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Cytokine Receptor CXCR4 Mediates Estrogen-Independent Tumorigenesis, Metastasis, and Resistance to Endocrine Therapy in Human Breast Cancer

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Abstract

Estrogen independence and progression to a metastatic phenotype are hallmarks of therapeutic resistance and mortality in breast cancer patients. Metastasis has been associated with chemokine signaling through the SDF-1–CXCR4 axis. Thus, the development of estrogen independence and endocrine therapy resistance in breast cancer patients may be driven by SDF-1–CXCR4 signaling. Here we report that CXCR4 overexpression is indeed correlated with worse prognosis and decreased patient survival irrespective of the status of the estrogen receptor (ER). Constitutive activation of CXCR4 in poorly metastatic MCF-7 cells led to enhanced tumor growth and metastases that could be reversed by CXCR4 inhibition. CXCR4 overexpression in MCF-7 cells promoted estrogen independence *in vivo*, whereas exogenous SDF-1 treatment negated the inhibitory effects of treatment with the anti-estrogen ICI 182,780 on CXCR4-mediated tumor growth. The effects of CXCR4 overexpression were correlated with SDF-1–mediated activation of downstream signaling via ERK1/2 and p38 MAPK (mitogen activated protein kinase) and with an enhancement of ER-mediated gene expression. Together, these results show that enhanced CXCR4 signaling is sufficient to drive ER-positive breast cancers to a metastatic and endocrine therapy-resistant phenotype via increased MAPK signaling. Our findings highlight CXCR4 signaling as a rational therapeutic target for the treatment of ER-positive, estrogen-independent breast carcinomas needing improved clinical management.

Introduction

Although endocrine therapy holds great promise in the treatment of hormone-dependent cancer, many patients with estrogen receptor (ER)-positive breast carcinoma exhibit *de novo* resistance to these therapies or will acquire resistance over the course of the disease. Resistance, whether *de novo* or acquired, primarily occurs through altered cellular signaling cascades leading to ligand-independent activation of ER-mediated gene expression and ultimately a hormone-independent phenotype (1). The progression to hormone independence and endocrine therapy resistance is a hallmark sign of progressive carcinoma (2, 3). At this time, all endocrine treatments currently approved for clinical use ultimately result in resistance, showing the ability of carcinoma cells to adapt by altering cellular signaling (4–6). Understanding the path to resistance is critical to developing better treatment options in the future.

The stromal cell–derived factor-1 (SDF-1)–CXCR4 chemokine signaling axis is a known mediator of metastasis in breast cancer as first shown by Muller and colleagues (7). Subsequent studies of clinical breast carcinoma tissues revealed CXCR4 expression in both primary invasive and *in situ* ductal carcinomas, suggesting a role for the SDF-1–CXCR4 axis at all stages of the disease (8). A link between hormone and chemokine signaling in breast carcinoma cells has been proposed, but not shown, based upon the association of a metastatic phenotype with a hormone-independent phenotype (2, 9). *SDF-1* is also an ER α -regulated gene, suggesting involvement of the SDF-1–CXCR4 axis on ER-mediated signaling (10). Furthermore, the biological effects of the SDF-1–CXCR4 axis are mediated through G-protein–coupled receptor (GPCR) signaling leading to activation of downstream signaling pathways including mitogen activated protein kinase (MAPK) family members and PI3K-AKT cascades (11, 12), which have been implicated in development of resistance to endocrine therapy in breast carcinoma (13, 14).

Though it is clear that the SDF-1–CXCR4 axis is a key player in breast cancer, particularly in regard to metastatic disease, its role in primary tumorigenesis and the progression to a

hormone-independent phenotype has yet to be determined. The purpose of this study was to determine the effects of CXCR4 expression, wild-type (WT) and constitutively active (Δ CTD), on the primary and metastatic tumorigenicity of the ER-positive hormone-dependent breast carcinoma cells. Further, the mechanisms of the hormone-independent and endocrine therapy-resistant attributes contributed by CXCR4 expression on MCF-7 and MDA-MB-361 cells were also examined.

Materials and Methods

Patient samples staining and analysis

The results of immunostaining for CXCR4 and ER α were examined using an immunohistochemical (IHC) histologic score (H-score) incorporating intensity and distribution of staining. The H-score is described by: $HS = (\pi * i) / 100$, where π denotes the percentage of stained cells and i denotes the intensity of the staining (15). The H-score scale was 0–3. Staining scale: 0, none; 1, weak; 2, moderate; and 3, strong. Scoring was performed blinded by trained pathologists (16).

Cells and reagents

MCF-7N cell variant (subclone of MCF-7 human breast adenocarcinoma line from American Type Culture Collection (ATCC) was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN) in 1996 (17). The MDA-MB-361 cell line (ER-positive human breast cancer cell line) acquired from ATCC in 2004. Liquid nitrogen stocks were made upon receipt and maintained until the start of each study. Estrogen response element-luciferase and/or qPCR for *ER* and progesterone receptor (*PgR*) were used to confirm cell lines sustained estrogen responsiveness. Morphology and doubling times were also recorded regularly to ensure maintenance of phenotype. Cells were used for no more than 6 months after being thawed. Cells were cultured as previously described (18, 19). AMD-3100 was purchased from Sigma-Aldrich, PD184352 from Santa Cruz Biotechnology, and RWJ-67657 and ICI 182,780 from Tocris Bioscience.

Generation of stable cell lines

Generation of MCF-7 stable cell lines with truncated CXCR4 (Δ CTD), wild-type CXCR4 (WT), or the vector was carried out as previously described (20). A series of stable cell clones were independently derived for confirmation of phenotypes. Each expression vector contained eGFP sequence for cell identification.

Western blot

Western blot analyses were conducted as published (21). After 72hrs in 5% charcoal-stripped fetal bovine serum (CS-FBS) DMEM phenol-free medium, cells were refed with medium-containing vehicle, SDF-1 (10 ng/mL), ICI (100 nmol/L), or SDF-1 + ICI. Membranes were probed with primary antibodies according to manufacturer's protocol. Antibodies: total ER α , CXCR4, corresponding total protein antibodies, α -tubulin (Millipore). Primary antibodies were purchased from Cell Signaling unless noted. IR-tagged secondary antibodies were purchased from LiCor Biosciences. Blots were analyzed by the Odyssey Infrared Imaging System (LiCor Biosciences). Experiments were conducted in triplicate.

Animal studies

Xenograft tumor studies were conducted as described (18, 19, 22). Detailed methods are available in Supplementary Materials and Methods section. For all groups, $n = 5$. All procedures involving animals were conducted in compliance with state and federal laws, the

U.S. Department of Health and Human Services, and guidelines established by the Tulane and Vanderbilt Universities Animal Care and Use Committees. The facilities and laboratory animal programs of the Universities are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Flow cytometry

Excised lungs were minced and pressed through 70- μ m nylon cell strainer (VWR) with 2 mL of HBSS. Single-cell suspension was collected by centrifugation and washed twice. RBCs were lysed with ACK Lysing Buffer (0.15 mol/L NH_4Cl ; 1 mol/L KHCO_3 ; 0.1 mmol/L Na_2EDTA , pH 7.2) followed by 2 washes. Pooled blood was separated on Ficoll-hypaque to obtain the cellular component and eliminate RBCs. Remaining RBCs were lysed with ACK. Cells labeled with CellTracker Violet (CTV) BMQC Dye (Invitrogen) in HBSS (5 μ mol/L per sample). Cells were incubated in CTV (15 minutes) at 37°C with rocking, washed, resuspended in 10% DMEM, incubated on a rocker (15 minutes). Cells were washed with HBSS before addition of 250 μ L of HBSS with 7-aminoactinomycin D (Invitrogen; 1 μ g/mL in PBS). Cells were analyzed by flow cytometry for GFP. Control tissues were taken from uninjected mice were processed at same time as experimental animal tissue.

Immunohistochemistry

Slides from FFPE lungs were deparaffinized and processed for antigen retrieval by heating in 10 mmol/L sodium citrate buffer (pH 6). Endogenous H_2O_2 quenched with 1% H_2O_2 in water. Slides were blocked with normal horse serum (Vector Labs) (1 hour) before application of primary antibody overnight (4°C): Zo1 (1:15; Zymed), eGFP (1:25), and/or E-cadherin (1:20; BD Biosciences). Universal secondary antibody (ABC Elite Substrate kit) and Nova Red color development system (Vector Labs) used per manufacturer's instructions. Slides viewed on a Zeiss Axioplan02 microscope with Olympus Q-Color3 camera.

Fluorescent immunostaining

Immunofluorescent staining was done as described previously and detailed in Supplementary Materials and Methods (20).

In vitro RNA isolation and quantitative real-time PCR

RNA was isolated from cultured cells using RNeasy (Qiagen) and evaluated spectrophotometrically by absorbance (260, 280 nm). About 2 μ g total RNA was reverse-transcribed (iScript kit; BioRad) and real-time PCR was carried out as published (23). Primer sequences are available in Supplementary Materials and Methods. Data analyses compare relative target expression to actin control and relative gene expression was analyzed using $2^{-\Delta\Delta\text{Ct}}$ method (24). Treatments and time points are specified in figure legends. Experiments were conducted in triplicate. Significance based on 95% CIs.

Reverse transcriptase PCR

In vitro: Total RNA was isolated as above. Two micrograms of cDNA was transcribed with SuperScript III (Invitrogen) and mRNA was amplified. Primers were used at 20 nmol/L final concentration.

Liver samples: DNA concentration of liver tissue was determined by spectrophotometry and adjusted to 5 ng per sample. PCR products were analyzed by 2% agarose gel electrophoresis. *GFP* fragment: 640 bp. Primer sequences available in Supplementary Materials and Methods.

Statistical analysis

Studies involving more than 2 groups were analyzed by 1-way ANOVA with Tukey's posttest; all others were subjected to unpaired Student's *t* test (GraphPad Prism V.4). Patient survival was estimated by Kaplan-Meier analysis and survival differences compared by log-rank test. A *P* < 0.05 value was considered statistically significant.

Results

CXCR4 expression effects on breast carcinoma patient survival

To verify a role for CXCR4 expression in patients, IHC staining of CXCR4 and ER α was conducted to determine expression in clinical primary breast tumor samples (Fig. 1A). Of the total 201 cases studied, 76 (37.8%) were positive for CXCR4, whereas 108 (53.7%) were ER positive. No significant correlation between ER status and CXCR4 expression was observed, suggesting that each represents an independent marker (Table 1).

The Kaplan-Meier analysis of patient survival was determined for CXCR4(+) and CXCR4(-) breast carcinomas based on ER status (Fig. 1B). As expected, ER(+) staining alone was a positive prognostic factor for overall patient survival compared with ER(-) tumors (*P* < 0.05). Among ER(+) breast carcinomas, CXCR4 expression was significantly correlated with poor patient survival (*P* < 0.05). Similarly, a significant correlation between poor patient survival among ER(-) and CXCR4(+) tumors was identified, suggesting that overexpression of CXCR4 is associated with decreased survival in breast carcinoma patients regardless of ER status. These results are consistent with previously published findings by Li and colleagues in which high CXCR4 expression was significantly correlated with poor overall survival of breast cancer patients (25).

Effects of constitutively active CXCR4 signaling on breast carcinoma growth *in vivo*

The carboxy terminal domain (CTD) of chemokine receptors play a crucial regulatory role associated with receptor desensitization and downregulation. Mutation of key regions of the CTD of CXCR4 results in constitutive activation. Previously, we had shown increased *in vitro* proliferation of MCF-7 cells expressing the truncated form of CXCR4 (MCF-7-CXCR4- Δ CTD cells) compared with MCF-7-CXCR4-WT and MCF-7-vector expressing cells (20). Orthotopic mouse mammary tumor models were used to assess the role of constitutively active CXCR4 on primary tumorigenesis and metastasis. MCF-7 cells stably expressing empty vector, CXCR4-WT, or CXCR4- Δ CTD were injected into the cleared mammary fat pad of female athymic nude mice and primary tumor growth examined. Mice injected with MCF-7-CXCR4- Δ CTD cells displayed palpable tumors 2 weeks after injection. Moreover, when orthotopic injections were conducted by mixing cells in a Matrigel solution, there was reduced tumor formation variability (19, 26). MCF-7-CXCR4- Δ CTD tumor growth was significantly enhanced compared with MCF-7-CXCR4-WT (*P* < 0.01), supporting previous *in vitro* findings that deletion of the CTD of CXCR4 enhances MCF-7 cell proliferation (20).

CXCR4 expressing breast carcinoma cells exhibit enhanced metastasis

Though MCF-7 cells are minimally invasive (27, 28), our previous *in vitro* data (20) indicated that expression of CXCR4- Δ CTD resulted in increased motility and a gene expression profile consistent with a metastatic phenotype. To examine the effects of CXCR4 expression on the spontaneous metastases *in vivo*, GFP-labeled MCF-7-vector, MCF-7-CXCR4-WT, or MCF-7-CXCR4- Δ CTD cells were injected orthotopically into immunocompromised mice. GFP-IHC staining of tissue sections revealed micrometastases in the lungs of MCF-7-CXCR4- Δ CTD injected mice (Fig. 2B). FACS analysis of GFP(+) cells revealed increases in MCF-7-GFP cells in the lungs of MCF-7-CXCR4- Δ CTD injected

mice ($0.704\% \pm 0.4\%$ viable GFP+ cells) compared with vector ($0.05\% \pm 0.03\%$) or CXCR4-WT ($0.125\% \pm 0.07\%$) injected mice (Fig. 2C).

To account for variable metastatic seeding due to tumor size, the direct tail vein model was used to determine the metastatic capacity of GFP-expressing MCF-7 lines (vector, WT-CXCR4 or CXCR4-ΔCTD). After 3 weeks, blood, lungs, bone marrow, and liver were harvested for analysis. Metastatic lesions were only visible in the lungs. Minimal metastases were observed on lung surfaces of MCF-7-WT-CXCR4 or MCF-7-vector-injected mice; however, numerous surface metastases were grossly visible in MCF-7-CXCR4-ΔCTD injected mice (Fig. 2D; $P < 0.01$). Animals injected with parental MCF-7 cells showed no visible metastases. FACS analysis revealed abundant viable GFP(+) cells in the lungs of MCF-7-CXCR4-ΔCTD injected mice ($0.53\% \pm 0.15\%$). Animals injected with vector ($0.01\% \pm 0.005\%$) or MCF-7-CXCR4-WT cells ($0.002\% \pm 0.002\%$) exhibited minimal GFP(+) cells (Fig. 2E; $P < 0.01$). RT-PCR analysis of liver samples from MCF-7-CXCR4-ΔCTD and MCF-7-CXCR4-WT injected animals revealed increased GFP expression compared with vector (Supplementary Fig. 1) indicating CXCR4-mediated-enhanced metastasis. There was no evidence of GFP(+) cells in bone marrow or blood (data not shown).

Effects of CXCR4 expression on epithelial cell markers

Cultured MCF-7-CXCR4-ΔCTD cells exhibit epithelial-mesenchymal transition (EMT)-like changes: stellate appearance, enhanced motility, and decreased cell adhesion protein expression (E-cadherin, ZO-1; ref. 20). To determine if this phenotype was retained *in vivo*, primary tumor sections were examined. The localization and expression of E-cadherin, was evaluated by comparing the staining pattern of E-cadherin (green) and β-catenin (red) in primary tumor sections by immunofluorescent microscopy. Both MCF-7-CXCR4-WT and vector control tumors showed high levels of membrane and cell junction expression of β-catenin and E-cadherin. Overlays show β-catenin and E-cadherin colocalized at epithelial cell junctions (arrows; Fig. 3A). In contrast, there was little evidence of membrane expression of β-catenin or E-cadherin on -CXCR4-ΔCTD tumors. Cells injected with and without Matrigel produced similar results. Zo1 expression was membrane associated in mouse epidermis from normal skin (+control) and MCF-7-vector cells, but both cytoplasmic and membrane-associated for MCF-7-CXCR4-WT cells, and MCF-7-CXCR4-ΔCTD cells (Fig. 3C).

Quantitative real-time PCR (qPCR) analysis have shown *E-cadherin* mRNA levels were significantly reduced in MCF-7-CXCR4-ΔCTD cells (−211.3 fold) compared with vector, whereas MCF-7-CXCR4-WT cells showed no significant difference from control (Fig. 3B). No statistical difference was found in *Zo1* mRNA expression between MCF-7-vector and MCF-7-CXCR4-WT or MCF-7-CXCR4-ΔCTD cells (Fig. 3D). In agreement with the EMT-like changes associated with increased CXCR4 expression, significant increases in vimentin were found in wild-type and truncated CXCR4-expressing cells by qPCR (data not shown).

To further investigate the involvement of CXCR4 expression on the EMT phenotype, MCF-7-CXCR4-WT cells were treated in culture with the CXCR4 inhibitor AMD-3100, a small molecule CXCR4 antagonist that competes with ligand for binding to the CXCR4 receptor (29, 30). Inhibition of CXCR4 signaling decreased gene expression of the EMT-associated marker Vimentin, indicating that by blocking CXCR4 signaling it is possible to reverse the CXCR4-induced progression of breast cancer cells to a mesenchymal phenotype ($P < 0.001$; Supplementary Fig. 2).

Hormone independent tumor growth of ER-positive breast cancer is associated with CXCR4 expression

EMT-associated changes (decreased E-cadherin, increased vimentin, decreased cell adhesion) are also correlated to decreased ER expression (31). Previously conducted microarray analyses that revealed reduced ER expression in MCF-7-CXCR4-ΔCTD cells compared with WT CXCR4 and vector control (20), were confirmed by qPCR to reveal a 141.6-fold and 9.6-fold decrease in *ER* expression in MCF-7-CXCR4-ΔCTD and MCF-7-CXCR4-WT cells, respectively ($P < 0.001$) compared with control (data not shown). Xenograft tumor samples stained for ER expression also showed reduced expression of ER in MCF-7-CXCR4-ΔCTD tumors compared with MCF-7-CXCR4-WT (data not shown).

Progressive loss of ER expression in CXCR4-WT and CXCR4-ΔCTD cells suggests a role for CXCR4 expression in ER(+) breast carcinoma growth and progression. Stable MCF-7-vector or MCF-7-CXCR4-WT cells were injected orthotopically into ovariectomized immunocompromised mice in the presence or absence of exogenous estrogen. Estrogen-dependent MCF-7-vector cells formed tumors only in the presence of estrogen (Fig. 4A). Overexpression of CXCR4-WT led to enhanced tumor growth in the presence of estrogen ($P < 0.05$), and estrogen-independent tumor formation ($P < 0.05$), showing that the CXCR4 axis drives MCF-7 cells toward a hormone-independent but estrogen-responsive phenotype. Additional stable clones tested exhibited the same results *in vivo* confirming maintenance of the phenotype across independently derived clones (Supplementary Fig. 3).

To validate its involvement in the promotion of breast cancer tumorigenesis the CXCR4 axis was disrupted. Tumors were established orthotopically by injection of MCF-7-CXCR4-WT cells mixed with Matrigel containing anti-CXCR4 antibodies that block ligand binding or isotype IgG control. Anti-CXCR4 antibodies suppress the hormone-independent tumorigenesis (Fig. 4B; $P < 0.001$) and estrogen-stimulated growth of MCF-7-CXCR4-WT xenografts (Fig. 4C; $P < 0.01$).

AMD-3100 was used to further validate that CXCR4 mediated the enhanced tumorigenesis observed *in vivo*. Animals injected with MCF-7-CXCR4-WT cells orthotopically were implanted with vehicle (Fig. 4D) or estrogen pellets (Fig. 4E) and treated twice daily with vehicle or AMD-3100 for 14 days beginning same day of cell injection. Treatment with AMD-3100 significantly suppressed estrogen-stimulated ($P < 0.01$) and hormone-independent tumor growth ($P < 0.001$).

CXCR4 expression alters ER-mediated gene expression and endocrine therapy response

Estrogen responsive gene expression of MCF-7-vector and MCF-7-CXCR4-WT cells was compared via RT-PCR. MCF-7-vector cells displayed expected expression in response to estrogen stimulation. Higher basal expression of ER-responsive genes (*SDF-1*, *PGR*, *BCL2*) was observed in MCF-7-CXCR4-WT cells (Supplementary Fig. 4), suggesting CXCR4-signaling targets estrogen responsive genes stimulating their expression in the promotion of tumor growth. The possibility arose of an autocrine SDF-1–CXCR4 loop within CXCR4 expressing cells where basal or estrogen-stimulated SDF-1 expression and secretion drives autocrine activation of CXCR4-mediated signaling and tumorigenesis.

Fulvestrant (ICI 182,780; ICI), an ER downregulator, was used to block ER signaling in MCF-7-CXCR4-WT cells resulting in suppressed estrogen stimulation of *SDF1* and *BCL2* gene expression (Fig. 5A). ICI did not completely suppress basal expression of *VEGFA* or *PGR*, suggesting that CXCR4 may also function to mediate target genes independent of ER. Western blot analysis of MCF-7-CXCR4-WT cells confirms ICI downregulation of ER expression, though no change was observed with SDF-1 treatment.

The effects of the SDF-1–CXCR4 cross-talk with ER on *in vivo* tumor formation was assessed using the MCF-7-CXCR4-WT cell line and the ER-positive, endogenously CXCR4(+) breast carcinoma cell line MDA-MB-361 (Supplementary Fig. 5). MCF-7-CXCR4-WT (Fig. 5C) or MDA-MB-361 (Fig. 5D) cells mixed with Matrigel containing SDF-1 or vehicle were injected orthotopically. ICI treatment (given once on day 7) resulted in reduced tumor volume and suppression of control tumor growth, whereas cells injected in the presence of exogenous SDF-1 treated with ICI continued to grow at control levels. This suggests that an intact SDF-1–CXCR4 axis counteracts the action of antiestrogen therapy in ER (+)/CXCR4(+) cells.

CXCR4 effects are mediated through ERK MAPK signaling

Many signaling pathways have been implicated in CXCR4 and chemokine signaling, particularly MAPKs and AKT (13, 14, 32, 33). We have previously shown that SDF-1 treatment induces a robust time-dependent increase in ERK1/2 activation in MCF-7-CXCR4-WT cells (increased phosphoprotein levels) (20). No significant changes in phospho-AKT levels were detected. To verify the involvement of ERK signaling in CXCR4(+) cells, animals were randomized into treatment groups after tumor formation and injected once/day (5 days) with the MEK1/2 (MAP/ERK kinase)-ERK1/2 inhibitor (PD184352) or vehicle. Treatment with PD184352 significantly suppressed tumor growth of MCF-7-CXCR4-WT (Fig. 6A; $P < 0.001$), suggesting a role for CXCR4-MAPK signaling in the hormone-independent growth of ER(+)/CXCR4(+) breast carcinoma.

ERK1/2 signaling was also tested in connection with SDF-1-mediated ICI resistance. MCF-7-CXCR4-WT cells mixed with Matrigel containing SDF-1 or vehicle were injected orthotopically. After tumor formation animals received daily injections (5 days) of vehicle or PD184135. On day 7, animals a single injection of ICI. Treatment of PD184135 reduced SDF-1-mediated ICI resistance significantly ($P < 0.05$); however, suppression of CXCR4-positive tumor growth was not sustained past day 30 post-cell injection (Fig. 6B).

Discussion

The association of CXCR4 expression and breast cancer metastasis has been well documented (34–36), however, the effects of SDF-1–CXCR4 signaling on primary tumorigenesis and disease progression is not well understood. We show here that CXCR4 expression is highly correlated with decreased breast carcinoma patient survival, as supported by others (25, 37–39), likely due in part to the prominent role of CXCR4 in cancer metastasis and chemotaxis. Downregulation of cadherins and other adhesion protein complexes involved in cell-cell junctions leads to increased cell motility and progression to metastasis (40, 41). Decreased membrane expression of E-cadherin in MCF7-CXCR4- Δ CTD primary tumors was observed. Additionally, treatment of MCF-7-CXCR4-WT cells in cultures with AMD-3100 significantly reduced the expression of vimentin. These results signify an association with CXCR4 expression and the progression of breast carcinoma cells toward EMT. Our orthotopic xenograft studies clearly show a role for CXCR4-mediated metastasis to SDF-1 rich organs (lung, liver) via regulation of the CXCR4 CTD, and in primary tumorigenesis of ER(+) breast carcinoma.

Although CXCR4 has been implicated in the proliferation and survival of primary breast carcinoma, these studies were conducted using highly aggressive cells that mimic advanced stage disease (8, 42). Here we utilized the MCF-7 breast cancer cell line as a model cell system for ER-positive, primary breast carcinoma. Interestingly, MCF-7-CXCR4- Δ CTD primary tumors showed reduced nuclear staining for ER α indicative of the progression to a more aggressive and less differentiated phenotype. Intrigued by these results, we sought to

determine what role CXCR4 signaling plays in primary tumor growth and progression to hormone independence.

MCF-7 cells are hormone-dependent and nontumorigenic in nonestrogenic xenograft environments (43). Initial *in vivo* studies conducted the ability of wild-type CXCR4 overexpression in MCF-7 cells to enhance tumor growth in the presence of estrogen. Here we showed the ability of CXCR4 overexpression to promote MCF-7 tumor growth in the absence of estrogen. This ability of CXCR4-mediated signaling to facilitate escape from hormone dependence has not been previously shown *in vivo*. The recent publication by Sauvé and colleagues showed ER–CXCR4 cross-talk in MCF-7 cells *in vitro* (9). It was shown that CXCR4 activates ER-mediated gene transcription via phosphorylation of ER β by members of the MAPK family. This ER–CXCR4 cross-talk bypasses the need for estrogen and initiates an autocrine feed-forward loop (9). This model is supported by our gene expression data showing higher basal expression for ER-responsive genes in the MCF-7-CXCR4 cells, which retained estrogen sensitivity. Our work further shows that estrogen activation of the ER increases SDF-1 gene expression, which may drive CXCR4 signaling and mediate estrogen-independent tumor growth. We have shown previously that MCF-7-CXCR4-WT cells secrete higher levels of SDF-1 compared with MCF-7-vector cells (99 ± 5.8 and 43 ± 3.8 pg per 10^5 cells, respectively) (20). However, ICI 182,780 is able to block CXCR4-induced hormone-independent growth and SDF-1 expression in MCF-7-CXCR4-WT and MDA-MB-361–derived tumors. Exogenous SDF-1 treatment is able to overcome the antitumor effects of ICI suggesting that the endocrine therapy-resistance observed seems to be achieved by ER-independent CXCR4 signaling mechanisms. Whereas we believe that estrogen-independence is in part due to ER-regulated gene expression (cross-talk) as suggested by Sauvé and colleagues (9), due to the ability of ICI to inhibit CXCR4-mediated tumorigenesis. Our data support a more complex model in which a combination of intrinsic CXCR4 signals respond to both autocrine produced and exogenously derived SDF-1 to drive progression to an estrogen-independent phenotype. These results support the idea that breast cancer progression may be associated with a switch from dependence upon estrogen stimulated production of SDF-1, to SDF-1 supplied by stromal cells (9). Although an autocrine loop may exist between ER and the SDF-1–CXCR4 axis, this system may also require the input of additional SDF-1 produced by tumor stromal cells to overcome endocrine therapies.

Additional inquiry into this cross-talk led to examination of specific signaling pathways. Both the MAPK and PI3K/AKT signaling pathways have been implicated in chemokine signaling (44–46) and are known mediators of ER activity (47, 48). These pathways are also involved in hormone-independent tumorigenesis and the development of endocrine resistance (14, 32, 33, 49, 50). Our data suggest that MAPK pathways, specifically ERK1/2, are involved in CXCR4-mediated progression to hormone independence; however, the SDF-1–CXCR4 axis seems to regulate progression to antiestrogen resistance through an alternate, non–ERK1/2-dependent, pathway *in vivo*.

We have shown a compelling role for CXCR4 signaling in the progression of ER-positive cancers to hormone-independent and therapeutic-resistant phenotypes. CXCR4 expressing cells retain ER expression and estrogen sensitivity, but have progressed to hormone independence and endocrine resistance. Blocking the SDF-1–CXCR4 axis through targeting of CXCR4 or downstream MAPK signaling proteins decreased these effects. Along with showing a novel role for CXCR4 signaling in primary breast carcinoma through cross-talk with the ER, our data provide insight into the signaling mechanisms that regulate progression to hormone independence. These data, by furthering our knowledge of the progression of breast carcinoma, provide novel therapeutic targets for the treatment of ER- and CXCR4-positive, hormone-independent breast disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CXCR4 expression in and impact on survival of clinical breast carcinoma. A, human breast carcinoma tissue samples were collected and stained with anti-CXCR4 or anti-human ER α . Scale bar, 60 μ m. B, the Kaplan–Meier analysis of overall survival of breast cancer patients was conducted and survival curves were stratified by expression status of CXCR4 and ER. Differences in survival distributions were evaluated by log-rank tests; $P < 0.05$.

Figure 2.

Constitutive activation of CXCR4 promotes tumorigenesis and metastatic potential *in vivo*. A, MCF-7-CXCR4-ΔCTD, MCF-7-CXCR4-WT, or MCF-7 empty vector control cells (5×10^6) mixed with Matrigel were injected (M.F.P) of ovariectomized 3- to 4-week-old female SCID/beige mice. Points represent mean TV $\pm$ SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. B, to evaluate spontaneous metastases, MCF-7-CXCR4-ΔCTD, MCF-7-CXCR4-WT, or MCF-7 empty vector cells (2×10^6) were injected into the cleared M.F.P. of 3- to 4-week-old female nude mice. After 2.5 months of tumor growth, lungs were harvested for fixation or flow cytometry. Images of lung sections immunostained with anti-IgG control or anti-GFP antibody. Scale bar, 125 μ m. Red arrows indicate GFP(+) lesions. C, detection of spontaneous metastases by flow cytometry for GFP(+) cells on endpoint lungs. Bars, mean percent live GFP(+) cells $\pm$ SEM. D, to evaluate experimental metastasis, nude mice were injected in the tail vein with MCF-7 parental cells or MCF-7 cells expressing WT CXCR4, vector, or ΔCTD-CXCR4 (1×10^6). Three weeks later, lungs and other organs were evaluated for visible appearance of metastatic lesions. Lung metastases appear as white lesions on the lung surface. Quantitation of surface lung metastases from triplicate experiments, scored independently by 3 individuals. Bars, mean count $\pm$ SEM; **, $P < 0.01$. E, quantitation of experimental metastases after tail vein injection by flow cytometry on larger lobe of the left lung (see Materials and Methods) to determine the number of GFP(+) cells. Bars, mean percent live GFP(+) cells $\pm$ SEM for triplicate experiments; **, $P < 0.01$.

Figure 3.

Constitutively active CXCR4 expression decreases epithelial cell markers. A, tumor tissue stained with antibodies to E-cadherin and β -catenin, with secondary antibodies visible in the green (λ_{488}) or red (λ_{594}) wavelength, respectively. Hoechst was used to stain nuclei (blue). Scale bar, 250 μ m. C, ZO1 immunostaining of tissue sections from normal mouse skin and tumors from vector control, MCF-7-CXCR4-WT, or MCF-7-CXCR4- Δ CTD cells. Scale bar, 60 μ m. qPCR of cultured cells for (B) *E-cadherin* or (D) *ZO1*. Bars, average fold-change in expression of triplicate experiments of MCF-7-CXCR4-WT and MCF-7-CXCR4- Δ CTD cells compared with vector, for each gene normalized to β -actin; *, $P < 0.05$.

Figure 4.

CXCR4 signaling promotes ER(+) breast tumorigenesis. A, female, 4- to 6-week-old, ovariectomized SCID/beige mice ($n = 5/\text{group}$) were injected in the mammary fat pad with 5×10^6 MCF-7-vector or MCF-7-CXCR4-WT cells. Estrogen treatment groups were implanted with a pellet of 17β -estradiol subcutaneously (0.72 mg, 60-day release). B–E, female, 4- to 6-week-old, ovariectomized Nu/Nu mice ($n = 5/\text{group}$) were injected (M.F.P.) with 5×10^6 MCF-7-CXCR4-WT cells suspended in 50 μL of Matrigel (reduced factor) containing isotype IgG or anti-CXCR4 antibodies (B and C) or treated with twice daily i.p. injections of vehicle or AMD-3100 (0.1 mg/animal/dose) for 14 days. D and E, estrogen groups were implanted with a pellet (0.72 mg 60-day slow release) of 17β -estradiol s.c. Points represent mean TV $\pm$ SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5.

CXCR4 autocrine loop drives hormone-independent growth of MCF-7 cell tumorigenesis. A, RT-PCR analysis of MCF-7-CXCR4-WT cells treated with DMSO, 100 pmol/L 17 β -estradiol (E2), 100 nmol/L ICI182,780 (ICI), or the combination E2 + ICI for 18 hours. Representative blots of triplicate experiments. B, Western blot analysis for total ER α in MCF-7-CXCR4-WT cells treated with DMSO, SDF-1 (10 ng/mL), ICI (100 nmol/L), or SDF + ICI for 18 hours. C and D, female, 4- to 6-week-old, ovariectomized SCID/beige mice ($n = 5$ /group) injected (M.F.P.) with 5×10^6 of MCF-7-CXCR4-WT (C) or MDA-MB-361 (D) cells suspended in 50 μ L of Matrigel (reduced factor) containing isotype control or exogenous SDF-1 (100 ng). ICI treatment groups were administered a single dose of ICI (5 mg) i.p. at day 7. Points, mean TV $\pm$ SEM; *, $P < 0.05$; ***, $P < 0.001$.

Figure 6.

MAPK signaling involved in CXCR4-mediated tumorigenesis and endocrine therapy resistance. A and B, female, 4- to 6-week-old, ovariectomized, nude mice ($n = 5/\text{group}$) were injected (M.F.P.) with 5×10^6 MCF-7-CXCR4-WT cells suspended in 50 μL of PBS and 50 μL of Matrigel. After tumor formation, animals were treated twice daily for 5 days with i.p. injections of vehicle or PD184352 (20 mg/kg/animal). All treatment groups were administered a single dose of ICI 182,780 (5 mg) i.p. at day 7. B, cells were mixed with exogenous SDF-1 (100 ng) or isotype control immediately prior to injection. Treatments and schedule as described above. Points represent mean TV $\pm$ SEM; *, $P < 0.05$; ***, $P < 0.001$.

Table 1Relationship between CXCR4 expression and ER α expression in 201 cases of primary breast carcinoma

	CXCR4		Total
	(-)	(+)	
ER			
(-)	59 (29.4%)	34 (16.9%)	93
(+)	66 (32.8%)	42 (20.9%)	108
Total	125	76	201 (100%)

NOTE: Two hundred one primary breast carcinoma samples were stained with anti-ER α and anti-CXCR4 antibodies as illustrated in Figure 1A. Receptor expression data are reported here. No significant correlation was found between CXCR4 and ER expression by chi-square analysis ($P > 0.05$).