Microenvironment and Immunology

Estrogen Promotes ER-Negative Tumor Growth and Angiogenesis through Mobilization of Bone Marrow-Derived Monocytes

Vandana Iyer1,2, Ina Klebba1,2, Jessica McCreary1,2, Lisa M. Arendt1,2, Monica Betancur-Boissel2, Meng-Fen Wu3,4, Xiaomei Zhang4, Michael T. Lewis4, and Charlotte Kuperwasser1,2

Abstract
Estrogen has a central role in the genesis and progression of breast cancers whether they are positive or negative for the estrogen receptor (ER). While therapies that disrupt estrogen biosynthesis or ER activity can treat these diseases in postmenopausal women, in younger women where ovarian function remains intact, these anti-estrogen therapies are not as effective. Moreover, emerging clinical evidence suggests that estrogen may promote other cancers. Thus, circulating estrogens may participate in cancer pathogenesis in ways that are not yet understood. In this study, we show that estrogen can promote the outgrowth of murine xenograft tumors established from patient-derived ER-negative breast cancer cells by influencing the mobilization and recruitment of a proangiogenic population of bone marrow–derived myeloid cells. ERα expression was necessary and sufficient in the bone marrow–derived cells themselves to promote tumor formation in response to estrogen. Our findings reveal a novel way in which estrogen promotes tumor formation, with implications for the development and application of anti-estrogen therapies to treat cancer in premenopausal women. Cancer Res; 1-9. ©2012 AACR.

Introduction
A wealth of evidence indicates that the steroid hormone estrogen contributes to the etiology and behavior of estrogen receptor (ER)-positive breast cancers; this has led to the successes in anti-estrogen therapies for the standard treatment of ER-positive breast cancer (1). However, estrogen has also been suggested to play a role in the genesis and behavior of breast cancers that lack ER expression or express ER in very few cells (2–4). Interestingly, anti-estrogen therapy has also been used in the treatment of cancers not traditionally considered hormone-responsive (e.g., lung cancer, hepatocellular carcinoma, desmoid tumors, malignant glioma, pancreatic carcinoma, melanoma, and renal cell carcinoma; refs. 5–7). The mechanism by which ER-negative tumors respond to anti-estrogen treatment is unknown, but ER is abundantly expressed in many stromal cells including endothelial cells, neurons, immune cells, bone mesenchyme, adipocytes, and even breast fibroblasts (8–13). Thus, it is highly plausible that estrogen can act on these stromal cells in many tissues, which could foster a microenvironment conducive for tumor growth.

All of the various physiologic actions of estrogen are thought to be mediated through ERα and ERβ, which function as ligand-activated transcription factors (14). Much of the effects of the ERs have been elucidated through examination of ER knockout mice (ERKO). Both ERKOα and ERKOβ mice have been generated through germ line deletion of ERα and ERβ and, while viable, they display significant reproductive abnormalities (8). In addition, ERKOα and ERKOβ mice also have defects in the cardiovascular, skeletal, and immune systems, underscoring the importance of ER in the physiology of other, nonreproductive, tissue types. Specifically, ERKOα mice are defective in estrogen-induced angiogenesis, vascular wound repair, and bone formation. They also exhibit a myeloproliferative disease and have alterations in neurologic behavior (8–12).

Many solid tumors, including those of the breast, are associated with a strong stromal response characterized by the infiltration of bone marrow–derived cells (BMDC; refs. 15, 16). Recruited BMDCs contribute to tumor growth and wound healing by providing epithelial cells with the growth factors and cytokines to stimulate proliferation and by enhancing vasculogenesis and angiogenesis (17–20). Most immune cells, including myeloid progenitors, monocytes, and macrophages, express ER, suggesting that these cells are sensitive to estrogen. However, it is not well understood which of these cells may increase tumorigenesis in response to estrogen. In this study,
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<th>With estradiol pellet</th>
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<tr>
<td></td>
<td>Frequency in the population (%)</td>
<td>Primary outgrowth take rate (%)</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>Positive</td>
<td>18/38 (47.4)</td>
<td>8/18 (44.4)</td>
</tr>
<tr>
<td>Negative</td>
<td>20/38 (52.6)</td>
<td>10/20 (50)</td>
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<tr>
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<td>4/12 (33.3)</td>
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<tr>
<td>Negative</td>
<td>26/38 (68.4)</td>
<td>14/26 (53.8)</td>
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*The frequency of tumor take is significantly different within the indicated parameter.*

**B**

<table>
<thead>
<tr>
<th>SUM1315</th>
<th>DU4475</th>
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**C**

<table>
<thead>
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we sought to determine the mechanism by which estrogen promotes ER-negative tumor formation and whether this activity is dependent on ERs.

Materials and Methods

Cell culture

SUM1315 cells were provided by Dr. Stephen Ethier (Karmanos Institute, Detroit, MI) and cultured in Ham/F12 media supplemented with 5% calf serum (CS; Thermo Scientific Hyclone), 5 μg/ml insulin (Sigma-Aldrich), 10 ng/ml hEGF (Sigma), and 1% antibiotic/antimycotic (Invitrogen). RAW264.7, DU4475, and PC-3 cells were purchased from the American Type Culture Collection. RAW264.7 and DU4475 cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimycotic. PC-3 cells were cultured in F12 supplemented with 10% FBS (Invitrogen) and 1% antibiotic/antimycotic. All cell lines were grown at 37°C and 5% CO₂ and passaged for less than 3 weeks. All cell lines were obtained in 2005 and tested negative for mycoplasma (MilliPROBE; Millipore); however the identity of each cell line was not authenticated in our laboratory.

Bone marrow transplantation and analyses

Bone marrow cells were flushed from donor femurs, red blood cells were lysed, and 5 x 10⁶ bone marrow cells were injected. Cells were injected into sublethally irradiated (3.50-Gy γ-radiation from a cesium source) mice by tail vein injection. Reconstitution was confirmed after 4 weeks by assaying the percentage of GFP⁺ or red fluorescent protein (RFP)⁺ cells in the blood by flow cytometry.

To quantitate leukocyte populations within the bone marrow, cells were incubated with conjugated antibodies (Supplementary Methods). Data were collected using a FACSCalibur flow cytometer (Beckton Dickinson) and cytospun for immunofluorescence.

In vivo experiments

Matrigel plug and tumorigenesis assays were completed as described in Supplementary Methods. GFP⁺ cells were FAC-sorted (BD Influx, Beckton Dickinson) and cytospun for immunofluorescence.

Histology and immunostaining

Immunofluorescence was conducted on frozen sections of tumors and Matrigel plugs (Supplementary Methods). Data were blinded and quantified across 4 fields from each frozen section (ImageJ).

Paraffin-embedded tumor tissues were stained for von Willebrand factor (vWF; DAKO), hematoxylin and eosin (H&E), and trichrome by the Histology Special Procedures Laboratory at Tufts Medical Center (Boston, MA) as previously described (16).

Statistical analyses

For tumor incidence, the χ² test was conducted. All other analyses were done using a 2-tailed Student t test. The Fisher exact test was used for comparison of rates of stable transplantation as xenografts with and without estrogen pellets using primary human breast cancers transplanted into the epithelium-free fat pad of SCID.Bg mice.

Results and Discussion

We previously reported that estrogen promotes the growth of human breast tumors that do not express either ERα or ERβ (16). This work showed that increased tumorigenesis in estrogen-treated mice was not due to a direct influence of estrogen on the breast epithelial tumor cells themselves. To exclude the possibility that this effect was unique to the engineered and somewhat artificial cell line used in this study, we examined whether estrogen could promote tumor formation using other human cancer cell lines that do not express ERα or that are insensitive to the mitogenic effects of estrogen (SUM1315, DU4475, PC-3 cells; Supplementary Fig. S1A and S1B). In addition, we conducted an interim statistical analysis of an ongoing study using patient-derived primary breast tumors to establish xenografts in which ER-positive and ER-negative breast cancers were transplanted into the epithelium-free mammary fat pad of mice, with (N = 72) and without supplemental E2 pellets (N = 38). Consistent with our previous findings, increasing the levels of circulating estrogen resulted in a marked acceleration of tumor growth (Fig. 1). In patient-derived xenografts, supplementation with 17β-estradiol (E2) had a significant effect on increasing both primary outgrowth rates and increasing rates of stable transplantation of ER-negative [and progesterone receptor (PR)-negative] xenografts (P = 0.0002). Only 1 of 20 (5%) ER-negative patient-derived tumors successfully transplanted without E2 supplementation yielding a stable xenograft whereas 17 of 31 (54.8%) of ER-negative patients yielded a stable xenograft in the presence of E2 supplementation (Fig. 1A). In contrast, E2 supplementation had no effect on the rate of stable transplantation of ER-positive human breast cancers (0 of 18 ER⁺ patients transplanted without E2 supplementation vs. 1 of 41 ER⁺ patients transplanted with E2 supplementation, P = 1.0). Likewise, ER-negative SUM1315 tumors grown in E2-treated mice showed a significant increase in tumor volume (P = 0.001) and tumor weight (P = 0.002) compared with those in placebo-treated mice (Fig. 1B). Similarly, E2 significantly increased incidence of ER-negative DU4475 tumors (P = 0.05; Fig. 1B); 75% (6 of 8) of tumors grew in E2-treated mice whereas only 14% (1 of 7) of tumors grew in placebo-treated mice.

Figure 1. Estrogen promotes growth, angiogenesis, and stromalization of ER-negative tumors. A, primary human breast tumor xenograft take rates based on hormone receptor status in mice implanted in the absence or presence of 17β-estradiol. B, growth curves and weights of SUM1315 and DU4475 tumors from placebo or 17β-estradiol-treated mice. C, representative images of H&E, trichrome, and vWF staining from DU4475 tumors. Arrows indicate vWF-stained vessels. D, representative immunofluorescence images of CD45 and αSMA expression in DU4475 tumors from 17β-estradiol-treated mice.
Because tumors in all of these various models were insensitive to estrogen-mediated proliferation (Supplementary Fig. S1), it was unlikely that E2 acted directly on the carcinoma cells to promote tumor growth. An extensive body of clinical and basic research has implicated estrogen (21) in promoting physiologic angiogenesis through a variety of mechanisms that operate independently of cancer growth. Therefore, we examined the tumor microenvironment for evidence that E2 may be altering the composition of the stroma. I&H, Masson trichrome, and staining for vWF confirmed that tumors from E2-treated mice showed increased matrix deposition, stromal condensation, and angiogenesis (Fig. 1C). Immunofluorescence confirmed the recruitment of hematopoietic (CD45) and smooth muscle/myofibroblastic (αSMA) lineages within the tumor-associated stroma of E2-treated mice (Fig. 1D).

Estrogen has been shown to mobilize bone marrow cells to sites of tumor growth and angiogenesis (16, 22). To determine which cells are recruited in response to E2, we used Matrigel plug assays, which model wound healing and neoangiogenesis (15, 16). We observed a 2.1-fold increase in the total number of stromal cells (P = 0.03) recruited in response to E2 as well as a 2.5-fold increase in the number of F4/80+ macrophages (P = 0.03) and a 2.3-fold increase in the number of CD31+ endothelial cells (Fig. 2A). To specifically address which BMDCs were recruited, mice were transplanted with GFP+ bone marrow cells and subcutaneously injected with Matrigel following confirmation of bone marrow engraftment. Consistent with these findings, we also observed an increase in the total number of GFP+ BMDCs recruited to the Matrigel plugs; the majority of these BMDCs were of the monocyte/myeloid lineage (Fig. 2B). Although E2 supplementation increased the total number of cells recruited to the Matrigel plugs, the characteristics of the cells that were recruited were not significantly different in the presence of E2 (Fig. 2B). Nearly all of the GFP+ BMDCs were F4/80+ macrophages, with a subpopulation of 41% also expressing LYVE-1, consistent with an activated macrophage phenotype (23). Hundred percent of GFP+ BMDCs expressed ERα, whereas 89% of GFP+ cells also expressed CD45 (Fig. 2B). Rarely did we observe differentiation of the recruited BMDCs into endothelial, smooth muscle, or myofibroblastic cells (Fig. 2B; Supplementary Fig. S2).

To examine the effect of E2 on BMDC recruitment in tumors, mice were transplanted with GFP+ bone marrow cells and orthotopically injected with DU4475 tumor cells. The resulting tumor-associated stroma showed GFP+ BMDCs that expressed ERα, CD45, and F4/80 but were negative for CD31 expression (Fig. 2C and D). Quantification of sorted and cytospun GFP+ BMDCs from tumors revealed that 78% of the recruited cells were CD45+ and 89% were F4/80+ (Fig. 2D, graph). Furthermore, we observed 2 distinguishable populations of F4/80+ macrophages that differed in LYVE-1 expression: LYVE-1+ macrophages localized at the periphery of the tumor and LYVE-1− macrophages within the tumor mass (Fig. 2C). Non-BMDCs were also found within the tumor-associated stroma: LYVE-1+ lymphatic endothelial cells that did not colocalize with either GFP or F4/80 and GFP-negative αSMA+ myofibroblasts (Fig. 2C). Similar results were obtained using the SUM1315 ER-negative tumor model (Supplementary Fig. S3). These findings, combined with our previous observations (16), reveal that estrogen accelerates the growth of ER-negative tumors by changing the composition and nature of the tumor microenvironment. Furthermore, these results suggest that E2 primarily promotes the recruitment of F4/80+ macrophages to the stromal microenvironment at distant sites, particularly those associated with stromalization/fibrosis, angiogenesis, and tumor formation.

On the basis of these findings, we examined the effect of E2 treatment on macrophage precursor cells within the bone marrow. Although treatment of mice with E2 resulted in a significant upregulation of both ERα and ERβ expression in bone marrow cells (P < 0.0001; Supplementary Fig. S4), there was a significant decrease in cells from the myeloid lineage in the bone marrow [P = 0.03; Fig. 3A (ii)]. Specifically, there was a significant decrease in the (CD31+/Gr1++) monocyte population (P = 0.005) as well as a significant increase in the (CD31+/Gr1−) early blast population [P = 0.05; Fig. 3A (iii)], suggesting that the myeloid lineage is mobilized in response to increases in circulating levels of E2.

To determine whether the response of the CD31−/Gr1++ monocyte population to E2 was dependent on ERα or ERβ, as monocytes/macrophages express both ERα and ERβ (Supplementary Fig. S5), we analyzed bone marrow from E2 or placebo-treated wild-type (WT), ERKOα, and ERKOβ mice by flow cytometry. There was a significant reduction in the proportion of CD31−/Gr1++ monocytes within the bone marrow of WT and ERKOβ mice (P = 0.001 and 0.003) in response to E2 (Fig. 3B), which was not observed in ERKOα mice (Fig. 3B), suggesting that ERα is necessary for E2-mediated mobilization.

To determine whether E2 can indeed increase macrophage migration, RAW264.7 macrophages were treated with vehicle (EtOH), bovine serum albumin (BSA), 1 nmol/L E2, or membrane-impermeable E2 (BSA-E2). Both E2 and BSA-E2 significantly increased migration of cells compared with either vehicle or BSA (P < 0.001 or P < 0.05, respectively; Fig. 3C), suggesting that E2 does increase migration of macrophages.

Given that matrix metalloproteinase (MMP)-9 expression has been reported to be induced upon estrogen treatment and mobilization of various cell types from the bone marrow is dependent on MMP-9 secretion (24), we examined whether mobilization of monocytes from the bone marrow in response to E2 was dependent on MMP-9 expression. Bone marrow from E2 and placebo-treated MMP-9KO mice revealed a similar depletion of CD31−/Gr1++ monocytes as compared with WT mice (P = 0.001; Fig. 3B), suggesting that the mobilization of monocytes in response to E2 does not require MMP-9.

To determine whether ERα expression in the bone marrow cells was necessary for the decrease in CD31−/Gr1++ population following E2 treatment, we conducted bone marrow transplants (BMT) in WT mice. In WT mice transplanted with WT bone marrow, treatment with E2 resulted in a significant increase in the proportion of CD31−/Gr1− early blast cells [P = 0.03; Fig. 3D (ii)] and a significant decrease in the proportion of monocytes [P = 0.001; Fig. 3D (iv)], consistent with findings in nontransplanted WT mice. However, E2 treatment of WT mice reconstituted with ERKOα bone marrow did not result in any
Figure 2. BMDCs recruited to Matrigel plugs and ER-negative tumors in response to estrogen are F4/80⁺ macrophages. A, quantification of cells recruited to Matrigel plugs in placebo or 17β-estradiol–treated mice. B, representative images and quantification of cells recruited to Matrigel plugs harvested from GFP⁺ BMT mice receiving 17β-estradiol or placebo. C, representative images of frozen sections of DU4475 tumors harvested from 17β-estradiol–treated NOD/SCID-GFP BMT mice. Arrows indicate colocalization with GFP⁺ bone marrow cells. D, representative images and quantification of sorted and cytospun GFP⁺ BMDCs isolated from DU4475 tumors harvested from 17β-estradiol–treated NOD/SCID-GFP BMT mice. NOD/SCID, nonobese diabetic/severe combined immunodeficient.
significantly changes within the monocyte/myeloblast lineage [Fig. 3D (i)]. This implies that expression of ERα in bone marrow cells is necessary for E2-mediated mobilization of macrophage precursor cells.

To determine whether the effects of E2 on macrophage recruitment, angiogenesis, stromalization, and tumor formation at distant sites were dependent on the expression of ERα, we conducted Matrigel plug and tumorigenesis assays in...
ERKOα mice treated with E2 or placebo pellets. Unlike plugs from WT mice in response to E2, Matrigel plugs from ERKOα mice did not show an increase in the number of recruited cells (Fig. 4A). In addition, quantification of CD45-, F4/80-, and CD31-expressing cells in plugs from ERKOα females showed no significant increase in bone marrow cells or angiogenesis in response to E2 (Fig. 4A). Moreover, unlike WT mice, SUM1315 tumors did not form in ERKOα females, nor were they accelerated in response to E2 \( P = 0.01 \); Fig. 4B(i) and (ii)], suggesting that ERα is critical in the recruitment of BMDCs for promotion of tumorigenesis.

To determine whether ERα expression within bone marrow cells was sufficient for E2-dependent tumor growth, we conducted tumorigenesis assays in mice that had received reciprocal BMT. WT mice received BMTs from either ERKOα GFP or WT GFP mice, whereas ERKOα mice received BMTs from WT GFP or ERKOα GFP mice. ER-negative cancer cells were injected subcutaneously, and mice were treated with E2. Although we were unable to maintain viable ERKOα mice reconstituted with ERKOα bone marrow, we found that tumors failed to grow in E2-treated WT mice reconstituted with ERKOα bone marrow (Fig. 4C), suggesting that ERα expression in bone marrow cells was necessary for E2-mediated ER-negative tumor growth. Tumor formation was comparable between ERKOα mice transplanted with WT marrow and WT mice transplanted with WT marrow (Fig. 4C). Taken together, these findings show that ERα expression in BMDCs, but not non-

Figure 4. Expression of ERα in BM cells is necessary and sufficient for accelerating macrophage recruitment, stromalization, and ER-negative tumor growth. A, quantification of total cells, hematopoietic (CD45−), macrophage (F4/80−), and endothelial cells (CD31−) cells recruited to Matrigel plugs in 17β-estradiol or placebo-treated WT or ERKOα mice. B, tumor growth curves (i) and final tumor volume (ii) of SUM1315 cells injected into WT or ERKOα mice treated with 17β-estradiol or placebo. C, tumor growth curves of PC-3 cells injected into BMT WT or ERKOα mice treated with 17β-estradiol. BM, bone marrow.
BMD host cells, is necessary and sufficient for E2-mediated tumor promotion of ER-negative cancers.

This study provides evidence to support a novel role for E2 in the pathogenesis of breast cancer through the mobilization of BMD monocytes to distant sites of angiogenesis and early tumor formation. Uptregulation of VEGF-A and/or SDF1α is a likely mechanism underlying this response as this is a well-established and documented downstream mediator of E2/ER-induced angiogenesis and macrophage chemotaxis, respectively (21, 25, 26). Until recently, the role of estrogen in the formation of breast cancer has been mainly attributed to its mitogenic, epigenetic, and genotoxic effects within ER-expressing breast cancer cells. This has limited the success of treatments that specifically target these mechanisms. However, recent studies have shown that estrogen can increase the inflammatory properties of tissue macrophages, an important cell type involved in the development and progression of cancers (27). Macrophage recruitment has been correlated with early relapse and poor prognosis in patients with breast cancer (19). Furthermore, macrophage infiltration is required for the angiogenic switch, and a failure to recruit macrophages delays angiogenesis and subsequent tumor progression (18). Therefore, targeting these cells or factors produced by these cells may improve the treatment of breast cancers in patients with elevated circulating estrogen.

Disclosure of Potential Conflicts of Interest
M.T. Lewis has ownership interest (including patents) from StemMed LP. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: V. Iyer, C. Kuperwasser
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Iyer, I. Klebba, J. McCready, L.M. Arendt, M. Betancur-Boissel, Z. Zhang, M.T. Lewis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Iyer, I. Klebba, J. McCready, L.M. Arendt, M.-F. Wu, M.T. Lewis, C. Kuperwasser
Writing, review, and/or revision of the manuscript: V. Iyer, I. Klebba, J. McCready, L.M. Arendt, M.T. Lewis, C. Kuperwasser
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Klebba, C. Kuperwasser
Study supervision: C. Kuperwasser

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