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# Sunitinib effects on the radiation response of endothelial and breast tumor cells

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ABSTRACT

*Background:* Endothelial cells are suggested regulators of tumor response to radiation. Anti-vascular targeting agents can enhance tumor response by targeting endothelial cells. Here, we have conducted experiments *in vitro* to discern the effects of radiation combined with the anti-angiogenic Sunitinib on endothelial (HUVEC) and tumor (MDA-MB-231) cells, and further compared findings to results obtained *in vivo*.
*Methods:* *In vitro* and *in vivo* treatments consisted of single dose radiation therapy of 2, 4, 8 or 16 Gy administered alone or in combination with bFGF or Sunitinib. *In vitro*, *in situ* end labeling (ISEL) was used to assess 24-hour apoptotic cell death, and clonogenic assays were used to assess long-term response. *In vivo* MDA-MB-231 tumors were grown in CB-17 SCID mice. The vascular marker CD31 was used to assess 24-hour acute response while tumor clonogenic assays were used to assess long-term tumor cell viability following treatments.
*Results:* Using *in vitro* studies, we observed an enhanced endothelial cell response to radiation doses of 8 and 16 Gy when compared to tumor cells. Administering Sunitinib alone significantly increased HUVEC cell death, while having modest additive effects when combined with radiation. Sunitinib also increased tumor cell death when combined with 8 and 16 Gy radiation doses. In comparison, we found that the clonogenic response of *in vivo* treated tumor cells more closely resembled that of *in vitro* treated endothelial cells than *in vitro* treated tumor cells.
*Conclusion:* Our results indicate that the endothelium is an important regulator of tumor response to radiotherapy, and that Sunitinib can enhance tumor radiosensitivity. To the best of our knowledge, this is the first time that Sunitinib is investigated in combination with radiotherapy on the MDA-MB-231 breast cancer cell line.


## Introduction

Recent studies suggest that tumor stroma plays an integral role in regulating tumor response to radiation therapy (Abdollahi and Folkman, 2010; Folkman and Camphausen, 2001; Franses and Edelman, 2011; Franses et al., 2011; Fuks and Kolesnick, 2005), challenging the canonical notion that tumor response is primarily dependent on the inherent radiosensitivity of its clonogenic cells. Furthermore, observed differences in tumor cell radiosensitivity *in vitro* and *in vivo* have been linked to the presence of host derived supporting cells *in vivo*, which include endothelial cells (Fenton et al., 2001; Fiebig et al., 2004; Garcia-Barros et al., 2003; García-Barros et al., 2010; Gerweck et al., 2006; Moeller et al., 2004; Ogawa et al., 2007; Ruka et al., 1996; Schwartz et al., 2011). Tumor endothelial cells have been demonstrated to undergo radiation-induced apoptosis faster (<6–20 h after treatment) than most other cancer cell lines (Fuks and Kolesnick, 2005; Garcia-Barros et al., 2003, 2004; García-Barros et al., 2010; Truman et al., 2006, 2010). This has been linked to a 20-fold enrichment of the ASMase enzyme in endothelial cell membranes compared to epithelial and tumor cells. High doses of radiotherapy (>8–10 Gy) are suggested to cause substantial damage to the endothelial cell membrane, leading to hydrolyzation by the ASMase enzyme, in turn releasing ceramide, which signals for apoptosis. At high radiation doses, endothelial cells can undergo apoptosis through two pathways: by DNA damage, or *via* a ceramide-signaling apoptosis pathway (Garcia-Barros et al., 2003; Gupta et al., 2001; Haimovitz-Friedman et al., 1994; Kolesnick and Fuks, 2003; Peña et al., 2000; Truman et al., 2006; Vit and Rosselli, 2003). Conventional radiation doses of 1.8–2 Gy are believed not to activate the ceramide-dependent apoptosis pathway. Basic fibroblast growth factor (bFGF) has been demonstrated to inhibit ceramide-dependent apoptosis messaging (Fuks et al., 1992, 1994; Gu et al., 2004; Peña et al., 2000; Truman et al., 2006), thus protecting endothelial cells from radiation induced cell death. Separate from endothelial effects, it is believed that epithelial and tumor cells are

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predominantly damaged through direct or indirect DNA damage, and undergo cell death or senescence only when they have accumulated sufficient DNA damage (Barcellos-Hoff et al., 2005; Park et al., 2012; Prise et al., 2005).

As stated above, enhanced radiation effects associated with rapid endothelial apoptosis have predominantly been observed at high radiation doses. It has been hypothesized that vascular targeting agents, which can target endothelial cells in a number of ways, can also radiosensitize whole tumors by altering the tumor micro-vasculature, the tumor microenvironment, or by directly radiosensitizing endothelial cells to clinical doses of radiotherapy (Bischof et al., 2004; El Kaffas et al., 2012; Kozin et al., 2001; Li et al., 2005; Maggiorella et al., 2009; McGee et al., 2010; O'Reilly, 2006; Schueneman et al., 2003; Senan and Smit, 2007; Timke et al., 2008; Wachsberger et al., 2003; Winkler et al., 2004; Zhang et al., 2011). Small-molecule receptor inhibitors such as Sunitinib have been suggested as radiosensitizing agents (Brooks et al., 2012; Cuneo et al., 2008; El Kaffas et al., 2013; Zwolak et al., 2008). Sunitinib is a small molecule tyrosine-kinase inhibitor (TKI), which prevents the activation of a wide-spectrum of receptors including vascular endothelial growth factor (VEGF) receptors (*i.e.* PDGFRα, PDGFRβ, VEGFR1, VEGFR2, VEGFR3, FLT3, CSF-1R, RET). It acts by inhibiting downstream signaling pathways rather than binding directly to VEGF, and is one of the few FDA approved anti-angiogenic agents for the treatment of renal cell carcinoma and gastro-intestinal stromal tumors (Faivre et al., 2007; Mendel et al., 2003; Osusky et al., 2004; Senan and Smit, 2007). Nevertheless, there remains unanswered questions in regard to how this type of agent should be administered, and whether it should be used alone or in combination with other forms of therapies (*i.e.* chemotherapy, radiation therapy) in order to optimize tumor responses.

On its own, Sunitinib has been reported to impede endothelial cell migration, tubule formation and blood vessel formation. It also impedes the induction of apoptosis in less mature endothelial cells, while having minimal effects on existing mature endothelial cells (Osusky et al., 2004). Combined with radiation, Sunitinib has been demonstrated to moderately enhance endothelial radiosensitivity *in vitro* and diminish micro-vascular densities with *in vivo* xenograft models (Kamrava et al., 2009; Senan and Smit, 2007; Zhang et al., 2011). Sunitinib has also been reported to induce a vascular remodeling process known as vascular 'normalization' (Czabanka et al., 2009; Griffioen et al., 2012; Hillman et al., 2009, 2010; Yoon et al., 2009; Zhou and Gallo, 2009, 2010). It has further been shown to induce *in vivo* and *in vitro* radiosensitization (for a limited number of single and fractionated radiation doses) of a range of cell lines, including endothelial cells (Brooks et al., 2012; Cooney et al., 2006; Cuneo et al., 2008; Gordon et al., 2010; Kamrava et al., 2009; Schueneman et al., 2003; Sun et al., 2012; Yoon et al., 2009, 2011). Nevertheless, the mechanism of synergy in such therapeutic combinations, and how it changes with radiation, remains unclear and controversial (Ebos et al., 2007, 2009; Gaustad et al., 2012). Potential mechanisms include vascular normalization resulting in increased radiosensitivity of the whole tumor due to enhanced oxygenation, and/or endothelial cell radiosensitization leading to complete or partial vascular collapse or destruction at the time of radiotherapy, and subsequent rapid tumor cell death. A number of parameters that may affect the mechanism by which this therapeutic combination acts still remain to be investigated. These parameters include: radiation dose, radiation fractions, drug dose and scheduling, amongst a number of other parameters (Kamrava et al., 2009; Kleibeuker et al., 2012; Suarez and Rini, 2012).

The primary objective of this study is to understand how endothelial and tumor cells respond to a range of radiation doses alone, or in combination with vascular targeting therapies, and to compare the responses of each of these cell lines. It is hypothesized that endothelial cells have a different dose dependent response to radiation therapy than tumor cells and that vascular targeting agents can alter endothelial cell response to single radiation doses. It is posited that the altered endothelial response directly affects tumor cell response to radiotherapy. In order to address this, we have investigated radiation responses *in vitro*, comparing endothelial cells *versus* breast cancer cells to a range of radiation doses between 0–16 Gy. Sunitinib was used as a vascular targeting agent and investigated as a potential tumor radiosensitizer. Additionally, bFGF was used to modulate endothelial responses to radiotherapy, and as a comparative condition to radiation–Sunitinib combination therapy. Experiments assessed the acute (24 hour cell death) and long-term response (clonogenic assay) of HUVEC cells compared to MDA-MB-231 cells. Results were compared to *in vivo* MDA-MB-231 tumor xenograft experiments (El Kaffas et al., 2013).

Results indicated an enhanced endothelial cell response to radiation therapy at doses greater than 8 Gy in comparison to breast cancer tumor cells. The use of bFGF resulted in minimized endothelial damage after radiation therapy, while having minimal effects on MDA-MB-231 cells. Sunitinib caused a significant endothelial response when administered alone, while having modest additive effects when combined with radiation. The combination of Sunitinib and radiation had minimal effects on MDA-MB-231 breast cancer cells. In animal studies, results indicated that Sunitinib acts as a tumor radiosensitizer and that tumor responses at higher doses of radiation alone are linked to endothelial cell responses to radiotherapy.

## Materials and methods

### *Cell culture*

MDA-MB-231 breast cancer cells were cultured in RPMI 1600 culture medium (ATCC, Manassas, VA), 5% fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin: Life Technologies, Grand Island, NY) to 80–90% confluence. Human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA) were cultured using endothelial cell basal medium-2 kit from Lonza (Walkersville, MD, USA). HUVEC cells are well-established in angiogenesis and radiation studies (Nakatsu et al., 2003; Tran et al., 2007). Both cell lines were allowed to undergo two passages before plating into treatment dishes and drug/radiation exposure. Passages were conducted following trypsinization using 0.05% trypsin EDTA (GIBCO; Invitrogen, Carlsbad, CA). Each cell line was treated with 15 different conditions: radiation alone at 0, 2, 4, 8 or 16 Gy, or in combination with bFGF, or with Sunitinib. On the day before treatment administration, $2 \times 10^5$ cells were plated in chamber-slides (Chamber Slides; Lab-Tek, Rochester, NY), for a total of 30 chamber-slides, 15 for MDA-MB-231 cells and 15 for HUVEC cells. Cells were also plated in petri dishes for clonogenic assays at 3 different cell concentrations accordingly ($10^1$, $10^2$ and $10^3$), for each of the treatment conditions.

### *Treatment*

Each treatment condition was comprised of a single dose of ionizing radiation therapy delivered alone, or combined with Sunitinib (Pfizer, New York, NY) or bFGF (R&D Systems, Minneapolis, MN). Cells plated on the day before experimentation received single doses of radiotherapy of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF or Sunitinib, delivered to plates at 0.75 ng/ml and 398.5 ng/ml, respectively, 1 h before irradiation; concentrations were based on literature to maximize effects (Fuks et al., 1994; Osusky et al., 2004; Truman et al., 2006). Cells were washed with PBS immediately before irradiation to remove bFGF or Sunitinib, and re-suspended in their respective medium as described in the cell culture section. Radiation therapy was administered using a Faxitron cabinet irradiator (Faxitron Bioptics, Lincolnshire, IL) with 160 kVp X-rays at an SSD of 35 cm, and a dose rate of 200 cGy/min.

*ISEL assay*

After radiation therapy, cells in chamber slides were washed again with PBS, re-suspended in their respective medium and left overnight in an incubator at 37 °C. The following day (at 24 h after irradiation), cells were fixed in 10% formalin for 1 h at room temperature and left in 70% ethanol until staining. Cells were then stained for DNA breaks using *in situ* end labeling (ISEL) stain as a cell death marker (Santa Cruz Biotechnology, Santa Cruz, CA). Once staining was complete, the total number of cells and the number of ISEL positive cells (nuclei brown staining) were counted for 8–10 fields of view (FOV) in the center of each slide with a 40× objective lens on an upright microscope (Carl Zeiss, Göttingen, Germany). The total number of cells and the number of ISEL positive cells (nuclei brown staining) were counted. A percent apoptotic index was determined by dividing the number of dead cells by the total number of cells in each FOV, and averaged over all 10–15 FOVs per condition. All counts from treatment conditions were normalized to respective controls.

*Clonogenic assay*

For clonogenic assays, cells plated in petri dishes were washed after irradiation and suspended in their respective medium. Dishes were left in an incubator for colony growth at 37 °C for approximately 2 weeks. Cells were then fixed, stained and counted. All plating efficiencies were normalized to control cells.

*Capillary-like structure assay*

Capillary-like structure (CLS; tube formation assay) assays were performed using a specialized kit (Cell Biolabs Inc.; San Diego, CA). As described above, cells were first treated with 0, 2 or 8 Gy radiation alone, or in combination with Sunitinib. Cells were then trypsinized and plated in a 96 well dish over a layer of extracellular matrix gel. Cells were plated in three wells per treatment condition. Tubes were allowed to form over 24 h. Cells were then fixed (10% formalin) and stained as per kit. An objective lens of 10× was used to take 5–8 images per treatment condition throughout all three wells. Quantification was achieved by counting the number of tubes formed per image.

*In vivo animal experiments*

All animal experiments presented in this work were conducted in compliance with internationally recognized guidelines specified in protocols approved by the Sunnybrook Health Science Centre Institutional Animal Care Committee. MDA-MB-231 breast cancer cells were cultured as described above for full confluence and $1 \times 10^6$ cells were injected subcutaneously into female CB-17 SCID mice (Charles River Laboratories International, Wilmington, MA). Tumors were grown in one hind leg of each mouse for a period of three weeks, reaching an average volume of 200 $mm^3$ before experimentation. Five animals per experimental condition were used (El Kaffas et al., 2013).

Animals bearing MDA-MB-231 breast cancer xenografts were treated with single radiation doses of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF (0.45 μg/ml) given one hour before irradiation. Another group was given Sunitinib daily for 14 days prior to radiation delivery at a dose of 30 mg/kg. Radiation was delivered as described above. Mice were euthanized 24 h after irradiation for immunohistochemistry and clonogenic assays. Tumor xenograft cross-sections were stained for tumor blood vessels using a cluster of differentiation-31 (CD31) antibody stain (Santa Cruz Biotechnology, Santa Cruz, CA) and standard hematoxylin and eosin (H&E), gamma-H2AX and VEGF receptor one (R1) and receptor two (R2). Three cross-sections per tumor were stained for both CD31 and H&E. Tumor cross-sections stained with CD31 were quantified for tumor micro-vascular density (MVD). Stained tissue section slides were digitized using a Mirax slide scanner (Carl Zeiss, Göttingen, Germany) after preparation. For each of the CD31 stained tumor cross-sections, 4 fields of view (FOV) were analyzed at 10× magnification to compute the density of vasculature for a total of 12 FOVs per tumor; the MVD was obtained as described in literature (Herbst et al., 1998; Sabo et al., 2001). For *in vivo* tumor clonogenic assays, cells from 18 tumors ($n = 3$ per treatment condition) were disaggregated, suspended in culture medium and plated into petri dishes for colony formation. Cells were fixed, stained and counted two weeks after plating. All plating efficiencies were normalized to the 0 Gy condition.

*Statistical analysis*

All comparisons of quantified *in vitro* and *in vivo* cell death, CD31 MVD and colony counts were evaluated for statistical significance using a Mann–Whitney test (two-tailed, assuming unequal variances; $\alpha < 0.05$ (*), 0.01 (**) or 0.001 (***). Each treatment condition was compared directly to the 0 Gy control condition, or as specified in the text. For clonogenic assays, radiation and bFGF or Sunitinib combination treatment conditions were tested against the equivalent irradiation dose in radiation only conditions. Statistical tests were conducted using Prism (GraphPad Software version 5, La Jolla, CA).

## Results

The *in vitro* dose-dependent radiation response of tumor and endothelial cells, treated with radiation alone or in combination with bFGF or Sunitinib, was assessed. Experiments evaluated the acute (24 hour cell death) and long-term viability (clonogenic assay) of HUVEC and MDA-MB-231 cells. Findings were then compared to *in vivo* treated MDA-MB-231 xenograft clonogenic assays (long-term response) and CD31 staining (acute response). Fig. 1 displays representative images of MDA-MB-231 and HUVEC radiation treated cells. The number of dead cells 24 h after a single dose of radiation therapy, as a percentage of all cells in a single FOV, is plotted in Fig. 2. Quantitative analysis of ISEL staining revealed that a single dose of 16 Gy caused the most significant amounts of acute HUVEC cell death, followed by the 8 Gy dose ($P < 0.001$). Almost no ISEL staining was observed in doses lower than 8 Gy. The addition of bFGF before high-dose radiotherapy protected endothelial cells from rapid 24-hour cell death. In HUVEC cells treated with Sunitinib alone, we observed significant acute cell death by 24 h (increase of 9.4%). Combining Sunitinib with radiation caused a moderate dose-dependent increase in acute cell death (2 Gy — 10.4%; 4 Gy — 14%; 8 Gy — 19.13%; 16 Gy — 29.1%). All were statistically significant when compared to control HUVEC cells ($P < 0.001$). However, combining Sunitinib with 2 or 4 Gy radiation did not significantly increase cell death in comparison to Sunitinib alone. We did however find a significant increase in cell death when Sunitinib was combined with 8 or 16 Gy, in comparison to Sunitinib alone; this effect was less substantial than radiation administered alone at doses greater than 8 Gy. In comparison, minimal (less than 7%) amounts of cell death were observed for MDA-MB-231 cells treated with radiation alone, or in combination with bFGF, for all radiation doses. Sunitinib significantly elevated the ISEL stained cell death count of tumor cells following radiotherapy to an average of ~ 15% for radiation doses greater than 8 Gy ($P < 0.05$ at 8 Gy and $P < 0.01$ at 16 Gy).

Colony counts for MDA-MB-231 and HUVEC cells exposed to 2–16 Gy are presented in Fig. 3. Respective plating efficiencies are presented in Fig. 7. From these results, we observed that administering bFGF before radiation treatment minimized the effects of 8 and 16 Gy doses on HUVEC cells significantly ($P < 0.05$). This was not the case with MDA-MB-231 cells. Sunitinib had minimal effects on the formation of MDA-MB-231 colonies and plating efficiency. It also did not decrease HUVEC colony formation. In contrast, a decrease in HUVEC plating efficiency was noted when Sunitinib was used. For example, for MDA-MB-231 cells, the normalized surviving fractions were 5.7%, 4.9% and 7.9% for 8 Gy alone, 8 Gy with Sunitinib and 8 Gy with bFGF, respectively.

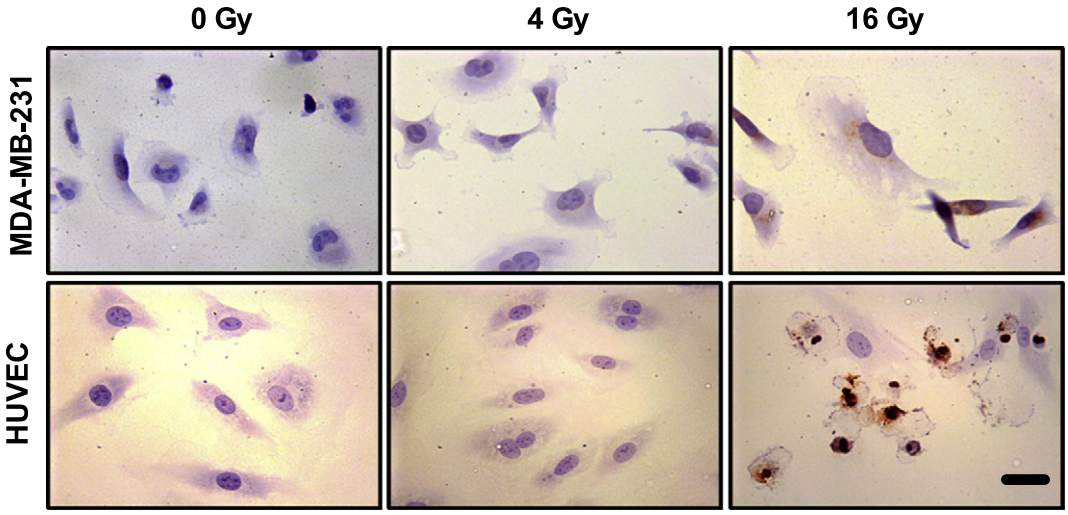


**Fig. 1.** Representative images of ISEL stained MDA-MB-231 cells (top) and HUVEC cells (bottom). MDA-MB-231 cells have minimal response to radiation therapy. 16 Gy treated MDA-MB-231 cells have slight staining, these are deemed viable when compared to cells undergoing apoptosis in the bottom panel. One can further observe enlargement of MDA-MB-231 cells after radiotherapy, likely associated with mitotic arrest. HUVEC cells respond by 24 h to 16 Gy irradiation. We can observe in the bottom right-hand image that many cells have stained nuclei after the single 16 Gy dose administration. Images were taken at 40× magnification. Scale bar is 12 μm.

In contrast, corresponding endothelial cell clonogenic surviving fractions were 6.7%, 8.4% and 12% respectively. These *in vitro* results suggest that Sunitinib minimally affects the long-term radiation response of HUVEC and MDA-MB-231 cells. However, we note that Sunitinib does affect the plating efficiency of HUVEC cells (Fig. 7), and not that of MDA-MB-231 cells.

Results from HUVEC-based CLS formation assays are presented in Fig. 4. We note a dose-dependent diminishment in the ability of these cells to form CLSs (Fig. 4A). This is further confirmed through quantification (Figs. 4B and C). Sunitinib alone also caused a decrease in CLSs; combining it with radiation caused complete CLS inhibition. These results suggest that combining Sunitinib with radiation completely inhibits angiogenesis *in vitro*.

For *in vivo* treatments, results from histological staining of tumor cross-sections and colony formation assays of MDA-MB-231 cells are presented in Figs. 5 and 6. After treatment administration of radiation alone, or in combination with bFGF or Sunitinib, animals were sacrificed and tumors were processed for immunohistochemistry (H&E, VEGF-R1/R2, gamma-H2AX and/or CD31) staining or clonogenic assay as described above. We first confirmed that Sunitinib does inhibit VEGF-R1 and R2; results are presented in Fig. S1. In H&E stained tumor cross-sections, we noted the most necrotic regions in tumors treated with Sunitinib and radiotherapy, while Sunitinib alone caused minimal tumor necrosis (Fig. 5). Gamma-H2AX staining confirmed this, where we observed the most fluorescent staining brightness in tumor cells treated with radiation and Sunitinib (Fig. S2). Clonogenic assay results (Fig. 6) indicated a dose dependent response to radiation doses. However, administering bFGF 1 h before doses of 8 or 16 Gy decreased the effects of high-dose radiation on tumor cell colony formation ($P < 0.05$). Sunitinib alone caused a decrease in colony formation plating efficiencies

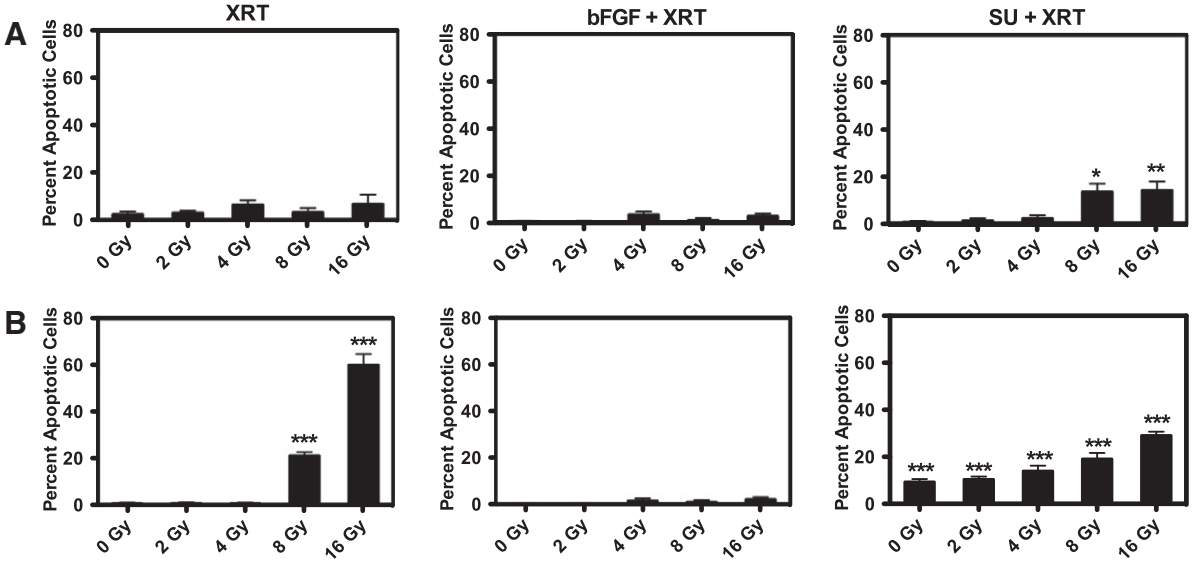


**Fig. 2.** Quantified ISEL stained cells as a percentage of the total number of cells in each FOV taken at 20× magnification. Each bar is an average of 8–10 FOVs. Top panel (A) is of MDA-MB-231 cells; bottom panel (B) is of HUVEC cells. The first column is of cells treated with radiation alone, the second column is of cells pre-treated with bFGF followed by radiotherapy and the third column is of cells pre-treated with Sunitinib (SU) followed by radiotherapy. While minimal cell death is observed in all treatment conditions for MDA-MB-231 cells, apparent amounts of cell death were quantified in HUVEC cells at 8 and 16 Gy and for all radiation conditions when combined with Sunitinib. For Sunitinib alone, we observed significant cell death by 24 h. Combining Sunitinib with radiation caused a moderate dose-dependent increase in cell death. Statistical significance is indicated for treatment condition compared to the control condition ($\alpha < 0.05$ (*), 0.01 (**) or 0.001 (***).

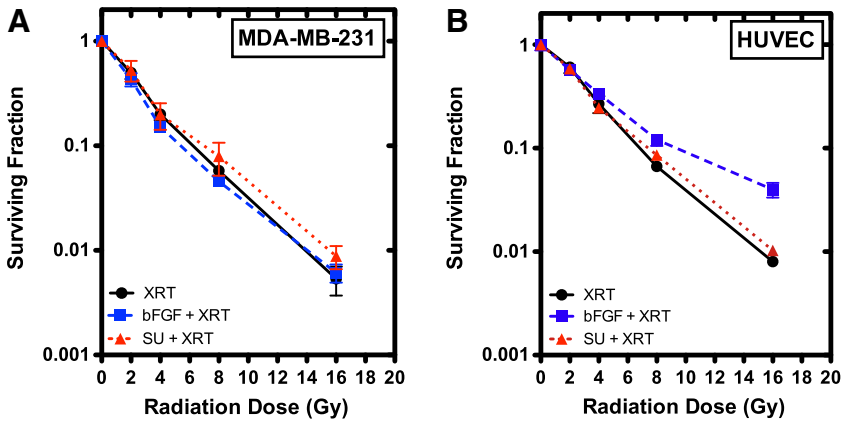


**Fig. 3.** Normalized colony counts from clonogenic assay experiments of MDA-MB-231 (A) and HUVEC (B) cells. Cells were plated 24 h before irradiation and left to form colonies for 14 days post-radiation. We observe that bFGF and Sunitinib had minimal effects on the dose-dependent response of MDA-MB-231 cells. In HUVEC cells, bFGF acted as a radio-protector at 8 and 16 Gy. Sunitinib had no effects on the dose-dependent response of HUVEC cells. Plating efficiencies are shown in Fig. 7.

compared to control animals (see *in vivo* tumor plating efficiencies: Fig. 7). On the other hand, in-advance treatment with Sunitinib combined with radiotherapy caused a decrease in colony formation at doses greater than 4 Gy. The quantified acute MVD from CD31 staining of tumor cross-sections significantly decreased ($P < 0.01$) at doses greater than 8 Gy; for instance, at 16 Gy, it decreased by ~75% compared to untreated animals. In contrast, a minimal MVD decrease following radiation was observed when tumors were pre-treated with bFGF. The use of Sunitinib in conjunction with radiotherapy enhanced overall tumor response for all radiation doses, but only moderately decreased the MVD when compared to control animals. An average MVD decrease of 45%–50% was noted for all Sunitinib treated animals in comparison to control animals; the MVD did not decrease further after single dose radiotherapy (Fig. 6).

## Discussion

We have investigated the acute and long-term effects of single dose radiation on tumor and endothelial cells *in vitro*. We have further conducted *in vivo* animal experiments to compare to our *in vitro* results. The aim of this work was to determine the relative temporal responses of tumor cancer cells and endothelial cells *in vitro*, in order to better understand which of the two tumor components predominantly determines tumor radiation-response *in vivo*. Endothelial cell radiation-

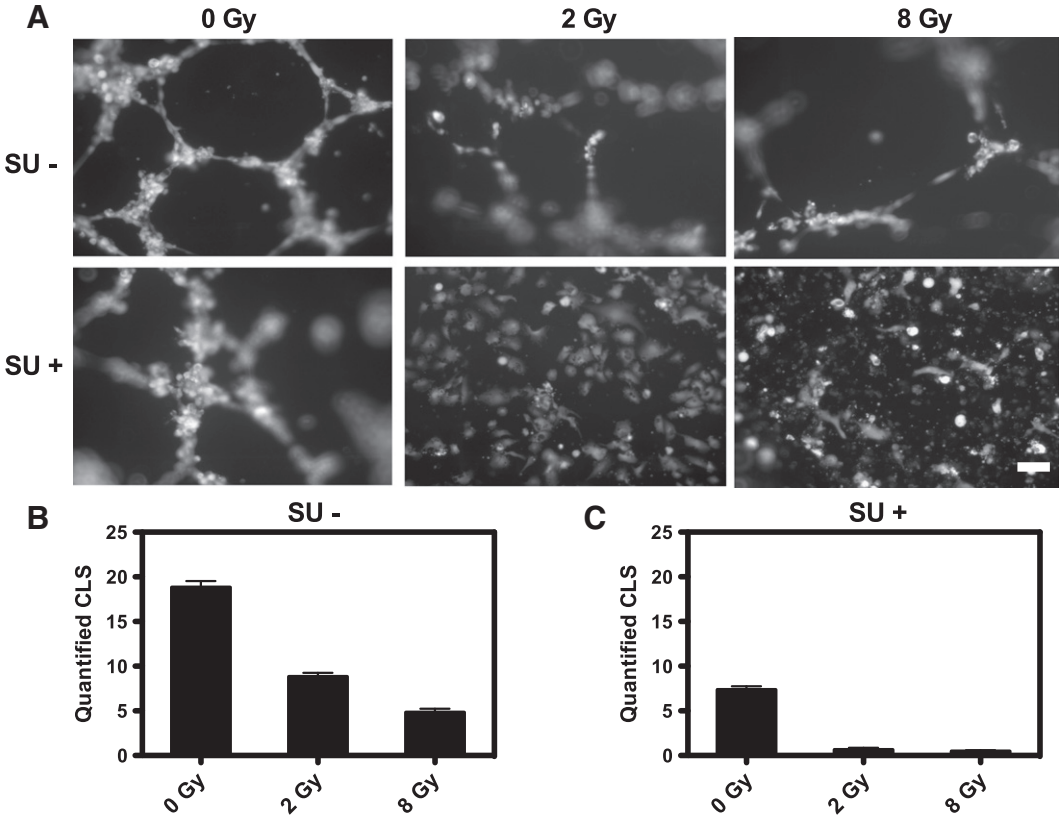


**Fig. 4.** A) Representative images of CLS formation assay of HUVEC cells treated with radiation with and without the addition of Sunitinib. B) Quantified CLS formation based on the number of tubes observed in 4–5 images per treatment condition. We observe a dose-dependent decrease in CLS formation in radiation treated HUVEC cells. Adding Sunitinib alone also decreases CLS formation, whereas combining Sunitinib and radiation completely blocks CLS formation. Scale bar is 30 μm.

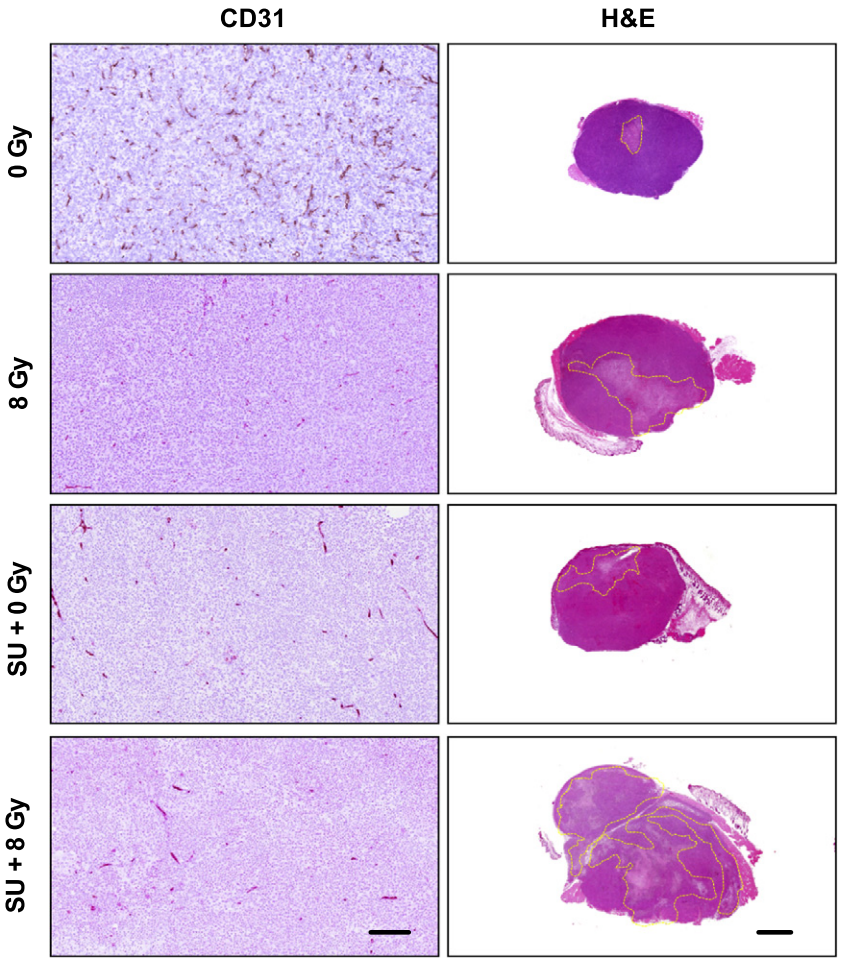


**Fig. 5.** CD31 (A) and H&E (B) staining of representative tumor cross-sections. In (A), while tumor vasculature is dense in MDA-MB-231 tumors, we note a decrease in vascular density when tumors are treated with 8 Gy. Treatment with Sunitinib diminishes the overall tumor vascular density, causing these blood vessels to appear less tortuous and less chaotic. It appears however that the administration of 8 Gy radiotherapy to tumors already treated with Sunitinib minimally alters the acute vascular density. In panel (B), representative H&E stained tumor cross sections are shown; we have outlined in yellow regions of extreme cell death in the tumor as observed at higher magnifications. Our results indicate that the greatest amount of cell death occurs in tumors treated with the combination of radiation and Sunitinib. Scale bar for CD31 is 500 μm, and 1 mm for H&E.

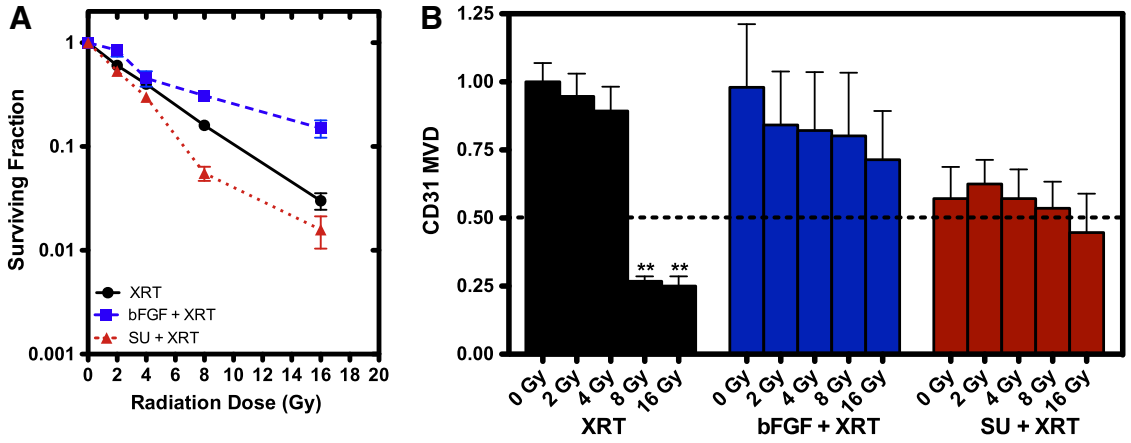


**Fig. 6.** Quantified results from colony formation assays (A) and CD31 staining of tumor cross-sections (B) of MDA-MB-231 tumors treated *in vivo*. After treatment administration of radiation alone, or in combination with bFGF or Sunitinib, animals were sacrificed and tumors were processed for histological staining or clonogenic assay as described in the methods section. Results indicate a dose dependent decrease in colony formation for cells treated with radiation alone. The administration of bFGF before radiotherapy caused tumor cells to be less responsive to ionizing radiation. On the other hand, pre-treatment with Sunitinib caused a decrease in colony formations at all radiation doses. CD31 staining demonstrated minimal MVD decrease at 2 and 4 Gy, and a significant decrease at 8 and 16 Gy. This was not observed when tumors were treated with bFGF before irradiation. Sunitinib decreased the overall MVD of tumors, however, no further decrease in MVD was observed when combined with radiotherapy. Statistical significance is indicated for treatment condition compared to control condition ($\alpha < 0.05$ (*), 0.01 (**) or 0.001 (***). Plating efficiencies for *in vivo* tumor clonogenic assays are shown in Fig. 6.

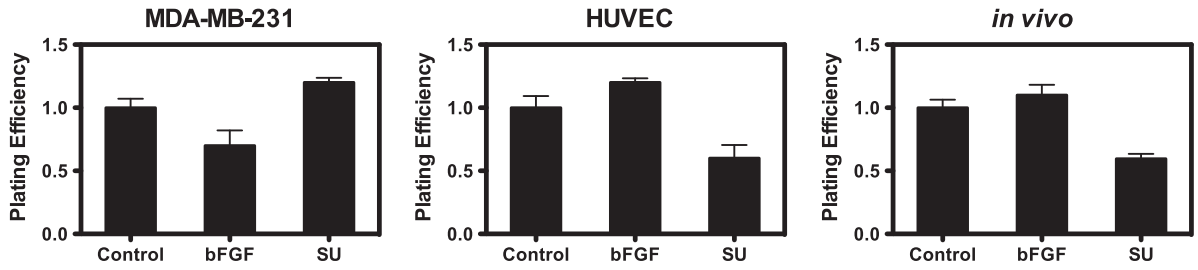


**Fig. 7.** Quantified plating efficiencies for *in vitro* clonogenic assay (MDA-MB-231 and HUVEC) and *in vivo* tumor clonogenic assays. The first group of columns is for MDA-MB-231 tumor cells, the second group is for HUVEC endothelial cells, and the third group is for MDA-MB-231 tumor cells treated *in vivo* as tumors. We note that the average control plating efficiency was always 1 for all three groups. From these, we observed a decrease in plating efficiency when MDA-MB-231 cells were treated with bFGF. This may be linked to reports that have suggested that bFGF decreases MDA-MB-231 aggressiveness. Contrary to this, bFGF slightly increased HUVEC cell plating efficiency, while having minimal effects on *in vivo* treated MDA-MB-231 tumor cells. Sunitinib had no effects on MDA-MB-231 cells, while decreasing the plating efficiency of both HUVEC and *in vivo* treated MDA-MB-231 cells.

responses were altered using pharmacological modulators of endothelial radiosensitivity *in vitro* and *in vivo*. Experiments were carried out using bFGF as an endothelial radio-protector to determine the extent of vascular-driven tumor response to a range of radiation doses. Sunitinib was used as a vascular targeting agent and investigated as a potential tumor radiosensitizer. Experiments with this anti-angiogenic agent aimed to better understand its effects when combined with a number of radiation doses *in vitro* and *in vivo*.

Results were consistent with previous findings that endothelial cells respond rapidly to single high doses of radiotherapy above 8 Gy (El Kaffas et al., 2013; Fuks and Kolesnick, 2005; Garcia-Barros et al., 2003; García-Barros et al., 2010). Cultured endothelial cells underwent cell death within 24 h when exposed to single radiation doses of 8 Gy or 16 Gy. The percentage of cells staining for ISEL, 24 h after irradiation, was found to be 21% and 60%, respectively. In contrast, minimal cell death was observed when HUVEC cells were exposed to bFGF before radiotherapy, which is consistent with previous studies demonstrating the efficacy of bFGF as a biological radio-protector (Fuks et al., 1994). Results from ISEL staining were in general agreement with gamma-H2AX staining. Studies have since demonstrated that this fibroblast growth factor has radio-protecting properties; it has been suggested to play a role in mitigating the initiation of a ceramide-signaling endothelial cell apoptosis pathway (Fuks et al., 1994; Maj et al., 2003; Paris et al., 2001; Rotolo et al., 2009; Truman et al., 2006). Contrary to HUVEC cells, MDA-MB-231 cells were minimally apoptotic 24 h after radiotherapy. We did find that some MDA-MB-231 cells had morphological changes, likely related to radiation-induced mitotic arrest. As anticipated, bFGF had minimal effects on the acute cell death of MDA-MD-231 cells.

The exposure of HUVEC cells to Sunitinib alone *in vitro* yielded a significantly elevated apoptotic index of 9% by 24 h. Combining Sunitinib with radiation did enhance cell death in comparison to control animals. The dose-dependent response was however moderate, especially when compared to 8 and 16 Gy alone. We interpret this to indicate that Sunitinib moderately radiosensitizes endothelial cells. It may be that Sunitinib rapidly prunes the less mature/proliferating, more radiosensitive endothelial cells. Sunitinib has been reported to have biophysical effects on endothelial cells, such as anti-proliferation and anti-migration effects, as well as causing the activation of rapid apoptotic pathways in less mature or proliferating cells (Osusky et al., 2004). This was confirmed in CLS assays, where we noted decreased tube formation when Sunitinib was used alone. In addition, we observed a complete inhibition in CLS formation when Sunitinib was combined with radiation. The addition of radiation to Sunitinib enhances its anti-angiogenic properties. From our results, we posit that Sunitinib is having minimal effects on existing mature endothelial cells, while inducing apoptosis in less mature or proliferating ones. These results also suggest that cells that undergo cell death due to Sunitinib alone may be the ones that are more sensitive to apoptosis by the ceramide pathway. This may then explain the lowered quantified apoptosis index when Sunitinib is combined with 8 or 16 Gy in comparison to delivering these doses alone. We anticipate that future *in vitro* studies would examine the rapid response of endothelial cells (<24 h) to the Sunitinib–radiation combination therapy and the involvement of the ceramide pathway in acute cell death.

Clonogenic assays were used to assess long-term viability of cells. In HUVEC cells, we noted a clonogenic difference between cells treated with and without bFGF at 8 and 16 Gy (Fig. 3). This was in agreement with ISEL assays, and consistent with the interpretation that endothelial cells have an enhanced response at 8 and 16 Gy radiotherapy, and that bFGF can act to prevent these effects at high radiation doses. The addition of Sunitinib to the cell medium before irradiation did not increase HUVEC radiosensitivity, although it did decrease its plating efficiency. In the radiation treated MDA-MB-231 tumor cells, we found that Sunitinib had minimal effects on their clonogenic proliferation. Interpreting *in vitro* clonogenic assay and ISEL staining results together, our results suggest that Sunitinib does not directly radiosensitize MDA-MB-231 cells in the long-term, but that it may have some acute radiosensitizing effects at high-radiation doses. We also find that Sunitinib causes a rapid wave of acute HUVEC cell death (pruning), while minimally affecting HUVEC radiosensitivity. Finally, we have noted that *in vitro* treatment of MDA-MB-231 cells with bFGF decreases their plating efficiencies; this can be linked back to studies which have suggested that bFGF alters the aggressiveness of MDA-MB-231 cells (Korah et al., 2000).

Experiments conducted *in vivo* with MDA-MB-231 tumor xenografts demonstrated that the clonogenic response of tumor cells treated with Sunitinib and radiation is greater than those treated with radiation alone. The administration of bFGF before irradiation reduced the clonogenic response of tumor cells to all radiation doses; this result directly supports the notion that endothelial response to radiation therapy is an important regulator of *in vivo* tumor response. Overall, the trends observed *in vivo* are more similar to those of HUVEC cells *in vitro* than tumor cells *in vitro*. Similarly, the quantification of an average CD31 MVD revealed an acute reduction in tumor micro-vasculature at high radiation doses, suggesting a rapid endothelial cell death mechanism at these doses. We also qualitatively observed increased cell death in tumor cross-sections stained with H&E. These results parallel those observed in endothelial cells *in vitro*. This suggests that the effects of radiation on endothelial cells and blood vessels contributed to overall tumor response, specifically at the higher radiation doses, and that the tumor endothelium bed is an important regulator of tumor response to radiotherapy. This may be in part, due to the high radiosensitivity of endothelial cells at higher radiation doses as demonstrated *in vitro* or due to effects of radiation on endothelial cells in the milieu of tumor cells.

Our results further demonstrate that Sunitinib has the potential to enhance tumor radiation response; MVD quantification indicate an initial pruning of tumor micro-vasculature after Sunitinib treatment alone. Meanwhile, the administration of radiation after Sunitinib had minimal effects on the MVD. These results are similar to our *in vitro* acute cell death findings, which suggest that Sunitinib causes rapid endothelial

cell death minimally enhanced with the addition of radiation therapy. We also observed increased areas of necrosis 24 h after radiation in combination with Sunitinib (Fig. 4). Finally, we found that clonogenic response of tumor cells treated with radiation and Sunitinib increased in comparison to radiation alone, while *in vitro* treatments of MDA-MB-231 cells demonstrated no Sunitinib-based radiation enhancement. This may be due to vascular re-modeling taking place in the tumor after Sunitinib treatment, in turn affecting the overall radiation response of tumor cells. These tumor radio-enhancement effects are potentially linked to some acute endothelial radiosensitization, as well as vascular 'normalization' effects such as described by Jain et al. (Jain, 2005). Future *in vitro* and *in vivo* studies will aim to give insight on mechanisms behind targeting tumor vasculature and maximizing tumor radiation response in tandem. Taking advantage of newly developed tumor and vascular imaging technologies could potentially facilitate such longitudinal studies.

Taken together, it is likely that the role of blood vessels in radiation response is not a straightforward issue, considering the complexity of the tumor microenvironment (El Kaffas et al., 2012). Nonetheless, our results support an ongoing paradigm shift from the accepted concepts of classic radiobiology advocating that tumor cells (and their innate radiosensitivity) as the primary regulator of tumor response to radiation therapy. This new paradigm assigns a more prominent role to the tumor's endothelium and stroma in radiation response. The *in vivo* results presented here have been conducted in SCID mice. There has been conflicting evidence regarding the use of these animals in experiments which investigate the relationship between vascular and tumor cell response (Brown et al., 2003; Garcia-Barros et al., 2003, 2004; Gerweck et al., 2006; Kolesnick, 2003; Ogawa et al., 2007; Suit and Willers, 2003). Recently published experiments have however demonstrated SCID animals are indeed a valid experimental model in the context of experiments such as those conducted here (García-Barros et al., 2010).

The results presented here suggest that there may be an array of vascular strategies waiting to be explored. Some strategies may include the combination of radiotherapy with chemical or biophysical vascular targeting agents to enhance tumor response. Our results support past findings suggesting that endothelial cells are important regulators of tumor response to radiation therapy at high radiation doses. We further give evidence that Sunitinib can alter endothelial radiosensitivity, while re-modeling the tumor microenvironment to enhance overall tumor response to radiation therapy. We anticipate that further experiments may help elucidate the relative contribution of cell death due to vascular destruction *versus* cell death occurring due to established radiobiological DNA damage. Finally, we anticipate that optimizing such therapies with fractionated radiation and potential anti-angiogenic 'normalization' windows can yield a greater tumor response.

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