Silencing of Cyclooxygenase-2 Inhibits Metastasis and Delays Tumor Onset of Poorly Differentiated Metastatic Breast Cancer Cells

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Abstract

Cyclooxygenases (COX) are rate-limiting enzymes involved in the conversion of PLA₂-mobilized arachidonic acid into prostaglandins and thromboxanes. COX-2 is a key mediator of inflammation during both physiologic and pathologic responses to endogenous stimuli and infectious agents. Its overexpression has been detected in different cancers, including that of the breast. Using RNA interference, we have reduced the expression of COX-2 in the highly malignant breast cancer cell line MDA-MB-231 below detectable levels in response to interleukin-1β or 12-O-tetradecanoylphorbol-13-acetate treatment. Microarray analysis showed that COX-2 silencing resulted in the loss of mRNA expression of several oncogenic markers, such as matrix metalloproteinase-1, chemokine (C-X-C motif) receptor 4, and interleukin-11, which have been correlated with poor disease outcome, and in the up-regulation of antimetastatic transcripts, such as thrombospondin-1 and Epstein-Barr-Induced 3. Cells lacking COX-2 were less able to invade reconstituted extracellular matrix than parental cells in vitro. Consistent with these changes, loss of COX-2 resulted in the abolition or the significant delay of tumor onset and metastasis to the lungs of severe combined immunodeficient mice. Finally, silencing of COX-2 resulted in the inhibition of metastasis to the lungs of severe combined immunodeficient mice after intravenous injection. These data show that silencing of COX-2 abolishes the metastatic potential of MDA-MB-231 cells in vivo. (Mol Cancer Res 2007;5(5):435–42)

Introduction

Cyclooxygenase (COX)-1 and COX-2 are cytoplasmic enzymes involved in the conversion of PLA₂-mobilized arachidonic acid into lipid signal transduction molecules known as prostaglandins and thromboxanes. The major product of the COX-2–catalyzed reaction is prostaglandin E₂ (PGE₂), an inflammatory mediator participating in many biological processes, including development, pain, immunity, and angiogenesis. COX-2 function has been the target of pharmaceutical intervention in a multitude of widespread degenerating conditions, including autoimmune diseases, gastric inflammation, and several different cancers, such as gastric, lung, breast, and colon cancer (2–7). Its expression is induced by proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, and its promoter contains a cyclic AMP response element, a nuclear factor-κB binding site, and two nuclear factors for IL-6 target sequences (8). Extensive expression of COX-2 has been observed in colon cancer (9) and in 37% to 43% of breast tumors (10, 11). Mechanisms responsible for the enhancement of tumor progression by COX-2 are thought to involve the promotion of invasion and angiogenesis, processes necessary for tumorigenesis and metastasis. COX-2 overexpression in the mammary glands of transgenic mice resulted in increased tumor formation (14). Studies on the possible role of COX-2 in cancer have predominantly been conducted using COX-2–selective inhibitors. Although the overall data arguing for the involvement of COX-2 in some human cancers are compelling, the possible COX-2–independent functions of COX-2–selective inhibitors have recently come under intense scrutiny (15). To our knowledge, this is the first study reporting that nonpharmacologically mediated loss of COX-2 delays tumor onset and inhibits metastasis formation in a metastatic human breast cancer xenograft.

Results

Generation of MDA-MB-231 Cells Lacking COX-2

We used RNA interference to study the effects of loss of COX-2 in the estrogen receptor/progesterone receptor–negative, poorly differentiated, malignant human breast cancer cell line MDA-MB-231. COX-2 protein was induced in response to IL-1β treatment in a dose-dependent manner in MDA-MB-231 cells with maximum stimulation observed at 10 ng/mL (Fig. 1A). Transient transfection experiments using COX-2 small interfering RNA (siRNA) showed specific inhibition of
COX-2 expression in response to IL-1β (Fig. 1A, lane 6). Additionally, a second COX-2 siRNA sequence described previously (16) also showed specific COX-2 down-regulation (Fig. 1A, lane 7). In contrast, a glyceraldehyde-3-phosphate dehydrogenase–specific siRNA duplex had no effect on the expression of COX-2 (Fig. 1A, lane 8). Stable clones expressing a short hairpin RNA complementary to COX-2 mRNA were obtained as described previously (17). To assess the extent of the loss of COX-2 expression following IL-1β stimulation, immunoassays for PGE2 were done on supernatants from individual clones (data not shown). Clone 2, which could not be induced to secrete PGE2, was selected for further experimentation. To avoid artifacts resulting from the use of single clones of transfected cells, we decided to simultaneously use a pool of four individual clones, which could also not be induced to secrete PGE2. Immunoblotting revealed no detectable COX-2 protein in the clone 2 and pooled clone cell lines induced with IL-1β or 12-O-tetradecanoylphorbol-13-acetate (TPA; 50 nmol/L; V, empty vector cells). Membranes were probed with a COX-2–specific antibody and with a glyceraldehyde-3-phosphate dehydrogenase–specific antibody (GAPDH).

**FIGURE 1.** Generation of MDA-MB-231 stable clones lacking COX-2. A, MDA-MB-231 (231) cells were induced with varying concentrations of IL-1β for 24 h to ensure that COX-2 was inducible. Two COX-2–specific siRNA molecules (–2, –2nv), one designed by us and the other described previously (16), severely down-regulated COX-2 expression in response to IL-1β. B, COX-2 short hairpin RNA–transfected clones were obtained using G418 as a selection marker and following standard protocols. One individual clone (c2) and four pooled clones (P) were induced with IL-1β (10 ng/mL) or 12-O-tetradecanoylphorbol-13-acetate (TPA; 50 nmol/L; V, empty vector cells). Membranes were probed with a COX-2–specific antibody and with a glyceraldehyde-3-phosphate dehydrogenase–specific antibody (GAPDH).

**Loss of COX-2 Does Not Alter Cell Growth Rate In vitro**

To measure cell growth, cells (1.5 × 10⁵) were plated on 35-mm dishes and trypsinized and counted at 24-, 48-, and 72-h time intervals. No significant differences in growth rate were seen between cells inducible for COX-2 (MDA-MB-231 and empty vector cells) and cells lacking COX-2 (clone 2 and pooled clones) as shown in Fig. 2, suggesting that COX-2 does not affect MDA-MB-231 doubling time in culture. Additional experiments showed that loss of COX-2 did not significantly affect cell size (data not shown).

**Cells Lacking COX-2 Have a Less Oncogenic Transcriptome**

PGE2 may promote cell growth by interfering with the β-catenin and Ras pathways (19, 20). We thus hypothesized that loss of COX-2 may result in alteration of the expression of factors responsible for many cellular functions. To assess differences between the transcriptomes of IL-1β–challenged and parental MDA-MB-231 cells, we did GeneChip expression arrays using the Affymetrix Human Genome U133 Plus 2.0 GeneChip. We sought to identify candidate genes whose expression was suppressed or increased in both the clone 2 and the pooled clone cells relative to parental MDA-MB-231 cells from two separate experiments. Results show that COX-2–lacking cells display altered expression of an array of genes coding for factors regulating gene expression, invasion, and metastasis as well as tumor markers, including matrix metalloproteinase-1 (MMP-1), chemokine (C-X-C motif) receptor 4 (CXCR4), SMA- and MAD-related protein 1 (SMAD1), IL-11, and thrombospondin-1 (THBS-1; Fig. 3A). Cells not induced with IL-1β also showed similar differences in transcript levels (Fig. 3B) with the exception of S100P, HAS2, TPM1, ROBO4, and LUM, which were not differentially expressed in pooled clone cells compared with the parental MDA-MB-231 cells to a significant extent. Only transcripts that were modified in both the single clone as well as the pooled clones are shown, as we hypothesized that transcripts responsible for a common phenotypic change would be modified in both the clone 2 and the pooled clone cells. To validate the results of the microarray data, quantitative PCR was done to probe RNA purified from MDA-MB-231– and COX-2–lacking cells clone 2 and pooled clones challenged with IL-1β (10 ng/mL) and probed for COX-2, CXCR4, JAG1, and SMAD1. No specific COX-2 or CXCR4 product was detected in the COX-2–silenced cells, which also displayed reduced levels of JAG1 (MDA-MB-231 versus clone 2, 3.9-fold; MDA-MB-231 versus pooled clones, 3.3-fold) and SMAD1 (MDA-MB-231 versus clone 2, 5.8-fold; MDA-MB-231 versus pooled clones, 3.2-fold). The values presented represent the mean from two independent experiments. These data validate the observations of the microarray analyses for COX-2, CXCR4, JAG1, and SMAD1.
Loss of COX-2 Reduces Invasion in vitro

MMPs are broad specificity enzymes that cancer cells use to degrade extracellular matrix. Because MMP-1 mRNA levels were significantly reduced in both clone 2 and pooled clone cells in two independent experiments, we examined the invasive potential of the cells lacking COX-2 using Matrigel-based invasion assays. Parental MDA-MB-231 cells, empty vector cells, clone 2 cells, and pooled clone cells (1.5 × 10^5) were plated on Matrigel-containing Transwell plates and allowed to invade for 24 h. COX-2–lacking cells (clone 2 and pooled clones) showed a significant reduction in invasion compared with MDA-MB-231 and empty vector cells that can be induced to express COX-2 (Fig. 3C). These results agree with a recent publication that suggested that nuclear factor for activated T cells can induce COX-2 expression, which correlated with increased invasiveness (12).

Loss of COX-2 Attenuates Tumor Onset

To assess the effect of loss of COX-2 on tumor onset in vivo, 2 × 10^6 of MDA-MB-231 cells (n = 10), empty vector cells (n = 9), clone 2 cells (n = 11), and pooled clone cells (n = 10), suspended in 50 μL HBSS, were inoculated in the mammary fat pad of severe combined immunodeficient (SCID) mice in at least two separate experiments. Inoculation of parental MDA-MB-231 and empty vector cells resulted in palpable tumors within 6 weeks after injection (Table 1). In contrast, of the 11 mice injected with clone 2 cells, only 1 mouse developed a palpable tumor within the first 10 weeks after injection. Similarly, of the 10 mice injected with pooled clone cells, 4 developed small but palpable tumors within the first 10 weeks after injection (summarized in Table 1). Average tumor volumes reflecting the differences in tumor onset are shown in Fig. 4A. The tumor from the mouse injected with clone 2 cells was excised and cells were isolated and cultured. To ascertain whether the clone 2 tumor-derived cells (c2T1205) had lost the siRNA-mediated COX-2 inhibition, immunoblotting was done. c2T1205 cells regained their ability to express COX-2 in the absence of, or in response to, exogenous IL-1β to a significant extent (Fig. 4B), suggesting that COX-2 is required for tumor initiation in vivo and that loss of COX-2 is sufficient for abolishing the tumorigenicity of MDA-MB-231 cells in vivo.

![FIGURE 2](Image 123x110 to 248x233)

**FIGURE 2.** Cells lacking COX-2 have similar growth characteristics with parental MDA-MB-231 cells. Cells (1.5 × 10^5) were seeded in 35-mm dishes and cells were trypsinized and counted 24, 48, and 72 h later. MDA-MB-231 (●), empty vector cells (■), clone 2 (▲), and pooled clones (■).

### Table 1. COX-2–Silenced MDA-MB-231 Cells Form Delayed, if Any, Tumors When Injected in the Mammary Fat Pad of SCID Mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. mice</th>
<th>Palpable tumors</th>
</tr>
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<tr>
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<td>30 D</td>
<td>60 D</td>
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<tr>
<td>231</td>
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<tr>
<td>V</td>
<td>9</td>
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<tr>
<td>c2</td>
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<td>P</td>
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Note: Summary of the tumor progression of mice orthotopically injected with cells with inducible COX-2, MDA-MB-231 and empty vector cells, and with COX-2–silenced cells, clone 2 and pooled clones, in the mammary fat pad. Abbreviations: 231, MDA-MB-231 cells; V, empty vector cells; c2, clone 2; P, pooled clones; N/A, not applicable. *Mice were sacrificed due to excessive tumor burden.

Loss of COX-2 Inhibits Extrapulmonary Metastasis

To examine the effect of loss of COX-2 expression on experimental metastasis from parental MDA-MB-231 cells (n = 5), empty vector cells (n = 5), clone 2 cells (n = 5), and pooled clone cells (n = 3), 2 × 10^6 cells resuspended in 50 μL HBSS were injected in the tail vein of SCID mice. Mice were sacrificed 5 weeks after injection and their lungs were infused with agarose to prevent collapse. A stark contrast in metastatic nodules was apparent both visually as well as in H&E-stained sections. Mice injected with parental MDA-MB-231 and empty vector cells had multiple visible metastatic nodules in the lungs, in contrast to COX-2–lacking cell lines, clone 2 and pooled clones, for which lungs appeared clear of any metastasis (Fig. 5A). H&E-stained lung sections showing multiple metastatic lesions from parental and empty vector cells, but none from COX-2–lacking cells, are shown in Fig. 5B. Images of H&E-stained lung sections representing the cross-sectional area from each pair of lungs were analyzed to determine the percentage area covered by metastases using ImageJ (NIH). The percentage areas (mean ± SE) were 4.2 ± 0.7, 24.9 ± 3.7, 0, and 0, for MDA-MB-231 cells (n = 5), empty vector cells (n = 5), clone 2 cells (n = 5), and pooled clone cells (n = 3), respectively. The two-tailed nonparametric Mann-Whitney U test was used to determine if there was a significant difference in the percentage areas between the cell lines using z of 0.05. A significant difference in the percentage area of metastases was evident between MDA-MB-231 and clone 2 (P = 0.007) or pooled clones (P = 0.032) and between empty vector cells and clone 2 (P = 0.007) or pooled clones (P = 0.032). These data suggest that COX-2 function is critical for metastasis of MDA-MB-231 cells in vivo.

### Discussion

Although the association of persistent inflammation and increased cancer risk has long been established, the mechanisms underlying it remain to be delineated. COX-2 is thought to promote tumorigenesis by the actions of lipid hydroperoxide formation and through the effects of its major product, PGE2. COX-2 lipid peroxidation products have been shown to result in the formation of DNA adducts (22), whereas PGE2 has been shown to affect a multitude of pathways promoting invasion
FIGURE 3. Loss of COX-2 alters the transcriptomes of MDA-MB-231 cells and reduces invasion. A, MDA-MB-231, clone 2, and pooled clone cells were induced with IL-1β for 24 h and total mRNA was subjected to hybridization on an U133 Plus 2.0 Affymetrix microarray chip. All represented transcripts were regulated in a similar manner in two independent experiments. Down-regulated or up-regulated transcripts are shown with negative or positive values, respectively. White columns and black columns, the differential expression of transcripts in clone 2 and pooled clone cells compared with MDA-MB-231 cells, respectively. PTGS2*, Affymetrix probe ID 1554997_a_at (National Center for Biotechnology Information public ID AY151286), which corresponds to the alternatively spliced COX-2b protein. Expression signals were obtained by Robust Multiarray Analysis (21). The posterior probabilities of the differential expression of genes between the treatments were estimated with an empirical Bayes method using Gamma-Gamma modeling by the bioconductor R package, EBarrays. The criterion of the posterior probability >0.5 was used to produce the differentially expressed gene lists shown. B, RNA from MDA-MB-231 and COX-2–silenced clone 2 and pooled clone cells, which were not challenged with IL-1β, was purified and subjected to microarray analyses as above. The criterion of the posterior probability >0.5 was used to produce the differentially expressed gene lists shown. C, Cells lacking COX-2 show significantly decreased invasion in vitro. MDA-MB-231 cells, empty vector cells, clone 2 cells, and pooled clone cells (1.5 × 10⁵) were plated onto a Biocoat Tumor Invasion System and allowed to invade the Matrigel for 24 h. The difference in serum concentration between the top and the bottom of the plate was only 2-fold (5-10%) to avoid any deleterious effects to the cells arising from serum starvation. Results are representative of six individual experiments done in triplicates for each cell line. Columns, relative invasion index; bars, SE. Statistical significance was assessed by doing a homoscedastic, two-tailed Student’s t test. P = 0.856205, empty vector cells; P = 0.006042, clone 2; P = 0.000026, pooled clones.
and angiogenesis (19, 20), including the induction of hypoxia-inducible transcription factor (23), a potent signaling molecule responsible for angiogenesis. Lewis lung carcinoma–derived tumors growing in COX-2 knockout mice had decreased vascular density and a significant decrease in the production of VEGF, suggesting that the absence of COX-2 from the tumor microenvironment affects tumor vasculature (24).

Deciphering the mechanism by which COX-2 promotes tumorigenesis is complicated by the fact that COX-2–selective inhibitors seem to also inhibit carcinogenic signaling independent of COX-2 at the concentrations used to study cell signaling in vitro (15). Additionally, COX-2–dependent pathways can vary depending on the tissue and the nature of the tissue microenvironment studied. For example, it was reported previously that celecoxib inhibits TNF-α–induced extracellular signal-regulated kinase phosphorylation in the non–small cell lung carcinoma cell line H1299 (25). However, in our cell lines that lack COX-2 expression, TNF-α (10 ng/mL) successfully induced mitogen-activated protein/extracellular signal-regulated kinase phosphorylation within 45 min.1

To investigate the effect of COX-2 in breast cancer, we decided to silence COX-2 expression using RNA interference in an effort to circumvent some of the nonspecific and transient effects associated with pharmacologic inhibitors. We have shown that loss of COX-2 in the metastatic human breast xenograft model MDA-MB-231 delayed or inhibited orthotopic tumor onset and inhibited extrapulmonary metastasis in a model of experimental metastasis. Interestingly, COX-2–silenced cells did not show any differences in growth rate in vitro. This is likely a result of the complex interactions required for tumor onset and growth in vivo in contrast to culture conditions. Such complex interactions may include alteration or loss of chemotactic signaling or other modifications of the tissue microenvironment that can only take place in an in vivo model of tumorigenesis. Cells depleted of COX-2 express messages that code for the production of tumor endothelial markers, such as THBS-1, which is thought to contribute to an environment less permissive to the establishment of metastasis (26), tropomyosin-1, which sensitizes breast cancer cells to apoptosis (27), IL-27 (EBI-3), which generates a Th1 immune response (28), and CSR1 (SCARA3), an oxygen scavenger down-regulated in prostate cancer (29). Additionally, MDA-MB-231 cells lacking COX-2 showed reduced mRNA expression of several oncogenic markers, including IL-11, a marker for metastasis of breast cancer to bone (30), the Notch1 receptor ligand JAG1, whose expression is correlated with poor breast cancer prognosis (31), CXCR4, a receptor involved in the regulation of the tumor microenvironment (32), and MMP-1, a secreted enzyme responsible for the degradation of the stroma during breast cancer cell invasion (33).

Our results indicate that COX-2 expression modulates the expression of many extracellular matrix components, including collagen, glycoproteins (e.g., THBS-1), hyaluronan, and proteoglycans (e.g., lumican). It is therefore possible that tumor-derived COX-2 modifies the extracellular matrix enabling tumors to successfully establish metastases. Modification of the extracellular matrix by microenvironment-derived COX-2 could also explain why tissues with active inflammatory processes, such as wounds, are sites of frequently successful metastases. The notion that sites of inflammation are highly permissive to successful metastasis was experimentally proven when Rous sarcoma virus–induced tumor metastasis was shown to be dependent on wound-induced inflammation (34). Further experimentation is required to prove the hypothesis that tumor- and microenvironment-derived COX-2 and the products of the COX reaction contribute significantly to the successful establishment of metastasis. With pharmacologic inhibition of COX-2 in vivo, it is difficult to determine the contribution of COX-2 secreted by stromal cells to tumor growth and metastasis. Here, we have shown that silencing COX-2 selectively in cancer cells, without directly altering COX-2 expression in stromal cells, has a profound effect on tumor onset and metastasis. Another critical difference between the use of pharmacologic agents and our model is that using RNA interference, we have reduced the level of COX-2 beyond the limit of detection. This extent of inhibition may not be easily achieved without associated side effects using pharmacologic agents. Silencing COX-2 expression selectively in tumor cells using cancer cell–targeted delivery of siRNA may minimize the side effects of COX-2 inhibition while achieving effective treatment of the tumor.

Taken together, our data indicate that COX-2 is important for tumorigenesis in the mammary fat pad in vivo. Additionally, COX-2 has a critical role in the establishment of metastasis in the lungs, a primary metastatic target of human breast cancers and associated with a large percentage of breast cancer–associated mortality. Microarray data suggest

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1 Unpublished observation.
that loss of COX-2 results in the reduction of the expression of proteins associated with poor breast cancer outcome and the up-regulation of angiostatic and antimetastatic transcripts. These data strongly suggest that at least in some cancers, COX-2 is a key mediator of the signaling interplay between the invading cancer and the tumor microenvironment, an event critical to tumor metastasis.

The effects of the COX-2–selective inhibitor celecoxib are currently studied in many clinical trials alone or in combination with chemotherapy and radiation.

Of particular interest is the clinical trial designed to test the effect of celecoxib in estrogen receptor–negative premenopausal women at high risk of breast cancer (National Cancer Institute identifier: NCT00056082). Our results suggest that COX-2 inhibition may be of particular clinical importance in poorly differentiated breast cancer tumors.

**Materials and Methods**

**Derivation of a COX-2–Specific siRNA Target Sequence**

The target siRNA sequence was determined by the online, freely available siRNA target finder search engine offered by Ambion and was synthesized by Dharmacon against the following COX-2 mRNA target sequence: AACAUUCCCUCUCCUGAAAU.

**Stable Expression of the COX-2 Short Hairpin RNA–Containing Plasmid in MDA-MB-231 Cells**

The COX-2 short hairpin RNA–coding plasmid was constructed and placed under the control of the U6 promoter as described previously by Glunde et al. (17). Cells were

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2 http://ambion.com/techlib/misc/siRNA_finder.html
transfected with COX-2–specific short hairpin RNA–bearing plasmid using LipofectAMINE 2000 as suggested by the manufacturer (Invitrogen). Individual clones were selected for G418 resistance using standard protocols. Several individual clones were analyzed for PGE2 production from the supernatant using the PGE2 EIA Kit-Monoclonal as described by the manufacturer (Cayman Chemical; data not shown). Parental MDA-MB-231 cells, empty vector cells (17), clone 2 cells, and cells pooled from four individual clones were selected for further experimentation.

Cell Culture and Immunoblotting

All cells were cultured with RPMI 1640 (Sigma-Aldrich) supplemented with 8.25% fetal bovine serum (Sigma-Aldrich) without penicillin or streptomycin. To establish single clones, cells were selected for G418 resistance (400 μg/mL; Invitrogen) according to standard protocols. Established COX-2–lacking clones were not further selected for G418 resistance; instead, cells were assayed functionally for loss of COX-2. MDA-MB-231, empty vector cells, clone 2 and pooled clone cells, and cT21205 cells were plated in 100-mm dishes (1.5 × 10^6) and induced with IL-1β (10 ng/mL) or 12-0-tetradecanoyl-phorbol-13-acetate (50 nmol/L) for all experiments involving immunoblotting for COX-2. Twenty-four hours later, cells were lysed using M-PER (Pierce) and a protease inhibitor cocktail (Sigma-Aldrich). Crude cell extracts were quantified using DC protein assay (Bio-Rad). Fifty micrograms of protein were subjected to SDS-PAGE and immunoblotting following standard protocols. Membranes were probed overnight with a COX-2–specific antibody (Oxford Biomedical) and with a glyceraldehyde-3-phosphate dehydrogenase–specific antibody (Santa Cruz Biotechnology), washed, and probed with antigen horseradish peroxidase–labeled secondary antibodies (Jackson ImmunoResearch) following standard protocols. Substrate (SuperSignal West Pico, Pierce) was added to the probed membranes and films were developed using an automatic film processor (Kodak and Agfa).

Biocat Tumor Invasion Assay

Cells (1.5 × 10^5) were plated on the top part of a rehydrated, Matrigel-containing Biocat Tumor Invasion System (Becton Dickinson) and allowed to invade for 24 h. Cells were labeled using calcein AM (Invitrogen) for 1 h as directed by the manufacturer. The difference in serum concentration between the top and the bottom of the plate was only 2-fold (5-10%) to avoid any deleterious effects to the cells arising from serum starvation. Fluorescence measurements were taken 1 h after labeling using a Victor plate reader set to read the bottom of the plate at 485/535 nm.

Cell Preparation for Injection into the Murine Fat Pad and Tail Vein

Cells were cultured as described above and trypsinized using standard protocols. The cells were then centrifuged and adjusted to a cell density of 2 × 10^6/50 μL HBSS (Sigma-Aldrich) without Ca^2+ or Mg^2+. The cells were then injected in the mammary fat pad or the mouse tail vein of anesthetized 5- or 6-week-old female SCID mice (National Cancer Institute) and mice were returned to their cages for further examination.

Mice Work Committee Approval

The authors of this publication state that all mice studies described were approved by the Animal Care and Use Committee and that all requirements for humane animal treatment dictated by the above committee were met.

Microarray Analysis

Cells used in the microarray experiment described in Fig. 3A and B were seeded (1 × 10^6) in 100-mm dishes and allowed to attach to the plate overnight. Cells were induced with IL-1β (10 ng/mL) for 24 h. RNA was purified using the RNeasy kit (Qiagen) as described by the manufacturer and the on-column DNase I digestion done as described by the manufacturer. Samples were subjected to agarose formaldehyde electrophoresis to determine the quality of the RNA by comparing the ratios of 18 S and 28 S RNA species. All samples were run in commercial arrays from Affymetrix, using Human Genome U133 Plus 2.0 GeneChip arrays as described in the Affymetrix Web site.3 Probe hybridization and analysis was run as suggested by the manufacturer in the aforementioned Web site. These descriptions include all information currently considered under the Minimum Information About a Microarray Experiment supportive guidelines, with which the JHMI Microarray Core Facility abides in all its procedures. The authors will repos the Minimum Information About a Microarray Experiment–compliant original data in the Gene Expression Omnibus database immediately following the acceptance of the manuscript.

Quantitative Real-time RT-PCR

Cells were induced with IL-1β (10 ng/mL) for 24 h and RNA was purified as above. Twenty nanograms of purified RNA were subjected to One-Step Real-time RT-PCR using SYBR Green (Qiagen) in a Bio-Rad iCycler as described by the manufacturer. Primers for COX-2, CXCR4, JAG1, and SMAD1 studies were obtained from Qiagen. Primer sequences for 18 S transcript used as a control: 5'-GGTTGATCTCTGCAGTAGC-3' and 5'-GCGACAAAGGAACCATAAC-3'. Fold differences were calculated using the 2^{ΔΔCt} method.

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