Molecular and Cellular Pathobiology

BMP4 Inhibits Breast Cancer Metastasis by Blocking Myeloid-Derived Suppressor Cell Activity
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Abstract

The TGFβ growth factor family member BMP4 is a potent suppressor of breast cancer metastasis. In the mouse, the development of highly metastatic mammary tumors is associated with an accumulation of myeloid-derived suppressor cells (MDSC), the numbers of which are reduced by exogenous BMP4 expression. MDSCs are undetectable in naïve mice but can be induced by treatment with granulocyte colony-stimulating factor (G-CSF/Csf3) or by secretion of G-CSF from the tumor. Both tumor-induced and G-CSF–induced MDSCs effectively suppress T-cell activation and proliferation, leading to metastatic enhancement. BMP4 reduces the expression and secretion of G-CSF by inhibiting NF-κB (Nfkb1) activity in human and mouse tumor lines. Because MDSCs correlate with poor prognosis in patients with breast cancer, therapies based on activation of BMP4 signaling may offer a novel treatment strategy for breast cancer. Cancer Res; 74(18); 5091–102. ©2014 AACR.

Introduction

Breast cancer is a common cancer in women and a major cause of cancer death among women of all races and populations, due to the development of secondary tumors in vital organs (1). While therapies for primary tumors are generally effective, metastatic disease is often refractory. Hence, there is an urgent need for new treatments that prevent the onset of secondary disease.

Metastasis is a multistep process involving interactions between tumor cells and stromal components, including fibroblasts, extracellular matrix, vasculature, and the immune system, all of which can contribute to the dissemination of tumor cells (2, 3). Normal tissue homeostasis is disrupted by the aberrant expression of factors by tumors and stromal elements, similar to those released during inflammation, wound healing, and immune responses (4, 5). For example, both in patients and in mice bearing metastatic tumors, there is a dramatic alteration in the leukocyte profile, particularly in myeloid-derived suppressor cells (MDSC), the levels of which are associated with the onset of metastatic disease and poor prognosis (6–10). MDSCs facilitate metastasis, in part, by suppressing antitumor immune responses. They inhibit T-cell activation and proliferation through release of immunosuppressive factors, such as reactive oxygen species and arginase-1 (7, 8). MDSCs also facilitate metastasis through immune-independent mechanisms, including enhancement of angiogenesis, extracellular matrix degradation, tumor cell invasion, and formation of the premetastatic niche (7, 10, 11). So far, there are no effective therapies that target the expansion or function of MDSCs in patients, although various approaches are being tested (10).

A role for BMP signaling in cancer, including breast cancer, has been reported (12–16). BMPs belong to the TGFβ superfamily and participate in developmental processes, in wound healing, and in homeostatic regulation (17, 18). BMP signaling is initiated by binding to heterodimeric transmembrane serine/threonine kinase type1/2 receptors. Receptor activation leads to phosphorylation of SMAD proteins (Smad1/5/8), resulting in canonical downstream signaling (17). BMPs can also signal through SMAD-independent pathways that interact with other signaling pathways, including MAPK and NF-κB to control vital processes during development and disease (17, 18).

Reports of the roles of BMPs in cancer are contradictory, and the impact on tumors or tumor cells differs, depending on the context of the experiment. In cell-based studies, BMPs are reported to inhibit proliferation of breast tumor cells (13, 14) and to either promote (15) or inhibit their migration (19). BMPs
are also reported to promote migration of colon cancer (20) and melanoma cells (21). However, different results are seen when BMP signaling is examined in vitro. Disruption of BMPR2, the receptor for BMP2, 4, and 7, leads to epithelial hyperplasia and formation of polyps in the colon (22) and to enhanced metastasis in mice transgenic for MMTV-PyMT (23). Consistent with this, expression of constitutively active BMPRIB in mammary tumor cells suppresses metastasis to lung (24). Furthermore, BMP inhibitors Coco and Noggin, respectively, promote the colonization of mammary tumor cells in the lung (24), and metastasis to bone (25). Thus, while BMPs may act on tumor cells in culture with a diversity of outcomes, paracrine signaling to the tumor environment is lacking in tissue culture and impact on metastasis can be shown only using in vivo models.

Here, we demonstrate that BMP4 acts as a metastasis suppressor in breast cancer. We show that BMP4 expression in tumor cells restricts the expansion and immunosuppressive capacity of MDSC in tumor-bearing mice. We have found that G-CSF, which is regulated by NF-κB, is a major factor driving MDSC formation and expansion, leading to enhancement of metastasis. BMP4 counteracts NF-κB activity in tumor cells, which results in a reduction in G-CSF secretion, leading to reduced numbers and activity of MDSC. In summary, BMP4 acts to suppress metastasis by regulating antitumor immune responses.

Materials and Methods

**Tumor lines and cell culture**

The 4T1 mammary tumor model comprises a series of isogenic lines, including nonmetastatic 67NR, the weakly metastatic 66c4 and 168FARN, and the highly metastatic 4T1.2 line derived from a spontaneous Balb/c/C3H mammary tumor (26). These lines, originally obtained from Dr. Fred Miller (Karnof Cancer Institute, Detroit, MI) or derived in our laboratory (4T1.2), were maintained in αMEM containing 5%FBS and 5 U/mL penicillin and streptomycin (P/S). No universal standards are available for authentication of these murine lines. In some experiments, lines with stable expression of mCherry fluorescent protein or with the neomycin resistance gene were used. NMuMG and E0771 cells (obtained from Dr. S. Tomlinson, Medical University, Charleston, SC) were cultured in DMEM/10%FBS, 0.2 IU/mL insulin, and 5 U/mL P/S). Human breast cancer cell lines MDA-MB-231-luc2 (authenticated by STR profiling in 2013), SKBr3, ESH172, MCF7 and HS587T (obtained directly from ATCC), and the viral packaging line PT67 (ATCC) were cultured in DMEM/10%FBS, 5 U/mL P/S. All cell lines were tested and validated to be mycoplasma free. Cells were maintained in culture for not more than 4 to 6 weeks. BAY11-7085 (Cayman CAS196309-76-9), BMP4 (R&D, 314-BP-010), TNFα (Cell Signaling, #8902), and G-CSF (Neupogen, Amgen) were used as indicated.

**Expression of BMP4, G-CSF, and the NF-κB reporter construct in cells**

Stable expression of BMP4 and G-CSF and transient knockdown of BMP4 was achieved as described in the Supplementary Data.

**Gene expression profiling**

Three 4T1.2 and 3 4T1.2-BMP4 primary tumors (~0.3 g) were excised from mice and total RNA isolated using RNeasy Mini Kits (Qiagen). Profiling using Affymetrix GeneChips is described in the Supplementary Data. Microarray profiling data were deposited into GEO (accession no. GSE51632).

**Immunohistochemistry**

All tissues were fixed in 10% neutral-buffered formalin for 24 hours, paraffin embedded, and sectioned. One section was retained for hematoxylin and eosin (H&E) staining, and serial sections were prepared for immunostaining. Antigen retrieval was achieved using citrate buffer (10 mmol/L trisodium citrate, pH 6.0) at 125°C for 3 minutes under pressure. Antibodies used at 1:100 dilution: E-cadherin (610181) and vimentin (550513) from BD BioSciences; pan-cytokeratin (C9927) from Sigma; N-cadherin (NB200-592) from Novus Biologicals; and Snail/Slug (Ab85931) from Abcam. ImmPRESS HRP Universal Antibody (Vector, MP-7500) and DAB (K3467) were used to visualize the primary antibodies, following the manufacturer’s instructions. Images were collected using Olympus BX-51 microscope.

**Flow cytometry**

Single-cell suspensions tagged with antibodies (1:200 dilution) as required were sorted on the FACS Diva Flow Cytometer or analyzed using BD FACScanto II flow cytometer. Data were analyzed using FCS Express (De Novo Software) or FlowJo. The antibodies used are as follows: TRC8-APC (17-5961-81), CD45-FITC (11-0454), Ly6c-APC (17-5933-80), CD4-APC (17-0042), Gr1-PE (2-5931), CD11b-PE (12-0112) from eBioscience; Ly6G-PE (130-093-139) from Miltenyi Biotech; CD8a-PerCP (553036), integrinβ4 (553745), integrinβ3 (553345) from BD Pharmingen; BMP2 (Ab10862) from Abcam; BMPRIA (AF346), BMPRIB (AF505) from R&D Systems; and integrinβ1β2 (Mab21412) from Chemicon.

**Measurement of BMP4 and G-CSF by ELISA**

Conditioned medium from cultured cells or plasma from mice was analyzed for BMP4 or G-CSF using kits from R&D Systems (Cat# LUCK-1G) and imaged 10 minutes later using the IVIS, Lumina II.

**Mammary tumor growth and luciferase imaging**

Female Balb/c and nu/nu mice, 6 to 8 weeks, from Walter and Eliza Hall Institute were used. All experiments had approval from the Peter MacCallum Animal Experimentation Ethics Committee. Cells (1 × 10^7/20 μL) were injected into the fourth mammary glands of Balb/c mice under brief anesthesia or via the tail vein (1 × 10^7/200 μL) for experimental metastasis assays. Human MDA-MB-231-luc2 cells (1 × 10^5/200 μL) were injected into the tail vein of nu/nu mice. Tumor dimensions were measured with electronic calipers, and volume was calculated as the (length × width^2)/2. In some experiments, primary tumors were resected. For tumors expressing luciferase, mice were injected subcutaneously with 15 mg/mL luciferin (200 μL/mouse, Gold Biotechnology, Cat#LUC-1G) and imaged 10 minutes later using the IVIS, Lumina II.
Measurement of metastatic burden

In some experiments, a TaqMan quantitative PCR (qPCR) assay was used to determine metastatic burden in tissues from mice inoculated with neomycin or mCherry-tagged tumor cells. Relative tumor burden was obtained from the ratio of tumor-specific neomycin or mCherry gDNA to mouse vimentin DNA in a multiplexed TaqMan qPCR assay as described previously (26). In other experiments, lung metastatic burden was obtained by counting the number of mCherry-positive cells by flow cytometry following disaggregation of the lungs in collagenase as described below. The incidence of mice with metastases was established by detection of mCherry fluorescence in organs using a dissection microscope (Olympus SXZ12).

rG-CSF treatment

Mice bearing 66cl4 tumors received rG-CSF treatment twice daily for 4 days per week by intraperitoneal injection at 125 μg/kg. In other experiments, mice were injected with rG-CSF 5 days before and/or after tail vein injection of tumor cells.

Cellular analysis of blood and tissues

Blood was collected using either submandibular or cardiac bleeds and analyzed using the ADVIA120 Hematology System. Tissues were chopped finely, digested in 3 mg/mL collagenase-A (Worthington), and filtered through a 70-μm nylon cell strainer. Erythrocytes were removed using lysis buffer (150 mmol/L NaCl, 1 mmol/L KHCO3, 100 mmol/L EDTA, pH 8). Cells were pelleted, resuspended in PBS, and analyzed by flow cytometry as specified.

T-cell suppression assay

The suppression of T-cell proliferation was evaluated using a standard assay as described previously (27). Details are provided in Supplementary Data.

Statistical analysis

For statistical analysis, the data analysis package within GraphPad Prism 5 for Windows was used (GraphPad Software). Tests comparing 2 means used a Student t test, with equal variance assumed. Error bars represent the SEM. Survival data were analyzed using a log-rank statistical test. Pearson correlation analysis was used to assess the relationship between two conditions.

Results

BMP4 suppresses leukocytosis and splenomegaly induced by metastatic tumors

An association between leukocytosis and malignancy, especially in more advanced tumors, has long been recognized in patients (28, 29) and is reported in preclinical tumor models (30). We compared the ability of a panel of genetically matched syngeneic mammary tumors with varying metastatic capacity (26) to induce alterations in leukocytic populations. Splenomegaly was observed in all tumor-bearing mice but was most pronounced in mice bearing highly metastatic 4T1.2 tumors (Fig. 1A). The increased spleen weight correlated with increased leukocytes in peripheral blood (Fig. 1B), and this elevation was also noted in C57Bl/6 mice bearing E0771 mammary tumors (Supplementary Fig. S1A and S1B). In both 4T1.2 and E0771 tumors, increased leukocytes were due primarily to the expansion of myeloid cells, not lymphocytes (Fig. 1G, Supplementary Fig. S1B).

Consistent with reports of the involvement of BMP signaling in tumor growth and metastasis (12, 15, 19, 22–25), we observed reduced levels of BMP4 in higher grade human breast cancers and noted that lower BMP4 mRNA expression in human breast tumors correlated with poor disease-free survival in patients (Supplementary Fig. S2A and S2B). We next determined the impact of BMP4 on tumor growth and metastasis using preclinical models. Aggressive 4T1.2 mammary tumor cells secrete very low levels of BMP4. Forced expression of BMP4 (4T1.2-BMP4 cells; Supplementary Fig. S2C and S2D) had no impact on primary tumor growth (Fig. 1C) or proliferation in vitro (data not shown). On seeking changes in phenotype of 4T1.2 cells expressing BMP4, we found some alterations in three integrin subunits (Supplementary Fig. S3A) and in expression of genes associated with epithelial–mesenchymal transition (EMT) but no change in their migratory capacity (Supplementary Fig. S3B—S3D).

Increased BMP4 markedly suppressed the extent and incidence of metastasis to lung and bone (Fig. 1D and E). The reduction in metastasis was associated with a reduction in both spleen weight and peripheral blood myeloid cells but not in circulating lymphocytes (Fig. 1F and G). On the other hand, a reduction of BMP4 in weakly metastatic 168FARN cells accelerated the onset of metastatic disease after primary tumor resection (Fig. 1H and J). These data confirm that BMP4 is able to suppress breast cancer metastasis and that the number of myeloid cells correlates with the extent of metastasis.

The kinetics of leukocytosis was measured in mice bearing 4T1.2 and 4T1.2-BMP4 orthotopic tumors. Peripheral blood samples were analyzed for the MDSC markers, CD11b and Gr1 (7), before and after primary tumor resection on day 24. A significant expansion of MDSC was apparent by day 3 and increased over time but was reduced by expression of BMP4 (Fig. 1K) despite the similarity in primary tumor growth (not shown). MDSC continued to accumulate after removal of the 4T1.2 primary tumors, presumably maintained by the expanding secondary lesions. In the primary tumors analyzed following resection and disaggregation on day 24, expression of BMP4 resulted in a reduction in MDSC and an increase in T lymphocytes (Fig. 1L). The increased T lymphocyte population observed within 4T1.2-BMP4 tumors indicates that BMP4 triggers an enhanced antitumor immune response. Increased tumor-infiltrating lymphocytes are indicative of a favorable prognosis in patients with breast cancer (31, 32).

Gene expression profiling reveals enhanced antitumor immune activity in mice bearing 4T1.2-BMP4 tumors

Gene expression profiles of 4T1.2 and 4T1.2-BMP4 primary tumors revealed differences in immune cell involvement. Supplementary Table S1 shows all genes altered more than 3-fold by expression of BMP4. While no significantly down-regulated gene ontology categories were obtained, many genes significantly up-regulated by BMP4 were involved in immune...
responses, including antigen processing and presentation, wound healing, T-cell differentiation and activation (Supplementary Table S2). This is consistent with the higher numbers of T lymphocytes detected in 4T1.2-BMP4 tumors (Fig. 1L). Upregulation of genes associated with maturation of antigen-presenting cells, such as CD86 (costimulatory signal) and MHC class II antigens (Supplementary Tables S1 and S2) indicates that the myeloid cells in 4T1.2-BMP4 tumors are more mature and may have a reduced capacity to suppress T-cell function (7).

To test the immunosuppressive activity of the MDSC, T-cell suppression was measured using enriched splenocyte-derived monocytic CD11b+Ly6G+ or granulocytic CD11b+Ly6G+ MDSC from mice bearing 4T1.2 or 4T1.2-BMP4 tumors (27). The granulocytic MDSC from 4T1.2 tumor–bearing mice exhibited potent inhibition of both CD4+ and CD8+ T-cell activation and proliferation, whereas those from 4T1.2-BMP4 tumor–bearing mice were much less active (Fig. 2A and C; Supplementary Fig. S4A and S4C). Monocytic MDSC isolated from 4T1.2 tumor–bearing mice also suppressed both CD4+ and CD8+ T-cell activity, whereas those from 4T1.2-BMP4 tumor–bearing mice had no suppressive activity (Fig. 2B and D; Supplementary Fig. S4B and S4D). These results indicate that BMP4 reduces not only the number of MDSC but also their immunosuppressive capacity, thereby restoring the activation and proliferation of T cells and possibly anti-metastatic immunosurveillance.

**BMP4 reduces secretion of G-CSF from mammary tumors**

The expansion and function of leukocytes are regulated by cytokines and chemokines released from tumors (4). BMP4 has...
restricted local diffusion (33) and, consistent with this, we have not been able to detect systemic BMP4 in plasma from mice bearing either 4T1.2 or 4T1.2-BMP4 tumors (data not shown). It is possible that myeloid cells respond directly to BMP4. However, we found that levels of BMP receptors were very low on myeloid cells from naïve or tumor-bearing mice and that BMP4 treatment of myeloid cells did not increase T-cell proliferation (Supplementary Fig. S5). Thus, another explanation for the reduction in MDSC is suppression by BMP4 of a factor released by tumor cells. MDSC are induced by inflammatory factors, including G-CSF, macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF; refs. 7, 34). We therefore hypothesized that BMP4-induced suppression of MDSC expansion could be mediated through regulation of one or more of these factors.

The expression of G-CSF, GM-CSF, and M-CSF was evaluated in a panel of 5 murine mammary tumor lines of varying metastatic potential. G-CSF expression was significantly higher in 4T1.2 cells than in the less metastatic lines and was reduced by enforced expression of BMP4 in 4T1.2 cells (Fig. 3A). In contrast, M-CSF and GM-CSF transcript levels did not correlate with metastatic potential, nor were they regulated by BMP4 (not shown). In human breast cancer lines, G-CSF expression was significantly higher in metastatic MDA-MB-231-luc2 than in the less metastatic lines, whereas BMP4 levels were lower (Fig. 3B and C), thus extending relevance to human tumors.

Addition of recombinant BMP4 (rBMP4) to 4T1.2 cells rapidly depleted G-CSF transcripts (Fig. 3D). Both ectopic BMP4 expression and treatment with rBMP4 decreased G-CSF secretion from 4T1.2 cells (Fig. 3E). G-CSF secretion from the less metastatic lines was low, consistent with the G-CSF expression data (Fig. 3A and E, Supplementary Fig. S1C). When plasma from mice bearing tumors of about 500 mm³ was analyzed, G-CSF levels (Fig. 3F) were proportional to the metastatic potential of the tumors (26). In mice bearing E0771 tumors, similar increases in plasma G-CSF were observed (Supplementary Fig. S1D). An inverse correlation between BMP4 and G-CSF was evident in both mouse and human tumor lines (Fig. 3G). Similarly, an inverse relationship was found between BMP4 and G-CSF in a cohort of invasive breast cancer samples (GEO microarray data accession number GSE6434; Fig. 3H). Downregulation of BMP4 in the 168FARN cells increased G-CSF secretion (Fig. 3E), consistent with their increased metastatic capacity (Fig. 1J). Similarly, downregulation of BMP4 in the human breast cancer lines, MCF7 and MDA-MB-231-luc2 cells (Fig. 3J) and in E0771 cells (Supplementary Fig. S1F and S1G), resulted in an increase in G-CSF expression. These data reinforce the view that the extent of G-CSF secretion is associated with metastatic capacity and that BMP4 can attenuate G-CSF expression. On restricted local diffusion (33) and, consistent with this, we have not been able to detect systemic BMP4 in plasma from mice bearing either 4T1.2 or 4T1.2-BMP4 tumors (data not shown). It is possible that myeloid cells respond directly to BMP4. However, we found that levels of BMP receptors were very low on myeloid cells from naïve or tumor-bearing mice and that BMP4 treatment of myeloid cells did not increase T-cell proliferation (Supplementary Fig. S5). Thus, another explanation for the reduction in MDSC is suppression by BMP4 of a factor released by tumor cells. MDSC are induced by inflammatory factors, including G-CSF, macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF; refs. 7, 34). We therefore hypothesized that BMP4-induced suppression of MDSC expansion could be mediated through regulation of one or more of these factors.

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the basis of these observations, the ability of BMP4 to regulate G-CSF is likely to be a critical factor for the expansion of immunosuppressive MDSC and for metastatic progression.

G-CSF induces MDSC expansion in nontumor-bearing mice, leading to enhanced spontaneous metastasis and lung colonization

G-CSF regulates hematopoiesis and stimulates the innate immune response of neutrophilic granulocytes (35). In mice, daily recombinant G-CSF (rG-CSF) administration enhances circulating myeloid cell numbers (36). However, it is not known whether G-CSF–mobilized granulocytes have the capacity to suppress acquired immune responses.

When rG-CSF was administered to naïve mice, we noted an increase in peripheral leukocytes, due mainly to an increase in CD11b+ Gr1+ cells (Fig. 4A and B). To measure their immunosuppressive activity, CD11b+ splenocytes were tested in the T-cell suppression assay. Myeloid cells from saline-treated mice
lacked suppressive activity on both CD4\(^+\) and CD8\(^+\) T cells. However, after rG-CSF treatment, CD11b\(^+\) splenocytes were able to suppress the proliferation of both CD4\(^+\) and CD8\(^+\) T cells (Fig. 4C and D). The results demonstrate that the myeloid cells from naïve mice have no immunosuppressive activity, but that rG-CSF treatment is able to transform them into MDSC.

Because rG-CSF may create an environment favorable for metastasis through the expansion of MDSC, the direct effect of rG-CSF therapy on tumor growth and metastasis was assessed. Mice bearing weakly metastatic 66cl4 mammary tumors were treated with rG-CSF or PBS. rG-CSF did not alter primary tumor growth (Fig. 4E), but enhanced spontaneous metastasis to lung (Fig. 4F). rG-CSF treatment also enhanced lung colonization of another mammary tumor line, 168FARN (Fig. 4G), and of the human MDA-MB-231-luc2 breast cancer cells (Fig. 4H and J) in experimental metastasis assays. After rG-CSF treatment, the number of MDSC in peripheral blood of the immunocompromised mice used for the MDA-MB-231-luc2 metastasis assay was also increased (not shown).

These results indicate that G-CSF alone can induce the transformation of myeloid cells into MDSC and, as a result, enhance both experimental and spontaneous metastasis of mouse and human tumors in immunocompetent or immunocompromised mice. Therefore, it is possible that one of the mechanisms exploited by tumors to metastasize is through secretion of G-CSF and subsequent induction of MDSC that occurs early in tumor growth (Fig. 1K). In addition to T-cell suppression, MDSC are involved in the establishment of the premetastatic niche (11, 37), thus explaining the G-CSF–mediated enhancement of metastasis in the immunocompromised mice. Hence, the action by BMP4 to suppress G-CSF secretion can explain the ability of BMP4 to inhibit metastasis.

**Enforced G-CSF re-expression in 4T1.2-BMP4 tumors restores metastatic capacity**

To determine whether the anti-metastatic effect of BMP4 is due to the inhibition of G-CSF secretion, 4T1.2-BMP4 cells with constitutive expression of G-CSF (4T1.2-BMP4/G-CSF) were

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**Figure 4.** rG-CSF treatment induces the expansion of MDSC in mice and facilitates experimental and spontaneous metastasis. Mice (n = 5/group) were treated with rG-CSF for 5 days. Control groups were either not treated (naïve) or received PBS. Leukocytes (A) and CD11b\(^+\)Gr1\(^+\) (B) cells in blood. Suppression of CD4\(^+\) (C) or CD8\(^+\) (D) T cells by CD11b\(^+\) splenocytes from mice (n=rG-CSF). E, 66cl4-mCherry mammary tumor growth in mice treated with rG-CSF (4 d/wk; n = 9) or PBS (n = 10) commencing 24 hours after tumor cell injection. F, incidence of metastases and metastatic burden in lung analyzed by flow cytometry for mCherry-positive cells. G, incidence of metastases and lung metastatic burden in mice (n = 9/group) treated with rG-CSF for 2 days before inoculation with mCherry-positive 168FARN cells by tail vein injection. rG-CSF treatment continued for the next 9 days and mice were culled on day 30. H and J, lung tumor burden (luciferase activity) of mice (n = 5 per group) inoculated with MDA-MB-231-luc2 cells via tail vein. Mice were treated with rG-CSF for 5 days before and 9 days after tumor cell injection. Representative results of repeated experiments are shown as the mean ± SEM. Significant differences are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.0001 following a 2-tailed Student t test.
generated. While secretion of BMP4 was unchanged in 4T1.2-BMP4/G-CSF cells, G-CSF was elevated (Fig. 5A and B). Expression of G-CSF did not alter primary tumor growth in the mammary gland (Fig. 5C) but resulted in significant increases in metastasis to bone and lung (Fig. 5D and E). In a separate experiment, after size-matched primary tumors (~0.35 g) were resected, the onset of metastatic disease was accelerated in mice bearing 4T1.2-BMP4/G-CSF tumors (Fig. 5F). Thus, the restored metastatic capacity of 4T1.2-BMP4/G-CSF tumors strongly supports the hypothesis that G-CSF secreted by 4T1.2 cells facilitates their metastasis and that BMP4 reduces metastasis by suppressing tumor cell secretion of G-CSF.

BMP4 reduces G-CSF secretion through inhibition of NF-κB activity

We next set out to establish the mechanism by which G-CSF secretion is suppressed by BMP4. Previous publications have shown that the expression of G-CSF is associated with stress and can be induced by inflammatory cytokines such as TNFα and IL1β via NF-κB activation (38, 39). Therefore, we hypothesized that BMP4 may suppress G-CSF secretion by tumor cells through inhibition of NF-κB activity. To address this, we first measured NF-κB activity in primary 4T1.2 and 4T1.2-BMP4 mammary tumors with stable integration of an NF-κB-luciferase reporter (4T1.2-NFκB-luc and 4T1.2-BMP4-NFκB-luc). Luciferase activity was detected at day 10 (Fig. 6A and B), demonstrating more NF-κB activity in 4T1.2 than in 4T1.2-BMP4-NFκB-luc cells (Fig. 6C). Similarly, NF-κB activity in response to TNFα treatment of 4T1.2-BMP4-NFκB-luc cells was reduced compared with that of the 4T1.2-NFκB-luc cells, indicating that BMP4 suppresses NF-κB activity (Fig. 6D). In 4T1.2-NFκB-luc cells, basal and TNFα-enhanced NF-κB activity was abrogated by treatment with rBMP4 (Fig. 6E). These data indicate that BMP4 acts as a negative regulator of NF-κB.

Enhanced G-CSF secretion from 4T1.2 cells by TNFα treatment could be blocked either by the NF-κB inhibitor BAY11-7085 or with rBMP4, consistent with the requirement for NF-κB activation (Fig. 6F). In contrast, the low G-CSF secretion from 4T1.2-BMP4 cells was not altered by TNFα or by BAY11-7085 (Fig. 6G), indicating that NF-κB is maximally suppressed by the expression of BMP4. In 66cl4 and E0771 cells and in immortalized mouse mammary epithelial NMuMG cells, rBMP4 was also able to inhibit TNFα-induced G-CSF production (Fig. 6H and J; Supplementary Fig. S1E). Knockdown of BMP4 in E0771 cells resulted in elevation of basal G-CSF secretion and of TNFα-induced G-CSF (Supplementary Fig. S1F and S1G). Consistent with these results, both basal and TNFα-stimulated expression of G-CSF in

Figure 5. Exogenous G-CSF expression in 4T1.2-BMP4 cells restores metastatic capacity. BMP4 (A) and G-CSF (B) levels secreted by 4T1.2-BMP4/G-CSF and 4T1.2-BMP4 cells. C, growth of 4T1.2-BMP4 and 4T1.2-BMP4/G-CSF mammary tumors (n = 10 per group). Incidence and metastatic burden in spine (D) and lung (E) quantified by qPCR for mCherry. F, in a separate experiment, primary tumors (n = 9 per group) were resected at about 0.35 g, and mice monitored for onset of secondary disease. Disease-free survival was analyzed using the log-rank test. Representative data of two independent experiments in A–E are shown as the mean ± SEM. Significant differences are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001. A–E, 2-tailed Student t test.
MDA-MB-231-luc2 cells was inhibited by rBMP4 (Fig. 6K).

Together, these data demonstrate a common mechanism of attenuation of NF-κB activity by BMP4, thereby restricting G-CSF expression.

Discussion

MDSC enhance metastasis both by contributing to the formation of the premetastatic niche and by suppressing antitumor immune responses (3, 6, 11, 37). In patients with solid tumors, levels of circulating MDSC correlate with clinical grade, the extent of metastatic burden, and response to therapy (9, 10). An increased granulocytelymphocyte ratio or increased neutrophils are found in patients with advanced cancer, including breast cancer (40–42). Here, we have shown that BMP4 can act through the NF-κB pathway to inhibit G-CSF secretion that otherwise promotes the number and the immunosuppressive activity of MDSC and, ultimately, the extent of metastasis.

The first indication of a paracrine action of BMP4 came from analysis of peripheral blood leukocytes, where the number of...
MDSC and their immunosuppressive activity was reduced in mice bearing BMP4-expressing tumors. We measured the levels of factors known to increase immature myeloid cells in circulation (7) and found that G-CSF expression and secretion correlated with the metastatic capacity of the tumor lines and was downregulated by BMP4. Mice bearing BMP4-expressing tumors also had lower levels of plasma G-CSF. Higher G-CSF levels have been reported in patients, including those with breast cancer, than in healthy volunteers (42, 43), and case reports reveal that patients with lung and gastric cancer with high G-CSF levels have aggressive tumors with poor outcome (44, 45).

We have demonstrated that, in the absence of G-CSF, myeloid cells have no immunosuppressive activity in naïve mice. However, rG-CSF treatment not only expanded the number of myeloid cells but also induced their T-cell immunosuppressive activity, leading to enhanced spontaneous and experimental metastasis of mouse and human breast tumors. Our data indicate that the MDSC expanded by G-CSF have similar properties to those described in previous studies where MDSC facilitate metastasis by establishing a premetastatic niche or by promoting the early stages of metastasis (3, 7, 37).

A previous report showed that G-CSF secretion by mammary tumors or treatment with rG-CSF contributes to the mobilization of MDSC and to the extent of experimental lung metastasis (34). Our data explain this observation by demonstrating the differential immunosuppressive activity of myeloid cells, depending on their exposure to G-CSF. In addition, we have demonstrated that BMP4 can suppress the expansion and immunosuppressive activity of MDSC, resulting in a reduction in the extent of metastasis.

G-CSF is expressed in human tumor lines, including MDA-MB-231-luc2 breast cancer (Fig. 3B), bladder carcinoma (46), hepatoma (47), squamous cell carcinoma (48), and gastric cancer (49). Under stressful or inflammatory conditions, G-CSF is expressed by normal cell lineages (35, 46) in response to endotoxins from infectious bacteria (43), iNOS (35), TNFα, IL1β, and NF-κB activation (35, 39). Tumors are in a chronic state of stress, due to their uncontrolled growth and disorganized tissue structure. Hypoxia, low pH, and nutrient deprivation are common features, leading to necrosis and to chronic inflammation that is associated with tumor progression (2, 50). As a classical stress response pathway, NF-κB activation is detected in most cancers, including breast cancer (38) and, as shown here, in 4T1.2 tumors. Hence, it is reasonable to propose that NF-κB activation, a known regulator of G-CSF (39), is responsible for the high levels of G-CSF secretion. We have shown that activation of NF-κB by TNFα enhances G-CSF secretion, which is blocked by an NF-κB inhibitor or by treatment with rBMIP4. This response was observed in several tumor and normal mouse and human breast lines, demonstrating the generality of the NF-κB response to BMP4.

On the other hand, NF-κB activity is required for maturation and differentiation of leukocytes involved in innate and adaptive immune responses that elicit antitumor responses, which could raise issues for its use as a direct therapeutic target (51). Here, BMP4 was shown to inhibit NF-κB activity in tumor cells while promoting the maturation of myeloid cells and the activation of T cells in stromal tissues. The dual roles of BMP4 in tumors and in stroma not only explain the mechanism by which BMP4 suppresses breast cancer metastasis but also offer a new strategy for targeting metastatic disease.

rG-CSF has been used for the treatment of patients with neutropenia, particularly during chemotherapy. Few deleterious effects of rG-CSF treatment have been reported, possibly due to the difference between controlled therapeutic application and uncontrolled pathologic states. G-CSF levels in human or mouse serum under normal conditions range from 30 to 163 pg/mL (35) but increase to more than 3,000 pg/mL under conditions of stress or inflammation (35). Patients receiving rG-CSF are given daily doses with a median concentration of less than 6 μg/kg. The peripheral neutrophil count is monitored carefully and treatment ceases when neutrophil levels return to baseline. This dosing strategy may minimize the deleterious effects of G-CSF.

In this study, we have revealed a paracrine mechanism by which BMP4 can suppress metastasis. It is likely that BMP4 also has direct effects on tumor cells, but we did not detect changes in proliferation, migration, or in genes associated with EMT. We have shown that BMP4 can block G-CSF secretion by tumor cells through inhibition of the NF-κB pathway and that the MDSC generated in the presence of BMP4 have diminished immunosuppressive capacity. These findings provide strong support for the use of BMP4, or activation of BMP4 signaling, as a therapeutic modality for the treatment of patients with breast cancer at risk of development of metastatic disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


