Invasion of Human Breast Cancer Cells In vivo Requires Both Paracrine and Autocrine Loops Involving the Colony-Stimulating Factor-1 Receptor

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Abstract

Colony-stimulating factor-1 (CSF-1) and its receptor (CSF-1R) have been implicated in the pathogenesis and progression of various types of cancer, including breast cancer. This is based on high levels of circulating CSF-1 in patient sera with aggressive disease and increased CSF-1R staining in the tumor tissues. However, there have been no direct in vivo studies to determine whether a CSF-1 autocrine signaling loop functions in human breast cancer cells in vivo and whether it contributes to invasion. Recently, in mouse and rat models, it has been shown that invasion and metastasis are driven by an epidermal growth factor (EGF)/CSF-1 paracrine loop between tumor cells and host macrophages. In this macrophage-dependent invasion, tumor cells secrete CSF-1 and sense EGF, whereas the macrophages secrete EGF and sense CSF-1. Here, we test the hypothesis that in human breast tumors, the expression of both the CSF-1 ligand and its receptor in tumor cells leads to a CSF-1/CSF-1R autocrine loop which contributes to the aggressive phenotype of human breast tumors. Using MDA-MB-231 cell-derived mammary tumors in severe combined immunodeficiency mice, we show here for the first time in vivo that invasion in a human mammary tumor model is dependent on both paracrine signaling with host macrophages as well as autocrine signaling involving the tumor cells themselves. In particular, we show that the autocrine contribution to invasion is specifically amplified in vivo through a tumor microenvironment-induced upregulation of CSF-1R expression via the transforming growth factor-β1. [Cancer Res 2009;69(24):OF1–9]

Introduction

Metastasis is a multistep process that requires increased motility of the tumor cells inside the primary tumor and invasion of surrounding tissues and blood vessels. The tumor microenvironment has an essential role in promoting these steps of motility and invasion in tumor cells, through either secretion of chemotactic factors or direct interactions with stromal cells. In rat and mouse mammary tumors, tumor-associated macrophages are essential for promoting angiogenesis, matrix remodeling, and chemotactic motility of the tumor cells (1). In particular, a paracrine interaction between macrophages and tumor cells that involves epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1) is the driving force for relay chemotaxis supporting macrophage-mediated invasion in both transgenic mouse and rat mammary tumors. During relay chemotaxis, tumor cells secrete CSF-1 and sense EGF, whereas the macrophages secrete EGF and sense CSF-1. This phenomenon has been studied extensively both by intravital imaging in living animals as well as by reconstituting the interaction of the two cell types in vitro (2–4). Additional studies support the importance of macrophages in invasion and metastasis of the tumor cells. Absence of CSF-1 in the mammary cancer–susceptible PyMT mice retarded tumor progression and metastasis but did not affect primary tumor development (5), directly implicating macrophages and CSF-1 as important regulators of invasion and metastasis. In later work with xenogenic tumors in mice, blockade of CSF-1 through antisense oligonucleotides or neutralizing anti–CSF-1 antibodies also reduced primary tumor growth and angiogenesis and prolonged long-term survival (6, 7).

In humans, patient sample data has suggested that CSF-1 and its receptor might play critical roles during progression of tumors of the female reproductive system and other solid tumors. Expression of the CSF-1 receptor (CSF-1R) has been associated with adverse clinicopathologic prognostic outcome in ovarian, endometrial, and breast carcinomas (8–10). Expression of CSF-1R has also been detected in prostate cancer cells in tumors with elevated Gleason scores (11). Interestingly, CSF-1 is also expressed in ovarian and endometrial tumors and cell lines (12) as well as in breast cancer (10, 13), whereas CSF-1 and the CSF-1R are coexpressed in >50% of mammary tumors (14). In addition, increased circulating CSF-1 levels are a prognostic marker for epithelial ovarian cancer (15, 16) and are elevated in a large proportion of endometrial cancers (16, 17). Elevated circulating CSF-1 was also suggested to be an indicator of early metastatic relapse in patients with breast cancer (13). These observations have led to the hypothesis that there may be an autocrine loop involving tumor cells expressing both CSF-1 and CSF-1R, which contributes to invasion and metastasis in human tumors (14). Several in vitro studies now support this hypothesis. Human lung cell lines and breast cell lines that express CSF-1R, but not the ligand, show increased invasion in vitro into an amniotic basement membrane upon stimulation with CSF-1 (18). Similarly, when the normal noninvasive mouse mammary cell line, HC11, which expresses CSF-1 but not the receptor, is forced to overexpress CSF-1R, it shows rapid growth and colony formation in soft agar, increased invasion through Matrigel, and a higher incidence of lung tumors after tail vein injections into BALB/c mice compared with the parental line (19). Additionally, when CSF-1 and its receptor are both stably overexpressed in the MCF10A human human...
mammary cell line, the cells exhibit abnormal acinar morphogenesis and increased motility in an in vitro wound-healing assay (20).

These latter in vitro studies were performed with cells in which CSF-1 or its receptor, or both, were overexpressed to mimic hypothesized autocrine signaling, and they did not assess the contribution of the tumor microenvironment to the onset of autocrine signaling. Indeed, there have been no direct in vivo studies to determine whether a CSF-1 autocrine signaling loop is induced in the microenvironment of the primary tumor and if it contributes to invasion in human breast cancer. Moreover, the contribution of autocrine CSF-1 signaling to invasion cannot be investigated in the popular transgenic mouse models because neither the mouse epithelial cells nor the mouse tumor cells express the CSF-1R.

Epithelial expression of the CSF-1R transcript in human mammary epithelium is hormonally regulated via a glucocorticoid response element that is absent from the murine locus (21). Therefore, additional models and studies are needed to test the hypothesis that CSF-1/CSF-1R autocrine signaling contributes to the invasion of human breast cancer cells in vivo.

Based on the rodent models and the patient data described above, CSF-1R could be involved in human breast tumor invasion in at least two ways. The rodent studies point to macrophage-assisted invasion involving an EGF/CSF-1 paracrine loop and the patient studies suggest that tumor cell invasion is regulated in an autocrine manner by CSF-1, provided that the tumor cells express both CSF-1 and CSF-1R. In this study, we directly address these two possibilities in vivo.

We used the human breast tumor cell line MDA-MB-231 as a model for our analysis, because its triple-negative status (estrogen/progesterone receptor negative, HER2 negative) categorizes it in the basal-like subtype of breast cancers (22). These are generally the most aggressive and highly metastatic breast tumors. As we show that MDA-MB-231 cells express both CSF-1 and the CSF-1R, this cell line, without further manipulation, is an appropriate one in which to study the role of autocrine signaling in aggressive breast tumors.

Materials and Methods

Cell culture. MDA-MB-231 cells (American Type Culture Collection) were cultured in DMEM (Invitrogen) with 10% fetal bovine serum. A stable green fluorescent protein (GFP)–expressing line was made by transfecting the MDA-MB-231 cells with the pEGFP-C1 vector (Clontech) using Amaxa CSF-1 (a gift from Chiron Corp.).

Based on the high expression of CSF-1 in the primary tumors, we showed that MDA-MB-231 cells express both CSF-1 and the CSF-1R, this cell line, without further manipulation, is an appropriate one in which to study the role of autocrine signaling in aggressive breast tumors.

RNA extraction and PCR amplification. For the in vitro stimulations, the cells were starved overnight in DMEM/0.3% bovine serum albumin, prior to the addition of cytokines for 1, 4, 7, 24, and 72 h. The results shown are for the 4-h stimulation, when the effect of transforming growth factor-β1 (TGF-β1) was maximal (EGF and CSF-1 were identical for all time points). RNA was extracted with the RNeasy Mini kit (Qiagen), and 1 μg of total RNA was reverse-transcribed using SuperScript II (Invitrogen). Total cDNA (2–5 ng) was used for real-time PCR reaction or standard PCR (30 cycles) with specific primers (see Supplementary Table for primer sequences). Electrophoresis of the PCR products was performed on 1.5% agarose gel and visualized by ethidium bromide staining.

For the comparison of the average primary tumor cells (APTC) with the cultured MDA-MB-231 cells, amplified total cDNA was used as input in the real-time PCR. The detailed protocol and validation of the technique has been published elsewhere (28, 29). Briefly, for the APTC sample, tumors from MDA-MB-231-GFP xenograft models were excised, mechanically dissociated into single cells in suspension on ice, sorted for the GFP-positive cells and lysed with the RNeasy Micro kit (Qiagen). Total RNA from APTC and from cultures of MDA-MB-231 cells was converted to cDNA and amplified with the SMART amplification kit (Clontech). The final amplified cDNA was purified with Qiagen MinElute columns and 2 ng was used in the real-time PCR per reaction.

Real-time PCR confirmation. Quantitative PCR analysis was performed as described previously (29), using the Power SYBR Green PCR Core Reagents system (Applied Biosystems). Each PCR reaction was performed in triplicate, and the mean threshold cycle (Ct) values were used for analysis. All the gene tested were compared with two housekeeping genes (β2-microglobulin and GAPDH) for the analysis. Results were evaluated with the ABI Prism SDS 2.1 software.

Western blot. For the Western blot analysis, whole cell lysates were prepared by washing the cells with cold PBS, followed by direct addition of SDS-PAGE sample buffer and sonication and boiling of the lysate. Western blots were carried out as follows: the samples were resolved by SDS-PAGE, transferred to nitrocellulose, blocked in Odyssey blocking solution (LiCor), and incubated for 24 h after which the assay was fixed with 4% formaldehyde. Where indicated, cells were pretreated with Iressa (1 μmol/L), or the CSF-1R blocking antibodies described above (final 10–25 μg/mL) for 2 h. The fixed assay was analyzed by confocal microscopy. Invasion of the tumor cells was quantified as the proportion of fluorescence >20 μm into the collagen.

Aging and immortalization. All cells were passage twice to assess their growth and viability. 

Determination of cell types collected. Typing of the collected cells was done as described previously (3), using cell type–specific antibodies rabbit anti-pancytokeratin (Santa Cruz Biotechnology, Inc.) for carcinoma cells and rat anti-F4/80 (27) for macrophages. 4′,6-Diamidino-2-phenylindole was used for counting total cells.

Collagen invasion assay. The assay was performed as described in ref. (4). Briefly, MDA-MB-231-GFP cells were plated in the presence or absence of BAC1.2F5 macrophages. The next day, they were overlaid with collagen I (BD Biosciences) and incubated for 24 h after which the assay was fixed with 4% formaldehyde. Where indicated, cells were pretreated with Iressa (1 μmol/L), or the CSF-1R blocking antibodies described above (final 10–25 μg/mL) for 2 h. The fixed assay was analyzed by confocal microscopy. Invasion of the tumor cells was quantified as the proportion of fluorescence >20 μm into the collagen.

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Statistical analysis. The results shown are representative of at least three experiments with duplicate plates of cells for the in vitro experiments and at least five different mice per point for the in vivo experiments. All statistical analyses were assessed using a two-tailed Student’s t test.

Results

Human breast tumor MDA-MB-231 cells express CSF-1R and its ligand. In previous studies, we have characterized a paracrine interaction between breast carcinoma cells and macrophages involving EGF and CSF-1, using both xenograft (MTLn3 rat breast tumor cells) and transgenic (MMTV-PyMT) mouse mammary tumors (3, 4). In an effort to test whether similar interactions exist in a human breast cancer cell–derived mammary tumor, we tested the expression of these growth factors and their ligands in the human breast cancer line MDA-MB-231 (Fig. 1A). Similar to the rodent breast carcinoma cells (4), the MDA-MB-231 cells express the mRNA for the EGFR but not for EGF. The MDA-MB-231 human breast tumor cells, like their rodent counterparts, also express mRNA for CSF-1, raising the possibility of an EGF/CSF-1 paracrine loop akin to the paracrine loop we have observed with rodent tumors, in which EGF is produced by tumor-associated macrophages (Fig. 1A). Based on this gene expression pattern, we used a three-dimensional in vitro invasion assay in which the tumor cells, either alone or in the presence of macrophages, are monitored for their invasive ability through a three-dimensional collagen matrix (4). The invasion of the MDA-MB-231 cells was enhanced in the presence of macrophages (Fig. 1C; and also in ref. 4), demonstrating that a EGF/CSF-1 paracrine interaction with macrophages exists for the human breast tumor cell–derived mammary tumors as seen in rat and mouse mammary tumors.

However, as described above, patient data have also shown concomitant expression of CSF-1 and its receptor in tumor cells of aggressive breast cancers (14) and unlike their rodent counterparts, the human MDA-MB-231 cells also express the CSF-1R as well as the mRNA for CSF-1 (Fig. 1A and B). Thus, the MDA-MB-231 cell line is a good model to test the potential of a CSF-1/CSF-1R autocrine contribution to invasion and metastasis in human breast cancer, without forced and artificial overexpression of either molecule.

The results shown in Fig. 1C also suggest that CSF-1/CSF-1R autocrine signaling is less potent at causing invasion in the three-dimensional in vitro invasion assay than in macrophage-supported paracrine signaling.

Macrophages are less involved in invasion in the human MDA-MB-231 tumors than in mouse and rat tumors. To investigate the relative contributions of autocrine and paracrine signaling to invasion and intravasation in vivo, we made mammary tumors using MDA-MB-231 cells in mice and we used the in vivo invasion assay (25) to measure the invasive potential of these cells. In this assay, as described in detail in Materials and Methods, microneedles containing Matrigel and a chemoattractant are inserted into the primary tumors while the animal is alive and under anesthesia. This assay mimics natural blood vessels inside the primary tumor in which EGF secreted by macrophages attracts the invasive tumor cells to these blood vessels, a movement that eventually will lead to intravasation and hematogenous metastasis (2). Using this assay in the MDA-MB-231 xenografts, we collected the invasive cells that responded to EGF (Fig. 2A), extruded them out of the needles and identified the cell types with cell type–specific antibodies. As expected from previous studies with rat MTLn3 xenografts and the MMTV-PyMT transgenic mouse models (3), the invasive subpopulation in MDA-MB-231 tumors was comprised of macrophages and tumor cells (Fig. 2B). However, the proportion of macrophages was less than observed with rodent tumors (~6% versus 25%, respectively; Fig. 2B and C), implying that MDA-MB-231 cell–derived mammary tumors are less dependent on macrophages for invasion and hematogenous dissemination. The possibility that the interaction between tumor cells and macrophages is hindered in this xenograft model due to species difference is unlikely; MDA-MB-231 cells respond to mouse EGF as well as to human EGF both in vitro and in vivo (Supplementary Fig. S1) and mouse macrophages respond similarly to both mouse and human CSF-1 (30).

Both autocrine and paracrine stimulation of the CSF-1R mediate invasion in the MDA-MB-231 mammary tumors. To further investigate the relative contributions of paracrine and autocrine signaling in vivo, we performed a series of experiments in the MDA-MB-231–derived primary mammary tumor using the in vivo invasion assay. First, we evaluated the contribution of EGF to invasion in primary MDA-MB-231 tumors (Fig. 3A). There was a decrease in the number of invasive cells collected in response to EGF in the presence of the EGFR inhibitor, Iressa (Fig. 3B). A similar inhibition by Iressa was observed when the cells were collected in response to CSF-1, suggesting a CSF-1/EGF paracrine loop between the human tumor cells and the macrophages in vivo. Interestingly, for either EGF- or CSF-1–driven invasion, the inhibition by Iressa was incomplete (Fig. 3B). In contrast, in an identical previous experiment in a rodent mammary tumor (3), there was complete inhibition by Iressa, which is consistent with a more significant involvement of macrophage-supplied EGF in the mouse tumors.

We next examined the contribution of CSF-1R to the paracrine and autocrine interactions in these human cell line–derived mammary tumors. To investigate the role of this receptor in tumor cells versus macrophages, we used an antibody that specifically blocks the mouse CSF-1R (ref. 3; Supplemental Fig. S2A) to block the paracrine interaction between the macrophages and the tumor cells (Fig. 3A). In contrast, to block only autocrine signaling in the human tumor cells (Fig. 3A), we used an antibody that specifically blocks the human (but not the mouse) CSF-1R (Supplemental Fig. S2A). In the MDA-MB-231 mammary tumors, blocking either the human or the mouse CSF-1R only partially decreased the number of total cells collected in response to EGF (Fig. 3C). However, a combination of both antibodies completely inhibited invasion to background levels (Fig. 3C, Matrigel alone control column). This is not an effect of doubling the total amount of antibody, as using twice the amount of each antibody alone did not produce this synergistic result (Supplementary Fig. S2B). Therefore, in MDA-MB-231 mammary tumors, a mix of paracrine signaling between tumor cells and macrophages and autocrine signaling in the tumor cells is involved in invasion in vivo.

The autocrine contribution to invasion via CSF-1R on MDA-MB-231 tumor cells is promoted specifically by TGFβ in the tumor microenvironment. We used the three-dimensional in vitro invasion assay, in which MDA-MB-231 cells alone or in coculture with macrophages are monitored for their invasion ability through a collagen matrix (Fig. 4A), with the specific inhibitors and the blocking antibodies described above, to investigate autocrine and paracrine contributions of EGFR and CSF-1R to in vitro invasion. In cocultures of MDA-MB-231 cells and macrophages, pretreatment with the EGFR-specific inhibitor, Iressa, dramatically decreased the ability of MDA-MB-231 cells to invade (Fig. 4B).
Additionally, incubation with the blocking antibody to the mouse (macrophage) CSF-1R substantially reduced invasion through the collagen matrix as well, almost to the background level of the MDA-MB-231 cells alone (Fig. 4C), whereas it had no effect on the migration of the tumor cells when alone in the collagen matrix (Supplemental Fig. S3). The requirement for the tumor cells’ EGFR function (Fig. 4B), together with the requirement for the mouse macrophage CSF-1R function (Fig. 4C), further supports the CSF-1/EGF paracrine interaction between the tumor cells and macrophages. However, incubation with the blocking antibody to the human tumor cell CSF-1R had no effect on the in vitro invasion of the tumor cells in the presence or absence of macrophages; i.e., invasion by the tumor cells was similar in the presence of the blocking or control antibodies (Fig. 4C; Supplemental Fig. S3). This result was unexpected, given that this same antibody inhibited invasion in vivo (Fig. 3C) and it suggests that autocrine CSF-1R signaling contributes more to invasion in vivo in the primary tumors than in vitro.

In Fig. 4A, we examined the gene expression pattern shown for the MDA-MB-231 cells in cell cultures. To test for gene expression changes that could account for the difference in behavior of these cells in vitro and in vivo, we also isolated RNA from the APTCs after their growth in the microenvironment of the primary tumor. Interestingly, when comparing the APTCs to the cultured MDA-MB-231, we found that the mRNA expression of CSF-1R in the MDA-MB-231 cells from tumors was 2.5-fold to 5-fold upregulated compared with the same cells in culture (Fig. 5A). We also tested the expression of the mRNAs for EGFR, CSF-1, and TGFα, but none showed such striking upregulation as did the CSF-1R mRNA (Fig. 5A). These results indicate that the CSF-1R mRNA upregulation is a phenotype specific to the growth of tumor cells in vivo in the primary tumor and not a direct result of stimulation by the growth factors involved in the paracrine/autocrine loops tested in this study.
study. In fact, stimulation of MDA-MB-231 cells with either EGF or CSF-1 was not sufficient to produce the CSF-1R mRNA upregulation seen in the invasive tumor cells isolated from the primary tumor (Fig. 5B and C).

To explore the microenvironmental signal that causes the upregulation of CSF-1R expression in tumor cells in vivo, we investigated the involvement of TGFβ1. Induction of the CSF-1R mRNA by TGFβ1 has been reported in vascular smooth muscle cells and in cervical cancer cell lines (31, 32). MDA-MB-231 cells express the receptors for TGFβ1 (33) and thus could be responsive to it. Considering the established role of TGFβ signaling in tumor progression in breast cancer (34, 35), we hypothesized that TGFβ1 is the microenvironmental signal that prompts the upregulation of CSF-1R in human breast tumor cells. Indeed, when we stimulated MDA-MB-231 cells in vitro with TGFβ1, we found a 2.5-fold to 3-fold upregulation of their CSF-1R mRNA expression. To address the role of TGFβ1 in the upregulation of CSF-1R in vivo, we treated MDA-MB-231 xenografts with SB431542, a specific inhibitor to the TGFβ receptor (32, 36). Treatment with this inhibitor significantly decreased the CSF-1R mRNA expression of the primary tumor cells (Fig. 6B). Additionally, invasion in the in vivo treated animals was significantly reduced compared with the animals injected with DMSO vehicle control (Fig. 6C), and was similar to the numbers observed when the human CSF-1R blocking antibody was used in the in vivo invasion assay (Fig. 3C). Our results suggest that TGFβ1 is the signal inside the primary tumor that promotes the in vivo autocrine CSF-1R–mediated invasion of the human breast tumor cells.

**Discussion**

Increased tumor expression of CSF-1 and its receptor has been observed in several types of solid tumors, including breast cancer (14). The correlation of expression of both the receptor and its ligand with cancer aggressiveness led to the hypothesis of autocrine signaling through CSF-1R in these tumors. In addition to this potential autocrine role, the elevated expression of CSF-1 by some tumors suggested that CSF-1 might contribute to increased infiltration of macrophages into the primary tumor and their interaction with the tumor cells, a phenomenon also linked to tumor aggressiveness (13, 37). We have used the highly metastatic human breast tumor line MDA-MB-231 to address the mechanism of...
contribution of CSF-1R in invasion by breast cancer cells. Here, we show that invasion of MDA-MB-231 cells in vitro is paracrine, requiring the presence of macrophages for optimum invasion. However, using MDA-MB-231–derived mammary tumors, we show that invasion in these human cell line–derived tumors in vivo involves both autocrine CSF-1R signaling, as well as macrophage-associated signaling through the EGF/CSF-1 paracrine loop. Interestingly, the autocrine contribution in human breast tumor cell invasion is greatly enhanced in vivo and this is associated with a TGFβ1-mediated increased expression of the CSF-1R mRNA by carcinoma cells in the microenvironment of the primary tumor.

As described in the Introduction, transgenic mouse models have failed to address the autocrine role of the CSF-1R in mammary tumor cells, because of the lack of CSF-1R expression in murine mammary epithelia. Notably, in a study by Kirma and colleagues, two transgenic mouse models were prepared in which MMTV

![Figure 4](image-url)
Promoter-driven transgenic mice were forced to overexpress either human CSF-1 or the mouse CSF-1R in the mammary epithelium (38). Both mouse models exhibited an increased rate of glandular dysplasia and ductal hyperplasia and an increased tissue macrophage infiltration. This model further illustrated the importance of CSF-1 and the CSF-1R in cancer progression, but did not evaluate the effects of autocrine regulation through their coexpression in the same tumor cells. In another study, Sapi and colleagues used tail vein injection in mice of CSF-1R overexpressing breast tumor cell lines to address a potential role of autocrine CSF-1R signaling in metastasis in vivo (19). The mice that received the CSF-1R-overexpressing cells showed increased lung metastasis compared with those receiving the parental cells not expressing the CSF-1R. However, this experimental metastasis system does not address the invasiveness of tumor cells in the primary tumor. In our study, we show a direct role for autocrine CSF-1R signaling in invasion in primary mammary tumors, resulting from spontaneous expression...

Figure 5. Expression of CSF-1R mRNA is upregulated in MDA-MB-231 cells in the microenvironment of the primary tumor. A, mRNA expression was analyzed by real-time PCR in MDA-MB-231 cells from cell culture (MDA-231), and compared with tumor cells isolated from primary mammary tumors (APTC). Results here are plotted as fold expression relative to the cultured MDA-MB-231 cells. B, MDA-MB-231 cells were stimulated in vitro with EGF, and total RNA was prepared and analyzed for mRNA expression levels of the same genes by real-time PCR. Results are plotted as fold expression relative to the no EGF control condition. C, MDA-MB-231 cells were stimulated with CSF-1 and analyzed as in B. Results are plotted as fold expression relative to the no CSF-1 control condition. Columns, mean; bars, SEM (*, P < 0.05).

Figure 6. TGFβ1 induces CSF-1R expression and in vivo invasion of MDA-MB-231 cells. A, MDA-MB-231 cells were stimulated in vitro with TGFβ1 and total RNA was prepared and analyzed for mRNA expression levels by real-time PCR. Results are plotted as fold expression relative to no TGFβ1 control condition. B, mRNA expression was analyzed by real-time PCR in MDA-MB-231 cells from cell culture (MDA-231) and tumor cells from xenografts treated with either DMSO or the TGFβ1 receptor-specific inhibitor SB431542. Results are plotted as fold expression relative to the cultured MDA-MB-231 cells. C, invasive cells from MDA-MB-231 xenograft mice that were treated with either DMSO or the SB431542 inhibitor were collected in response to EGF with the in vivo invasion assay. Columns, mean; bars, SEM (*, P < 0.05).
of CSF-1/CSF-1R in human breast cancer cells. Considering that invasion is an early step in the metastatic cascade, our results indicate that both autocrine and paracrine CSF-1 loops significantly contribute to dissemination of cancer cells and progression in human breast cancer. In addition, it is possible that different microenvironments of the same tumor have different contributions to paracrine and autocrine invasion depending on local TGFβ levels and local macrophage densities. The important clinical implication of our findings is that because the mammary tumor microenvironment is inducing the elevated expression of CSF-1R, blocking CSF-1 or its receptor would not only suppress macrophage infiltration in breast tumors and the EGF/CSF-1 paracrine loop, but also the CSF-1/CSF-1R autocrine-mediated invasion by carcinoma cells.

Although MDA-MB-231 cells do not express significant amounts of EGF mRNA, they do express the mRNA for TGFα, another EGFR ligand (Fig. 1A). However, by MDA-MB-231 cells does not seem to be regulated in an autocrine fashion by TGFα. In particular, the EGFR inhibitor, Iressa, did not affect the ability of the cells to move by themselves in the *in vitro* invasion assay (data not shown), but decreased their invasive ability when they were in the presence of macrophages, due to the effect on the paracrine loop (Fig. 4B). Furthermore, simultaneously blocking both the tumor cell and macrophage CSF-1Rs brings *in vivo* invasion to background levels (Fig. 3C), showing that there is no additional contribution from autocrine activation of the EGFR.

Interestingly, the human MDA-MB-231 breast tumor cells are more autocrine for CSF-1 *in vitro* than *in vivo*, a finding explained by the upregulation of the CSF-1 mRNA in tumor cells within the microenvironment of the primary tumor. We also show that TGFβ1 signaling is likely the microenvironmental signal that causes this *in vivo* CSF-1R upregulation in the human tumor cells. Tumors are regularly infiltrated by leukocytes, macrophages, myeloid precursors, and other cells, which are the source of TGFβ secretion and accumulation in primary tumors (39). TGFβ is associated with tumor progression (40) through several mechanisms such as induction of epithelial-mesenchymal transition, myofibroblast generation, inhibition of host immunosurveillance, and others (35). Our study suggests an additional role for TGFβ signaling in invasion and metastasis of human breast cancer cells, through an upregulation of the CSF-1R and induction of the CSF-1/CSF-1R autocrine loop in human primary tumor cells.

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

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**References**


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