Dysregulated Hematopoiesis Caused by Mammary Cancer Is Associated with Epigenetic Changes and \textit{Hox} Gene Expression in Hematopoietic Cells

Alexander Sio\textsuperscript{1}, Manreet K. Chehal\textsuperscript{1}, Kevin Tsai\textsuperscript{1}, Xueling Fan\textsuperscript{1}, Morgan E. Roberts\textsuperscript{1}, Brad H. Nelson\textsuperscript{2}, Jolanta Grembecka\textsuperscript{3}, Tomasz Cierpicki\textsuperscript{3}, Danielle L. Krebs\textsuperscript{1}, and Kenneth W. Harder\textsuperscript{1}

Abstract

Cancer is associated with immune dysfunction characterized by the presence of proinflammatory and immunosuppressive cells and factors that contribute to tumor growth and progression. Here we show that mammary tumor growth is associated with defects in hematopoiesis, leading to myeloproliferative-like disease (leukemoid reaction), anemia, and disruption of the bone marrow stem/progenitor compartment. The defects we characterized included impaired erythropoiesis, leukocytosis, loss of early progenitor cells in the bone marrow, and splenic extramedullary hematopoiesis. We established an \textit{in vitro} model to dissect interactions between mammary cancers and the hematopoietic system. Investigations in this model revealed that granulocyte colony-stimulating factor (G-CSF) produced by mammary tumors can synergize with FLT3L and granulocyte macrophage CSF (GM-CSF) to expand myeloid progenitors and their progeny in culture. Mammary tumor growth was associated with histone methylation changes within lineage-negative c-Kit-positive hematopoietic cells within the bone marrow of tumor-bearing mice. Similarly, parallel histone methylation patterns occurred in cultured bone marrow cells exposed to mammary tumor-conditioned cell culture media. Notably, changes in histone methylation in these cell populations correlated with dysregulated expression of genes controlling hematopoietic lineage commitment and differentiation, including \textit{Hox} family genes and members of the Polycomb repressive complex 2 (PRC2) chromatin-remodeling complex. Together, our results show that mammary tumor-secreted factors induce profound perturbations in hematopoiesis and expression of key hematopoietic regulatory genes.

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Introduction

The link between cancer and inflammation has been recognized for almost 150 years (1). However, the complexity of the interactions between tumors and the immune system is only beginning to be appreciated. Solid tumor development is often associated with cytokine and metabolite upregulation, and immunosuppressive cell expansion [including myeloid-derived suppressor cells (MDSC), T regulatory cells (Treg), tumor-associated macrophages (TAM), and tumor-associated dendritic cells (tDC)]. These cells facilitate tumor growth, metastasis, and immunological escape (2). Cancer is also frequently associated with perturbations in hematopoiesis manifested as disturbed myelopoiesis, leukocytosis (leukemoid reaction), cytopenia, and/or anemia, which may predict adverse patient outcome (3, 4). As the nature of antitumor immune responses may hold better prognostic value than standard scoring regimes, a better understanding of tumor-induced changes to the immune response and hematopoietic system may lead to improved cancer diagnosis and treatment (5). Indeed, recent studies show that manipulation of tumor-induced stromal cells can impair tumor growth and elicit antitumor immunity (6).

Development of blood cell lineages is controlled by sequential differentiation of progressively restricted progenitor populations derived from hematopoietic stem cells (HSC). Gene expression patterns governing blood cell production and lineage commitment are regulated by transcription factors and epigenetic control of chromosomal organization involving histone modifications and DNA methylation (7). Epigenetic changes are linked to control of cell fate, stem/progenitor cell function and tumorigenesis. In leukemia and myeloid neoplasia, epigenetic dysregulation induced by mutations in DNA- or histone-modifying genes is a significant step in disease progression. Mutations in genes encoding Trithorax group (TrxG) and Polycomb group (PcG) proteins regulating histone H3 lysine-4 (H3K4) and lysine-27 (H3K27) methylation,
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respectively, are frequently observed in hematologic malignancies and myeloproliferative/dysplastic neoplasms (8, 9). H3K4 trimethylation (H3K4me3) correlates with enhanced gene expression, whereas H3K27 trimethylation (H3K27me3) is generally linked to suppression of gene expression. Enhancer of zeste homolog 2 (EZH2), the H3K27 methyltransferase component of Polycomb repressive complex 2 (PRC2), plays complex roles in leukocyte neoplasms with both loss- and gain-of-function mutations in Ezh2 associated with disease. EZH2 catalyzes progressive H3K27 methylation, resulting in suppression of genes including Hoxa9 (10, 11), with recent work revealing a role for PRC2 in HSC physiology (12, 13). Hoxa genes regulate steady-state hematopoiesis governing stem/progenitor cell function. As blood cells differentiate, expression of Hoxa genes decreases, a process involving changes in H3K27 methylation catalyzed by enzymes like JMJD3 (14, 15). Hoxa gene family upregulation, particularly Hoxa7 and Hoxa9, promote hematopoietic stem/progenitor cell (HSPC) expansion and contribute to leukemogenic transformation (16, 17).

Here, we assessed the HSPC compartments in tumor-bearing mice and found that mammary tumors (MT) induced greater changes in hematopoiesis than previously appreciated. All mammary tumors examined affected primitive HSPC compartments, leading to myeloproliferative-like disease (leukocytosis) and anemia. Mammary tumor growth was associated with diminished Ezk2 expression, reduced H3K27me3, and Hoxa9 upregulation in lineage- (Lin-) c-Kit+ (LK) hematopoietic progenitors in the bone marrow.

We established an in vitro model to decipher the tumor-secreted and endogenous factors that might contribute to dysregulation of hematopoiesis, and found that the hematopoietic regulatory cytokines FLT3L and granulocyte macrophage colony-stimulating factor (GM-CSF) synergized with mammary tumor–produced granulocyte colony-stimulating factor (G-CSF) to enhance HSPC production. In addition, mammary tumor–secreted factors led to global and gene-specific changes in histone methylation patterns associated with enhanced Hoxa9 gene expression. Taken together, our data suggests that mammary tumor–secreted factors such as G-CSF lead to epigenetic changes and altered expression of key hematopoietic regulatory genes, ultimately resulting in leukocytosis, anemia and HSPC defects.

Materials and Methods

Mice

MMTV-neu<sup>OTIS/OTII</sup> mice were previously described (18, 19). Mice were C57BL/6 genetic background (>10 generations). Animal experiments were conducted according to Canadian Council for Animal Care and the University of British Columbia Animal Care Committee guidelines. Mice were age/sex matched and analyzed between 9 and 14 weeks of age.

Tumor cell lines

NOP cell lines express Neu linked to OVA<sup>OTI/OTII</sup> peptide sequences and a dominant-negative p53 transgene (MMTV-neu<sup>OTIS/OTII</sup>, refs. 18, 19). NOP cells were maintained in RPMI-1640 (Invitrogen), 10% heat-inactivated (HI)-FBS, 100 U/mL penicillin-G, 100 μg/mL streptomycin, 2 mmol/L glutamax, 50 μmol/L 2-mercaptoethanol, and insulin/transferrin/selenium (Lonza). NOP12, 18, 21 and 23 lines are referred to as MT-1, 2, 3 and 4. CMT-93 (ATCC-CLL-223), 4T1 (ATCC-CLL-6323), and B16-F1 (ATCC-CLL-6323) cells were maintained in Dulbecco’s Modified Eagle Medium containing 10% HI-FBS. Tumor-conditioned media (T-CM) was produced by culturing cells at 5 × 10<sup>6</sup> cells/25 mL for 4 days. All cell lines were screened by PCR and determined to be free of 20 viral pathogens (Impact-I PCR profile, RAADIL).

In vitro bone marrow analysis

Bone marrow was cultured in NOP medium without insulin/transferrin/selenium in the presence of cytokines at 1 × 10<sup>6</sup> cells/mL for 3 to 8 days. For GM-CSF experiments, bone marrow was cultured at 2 × 10<sup>5</sup> cells/mL unless otherwise indicated. For T-CM treatments, bone marrow was supplemented with 25% T-CM unless otherwise indicated.

Tumor injection and tissue processing

Mice were injected with 1 to 5 × 10<sup>6</sup> mammary tumor cells subcutaneously (s.c.) or into the mammary fat-pad (MFP), 1 × 10<sup>3</sup> 4T1 cells s.c. or 7.5 × 10<sup>5</sup> B16 cells s.c. Spleens, femurs, and blood were analyzed once tumors reached 1 cm<sup>3</sup>. Blood was analyzed on the scil Vet abc hematology analyzer (scil animal care company) or treated with NH<sub>4</sub>Cl to lyse RBCs before flow cytometry.

Flow cytometry and fluorescence-activated cell sorting

Cells were incubated with 2.4G2 mAb (Fc block), before Ab addition. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo (TreeStar). Dead cells were excluded using PI or DAPI, RBCs were excluded by lysis/size.

Menin inhibitor treatment of bone marrow cultures

Bone marrow was treated with menin inhibitors, MI-2/MI-3, on d0 of culture and analyzed on d3 for colony forming cells (CFC). Alternatively, inhibitors were added on d0 and cells were analyzed on d8 by flow cytometry and qPCR. For Hoxa9 qPCR, cells were treated for 24 hours with MI-2/MI-3.

Statistical analysis

Statistical comparisons were conducted using unpaired t test. Error bars and ± symbols represent SEM. *, P < 0.05, **, P < 0.01, ***, P < 0.001, and ****, P < 0.0001.

Reagents, antibodies, competitive-reconstitution, CFU-S<sub>5/6</sub> assay, chromatin immunoprecipitation (ChIP), qPCR, methylcellulose assay, and Western blot

See supplementary materials and methods.

Results

Mammary tumors induce widespread changes in hematopoiesis

Cancer is frequently associated with immune system perturbations, with the tumor microenvironment known to reprogram myeloid cells into MDSCs, TAMs, and/or DCs. We used previously described MMTV-neu<sup>OTIS/OTII</sup> mammary tumor lines (MT1–4) that are syngeneic to C57BL/6-MMTV-neu<sup>OTIS/OTII</sup> transgenic mice (18, 19). Tumor development was associated...
with a 3- to 7-fold increase in white blood cells (WBC) due to a 7- to 10-fold increase in granulocytes. We also observed decreased hematocrit, red blood cells (RBC), hemoglobin (Hgb), and platelets (PLT) in mammary tumor-bearing mice (Table 1). Changes in monocyte and granulocyte frequency/numbers were confirmed by flow cytometry of blood, bone marrow, and spleen (Supplementary Fig. S1A). We observed increased splenic cellularity, monocytes (CD11b<sup>hi</sup>/Gr-1<sup>low/neg</sup>) and granulocytes (CD11b<sup>low/med</sup>/CD11b<sup>hi</sup>/Gr-1<sup>low</sup>) (Supplementary Fig. S1B). By contrast, mammary tumor-bearing mouse bone marrow was hypocellular, with fewer monocytes (Supplementary Fig. S1B). Increased proportions and numbers of granulocytes and WBCs were observed in immunocompetent (syngeneic MMTV-neuOTI/OTII) or noncompromised (RAG1<sup>–/–</sup>) mammary tumor-bearing mice independent of injection site (MFP or s.c.; Supplementary Fig. S1A) and spleen also possessed more long-term HSCs (LT-HSCs) (LSK, c-Kit<sup>hi</sup>/Sca-1<sup>hi</sup>/Flt3<sup>lo</sup>) were increased in spleens, bone marrow and spleen of mammary tumor-bearing mice (Table 1). Blood was obtained from cardiac puncture and analyzed on a scil Vet abc hematology analyzer, scil animal care company, Gurnee, IL.

### Table 1. Hematologic analyses of MT-1-bearing mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MT-1</th>
<th>B6</th>
<th>MT-1</th>
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<tbody>
<tr>
<td>WBC, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>3.7 ± 0.5</td>
<td>19.8 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 0.9</td>
<td>30.8 ± 6.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBC, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>9.9 ± 0.1</td>
<td>8.3 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.1</td>
<td>9.2 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hgb, g/dL</td>
<td>14.9 ± 0.1</td>
<td>12.6 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.0 ± 0.2</td>
<td>13.5 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCT, %</td>
<td>46.4 ± 0.6</td>
<td>39.1 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.8 ± 1.1</td>
<td>41.7 ± 1.7 (P = 0.057)</td>
</tr>
<tr>
<td>PLT, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>1103 ± 59</td>
<td>884 ± 57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1119 ± 30</td>
<td>866 ± 46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LYM, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>4.7 ± 0.8</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>MON, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>0.3 ± 0.0</td>
<td>0.7 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8 ± 0.1</td>
<td>1.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GRA, 10&lt;sup&gt;3&lt;/sup&gt;/μL</td>
<td>2.5 ± 0.3</td>
<td>18.3 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.2</td>
<td>23.9 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

**NOTE:** RAG1<sup>–/–</sup>: Control n = 13, MT-1 n = 12; B6: Control n = 4, MT-1 n = 4. Blood was obtained from cardiac puncture and analyzed on a scil Vet abc hematology analyzer, scil animal care company, Gurnee, IL. Abbreviations: GRA, granulocyte; HCT, hematocrit; Hgb, hemoglobin; LYM, lymphocyte; MON, monocyte; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

<sup>a</sup>P < 0.0001.
<sup>b</sup>P < 0.05.
<sup>c</sup>P < 0.001.
<sup>d</sup>P < 0.01.
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Figure 1. Mammary tumor development is associated with impaired bone marrow erythropoiesis and compensatory splenic erythropoiesis. A, femurs from control and MT-1-bearing mice. H&E stained bone marrow sections imaged at ×10 and ×40 original magnification. B, frequencies of CD71+/Ter119−, CD71+/Ter119+, CD71−/Ter119−, and CD71−/Ter119+ (erythroid populations) in bone marrow of control and MT-1-bearing mice. C, frequencies of splenic erythroid populations in control and MT-1-bearing mice. D and E, numbers of erythroid populations in the bone marrow of RAG1−/− mice (D) or MMTV-neuOTI/OTII mice (E). F, numbers of erythroid populations in spleens of control and MT-1 (MMTV-neuOTI/OTII, RAG1−/−) or control and B16 tumor-bearing (C57BL/6, RAG1−/−) mice. B6, MMTV-neuOTI/OTII or C57BL/6 for MT-1 or B16 tumor-bearing mice, respectively. Data represent 3 experiments, n = 3–4.

(CMP), or granulocyte/macrophage progenitors (GMP; Figs. 2B and 1B, D, and E). By contrast, spleens contained abundant MEPs correlating with increased erythroblasts (Figs. 2C and 1C and F). Changes in HSPCs were observed in mice bearing each of the 4 mammary tumors and 4T1s, irrespective of injection site or genetic background (C57BL/6 or RAG1−/−; Supplementary Fig. S2C, data not shown). B16 tumor-bearing mice did not exhibit changes in HSPC frequency/numbers (Fig. 2A and Supplementary Fig. S3B, data not shown).

To further investigate the impact of mammary tumor growth on HSPCs, we assessed progenitor numbers using the methylcellulose colony-forming and CFU-S12 assays, and stem cell functionality by competitive reconstitution in radiation chimeric mice. Methylcellulose assays revealed increased splenic myeloid progenitors in mammary tumor–bearing mice, correlating with increased LKs (Figs. 3A and 2C). By contrast, bone marrow CFU-G/M/GM numbers were similar in control and mammary tumor–bearing mice (Fig. 3A). The CFU-S12 assay identifies primitive multipotent progenitor populations (21). Surprisingly, bone marrow from mammary tumor–bearing mice exhibited a 5- to 10-fold loss in primitive progenitor activity (Fig. 3B), whereas spleens from mammary tumor–bearing mice possessed large numbers of progenitors, correlating with increased splenic HSPCs (Figs. 3B and 2C). Competitive reconstitution experiments with mixtures of control and mammary tumor–bearing mouse bone marrow or splenocytes revealed that the bone marrow LT-HSC population was intact in mammary tumor–bearing mice, with equal contributions from cells of control or mammary tumor–bearing mice observed 3 to 20 weeks following reconstitution (Fig. 3C). By contrast, whereas control splenocytes provided only short-term repopulation, mammary tumor–bearing mouse splenocytes contained multipotent stem cells capable of myeloid/lymphoid long-term reconstitution (Fig. 3C). Therefore, mammary tumor growth leads to perturbations in the bone marrow HSPC compartment with accompanying extramedullary hematopoiesis.

Conditioned media from mammary tumors, but not other tumor types, induces bone marrow proliferation and expansion of hematopoietic progenitors in vitro

Our results suggested that either mammary tumors impacted HSPCs at sites distant from the tumor or that mammary tumors had metastasized to bone marrow. Histologic examination of bone marrow, and PCR for the tumor-specific neuONMT transgene in RAG1−/− recipients, failed to reveal bone marrow metastasis (Fig. 1A, data not shown), suggesting that mammary tumors influenced stem/progenitor numbers and functionality from a distance, that is via tumor-derived soluble factors. Indeed, recent research suggests that breast cancers can induce changes in HSPC gene expression in a systemic manner (22). We therefore investigated whether culturing bone marrow with tumor cells, or in medium supplemented with mammary tumor–conditioned media (MT-CM), would impact stem/progenitor cell numbers. Culture of bone marrow with mammary tumor cells or MT-CM in the absence of supportive cytokines led to a 2- to 3-fold increase in cellularity but did not increase LSK/LKs or progenitor activity (Fig. 4A, data not shown).
We next investigated whether MT-CM could synergize with cytokines known to facilitate HSPC survival and/or expansion, that is FLT3L, GM-CSF, IL-3, IL-6, TPO, M-CSF, G-CSF, or a combination of IL-3/IL-6/SCF. Analysis of bone marrow cultures revealed that FLT3L synergized with MT-CM, dramatically increasing culture cellularity and LSK/LK numbers (Fig. 4A and E). Increased total cellularity, LSKs, LKs, CMPs, and GMPs in cultures supplemented with FLT3L and MT-CM was detected by day 3 (Fig. 4E). GM-CSF synergized with MT-CM less effectively than FLT3L, where total cellularity increased from day 3 to 9, whereas LSKs peaked at day 3 (Supplementary Fig. S4A). The ability of MT-CM to increase cellularity, LSKs and LKs in FLT3L bone marrow cultures was shared with T-CM from all analyzed mammary tumor cell lines (MT-1, 4T1), whereas melanoma (B16) and colon cancer (CMT93) T-CM was ineffective (Fig. 4B, data not shown).

To determine whether the appearance of bone marrow cells with stem/progenitor cell markers correlated with progenitor activity, we cultured bone marrow in MT-CM and GM-CSF or FLT3L for 3 or 8 days and enumerated methylcellulose CFU-G/M colonies. FLT3L bone marrow cultures containing MT-CM had a ≥400-fold increase in CFCs compared with FLT3L.

Figure 2. Perturbations in hematopoietic stem/progenitor cell numbers and location in mammary tumor-bearing mice. A, classification of HSPCs. Bone marrow from control and MT-1 or B16 tumor-bearing RAG1−/− mice was stained with mAbs recognizing Lin-specific antigens, as well as Sca-1, c-Kit, CD34, FcγRII/III and Flt3, and Lin−DAPI− cells were analyzed by flow cytometry. B, total numbers of bone marrow and HSPCs subsets; LSK (Lin−Sca−1−c-Kit−), LK (Lin−Sca−1−c-Kit−), LT-HSC (LSK,Flt3+CD34+), ST-HSC (LSK,Flt3−CD34+), MPP (LSK,Flt3−CD34−), CMP (LK, FcγRII/III−CD34+), GMP (LK, FcγRII/III−CD34−), and MEP (LK, FcγRII/III−CD34−). C, splenocytes and HSPCs were analyzed as in B. B6, MMTV-neu+/+Data represent ≥3 experiments, n = 3.
alone cultures, and a >20-fold increase in CFCs compared with an equivalent number of freshly harvested and plated bone marrow cells, showing expansion of progenitors upon exposure to MT-CM (Fig. 4D). CFCs in GM-CSF and MT-CM bone marrow cultures were increased 3- to 4-fold (Fig. 4D). To investigate whether MT-CM was regulating progenitor survival, apoptosis, and/or proliferation, we analyzed Ki67 expression or Annexin V and DAPI staining in day 3 MT-CM-treated FLT3L and GM-CSF cultures. MT-CM treatment led to increased Ki67-expressing LSKs and LKs (FLT3L cultures) without affecting HSPC apoptosis (Supplementary Fig. S5A and Fig. 5B). However, a small but significant reduction in DAPI+ LSKs was evident in MT-CM treated GM-CSF and FLT3L cultures (Supplementary Fig. S5C).

Multilineage hematopoietic system reconstitution is the defining characteristic of HSCs, therefore LSKs or unfractionated bone marrow cells from day 8 FLT3L MT-CM-treated cultures were injected into lethally irradiated C57BL/6 or sublethally irradiated RAG1−/− mice were transplanted into lethally irradiated CD45.1+ recipients at a 1:1 ratio with CD45.1+ carrier cells. Engraftment efficiency was assessed by the relative frequency of CD45.2+ versus CD45.1+ cells in blood at 3, 8, and 20 weeks. Percentages of CD45.2+ nucleated P1+ cells are shown. *, euthanized due to lack of reconstitution. Data represent ≥ 2 experiments, n = 3–5.

Mammary tumors alter histone methylation patterns associated with hematopoietic regulatory genes

The homeobox gene cluster plays key roles in hematopoiesis and leukemic transformation. Tumors have been shown to
Table 1. Summary of cell numbers in different conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells (x10^6)</th>
<th>LSKs</th>
<th>LKs</th>
<th>CMPs</th>
<th>GMPs</th>
<th>CD11b^+CD115^hi</th>
<th>CD11b^+Gr-1^hi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>MT-1</td>
<td>4.0</td>
<td>3.0</td>
<td>2.0</td>
<td>1.5</td>
<td>0.8</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>FLT3L</td>
<td>5.0</td>
<td>4.0</td>
<td>3.0</td>
<td>2.5</td>
<td>1.6</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6.0</td>
<td>5.0</td>
<td>4.0</td>
<td>3.5</td>
<td>2.8</td>
<td>1.5</td>
<td>0.8</td>
</tr>
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</table>

Figure 4. Mammary tumor-conditioned media expands hematopoietic progenitors in bone marrow cultures supplemented with FLT3L or GM-CSF. A, total cells and LSKs in bone marrow cultured in media containing the indicated cytokines, plated at 1 x 10^6 cells/mL, supplemented with MT-1-conditioned media (MT-1-CM). B, bone marrow was cultured in FLT3L and GM-CSF from the indicated tumor cell lines and total cellularity and LSKs were enumerated. C, as in A, except bone marrow was cultured with FLT3L and the indicated cytokines or MT-1-CM. D, total CFCs in FLT3L or GM-CSF bone marrow cultures supernatant over a period of 9 days. E, total cells, HSPCs (LSK, LK, CMP, GMP), CD11b^+CD115^hi, and CD11b^+Gr-1^hi cells in FLT3L bone marrow cultures supplemented with MT-1-CM over a period of 9 days. F, total cells, LSKs, and LKs in FLT3L bone marrow cultures supplemented with 1.6% MT-1-CM, treated with G-CSF or IL-6 neutralizing Abs (or isotype-control Abs, iso). G, CFCs were enumerated in control and G-CSF or IL-6 neutralizing Ab treated cultures as in D, except with 1.6% MT-1-CM. Data represent ≥ 3 experiments. MT-1-CM is designated as MT-1.
alter gene expression in bone marrow myeloid cells (25). The similarities between mammary tumor–bearing mice and mice with leukemia/myeloproliferative disease led us to examine tumor induction of Hox genes. Hoxa7, Hoxa9, and Hoxa10 mRNAs were overexpressed in MT-CM-treated FLT3L and GM-CSF bone marrow cultures (Fig. 5A). Importantly, Hoxa9 expression in Lin− bone marrow cells from mammary tumor–bearing mice was also greatly elevated in vivo (Fig. 5B).

Activating and inhibitory epigenetic regulation controls Hoxa9 expression. Interestingly, expression of Ezh2, the methyl-transferase component of PRC2, was decreased in MT-CM-treated FLT3L and GM-CSF bone marrow cultures (Supplementary Fig. S6A). Furthermore, expression of the PRC2 complex components Ezh1, Suz12, and Eed were reduced in MT-CM-treated FLT3L cultures (Supplementary Fig. S6A). RNA levels of the H3K27 demethylase, Jmjd3, and H3K4 methyltransferases, Mll1-5, and the Mll complex component, Rbbp5, were decreased in MT-CM-treated FLT3L bone marrow cultures (Fig. 5A and Supplementary Fig. S6A). Conversely, in GM-CSF cultures, all other epigenetic regulators, including Mll1 and Mll3, were unchanged when treated with MT-CM (Supplementary Fig. S6A). We also detected reduced Ezh2 mRNA in Lin− bone marrow cells from tumor-bearing mice, and a corresponding reduction in the EZH2 product H3K27me3 in bone marrow LKs (MFI ± SD, control: 5.526 ± 864; MT-1-bearing mice: 2.996 ± 706, P = 0.018; Fig. 5B). By contrast, H3K4me3 levels were indistinguishable in LKs from control and tumor-bearing mice (Fig. 5B). Neutralization of G-CSF, but not GM-CSF or M-CSF, in bone marrow cultures containing 1.6% MT-CM reduced Hoxa9 mRNA levels and partially restored Ezh2 mRNA to control levels, suggesting that G-CSF in MT-CM regulates Hoxa9 expression (Fig. 5C and Supplementary Fig. S4D). Importantly, analysis of total H3K27me3 in MT-CM-treated GM-CSF bone marrow cultures revealed a dose-dependent reduction in H3K27me3, correlating with reduced H3K27me3 levels in tumor-bearing mouse bone marrow LK cells (Fig. 5D). Similarly, H3K27me3 was reduced in FLT3L bone marrow cells cultured in MT-CM, which correlated with reduced EZH2/1 protein (Supplementary Fig. S6B and S6C).

Loss of the PRC2 (EZH2)-dependent epigenetic mark H3K27me3, and/or gain of H3K4me3 associated with the Hoxa9 locus correlate with Hoxa9 expression (26, 27). Consistent with diminished Ezh2 expression in bone marrow cultured with FLT3L or GM-CSF and MT-CM, H3K27me3 associated with the Hoxa9 locus (11) was decreased at amplicon A in FLT3L and GM-CSF cultures (Fig. 5E and F) and variably decreased at the other amplicons (Supplementary Fig. S7A). Conversely, we observed increased H3K4me3 associated with multiple regions of the Hoxa9 locus (amplicons A–E; refs. 28, 29) correlating with enhanced Hoxa9 expression (Fig. 5E and F and Supplementary Fig. S7A).

Neutralization of G-CSF in FLT3L bone marrow cultures treated with 1.6% MT-CM (Supplementary Fig. S4D) reduced H3K4me3 at amplicons A and C (Supplementary Fig. S7B). However, 1.6% MT-CM did not attenuate H3K27me3 at amplicon A, nor was neutralization of G-CSF able to restore H3K27me3 at amplicon C (Supplementary Fig. S7B). Addition of G-CSF to FLT3L and GM-CSF bone marrow cultures decreased H3K27me3, enhanced Hoxa9 expression, and increased CFC progenitor content, suggesting that mammary tumor–secreted G-CSF is responsible for the majority of MT-CM bioactivity (Fig. 6A and B, data not shown).

To determine whether histone methylation changes were required for MT-CM bioactivity, we used recently described menin inhibitors MI-2 and MI-3, which attenuate H3K4me3 and suppress Hoxa9 expression and leukemic transformation (30). Menin is a key protein required to assemble SET1-like histone methyl-transferase complexes required for H3K4me3 epigenetic remodeling of the Hoxa9 locus (31). We confirmed that MI-3 treatment of bone marrow cultures blocked H3K4me3 (Fig. 6C inset). We detected a dose-dependent reduction in total cellularity and LSK/LKs in MT-CM-treated FLT3L and GM-CSF bone marrow cultures upon treatment with MI-2/MI-3 compared with control inhibitor (MI-nc; Fig. 6C–E, data not shown). In addition, MT-CM-treated bone marrow cultures revealed a diminution of myeloid progenitors, and a reduction in Hoxa9 expression, following inhibitor addition (Fig. 6F and G). Collectively, these results show that G-CSF secreted by mammary tumor cells can synergize with FLT3L and GM-CSF to induce expansion of hematopoietic progenitors with the effects of G-CSF correlating with changes in histone methylation patterns required for Hox gene expression and tumor-induced HSPC expansion.

Discussion

Tumors harness elements of both the innate and adaptive immune systems to facilitate their growth and metastasis. Perturbations in myelopoiesis are common in neoplastic and chronic inflammatory diseases, and may be critically important in disease etiology. Many cancers induce tumor-promoting TAMs, MDSCs, and/or IDCs, and recent reports have revealed how significant these tumor-induced perturbations in the immune system are and have highlighted the need to better understand these perturbations to improve strategies to initiate or reinvigorate antitumor immune responses (32). In the case of breast cancer, the immune cell composition of the tumor may predict treatment outcome (33). As different cancers affect the immune system uniquely, understanding these interactions will help predict patient responses to therapy and to advance immunotherapy strategies.

Here we show that mammary cancer affects the primitive HSPC compartments of the hematopoietic system, leading to a myeloproliferative-like disease characterized by neutrophilia, anemia, and HSPC defects. Importantly, tumor-induced changes were observed using 5 different mammary tumor lines in mice of immune-deficient and -sufficient backgrounds. We established an in vitro system to investigate regulation of HSPCs by mammary tumors and found that MT-CM synergized with FLT3L or GM-CSF to enhance bone marrow proliferation and progenitor cell expansion, an ability shared with every mammary tumor line analyzed. By contrast, melanoma and colon cancer cells lacked this activity in vitro and melanoma cells did not significantly perturb myelopoiesis in vivo. Our data indicates that different tumor types interact with the immune system in unique ways, and that disruption of hematopoiesis is a common mammary tumor characteristic.
Figure 5. Mammary tumor growth is associated with enhanced Hoxa family gene expression and changes in histone methylation at the Hox9 locus. A, Ezh2, Jmjd3, Hoxa7, Hoxa9, and Hoxa10 expression in FLT3L and GM-CSF bone marrow cultures + MT-1-CM. B, Hox9 and Ezh2 expression in sorted Lin- bone marrow cells (upper), and H3K4me3 and H3K27me3 levels in bone marrow LKS from control and MT-1-bearing mice analyzed by intracellular flow cytometry (lower). C, Hox9 and Ezh2 expression in FLT3L bone marrow cultures treated with 1.6% MT-1-CM and isotype control (iso) or neutralizing Abs against G-CSF. D, lysates of GM-CSF bone marrow cells treated with an increasing concentration of MT-1-CM for 8 days were Western blotted for H3K27me3, histone H3, and β-actin. E, depiction of amplicons (A–E) in the Hox9 locus for ChIP analysis. F, repressive (H3K27me3-amplicon A) and activating (H3K4me3-amplicons B–E) histone modifications in FLT3L or GM-CSF bone marrow cultures + MT-1-CM were investigated by ChIP. Amplicons A–E were analyzed by qPCR. MT-1-CM is designated as MT-1. Data represent ≥ 3 experiments.
Identification of HSPCs requires functional and phenotypic assessment. We ensured that LSK/LKs were not contaminated with differentiated cells such as dendritic cells or mast cells, however it was possible that LSK/LKs might express HSPC markers but lack HSPC activity. HSPC competitive reconstitution in chimeric mice showed that the bone marrow LT-HSC compartment was intact in tumor-bearing mice, consistent with LT-HSC numbers in the bone marrow. However, restricted primitive bone marrow progenitors from the CFU-S<sub>12</sub> assay were greatly reduced by mammary tumor growth. By contrast, spleens of mammary tumor–bearing mice contained large numbers of LSK/LKs that could reconstitute the myeloid and lymphoid compartments of lethally irradiated recipients, and contained abundant primitive CFU-S<sub>12</sub> colony-forming progenitors and more restricted myeloid progenitors. Therefore, LSK/LKs in tumor-bearing mice possessed HSPC activity. Although cells propagated for 8 days in vitro in MT-CM and FLT3L expressed LSK/LK markers and included large numbers of myeloid CFCs, they could not reconstitute lethally or sublethally irradiated mice. Notably, actively cycling HSCs and HSCs in inflammatory environments may be functionally defective (34, 35).

Our results suggest that tumor development induces mobilization and proliferation of the stem cell compartment with inappropriate seeding of spleen. This deviation in hematopoiesis by tumor-secreted factors, and compensatory splenic erythroid output, may partially explain the HSPC irregularities (36). Similar to our findings, an investigation of murine lung adenocarcinoma revealed tumor-induced extramedullary hematopoiesis in the spleen through migration of HSCs from the bone marrow to the spleen, resulting in production of TAMs and neutrophils (37). However, tumor-induced effects on erythropoiesis were not examined.

An alternative explanation for HSPC mobilization to spleen may correspond to tumor-induced loss of erythropoietic potential in the bone marrow, as bone marrow MEPs and erythroblasts at various stages of development were dramatically reduced in mammary tumor–bearing mice. Interestingly, patients with preexisting anemia before therapy exhibit reduced survival following treatment for breast, lung, prostate, and other cancers (4, 38). Our results support the hypothesis that breast cancer patients may develop anemia concomitant with disease severity or progression, a characteristic that may impact treatment options.

Tumors can express cytokines and factors important for tumor growth, survival, or spread, for example the 4T1 mammary tumor line expresses GM-CSF, G-CSF, and M-CSF associated with a leukemoid reaction (39). We showed that G-CSF...
was an abundant factor secreted by mammary tumors, and used loss- and gain-of-function experiments to confirm that G-CSF present in MT-CM synergized with FLT3L and GM-CSF to stimulate increases in LSKs, Lks, CFCs, and Hoxa9 expression, in vitro. Interestingly, Waight and colleagues used loss- and gain-of-function experiments to show that tumor-secreted G-CSF increases tumor growth, splenomegaly, and MDSC production in mice, although the underlying mechanisms leading to these changes were not explored (40). G-CSF can also impair erythropoiesis and synergize with FLT3L to mobilize stem cells (41).

G-CSF is produced by many human tumors, including pancreatic, cervical, bladder, and breast (42, 43). G-CSF is elevated in breast carcinomas compared with healthy breast tissue (44). Similarly, G-CSF mRNA is elevated in breast cancer tissues compared with surrounding healthy tissues and G-CSF levels were higher in the serum of breast cancer patients compared with healthy controls (45). The association between increased G-CSF and breast cancer clinical grade and lymph node infiltration has been established, further supporting its role in tumor progression and disease severity (46, 47). Therefore, G-CSF may be useful in the diagnosis of breast cancer (45). Breast carcinoma activation of bone marrow cells and progenitors of hematopoietic origin can instigate the growth of indolent tumors and metastases, therefore our results suggest that the effect of G-CSF on HSPCs and myeloid cells may enhance metastases (48). In addition, mammary tumor-derived G-CSF may be important in regulating bone marrow and spleen stromal elements controlling HSPC release or receptivity, a possibility we are exploring.

G-CSF is a secreted glycoprotein that mobilizes hematopoietic stem cells, progenitors, mature cells, and stimulates granulocyte differentiation. G-CSF has been used to treat radiation- and chemotherapy-induced neutropenia. However, G-CSF promotes the proliferation and invasion/angiogenesis of various human tumors (45) and G-CSF-producing malignant tumors are associated with poor clinical outcome (49). The use of G-CSF to treat cancer patients underscores the need to better understand its effect on hematopoiesis, metastasis, and antitumor immune responses.

The phenotypic similarities between mammary tumor-bearing mice and mice with myeloproliferative/myelodysplastic syndrome (leukemia reaction, HSPC defects) suggested that mammary tumors modulate key hematopoietic regulatory genes. Ezh2 loss-of-function mutations are associated with myeloid disorders (8) and Hox genes are critical regulators of HSC renewal, HSPC expansion (50), and differentiation repression (16). HOXA9 downregulation promotes hematopoietic cell differentiation whereas its overexpression is linked to acute myeloid leukemia (16, 26). We showed that mammary tumor growth was associated with enhanced Hoxa9 expression in bone marrow Lin^{-} cells. Moreover, Hox genes were overexpressed, whereas Ezh2 was reduced in MT-CM-treated bone marrow cultures. Diminished Ezh2 expression correlated with reduced H3K27me3 in vitro and in vivo. In addition, H3K27me3 associated with the Hoxa9 locus (11) was diminished correlating with enhanced Hoxa9 expression.

H3K4me3 is an activating epigenetic histone modification catalyzed by MLL family members and coordinated by menin. Hoxa9 expression correlated with increased H3K4me3 at multiple regions of the Hoxa9 locus in MT-CM-treated cells, suggesting that increased H3K4me3 and diminished H3K27me3 are associated with tumor-induced changes in Hox expression. Addition of H3K4me3 inhibitors (30) to MT-CM-treated bone marrow cultures reduced culture cellularity and progenitor content. Taken together, our data links H3K4me3 regulation of the Hoxa9 locus with the induction of HSPC expansion by tumor-secreted factors such as G-CSF. Our results suggest that therapeutic strategies for treating myeloid neoplasia may also be effective at attenuating tumor-induced hematological defects.

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

Authors’ Contributions
Conception and design: A. Sio, M.K. Chehal, K. Tsai, X. Fan, J. Grembecka, D.L. Krebs, K.W. Harder
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Sio, M.K. Chehal, K. Tsai, X. Fan, B.H. Nelson, D.L. Krebs, K.W. Harder
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Sio, M.K. Chehal, K. Tsai, X. Fan, D.L. Krebs, K.W. Harder
Writing, review, and/or revision of the manuscript: A. Sio, M.K. Chehal, K. Tsai, M.E. Roberts, D.L. Krebs, K.W. Harder
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.K. Chehal, D.L. Krebs, K.W. Harder
Study supervision: K.W. Harder

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Dysregulated Hematopoiesis Caused by Mammary Cancer Is Associated with Epigenetic Changes and Hox Gene Expression in Hematopoietic Cells

Alexander Sio, Manreet K. Chehal, Kevin Tsai, et al.


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