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Tumor cells but not endothelial cells mediate the eradication of primary sarcomas by stereotactic body radiation therapy[#]

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Abstract

Cancer clinics currently use high-dose stereotactic body radiation therapy as a curative treatment for several kinds of cancers. However, the contribution of vascular endothelial cells to tumor response to radiation remains controversial. Using dual-recombinase technology, we generated primary sarcomas in mice with targeted genetic mutations specifically in tumor cells or endothelial cells. We selectively mutated the proapoptotic gene *Bax* or the DNA damage-response gene *Atm* to genetically manipulate the radiosensitivity of endothelial cells in primary soft tissue sarcomas. *Bax* deletion from endothelial cells did not affect radiation-induced cell death in tumor endothelial cells or sarcoma response to radiation therapy. Although *Atm* deletion increased endothelial cell death after radiation therapy, deletion of *Atm* from endothelial cells failed to enhance sarcoma eradication. In contrast, deletion of *Atm* from tumor cells increased sarcoma eradication by radiation therapy. These results demonstrate that tumor cells, rather than endothelial cells, are critical targets that regulate sarcoma eradication by radiation therapy. Treatment with BEZ235, a small-molecule protein kinase inhibitor, radiosensitized primary sarcomas more than hearts. These results suggest that inhibiting ATM kinase during radiation therapy is a viable strategy for radiosensitization of some tumors.

Introduction

Approximately half of all cancer patients are treated with radiation therapy (1), which may be given with palliative intent in cases where a delay in tumor regrowth (growth delay) can

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be clinically meaningful. However, the majority of cancers treated with radiation therapy are treated with the intent to cure, where the goal of radiation therapy is to achieve complete and permanent tumor regression (local control). When patients with cancer are treated with radiation, they usually receive relatively small (1.8–2.0 Gy) daily fractions over 1 to 2 months. Recently, advances in radiation-treatment planning and delivery have made it possible to safely deliver a small number of high radiation doses (15–24 Gy), termed stereotactic body radiation therapy (SBRT) or radiosurgery, to improve the local control of some tumors (2).

The tumor microenvironment of human cancers consists of blood vessels, fibroblasts, and immune cells that modulate cancer development, progression, and response to therapy (3). However, whether or not stromal cells, such as endothelial cells, are critical targets of radiation therapy remains controversial. Indeed, experiments using transplanted tumors in mice with radiosensitive stroma have suggested that tumor stromal cells do not contribute to local control of cancer by radiation therapy (4).

Recently, endothelial cell apoptosis and microvascular collapse were reported to contribute to the radiation response of transplanted melanoma and fibrosarcoma cell lines (5). Endothelial cell apoptosis can occur because of membrane damage, which triggers rapid ceramide-mediated apoptosis after high doses of radiation exposure (6, 7). As a result, transplanted tumors with *acid sphingomyelinase* or *Bcl-2 associated X protein (Bax)*-deficient stroma, which have defective radiation-induced endothelial cell apoptosis, grow 200 to 400% faster than transplanted tumors with wild-type stroma and have a decreased growth delay after radiation doses of up to 20 Gy (5). Notably, endothelial cell apoptosis has been proposed to occur at a threshold of 8 to 10 Gy and to increase up to 20 to 25 Gy (7), suggesting that endothelial apoptosis may be contributing to tumor cure by SBRT (8). However, the conclusion that microvascular damage regulates tumor response to radiation has been challenged (9, 10), and additional experiments using transplant model systems have failed to resolve the controversy (11–14).

Unlike transplanted tumor models, which may not fully recapitulate the vasculature and immune surveillance of autochthonous tumors (15, 16), genetically engineered mouse models (GEMMs) develop tumors within the native microenvironment in immunocompetent mice (17) and may more faithfully recapitulate the tumor stroma and microenvironment of human cancers (18). In addition, the response of these primary mouse cancer models to therapeutics might mimic the response of human cancers in clinical trials (19, 20).

To investigate the contribution of endothelial cells to the radiation response of primary sarcomas, we developed the technology to contemporaneously mutate different genes specifically in the tumor cells versus the endothelial cells of primary sarcomas (21). In this system, an adenovirus expressing FlpO (adeno-FlpO) activates a conditional allele of oncogenic *Kras* (*FSF-Kras^{G12D}*) and deletes both copies of a conditional allele of *p53* (*p53^{FRT}*), while a tissue-specific Cre driver mutates floxed alleles specifically in endothelial cells. We recently used this dual recombinase technology to demonstrate that selectively sensitizing endothelial cells to mitotic cell death by deleting the DNA damage response gene *ataxia telangiectasia mutated (Atm)* (22) prolongs sarcoma growth delay after SBRT (23).

However, an increase in growth delay does not necessarily translate into improved local control (4, 24, 25).

Here, we irradiated sarcomas with *Atm* deleted in endothelial cells with a curative dose of radiation. We also used dual recombinase technology to selectively protect endothelial cells from apoptosis by deleting the proapoptotic gene *Bax* (26). We found that tumor endothelial cells in primary sarcomas did not die via apoptosis within four hours of SBRT. In addition, endothelial cell death did not contribute to sarcoma eradication by radiation therapy. In contrast, radiosensitizing tumor cells by deleting *Atm* increased local control of primary sarcomas after radiation therapy. These results demonstrate that tumor cells, but not endothelial cells, are critical targets of curative radiation therapy in primary sarcomas. Also, to test whether ATM inhibition can improve the therapeutic ratio during SBRT, we compared the radiation response of primary sarcomas and hearts after *Atm* inhibition with BEZ235 to demonstrate that targeting ATM during radiation therapy might be a viable approach for radiosensitization of tumors at certain anatomic sites.

Results

Bax and Bak do not regulate endothelial cell death after SBRT in primary sarcomas

Because apoptosis of tumor endothelial cells is dependent on Bax in transplanted tumor models (5), we used (i) dual recombinase technology to initiate primary sarcomas in conditional *FSF-Kras^{G12D}; p53^{FRT/FRT} (KP^{FRT})* mice with adeno-FlpO (21) and (ii) *VE-Cadherin-Cre* (27) to delete *Bax* floxed alleles (*Bax^{FL}*) (28) specifically in endothelial cells. We recently demonstrated that this approach efficiently deletes floxed alleles in endothelial cells of primary sarcomas (23). We confirmed by quantitative polymerase chain reaction (qPCR) that *VE-Cadherin-Cre* efficiently deleted *Bax* in endothelial cells (fig. S1A).

We next investigated the effect of *Bax* deletion specifically in endothelial cells on sarcoma initiation and growth by generating primary sarcomas in *KP^{FRT}; VE-Cadherin-Cre; Bax^{FL/+}* (*KP^{FRT}VBax^{FL/+}*) and *KP^{FRT}; VE-Cadherin-Cre; Bax^{FL/FL}* (*KP^{FRT}VBax^{FL/FL}*) mice. In contrast to previous reports with tumor cells transplanted into *Bax* null mice (5), we observed no change in primary sarcoma initiation or growth in mice with deletion of *Bax* specifically in endothelial cells (fig. S1, B–D). To determine if *Bax* deletion from endothelial cells protected tumor endothelial cells from radiation in an autochthonous model system, we irradiated the sarcomas in *KP^{FRT}VBax^{FL/+}* and *KP^{FRT}VBax^{FL/FL}* mice with a single dose of 20 Gy using fluoroscopy-guided radiation therapy and examined endothelial cell death via TUNEL staining at various time points after irradiation. In transplanted tumor models, tumor endothelial cell apoptosis peaks 4 to 6 hours after radiation exposure (5). Consistent with our previous results (23), we did not observe a significant change in apoptotic endothelial cell death 4 h after irradiation of primary sarcomas, but endothelial cell death did increase 48 h after radiation likely due to mitotic catastrophe (Fig. 1, A and B). Furthermore, deletion of *Bax* did not affect endothelial cell death, suggesting that endothelial cells in primary sarcomas do not undergo Bax-mediated apoptosis after irradiation.

We next evaluated whether *Bax* in endothelial cells contributed to the growth delay of primary sarcomas. To this end, we irradiated sarcomas in $KP^{FRT}VBax^{FL/+}$ and $KP^{FRT}VBax^{FL/FL}$ mice with a single dose of 20 Gy and monitored tumor growth until the sarcomas tripled in size. In contrast to transplanted tumors in *Bax* null mice (5), primary sarcomas in $KP^{FRT}VBax^{FL/FL}$ mice displayed a growth delay after irradiation similar to sarcomas in $KP^{FRT}VBax^{FL/+}$ mice (Fig. 1, C and D). To investigate if there was a difference in local control in this model, we irradiated primary sarcomas in $KP^{FRT}VBax^{FL/+}$ and $KP^{FRT}VBax^{FL/FL}$ mice with 50 Gy, which we determined to be the maximally tolerated dose in our system. Although 50 Gy was able to eradicate a small percentage of primary sarcomas, there was no difference in local control or growth delay between the two genotypes (Fig. 1E and fig. S2).

Because the pro-apoptotic genes *Bax* and *Bcl-2* homologous antagonist killer (*Bak*) can be redundant for executing programmed cell death in some settings (29), we also deleted the *Bax* gene from the endothelial cells of *Bak* null ($Bak^{-/-}$) mice (30). Primary sarcomas in $KP^{FRT}; VE-Cadherin-Cre; Bak^{-/-}; Bax^{FL/+}$ ($KP^{FRT}VBak^{-/-}Bax^{FL/+}$) and $KP^{FRT}; VE-Cadherin-Cre; Bak^{-/-}; Bax^{FL/FL}$ ($KP^{FRT}VBak^{-/-}Bax^{FL/FL}$) mice developed at the same time after injection of adeno-FlpO and grew at the same rate in the absence of radiation (fig. S3). After irradiation with 20 Gy, we observed no difference in endothelial cell death or growth delay for primary sarcomas in the $KP^{FRT}VBak^{-/-}Bax^{FL/+}$ and $KP^{FRT}VBak^{-/-}Bax^{FL/FL}$ mice (fig. S4), suggesting that endothelial cell apoptosis does not contribute to the radiation response of primary sarcomas. Because previous studies have demonstrated differences between the vasculature of transplanted and primary cancers (15, 31, 32), our discordant results on the contribution of endothelial cell death might reflect differences in the vasculatures of tumors derived from transplanted cell lines versus the autochthonous tumors studied herein. Taken together, these findings illustrate the importance of studying the tumor microenvironment using multiple complementary models, including GEMMs.

Endothelial-cell death does not contribute to eradication of primary sarcomas by SBRT

We showed recently that deleting *Atm* specifically in endothelial cells radiosensitizes these cells in autochthonous sarcomas, causing them to undergo a delayed mitotic cell death that increases tumor growth delay after radiation therapy with 20 Gy (23). To determine whether *Atm* deletion increased endothelial cell death after a curative dose of radiation, we treated primary sarcomas in $KP^{FRT}; VE-Cadherin-Cre; Atm^{FL/+}$ ($KP^{FRT}VAtm^{FL/+}$) and $KP^{FRT}; VE-Cadherin-Cre; Atm^{FL/FL}$ ($KP^{FRT}VAtm^{FL/FL}$) mice with 50 Gy. Consistent with our previous results, deletion of *Atm* significantly increased endothelial cell death 24 h after irradiation (Fig. 2, A and B).

To investigate whether endothelial cell death contributes to the local control of primary sarcomas, we irradiated sarcomas in $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice with 50 Gy. Although 50 Gy was able to eradicate primary sarcomas in both $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice, there was no difference in local control between the two genotypes (Fig. 2C). Moreover, at the curative dose of 50 Gy, there was no difference in growth delay for primary sarcomas in $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice (fig. S5, A and B).

Because sarcomas in $KP^{FRT}VAtm^{FL/FL}$ mice have a prolonged growth delay after 20 Gy compared to sarcomas in $KP^{FRT}VAtm^{FL/+}$ mice (23), we repeated this experiment with 4 daily fractions of 20 Gy. Similar to a single dose of 50 Gy, we observed no difference in local control or growth delay for sarcomas in $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice after 80 Gy of radiation was delivered in four 20 Gy fractions (Fig. 2D and fig. S5, C and D). These results demonstrated that although endothelial cell death can delay the regrowth of primary sarcomas at noncurative doses of radiation, endothelial cell death is not rate-limiting for sarcoma regrowth after curative doses of radiation and does not contribute to local control of primary soft-tissue sarcomas.

Tumor cells within primary sarcomas are critical targets that mediate local control by SBRT

For comparison, we also deleted *Atm* in tumor cells. First, we injected the muscle of $LSL-Kras^{G12D}; p53^{FL/FL}; Atm^{FL/+}$ ($KP^{loxP}Atm^{FL/+}$) and $LSL-Kras^{G12D}; p53^{FL/FL}; Atm^{FL/FL}$ ($KP^{loxP}Atm^{FL/FL}$) mice with adeno-Cre. However, the sarcomas that developed did not reliably delete both alleles of *Atm* (fig. S6A). Therefore, we crossed $KP^{loxP}Atm^{FL/+}$ and $KP^{loxP}Atm^{FL/FL}$ mice to $Pax7-CreER$ ($P7$) mice, which express a tamoxifen-inducible Cre recombinase in muscle satellite cells (33). $P7KP^{loxP}$ mice develop sarcomas after administration of systemic tamoxifen (34). In contrast to the $KP^{loxP}Atm^{FL/FL}$ mice injected with adeno-Cre, intramuscular injection of $P7KP^{loxP}Atm^{FL/FL}$ mice with 4-hydroxy-tamoxifen generated primary sarcomas at the site of injection with efficient deletion of both *Atm* alleles (fig. S6B).

To determine if *Atm* deletion sensitized sarcoma cells to radiation, we irradiated cell lines isolated from sarcomas in $P7KP^{loxP}Atm^{FL/+}$ and $P7KP^{loxP}Atm^{FL/FL}$ mice. Sarcoma cells from $P7KP^{loxP}Atm^{FL/FL}$ mice were more sensitive to radiation in clonogenic survival assays compared to sarcoma cells from $P7KP^{loxP}Atm^{FL/+}$ control mice that retained one allele of *Atm* (Fig. 3A). Next, we irradiated primary sarcomas in $P7KP^{loxP}Atm^{FL/+}$ and $P7KP^{loxP}Atm^{FL/FL}$ mice with 50 Gy and monitored the sarcomas for local control. Deletion of *Atm* in tumor cells significantly improved local control of primary sarcomas (Fig. 3, B and C), suggesting that direct tumor cell death can mediate tumor eradication by SBRT. Prior to local recurrence, several mice developed second sarcomas at other locations in the body presumably from systemic tamoxifen-mediated Cre recombination. When we analyzed the histology from the site of the irradiated primary sarcomas in nine $KP^{loxP}Atm^{FL/FL}$ mice that developed second-site sarcomas, there were no tumor cells present in five mice and a few scattered histologically intact tumor cells in three mice (fig. S7).

The protein kinase inhibitor BEZ235 preferentially radiosensitizes primary sarcomas

Deletion of the *Atm* gene from tumor cells increased the probability of tumor eradication after SBRT, suggesting that therapeutic targeting of the ATM protein could improve tumor response to radiation therapy. However, systemic targeting of ATM in normal tissues might also increase radiation toxicity. To begin to address whether targeting ATM during radiation therapy can improve the response of tumors relative to some normal tissues (i.e. enhance the therapeutic ratio), we showed recently that deleting *Atm* preferentially radiosensitizes proliferating tumor endothelial cells compared with quiescent heart endothelial cells (23).

Here we used a pharmacological approach to investigate whether a therapeutic window exists for inhibiting ATM during radiation therapy. To this end, we treated *KP^{loxP}* mice that had primary sarcomas with the phosphoinositide 3-kinase (PI3K)–like kinase (PI3KK) inhibitor BEZ235, which potently inhibits human ATM(35). Pretreatment of the mice with BEZ235 significantly decreased autophosphorylation of mouse Atm and phosphorylation of the Atm target Kap1 in primary sarcomas after irradiation with 20 Gy (fig. S8, A–D).

To investigate whether BEZ235 can selectively radiosensitize sarcomas, we collected hearts and primary sarcomas from vehicle and BEZ235-treated *KP^{loxP}* mice 24 h following whole-body irradiation with 20 Gy. Treatment with BEZ235 significantly increased cell death in sarcomas but not hearts (Fig. 4, A and B). Next, we monitored tumor growth in *KP^{loxP}* mice treated with BEZ235 alone or in combination with focal 20 Gy irradiation. Although a single dose of BEZ235 alone did not delay tumor growth (fig. S8, E and F), BEZ235 treatment significantly delayed primary sarcoma regrowth after 20 Gy (Fig. 4C and D).

We next investigated the effect of BEZ235 treatment on the development of radiation-induced heart disease. We showed previously that deletion of *Atm* radiosensitizes *p53* null cardiac endothelial cells but not cardiac endothelial cells with intact *p53* (23). Therefore, we treated *VE-Cadherin-Cre; p53^{FL/+}(VP^{FL/+})* mice with one allele of *p53* deleted in endothelial cells and *VE-Cadherin-Cre; p53^{FL/FL}(VP^{FL/FL})* mice with two alleles of *p53* deleted in endothelial cells with either vehicle or BEZ235 2 h before whole-heart irradiation with 12 Gy and monitored the mice for the development of radiation-induced heart disease. BEZ235 shifted the myocardial necrosis-free survival curve to the left in *VP^{FL/FL}* mice but did not promote the development of radiation-induced myocardial necrosis in *VP^{FL/+}* mice (Fig. 4E). Because we utilized BEZ235 with a single dose of radiation, we cannot exclude the possibility that ATM inhibition has some effect on radiation-induced heart disease with other radiation doses. However, taken together with the genetic studies, these experiments with a pharmacological inhibitor of ATM suggest that inhibiting ATM may preferentially radiosensitize sarcomas compared with quiescent normal tissues, such as the heart.

Discussion

In this study, we used a new dual recombinase technology to manipulate the radiosensitivity of endothelial cells in primary sarcomas. In contrast to results with transplanted tumor models (5), we found that tumor endothelial cells in primary sarcomas did not die via apoptosis within 4 h after irradiation. Although endothelial cells can contribute to primary sarcoma growth delay after noncurative radiation therapy (23), radiosensitizing endothelial cells did not increase local control. In contrast, radiosensitizing of tumor cells increased the probability of sarcoma eradication by SBRT. Therefore, tumor cells but not endothelial cells are critical targets of SBRT that mediate sarcoma eradication.

These results have important clinical implications, suggesting that increased endothelial cell death does not contribute to the improved local control of some tumors after SBRT. Instead, the increased efficacy of SBRT is likely the result of delivering larger, biologically effective doses than are delivered with standard radiation therapy (36). Our results are consistent with the previous observation that local control is not affected when tumors are transplanted into

radiosensitive mice (4) and extend these findings to primary sarcomas. Despite the extensive endothelial cell death observed after a dose of 50 Gy (Fig. 2, A and B), the majority of sarcomas in $KP^{FRT}VAtm^{FL/FL}$ mice recurred (Fig. 2C). These results raise the possibility that the vasculature in recurrent sarcomas may have come from outside of the irradiation field (37). Although the source of these newly formed blood vessels remains an area of ongoing debate (38), targeting vascular recruitment could improve local control.

Our results do not exclude a contribution from other stromal cells to tumor eradication with SBRT. Indeed, recent studies suggest that the recruitment of macrophages from outside the radiation field via a stromal cell-derived factor 1 (SDF-1)/C-X-C chemokine receptor 4 (CXCR4) axis might participate in tumor-cell repopulation after radiotherapy (37). In addition, a growing body of data suggests that the immune system can contribute to the killing of tumor cells after radiation therapy (39, 40), and this response may be greater after SBRT than after standard radiation therapy. Furthermore, our experiments focused on soft-tissue sarcomas, and additional experiments are needed to determine whether endothelial-cell death contributes to the radiation response of other tumor types. In the future, dual recombinase technology can be applied to other primary tumor model systems and stromal cell populations to further explore how the tumor microenvironment contributes to curative radiation therapy.

Although most patients are treated with curative intent, for patients receiving palliative radiation therapy, an increased growth delay can be clinically meaningful. For example, radiation therapy is the standard of care for children with diffuse intrinsic brainstem gliomas and provides many months of relief from severe neurological symptoms (41). Targeting tumor endothelial cells during radiation therapy could prolong the time that these patients remain neurologically intact. However, our results suggest that this approach may not improve tumor eradication. Instead, targeting tumor cells with radiosensitizers, such as inhibitors of ATM, might be a more promising treatment approach to achieve local control.

Materials and Methods

Study design

The goal of this controlled laboratory experiment was to determine the relative contribution of endothelial cells and tumor cells to primary sarcoma response to radiation therapy. We used genetically engineered mice and observed histological, growth delay, and local control endpoints. Sample sizes were selected prior to initiating the study on the basis of prior primary sarcoma radiation response data from the lab and power calculations performed as described previously (42); data collection was stopped if a smaller sample size achieved statistical significance. Outliers were defined prior to initiating the study as falling greater than two standard deviations from the mean. No outliers were excluded from this study. Sarcoma eradication was assumed if a sarcoma failed to triple in size 18 weeks after radiation treatment. Assuming a constant sarcoma doubling time of 4 days and tumor cell diameter of 25 μm (volume = $8.18 \times 10^{-6} \text{ mm}^3$), it would take a single tumor cell 100 days (~14 weeks) to reach 300 mm^3 . Mice that died prior to sarcoma tripling as a result of metastasis or sarcoma development at a distant site were censored at the time of death and were scored as locally controlled until this point. The mice in this study were not

randomized to their treatments and were selected based on availability. The investigators were not blinded when performing sarcoma measurements. Histological quantification was performed by an observer blinded to treatment and genotype. Histological assessment of residual sarcoma cells in the legs of irradiated mice was assessed by a sarcoma pathologist (D.M.C.) who was blinded to treatment and genotype.

Mouse strains and sarcoma induction

All animal studies were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee. All of the mouse strains used in this study have been described previously, including *LSL-Kras^{G12D}*, *p53^{FL}*, *FSF-Kras^{G12D}*, *p53^{FRT}*, *VE-Cadherin-Cre*, *Atm^{FL}*, *Bax^{FL}*, *Bak^{-/-}*, and *Pax7-CreER* (21, 27, 28, 30, 33, 43–46). *LSL-Kras^{G12D}* and *FSF-Kras^{G12D}* mice were provided by Tyler Jacks, *Atm^{FL}* mice were provided by Shan Zha and Frederick Alt, *Bax^{FL}* and *Bak^{-/-}* mice were provided by Stanley Korsmeyer, *p53^{FL}* mice were provided by Anton Berns, and *Pax7-CreER* mice were provided by Chen-Ming Fan. *VE-Cadherin-Cre* mice were obtained from the Jackson Laboratory. Primary sarcomas were generated in the right hind leg of *KP^{loxP}* or *KP^{FRT}* mice between 6 and 10 weeks of age as described previously (21, 47). Tamoxifen-induced primary sarcomas were generated by injecting 50 µl of 5 mg/ml 4-hydroxy-tamoxifen (Sigma) in DMSO into the right hind leg of *P7KP^{loxP}* mice. All mice were on a mixed genetic background. To minimize the effect of genetic background, age-matched littermate controls were used for every experiment so that potential genetic modifiers would be randomly distributed between the experimental and control groups.

Radiation treatment

Sarcoma and whole-heart irradiations were performed using the X-RAD 225Cx small animal image-guided irradiator (Precision X-Ray). The irradiation field was centered on the target via fluoroscopy with 40 kVp, 2.5 mA X-rays using a 2 mm Al filter. Sarcomas were irradiated with parallel-opposed anterior and posterior fields with an average dose rate of 300 cGy/min prescribed to mid-plane with 225 kVp, 13 mA X-rays using a 0.3 mm Cu filter and a collimator with a 40 x 40 mm square radiation field at treatment isocenter. Whole-heart irradiation was performed using a collimator to produce a 15 mm circular radiation field at treatment isocenter. Dose rates were measured with an ion chamber by the Radiation Safety Division at Duke University. Sarcomas were irradiated at ~250 mm³ by caliper measurement for growth delay and histological endpoints and ~100 mm³ for local control endpoints. After sarcoma irradiation, sarcomas were measured three times per week for growth delay and once per week for local control experiments until they tripled in size from the initial volume measured at the time of radiation. Following whole-heart irradiation, mice were monitored daily for symptoms of heart disease. Myocardial necrosis was confirmed histologically by hematoxylin and eosin staining of heart sections.

Histological analysis

Hematoxylin and eosin staining was performed on paraffin-embedded tissue sections. Tissue specimens were fixed in 10% neutralized formalin overnight and preserved in 70% ethanol until paraffin embedding. Five micron sections were deparaffinized with xylene and

rehydrated with a graded series of ethanol and water washes prior to performing hematoxylin and eosin staining. Sarcoma and heart immunohistochemistry was performed on frozen tissue sections. Specimens were embedded directly in OCT compound (Sakura Fintek) by snap freezing in a dry ice/isopentane slurry and stored at -80°C until sectioning. Ten micron sections were fixed in 4% paraformaldehyde prior to immunofluorescence staining. The primary antibody was rat anti-mouse CD31 (1:250, BD Pharmingen, #553370). The secondary antibody was Alexa Fluor 488-conjugated donkey anti-rat IgG (1:500, Invitrogen, #A21208). Nuclear staining was performed using Hoechst 33342 (10 μM , Sigma). TUNEL staining was performed with the In Situ Cell Death Detection Kit, TMR Red (Roche) according to the manufacturer's instructions. Pictures were acquired with a Leica DFC340 FX fluorescence microscope (Leica Microsystems) using Leica Suite software (Leica Microsystems). Quantification was performed using ImageJ (NIH). Each data point represents the average of eight randomly selected 200X fields per sample.

Flow sorting of endothelial cells

Lungs were dissected, washed in PBS, homogenized, and digested in 0.8 mg/ml type I collagenase (Worthington) for one hour at 37°C . Digested tissues were filtered and red blood cells were lysed with ACK lysing buffer (Lonza). Total number of cells was counted by Coulter counter (Beckman Coulter). Three million cells were stained with PE-conjugated anti-mouse CD31 (Biolegend, # 102407), PE-Cy5-conjugated anti-mouse CD45 (eBioscience, # 15-0451) and eFluor 660-conjugated anti-mouse CD34 (eBioscience, # 50-0341) antibodies. Dead cells were excluded by staining with 7-AAD (BD Pharmingen). Viable CD45 negative and CD31 and CD34 double positive cells were sorted by FACSVantage (BD Pharmingen) and used for RNA isolation.

Quantitative RT-PCR analysis

Total RNA was extracted from sorted lung and tumor endothelial cells with the RNAqueous-Micro Kit (Ambion), and reverse transcription was performed with the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Taqman universal PCR master mix (Applied Biosystems) and TaqMan Gene Expression Assay Mix (Applied Biosystems) for *Bax* (Mm00432050_m1) or *Hprt* (Mm0446968_m1). *Hprt* was used as an internal control to correct for the concentration of cDNA in different samples. Each experiment was performed with three replicates from each sample, and the results were averaged.

Cell line experiments and clonogenic survival assays

Cell lines were isolated by digestion of primary sarcomas with trypsin, type IV collagenase, and dispase (Invitrogen). Cells were cultured in Dulbecco's modified Eagle's medium with high glucose and pyruvate (Gibco) supplemented with 10% fetal bovine serum. Cells were passaged five times to deplete stromal cells prior to isolation of genomic DNA for PCR or clonogenic survival assays. Deletion of *Atm* was verified by PCR using primers flanking the 3' *loxP* site: sense 5'-GGGCTACGAAATGAGACACACAC-3' and anti-sense 5'-CTTCCCTGTTCAAAAGCCACTC-3' and primers flanking the recombined *loxP* site: sense 5'-TGAGTTCAAATCCCAGGAGCCAG-3' and anti-sense 5'-

CTTCCCCTGTTCAAAAGCCACTC-3'. For clonogenic survival assays, cells were plated in triplicate and allowed to adhere overnight prior to irradiation with an X-RAD 320 biological irradiator (Precision X-Ray). Cells were placed 50 cm from the radiation source and were irradiated with a dose rate of 161 cGy/min using 320 kVp, 10 mA X-rays and a 2 mm Al filter. Following development of colonies, cells were fixed with 70% ethanol, stained with Coomassie Brilliant Blue (Bio-Rad), rinsed with deionized water, and dried. Populations of greater than 50 cells were counted as one colony, and surviving fractions were calculated relative to unirradiated controls.

BEZ235 treatment

Mice were treated by oral gavage with a single dose of 50 mg/kg BEZ235 (Novartis) dissolved in 10% N-methyl-2-pyrrolidone/90% polyethylene glycol 300 (Sigma) two hours prior to radiation therapy.

Statistics

Results are presented as mean $\pm$ standard error of the mean (SEM). Two-tailed student's *t* test was performed to compare the means of two groups. Two-way ANOVA was performed to examine the interaction between genotypes and treatments followed by Bonferroni's post hoc tests for pairwise comparisons of individual treatments or genotypes. Non-normally distributed data were log-transformed before applying statistical tests. For survival studies, Kaplan-Meier analysis was performed, followed by the log-rank test for statistical significance. Significance was assumed at $P < 0.05$. All calculations were performed using Prism 5 (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Deletion of *Bax* from mouse endothelial cells does not affect primary sarcoma response to radiation therapy. **(A)** Representative immunofluorescence for CD31 and TUNEL in sarcomas from KP^{FRT}VBax^{FL/+} and KP^{FRT}VBax^{FL/FL} mice 4 and 48 h after irradiation with 20 Gy. Areas enclosed by dashed lines are shown at higher magnification in the insets. Scale bar, 100 μm. **(B)** Quantification of CD31 and TUNEL double-positive cells in sarcomas from KP^{FRT}VBax^{FL/+} and KP^{FRT}VBax^{FL/FL} mice at the indicated time points after irradiation with 20 Gy (*n*=4 mice per group). Two-way ANOVA for genotype and time interaction followed by Bonferroni's post hoc tests for pairwise comparisons between genotypes not statistically significant. **(C)** Tumor growth curves and **(D)** time to volume tripling for sarcomas in KP^{FRT}VBax^{FL/+} and KP^{FRT}VBax^{FL/FL} mice after irradiation with 20 Gy (*n*=8 mice per group). Two-tailed student's *t* test not statistically significant. **(E)** Kaplan-Meier plot of local sarcoma control defined as the absence of tumor volume tripling for sarcomas in KP^{FRT}VBax^{FL/+} and KP^{FRT}VBax^{FL/FL} mice following irradiation with 50 Gy (*n*=18 and 20 mice per group). Log rank test not statistically significant.

Fig. 2.

Sensitizing sarcoma endothelial cells to radiation does not affect local control of primary sarcomas. **(A)** Representative immunofluorescence for CD31 and TUNEL in sarcomas from $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice 24 h after irradiation with 20 Gy. Areas enclosed by dashed lines are shown at higher magnification in the insets. Scale bar, 100 μ m. **(B)** Quantification of CD31 and TUNEL double-positive cells in sarcomas from $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice 24 h after irradiation with 20 Gy ($n=6$ mice per group). Two-tailed student's t test, $P<0.05$. Kaplan-Meier plot of local sarcoma control (defined as the absence of tumor volume tripling) for sarcomas in $KP^{FRT}VAtm^{FL/+}$ and $KP^{FRT}VAtm^{FL/FL}$ mice after irradiation with **(C)** 50 Gy ($n=22$ and 23 mice per group) or **(D)** four daily fractions of 20 Gy ($n=23$ and 25 mice per group). One $KP^{FRT}VAtm^{FL/FL}$ mouse developed an abdominal metastasis 8 weeks after irradiation with 50 Gy, and two $KP^{FRT}VAtm^{FL/+}$ mice died of unknown causes at 7 and 10 weeks after irradiation with 4 daily fractions of 20 Gy prior to sarcoma tripling. Thus, these mice were scored as locally controlled until the time points at which they either developed metastasis or died. Log rank tests not statistically significant. Asterisk represents a statistically significant difference between the indicated groups.

Fig. 3.

Sensitizing tumor cells to radiation increases local control of primary sarcomas after radiation therapy. (A) Clonogenic survival of primary sarcoma cell lines from $P7KPlloxPAtm^{FL/+}$ and $P7KPlloxPAtm^{FL/FL}$ mice ($n=3$ independent cells lines per genotype). Two-way ANOVA for genotype and dose interaction, $P<0.05$, followed by Bonferroni's post hoc tests for pairwise comparisons between genotypes after 2 and 4 Gy, $P<0.05$. (B) Sarcoma growth curves and (C) Kaplan-Meier plot of local sarcoma control defined as the absence of tumor volume tripling for primary sarcomas generated by intramuscular 4-hydroxy-tamoxifen injection into $P7KPlloxPAtm^{FL/+}$ and $P7KPlloxPAtm^{FL/FL}$ mice after irradiation with 50 Gy ($n=13$ mice per group). Several mice were euthanized prior to sarcoma tripling because of the development of second sarcomas at other locations in the body, presumably from systemic tamoxifen-mediated Cre recombination. These mice were scored as locally controlled until the time point of second tumor formation. Log rank test, $P<0.05$. Asterisks represent a statistically significant difference between the indicated groups.

Fig. 4.

The PI3KK inhibitor BEZ235 preferentially radiosensitizes primary sarcomas compared to mouse heart tissue. **(A)** Immunofluorescence and **(B)** quantification of TUNEL-positive cells in hearts and sarcomas from vehicle- or BEZ235-treated KP^{loxP} mice 24 h after irradiation with 20 Gy ($n=5$ mice per group). Two-way ANOVA for tissue and treatment interaction, $P<0.05$, followed by Bonferroni's post hoc tests for pairwise comparison between treatments in hearts not statistically significant, but in sarcomas, $P<0.05$. Scale bar, 100 μ m. **(C)** Tumor growth curves and **(D)** time to volume tripling of primary sarcomas in KP^{loxP} mice treated on day 0 with vehicle or a single dose of 50 mg/kg BEZ235 2 h before irradiation with 20 Gy ($n=8$ mice per group). Two-tailed student's t test, $P<0.05$. **(E)** Kaplan-Meier plots of myocardial necrosis-free survival for mice that lacked p53 in endothelial cells ($VP^{FL/FL}$) or mice that retained p53 in endothelial cells ($VP^{FL/+}$), both of which were treated with vehicle or 50 mg/kg BEZ235 2 h before whole-heart irradiation with 12 Gy ($n=7$ to 9 mice per group). Two $VP^{FL/FL}$ mice treated with vehicle were censored because they developed tumors before developing myocardial necrosis. Log rank test $VP^{FL/+}$ Vehicle vs. $VP^{FL/FL}$ Vehicle, $VP^{FL/+}$ Vehicle vs. $VP^{FL/FL}$ BEZ235, $VP^{FL/+}$ BEZ235 vs. $VP^{FL/FL}$ Vehicle, and $VP^{FL/+}$ BEZ235 vs. $VP^{FL/FL}$ BEZ235, $P<0.05$. Asterisks represent a statistically significant difference between the indicated groups.