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Recruitment of Circulating Breast Cancer Cells Is Stimulated by Radiotherapy

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SUMMARY

Radiotherapy (RT) is a localized therapy that is highly effective in killing primary tumor cells located within the field of the radiation beam. We present evidence that irradiation of breast tumors can attract migrating breast cancer cells. Granulocyte-macrophage colony stimulating factor (GM-CSF) produced by tumor cells in response to radiation stimulates the recruitment of migrating tumor cells to irradiated tumors, suggesting a mechanism of tumor recurrence after radiation facilitated by transit of unirradiated, viable circulating tumor cells to irradiated tumors. Data supporting this hypothesis are presented through in vitro invasion assays and in vivo orthotopic models of breast cancer. Our work provides a mechanism for tumor recurrence in which RT attracts cells outside the radiation field to migrate to the site of treatment.

INTRODUCTION

Over 200,000 breast cancers are diagnosed every year in the United States (American Cancer Society, 2013). One-third of all breast cancer patients will suffer local recurrence after their initial treatment. The addition of radiotherapy (RT) to breast cancer treatment regimens has been demonstrated in numerous trials to reduce the rate of local recurrence (Whelan et al., 2010). However, there remains concern over the persistence of in-field recurrences, particularly in aggressive tumors such as triple-negative breast cancer (Abdulkarim et al., 2011). Radiation is known to exert both short- and long-term biological effects beyond simply killing tumor cells. On the microscopic scale, radiation alters blood vessel permeability and integrity early after treatment (Dewhirst et al., 1990). The benefits of RT to breast cancer patients generally outweigh the side effects of this procedure in normal tissue, but it is critical to understand the full spectrum of radiation effects so that this therapy can be optimally applied.

Circulating tumor cells (CTCs) have been identified in the peripheral blood of breast cancer patients. Previous studies have proposed that the levels of CTCs can be a predictor of outcome (Cristofanilli et al., 2004). Although CTC burden does not correlate with the size of the primary tumor (Hüsemann et al., 2008), it has been observed that the number of CTCs present in the blood is indicative of an unfavorable prognosis for both tumor recurrence and metastasis in breast cancer patients (Graves and Czerniecki, 2011). It has also been shown that CTCs can return to and colonize their tumors of origin in addition to seeding metastasis in distant organs, in a process that has been termed tumor self-seeding. Tumor self-seeding has been shown to be mediated by both CTC attraction to the parent tumor and the infiltrative properties of the CTC itself (Kim et al., 2009; Norton and Massagué, 2006). This process has been postulated to contribute to tumor aggressiveness, since CTCs can survive and proliferate in the supportive environment of a primary tumor more so than in a foreign tissue type.

The relationship between tumor self-seeding and anticancer therapies has not yet been investigated. Because of the focal nature of RT, we hypothesized that transit of tumor cells outside the radiation field at the time of treatment back to the primary tumor may provide a previously unconsidered mechanism of tumor regrowth. The aim of this study was to assess the incidence of tumor cell migration in the context of RT and specifically to evaluate whether radiation influences this process.

RESULTS

Radiation Increases Tumor Cell Invasion, but Not Cell Growth, In Vitro

To investigate the effects of cell irradiation on invasion in vitro, we performed transwell invasion assays using conditioned media as an attractant, harvested from cells treated with different doses of radiation. Both murine (4T1) and human (A549, A375, and MDA-MB-231) cell lines showed an increase in cell invasion (Figures 1A and 1B) toward media collected after irradiation to a dose of 20 Gy. We observed differences in the number of invading cells based on the time of supernatant (SN) collection postirradiation and the cell type (data not shown). When we applied different radiation doses and performed invasion assays using the harvested SN, we observed a dose-threshold effect in the number of invading cells between doses from 0 to 5Gy but no difference with higher doses (Figures 1C and 1D). In vitro cell proliferation measurements using control and irradiated (IR) SN did not show any significant difference in cell growth induced by IR SN (Figures 1E and 1F). Taken together, these results suggest that radiation induces the production of a secreted factor that attracts tumor cells but does not affect cell proliferation.

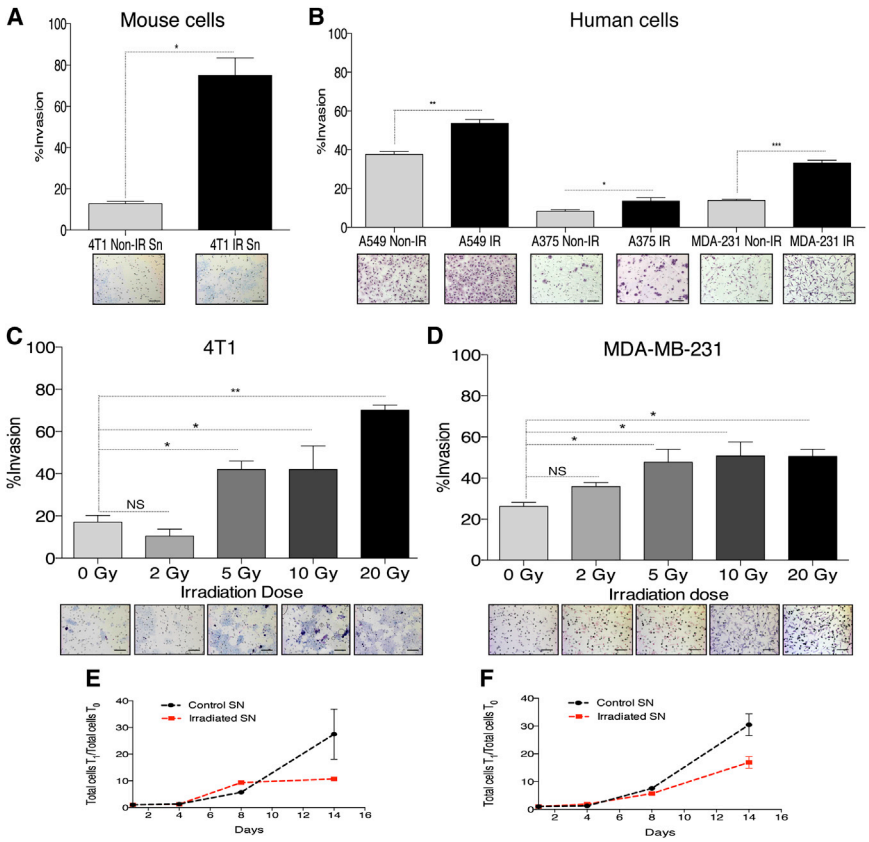

Figure 1. Supernatant from Irradiated Cells Promotes Cell Invasion, but Not Cell Proliferation, in Both Murine and Human Cell Lines

(A) Transwell migration assays using supernatant (SN) from a murine breast cancer cell line (4T1) collected 2 days after irradiation (IR) to a dose of 0 Gy (non-IR) or 20 Gy (IR).

(B) Transwell migration assays using SN from human lung (A549), melanoma (A375), and breast cancer cells (MD-MB-231) collected 7 days after IR to a dose of 0 Gy (non-IR) or 20 Gy (IR).

(C and D) Transwell migration assays using SN from 4T1 and MDA-MB-231 cells treated with increasing doses of radiation (0, 5, 10 and 20Gy).

(E and F) Proliferation of 4T1 and MDA-MB-231 cells grown with SN from control and IR cells, respectively.

Scale bars represent 100 μm (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Error bars represent SD.

Radiation Enhances Tumor Cell Recruitment In Vivo

To study the effects of radiation on tumor cell migration in vivo, tumors were grown in mice using mouse mammary carcinoma 4T1 and luciferase-expressing 4T1 (4T1-luci) cell lines and the human breast adenocarcinoma MDA-MB-231 and luciferase-expressing MDA-MB-231 (MDA-MB-231-luci) cell lines. The parental and labeled cell lines were injected into contralateral mammary fat pads in order to create a bioluminescent "donor" tumor and an unlabeled "recipient" tumor (Figure 2A). The recipient tumors in one group of mice were treated with a single dose of 20 Gy, with another group of mice with equivalent tumors acting as unirradiated controls (non-IR). A 3.2 mm lead shield was used to conform radiation to the targeted recipient tumor while sparing the donor tumor and other tissues (Li et al., 2011). After sacrificing the mouse and excising the recipient tumor at 10 days postirradiation, ex vivo bioluminescence imaging (BLI) was performed. A significant increase in the number of photons released from IR recipient 4T1 tumors (n = 22) was observed relative to non-IR control 4T1 (n = 20) (Figures 2B and S1F), indicating an increased number of labeled tumor cells invading the IR tumors. When only an unlabeled recipient tumor was injected into the mammary fat pad, followed by an intravenous (i.v.) injection of labeled cells, we also observed a significant increase of light photons from the IR recipients (Figure 2D).

The kinetics of this CTC recruitment by IR tumors was studied by harvesting tumors at different time points after irradiation of the recipient to a dose of 20 Gy (Figure 2E). In the 4T1 tumor model, radiation-induced tumor reseeding is initially minimal but increases to a measured maximum at 10 days postirradiation. To investigate the dose dependence of this process, the recipient tumors were treated with doses up to 20 Gy and ex vivo BLI was performed 10 days after treatment (n = 5 for dose) (Figure 2F). We observed statistically significant differences between the non-IR tumors and the tumors IR to doses of 10, 15, and 20 Gy; however, this effect appears to plateau for doses of 10 Gy and higher as also shown in vitro.

These experiments were repeated in analogous model systems employing the human MDA-MB-231 cell line (Figure 2C). As in the 4T1 model, there was an increase in the number of photons released from the IR recipient tumor relative to the control tumor. This difference was statistically significant, although the magnitude of the difference was less pronounced than in the 4T1 model, consistent with the relative results obtained in vitro for these cell lines (Figure 1). These data demonstrate that irradiation of a tumor in vivo stimulates the recruitment of migrating cancer cells after a dose threshold.

GM-CSF Is Overexpressed after Irradiation in Both Human and Murine Tumor Cell Lines

To identify the factor(s) responsible for the increase in tumor cell recruitment after IR, we probed the SN of 4T1 cells collected 2 days after irradiation to doses up to 20 Gy to evaluate the levels of 62 secreted cytokines (Figures 3A, S2, and S3). Within this panel of cytokines, radiation increases were observed in granulocyte-macrophage colony-stimulating factor (GM-CSF). Western blots of SN from 4T1 and MDA-MB-231 cells confirmed that the secretion of GM-CSF is increased in response to radiation (Figures 3B and 3D). A panel of SN from cells treated with lower doses showed a threshold in the amount of expression of GM-CSF between 4 and 5 Gy, which then was stable at higher

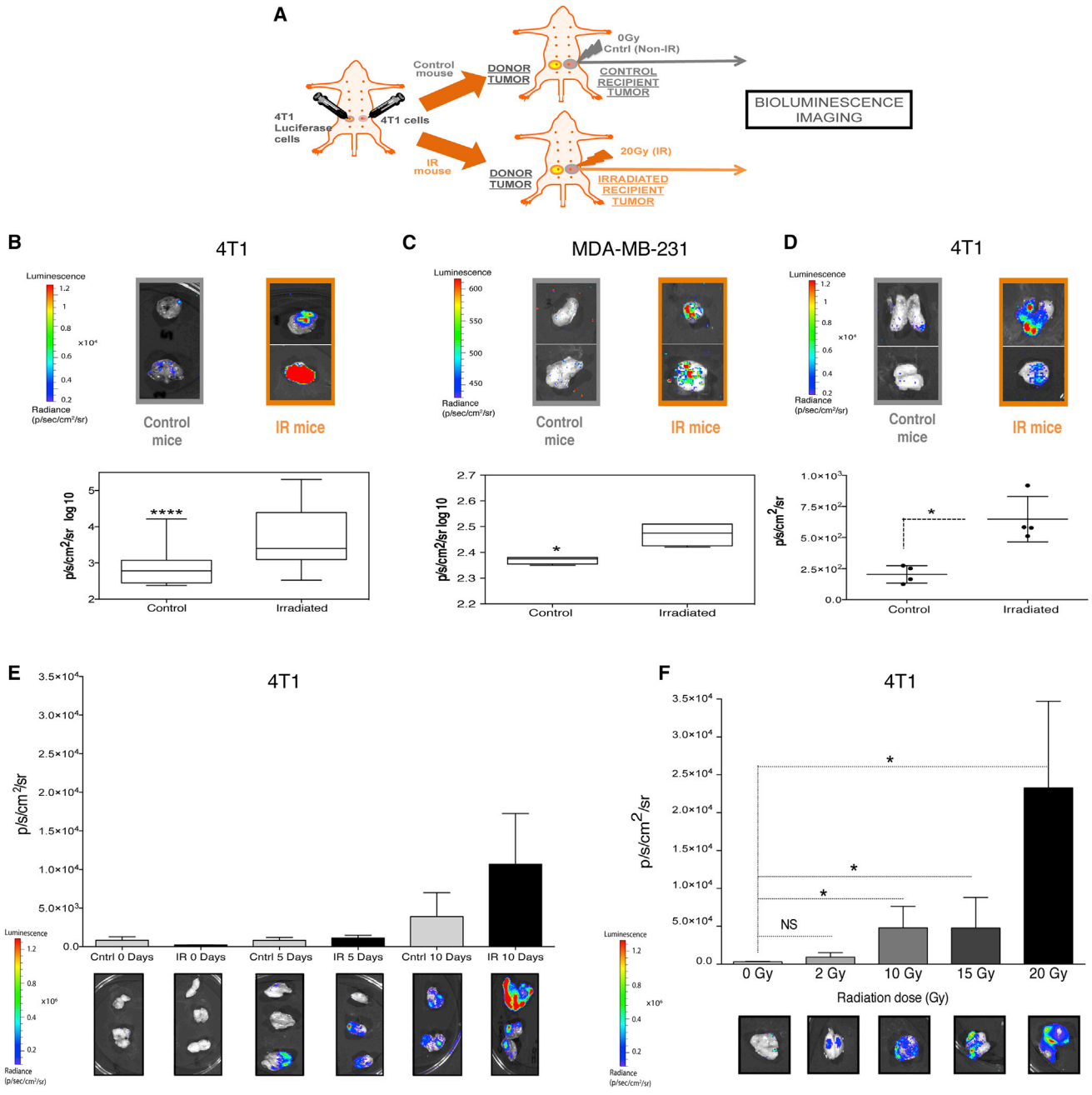

Figure 2. Seeding of Tumors by Migrating Tumor Cells Is Enhanced by Irradiation In Vivo in Both Murine and Human Tumor Models

(A) Schematic of the donor-recipient experimental protocol.

(B) Ex vivo bioluminescence images of 20 Gy irradiated (IR) and unirradiated (non-IR) 4T1 recipient tumors from two representative mice from each group. Quantification of the ex vivo bioluminescence images from 22 IR 4T1 recipient tumors and 20 non-IR 4T1 recipient tumors. Values are represented on a logarithmic scale.

(C) Ex vivo bioluminescence images of IR and non-IR MDA-MB-231 recipient tumors from 2 representative mice from each group. Quantification of the images in from 5 IR MDA-MB-231 recipient tumors and 5 non-IR MDA-MB-231 recipient tumors.

(D) Ex vivo bioluminescence images of non-IR and 20Gy IR 4T1 recipient tumors 10 days after intravenous injection of 4T1-luci cells. Quantification of the images in from 5 IR 4T1 recipient tumors and 5 non-IR 4T1 recipient tumors.

(E) Quantification of photons released from ex vivo BLI of 4T1 recipient tumors IR or non-IR after 0, 5, and 10 days (n = 5 mice per group).

(F) Quantification of ex vivo BLI of 4T1 recipient tumors 10 days after treatment with a variety of radiation doses (0, 2, 10, 15, and 20 Gy) (n = 5 mice per group).

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0005. Error bars represent SD.

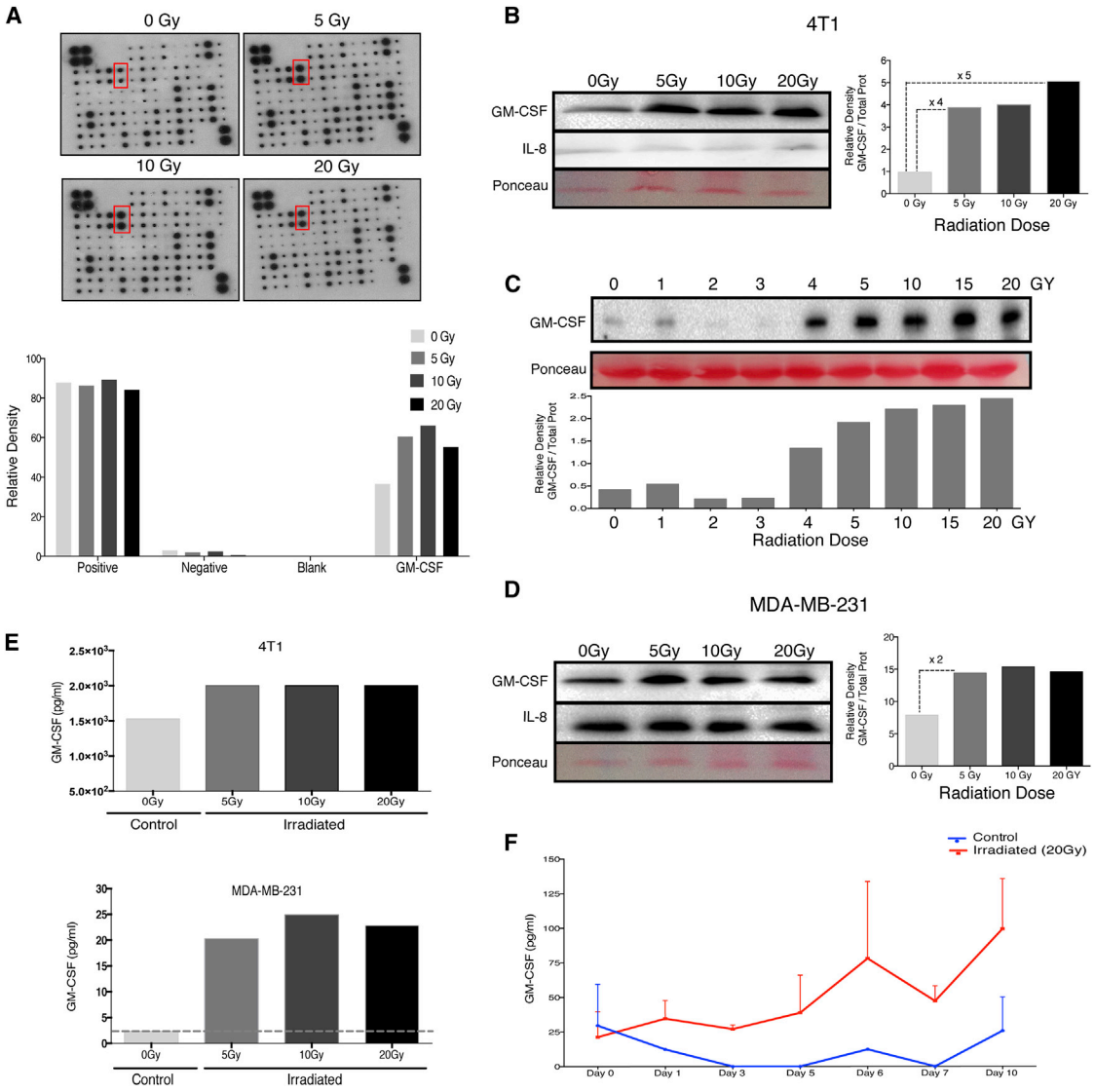

Figure 3. GM-CSF Is Overexpressed after Irradiation in Both Human and Murine Cell Lines

(A) Membranes from cytokine arrays probed with SN from 4T1 cells IR to doses of 0, 5, 10, or 20 Gy and its quantification.

(B) Western blot (WB) for GM-CSF and IL-8 from SN from 4T1 cells IR to doses of 0, 5, 10, and 20 Gy and its quantification relative to total protein loaded (Ponceau).

(C) GM-CSF WB from 4T1 IR SN with lower doses (0, 1, 2, 3, 4, 5, 10, 15, and 20 Gy). Quantification is relative to the total protein loaded.

(D) WB analysis for GM-CSF and IL-8 in the SN of MDA-MB-231 cells treated with different doses (0, 5, 10, and 20 Gy) and its quantification relative to total protein loaded.

(E) ELISA analysis of GM-CSF in SN of 4T1 and MDA-MB-231 IR cells with different doses (0, 5, 10, and 20 Gy).

(F) ELISA analysis of GM-CSF in the serum of mice bearing IR and control orthotopic 4T1 tumors.

See also Figures S2 and S3. Error bars represent SD.

doses (Figure 3C). Furthermore, ELISA analysis of the IR SN confirmed the increase of GM-CSF expression in both 4T1 and MDA-MB-231 cell lines (Figure 3E). Thus, the level of induction of GM-CSF correlates with the level of increase in tumor cell migration seen for 4T1 and MDA-MB-231 cells in Figures 1 and 2. Increased GM-CSF was also detected by ELISA in the serum of tumor-bearing mice treated with radiation as compared to untreated controls, indicating that this effect occurs in vivo and that the GM-CSF enters the circulation (Figure 3F).

Silencing of GM-CSF Inhibits Cell Invasion In Vitro and Tumor Self-Seeding In Vivo

To investigate the role of GM-CSF in cell invasion and tumor cell migration after IR, knockdown (KD) 4T1 clones were obtained after transfection with small hairpin RNA (shRNA) constructs targeting GM-CSF (Figure 4A). The in vitro growth rate of cells expressing these constructs was not significantly different from that of parental cells (Figure S1C). The role of GM-CSF in invasion was evaluated through transwell assays using SN collected

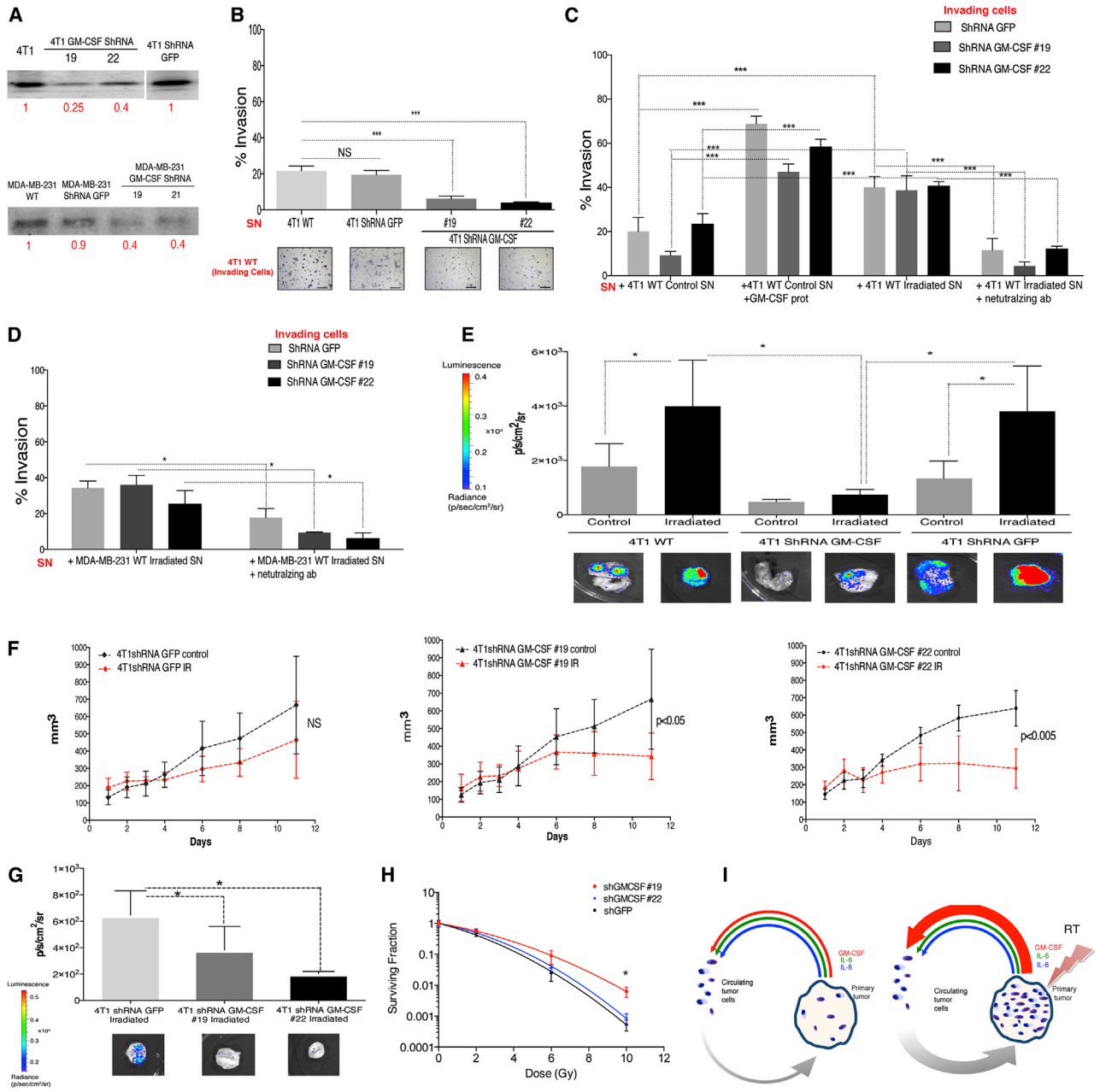

Figure 4. GM-CSF Promotes Cell Invasion In Vitro and Tumor Seeding In Vivo

(A) Western blot analysis of GM-CSF in 4T1 and MDA-MB-231 cells after transfection with an shRNA targeting GM-CSF.

(B) Transwell migration assay of 4T1 WT cells toward SN collected from 4T1 WT, 4T1 shRNA GFP, and 4T1 shRNA GM-CSF clones.

(C) Transwell migration assay using GM-CSF KD 4T1 cells and 4T1 shRNA GFP cells. SN from control or IR 4T1 WT cells was used as chemoattractant. GM-CSF neutralizing antibody or recombinant GM-CSF protein was added to IR and control SN.

(D) Transwell migration assay using GM-CSF KD MDA-MB-231 cells and MDA-MB-231 shRNA GFP cells. SN from IR MDA-MB-231 WT cells was used as chemoattractant and GM-CSF neutralizing antibody was added.

(E) Ex vivo bioluminescence images and corresponding quantification for recipient tumors IR to a dose of 0 or 20 Gy from WT, shGFP-expressing, or shGM-CSF-expressing 4T1 cells.

(F) Tumor growth curves from 4T1 shGFP-expressing and 4T1 shGM-CSF clones (#19 and #22) after 0 or 10 Gy irradiation.

(G) Ex vivo bioluminescence images and corresponding quantification of recipient tumors IR to a dose of 0 or 10 Gy, after subsequent intravenous delivery of 4T1-luci cells, from WT, shGFP-expressing, or shGM-CSF-expressing 4T1 cells.

(H) Clonogenic survival of 4T1 shRNA GFP and 4T1 GM-CSF KD clones after RT.

(I) A model of baseline and radiation-induced tumor reseeding.

Scale bars represent 100 μm (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0005$). Error bars represent SD.

from the different silenced clones. A clear relationship between the level of GM-CSF in the SN and cell invasion was observed (Figure 4B). When these KD cells were used as invading cells toward control or IR SN, no significant difference was observed when compared to parental cells (Figure 4C). Adding recombinant GM-CSF to the control medium was sufficient to stimulate invasion of 4T1 cells and KD clones (Figure 4C). We also investigated the use of neutralizing antibody added to the IR SN, which produced a large decrease in cell invasion in both 4T1 and MDA-MB-231 cells (Figures 4C, 4D, S1D, and S1E). GM-CSF is therefore necessary and sufficient to induce tumor cell invasion in vitro.

To determine whether GM-CSF plays a role in tumor recruitment, we conducted an in vivo donor/recipient experiment. The donor was a 4T1-luci tumor, while the recipient was grown from 4T1 wild-type (WT), 4T1-shGM-CSF (clone #19) or 4T1-shGFP cells. A portion of the recipient tumors of each type was treated to a single dose of 20 Gy, while others were kept as controls. We observed the same degree of ex vivo BLI signal in the 4T1-WT and 4T1-shGFP recipient tumors and the same increase in signal in these tumors after irradiation. However, seeding of 4T1-shGM-CSF recipient tumors was significantly reduced relative to the 4T1-WT recipient tumors (Figure 4E). This was true for both the IR and non-IR recipients. Moreover, no significant stimulation of tumor cell recruitment by radiation was observed in the 4T1-shGM-CSF tumors. While the difference between IR and non-IR tumors from each cell type was very evident, there was no significant difference in final tumor volume when comparing each condition (IR and non-IR) within the different tumor types (Figure S1G). Using the single recipient tumor and i.v. donor cell delivery model, we found that regrowth of the recipient tumor after a single dose of 10 Gy was significantly inhibited in the GM-CSF KD clones relative to the 4T1 shRNA GFP control (Figures 4F and 4G). No corresponding difference in intrinsic cell radiosensitivity was observed between these cells in vitro (Figure 4H). These findings demonstrate the necessity of GM-CSF in the stimulation of tumor cell migration and recruitment by radiation.

DISCUSSION

Tumor self-seeding has been demonstrated in models of breast, colon, and skin cancer (Kim et al., 2009). We found that in preclinical models of breast cancer, irradiation of a tumor provokes a significant increase in seeding by tumor cells migrating from another preexisting tumor. Similarly, we observed an enhancement in seeding of IR tumors when CTCs were introduced directly into the circulation by i.v. injection, suggesting that this effect is not specific to the mechanism of introduction of CTCs. Our findings agree with previous studies that have shown that sublethal doses of irradiation can promote migration and invasiveness of breast (Paquette et al., 2013), glioma (Wild-Bode et al., 2001), pancreas (Qian et al., 2002), liver (Zhou et al., 2012), and melanoma (Rofstad et al., 2004) cancer cells. These previous studies, however, relied primarily on in vitro experiments and employed in vitro preirradiation prior to tumor inoculation in an animal when performing in vivo studies. Clinically, it has recently been demonstrated for the first time that sublethal doses of RT result in mobilization of viable CTCs into the circulation of patients with non-small cell lung cancer (Martin et al., 2014), which highlights the need to further monitor the behavior of CTCs during RT. The aim of this study was to replicate the clinical situation where a patient has a primary tumor and a burden of CTCs, which in our model were produced either by another preexisting tumor or through direct i.v. injection. While the presence of metastasis generally limits patient outcome more so than control of the primary tumor by radiation, it is important to note that migrating tumor cells may exist in the absence of secondary lesions and that occult metastatic cells may lay dormant, in a growth-arrested state, for months or years (Schmidt-Kittler et al., 2003). Under these circumstances, patients without frank metastatic disease may be susceptible to radiation-induced tumor cell reseeding and subsequent recurrence.

We observed an increase in the rate of invasion of cells toward the SN of IR cells above a threshold dose of approximately 4 Gy in vitro, suggesting that the factors produced in response to irradiation may serve as chemoattractants. From a cytokine array, we observed that GM-CSF was overexpressed in the SN of 4T1 cells after irradiation. Dose- and time-dependent increases in the expression of GM-CSF by human fibroblasts in response to irradiation have been reported, in which expression is regulated at both the transcriptional and posttranscriptional levels (Hachiya et al., 1994). More recent studies have also demonstrated that GM-CSF is expressed in the skin after irradiation (Müller and Meineke, 2007). SN from 4T1 cells in which GM-CSF has been knocked down had a diminished ability to provoke cell invasion in vitro and tumor recruitment in vivo, suggesting that GM-CSF acts as a paracrine signal in this model to attract CTCs to an IR tumor.

GM-CSF is administered to patients clinically under the trade name Leukine to stimulate production of white blood cells during fungal infections (Safdar, 2007), Crohn disease (Korzenik et al., 2005), bone marrow transplant, and chemotherapy (Hsu and Link, 2012). The data presented here demonstrate that overexpression of GM-CSF recruits CTCs to IR tumors. A similar paracrine function of this factor was described in a study in which a panel of cytokines including GM-CSF produced by both MDA-MB-231 and MCF-7 cells attracted adipose tissue-derived mesenchymal stem cells (AMSCs) toward the tumor microenvironment (Senst et al., 2013). These same AMSCs were also induced to migrate toward conditioned media of both MDA-MB-231 and 4T1 cells (Gehmert et al., 2010).

It is important to note that tumor cell migration toward media from non-IR cells as well as toward non-IR tumors was observed in this study. This suggests that radiation is not necessary for cancer cells to home to primary tumors, but that it stimulates this process. We propose that different cytokines may mediate tumor seeding under normal conditions, such as interleukin-6 (IL-6) and IL-8 (Kim et al., 2009), and in response to genotoxic stress, such as GM-CSF, identified here. IL-6 and IL-8, along with baseline levels of GM-CSF, may promote tumor cell migration in the pretreatment setting, while increases in GM-CSF levels give rise to the increase in this process after RT (Figure 4I). This model is supported by the present observations that radiation did not modulate levels of IL-6 or IL-8 in vitro and that KD of

GM-CSF inhibited tumor seeding both with and without radiation.

Tumor self-seeding has been shown to involve factors in both the recruiting tumor and the CTCs. In this work, we focused on the chemoattractive ability of the primary tumor and the mechanisms by which radiation may modulate it. However, the incidence of radiation-induced tumor seeding in the 4T1 and MDA-MB-231 models studied here is associated with the aggressiveness of each of these tumor systems. The 4T1 model is extremely aggressive and metastatic, resulting in widespread metastasis and mouse death within a month of orthotopic inoculation, while the MDA-MB-231 is slower growing. This observation highlights the role of the CTCs in this process, in addition to that of the primary tumor (Kim et al., 2009). In addition, it is known that accelerated repopulation occurs after RT, and this may be exacerbated in the case of unirradiated CTCs infiltrating an irradiated tumor. However, the sensitivity of this process to KD of GM-CSF indicates that recruitment of CTCs and not proliferation is the critical aspect of this process.

Triple-negative breast cancers exhibit increased local and distant recurrence after treatment (Dent et al., 2007). This may be due in part to a propensity for tumor self-seeding, because of both the aggressiveness of this disease and the presence of CTCs, and in part to the stimulation of CTCs migration by radiation as shown here. Clinical studies evaluating this hypothesis are complicated by the lack of technology to track CTCs in vivo. Further work is needed to evaluate the association between clinically measurable variables including CTC number, GM-CSF levels, exogenously administered GM-CSF, and tumor recurrence. In addition, the development of therapies blocking the effects of radiation-induced overexpression of GM-CSF, such as neutralizing antibodies, may be of great clinical interest in order to minimize recruitment of CTCs to the primary tumor after RT. Finally, the sensitivity of this process to fractionated RT regimens should be investigated.

EXPERIMENTAL PROCEDURES

Cell Culture

Human lung (A459), melanoma (A375), and breast cancer (MDA-MB-231) cell lines and a mouse breast cancer cell line (4T1) were grown following ATCC specifications (American Type Culture Collection). 4T1 and MDA-MB-231 cells were transduced with retrovirus particles encoding for the expression of firefly luciferase gene (FLuc) and with retroviruses produced from mouse shRNA clones targeting GM-CSF gene (TRCN0000054618, TRCN0000054619, TRCN0000054620, TRCN0000054621, and TRCN0000054622) (Thermo Scientific). A vector encoding shRNA GFP was used as a control. FLuc expression was determined both in vivo and in vitro (Figures S1A and S1B).

Transwell Invasion Assays

A total of 1×10^6 cells of each cell type were plated on a 10 cm dish and IR with different doses using a cesium source. Media from IR cells was collected at different time points and used as conditioned media in transwell migration assays (BD Biocat Matrigel Invasion Chamber). A total of 1×10^5 cells were placed in the upper chamber of the transwell setup.

Cytokine Antibody Array

Proteins secreted from 4T1 cells were probed using mouse cytokine antibody arrays (Mouse Cytokine Antibody Array 3, Raybiotech), using conditioned media from IR and non-IR cells. All the steps were done according to the manufacturer's protocols.

Animal Studies

All animal experiments were done according to a protocol approved by the institutional animal care and use committee. Seven-week-old nude female mice (Charles River Laboratories) were inoculated with 5×10^4 4T1 and 5×10^4 4T1-luci cells or 1×10^6 MDA-MB-231 and 1×10^6 MDA-MB-231-luci cells, suspended in 50 μl of PBS, in contralateral mammary glands. Once tumors reached 7 mm, the unlabeled tumor was IR to a dose from 0 to 20 Gy. IR was performed using a 225 kVp cabinet X-ray system filtered with 0.5 mm Cu. Animals were anesthetized and placed under a 3.2 mm lead shield with 1 cm circular apertures where the tumors were placed. Ex vivo BLI was performed 10 days after IR for the 4T1 tumor model or 20 days for the MDA-MB-231 tumor model. Animals were injected with 100 μg/g of D-luciferin, and the unlabeled 4T1 tumor was excised. Tumors were imaged using an IVIS 200 (PerkinElmer).

Western Blot

For protein detection, cell supernatant was collected and concentrated with Ultracel-3K centrifugal filters (EMD Millipore). A total of 10 μg of protein was separated using a 12% SDS-PAGE gel and further detected with a GM-CSF antibody (Lifespan BioSciences) and a CXCL8/IL-8 antibody (R&D Systems).

Clonogenics

4T1 shRNA GFP and 4T1 shRNA GM-CSF (clones 19 and 22) cells were IR up to 10 Gy using a cesium source and plated in 6 cm dishes at appropriate concentrations. After 12 days, colonies were stained and counted. The survival data were fitted with the linear-quadratic model ($Surviving\ Fraction = e^{-(\alpha * Dose + \beta * Dose^2)}$).

Statistical Analysis

Statistical significance was evaluated using an unpaired Student's t test to compare two different groups and one-way nonparametric ANOVA test, accounting for multiple comparisons using the Tukey method, to compare multiple doses or conditions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.011.

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