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Systemic Endocrine Instigation of Indolent Tumor Growth Requires Osteopontin

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SUMMARY

The effects of primary tumors on the host systemic environment and resulting contributions of the host to tumor growth are poorly understood. Here, we find that human breast carcinomas instigate the growth of otherwise-indolent tumor cells, micrometastases, and human tumor surgical specimens located at distant anatomical sites. This systemic instigation is accompanied by incorporation of bone-marrow cells (BMCs) into the stroma of the distant, once-indolent tumors. We find that BMCs of hosts bearing instigating tumors are functionally activated prior to their mobilization; hence, when coinjected with indolent cells, these activated BMCs mimic the systemic effects imparted by instigating tumors. Secretion of osteopontin by instigating tumors is necessary for BMC activation and the subsequent outgrowth of the distant otherwise-indolent tumors. These results reveal that outgrowth of indolent tumors can be governed on a systemic level by endocrine factors released by certain instigating tumors, and hold important experimental and therapeutic implications.

INTRODUCTION

The tumor microenvironment has been the subject of intensive investigation in recent years. A tumor-supportive role for many of the host-derived stromal cells that form the tumor microenvironment has been demonstrated in a number of studies (Tlsty and Coussens, 2006; Yang et al., 2008). For example, we and others demonstrated that stromal fibroblasts and myofibroblasts within the tumor environment facilitate tumor growth and progression (Bhowmick et al., 2004; Olumi et al., 1999; Orimo et al., 2005).

Far less is known about the contribution to tumor growth of the host systemic environment. Several reports have shown that assorted bone marrow-derived cells can be incorporated to various extents into the tumor-associated stroma (Direkze and Alison, 2006; Song et al., 2005; Udagawa et al., 2006). Most of these studies suggest that bone marrow-derived cells support tumor angiogenesis in one fashion or another, although the extent to which they do so is debated (Kopp et al., 2006; Lamagna and Bergers, 2006).

While the presence of both locally and distantly derived cell types has been observed in a number of tumors, it is not clear whether tumors actively elicit stromal cell recruitment by directly perturbing host cell reservoirs, such as the bone marrow (Shojaei et al., 2007), or whether tumors are passive recipients of stromal cell precursors that normally circulate throughout the body. We therefore undertook to determine if engrafted human carcinomas actively perturb the host systemic environment as a means of facilitating tumor outgrowth.

RESULTS

Primary Human Tumors Facilitate Growth of Distant Indolent Tumors

In order to study systemic mechanisms that might facilitate tumor progression, we constructed an in vivo experimental model utilizing two human tumor cell lines. The first, termed here an "instigator," is the experimentally transformed human mammary epithelial BPLER cell line (Ince et al., 2007), which yields vigorously growing tumor xenografts that histopathologically resemble invasive ductal adenocarcinomas commonly encountered in breast cancer patients. These xenografts contain

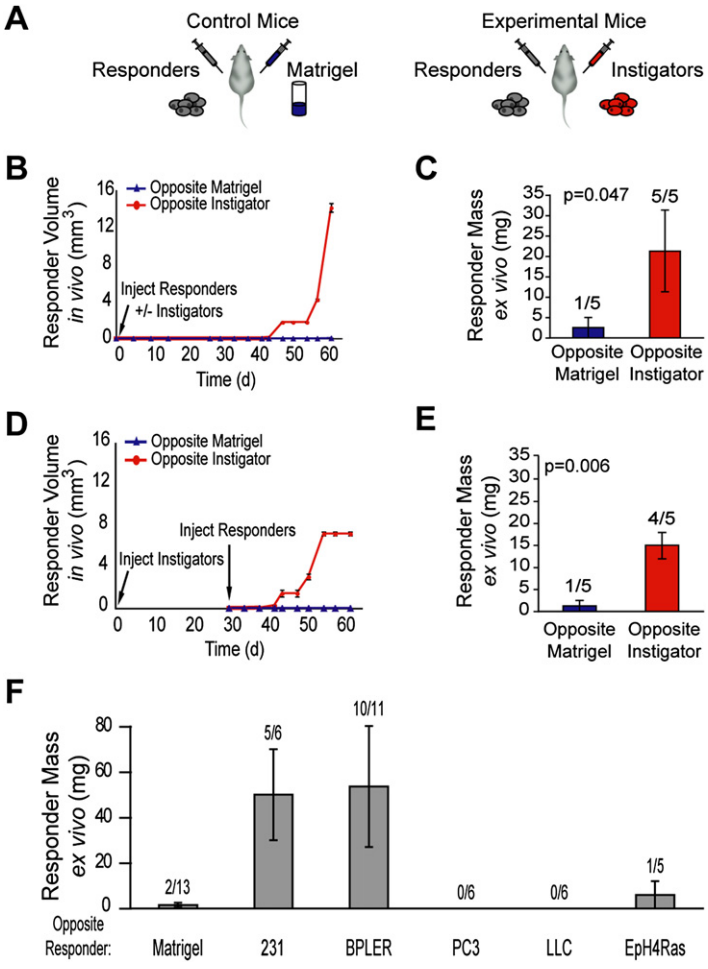


Figure 1. Primary Human Breast Carcinomas Instigate Growth of Distant Indolent Tumors

(A) Scheme of bilateral implantation system. Indolently growing HMLER-HR transformed cells (Responders) are implanted subcutaneously into one flank of host mice and either Matrigel control or vigorously growing tumor cell lines (Instigators) are implanted into the contralateral flank.

(B) In vivo growth kinetics of responder cells when implanted contralaterally to either Matrigel (blue) or BPLER instigators (red); n = 5 per group.

(C) Final mass of responders from (B) 9 weeks after implantation opposite either Matrigel or instigating BPLER tumors. Incidence of tumor formation is shown above data bars; data include injections not resulting in responding tumor growth.

(D) In vivo growth kinetics of responders when injected 30 days after implantation of either Matrigel plugs (blue) or instigating BPLER tumor cells (red); n = 5 per group.

(E) Average final mass of responding tumors from (D) recovered opposite either Matrigel or BPLER instigators; incidence of responder tumor formation is indicated above data bars.

(F) Final mass of responding tumors recovered opposite Matrigel or indicated tumor cell lines 9 weeks after implantation. Incidence of responding tumor formation is indicated above bars; data include mice from three separate experiments. Mass of responders recovered opposite Matrigel is significantly different from those opposite 231 ($p = 0.031$) and BPLER ($p = 0.039$).

abundant stroma, indicating that they are capable of recruiting murine stromal cells. The second, termed here a "responder," is the experimentally transformed human mammary epithelial cell line, HMLER hygro-H-*ras*V12 (HMLER-HR) (Elenbaas et al., 2001). Only ~25% of the mice injected with these indolent cells form observable tumors when examined 9 weeks after implantation.

In an initial experiment, instigating BPLER tumor cells were injected subcutaneously into the right flanks of Nude mice, while indolent responding HMLER-HR cells were injected into the contralateral flanks of these mice (Figure 1A). In a control group, responder cells were injected contralaterally to Matrigel vehicle; these responder cells did not form externally palpable tumors during the subsequent experimental time period (Figure 1B); however, after surgical exposure of the subcutaneous site at the experimental end point, small masses were apparent in 20% of the injected mice (Figure 1C).

In contrast, when the responder cells were injected opposite instigating tumors, the responder cells formed actively growing tumors after a lag phase of ~40 days (Figure 1B), at which time the instigating tumors were only ~3.7 mm in diameter (Figure S1A). These responding tumors followed a progressive increase in their growth that paralleled the growth of the contralateral instigating tumors (Figure S1A). The average final mass of these responding tumors was ~10-fold that of the responding cell plugs recovered opposite Matrigel, and the incidence of responding tumor formation was increased from 20% to 100% (Figure 1C). We confirmed that instigating cells did not metastasize to sites of responding cell implantation and that the neoplastic cells in the responding tumors were derived exclusively from responder cells (Figures S1D and S1E).

We also determined that instigating tumors established a supportive systemic environment soon after their implantation. Thus, when responding cells were implanted following 30 days of instigating BPLER tumor growth, the first palpable responding tumors were evident 5 days later (Figure 1D). Notably, the instigating tumors were only ~2 mm in diameter when the responder cells were injected, and the growth of responding tumors was accompanied by a constant rate of growth of the contralateral instigating tumors (Figure S1B). The average mass of these responding tumors was ~10-fold greater than that of the responder cell plugs recovered opposite Matrigel (Figure 1E). Moreover, the presence of contralateral instigating tumors increased the incidence of responder tumor formation from 25% to 80%.

In a set of in vitro cell culture experiments, we determined that the conditioned media taken from instigating BPLER tumor cells did not alter the growth rate of responding HMLER-HR cells (data not shown). These findings suggested that the outgrowth of responding tumors in vivo was not a consequence of instigating tumor-derived growth factors stimulating responder cell proliferation.

We next asked whether the capacity for systemic instigation was unique to the BPLER cell line. We found that MDA-MB-231 human breast cancer cells (231) also instigated

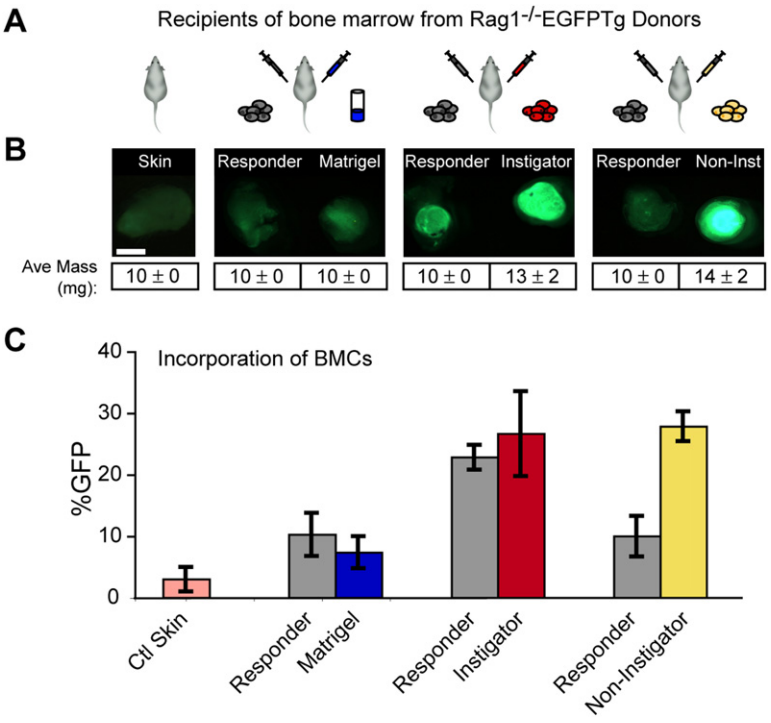


Figure 2. Bone Marrow-Derived Cells Are Incorporated into Responding Tumor Stroma

(A) Indicated tumor cells were injected into mice that had previously been engrafted with GFP$^+$ BMCs.

(B) Whole mount fluorescence photomicrographs to visualize GFP$^+$ BMCs recruited to indicated tumors and control tissues 4 weeks after tumor cell injections; scale bar = 2 mm. Average mass of all tissues is indicated; n = 10 mice per group.

(C) Average contribution of GFP$^+$ cells as a percentage of total cells (flow cytometric analysis); n = 7 tumors/tissues per group. Responding tumors opposite BPLER instigators incorporated significantly more GFP$^+$ BMCs than those opposite Matrigel ($p = 0.039$) and PC3 noninstigating tumors ($p = 0.042$); responders were not statistically different from their contralateral BPLER instigators; instigating BPLER tumors were not statistically different from noninstigating PC3 tumors.

HMLER-HR responding tumors that were ~25-fold larger than those injected opposite Matrigel (Figure 1F); as before, the neoplastic cells in responding tumors were formed exclusively from the HMLER-HR responders (Figure S1E). In contrast, three other vigorously growing tumor types all failed to instigate responder tumor growth (Figure 1F). Hence, the ability to systemically instigate indolent tumor outgrowth was uncoupled from the ability of transformed cells to form vigorously growing primary tumors.

Examination of tissues recovered from responding cell injection sites located opposite Matrigel plugs revealed a high degree of necrosis (Figure S1C). Responding tumors growing opposite instigating BPLER or MDA-MB-231 tumors, however, closely resembled adenocarcinomas (Figure S1C). The latter observations indicated that instigating tumors have a profound systemic effect on both the viability and histopathology of distantly located responding tumors.

Incorporation of Bone Marrow-Derived Cells into Instigated Tumors

Vigorous growth of responding tumors occurred only when the responder cells were implanted contralaterally to instigators in vivo. This fact, taken together with reported observations that certain types of stromal cells have origins in the bone marrow (Direkze and Alison, 2006), caused us to speculate that instigating tumors might mobilize stromal cell precursors from the bone marrow into the circulation, thereby making them available for recruitment by responding tumors.

In order to determine whether instigating tumors mediated recruitment of bone marrow cells (BMC) into the responding tumor stroma, we transplanted BMCs from immunocompromised mice that ubiquitously express green fluorescent protein (*Rag1*$^{-/-}$*EGFPTg* mice) into irradiated mice that were used as hosts in subsequent experiments. After confirming successful engraftment of GFP$^+$ BMCs (Figure S2A), various human tumor cells were implanted into these mice using the contralateral injection protocol (Figure 2A).

Responder growths were surveyed for GFP$^+$ BMC incorporation 4 weeks after tumor cell injections, a time when all xenografted masses were of comparable size (~10 mg, ~0.04% total body weight; Figure 2B). This time period was dictated by previous observations that small responder cell plugs implanted opposite noninstigating tumor cells could be recovered within ~4 weeks of their initial implantation, but thereafter, were no longer visible at the injection site (data not shown).

Incorporation of GFP$^+$ BMCs into responder grafts injected opposite Matrigel (10% ± 4%) were not statistically significantly greater than those of control subcutaneous tissues taken from mice that had been transplanted with GFP$^+$ BMCs but had not been injected with tumor cells (3% ± 3%; Figure 2C). Moreover, in spite of the significant recruitment of GFP$^+$ BMCs to noninstigating PC3 tumors (28% ± 2%), contralateral responding grafts failed to incorporate GFP$^+$ cells to any extent above that of controls (10% ± 3%; Figure 2C). In contrast, GFP$^+$ cells accounted for ~23% of the total cells present in the responding tumors growing opposite BPLER-instigating tumors, which were comprised of ~27% GFP$^+$ cells (Figure 2C).

We also analyzed incorporation of GFP$^+$ BMCs into tumors that had grown as xenografts for 9 weeks in such host mice. The responding tumors were comprised of ~24% GFP$^+$ BMCs, while the contralateral instigating tumors consisted of ~31% GFP$^+$ BMCs (Figures S2B and S2C). These results indicated that the contribution of bone marrow-derived cells to both instigating and responding tumor stroma remained relatively constant during tumor growth. Moreover, the fact that certain noninstigating tumors, despite their own rapid growth and BMC recruitment, were unable to mobilize BMCs to the stroma of the contralateral responders, suggested that only instigating tumors could provide the distant responding tumors with stromal cells that they require for growth.

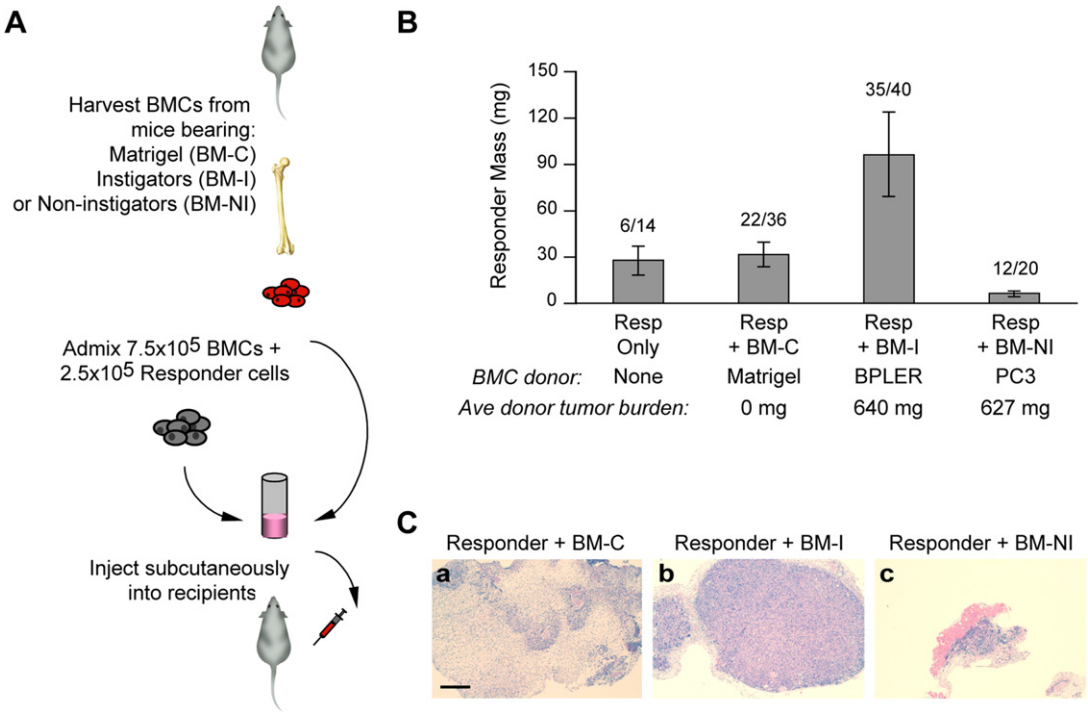


Figure 3. Instigators Functionally Activate the Bone Marrow

(A) Experimental scheme for implantation of BMCs/responding tumor cell admixtures.

(B) Average mass of resulting tumors 12 weeks after implantation of mixtures of responder cells with indicated BMCs. Tumor incidence is indicated above bars; data represent mean of four separate experiments. $p = 0.012$ comparing BM-I with responders alone; $p = 0.015$ comparing BM-I with BM-C; $p = 0.001$ comparing BM-I with BM-NI.

(C) Hematoxylin and Eosin stain to visualize histopathology of resulting tumors/tissues; scale bar = 400 μm.

Instigating Tumors Functionally Activate the Bone Marrow Prior to Mobilization of Cells

The above results suggested that instigating tumors might perturb the bone marrow, mobilizing BMCs into the circulation that then become available for recruitment by responding tumors. We therefore devised a test to determine whether there were ascertainable functional differences between the BMC populations of various types of tumor-bearing hosts. We isolated BMCs from mice bearing either instigating BPLER tumors or size-matched, noninstigating PC3 tumors and mixed each of these BMC populations with responder cells prior to injection (Figure 3A). As controls, we mixed responder cells with BMCs from mice bearing only Matrigel plugs. We then gauged the relative abilities of these various admixed BMC populations to promote responder tumor growth over a period of 12 weeks. We confirmed that instigating tumor cells were absent from the BMC populations used in these experiments (Figures S3A and S3B).

When mixed with responder cells, BMCs from control, nontumor bearing mice (BM-C) and from mice bearing noninstigating PC3 tumors (BM-NI) both failed to enhance responder tumor growth to any significant extent (Figure 3B). Histological observation of the resulting masses revealed either large necrotic areas or only small clusters of apparently viable cells (Figures 3Ca and 3Cc). However, admixture of BMCs from mice bearing BPLER-instigating tumors (BM-I) greatly enhanced both the size and incidence of responder tumor formation (Figure 3B); these tumors formed with a histopathology similar to that of responding tumors growing opposite instigating tumors (Figure 3Cb). Moreover, in a separate set of experiments, mixing BMCs from instigator-bearing *Rag1*$^{-/-}$*EGFPTg* mice with responder cells revealed that the admixed GFP^{+} marrow-derived cells were retained within the responding tumor stroma after 9 weeks of growth (Figure S3C).

We also functionally tested BMCs prepared from mice bearing relatively small instigating BPLER tumors (20–60 mg) and found that even these admixed BMCs enhanced responding tumor formation compared with control BMCs (Figure S3D). These results indicated that instigating tumors as small as 20 mg—only ~0.08% of total body weight—are capable of functionally activating the bone marrow and extended our earlier results by demonstrating that instigating tumors established a tumor-promoting systemic environment relatively early in their growth. Moreover, since the admixed BMCs from instigator-bearing mice recapitulated the effects of an implanted instigating tumor, we concluded that most if not all of the tumor-promoting effects of systemic instigation are achieved via endocrinal perturbation of the host bone marrow.

Instigators Perturb Primitive Hematopoietic BMCs

In order to determine how instigating tumors might affect bone marrow physiology, we analyzed the hematopoietic cell types

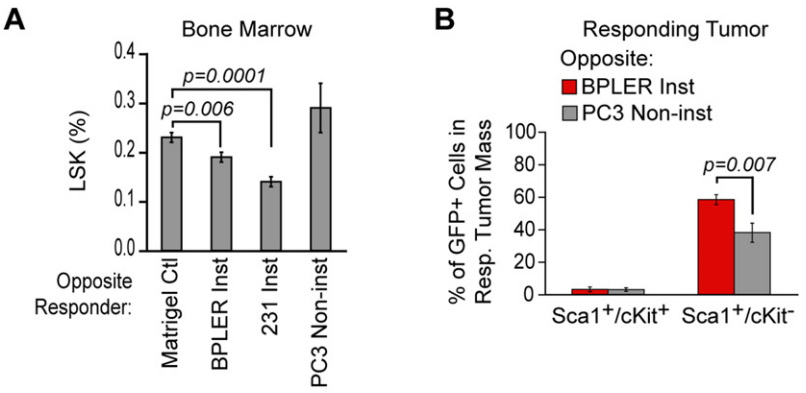
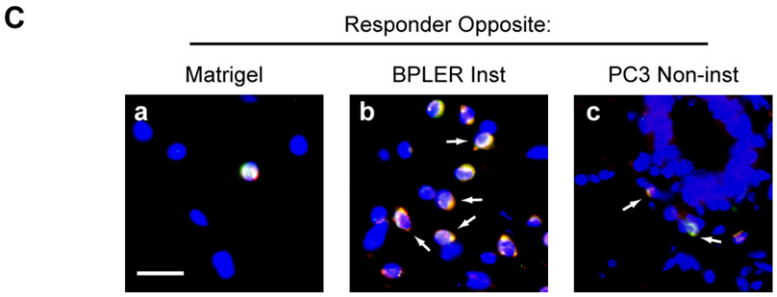


Figure 4. Instigating Tumors Perturb Bone Marrow Lin⁻/Sca1⁺/cKit⁺ (LSK) Cells while Sca1⁺/cKit⁻ Cells Are Incorporated into Responding Tumors

(A) Flow cytometric analysis of LSK cells in the bone marrow of various tumor-bearing mice; n = 4 per group.

(B) Quantification of Sca1⁺/cKit⁺ and Sca1⁺/cKit⁻ cells as percentage of total GFP⁺ cells in responding tumors recovered opposite instigating BPLER tumors (red) or noninstigating PC3 tumors (gray); n = 7 per group.

(C) Merged photomicrographs of indicated responding tumors stained for GFP (green), Sca1 (red), and cell nuclei (blue); GFP⁺/Sca1⁺ cells appear yellow (arrows); scale bar = 25 μm.



in the marrow of these immunocompromised host mice. When we surveyed expression of a number of cell-surface antigens, including CD11b, CD45, Gr1, VEGFR1, VEGFR2, and CD31, we detected no significant changes in the representation of cell types in the marrow of the instigator-bearing mice, as compared to Matrigel-bearing mice (data not shown). However, we repeatedly observed a subtle yet significant decrease in the frequency of Lin⁻/Sca1⁺/cKit⁺ (LSK) cells in the marrow of mice bearing instigating tumors when compared with mice bearing only Matrigel plugs (Figure 4A). Such LSK cells have been shown by others to contain the hematopoietic stem cells in mice (Kondo et al., 2003). This decrease in the LSK population was not manifest in the bone marrow of mice bearing noninstigating PC3 tumors (Figure 4A). In separate experiments, we saw similar reductions in the LSK population in the marrow of mice bearing BPLER-instigating tumors when compared with mice injected only with Matrigel (Fig S4A).

Bone marrow-derived hematopoietic cells have been observed within primary tumor stroma and are thought to play a role in promoting tumor progression (Kopp et al., 2006). Accordingly, we undertook to determine whether the observed decrease in LSK cells in the bone marrow correlated with the presence of Sca⁺/cKit⁺ hematopoietic cells in the responding tumor stroma. To do so, we measured the cell-surface expression of Sca1 and cKit on the GFP⁺ bone marrow-derived cells present in 4 week responding tumor masses that were implanted contralaterally to either BPLER instigators or PC3 noninstigators.

The contribution of primitive Sca1⁺/cKit⁺ cells to the total GFP⁺ bone marrow-derived responder stromal compartment was minimal and equivalent between the two groups (~3%; Figure 4B). We found, instead, that the majority of the GFP⁺/Sca⁺ bone marrow-derived cells were negative for cKit and that these Sca1⁺/cKit⁻ cells comprised ~66% of the GFP⁺ cells recruited to the responding tumors that had grown opposite instigating tumors; the GFP⁺ cells from indolent cell clusters recovered opposite noninstigators contained only ~38% of these Sca1⁺/cKit⁻ cells (Figure 4B). Cells in the bone marrow that are Sca1⁺/cKit⁻ have been described as a quiescent population of hematopoietic progenitor cells that are resistant to the cytotoxic effects of 5-FU and, under certain conditions, have reconstitution capability (Klarman et al., 2003; Randall and Weissman, 1998).

Upon immunohistological examination of responding tumors for the colocalization of GFP and Sca1 antigens, we found that bone marrow-derived GFP⁺/Sca1⁺ cells (which were almost entirely cKit⁻, see above) were rarely observed in the responder cell plugs recovered opposite Matrigel or noninstigating tumors and, when present, were localized largely near blood vessels of the normal skin (Figures 4Ca and 4Cc). In contrast, when growing opposite instigating tumors for either 4 or 8 weeks, bone marrow-derived GFP⁺/Sca1⁺ cells were found intermingled with the neoplastic HMLER-HR responding cells (Figures 4Cb and S4B).

Collectively, these results demonstrate that instigating tumors specifically decrease the primitive hematopoietic cell compartment (LSK) in the host marrow. This perturbation is not associated with a concomitant recruitment of Sca1⁺/cKit⁺ cells to the responding tumor stroma but, rather, correlates with enhanced recruitment of Sca1⁺/cKit⁻ cells into the responding tumor stroma.

Tumor-Derived Osteopontin Is Necessary for Systemic Instigation

We wished to learn how instigating tumors communicate with the host bone marrow. We therefore analyzed the plasma of mice bearing instigating tumors, noninstigating tumors, or Matrigel control plugs in order to determine whether we could detect alterations in the levels of various human cytokines that were secreted by the engrafted tumor cells (Table S1). There were no significant differences in the plasma levels of 80 distinct human cytokines in mice bearing instigating tumors compared with those bearing noninstigating tumors (data not shown),

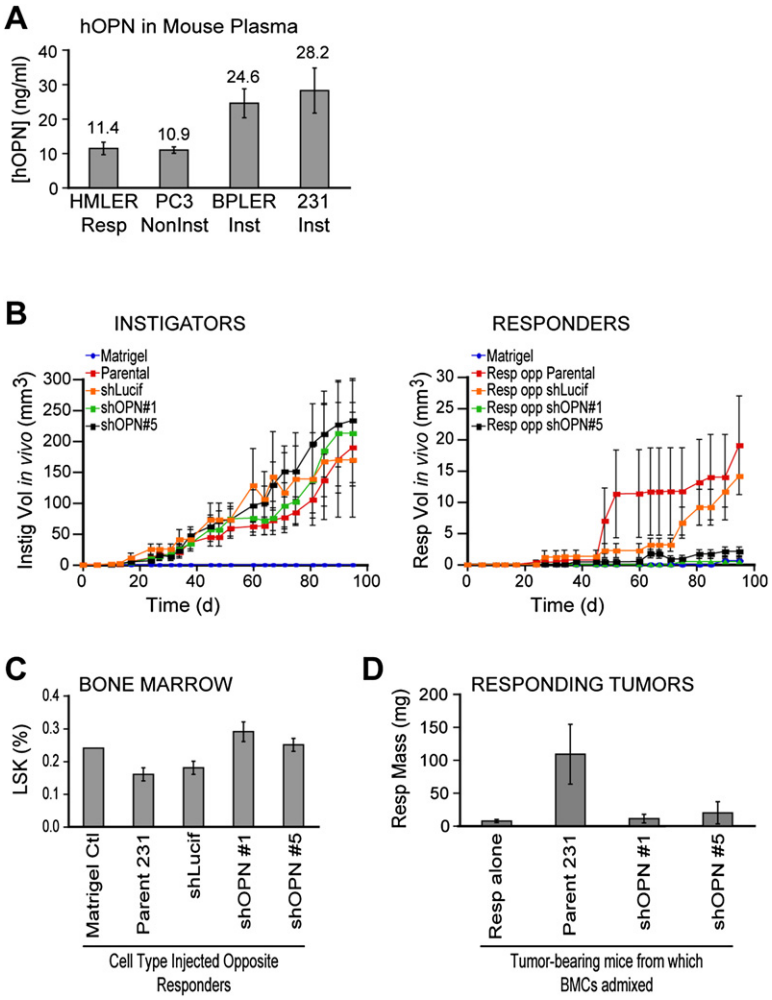


Figure 5. Tumor-Derived Osteopontin (hOPN) Is Necessary for Systemic Instigation

(A) Concentration of hOPN in plasma of mice 9 weeks after injection of indicated cells; n = 14 for responder group; n = 5 for all other groups. Plasma OPN was significantly elevated in mice bearing BPLER ($p = 0.02$) and 231 ($p = 0.01$) tumors compared with plasma from mice injected only with responder cells.

(B) The left shows in vivo growth kinetics of instigating MDA-MB-231 (parental), control 231 cells expressing shRNA against Luciferase (shLucif), and 231 derivatives expressing shRNAs against hOPN; n = 9 for parental group; n = 5 for all other groups. The right shows in vivo growth kinetics of the responding cells injected opposite the indicated tumor cell lines.

(C) Flow cytometric analysis of LSK cells in the bone marrow of mice with indicated bilateral tumors; n = 4 per group.

(D) Admixtures of responder cells with BMCs from mice bearing 231 instigators yielded tumors that were significantly larger than those resulting from admixtures of BMCs from mice bearing shOPN 1 tumors ($p = 0.030$), shOPN 5 tumors ($p = 0.047$), or responder cells alone ($p = 0.026$); n = 4 per group.

with one exception: osteopontin (hOPN). In an initial experiment, plasma levels of tumor-derived hOPN in mice bearing instigating tumors were elevated ~3-fold relative to noninstigating tumors (data not shown).

Osteopontin (OPN) is a secreted glycoprotein having pleiotropic effects on inflammation, angiogenesis, fibrosis, and tumor metastasis, and carries functional domains that are conserved across mouse and human species. (Cook et al., 2005). A number of studies have shown that OPN is secreted in a soluble form by a variety of tumor cell types and is elevated in the blood of many cancer patients with metastatic disease (Mor et al., 2005; Tuck et al., 2007); however, such studies have not revealed a physiologic role for circulating OPN in tumor pathogenesis.

We therefore expanded our analysis of plasma hOPN to include mice bearing various instigating and noninstigating tumors. Plasma levels of tumor-derived hOPN in mice bearing instigating tumors—BPLER and MDA-MB-231—were elevated at least 2.2-fold, and up to 2.6-fold, above those of control mice injected with either HMLER-HR responders or noninstigating PC3 tumors (Figure 5A). In contrast, there were no significant differences in plasma hOPN levels between mice injected with the responding cells and those injected with PC3 noninstigating tumors (Figure 5A). We also determined that none of the tumor types that we implanted altered the plasma levels of murine osteopontin (Figure S4C).

The relative increases in plasma hOPN that we observed in instigator-bearing mice were comparable to increases that we observed in the plasma of patients with metastatic breast cancer compared to cancer-free subjects (2.2- to 2.8-fold; Figure S4D). Indeed, these elevations are consistent with previously published reports and are considered to represent a clinically significant parameter that is associated with reduced patient survival (Furger et al., 2001).

OPN is reported to restrict LSK numbers in the bone marrow through its actions on the hematopoietic cell niche as well as on the LSK cells themselves (Nilsson et al., 2005; Stier et al., 2005); this was reminiscent of the decline in LSK numbers we observed in the marrows of mice bearing instigating tumors (Figure 4A). Furthermore, hematopoietic stem cells express the OPN receptors, CD44 and α4 integrin, and the differentiation status of these cells can be altered by OPN signaling (Iwata et al., 2004; Schmits et al., 1997; Scott et al., 2003).

We therefore tested the notion that circulating hOPN released by instigating tumors perturbs the host bone marrow, thereby facilitating systemic instigation. In order to do so, we suppressed hOPN expression in MDA-MB-231 (231) instigating tumor cells using shRNA; such suppression would presumably allow us to determine whether OPN deficiency would affect their ability to function as instigators. We identified two shRNA constructs that reduced OPN secretion at least 23-fold and did not significantly alter either cell morphology or growth kinetics in culture (Figures S5A–S5D).

We injected either 231 cells or their OPN-deficient derivatives contralaterally to HMLER-HR responder cells in order to gauge the ability of these 231 variants to function as instigators. We

observed that OPN deficiency did not impair in vivo growth kinetics of implanted instigating 231 cells in which the shRNAs were expressed (Figures 5B [left] and S5E). As expected, the parental 231 tumors, as well as control tumors expressing shRNA against luciferase, instigated growth of contralateral responding tumors (Figure 5B; right). In striking contrast, responding tumors did not grow when the contralateral 231 tumors were deficient for OPN (Figure 5B; right). Hence, despite the fact that OPN-deficient 231 cells continued to form vigorously growing tumors, OPN deficiency abolished their instigating ability.

We used mice engrafted with GFP^+ BMCs to determine whether suppressing OPN in instigating tumors had any effect on incorporation of BMCs into the contralateral responder cell plugs. Responder tumors grown opposite the parental 231 instigators were comprised of ~28% bone marrow-derived cells, while responder cell plugs recovered opposite OPN-deficient tumors contained only ~3% GFP^+ bone marrow-derived cells (Figure S5F).

The reduction in LSK cells that was reproducibly observed in the mice bearing parental 231 instigating tumors was absent in mice bearing OPN-deficient 231 tumors (Figure 5C). Moreover, when we mixed responding BMCs from mice bearing OPN-deficient 231 tumors with responding cells prior to injection, responding tumor growth was not facilitated, whereas the admixed BMCs from mice bearing the parental 231 tumors, as before, supported tumor growth (Figure 5D). These results indicate that instigating tumor-derived OPN is required to perturb the host bone marrow in order to mediate systemic instigation.

Finally, we expressed hOPN in the noninstigating PC3 cells (PC3OPN), which normally secrete hOPN to negligible levels (Figures S5B and S6A), in order to determine whether hOPN expression might confer upon these cells an ability to systemically instigate responder growth. We identified a population of PC3OPN cells that secreted hOPN at levels comparable to those of the instigating 231 cells and demonstrated growth kinetics that were no different from the parental PC3 cells in vitro (Figures S6A–S6C). These PC3OPN cells were therefore implanted into host mice contralaterally to the responder cells. Although hOPN concentrations in the plasma of mice bearing the PC3OPN tumors were comparable to those of instigator-bearing mice (Figure S6D), and both PC3 parental and PC3OPN tumors grew with similar kinetics in vivo (Figure S6E; left), the PC3OPN tumors, like the PC3 parental tumors, did not instigate outgrowth of the contralateral responder cells (Figure S6E; right). Collectively, these results reveal that OPN secretion, while necessary, is not sufficient for systemic instigation.

Systemic Instigation of Metastatic Outgrowth

Taken together, these observations suggested that mechanisms of systemic instigation might also serve to facilitate the outgrowth of disseminated metastatic cells. To test this notion, we used a model of experimental metastasis to gauge the effects of systemic instigation on this process. Accordingly, we simultaneously injected GFP^- instigating BPLER tumor cells into subcutaneous sites and GFP^+ MDA-MB-231 cells (231+GFP) into the tail veins of mice (Figure 6A). These 231+GFP cells, which serve as responders in these experiments, are only weakly metastatic, and nearly 90% of the cells are cleared from the lungs within 1 day of intravenous injection (data not shown).

There was no significant difference between mice bearing subcutaneous instigating tumors and those bearing only Matrigel plugs when the numbers of 231+GFP micrometastatic lung foci were counted at days 2 and 16 following injections (Figure 6B). Furthermore, the numbers of lung metastases decreased between days 2 and 16 in both groups, even though instigating tumors had started to grow within this time period (Figure 6B). These results suggested that the presence of a growing instigating tumor at a distant site did not affect the initial survival or retention of the weakly metastatic cells in the lungs of host mice.

In stark contrast, at the 30 and 84 day time points, the numbers of 231+GFP foci in the lungs of mice bearing primary subcutaneous instigating tumors were elevated 3- and 9-fold, respectively, over the basal level of foci observed in lungs of control mice (Figures 6B, 6C, and S7A). Importantly, no instigating BPLER cells were observed in these instigated lung foci, indicating that the lung metastases were derived from the responding 231+GFP cells (Figures 6D and S7B). Moreover, we observed $Sca1^+$ cells intermingled with metastatic tumor cells only in the presence of subcutaneous instigating tumors (Figure S7C). These data indicate that primary instigating tumors had a profound systemic effect on the outgrowth of otherwise-weakly metastatic cells that were present in the lungs.

We next tested whether OPN was necessary for the effect of primary instigating tumors on the outgrowth of lung metastases in a model that mimics dissemination of metastatic cells from a primary tumor. Accordingly, we injected either Matrigel, the parental GFP^- 231 cells, or GFP^- 231-shOPN cells, into subcutaneous sites and, at the same time, injected the weakly metastatic 231+GFP cells into the tail vein of hosts. Lungs were examined 4 weeks later—a time prior to which the parental 231 cells are capable of metastasizing to the lungs from subcutaneous sites (data not shown).

As before, the presence of subcutaneous instigating tumors enhanced the numbers of 231+GFP lung micrometastases nearly 3-fold when compared to lungs of mice bearing only Matrigel plugs (Figure 6E). Importantly, subcutaneous OPN-deficient tumors lost their ability to enhance lung metastases (Figure 6E). Moreover, macrometastases were present in 100% of the lungs from mice bearing subcutaneous instigating tumors, while none of the mice bearing tumors deficient for OPN had macrometastatic lung tumors (Figure 6F). The average primary subcutaneous tumor burden was approximately equivalent in both groups of mice; these tumors were small (~40 mg) and represented ~0.16% of total body mass (Figure 6E). These data demonstrate that secretion of OPN is necessary for the ability of instigating tumors to systemically instigate outgrowth of distant responding tumor cells, independent of whether they are implanted into subcutaneous sites or have arrived in the lungs via the circulation.

Systemic Instigation of Human Colon Tumor Surgical Specimens

The present results demonstrate that actively growing tumors, even when relatively small, can exert systemic effects that are

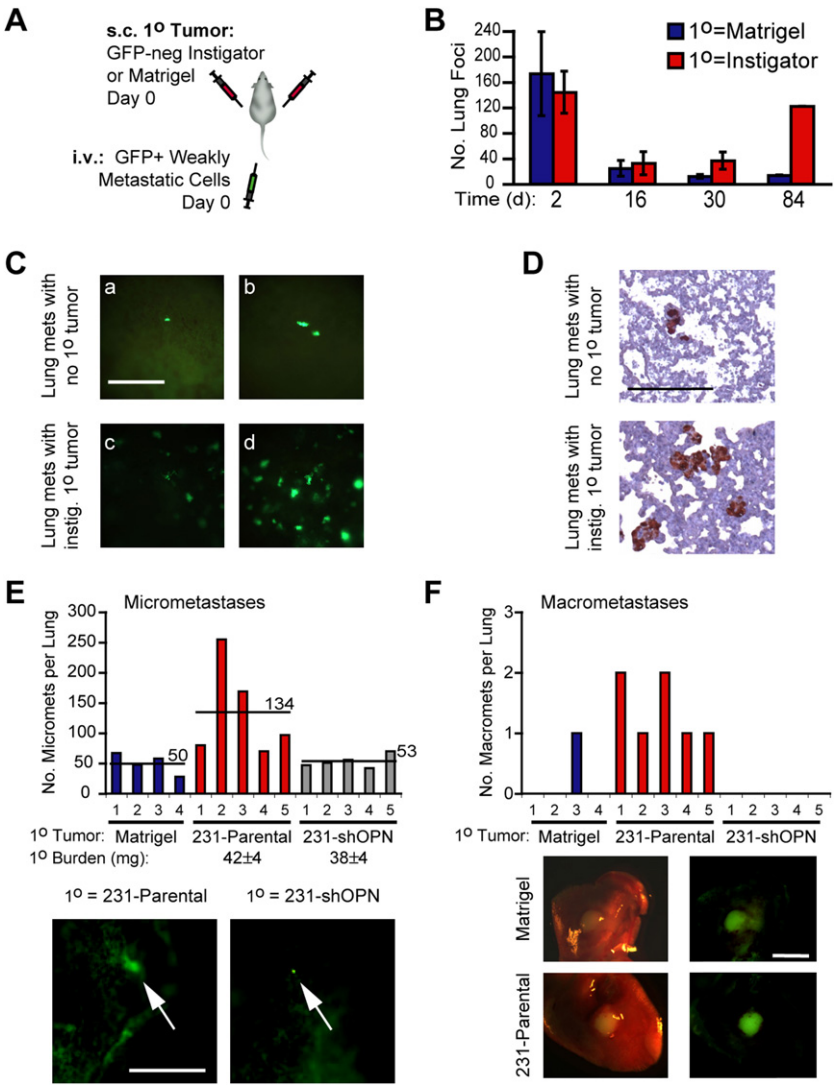


Figure 6. Instigating Tumors Instigate Outgrowth of Disseminated Lung Metastases

(A) Model of metastatic systemic instigation: GFP$^-$ instigating tumor cells (1° tumor) or Matrigel control are subcutaneously (s.c.) injected into both flanks of host mice while weakly metastatic GFP$^+$ tumor cells are injected intravenously (i.v.).

(B) Average numbers of micrometastatic lung foci after concurrent injection of weakly metastatic 231 cells i.v. and either instigating BPLER tumors or Matrigel control plugs s.c.; n = 4 mice per group.

(C) Whole-mount fluorescent photomicrographs of 231+GFP responder lung foci in mice bearing either Matrigel control plugs (a, b) or GFP$^-$ instigating 1° tumors (c, d); scale bar = 0.5 mm.

(D) Immunohistochemical staining of lung sections for GFP$^+$ responder cells (red); nuclei stain, blue; scale bar = 200 μm.

(E) For (E) and (F), mice received s.c. injections of either Matrigel, GFP$^-$ parental 231 cells or GFP$^-$ shOPN 231 cells and i.v. injection of weakly metastatic 231+GFP cells. Graph depicts numbers of micrometastatic 231+GFP lung foci from each mouse after 4 weeks; lines denote average number of foci per group; average 1° tumor burden is indicated. Whole-mount fluorescent photomicrographs depict micrometastatic foci (arrows); scale bar = 0.5 mm.

(F) Numbers of macrometastatic 231+GFP foci counted by eye in the lungs of each mouse. Whole-mount images of lungs are shown with corresponding fluorescent images; scale bar = 2 mm.

sufficient to induce distantly implanted, transformed cells to progress from an indolent state to one yielding vigorous tumor growth. We were therefore curious if systemic instigation could be used to facilitate the growth of human tumor surgical specimens. Some surgical samples of human tumors are capable of growing as xenografts after serial passage through SCID mice (Hylander et al., 2005; Naka et al., 2002), while the majority of human tumors samples are incapable of doing so. Accordingly, in order to select a human tumor sample that would serve as an indolent responder in our systemic instigation model, we first screened a number of surgical samples for their growth as xenografts in SCID mice.

In all of these experiments, we prepared ~2 mm fragments of tumors that were surgically removed from patients with colon cancer and implanted them beneath the skin of SCID mice (Figure S8A). After two serial passages through the mice, we identified a patient tumor xenograft that exhibited slow growth kinetics and yielded histopathology involving widespread necrosis (Figure S8B). This tumor sample was therefore designated as a candidate responder in our systemic instigation model. Consequently, 1–2 mm fragments of this human colon carcinoma xenograft were implanted subcutaneously into NOD-SCID mice contralaterally to either Matrigel or to instigating BPLER human mammary carcinoma cells.

Segments of the colon tumor that were implanted contralaterally to Matrigel were unable to grow during the 40 day experimental time period (Figure 7A; left). In striking contrast, all of the colon tumor segments (three of three) implanted contralaterally to the BPLER instigators displayed robust growth kinetics; growth of these responding colon tumor specimens was first observed 27 days after implantation and lagged the first growth of the contralateral instigators by only 15 days (Figure 7A; center). As a control, we determined that neither of the responder types—HMLER-HR responder or colon surgical sample responder—could instigate the growth of the other when implanted contralaterally (Figure 7A; right).

The responding colon tumors that grew opposite instigating BPLER tumors displayed histopathology consistent with that of adenocarcinomas (Figure 7B), and the associated neoplastic cells were actively proliferating (Figure 7C). As before, Sca1+ cells were observed in these tumor stromata and were localized near areas of proliferating tumor cells (Figure S8C).

These findings hold at least two implications. First, mammary tumors can act across tissue lineages to systemically instigate

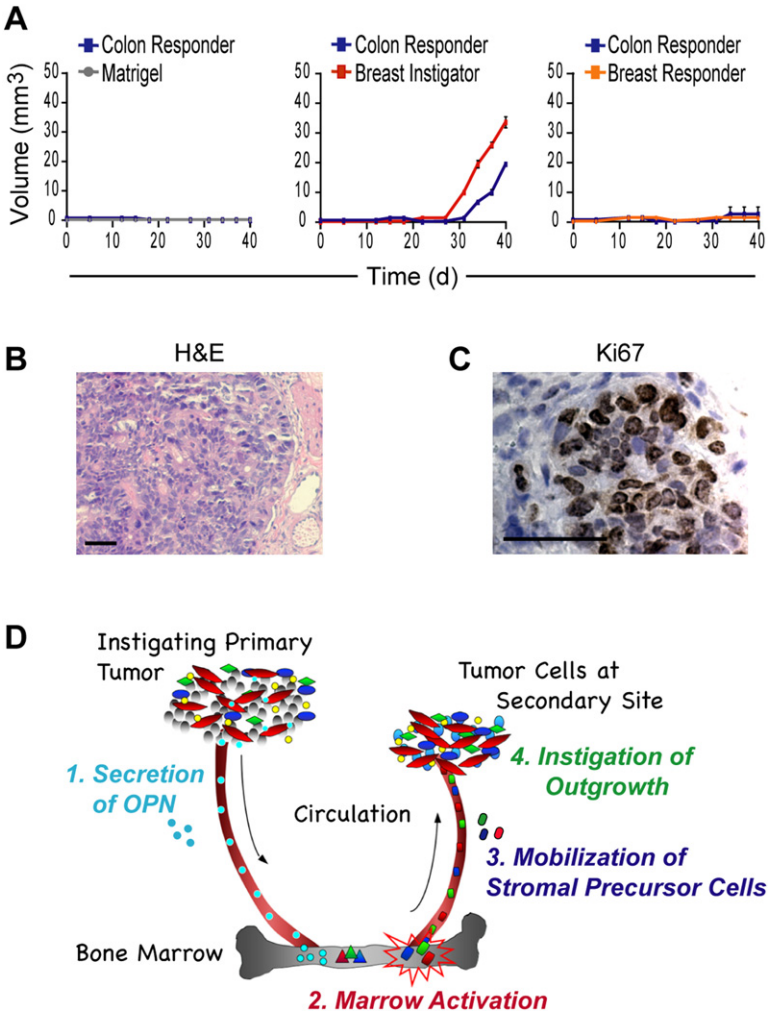


Figure 7. Human Colon Tumor Surgical Specimen Responds to Systemic Instigation

(A) The left shows human colon tumor segments ("colon responder"; blue line) dissected from a single patient's surgical specimen and implanted opposite Matrigel plugs (gray line). The center shows in vivo growth kinetics of colon responder tumor segments (blue line) implanted opposite instigating BPLER breast carcinoma cells (red line). The right shows that neither the human colon tumor segments (blue line) nor the HMLER-HR breast responder cells (orange line) are able to grow when implanted opposite one another. n = 3 mice per group.

(B) Hematoxylin and Eosin stain of a colon responder recovered opposite a breast instigator, scale bar = 50 μm.

(C) Staining for Ki67 (brown) to reveal proliferating responding colon tumor cells implanted opposite BPLER breast instigators, scale bar = 50 μm.

(D) Model of Systemic Instigation: Instigating tumors secrete osteopontin (OPN), which perturbs primitive hematopoietic cells in the host bone marrow; cells in the bone marrow are functionally activated prior to mobilization into the circulation; release of activated bone marrow-derived cells into the circulation and their subsequent incorporation into distant responding tumor stroma serve to foster outgrowth of the once-indolent cells into growing adenocarcinomas.

growth of colon tumors, indicating the generality of this physiologic signaling. Second, the presently described procedure, or derivatives thereof, can be used to study the growth of some human tumor specimens and tumor cell lines that might otherwise grow very slowly or not at all as xenografts in vivo.

DISCUSSION

The mechanisms of systemic instigation presented here operate in a fashion opposite to that occurring when primary tumors release antiangiogenic factors, which function to suppress metastatic outgrowth (Gohongi et al., 1999; O'Reilly et al., 1994, 1997). Moreover, systemic instigation is quite different from more recently reported processes by which primary murine tumors affected the host lung environment prior to the dissemination of metastatic tumor cells (Hiratsuka et al., 2002, 2006; Kaplan et al., 2005). The work presented here, instead, describes the ability of instigating tumors, even when relatively small (less than 0.08% of total body mass), to facilitate outgrowth of already-established, otherwise-indolent tumor cells located at distant sites (Figure 7D). At present, it is unclear which of these systemic signaling mechanisms is more likely to govern the behavior of commonly occurring human tumors, as remarkably little is known about the effects of primary tumors on their hosts and on derived metastases.

The relevance of OPN expression to human cancer pathogenesis has been revealed by studies showing that OPN levels are elevated in aggressive tumors types when compared with counterpart normal tissue or low-grade tumors, correlates with the presence of metastatic disease, and is included among lists of genes that predict poor prognosis in patients with various types of cancer (Graudens et al., 2006; Minn et al., 2005; Richardson et al., 2006; van de Vijver et al., 2002). Yet, other studies have shown that soluble OPN is detected at elevated levels in the blood of many cancer patients with metastatic disease (Ramankulov et al., 2007; Tuck et al., 2007). Despite these findings, the mechanism by which OPN supported metastasis was not clear.

It had been reported that OPN deficiency does not affect the growth of certain primary tumors, yet it significantly reduced their metastatic potential (Cook et al., 2005; Feng and Rittling, 2000; Wai et al., 2005). In these earlier reports, the ability of OPN to enhance cell motility and invasion in vitro was presented as one explanation for the role of OPN in furthering metastasis. Here, however, we identify an alternative, novel role for tumor-derived OPN in supporting distant tumor outgrowth: release of soluble OPN enables instigating tumors to communicate with and perturb the bone marrow. Indeed, BMCs from hosts bearing OPN-deficient tumors, unlike their OPN-secreting counterparts, were not functionally activated to support tumor growth when admixed with responder cells, indicating that instigator-derived circulating OPN is acting on the bone marrow rather than directly

on the distantly located responder cells. Our observations that forced hOPN expression was not sufficient to convert non-instigators into instigators suggest that other, still-unidentified instigator-specific factors cooperate with hOPN to promote systemic instigation. Nonetheless, the fact that hOPN is necessary for all of the currently identified aspects of systemic instigation supports the notion that OPN signaling is an attractive therapeutic target (Rittling and Chambers, 2004).

Other studies first alerted us to the possibility that OPN signaling might mediate at least part of the systemic instigation mechanism. Such studies demonstrated that OPN signaling regulates the fate of primitive hematopoietic cells in vitro and perhaps more importantly, that *OPN*$^{-/-}$ mice have significantly elevated levels of LSK cells in their bone marrow (Iwata et al., 2004; Nilsson et al., 2005; Stier et al., 2005). While our results generally concur with these studies, our observations do not reveal whether the significant incorporation of Sca1^{+}/cKit^{-} bone marrow-derived cells into the instigated tumor stroma is directly related to the reduction in marrow LSK cell numbers that we observed. In fact, little is known about Sca1^{+}/cKit^{-} cells; they have been referred to as a "mystery population" in the bone marrow (Randall and Weissman, 1998). In any event, our results provide evidence that bone marrow functional activation can be governed on a systemic level by endocrine factors that are released by certain instigating tumors. These results therefore move beyond existing studies, which largely address bone marrow cell recruitment to primary tumors.

The ability of instigating tumors to foster the growth of a human colon tumor surgical specimen underscores the powers of systemic instigation. Indeed, to our knowledge, methods to expedite the growth of human tumor surgical specimens in vivo have not been previously described. These results suggest that the presently described procedure can be used to study aspects of human tumor biology that would otherwise be difficult if not impossible to study. In the longer term, identification of additional tumor-derived factors that perturb the host systemic environment in one way or another may allow one to predict the effects that a given primary tumor type has on the outgrowth of indolent cancer cells that have disseminated to distant sites.

EXPERIMENTAL PROCEDURES

Cell Lines

Generation of HMLER hygro-H-*ras*V12 and BPLER human mammary epithelial tumor cells has been described (Elenbaas et al., 2001; Hahn et al., 1999; Ince et al., 2007). EpH4Ras murine mammary carcinoma cells were a gift from E. Reichmann (Oft et al., 1996). Human mammary carcinoma MDA-MB-231, human prostate carcinoma PC3, and murine Lewis lung carcinoma LLC were obtained from the ATCC (Manassas, VA) and cultured under standard conditions.

Animals and Tumor Xenografts

Female Nude mice were purchased from Taconic (Hudson, NY); a colony of NOD-SCID mice was maintained in-house. All experiments were performed in accordance with regulations of MIT Committee on Animal Care protocol (1005-076-08). Tumor cells were injected subcutaneously into nonirradiated mice (see Supplemental Data) and tumor diameter was measured on the flanks of live Nude mice using calipers; volume was calculated as $4/3\Pi r^3$.

Bone Marrow Harvest and Transplantation

BMCs were harvested from donor mice by flushing femurs with sterile Hanks' balanced salt solution (HBBS; GIBCO) with penicillin/streptomycin/fungisone. Cells were washed 2× with sterile HBBS, dissociated with 18 g needles, and filtered through 70 μm nylon mesh. For transplantation experiments, 2 × 10^6 BMCs from *Rag1*$^{-/-}$*xEGFPTg* donor mice were injected into the retroorbital sinus 8–10 hr after irradiation of recipient mice (600 rads for Nude mice; 350 rads for NOD-SCID mice). Antibiotics were added to drinking water for 14 days following the procedure. At the end of each experiment, recipient mice were anesthetized by i.p. injection of Avertin, and vasculature was exsanguinated by perfusion of sterile PBS through the left ventricle.

Immunohistochemistry

Dissected tissues were fixed in 4% (WT/vol) paraformaldehyde 16–18 hr, embedded in paraffin, and sectioned onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA) for immunohistochemistry using Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) as described (Sendobry et al., 1997) (see Supplemental Data).

Flow Cytometric Analysis

Freshly harvested tissues were digested in 1 mg/ml collagenase A for 1–4 hr at 37°C with continuous rotation. Resulting cell suspensions were dispersed with an 18 g needle, washed 2× with resuspension buffer (2% heat-inactivated fetal calf serum in sterile HBBS) and filtered through 70 μm nylon mesh. Tissue cells or BMCs were labeled for flow cytometry by incubation with appropriate antibodies for 30 min, 1 hr at 4°C with continuous rotation. Antibodies are PE-anti-Ly-6A/E/Sca-1 clone E13-161.7 (400 ng/10^6 cells), APC-anti-CD117/c-Kit 2B8 (400 ng/10^6 cells), and mouse lineage panel kit (BD PharMingen).

ELISA

Mouse plasma was prepared by centrifugation of whole blood collected into EDTA Microtainer tubes (BD PharMingen). Human OPN levels were measured using species-specific TiterZyme EIA kits (Assay Designs) according to manufacturer's instructions.

OPN shRNA Plasmids

Five sequence-verified shRNA clones specific to human OPN were provided in the pLKO.1-Puro lentivirus expression plasmid (Mission shRNA; Sigma, St. Louis, MO). Infection and selection of target cells was performed as described (Stewart et al., 2003). Only hairpins that suppressed OPN expression without altering cell growth or morphology were used in subsequent experiments (see Supplemental Data).

Analysis of Lung Metastases

Entire lungs were harvested and placed directly into sterile HBS, and GFP^{+} surface foci were counted under a dissecting microscope with fluorescence capability. Discreet GFP^{+} foci that were visible under 4× magnification were counted as micrometastases; GFP^{+} foci that were visible by naked eye were counted as macrometastases.

Human Colon Tumor Specimens

Fresh surgical specimens of patient tumors were obtained with patient consent from the Tissue Procurement Facility at Roswell Park Cancer Institute shortly after resection and applied to the SCID mouse xenograft model as described (Hylander et al., 2005; Naka et al., 2002) (Supplemental Data). The tumor specimen that was used as a responder in the bilateral instigation system was quickly thawed at 37°C, washed 2× with RPMI, cut into 1–2 mm segments and surgically implanted beneath the skin of NOD-SCID mice.

Statistical Analysis

Data are expressed as mean ± SEM. Data were analyzed by Student's t test and were considered statistically significant if $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include eight figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/133/6/994/DC1/.

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