Bone Marrow–Derived Gr1+ Cells Can Generate a Metastasis-Resistant Microenvironment Via Induced Secretion of Thrombospondin-1

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ABSTRACT
Metastatic tumors have been shown to establish permissive microenvironments for metastases via recruitment of bone marrow-derived cells. Here, we show that metastasis-incompetent tumors are also capable of generating such microenvironments. However, in these situations, the otherwise prometastatic Gr1+ myeloid cells create a metastasis-refractory microenvironment via the induction of thrombospondin-1 (Tsp-1) by tumor-secreted prosaposin. Bone marrow–specific genetic deletion of Tsp-1 abolished the inhibition of metastasis, which was restored by bone marrow transplant from Tsp-1+ donors. We also developed a 5-amino acid peptide from prosaposin as a pharmacologic inducer of Tsp-1 in Gr1+ bone marrow cells, which dramatically suppressed metastasis. These results provide mechanistic insights into why certain tumors are deficient in metastatic potential and implicate recruited Gr1+ myeloid cells as the main source of Tsp-1. The results underscore the plasticity of Gr1+ cells, which, depending on the context, promote or inhibit metastasis, and suggest that the peptide could be a potential therapeutic agent against metastatic cancer.

SIGNIFICANCE: The mechanisms of metastasis suppression are poorly understood. Here, we have identified a novel mechanism whereby metastasis-incompetent tumors generate metastasis-suppressive microenvironments in distant organs by inducing Tsp-1 expression in the bone marrow–derived Gr1+ myeloid cells. A 5-amino acid peptide with Tsp-1–inducing activity was identified as a therapeutic agent against metastatic cancer. Cancer Discov; 3(5); 578–89. ©2013 AACR.

INTRODUCTION
The vast majority of cancer-related deaths are caused by organ failure brought about by metastatic dissemination of tumor cells. At the metastatic site, the disseminated tumor cells proliferate and induce angiogenesis to allow further tumor growth to form lethal macrometastases (1–3). However, despite our increased understanding of the physiologic processes involved in tumor metastasis, no clinically approved drugs have shown significant efficacy at treating advanced, metastatic cancer. As early as 1889, Stephen Paget set forth his “seed” and “soil” hypothesis establishing the concept that breast cancer metastasizes to specific organs that harbor a receptive microenvironment (4). Experimental support for this hypothesis has been provided by the demonstration that primary tumors release specific cytokines, such as VEGF, stromal cell–derived factor 1 (SDF-1), TGF-β, and TNF-α, which systemically initiate premetastatic niches, characterized by the accumulation of bone marrow–derived cells such as VEGF receptor (VEGFR)1+ hematopoietic cells and CD11b+ myeloid cells (5). Furthermore, these premetastatic niches are characterized by the selective induction of organ-specific chemoattractants, growth factors, and extracellular matrix–related proteins, including fibronectin, lysyl oxidase, and S100A8 (6–9). Although these studies have provided key insights into metastasis-promoting niches, the existence of niches that may confer metastasis suppression, has not been examined.

In this study, we show that tumors that are deficient in metastatic potential are as capable of recruiting bone marrow–derived myeloid cells to potential metastatic organs as highly metastatic tumors. However, metastasis-incompetent tumors systemically stimulate expression of the antimetastatic factors Tsp-1 in the recruited CD11b+Gr1+ cells, converting these prometastatic cells into metastasis-inhibitory cells that are refractory to the outgrowth of metastatic tumor cells. As such, our study provides a novel insight into the exquisite functional plasticity of the Gr1+ cells previously shown to enhance carcinogenesis (10–12). Moreover, we describe the development of a novel peptide that stimulates Tsp-1 in Gr1+ cells and blunts metastasis when administered systemically.

RESULTS
Poorly Metastatic Tumors Recruit Bone Marrow–Derived Cells to the Premetastatic Microenvironment in the Lungs
It has been shown that metastatic tumors are able to induce the recruitment of bone marrow–derived cells to future sites of metastasis, creating a permissive microenvironment for colonization (5). However, no analysis has determined whether this process is impaired in metastasis-incompetent tumors or if...
these tumors create metastasis-refractory microenvironments. To determine whether tumors that lack robust metastatic capabilities are able to generate metastasis-suppressive niches in distant organs, we examined human prostate and breast cancer models that exhibit varying grades of metastatic potential (13, 14). These included weakly metastatic parental cells, PC3 or MDA-MB-231, and their highly metastatic variants, PC3M-LN4 (LN4) and MDA-MB-LM2 (LM2), respectively. To mimic the paracrine and endocrine effects of tumor-secreted factors on the premetastatic niche in the lung microenvironment in vivo, we administered conditioned media (CM) prepared from either the prostate or breast cancer cells to mice. To visualize the bone marrow–derived metastatic microenvironment, we conducted bone marrow transplantation (BMT) of wild-type (WT) mice with syngeneic GFP+ bone marrow (2, 15). The CM from metastatic LN4 cells was able to efficiently increase the recruitment and formation of a GFP+ bone marrow–derived premetastatic microenvironment, as would have been predicted (Fig. IA and B). Strikingly, the CM from weakly metastatic PC3 tumor cells was also able to generate these microenvironments with similar efficiency (Fig. IA and B). Further analysis revealed that the GFP+ bone marrow cells recruited in both cases were predominantly CD11b+ myeloid cells (Fig. 1A–C, Supplementary Figs. S1A and S1B).

Poorly Metastatic Tumors Induce Tsp-1 Expression in the Bone Marrow–Derived Cells in the Premetastatic Microenvironment

Having observed no differences in the cellular composition of premetastatic lungs generated by metastasis-competent and incompetent tumors, we postulated that the bone marrow–derived cells recruited by the different tumor types might exhibit molecular differences that would provide insights into metastasis-resistant microenvironments. As such, we conducted gene expression analysis of lungs of PC3 and LN4 CM-treated mice (Supplementary Fig. S2). Among the genes differentially regulated by PC3 CM, we focused on those encoding secreted proteins that are most likely to reprogram the local microenvironment and exert a negative impact on metastatic outgrowth of tumor cells. Among candidate genes, thrombospondin-1 (Tsp-1) was selected for further analysis, as it encodes a secreted...
Tumor Instigated Bone Marrow–Derived Metastasis-Resistant Microenvironment

**Figure 2.** Tsp-1 expression is mainly confined to bone marrow-derived Gr1+ cells. A, quantitative RT-PCR analysis of Tsp-1 expression in different subsets of flow sorted CD45+ bone marrow hematopoietic cells isolated from the lung. Of these, the Gr1− cells showed elevated Tsp-1 compared with Gr1+ cells or other CD45− cells. Total lung, all cells from the lung. B, Western blot analysis of Tsp-1 levels in cellular hematopoietic compartments in the peripheral blood from WT mice and Tsp-1−/− mice show Gr1+ cells as a major source of Tsp-1. WBC, white blood cells; PRP, platelet-rich plasma. C, immunofluorescence staining of lungs showing expression of Tsp-1 by Gr1+ cells. The boxed area highlights Tsp-1–expressing Gr1+ cells depicted by arrows. D, quantitative RT-PCR analysis of Tsp-1 expression in different subsets of sorted bone marrow (BM)-hematopoietic cells from the lung. Both subsets of Gr1− cells, Ly6C+ and Ly6G− cells express Tsp-1. E, Western blot analysis of Tsp-1 levels in lungs of mice after bone marrow transplantation. Transplantation of Tsp-1−/− bone marrow (BM) to WT recipients (Tsp-1 knockout [KO] BM > WT) rendered lungs devoid of Tsp-1. Conversely, transplantation of WT BM to Tsp-1−/− recipients (WT BM > Tsp-1 KO) restored Tsp-1 expression in the lung. WT BM > WT. Transplantation of WT BM into WT recipients. Tsp-1 KO BM > Tsp1 KO; transplantation of Tsp-1−/− BM into Tsp-1−/− recipients.

extracellular matrix protein and one of the most potent inhibitors of tumor angiogenesis and growth (16, 17). Both cell count and real-time (RT)-PCR analysis confirmed that Tsp-1 was upregulated in the lungs of PC3 CM-treated mice compared with controls or LN4 CM-treated mice (Fig. 1D and E).

**Bone Marrow–Derived CD11b+Gr1+ Myeloid Cells Are the Major Contributors of Tsp-1**

We next conducted an immunostaining analysis to determine the source of Tsp-1 in the lung stroma and observed that Tsp-1 expression was mainly confined to the bone marrow–derived CD11b+ cells recruited in the lungs of mice treated with PC3 CM (Fig. 1A, Supplementary Fig. S1A). Conversely, we observed no discernible induction of Tsp-1 expression in the lungs of mice treated with LN4 CM (Fig. 1A). To further assess which bone marrow cell populations expressed Tsp-1, we analyzed specific flow-sorted bone marrow cell populations from the lungs by RT-PCR (Fig. 2A) and observed that Tsp-1 expression was largely confined to Gr1+ myeloid cells and not the Gr1− myelocytes or other hematopoietic cells (Fig. 2A). To further exclude the possibility that other cell types might also express Tsp-1, we conducted a Western blot analysis of different cellular components from peripheral blood harvested from WT mice or Tsp-1 knockout (KO) (Tsp-1−/−) mice. Notably, the Gr1+ cells expressed abundant Tsp-1 compared with Gr1− cells (Fig. 2B; Supplementary Fig. S3A). As expected, there was a complete lack of Tsp-1 in all compartments examined in Tsp-1−/− mice (Fig. 2B).

Further analysis of the peripheral blood showed that Gr1+ cells express predominantly the higher-molecular-weight isoforms of Tsp-1 (160–170 kDa), whereas the platelets express a lower-molecular-weight isoform (135–140 kDa; Fig. 2B, right). Indeed, both forms have been observed previously in platelets (18). Importantly, Gr1+ cells expressed higher levels of Tsp-1 compared with the platelets. Taken together, these data indicate that the bone marrow–derived Gr1+ cells are...
the major source of Tsp-1 in the premetastatic microenvironment generated by weakly metastatic tumors.

Given that immunostaining analysis confirmed that Tsp-1 expression was confined to Gr1$^+$ cells (Fig. 2C) and the Gr1$^+$ population is composed of 2 major subpopulations; Ly6G$^+$ granulocytic myeloid cells and Ly6C$^{high}$ myelomonocytic cells, we further analyzed these Gr1$^+$ subpopulations (Supplementary Fig. S3B). Our analyses revealed that both subsets of Gr1$^+$ cells expressed Tsp-1 (Fig. 2D). To conclusively determine that the chief source of Tsp-1 expression in the lungs was the Gr1$^+$ bone marrow cells and not other lung stromal cells, we conducted BMT experiments. We observed Tsp-1 expression in the lungs of Tip$^{1^-/-}$ mice that were transplanted with WT bone marrow but not in WT mice transplanted with Tip$^{1^-/-}$ bone marrow (Fig. 2E).

To exclude the possibility that these findings were somehow unique to the prostate cancer model, we examined a breast cancer model as well. As described above, we used the weakly metastatic parental cells, MDA-MB-231 (MDA), and their highly metastatic variants, MDA-MB-LM2 (LM2). Consistent with the observations in the prostate cancer model, CM from weakly metastatic MDA breast cancer cells also induced upregulation of Tsp-1 expression in bone marrow–recruited Gr1$^+$ cells as compared with CM from highly metastatic LM2 breast cancer cells (Supplementary Fig. S4). These results, when taken together, strongly indicate that bone marrow–derived cells are the exclusive source of Tsp-1 induced by weakly metastatic tumors in the lungs.

Genetic Deletion of Tsp-1 in the Bone Marrow Results in Increased Metastasis

We next postulated that expression of Tsp-1 in recruited Gr1$^+$ myeloid cells in the lungs could generate a metastasis-refractory environment. To test the significance of bone marrow–derived Tsp-1 in modulating metastasis, we generated Tsp-1 deficiency in the bone marrow. Specifically, we harvested bone marrow cells from Tip$^{1^-/-}$ mice and transplanted these cells into irradiated syngeneic WT mice to generate a cohort of Tip$^{1^-/-}$ BMT mice. As controls, bone marrow from WT mice was transplanted into irradiated WT mice to generate a cohort of WT BMT control mice. As expected of a successful BMT and engraftment of recipient mice with donor bone marrow, Western blot and RT-PCR analysis of peripheral blood leukocytes from Tip$^{1^-/-}$ BMT mice showed undetectable Tsp-1 protein levels and low mRNA levels compared with WT BMT controls (Supplementary Figs. SSA and SSB).

We then sought to determine the impact on metastasis of bone marrow–derived Tsp-1 in the lung microenvironment. As such, we examined the ability of Lewis lung carcinoma cells (LLC) to colonize the lungs of mice that received a bone marrow transfer from WT mice or Tip$^{1^-/-}$ mice. Specifically, we injected LLC cells stably expressing the firefly luciferase gene (LLC-luc) (2, 14) via tail vein and monitored lung metastases. Strikingly, we observed accelerated metastases in Tip$^{1^-/-}$ BMT mice compared with WT BMT mice (Fig. 3A and B). Western blot analysis of the total lung lysates showed complete absence of Tsp-1 in the lungs of Tip$^{1^-/-}$ BMT mice (Fig. 3C), indicating that in a metastatic setting the bone marrow–derived cells are the main source of Tsp-1 in the lung parenchyma. Moreover, as expected, in Tip$^{1^-/-}$ BMT mice, the Gr1$^+$ cells were devoid of Tsp-1 expression (Supplementary Fig. S6). Importantly, Tsp-1 deficiency in the bone marrow cells in Tip$^{1^-/-}$ BMT mice did not perturb the recruitment of Gr1$^+$ cells in the lung microenvironment compared with WT BMT mice (Supplementary Fig. S6), nor did the lack of Tsp-1 perturb the biogenesis of other bone marrow hematopoietic cells (Supplementary Fig. SSC). To exclude the possibility that enhanced metastasis was due to differential tumor cell seeding in the lung parenchyma of Tip$^{1^-/-}$ BMT mice, we conducted a seeding experiment. We found, via a time course evaluation of the lungs following administration of tumor cells, that seeding efficiency was comparable in the lungs of BMT mice regardless of the donor source (Supplementary Fig. S7).

To provide further evidence that the observed antimetastatic effect is due to Tsp-1 production in Gr1$^+$ cells, we conducted a reverse BMT experiment. Specifically, we transplanted bone marrow from WT mice into Tip$^{1^-/-}$ mice. As a control, we transplanted bone marrow from Tip$^{1^-/-}$ donors into Tip$^{1^-/-}$ mice to account for any possible effects of the transplantation procedure. Strikingly, transplantation of WT bone marrow into Tip$^{1^-/-}$ mice resulted in complete suppression of metastasis (Fig. 3D and E). This phenomenon was most likely due to the dramatically increased expression of Tsp-1 in the lungs of these mice compared with WT mice transplanted with WT bone marrow (Fig. 3F; Supplementary Fig. S8). Consistent with previous observations, Tsp-1 expression was restored in the Gr1$^+$ cells in Tip$^{1^-/-}$ mice transplanted with WT bone marrow (Supplementary Fig. S8). These findings, support the hypothesis that supraphysiologic induction of Tsp-1 in Gr1$^+$ cells can blunt metastasis as well as the possibility that a therapeutic agent with such activity could be effective at treating advanced metastatic cancer.

Bone Marrow–Derived Tsp-1 Inhibits Metastatic Outgrowth through Inhibition of Tumor Cell Proliferation

The antitumor activity of Tsp-1 has been shown to be involved in tumor growth blockade through diverse mechanisms: angiogenesis inhibition, blockade of cell proliferation, and induction of apoptosis both in tumor cells and endothelial cells (19, 20). Consistent with these functions, the metastatic lesions in the lungs of Tip$^{1^-/-}$ BMT mice showed elevated proliferation of tumor cells compared with control WT BMT mice ($P = 0.002$; Fig. 3G, Supplementary Fig. S9), whereas the tumor cell apoptotic index remained unperturbed in the lungs of Tip$^{1^-/-}$ BMT mice compared with control WT BMT mice, ($P = 0.09$, Fig. 3H). Taken together, these results show that Tsp-1 in recruited bone marrow–derived Gr1$^+$ cells did not affect metastatic seeding of tumor cells or their ability to stimulate angiogenesis, but impaired tumor outgrowth at the metastatic site by inhibiting tumor cell proliferation.

Stimulation of Tsp-1 in the Lung Microenvironment as a Potential Antimetastatic Approach

We next speculated about whether administration of Tsp-1 would constitute a potential antimetastatic strategy, as it has been postulated that Tsp-1 could be a potent clinical inhibitor of tumor progression and metastasis (16, 21, 22). However, the translational relevance of this approach has been
minimized by the fact that Tsp-1 is an extremely large protein that is not feasible to use as a therapeutic agent (23). Indeed, a mimetic nonapeptide derived from the type I repeat of Tsp-1, which contains the antiangiogenic activity of the endogenous protein, has been developed. However, in clinical trials, this drug showed little efficacy due, in part, to its inability to fully replicate the activity of the full-length protein (24, 25). We reasoned that to effectively use Tsp-1 as a therapeutic agent, it would be necessary to induce the expression of full-length Tsp-1, containing all of its inhibitory activity, in the premetastatic lung, particularly in the bone marrow cells. We therefore turned our attention to prosaposin (Psap), a precursor of sphingolipid activator proteins, which was previously shown to stimulate the expression of Tsp-1 in human lung fibroblasts in vitro (14). Indeed, breast cancer CM from MDA cells stimulated Tsp-1 in the Gr1+ cells in the lungs, as expected. However, CM from MDA cells expressing Psap-short hairpin RNA (shRNA) failed to induce Tsp-1 expression in the Gr1+ bone marrow–derived Gr1+ cells confer a metastasis-suppressive phenotype through the secretion of Tsp-1. A, quantitation of pulmonary metastases in WT BMT mice and Tsp-1−/− BMT mice (n = 7 per group). BLI estimates were obtained at days 0, 8, 11, 14, and 19 following tail vein injections of 1 × 10⁶ LLC cells. P = 0.0013 between WT BMT and Tsp-1−/− BMT groups at day 19. B, representative BLI images showing metastasis suppression in WT BMT mice compared with Tsp-1−/− BMT mice depicted in A. C, Western blot analysis of Tsp-1 from the lungs of WT BMT mice and Tsp-1−/− BMT mice. Lung from whole-body Tsp-1−/− mice was used as a control. D, quantitation of pulmonary metastases in Tsp-1−/− mice transplanted with WT bone marrow and Tsp-1−/− mice transplanted with Tsp-1−/− bone marrow (n = 5 per group). BLI estimates were obtained following tail vein injections of 1 × 10⁶ LLC cells. P = 0.033 between the 2 groups at day 30. E, representative BLI images showing metastasis suppression in Tsp-1−/− mice transplanted with WT bone marrow compared with Tsp-1−/− mice transplanted with Tsp-1−/− bone marrow depicted in D. F, quantitation of Tsp-1 protein expression in the lungs of mice following BMT. G, left, representative immunofluorescence images showing Ki67+ cells in the metastatic lungs of WT BMT mice and Tsp-1−/− BMT mice. Right, quantitation of Ki67+ cells. Sections were evaluated from 6 animals per group. H, left, representative immunofluorescence images showing active caspase-3+ cells in the metastatic lungs of WT BMT mice and Tsp-1−/− BMT (P = 0.0903). Right, quantitation of active caspase-3+ cells. Sections were evaluated from 6 animals per group. White dotted line in G and H is the demarcation of the tumor/normal tissue.
cells (Supplementary Fig. S10A and S10B). We also examined the prostate cancer model, and as expected, Western blot analysis showed elevated Tsp-1 levels in the lungs of PC3 CM-treated mice compared with LN4 CM treatment (Supplementary Fig. S10C). However, CM from isogenic PC3 cells in which Psap expression was silenced by shRNA (PC3-shPsap) failed to enhance Tsp-1 levels in the lungs (Supplementary Fig. S10C).

We also examined whether overexpression of Psap in metastatic prostate cells from the orthotopic site would stimulate Tsp-1 in the lungs. Orthotopic prostate tumors were generated by injecting either metastatic LN4 cells or LN4 cells overexpressing Psap (LN4-Psap) in the prostate gland of mice. As expected, LN4-Psap tumors induced robust Tsp-1 expression in the premetastatic lungs compared with LN4 tumors (Supplementary Fig. S10D). Taken together, these results suggest that nonmetastatic tumor cells, by virtue of expressing Psap, systemically elevate Tsp-1 in the recruited bone marrow–derived Gr1+ cells in an endocrine fashion.

We next postulated that Psap might directly enhance the expression of Tsp-1 in recruited Gr1+ myeloid cells in the lungs to generate a metastasis-refractory environment. To determine whether the ability of Psap to inhibit metastasis via stimulation of Tsp-1 could be translated into a smaller, more easily deliverable agent, we sought to determine the minimal active region of the Psap protein. We first generated Psap truncation mutants, which contain saposin A, saposin AB, saposin ABC, and saposin BCD (Supplementary Fig. S11A) and ectopically expressed these mutants in LN4 cells. We then analyzed CM from these cells to determine which version harbored Tsp-1–stimulating activity. All truncation mutants except saposin BCD were able to stimulate Tsp-1 activity to the same level as the full-length protein (Supplementary Fig. S11A). Thus, we concluded that saposin A contains the Tsp-1–stimulating activity of Psap. We next sought to determine whether there was a smaller domain within saposin A that contained the Tsp-1–stimulating activity. Accordingly, we generated seven 20-amino acid overlapping peptides spanning the 81-amino acid sequence of saposin A (Fig. 4A). We observed that a 20-amino acid peptide spanning residues 31–50 of saposin A was the only peptide capable of stimulating the expression of Tsp-1 (Fig. 4B). We then examined the crystal structure of saposin A and determined that residues 31–50 reside in the hairpin region between helices 2 and 3 (Supplementary Fig. S11B; ref. 26). Accordingly, we tested a cyclic 13-amino acid peptide, corresponding to residues 35–47, constrained by a disulfide bond between cysteine residues at each end of the
peptide. The cyclic peptide was able to stimulate Tsp-1 (Fig. 4C). We then tested a 6-amino acid peptide, CDWLPK, corresponding to the first 6 residues of the cyclic peptide and found that it also was able to stimulate Tsp-1 (Fig. 4D). Finally, we tested 6 peptides from within the 6-amino acid sequence of CDWLPK. Of these peptides, we found that both a 5-amino acid peptide (DWLPK) and a 4-amino acid peptide (DWLP) were able to stimulate Tsp-1 expression to an even greater extent than CDWLPK (Fig. 4D). We then tested the 5-amino acid peptide in the in vivo assay by administering it to mice in combination with CM from LN4 cells and observed that it was able to stimulate Tsp-1 in the lungs of these mice (Fig. 4E).

Having determined that the 5-amino acid DWLPK peptide derived from Psap retains the Tsp-1–stimulating activity of the full-length Psap both in vitro and in vivo, we sought to determine whether it also targeted bone marrow–derived cells. As such, we treated mice with CM from LN4 cells alone to simulate a systemic tumor-induced recruitment of bone marrow–derived stromal cells in the lungs, or CM in combination with DWLPK peptide. As expected, the recruitment of Gr1+ cells was identical regardless of the treatment (Supplementary Fig. S12A). However, administration of the DWLPK peptide stimulated Tsp-1 expression in the Gr1+ cells by more than 2-fold, whereas a peptide composed of the same amino acids in a scrambled sequence failed to stimulate Tsp-1 (Fig. 4F, Supplementary Fig. S12B). The induction of Tsp-1 occurred systemically, as shown by Western blot analysis of peripheral leukocytes from peptide-treated mice (Supplementary Fig. 12C).

These results suggest that the Tsp-1–enhancing activity of Psap is confined to a 5-amino acid peptide and that administration of this peptide could create a Tsp-1–mediated metastasis-suppressive microenvironment.

Psap Mimetic Peptide (DWLPK) Inhibits Metastases through Stimulation of Tsp-1 in the Gr1+ Cells

To determine whether Psap peptide-mediated stimulation of Tsp-1 by bone marrow Gr1+ cells in the lung parenchyma contributes to a metastasis-resistant niche, we administrated LLC-luc cells via tail vein into WT mice treated with either DWLPK peptide or scrambled control. Strikingly, administration of the DWLPK peptide dramatically reduced lung metastases compared with the scrambled peptide, as measured by bioluminescence imaging (BLI; Fig. 5A and B). Notably, metastatic lungs from mice (day 30) treated with DWLPK peptide showed persistent Tsp-1 upregulation in the Gr1+ myeloid compartment compared with treatment with scrambled peptide (Fig. 5C, Supplementary Fig. S12B). In agreement with previous observations, immunostaining and RT-PCR analysis showed that the Tsp-1 expression was confined to the Gr1+ cells and not to the Gr1− stromal cells, which included F4/80+ macrophages (Fig. 5C, Supplementary Fig. S13).

To confirm that the DWLPK peptide-induced Tsp-1 expression in the bone marrow cells was directly responsible for the antitumorigenic phenotype, we conducted genetic deletion of Tsp-1 in the bone marrow by generating Tsp-1−/− BMT mice and used WT BMT mice as controls. Strikingly, the DWLPK peptide failed to inhibit metastases in Tsp-1−/− BMT mice, whereas it potently inhibited lung metastases in WT BMT mice (Fig. 5D and E). Histologic examination of the lungs showed increased size of nodules (P = 0.0317), as well as a higher percentage of macrometastases (>1 mm of diameter) in Tsp-1−/− BMT mice compared with control WT BMT mice treated with the Psap peptide (Fig. 5F and H).

Finally, we sought to determine whether our observations with the experimental metastasis models could be recapitulated with tumors generated in the orthotopic site. As such, we tested the ability of the DWLPK peptide to inhibit metastasis in an orthotopic, clinically relevant model of breast cancer metastasis to the lung. Metastatic MDA-MB-LM2 breast cancer cells expressing luciferase were injected orthotopically into mammary glands of SCID mice. Metastatic burden in the lungs was significantly reduced by 50% compared with scrambled peptide-treated mice (Fig. 6B). Consistent with the mechanism of action of the peptide, Tsp-1 levels were elevated in the lungs after systemic DWLPK administration, as shown by Western blot and immunofluorescence in lung extracts of these mice (Fig. 6C and D). Taken together, these results indicate that the 5-amino acid DWLPK Psap peptide could have significant efficacy in treating metastatic cancer via induction of Tsp-1 in bone marrow–derived cells in the lung microenvironment.

DISCUSSION

In this study, we have shown that metastasis can be acutely affected by the tumor-induced systemic reprogramming of the bone marrow–derived cells in the lung microenvironment. Strikingly, we observed that the very same bone marrow–derived myeloid cells that have previously been shown to promote metastasis can also be induced to inhibit metastasis by the endocrine activity of metastasis-incompetent primary tumors. Research in metastasis suppression has been generally focused on cancer cell autonomous properties, and suppressors of metastatic tumor outgrowth, such as KISS1, MKK4, p38, and MKK7, have been identified (27, 28). Here, we have identified a novel mechanism whereby primary tumors systematically modulate the microenvironment in distal target organs to inhibit metastasis (Fig. 7A). Significantly, we show that tumors that are deficient in metastatic potential are as capable of recruiting bone marrow–derived CD11b+Gr1+ hematopoietic cells to potential metastatic organs as highly metastatic tumors. However, metastasis-incompetent tumors systemically stimulate expression of the antitumorigenic factor Tsp-1 in the recruited bone marrow cells, converting these cells, which would otherwise serve to stimulate metastasis, into metastasis-inhibitory cells. The Tsp-1–expressing bone marrow–derived cells thus establish a microenvironment in the potential metastatic site that is refractory to the outgrowth of metastatic tumor cells. Of significant interest is the fact that metastasis suppression was abrogated in Tsp-1 knockout BMT mice. The requirement of Tsp-1 expression in bone marrow–derived cells suggests a novel mechanism of action in which recruited bone marrow–derived Gr1+ cells are the chief source of Tsp-1 in the metastatic lung. This is
consistent with published reports showing Tsp1 expression in monocye/macrophage populations (29, 30).

Interestingly, we have previously shown that metastatic tumors induce metastasis-promoting potential in Gr1+ cells recruited to the premetastatic lungs (31). However, depending on the nature of the tumor-derived systemic factor, the same cells can be modulated to exhibit metastasis-suppressive potential as shown in this study. It is thereby of great importance to decipher these pathways in the tumor milieu to further refine current therapies. Here, we show an example of an important antimetastatic effect of Gr1+ cells.

Previously, we identified Psap as a tumor-secreted factor with antimetastatic activity. On the basis of these findings, a therapeutic strategy such as the 5-amino acid DWLPK peptide, which targets Gr1+ bone marrow–derived cells in the microenvironment, could have significant efficacy in treating advanced metastatic cancer and ultimately in reducing the lethality of the disease.

From the perspective of targeting metastases, it has been emphasized that therapy should be targeted not only against tumor cells but also against the host microenvironment, which contributes to, and supports the progressive growth and survival of metastatic cancer cells (32). Given that the progression of human cancer to metastatic disease is the major contributing factor to its lethality, elevating Tsp-1 levels in the metastatic organ microenvironment to suppress metastasis may have clinical value. The Psap–Tsp-1 axis thus seems to be relevant in vivo. Significantly, examination of a tumor tissue microarray compiled from 103 patients with prostate cancer with long and complete follow-up revealed that Psap expression was positively correlated with increased overall survival. Specifically, patients with higher Psap levels in their primary
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METHODS

Mice and Cell Lines

All animal work was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Wild-type C57BL/6J, and GFP transgenic C57BL/6-Tg (ACTB-EFPR) 1Osb/J mice were obtained from The Jackson Laboratory. CB-17 SCID mice were obtained from Charles River. Tsp1 knockout mice in the C57BL/6J background, a kind gift from Dr. Shahin Rafii (Weill Cornell Medical College, New York, NY) were bred in the laboratory and genotyped according to standard protocols.

The cell lines PC3 and PC3M-LN4 are previously described (14). Human breast cancer cell lines MDA-MB-231 and MDA-MB-LM2 are described previously (13). The murine Lewis lung carcinoma cell line LLC/D122 (provided by Lea Eisenbach, Weizmann Institute of Science, Rehovot, Israel) stably expressing RFP and firefly luciferase (1–3), was described previously (13). The murine Lewis lung carcinoma cell line LLC/D122 (provided by Lea Eisenbach, Weizmann Institute of Science, Rehovot, Israel) stably expressing RFP and firefly luciferase (1–3), was provided by Lea Eisenbach, Weizmann Institute of Science, Rehovot, Israel, and used to treat WI-38 lung fibroblasts for 16 hours. After CM treatment, cells were harvested, lysed, and protein expression analyzed by Western blotting as previously described (14).

Bone Marrow Isolation and Transplantation

Bone marrow harvest and transplantation were conducted using our protocol approved by the Institutional Animal Care and Use Committee. Wild-type C57BL/6J, and GFP transgenic C57BL/6-Tg (ACTB-EFPR) 1Osb/J mice were obtained from The Jackson Laboratory. CB-17 SCID mice were obtained from Charles River. Tsp1 knockout mice in the C57BL/6J background, a kind gift from Dr. Shahin Rafii (Weill Cornell Medical College, New York, NY) were bred in the laboratory and genotyped according to standard protocols.

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Bone marrow harvest and transplantation were conducted using our protocol approved by the Institutional Animal Care and Use Committee. Wild-type C57BL/6J, and GFP transgenic C57BL/6-Tg (ACTB-EFPR) 1Osb/J mice were obtained from The Jackson Laboratory. CB-17 SCID mice were obtained from Charles River. Tsp1 knockout mice in the C57BL/6J background, a kind gift from Dr. Shahin Rafii (Weill Cornell Medical College, New York, NY) were bred in the laboratory and genotyped according to standard protocols.

The seven 20-amino acid peptides spanning the length of saposin A were composed of the following sequences:

- Psap generated by PCR amplification using the following strategy: ggcggcgctcggagatttttgatgtcagc. The PCR products were then cleaved with Xba1 and Sal1 and cloned into pCMVneo.

- The Psap truncation constructs were then used to transfect PC3M-LN4 using the Fugene6 transfection reagent and protocol (Roche). CM of transfected cells was harvested 48 hours after transfection and used to treat WI-38 lung fibroblasts for 16 hours. After CM treatment, cells were harvested, lysed, and protein expression analyzed by Western blot analysis as previously described (14).

- The seven 20-amino acid peptides spanning the length of saposin A were composed of the following sequences:

  - 1–20-SLPCDICKDVVTAAGDMLKD; 11–30-VTAAGDMLKDNAT
  - 21–40- NATEEEILVYLEKTCDWLPK; 31–50- LEKTCDWLPK
  - 41–60-PNMSASCKEIVDSYLPVILD
  - 51–70-VDSYL
  - 61–81-IIKGEMSRPGEVCSALNLCES. All

Figure 6. DWLPK peptide-mediated stimulation of Tsp-1 in the Gr1+ cells results in suppression of lung metastases in an orthotopic breast cancer model. A, left, representative BLI images of animals showing orthotopic primary breast tumors derived from MDA-LM2 cells in the mammary gland of SCID mice (day 21). Right, quantitation of BLI reads from mice shown at left (n = 6). B, left, representative BLI images of lungs in mice from A treated with either a scrambled peptide or DWLPK peptide (images taken 3 weeks after surgical removal of primary tumors). Right, quantitation of BLI reads from mice shown at left (n = 6; P = 0.037, Mann–Whitney U test). C, Western blot analysis showing induction of Tsp-1 in the premetastatic lungs of LM2 tumor-bearing mice (3 weeks after primary tumor injection) treated with scrambled (Scr), or DWLPK peptide. Two representative mice per group shown; experiment was repeated and identical results obtained. D, representative images of premetastatic lungs immunostained for Gr1 and Tsp-1 showing upregulation of Tsp-1 in lungs from MDA-LM2 tumor-bearing mice treated with DWLPK peptide compared with animals treated with scrambled peptide.
Figure 7. Expression of Psap positively correlates with increased overall survival in prostate cancer. **A,** model showing how metastasis-incompetent primary tumors, by expressing Psap, can systemically generate a metastasis-suppressive microenvironment in the lungs by inducing Tsp-1 expression in the bone marrow-derived CD11b+ Gr1+ myeloid cells. BMDC, bone marrow-derived cells. **B,** immunohistochemical analysis of Psap protein expression in a tissue microarray showing time to biochemical failure in patients with prostate cancer following radical prostatectomy. **C,** immunohistochemical analysis of Psap protein expression in a tissue microarray showing cancer-specific survival in patients with prostate cancer following radical prostatectomy. *P* values indicate significant difference for both endpoints, time to biochemical failure and overall survival (*P* = 0.027 and *P* = 0.036, respectively). **D,** examples of high and low Psap-staining patterns.

For in vitro analysis of peptide activity WI-38 lung fibroblasts were treated with peptide for 16 hours at a concentration of 5 μg/mL. The cells were then harvested and lysed for Western blot analysis as previously described (14).

For in vivo analysis of peptide activity 8-week-old C57/BL6 mice were treated with peptide, diluted in PBS, at a dose of 30 mg/kg/d via intraperitoneal injection for 4 days. After 4 days, the mice were euthanized by cervical dislocation under isoflurane anesthetic. The lungs were harvested, lysed, and analyzed for protein expression as previously described (14).

For metastasis experiments, the Psap peptide DWLPK and a scrambled peptide LPKDW were synthesized by AnaSpec. Peptide diluted in saline was administered to mice (30 mg/kg) via intraperitoneal injection on a daily basis for 6 days before tumor cell injection and until the end of the metastasis assays.

**Conditioned Medium Experiments**

PC3 or PC3M-LN4 cells were cultured in RPMI with 10% FBS. A total of 5 × 10⁶ cells were then subcultured in serum-free medium in 10-cm plates for 24 hours to generate CM. Harvested media was centrifuged and filtered through 0.22 μmol/L pore-size filters to remove any cells or cell debris.

WT BMT and Tsp-1−/− BMT C57BL/6j mice were pretreated with 200 μL serum-free CM from PC3 or LN4 cells or serum-free RPMI media daily for 6 days via intraperitoneal injection.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Tumor Instigated Bone Marrow–Derived Metastasis-Resistant Microenvironment

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Acknowledgments

The authors thank Bruce Zetter (Children’s Hospital, Boston, MA) for comments on the manuscript, and Jenny Xiang of the Genomics Resources Core Facility of Weill Cornell Medical College for the microarray experiments, and Sharrlee Lee, Mary Hahn, Gerd Lillian Hallseth, and Bendik Nordanger for technical support.

Grant Support

This study was supported by NIH grant CA135417 (to V. Mittal and R.S. Watnick) and by grants from the Norwegian Cancer Society and the Norwegian Research Council (to L.A. Akslen). This study was also partially supported by the Cornell Center on the Microenvironment and Metastasis through Award Number U54CA143876 from the NCI and Robert J. Goldman Foundation (to V. Mittal) and by the Elsa U. Pardee Foundation and Boston Children’s Hospital Technology Development Grant (to R.S. Watnick). R. Catena was supported by fellowships from the “Government of Navarra” and the “Cámara Navarra de Comercio” (Navarra, Spain).

Received October 15, 2012; revised February 20, 2013; accepted February 21, 2013; published OnlineFirst April 30, 2013.

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