B) Representative snapshots of live C-Arm imaging depicting optimal tumor fill of contrast dye with 1Gy x 2 and 12Gy x 3 radiation doses, while detecting subcutaneous leak in control group due to intratumoral pressure. Percent tumor fill was calculated by dividing contrast volume by the tumor volume multiplied by 100. (C) Percent tumor fill were then graphed for 3 days post XRT. (D) Percent tumor fill graphed for 6 days post XRT. $P \leq 0.05$ was considered significant.
Fig. 4. Low-dose XRT downregulates the extracellular matrix (ECM) in 344SQ-P tumors depicted by Masson’s trichrome staining for collagen fibers. (A) Tumors were harvested 2 days after the last dose of low-dose XRT and 4 days after the last dose of high-dose XRT to conduct the staining. Percent stain positivity was reported for control, 1Gy x 2, and 12Gy x 3 treatments (n = 5 mice per group). Groups were compared using Student’s t-tests, * P ≤ 0.05. (B) Left column: representative hematoxylin and eosin (H&E) stained images. Middle column: representative trichrome stained images. Right column: histochemical images shown at 5X magnification for the specified XRT doses.