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Tumor Self-Seeding by Circulating Cancer Cells

Mi-Young Kim,[1] Thordur Oskarsson,[1] Swarnali Acharyya,[1] Don X. Nguyen,[1] Xiang H.-F. Zhang,[1] Larry Norton,[2] and Joan Massagué[1,3,*]
[1]Cancer Biology and Genetics Program
[2]Department of Medicine
[3]Howard Hughes Medical Institute
Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA
*Correspondence: massaguj@mskcc.org
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SUMMARY

Cancer cells that leave the primary tumor can seed metastases in distant organs, and it is thought that this is a unidirectional process. Here we show that circulating tumor cells (CTCs) can also colonize their tumors of origin, in a process that we call "tumor self-seeding." Self-seeding of breast cancer, colon cancer, and melanoma tumors in mice is preferentially mediated by aggressive CTCs, including those with bone, lung, or brain-metastatic tropism. We find that the tumor-derived cytokines IL-6 and IL-8 act as CTC attractants whereas MMP1/collagenase-1 and the actin cytoskeleton component fascin-1 are mediators of CTC infiltration into mammary tumors. We show that self-seeding can accelerate tumor growth, angiogenesis, and stromal recruitment through seed-derived factors including the chemokine CXCL1. Tumor self-seeding could explain the relationships between anaplasia, tumor size, vascularity and prognosis, and local recurrence seeded by disseminated cells following ostensibly complete tumor excision.

INTRODUCTION

Cancer progression is commonly segregated into processes of primary tumor growth and secondary metastasis. In this conventional model, the high cell density and rapid growth rate of primary tumors are attributed to an ability of cancer cells to sustain unlimited proliferation and to favorably influence their microenvironment. In contrast, metastasis is thought to depend on cancer cell dissemination and adaptation to distant organs (Chiang and Massagué, 2008; Langley and Fidler, 2007; Scheel et al., 2007). Shedding of tumor cells into the circulation may occur in large numbers and from early stages of tumor formation (Husemann et al., 2008; Pantel and Brakenhoff, 2004; Stoecklein et al., 2008). Yet overt metastasis is achieved only by a minority of these dispersed cells. Tight vascular wall barriers, unfavorable conditions for survival in distant organs, and a rate-limiting acquisition of organ colonization functions are just some of the impediments to the formation of distant metastasis (Nguyen et al., 2009). However, these impediments may be less stringent as regards the ability of CTCs to reinfiltrate their tumors of origin. The neovasculature of tumors is typically leaky (Carmeliet and Jain, 2000; Rafii et al., 2003), a feature that would facilitate not only the passage of tumor cells into the circulation but also their entry from the circulation back into the tumor. CTCs would likely need no further adaptation to thrive in the microenvironment of their source tumor. Based on these theoretical considerations, we have postulated that CTCs may reinfiltrate an established tumor, enriching it with aggressive cells that have withstood a period of dissemination. This process, which we refer to as "tumor self-seeding," could have consequences for tumor growth and the breeding of metastatic cell progenies (Norton and Massague, 2006).

In the present studies we sought experimental evidence for the existence, the features, the mediators, and the potential consequences of tumor self-seeding. Using human breast, colon, and melanoma cancer cells, we investigated in mice the ability of malignant cells to seed a tumor from the circulation. The results led us to investigate whether metastatic cells have a superior ability to seed an established tumor, and whether tumor self-seeding depends on attraction signals from the tumor mass and infiltrative functions in the circulating seeds. Our results uncovered various mediators of such attraction and infiltration functions, including factors whose expression in primary tumors is associated with relapse in patients. These insights dissect tumor self-seeding into steps of CTC attraction and tumor infiltration and highlight the implications of tumor self-seeding for cancer biology and clinical oncology.

RESULTS

Seeding of Established Tumors by CTCs

To investigate whether cancer cells that are shed into the circulation can reinfiltrate a primary tumor mass, we first used MDA-MB-231 (MDA231 for brevity), a breast cancer cell line from which metastatic subpopulations were previously isolated and characterized (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a). The lung-metastatic derivative line MDA231-LM2 (Minn et al., 2005a) was transduced with a GFP-luciferase fusion vector

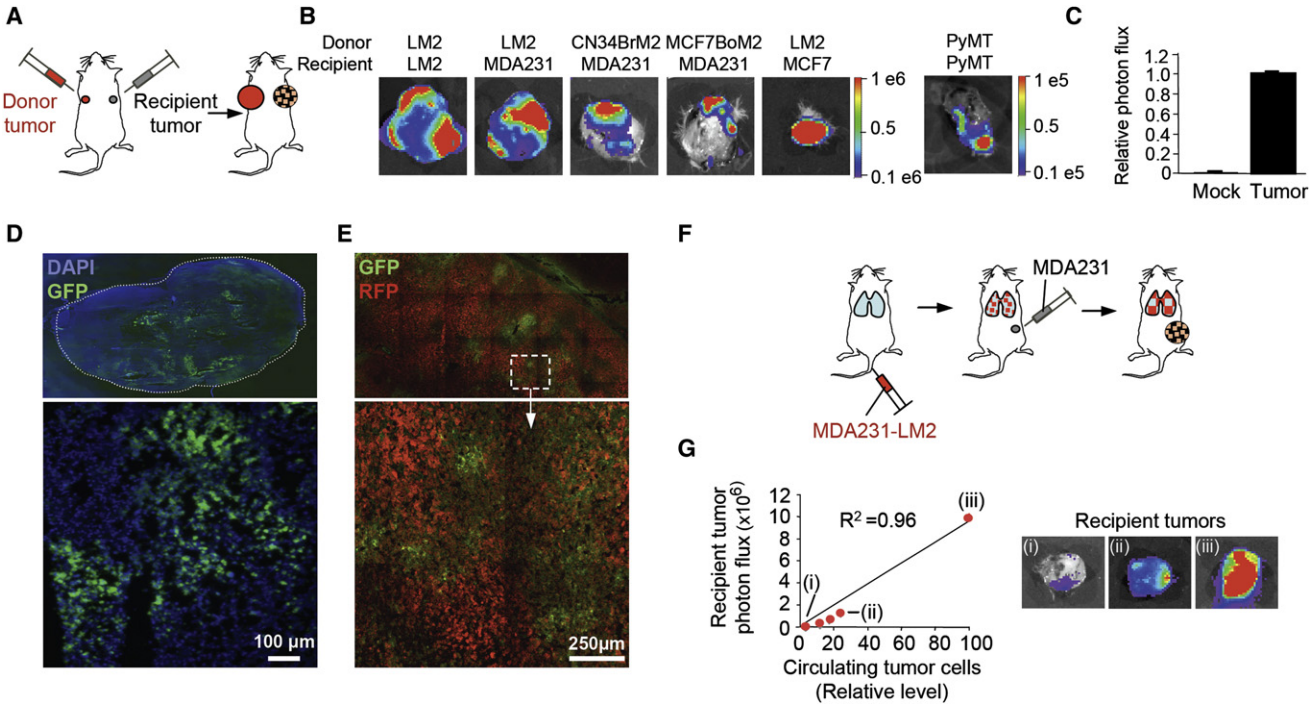

Figure 1. Seeding of Established Tumors by CTCs

(A) A diagram of contralateral seeding experiment. Unlabeled and GFP/luciferase-expressing breast cancer cells were injected into contralateral No. 2 mammary glands as a "recipient tumor" and a "donor tumor," respectively.

(B) BLI of recipient tumors extracted from mice bearing the indicated GFP/luciferase-expressing donor tumors. Color-range bars: photon flux. LM2: a lung-metastatic derivative of MDA231. MCF7-BoM2: a bone-metastatic derivative of MCF7. CN34-BrM2: a brain-metastatic derivative of pleural effusion CN34. PyMT: cells derived from mammary tumors developed in MMTV-PyMT transgenic mice.

(C) BLI of tumor-free and tumor-bearing mammary glands from mice bearing GFP/luciferase-expressing donor tumors. n = 9–18. Error bars represent SEM.

(D) Frozen sections of seeded MDA231-LM2 tumors were visualized by fluorescence microscopy. An entire tumor section and a higher-magnification image (×10) of a selected field are shown.

(E) A contralateral seeding experiment was performed with RFP- and GFP-expressing MDA231-LM2 cells. Frozen sections from RFP-labeled tumors were visualized under confocal microscopy at ×20.

(F) A diagram to test mammary tumor seeding from lung metastases. GFP/luciferase-expressing MDA231-LM2 cells were injected intravenously. Once lung metastases were established, unlabeled MDA231 cells were injected into a mammary gland No. 2.

(G) Left: burden of CTCs derived from lung metastases in mice described in (F). Relative levels of CTC were plotted against the luminescent signals of recipient tumors. Right: BLI of three representative recipient tumors (i, ii, and iii) identified in the graph.

and inoculated into one mammary gland in mice to form a "donor" tumor mass. Unlabeled MDA231-LM2 cells were inoculated into a contralateral mammary gland to form a "recipient" mass of the same tumor (Figure 1A). After 60 days, the recipient tumors were excised and examined for the presence of seeding cells by means of ex vivo bioluminescence imaging (BLI). A majority (85%) of the recipient tumors showed extensive seeding by MDA231-LM2 cells (Figure 1B and Table 1). Tumors formed by the more indolent MDA231 parental population were as effective as MDA231-LM2 tumors at capturing seed cells (Figure 1B and Table 1). No seeding was observed in mock-inoculated mammary glands within the same time period (Figure 1C).

Fluorescence microscopy analysis of MDA231 recipient tumors confirmed the presence of numerous GFP+ MDA231-LM2 seeding cells as distinct patches typically encompassing less than a quarter of a tumor section (Figure 1D and data not shown). When recipient tumors were generated using red-fluorescent protein (RFP)-labeled cells, the infiltrating GFP+ cells were observed intermingling with resident RFP+ cancer cells and with unlabeled areas of presumptive tumor stroma (Figure 1E). Quantitative RT-PCR analysis of firefly-luciferase mRNA level in seeded recipient tumors revealed that seeder cells accounted for 5%–30% of the recipient tumor mass (data not shown).

To establish the generality of this seeding phenomenon, we performed similar experiments with different cancer cell lines. Recipient mammary tumors became seeded with high frequency (53% to 100% of mice) by donor tumors that were formed with bone-metastatic (MCF7-BoM2), lung-metastatic (MDA231-LM2), or brain-metastatic (CN34-BrM2) cells from different subtypes of breast cancer (basal, estrogen receptor-negative MDA231 cells versus luminal, estrogen receptor-positive MCF7 cells) or patient-derived malignant cell cultures (CN34 cells) (Figure 1B and Table 1). Seeding of a recipient tumor by its own aggressive progeny was also observed between subcutaneous tumors formed by the human colon carcinoma line SW620 and its lung-metastatic derivative SW620-LM1, and between the

Table 1. Tumor-Seeding Activity of Various Carcinoma and Melanoma Cell Lines

Cancer	Relationship	Species	Donor Tumor	Recipient Tumor	Site	Seeding (%)
Breast	Homotypic	Human	MDA231-LM2	MDA231-LM2	MFP	11/13 (85)
Breast	Homotypic	Human	MDA231-LM2	MDA231	MFP	39/47 (83)
Breast	Homotypic	Mouse	4T1	4T1	MFP	3/5 (60)
Breast	Homotypic	Mouse	4T1	67NR	MFP	11/11 (100)
Breast	Homotypic	Mouse	PyMT	PyMT endogenous	Breast	8/11 (73)
Breast	Heterotypic	Human	CN34-BrM2	MDA231	MFP	10/19 (53)
Breast	Heterotypic	Human	MCF7-BoM2	MDA231	MFP	6/7 (86)
Breast	Heterotypic	Human	MDA231-LM2	MCF7	MFP	7/7 (100)
Melanoma	Homotypic	Human	A375-BoM2	A375	SubQ	8/11 (73)
Colon	Homotypic	Human	SW620-LM1	SW620	SubQ	7/9 (77)

The table indicates the type of cancer, the relationship, species, and name of the cell lines used as donor and recipient pairs, the tumor site (MFP: mammary fat pad, SubQ: subcutaneous), and the fraction of tumors that showed seeding as determined by BLI. MDA231-LM2, a lung-metastatic derivative of MDA231; MCF7-BoM2, a bone-metastatic derivative of MCF7; CN34-BrM2, a brain-metastatic derivative of pleural effusion CN34; SW620-LM1, a lung-metastatic derivative of SW620; A375-BoM2, a bone-metastatic derivative of A375. PyMT: cells derived from a mammary tumor developed in MMTV-PyMT transgenic mice.

human melanoma line A375 and its bone-metastatic derivative A375-BoM2 (Table 1). These experiments with human cell lines required the use of immunodeficient mice. However, seeding was also observed in immunocompetent mice using the syngeneic cell lines 4T1 and 67NR (Table 1). Derived from a spontaneous mouse mammary tumor, 4T1 is highly metastatic to lungs, liver, bones, and brain whereas 67NR is poorly metastatic (Aslakson and Miller, 1992). Moreover, endogenous mammary tumors driven by the polyoma middle T oncogene (PyMT) in mice were efficiently seeded by PyMT tumor-derived cells placed in the circulation (Figure 1B; Table 1).

To determine if mammary tumors may be seeded with cells shed from metastatic lesions, we generated lung tumor colonies by tail-vein inoculation of labeled MDA231-LM2 cells and then implanted unlabeled MDA231 mammary tumors in the same mice (Figure 1F). Seeding of these mammary tumors by lung metastasis-derived cells was observed in 10/11 (91%) mice (Figure 1G). The extent of seeding was proportional to the level of donor cells in the circulation (Figure 1G). Collectively, these results indicate that aggressive carcinoma and melanoma cells shed into the circulation can avidly seed an established tumor mass.

Preferential Tumor Seeding by Metastatic Cell Progenies

We compared the ability of metastatic derivatives from SW620, A375, and MDA231 with their corresponding parental lines to act as donor tumors. In contralateral homotypic tumor seeding assays, the metastatic derivatives showed an 8- to 35-fold higher seeding ability than their parental counterparts, as determined by BLI of homotypic recipient tumors (Figure 2A). Next we carried out in vivo selection experiments to isolate, from the parental MDA231 and A375 cell lines, the cells that most actively seed a tumor mass from the circulation. We inoculated contralateral mammary glands (MDA231) or contralateral flanks of mice (A375) with unlabeled (recipient) or GFP/luciferase-labeled (donor) cells, respectively (Figure 2B). Seeding of recipient tumors was detectable by BLI within 50 days of the inoculation. The seeded tumors were excised, dissociated, and placed in culture, and the GFP-positive seeder cell populations (MDA231-S1a, MDA231-S1b, and A375-S1) were obtained from these cultures by fluorescence-activated cell sorting (FACS). The tumor seeding ability of MDA231-S1a and MDA231-S1b as contralateral donor tumors was >100-fold higher than that of parental MDA231 (Figure 2C). The seeding ability of MDA231-S1a and A375-S1 from the circulation was 6-fold higher than that of the parental populations (Figure 2D). The S1 populations additionally showed an increased ability to pass across an endothelial cell layer in trans-well migration assays (Figure S1 available online).

We chose MDA231 for further analysis because the metastatic composition of this cell line has been extensively characterized. MDA231 was derived from the pleural fluid of a breast cancer patient with advanced metastatic disease, and it contains minority subpopulations that are highly metastatic to either the bones, the lungs, or the brain (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a). These subpopulations may represent the disseminated descendants of different metastatic lesions in the source patient and are characterized by a differential expression of distinct gene sets. Several of these genes act as mediators of organ-specific metastasis in experimental systems and are associated with organ-specific relapse in independent cohorts of breast cancer patients (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a, 2005b).

A comparison of transcriptional profiles uncovered 72 genes (78 probe sets) whose mRNA level was at least 3-fold higher in the MDA231-S1a and -S1b cells than in parental MDA231 (Table S1). Interestingly, many of these are genes whose expression is characteristic of bone, lung, and/or brain-metastatic cell populations (Table S1) (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a). The antiapoptotic gene BCL2A1 was also highly expressed in S1 cells (Table S1). Furthermore, the MDA231-S1a and -S1b transcriptomes were significantly enriched for the expression of bone metastasis (BoMS), lung metastasis (LMS), and brain metastasis (BrMS) gene expression classifiers (Figure 2E). These signatures were previously derived from human breast cancer cells that are selectively metastatic to one of these organs in mouse models and in patients (Bos et al., 2009; Kang

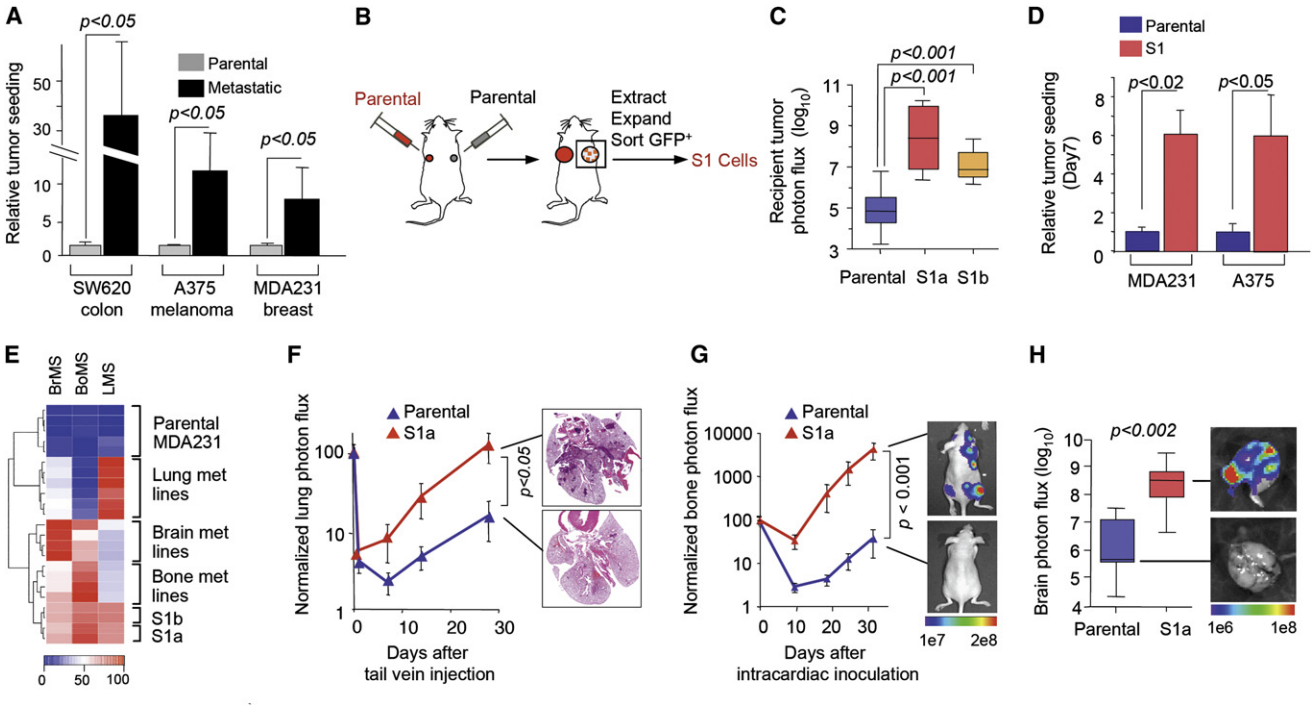

Figure 2. Preferential Tumor Seeding by Metastatic Cell Progenies

(A) Comparison of seeding activity of highly and poorly metastatic cells. Contralateral seeding experiments were performed with the GFP/luciferase-expressing parental cancer cell lines, or with their lung-metastatic (SW620-LM1, MDA231-LM2) or bone-metastatic derivatives (A375-BoM2). Relative luminescent signals are plotted.

(B) A schematic diagram of isolation of seeder cell populations. For details, see Experimental Procedures.

(C) Comparative tumor-seeding ability of in vivo-selected seeder cells (MDA231-S1a and S1b) and parental MDA231 cells in contralateral seeding experiments as described in Figure 1A. n = 6–8.

(D) Comparative tumor-seeding ability between parental and in vivo-selected seeder cells (MDA231-S1 and A375-S1) from the circulation as described in Figure 3A. n = 8–10.

(E) The gene expression profiles of parental MDA231, various metastatic derivatives, and the seeder lines S1a and S1b were scored with gene expression classifiers for metastasis to lung (LMS), bone (BoMS), or brain (BrMS). Raw scores were scaled between 0 and 100.

(F) Left: Parental or MDA231-S1a cells were inoculated intravenously into mice. Lung colonization was measured by BLI and quantified. n = 6–7. Right: representative histological staining (H&E) of lung sections from the experiment on the left.

(G and H) Cells were inoculated into the cardiac left ventricle. Bone colonization was measured by BLI and quantified; n = 12. Brains were extracted and colonization was measured by BLI; n = 5–7.

Error bars in all cases represent SEM and p values were based on two-tailed Mann-Whitney test; for details see Experimental Procedures.

et al., 2003; Minn et al., 2005a, 2005b). Indeed, when compared to parental MDA231 in metastasis assays in mice, the MDA231-S1a cells demonstrated a higher ability to colonize the lungs (Figure 2F), the bones (Figure 2G), and the brain (Figure 2H) from the circulation. Collectively, these results suggest that tumor self-seeding preferentially involves metastatic cancer cell progenies irrespective of their organ tropisms.

Tumor Attraction and Infiltration Functions

To define the basic functions required for tumor seeding by CTCs, we inoculated LacZ/GFP/luciferase-expressing MDA231 cells (either parental or LM2) into the arterial circulation (via the left cardiac ventricle) of mice bearing unlabeled MDA231 mammary tumors (Figure 3A). This experimental protocol obviates possible differences in donor tumor growth rate or in donor tumor-derived systemic signals (McAllister et al., 2008) that might confound the interpretation of our results. The inoculated cells were rapidly distributed throughout the body (Figure 3B, day 0) and became extensively cleared within a few days except for cells that infiltrated the mammary tumors (Figure 3B, day 7 and beyond). These cells readily seeded the established mammary tumors but not the intact or mock-inoculated mammary glands (Figure 3B, day 42). Furthermore, the highly metastatic MDA231-LM2 cells were more effective at seeding the recipient tumors from the circulation than were the parental MDA231 cells (Figure 3C). This effect was already apparent within 10 days after inoculation (Figure 3D), before a marked outgrowth of the seeding cells took place within the recipient tumor mass. In similar experiments, the metastatic derivative A375-BoM2 seeded subcutaneous A375 melanoma tumors from the circulation more effectively than did the parental A375 cells (Figure 3D). These results suggested that tumor seeding by CTCs involves two distinct functions, namely, an ability of tumors to attract their own circulating progeny and an ability of

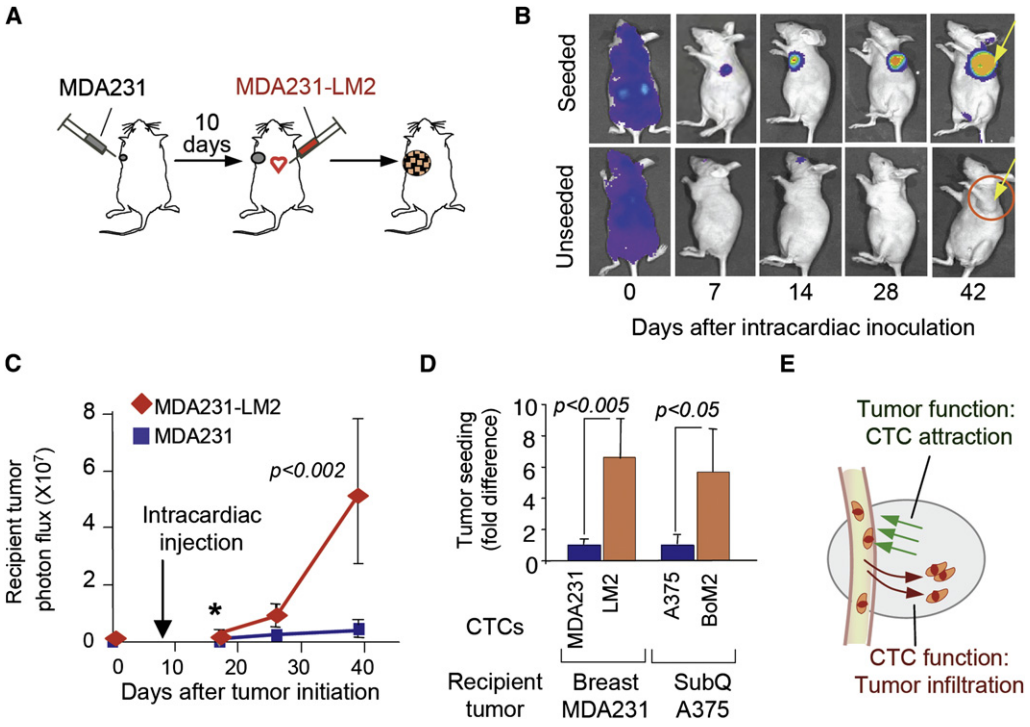

Figure 3. Tumor Attraction and Infiltration Functions

(A) Unlabeled MDA231 cells were injected into a mammary gland No. 2. When tumors became palpable, LacZ/GFP/luciferase-expressing MDA231-LM2 cells were introduced into the circulation by intracardiac injection.

(B) BLI of mice with seeded and unseeded tumors. Arrow, recipient tumor.

(C) Comparative tumor-seeding ability of MDA231 and MDA231-LM2 cells from the circulation. Luminescent signals from recipient tumors at the indicated time points are shown.

(D) Luminescent signals of recipient tumors from mice injected with indicated cell lines were quantified 10 (MDA-231) and 5 (A375) days after injection. n = 6–10.

(E) A diagram summarizing two functions involved in tumor self-seeding.

Error bars in all cases represent SEM and p values were based on two-tailed Mann-Whitney test.

CTCs to infiltrate tumors in response to this attraction (Figure 3E).

To gain further insight into these attraction and infiltration functions, we performed a trans-endothelial migration assay in which tumor cell-conditioned media were placed in the bottom well of the chamber (Figure 4A). Media conditioned by MDA231 breast carcinoma or A375 melanoma cells were several-fold more active at stimulating the trans-endotheilal migration of MDA231-LM2 cells than were media conditioned by MCF10A cells, a human breast epithelial cell line derived from untransformed tissue (Figure 4B). Similarly, A375-BoM2 melanoma cells migrated through endothelial cell layers more actively in response to these cancer cell-conditioned media than to media conditioned by HaCat cells (Figure 4B), a human keratinocyte cell line representing the most abundant cell type in skin epidermis. Media from MDA231 and MDA231-LM2 cultures were equivalent as a source of attraction in these experiments (Figure 4C), which is consistent with the equivalent ability of these two cell lines to act as recipient tumors in self-seeding assays (refer to Figure 1B and Table 1).

MDA231-LM2 cells are more active at migrating through endothelial cell layers compared to parental MDA231 cells (Gupta et al., 2007). Conditioned media from either MDA231-LM2 or MDA231 cells further stimulated the trans-endothelial migration of MDA231-LM2 cells (Figure 4C). Parental MDA231 cells showed low trans-endothelial migration activity even in the presence of media conditioned by tumor cells (Figure 4C). Similarly, the migration of A375-BoM2 cells through endothelial layers was several-fold more efficient than that of the parental A375 cells in the presence of conditioned media from A375 or A375-BoM2 (Figure 4C). These results demonstrated that cancer cells release signals that attract their progeny across endothelial layers. In addition, these results suggest that aggressive cancer cells are superior to their more indolent counterparts in their ability to migrate in response to these signals.

Tumor-Derived Mediators of Cancer Cell Attraction

To identify candidate tumor-derived attractants for CTCs, we compared the secreted levels of 180 cytokines in conditioned media. This analysis uncovered several cytokines whose production was higher (IL-6, IL-8, oncostatin M, and vascular endothelial growth factor [VEGF]) or lower (CCL2) in MDA231 and its derivatives than in MCF10A cells (Figures 5A, S2A, and S2B). IL-6 and IL-8 showed the sharpest increase. IL-8 was also the most abundantly secreted cytokine in A375 melanoma cells compared to the HaCat cells. IL-6 and IL-8 are regulators

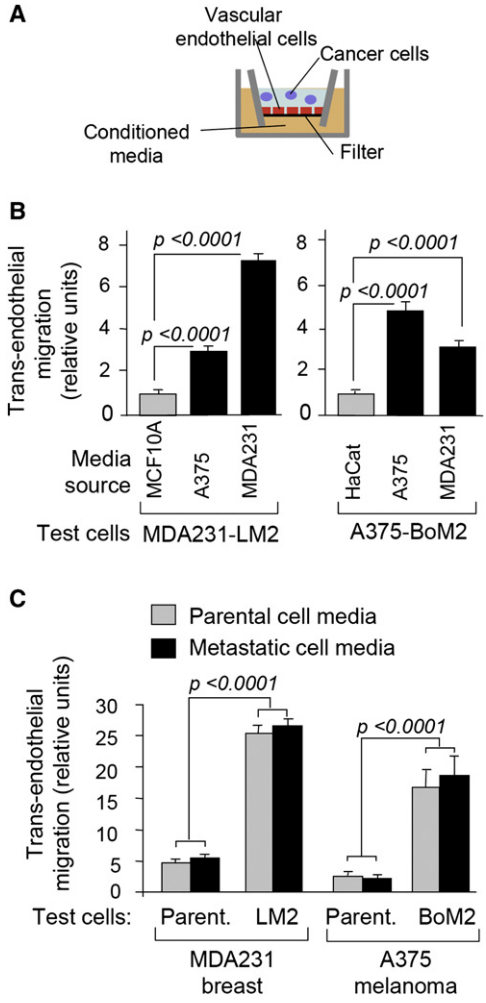

Figure 4. Tumor-Derived Mediators of Cancer Cell Attraction

(A) Schematic diagram of the in vitro trans-endothelial migration assay. Conditioned media were placed in the bottom well. Test cells were plated in the top trans-well chamber. A confluent monolayer of human endothelial cells was present as indicated. Quantification was performed as described in the Experimental Procedures.

(B) Trans-endothelial migration of MDA231-LM2 and A375-BoM2 cells in the presence of media conditioned by the indicated cell lines. n = 60–150.

(C) Trans-migration activity of tumor cells under indicated conditions was expressed as a fold difference relative to migration of parental MDA231 or A375 cells in the presence of media from nontumorigenic cells (MCF10A conditioned media for MDA231; HaCat conditioned media for A375). n = 40–70.

Error bars in all cases represent SEM and p values were based on two-tailed Mann-Whitney test.

of immune and inflammatory responses and have been implicated in tumor progression (Hodge et al., 2005; Kishimoto, 2005). Focusing on these two cytokines, the addition of recombinant IL-6 or IL-8 to media from the corresponding nontumorigenic cells (MCF10A and HaCat, respectively) stimulated the trans-well migration of MDA231-LM2, MDA231-S1a, and A375-BoM2 cells (Figure 5B). These effects were maintained or further increased in the presence of an endothelial cell layer (Figure 5C). IL-8 had little effect on MDA231-LM2 migration (data not shown), but these cells lack IL-8 receptors (CXCR-1 and 2) (Helbig et al., 2003).

To determine the role of IL-6 on the attraction of MDA231 CTCs in vivo while averting the confounding effects of IL-6 on tumor vessels, we tested MDA231-S1a cells whose IL-6 receptor (*IL6R*) expression was inhibited with RNAi (Figure S3A). The knockdown of *IL6R* expression significantly decreased the self-seeding ability of seeder cells (Figure 5D). The knockdown of *IL6ST* (gp130) (Figure S3A), a shared signal transducer for IL-6 cytokine family members including oncostatin M, strongly inhibited the seeding activity of MDA231-S1a (Figure 5D). Taken together, these results suggest that tumor-derived IL-6 and IL-8 can facilitate the self-seeding in these breast carcinoma and melanoma models by functioning as chemoattractants for circulating tumor cells.

Mediators of Cancer Cell Infiltration of Tumors

The enriched expression of various metastasis-associated genes in the MDA231-S1a and S1b populations indicated a preferential seeding of tumors by their metastatic cell progenies (refer to Figure 2E and Table S1). But this alone did not specifically link these genes to the ability of CTCs to infiltrate a tumor mass. To identify candidate mediators of this function, we searched this list for proinvasive genes whose expression in primary breast tumors has been previously associated with relapse in breast cancer. These criteria were based on the rationale that expression of such mediators in primary tumor cells would endow these cells with a potential infiltrative advantage as they pass into the circulation.

These criteria were fulfilled by three genes (Table S1), namely, *collagenase 1* (*matrix metalloproteinase 1*, *MMP1*), *FASCIN 1* (*FSCN1*), and *CXCL1*. All three genes are implicated in invasion and infiltration and, most importantly, their expression in breast tumors is associated with priming of breast cancer cells for seeding of the lungs (*MMP1*, *FSCN1*, and *CXCL1*) and the brain (*MMP1*, *FSCN1*) (Bos et al., 2009; Minn et al., 2005a). These are among the top-ranked genes by association with lung relapse in the LMS signature in breast cancer patients (Minn et al., 2005a). MMP1 has been implicated in tumor cell invasion, vascular remodeling, and pulmonary extravasation (Egeblad and Werb, 2002; Gupta et al., 2007). Fascin-1 is an actin crosslinking protein that functions in the organization of cytoplasmic microfilament bundles and dynamic, cortical cell protrusions including filopodia, lamellipodia, and dendrites (Adams, 2004; Hashimoto et al., 2005). Fascin-1 has been implicated in the migration and invasiveness of malignant glioma and colorectal carcinoma cells (Hwang et al., 2008; Vignjevic et al., 2007). CXCL1 is a powerful mediator of leukocyte influx into sites of inflammation (Dhawan and Richmond, 2002; Kobayashi, 2008) and also participates in the recruitment of endothelial precursor cells for angiogenesis (Hristov et al., 2007).

Using qRT-PCR, we confirmed that the expression level of *FSCN1, CXCL1*, and *MMP1* was 11-fold to >40-fold higher in the S1a and S1b seeder populations than in parental MDA231 (Figure S3B). To investigate the role of these genes in tumor seeding, we conducted trans-endothelial migration assays with MDA231-S1a cells in which the expression of these genes was inhibited with RNAi vectors (Figures S3C–S3E). Whereas the

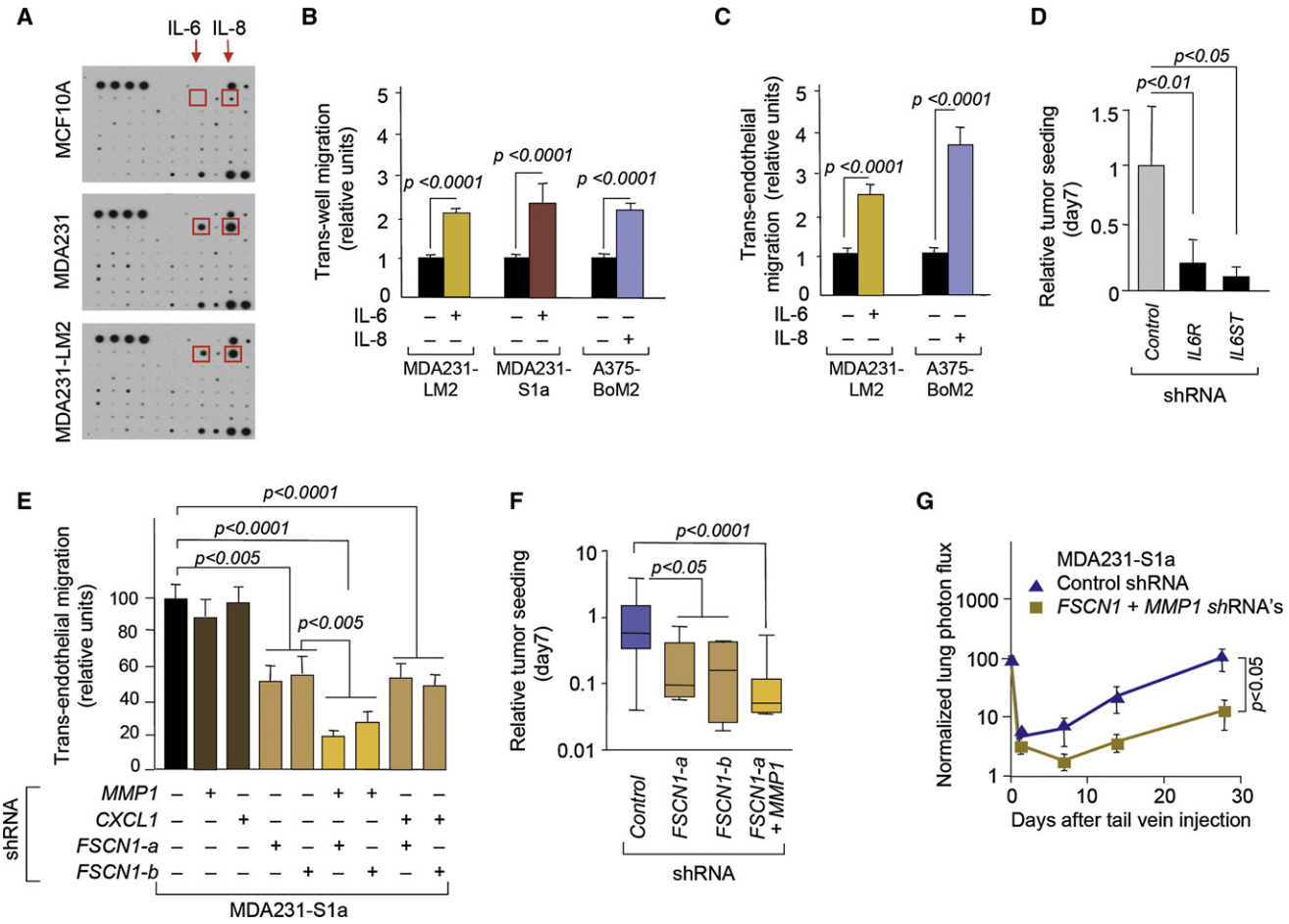

Figure 5. Mediators of Tumor Infiltration by Cancer Cells

(A) Cytokine antibody arrays (Raybiotech) probed with MCF10A, MDA231, and MDA231-LM2 conditioned media. The positions of IL-6 and IL-8 are indicated. For additional annotation see Figure S2.

(B) Relative trans-well migration activity (without endothelial cell layer) of the indicated cell lines, with or without IL-6 or IL-8. n = 89–110.

(C) Relative trans-endothelial migration activity of indicated cells with or without IL-6 or IL-8. n = 84–124.

(D) Relative seeding activity of MDA231-S1a cells expressing the indicated shRNA from the circulation. n = 5–10.

(E) Trans-endothelial migration activity of indicated cell lines. n = 30–45.

(F) Relative seeding activity of MDA231-S1a cells expressing indicated shRNA from the circulation. n = 5–8; error bars, maximum and minimum values.

(G) Lung colonization activity of MDA231-S1a cells expressing indicated shRNAs. n = 6–7.

Error bars in all cases represent SEM and p values were based on two-tailed Mann-Whitney test unless indicated otherwise.

knockdown of *MMP1* or *CXCL1* singly had no effect on trans-endothelial migration, the knockdown of *FSCN1* expression significantly decreased this activity (Figure 5E). The combined knockdown of *MMP1* and *FSCN1* further decreased this migration, whereas the combined knockdown of *CXCL1* and *FSCN1* did not (Figure 5E). Correlating with these effects, the ability of MDA231-S1a cells to infiltrate MDA231 mammary tumors from the circulation was significantly diminished by *FSCN1* knockdown and was further decreased by a combined knockdown of *MMP1* and *FSCN1* (Figure 5F). The knockdown of *MMP1* (Gupta et al., 2007) or *FSCN1* (Figure S3F) had no effect on the growth of MDA231-LM2 cells as mammary tumors. The combined knockdown of *MMP1* and *FSCN1* in MDA231-S1a cells also inhibited the ability of these cells to colonize the lungs of mice from the venous circulation (Figure 5G). These results suggest that *FSCN1* and *MMP1*, two genes whose expression in primary tumors is associated with distant relapse to lung and brain in breast cancer patients (Bos et al., 2009; Minn et al., 2005a), may also mediate the reinfiltration of mammary tumors by CTCs.

Promotion of Tumor Growth and Stroma Recruitment by Self-Seeding

Aggressive cancer cells may promote tumor growth through the release of paracrine signals that enhance tumor angiogenesis and the recruitment of a supportive stroma (Carmeliet, 2005; Coussens and Werb, 2002; Joyce and Pollard, 2009; Tisty and Coussens, 2006). To determine if the seeding of a tumor mass by aggressive CTCs could affect the rate of tumor growth, we implanted MDA231 mammary tumors in mice and 10 days later we

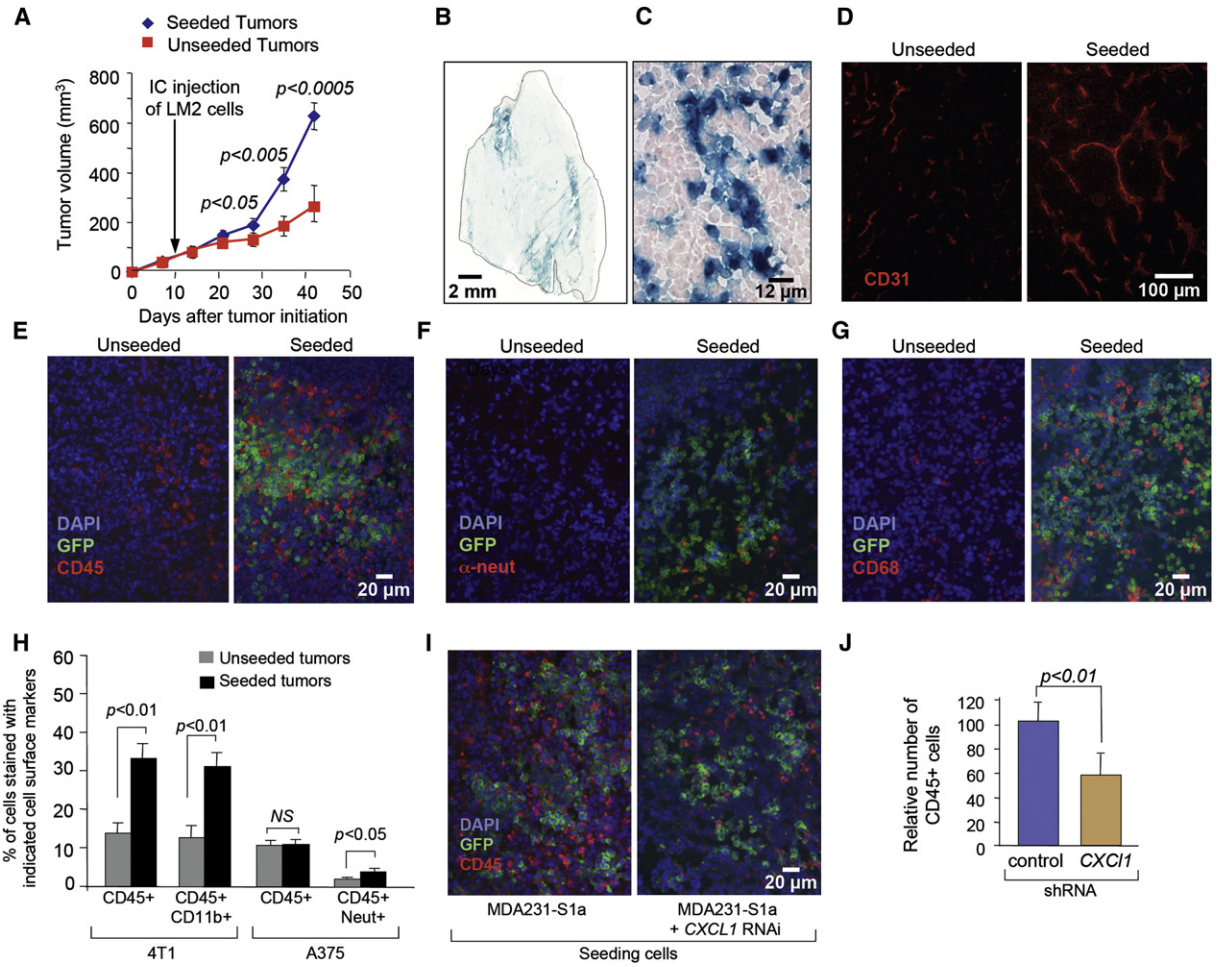

Figure 6. Promotion of Tumor Growth and Stroma Recruitment by Self-Seeding

(A) Tumor volumes of MDA231-LM2-seeded and -unseeded tumors at the indicated time points. n = 13–14.

(B and C) β-galactosidase staining of a typical tumor seeded with LacZ+ MDA231-LM2 cells. An entire tumor section (B) and a ×40 magnification (C) are shown.

(D) CD31 staining of sections from unseeded and seeded tumors.

(E) CD45 staining of sections from unseeded and seeded tumors.

(F) Anti-neutrophil staining of sections from unseeded and seeded tumors.

(G) CD68 staining of sections from unseeded and seeded tumors.

(H) Seeding experiments from the circulation were performed with 4T1 in syngeneic mice and with A375-BoM2 cells. Recipient tumors were analyzed by FACS. The percentage of stromal cells positively stained for the indicated markers are shown. p values were calculated based on two-tailed student's t test.

(I) Seeding experiments from the circulation were performed as described in Figure 3A with MDA231-S1a cells expressing indicated shRNAs. CD45 staining was performed on recipient tumors 19 days after the injection.

(J) Quantification of CD45-positive cells in tumors seeded by MDA231-S1a control and *CXCL1* knockdown cells. n = 9.

Error bars in all cases represent SEM and p values were based on two-tailed Mann-Whitney test unless indicated otherwise.

injected LacZ/GFP/luciferase-expressing MDA231-LM2 cells into the circulation of these mice. As these tumors became seeded with MDA231-LM2 cells, the rate of tumor growth was accelerated compared to that of unseeded tumors (Figure 6A). The increase in the size of seeded tumors was reflected in an increase in the number of tumor cells and was not due to the less dense packing of cells (Figures S4A and S4B). Notably, β-galactosidase staining of histological sections revealed that the LacZ+ seeder cells represented 7% to 19% of the entire tumor mass, which could not fully account for the larger size of these tumors (Figures 6B and 6C and data not shown). Of note, the intrinsic proliferation rate of MDA231-LM2 is similar to that of the parental MDA231 population (Gupta et al., 2007), and the same applies to the MDA231-S1a and S1b lines (data not shown). These results argue that a small proportion of infiltrated MDA231-LM2 cells in a mammary tumor can augment the growth rate of the overall tumor cell population by paracrine effects on the stroma.

The presence of seeder MDA231-LM2 cells in MDA231 tumors increased the average length and the extent of branching of tumor capillaries as determined subjectively (Figure 6D) and by quantitative digital imaging analysis of CD31-stained vasculature

(Figure S4C). Moreover, the seeded tumors contained elevated numbers of infiltrating leukocytes, as determined by immunostaining for the pan-leukocyte marker CD45 (Figure 6E) (Charbonneau et al., 1988). Further analysis of recruited leukocytes revealed increased numbers of neutrophils as well as macrophages (CD68-positive cells) in the seeded tumors (Figures 6F and 6G).

We also investigated stroma recruitment in tumors seeded with 4T1 cells in a syngeneic mouse mammary tumor model and A375-BoM2 cells in a melanoma model. FACS analysis of recipient tumors revealed that GFP+ A375-BoM2 seeder cells represent about 18%–33% of the cells in seeded tumors whereas GFP+ 4T1 seeder cells account for 3%–8% (Figure S5A). Tumors seeded by 4T1 cells showed an increase in leukocyte recruitment (CD45+), the majority of which were CD11b+ myeloid cells (Figure 6H). Over 70% of the CD11b+ cells were also stained with F4/80, a pan-macrophage marker (Figure S5B), which is consistent with previous analysis of stromal composition of 4T1 tumors (DuPre et al., 2007). Analysis of tumors seeded by A375-BoM2 revealed a limited but significant increase in neutrophil recruitment (CD45+ Neut+) (Figure 6H).

To provide proof of principle that seeding cells can affect their host tumor through the release of specific leukocyte-recruiting signals, we turned our attention to the MDA231 model system and the seeder-derived chemokine *CXCL1*, which is a mediator of leukocyte influx into sites of inflammation (Galkina and Ley, 2007). Unlike the knockdown of *MMP1* and *FSCN1* expression, the knockdown of *CXCL1* did not diminish the trans-endothelial migration of MDA231 cells (refer to Figure 5E) or the extent of seeding of MDA231 tumors with GFP+ MDA231-S1a cells (Figure 6I). However, *CXCL1* knockdown in MDA231-S1a cells caused a significant decrease in the recruitment of CD45+ cells into the stroma of seeded MDA231 tumors (Figures 6I and 6J). These results are consistent with the expected consequences of an enrichment of a tumor mass with aggressive cancer cells that secrete stroma-recruiting signals.

DISCUSSION

Based on previous considerations (Norton and Massague, 2006), here we present evidence that tumor self-seeding is a general phenomenon in experimental models of breast carcinoma, colon carcinoma, and malignant melanoma. In these models, tumor masses become readily seeded by CTCs derived from a separate tumor mass, from metastatic lesions, or from direct inoculation. We observed self-seeding with homotypic models using the same cancer cell population as the recipient tumor mass, and with heterotypic models using cell lines from different patients and different breast cancer subtypes. Although our focus is on seeding of a tumor by its own circulating progeny, we also observed cross-seeding between mammary and melanoma tumors (our unpublished results).

The features of tumor self-seeding indicate a process in which CTCs reinfiltrate a tumor mass based on distinct biological functions, a process that may foster tumor growth and the breeding of metastatic progenies. Unlike colonization of distant organs, self-seeding requires little, if any, additional adaptation of CTCs to the recipient microenvironment. However, self-seeding does select for cancer cell populations that are more aggressive than the bulk population of the primary tumor. As CTCs, the seeds have undergone selection for movement into and survival in the circulation. Moreover, self-seeding is actively driven by the ability of CTCs to sense attraction signals from the tumor and to extravasate in response to such signals. These functions are represented in the most aggressive segment of a CTC population, including CTCs that may have already acquired a full complement of metastatic functions.

Tumor self-seeding selects for highly aggressive CTCs, as shown by our consistent observation that metastatic cell subpopulations are more efficient as seeders than their parental populations. Moreover, MDA231 seeder populations recovered from mammary tumors are a mixed population with a gene expression profile and a multi-organ metastatic phenotype that recapitulate those of various site-selective metastatic entities present in the MDA231 cell population. Bone-, lung-, or brain-metastatic subpopulations that can be segregated from each other in experiments of organ-specific metastases (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a) emerged as a mixed population when we selected for tumor-seeding cells. If seeding selects for highly aggressive segments of a CTC population, then seeding may foster the expansion of potentially metastatic populations in the compatible soil of the primary tumor.

Tumor self-seeding in mice carrying a large load of CTCs was not accompanied with de novo tumor formation in orthotopic sites (mammary glands or skin), suggesting that self-seeding requires tumor-derived attraction signals. Our evidence points at IL-6 and IL-8 as tumor-derived attractants of CTCs in breast carcinoma and melanoma models. IL-6 and IL-8 have been implicated in several tumorigenic processes including cancer cell chemoattraction (Arihiro et al., 2000; Wang et al., 1990; Waugh and Wilson, 2008). High serum levels of IL-6 indicate poor prognosis in breast, colon, and lung cancer (Esfandi et al., 2006; Knupfer and Preiss, 2007; Schafer and Brugge, 2007), and high expression of IL-8 in metastatic melanoma is associated with tumor load (Scheibenbogen et al., 1995; Ugurel et al., 2001). Inflammatory cells recruited to the tumor site can also be sources of IL-6 (Balkwill et al., 2005; Grivennikov et al., 2009). Thus, stroma-derived and cancer cell-derived factors may function in combination to attract CTCs back to a primary tumor.

The superior ability of aggressive cancer cells to infiltrate a tumor in response to this attraction argues that self-seeding also requires infiltration functions on the part of the CTCs. We show that MMP1 and fascin-1 expressed by breast cancer cells act as mediators of trans-endothelial migration and tumor seeding. Expression of *MMP1* and *FSCN1* in estrogen receptor-negative (ER^-) breast tumors is associated with relapse to lungs and brain (Bos et al., 2009; Minn et al., 2005a). Our present and previous results (Gupta et al., 2007) are consistent with roles of MMP1 and fascin-1 in cancer cell extravasation—extravasation into distant organs for the development of metastases but also, as our present results suggest, extravasation into the tumor of origin.

The mediators of seed attraction and tumor infiltration involved in self-seeding may well be different depending on the tumor type. For example, although IL-6 secretion occurs in ER^- breast cancer cells, no IL-6 expression is detected in various ER+ breast cancer cell lines (Sasser et al., 2007) or in A375 melanoma. Similarly, *MMP1* and *FSCN1* expression is associated

with distant relapse in patients with ER$^-$ breast cancer but not with ER+ breast cancer (Minn et al., 2005a, 2007). In principle, seed-attracting signals could include chemoattractants secreted by tumor cells and/or by inflammatory cells, and tumor infiltration could involve any mediator of extravasation expressed in CTCs.

The interaction of aggressive cancer cells with the tumor stroma results in the release of signals that foster tumor growth, angiogenesis, invasion, and metastasis. These signals prominently include factors that recruit and activate inflammatory cells. Therefore, to the extent that tumor self-seeding recaptures highly aggressive segments of a CTC population it may result in a further enhancement of tumor growth through the action of seed-derived signals. Indeed, the seeding pattern of recipient tumors in our experiments was typically uneven and diffuse, with the seeding cells remaining a minority that mingled with resident cancer cells and tumor stroma. The seeding cells did not have an intrinsic proliferative advantage over the bulk population. Yet, seeded MDA231 mammary tumors grow faster, an increase that is not fully explained by the added mass of the seeder cells. Enhanced angiogenesis and increased recruitment of neutrophils and macrophages accompanied the seeded areas of these tumors, and seeder-derived CXCL1, which is another marker of poor prognosis in ER$^-$ breast cancer (Minn et al., 2005a, 2007), was partly responsible for this recruitment.

It would be premature to conclude at present that enhanced tumor growth is an obligate outcome of tumor self-seeding. The net effect of self-seeding would likely depend on variables such as the ratio of the tumor size to the size of the CTC population, the aggressiveness of the CTCs, the vascularity of the tumor, tumor microarchitecture, and other factors that may change in the course of the disease. Self-seeding may provide harbor in a primary tumor for the expansion of cancer cell subpopulations that are primed for metastasis. As the shedding and attraction of CTCs by a tumor mass is a dynamic process, it is also conceivable that the presence of a substantial tumor mass could transiently decrease the load of aggressive cells in the circulation owing to their recapture by the tumor.

The present evidence provides clues that could elucidate certain enigmas in clinical oncology. The long-established association of large primary tumor size with poor prognosis in many types of cancer, thought to reflect the ability of larger cancers to release more cells of metastatic potential, may in addition reflect the ability of such aggressive cells to self-seed, promoting local-regional growth, acting in turn as a locus of expansion of these cells and priming for distant metastases. Similarly, the association of anaplasia with poor prognosis may be because micro-anatomical disorganization is a consequence of—and hence a marker of—assertive self-seeding. The hypervascularity of many cancers—and the association of such hypervascularity with poor prognosis—may similarly be explained. Our observation that a mammary tumor can be seeded by CTCs derived from lung-metastatic nodules raises the possibility of reseeding after tumor excision as a potential cause of eventual local recurrence. Moreover, that the phenomenon of self-seeding is hereby linked to tumor-specific and circulating cell-specific factors may create opportunities for the development of targeted therapies for the attrition of residual neoplastic cells from the breast and other organs.

EXPERIMENTAL PROCEDURES

Additional methods can be found in the Supplemental Data.

Animal Studies

All animal work was done in accordance with a protocol approved by the MSKCC Institutional Animal Care and Use Committee. Xenografts were performed on BALB/c nude, Athymic nu/nu (for reseeding experiments from the circulation) and NOD/SCID mice (for contralateral seeding experiments) were age-matched between 5–8 weeks. Wild-type BALB/c mice were used for studies in syngeneic model. For contralateral seeding experiments, typically 5×10^5 unlabeled and GFP/luciferse/TK-expressing tumor cells, unless noted, were resuspended in a 1:1 mixture of PBS and growth-factor-reduced Matrigel (BD Biosciences) and injected into contralateral No. 2 mammary glands (MDA231) or into flanks of NOD/SCID mice (A375 and SW 620) in a total volume of 50 μl. At necropsy, recipient tumors were extracted and imaged for luminescent signals with a Xenogen IVIS system. For experiments with CN34.BrM2, mice were intraperitoneally injected with etoposide (30 mg/kg dissolved in DMSO, Sigma) 2 days prior to mammary gland injection. For reseeding experiments from the circulation, 2.5×10^5 and 5×10^5 unlabeled parental MDA231, 67NR, and A375 cells were injected into a mammary gland No.2 or subcutaneously, respectively. Once the tumors became palpable (50–100 mm^3), 1×10^5 GFP/luiferase-expressing MDA231-LM2, 4T1, or A375-BoM2 cells were injected into the cardiac left ventricle in a total volume of 100 μl. Reseeding was monitored by BLI. Recipient tumors were extracted and imaged at the necropsy, typically 6–7 weeks after mammary gland injection. MMTV-PyMT transgenic mice in which mammary tumors are developed by the MMTV promoter-driven expression of the polyoma middle-T oncogene were used for reseeding experiment from the circulation. Cells derived from one such tumor were transduced with lentivirus encoding GFP/luciferase/TK and used for intracardiac injection. Tumors were harvested and imaged after 3 weeks. For reseeding from lung metastases, 2×10^5 MDA231-LM2 cells were injected into the lateral tail vein. After 3 weeks, 5×10^5 unlabeled parental MDA-MB-231 cells were injected into a mammary gland No. 2. After 3–4 weeks, tumors were extracted and imaged. Relative numbers of CTC from these mice were analyzed as previously described (Gupta et al., 2007) except that primers against luciferase were used to detect CTCs. Experimental metastasis assays and calculation of tumor size were done as previously described (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005a).

In Vivo Selection of Seeder Cells

One million unlabeled and GFP/luciferase/TK-expressing parental MDA-MB-231 or A375 cells were injected as in contralateral seeding experiments. Fifty days after the injection, recipient tumors were extracted and imaged by BLI as previously described (Minn et al., 2005a). Two independent seeded tumors by MDA-MB-231 cells and one seeded tumor by A375 cells were minced and centrifuged in PBS-containing antibiotics. Samples were then resuspended in DMEM containing 0.125% collagenase III and 0.1% of hyaluronidase and incubated at 37°C for 2.5 hr with occasional trituration. Subsequently, samples were trypsinized for 5 min, followed by centrifugation in DMEM with 10% FBS. Cells were filtered through a 40 μm strainer and plated in a T75 flask with DMEM containing 10% FBS. Cells were expanded in culture for two passages and GFP-positive cells were isolated by FACS.

Trans-Well Migration Assays

To generate conditioned media, 10^6 cells were plated on a 6 cm dish. The next day media were replaced with 0.2% FBS media without growth factors. After 2 days, media were collected, centrifugated, and used in trans-well migration assays as described previously (Gupta et al., 2007). Recombinant human IL-6 and IL-8 were purchased from R&D systems.

Cytokine Antibody Array

Cytokine antibody array was performed with conditioned media from MCF10A, MDA231, MDA231-S1a, MDA231-S1b, MDA231-LM2, MCF7, HaCat, A375, and A375-BoM2 according to manufacturer's protocol. The complete array maps (Array 5, 9, and 10) can be found at http://www.raybiotech.com/map_all_m.asp#8.

Statistical Analysis

For trans-well migration assays and animal studies in which a value is quantified relative to a normalized standard, standard deviation was calculated based on the following formula:

$$sd\{f/g\} = (f/g)\sqrt{\left[sd\{f\}^2/f^2\right] + \left[sd\{g\}^2/g^2\right]}$$

where *f* and *g* represent the mean of experimental and control data points, respectively. *sd*, standard deviation. Standard error of the mean (SEM) was then calculated by using sd{f/g} /√ n.

ACCESSION NUMBERS

Gene expression data of MDA231-S1 cells are deposited at GEO (GSE18833).

SUPPLEMENTAL DATA

Supplemental Data include five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01437-8.

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