CXCR4 and Matrix Metalloproteinase-1 Are Elevated in Breast Carcinoma–Associated Fibroblasts and in Normal Mammary Fibroblasts Exposed to Factors Secreted by Breast Cancer Cells

Sarah M. Eck, Anik L. Côte, William D. Winkelman, and Constance E. Brinckerhoff

Abstract

The complex molecular communications that occur between neoplastic and stromal cells within the tumor microenvironment play an integral role in breast cancer pathogenesis. Cancer-associate fibroblasts (CAF) produce tumor-enhancing factors and have been strongly implicated in breast cancer development. Similar to the way in which tumors have been compared with "wounds that never heal," CAFs have been equated to activated fibroblasts, which are present in inflammatory environments, in which they aid in wound healing through tissue remodeling and repair. Matrix metalloproteinase-1 (MMP-1) and G protein–coupled receptor, CXCR4, are elevated in these activated fibroblasts, in which they facilitate angiogenesis and matrix degradation, processes that are also vital to breast cancer metastasis. In this study, we investigated MMP-1 and CXCR4 expression in normal human mammary fibroblasts (HMF) exposed to soluble breast cancer factors. Historically, elevated CXCR4 expression is associated with breast cancer cells. However, we show that soluble factors secreted by SUM102 breast cancer cells stimulated the expression of MMP-1 and CXCR4 in HMFs. As a result, these stromal cells acquired an invasive and migratory phenotype. To confirm the clinical relevancy of our findings, we analyzed CAFs obtained from primary breast cancers. These cells also displayed elevated MMP-1 and CXCR4 levels compared with counterpart fibroblasts, and were more invasive and migratory. Together, our data suggest that soluble breast cancer factors initiate the transdifferentiation of normal HMFs to tumor-promoting CAFs, and that through the induction of MMP-1 and CXCR4 levels, these cells exhibit an invasive and migratory phenotype. (Mol Cancer Res 2009;7(7):1033–44)

Introduction

For the past three decades, cancer research has focused predominantly on the characteristics of malignant epithelial cells. However, recently, clinical and experimental evidence have revealed that tumor development is intimately related to the complex interactions that transpire within the tumor microenvironment (1, 2). Neoplastic epithelial cells engage in reciprocal molecular dialogues with surrounding stromal cells, including inflammatory cells, vascular cells, and fibroblasts, resulting in the production of stromal-derived tumor-aiding factors, such as growth factors, chemokines, cytokines, proteases, and vascular-stimulating factors (3, 4). These molecular communications at the site of the primary tumor enable its growth and dissemination.

Breast cancer represents one example of a carcinoma in which the collaborative efforts from malignant epithelial cells and stromal cells within the primary tumor coordinate carcinogenesis, and ultimately, metastasis to distant organs. Women have a 12% risk of developing breast cancer during their lifetime, and a 5% risk of breast carcinoma–related death (5). Mortality results from frequent metastasis, which reduces long-term survival (+5 years) from ~90% to 5% (6). A xenograft model of breast cancer has shown that breast carcinoma cells depend on stromal cells to metastasize (7). This may be explained by the finding that the stroma plays a paramount role in supporting breast tumor growth and vascularization (8), both of which are prerequisites to metastasis (9). Because of the importance of the tumor/stromal interactions in promoting breast cancer metastasis, it is imperative to elucidate the molecular events that occur between cancer cells and adjacent stroma at the site of the primary tumor.

Of particular interest are the stromal fibroblasts found within mammary carcinomas, which by volume constitute the major cellular component of the tumor microenvironment (1, 10). Termed carcinoma-associated fibroblasts (CAF), these cells have been connected to several tumor-promoting events, including: angiogenesis (8), antitumor immunosuppression (11), genetic instability (12), epithelial-to-mesenchymal transition (13), and metastasis (7). Although the importance of these cells in tumor progression has been acknowledged, CAFs have not been clearly and universally defined, and are only roughly characterized by their enhanced production of tumor-promoting factors.

Similar to the way in which a tumor is equated to a "wound that never heals" (14), CAFs have been compared with "activated..."
fibroblasts" or myofibroblasts, which are present in inflammatory environments, in which they play a critical role in angiogenesis and the production of components of the extracellular matrix (3, 4). Several studies suggest that myofibroblasts constitute the majority of CAFs, as determined by α-smooth muscle actin (αSMA), a marker for differentiated myofibroblasts (15, 16). In contrast, other data suggest that not all CAFs within the tumor microenvironment are myofibroblasts. For example, Holliday and colleagues established that only 35% of CAFs isolated from primary breast carcinomas were αSMA-positive, and that this mirrored the expression of αSMA in normal breast fibroblasts (17). Yet other studies characterize CAFs by elevated stromal-derived factor-1 (SDF-1) expression (8, 18). Sugimoto and colleagues show that the expression of commonly used "CAF markers", including αSMA, vimentin, and fibroblast activation protein (FAP) do not overlap with each other. Thus, they conclude that CAFs are a heterogeneous population and that the use of these markers alone will not suffice to identify all CAFs (19). We speculate, therefore, that multiple CAF subpopulations exist and suggest that they evolve from a variety of origins.

Indeed, Orimo and Weinberg have proposed three models by which CAFs are derived in human carcinomas, (a) clonal selection of a group of fibroblasts or progenitors that have acquired genetic alterations; (b) transdifferentiation of normal stromal fibroblasts, without acquiring genetic alterations; and (c) differentiation of stromal fibroblasts recruited from specialized progenitor cells in the circulation (20). Studies support the notion that CAFs are either derived from bone marrow progenitor cells (7, 21, 22) and/or that they evolve from the conversion of normal mammary fibroblasts (8) or other resident cells, such as endothelial cells or epithelial cancer cells (23).

We are interested in the mechanism(s) by which breast cancer cells solicit normal, resident mammary fibroblasts (HMF) to produce inflammatory and tumor-promoting factors, and thus become activated and CAF-like, and/or mature CAFs. Little is known about the molecular determinants of the transformation of an HMF to a CAF (8, 24). In the current study, we examined the transdifferentiation of normal primary resident HMFs to activated fibroblasts, or to acquire CAF-like phenotype, in response to breast cancer–secreted factors. We measured factors that have previously been affiliated with CAFs and/or the activated fibroblasts found in sites of inflammation (8, 25, 26). Our data show that SUM102 breast cancer cells, which were derived from an intraductal breast carcinoma, secrete factors that stimulate normal HMFs to express tumor-enhancing, inflammatory-related factors, including matrix metalloproteinase-1 (MMP-1) and the G protein–coupled receptor, CXCR4.

The neutral protease, MMP-1, is recognized as a critical factor in breast cancer metastasis (27-29), and its roles as a mediator of matrix degradation and as a modulator of cell behavior have been established (30-33). However, no study has examined MMP-1 expression and function during the transition from an HMF to a CAF; nor has the function of this protease been identified in CAF isolates from patients with breast cancer, although it has been associated with matrix remodeling by activated fibroblasts in inflammation (34).

The overexpression of chemokines and their receptors, such as CXCR4, is also well documented as a key stimulus in promoting breast cancer tumorigenesis (35). Classically, CXCR4 is markedly up-regulated on breast cancer cells, allowing them to migrate to targeted distant organs containing cells that secrete its ligand, SDF-1 (36). However, it has also been documented that CXCR4 expression is elevated in fibroblasts in inflammatory environments other than cancer. For example, synovial fibroblasts from patients with rheumatoid arthritis display constitutively expressed CXCR4 mRNA and cell surface protein (37). Similarly, studies show that fibroblasts from mice with pulmonary fibrosis express CXCR4 and respond chemotactically to SDF-1 (38, 39). Because the cancer microenvironment has been equated to a chronic site of inflammation (14), we propose that similar to activated fibroblasts in inflammatory conditions, CAFs also increase CXCR4 levels and that this contributes to breast cancer progression.

Our study illustrates that MMP-1 and CXCR4 mRNA and protein levels are increased in primary HMFs treated with factors secreted by breast cancer cells, and that MMP-1 and CXCR4 are functionally important for downstream signaling, cell migration, and matrix degradation. Notably, we use normal, primary HMFs in these studies to avoid potential artifacts, such as a spontaneous increase in CXCR4 following immortalization with telomerase (40). Importantly, we also show increased MMP-1 and CXCR4 expression and function in CAF isolates from primary breast tumors, compared with counterpart, non–tumor-associated fibroblasts from the same patient, signifying that our findings are clinically relevant. Together, our data suggest that breast cancer cells secrete growth factors and chemokines that stimulate normal resident HMFs to become CAF-like.

Results

HMFs Promote Tumor Engraftment and Growth of 102 Breast Cancer Cells

SUM102 (102) breast cancer cells were derived from an intraductal breast carcinoma and are estrogen receptor–negative/progesterone receptor–negative, suggesting that they are phenotypically aggressive (41, 42). Because MMP-1 and CXCR4 mediate breast cancer invasion and metastasis by MDA-MB-231 (231) breast cancer cells in vivo (43), we compared MMP-1 and CXCR4 gene expression in 102 breast cancer cells to that of the well-established, aggressive 231 breast cancer cells. Compared with 102 cells, MMP-1 and CXCR4 mRNA was greater in 231 cells by approximately 35-fold and 70-fold, respectively (Fig. 1A), indicating that the 102 cells do not express the same parameters as the 231 cells. Unlike 231 cells, which readily form tumors in vivo, when 102 cells were injected orthotopically into the fourth mammary fat pad of nude mice, they were weakly tumorigenic (Fig. 1B), suggesting that tumorigenesis in these cells may depend on stromal/tumor cell interactions.

Reports have documented that either non–cancer associated, normal HMF or CAFs enhance breast tumor growth when injected simultaneously with mammary carcinoma cells into nude mice using an orthotopic model (8, 44). We used this model to examine the functional ramifications of the molecular interactions between weakly tumorigenic 102 breast cancer cells and HMFs isolated from cancer-free mammary tissues: 102 breast cancer cells were injected orthotopically into nude mice in the
or 1 × 106 of each cell line (102 + HMF). Points, average tumor diameter calculated using the two levels than SUM102 breast cancer cells. Values were normalized to HPRT relatively higher MMP-1 (**, P \leq 0.01) and CXCR4 (*, P > 0.05) mRNA levels than SUM102 breast cancer cells. Values were normalized to HPRT and calculated using the 2ΔΔCT method (see Materials and Methods). Columns, mean fold difference; bars, SD.

To determine whether factors produced by 102 carcinoma cells affect HMF gene expression, causing these stromal cells to gain an activated, CAF-like phenotype, we used real-time reverse transcription (RT)-PCR to measure the expression of a panel of genes known to be associated with CAFs and/or activated fibroblasts. Compared with control HMFs, treated with HMF cell–conditioned media (HMFCM), HMFs treated with 102 cell-conditioned media (102CM) exhibited significantly increased levels of several genes that have previously been associated with activated fibroblasts present in inflammatory situations and CAFs: MMP-1, MMP-3, IL-8, and SDF-1 (refs. 3, 8, 18, 45, 46; Fig. 2A). Notably, these genes also promote breast cancer invasion and metastasis (13, 36, 43, 47). As expected, when studying primary cells from an outbred population, we observed inherent differences in the responsiveness of HMFs treated with 102CM. Nonetheless, of the 12 primary HMF samples tested, ~70% were responsive.

These data suggest that HMFs become activated or CAF-like in response to 102CM, and thus, acquire an enhanced tumor-promoting phenotype following exposure to soluble breast cancer factors. However, 102CM did not induce other genes, including several classic CAF-related genes: FAP, αSMA, vimentin, and VEGF within 24 hours (data not shown; refs. 19, 48). This finding suggests that resident HMFs, which become activated and CAF-like, may represent a distinct subpopulation of CAFs in the tumor microenvironment. It is also feasible that HMFs may require additional environmental cues, which are absent in vitro, in order to transform into mature CAFs. Alternatively, other studies have shown that fibroblast precursor cells only express CAF-related genes, such as SDF-1, αSMA and FAP, after 30 days of exposure to breast cancer conditioned medium (18).

FIGURE 1. HMFs enhance tumor engraftment and growth of SUM102 breast cancer cells in vivo. A. SUM102 and MDA-231 cell lines were serum-starved for 24 h, and total RNA was collected for real-time RT-PCR quantification of MMP-1 and CXCR4 mRNA. MDA-231 cells display relatively higher MMP-1 (**, P \leq 0.01) and CXCR4 (*, P > 0.05) mRNA levels than SUM102 breast cancer cells. Values were normalized to HPRT and calculated using the 2ΔΔCT method (see Materials and Methods). Columns, mean fold difference; bars, SD. B. Nude mice were injected orthotopically with 2 × 106 SUM102 breast cancer cells, 2 × 106 HMFs, or 1 × 106 of each cell line (102 + HMF). Points, average tumor diameter (mm) 6 mo following injection; bars, mean (***, P \leq 0.0006 compared with tumors formed by injection of SUM102 cells alone).

Table 1. Tumor Formation and Diameter in Mice Injected with HMFs, 102 Cells, or Both Simultaneously

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<tr>
<th>Tumor Formation (%)</th>
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<tr>
<td>102</td>
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102 Breast Cancer Cells Stimulate the Expression of Tumor-Promoting Genes That Are Associated with Cancer and Inflammation in Normal Mammary Fibroblasts

To determine whether factors produced by 102 carcinoma cells affect HMF gene expression, causing these stromal cells to become activated or CAF-like, we used real-time reverse transcription (RT)-PCR to measure the expression of a panel of genes known to be associated with CAFs and/or activated fibroblasts. Compared with control HMFs, treated with HMF cell–conditioned media (HMFCM), HMFs treated with 102 cell-conditioned media (102CM) exhibited significantly increased levels of several genes that have previously been associated with activated fibroblasts present in inflammatory situations and CAFs: MMP-1, MMP-3, IL-8, and SDF-1 (refs. 3, 8, 18, 45, 46; Fig. 2A). Notably, these genes also promote breast cancer invasion and metastasis (13, 36, 43, 47). As expected, when studying primary cells from an outbred population, we observed inherent differences in the responsiveness of HMFs treated with 102CM. Nonetheless, of the 12 primary HMF samples tested, ~70% were responsive.

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Perhaps most interestingly, we found that HMFs exhibited elevated CXCR4 mRNA levels in response to 102CM (Fig. 2B), because overexpression of this receptor is usually associated with invasive breast cancer cells (ref. 36; see Fig. 1A), whereas CAFs are recognized for secreting increased amounts of its ligand, SDF-1 (8). As noted earlier, we also detected SDF-1 gene induction in HMFs exposed to 102CM (see Fig. 2A). Elevated CXCR4 expression on breast cancer cells has been correlated with augmented angiogenesis and directional migration (8, 36). Thus, CXCR4 induction in resident fibroblasts implies that these cells may also contribute to tumorigenesis, invasion, and migration. We conclude that 102CM induces the expression of genes associated with activated fibroblasts and/or CAFs, and we will focus on MMP-1 and CXCR4 because of their documented importance in breast cancer progression (8, 36, 49, 50). To emphasize the broad significance of this finding, medium conditioned by 231 breast cancer cells, or their lung metastatic derivative, LM2 cells, also induced MMP-1 (top) and CXCR4 (bottom) mRNA in HMFs within 24 hours (Fig. 2C). Due to
the low levels of MMP-1 and CXCR4 in the 102 cells, compared with those in the aggressive 231 cells (see Fig. 1A) and LM2 cells (data not shown), the 102 cells represent a stronger model for examining the functional contribution of stromal-derived MMP-1 and CXCR4 in breast cancer progression. Thus, we focus on the effects made by 102 breast cancer cells on surrounding fibroblasts.

Time Course of MMP-1 and CXCR4 mRNA and Protein Levels in HMFs Exposed to Soluble 102 Breast Cancer Factors

To define the time course of MMP-1 and CXCR4 induction in HMFs, mRNA was measured following treatment of these cells with 102CM or control HMFCM. Both MMP-1 and CXCR4 mRNA in HMFs increased steadily from 3 to 24 hours of exposure to 102CM (Fig. 3A), whereas levels of these mRNAs were unchanged in HMFs treated with control medium, HMFCM. Both MMP-1 and CXCR4 gene expression returned to original levels within 3 hours upon removal of the breast cancer cell-conditioned medium (data not shown). Depending on the HMF sample, the relative fold change in CXCR4 between 102CM-treated HMFs and control HMFs ranged from 10-fold to >50-fold, whereas fold change in MMP-1 induction ranged from 2-fold to 10-fold (data not shown).

Western blot analysis confirmed that protein levels correlated with gene induction. Neither HMFCM nor 102CM alone generated measurable levels of latent or active MMP-1 (Fig. 3B, left), confirming that basal MMP-1 levels in HMFs and 102 cells are low. However, 102CM triggered the secretion of both latent and active MMP-1 in HMFs, compared with HMFs treated with...
control medium, HMFCM (Fig. 3B, right). Bands larger than 52 kDa in the conditioned medium represent glycosylated MMP-1 (51). Similarly, high levels of CXCR4 protein were detected in HMFs treated with 102CM after 12 hours, whereas little CXCR4 was detected in HMFs treated with HMFCM (Fig. 3C). HMF-derived CXCR4 protein diminished by 24 hours of treatment with 102CM (Fig. 3C), possibly due to the increase in SDF-1 mRNA (Fig. 3D), which typically ranged from 2-fold to 10-fold (data not shown). Indeed, the kinetic profile of SDF-1 is such that if SDF-1 levels begin to increase at 16 hours, it is possible that this chemokine is generated and binds CXCR4, causing internalization and degradation of this receptor by 24 hours (ref. 52; see Discussion). Medium conditioned by 231 and LM2 breast cancer cells also induced MMP-1 and CXCR4 protein levels in HMFs within 24 hours (data not shown).

**Functional Significance of Increased Stromal MMP-1 in the Absence of Breast Cancer Cell-Derived MMP-1**

To show the functional importance of MMP-1 in HMFs, we used an *in vitro* collagen degradation assay (32, 50), in which HMFs were embedded in a collagen gel and incubated with control HMFCM or 102CM. Immunohistochemistry detected MMP-1 protein produced by HMFs embedded in a collagen matrix when subjected to either HMFCM or 102CM for 6 hours (Fig. 4A). Although HMFs treated with 102CM stained positively for MMP-1, only a few cells treated with HMFCM were partially stained (see arrows), despite the equal number of cells per gel. Furthermore, collagen surrounding HMFs treated with 102CM displayed punctate MMP-1 staining throughout, indicating that these cells actively secreted MMP-1. In contrast, MMP-1 was not present in HMF-embedded gels treated with HMFCM.

Although very little degradation resulted when collagen-embedded HMFs were treated with HMFCM for 48 hours, collagen degradation by HMFs treated with 102CM was extensive, as measured by the amount of medium liberated from the collagen as it was degraded (***, P < 0.0001; Fig. 4B). The negative values on the Y-axis result from evaporation of medium. To confirm MMP-1 as the agent responsible for the collagen degradation, an MMP-1 neutralizing antibody was added to the collagen, and HMF-driven degradation of collagen was prevented (Fig. 4C). Similarly, the addition of a general MMP inhibitor, Ilomastat, abrogated collagen degradation (*, P < 0.05; Fig. 4C). Again, the negative values on the Y-axis result from evaporation of medium. These data imply that weakly invasive 102 breast cancer cells, which do not produce MMP-1 (Fig. 3B),

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**FIGURE 3.** Time course of MMP-1 and CXCR4 mRNA and protein levels in HMFs exposed to factors from SUM102 breast cancer cells. **A.** HMFs were treated with control HMFCM or 102CM for the indicated times, and then MMP-1 and CXCR4 mRNA was measured using real-time RT-PCR. Values were normalized to HPRT and calculated using the $2^{-\Delta\Delta C(t)}$ method (*, P = 0.05; **, P < 0.01; ***, P < 0.005). **B.** HMFs were treated with HMFCM or 102CM for 24 h, then proteins in the medium were precipitated and immunoblotted with antibodies against MMP-1. Recombinant MMP-1 (rMMP-1) and each conditioned medium alone serve as controls (left). Multiple banding patterns around latent MMP-1 represent glycosylated forms of MMP-1. **C.** Lysates were harvested from HMFs treated with HMFCM or 102CM for 12 or 24 h and immunoblotted with anti-CXCR4 antibodies. Multiple banding patterns represent glycosylated forms of CXCR4. Nonspecific (NS) bands serve as a loading control. **D.** HMFs were treated with control HMFCM or 102CM for the indicated times and then SDF-1 mRNA was measured using real-time RT-PCR. Values were normalized to HPRT and calculated using the $2^{-\Delta\Delta C(t)}$ method. Columns, mean; bars, SD (*, P < 0.05). Experiments were conducted thrice.
known ligand for CXCR4 (54, 55), we determined whether SDF-1 successfully signaled through HMF CXCR4 by measuring the activation of p44/42 (Erk1/2; ref. 56), a component of the mitogen-activated protein kinase pathway. The addition of SDF-1 to HMFs for 5 minutes stimulated the phosphorylation of p44/42, indicating the activation of this pathway (Fig. 5A, left). To confirm SDF-1 signaling through activation of CXCR4, cells were pretreated with a CXCR4 inhibitor, AMD3100, and subsequently treated with SDF-1 for 0 or 5 minutes. Inhibition of CXCR4 blunted p44/42 phosphorylation by SDF-1 (Fig. 5A, right), allowing us to conclude that upon binding to CXCR4, SDF-1 stimulates downstream signal transduction in HMFs. The partial inhibition observed with AMD3100 most likely reflects the fact that this mitogen-activated protein kinase pathway is activated by other mechanisms in these cells (ref. 57; data not shown).

Standard chemotaxis assays were conducted to investigate the downstream function of the SDF-1/CXCR4 signaling axis in HMFs stimulated with HMFCM or 102CM. These functional experiments also confirm that the increased total CXCR4 protein observed by Western blot analysis results in increased surface CXCR4. Figure 5B represents a qualitative analysis of these results, which are quantified in Fig. 5C. As expected, HMFs migrated toward fetal bovine serum (FBS; positive control), but not toward bovine serum albumin (negative control). Although HMFs treated with control HMFCM failed to migrate toward SDF-1, HMFs exposed to 102CM, and which, therefore, have increased CXCR4 levels (see Fig. 3), migrated readily toward SDF-1 (**, P = 0.0001; Fig. 5B and C). To corroborate CXCR4 as the key mediator in HMF migration toward SDF-1, the CXCR4 antagonist, AMD3100, was used to block CXCR4 signaling. Blocking CXCR4 reduced HMF migration toward SDF-1 by ~2-fold to 3-fold, as is evident in Fig. 5B and C (***, P = 0.0016). Taken together, these data confirm that breast cancer cells secrete factors that not only induce CXCR4 protein levels in HMFs, but also that this receptor responds to its ligand by transmitting downstream signals and enhancing migration.

FIGURE 4. HMF-derived MMP-1 degrades type I collagen. A, HMFs were embedded into a type I collagen matrix, treated with HMFCM or 102CM for 6 h, and then were stained using immunohistochemistry and anti-MMP-1 antibodies. B, Collagen-embedded HMFs were treated with HMFCM or 102CM for 48 h. Collagen degradation was calculated by weighing the medium released from the collagen gels (see Materials and Methods). C, Collagen-embedded HMFs were treated with 102CM in the presence of control anti-FLAG antibody (1 μg/mL), an MMP-1 neutralizing antibody (1 μg/mL), or Ilosmastat (25 μmol/L) for 48 h. Collagen degradation was calculated by weighing the medium released from the collagen gels (see Materials and Methods). The negative values on the Y-axis result from evaporation of the medium. Columns, mean; bars, SD. One out of three representative experiments is presented. [***, P < 0.0001 compared with controls (B) and *, P < 0.05 versus anti-FLAG control condition (C)].

MMP-1 and CXCR4 mRNA and Protein Are Increased in Primary CAFs Compared with Their Counterpart Fibroblasts and HMFs

To ascertain whether our observations were clinically relevant, we investigated MMP-1 and CXCR4 expression and function in CAFs isolated from primary human breast tumors, their respective (non–cancer-associated) counterpart fibroblasts (CPF), and age-matched, HMFs from patients without cancer (Fig. 6A). Basal MMP-1 and CXCR4 mRNA levels in CPFs were similar to those in normal, unstimulated HMFs, suggesting that non–tumor-associated fibroblasts collected from patients with breast cancer have comparable MMP-1 and CXCR4 gene expression to those observed in HMFs taken from patients not afflicted with cancer. In contrast, basal expression of these genes was significantly higher in CAFs, demonstrating that clinically derived CAFs have constitutively increased levels of MMP-1 and CXCR4 mRNA (*, P < 0.05; Fig. 6A). 102CM further stimulated MMP-1 and CXCR4 mRNA levels in HMFs, CPFs, and CAFs, suggesting that regardless of cell type, soluble factors from breast cancer cells can exploit nearby fibroblasts to produce MMP-1, resulting in the degradation of surrounding extracellular matrix.

CXCR4 on HMFs Stimulates Downstream Signaling and Allows Cells to Migrate in Response to Exogenous SDF-1

It has been shown that cell surface CXCR4 may be present on cells, but unresponsive to SDF-1 stimulation, as measured by downstream signaling (53). Because SDF-1 is the only
stimulate the expression of these tumor-promoting genes (Fig. 6A). To confirm their status as CAFs, we examined these cells for increased expression of a CAF-recognized gene, FAP, and found it to be elevated compared with FAP mRNA in CPFs (data not shown).

Constitutive MMP-1 (top) and CXCR4 (bottom) protein levels were also increased in CAFs compared with HMFs and CPFs (Fig. 6B). The multiple banding pattern for both MMP-1 and CXCR4 protein results from glycosylation (51, 58). Flow cytometry detected increased CXCR4 surface expression on ∼1.0% of CAFs, compared with ∼0.1% of HMFs (Fig. 6C). The percentage of cells expressing CXCR4 over time is likely higher than the values obtained because CXCR4 is rapidly internalized (52), making it difficult to detect. Importantly, flow cytometry revealed that 10-fold more CAFs express CXCR4 than HMFs, and of these cells, the mean fluorescence intensity of CAFs was consistently greater than the mean fluorescence intensity of HMFs (data not shown). Because CXCR4 is rapidly internalized, this method of determining CXCR4 surface expression may be less definitive than a functional assay (see below).

Migratory and Invasive Capabilities Are Increased in Primary CAFs Compared with Their CPFs and HMFs

To measure the functionality of CAF-derived MMP-1, we assessed the ability of these cells to degrade collagen in the collagen degradation assay. HMFs and CPFs treated with naïve, serum-free medium did not degrade the type I collagen. In contrast, CAFs readily degraded the collagen matrix, except when MMP-1 was neutralized with an antibody (Fig. 7A). The negative values on the Y-axis result from evaporation of medium. These findings confirm and expand on previous studies, demonstrating that CAFs derived from clinical specimens not only maintain increased MMP-1 levels, but also that this fibroblast-derived enzyme degrades the surrounding matrix, suggesting a role for these cells in vivo.

To investigate whether CAF-expressed CXCR4 promoted chemotaxis toward SDF-1, these cells were examined in a standard migration assay. CAFs migrated readily toward SDF-1, whereas CPFs and HMFs did not (**, \( P < 0.001 \); Fig. 7B), and that the degree of migration corresponded with the relative levels of CXCR4 in these cells. Each of the primary cell lines migrated toward 10% FBS, the positive control, but were not attracted toward the negative vehicle control, 0.1% bovine serum albumin (Fig. 7B). Importantly, blocking CXCR4 function with AMD3100 abrogated CAF migration (***, \( P = 0.001 \); Fig. 7C), indicating that CAF-derived CXCR4 is necessary for the migration toward SDF-1 and demonstrating that CXCR4 expressed on these cells is functional.

Thus, we conclude that CAF specimens derived from clinical material express increased MMP-1 and CXCR4, which potentiate the invasive and migratory phenotype of these cells. These results validate the clinical relevancy of our findings with HMFs treated with 102CM because HMFs treated with 102CM share characteristics with CAFs obtained from patient

![Figure 5](https://mcr.aacrjournals.org/figs/20_1129_f5.png)

**FIGURE 5.** HMF CXCR4 stimulates downstream signaling in response to SDF-1 and allows cells to migrate toward the ligand, SDF-1. **A**, HMFs were exposed to 102CM for 24 h and then treated with 100 ng/mL of SDF-1 for the indicated amounts of time or pretreated with AMD3100 (5 μg/mL) for 30 min and then treated with SDF-1 for 0 or 5 min. Whole cellular lysates were analyzed by immunoblotting for phosphorylated p44/42. **B**, HMFs were plated in FluoroBlok transwells and subjected to 102CM for 12 h, after which BSA (negative vehicle control), FBS (positive control), or SDF-1 was added to the bottom chamber. Sixteen hours later, cells were stained with Calcein AM and migrated cells were visualized using a mercury bulb. Bars, 25 μm. **C**, HMFs were plated in FluoroBlok transwells and subjected to 102CM for 12 h, after which time, cells were pretreated with or without 5 μg/mL of AMD3100 for 30 min and then 0.1% BSA (negative control for SDF-1), FBS (positive control), or SDF-1 (see Materials and Methods) was added to the bottom chamber. Eighteen hours later, cells were stained with Calcein AM and cells were enumerated in six fields of view (×40) per condition to quantify migration. Columns, mean; bars, SD (***, \( P < 0.005 \)). Data are representative of three individual experiments.
specimens. Furthermore, the data support the concept that inflammatory factors provided by breast cancer cells might initiate the HMF-to-CAF transdifferentiation.

**Discussion**

Some CAFs have been compared with the activated fibroblasts, or myofibroblasts, present in sites of wound healing and chronic inflammation. Myofibroblasts at sites of inflammation due to rheumatoid arthritis and idiopathic pulmonary fibrosis have increased MMP-1 (59, 60) and CXCR4 expression (37, 61), which facilitate cellular invasion and migration. Taken together, these observations support the hypothesis that MMP-1 and CXCR4 may also be elevated in fibroblasts that are found within the breast tumor microenvironment and suggest that these factors are induced as an HMF becomes more activated and CAF-like.

In this report, we provide evidence to support this hypothesis. First, our data show that SUM102 breast cancer cells, which express low levels of MMP-1 and CXCR4, require mammary fibroblasts to grow in vivo (Fig. 1). Second, the SUM102 cells secrete factors that stimulate the expression of several inflammatory factors, including MMP-1 and CXCR4, in adjacent stromal cells (Figs. 1 and 2). This suggests that 102 breast cancer cells may solicit normal, resident HMFs to facilitate tumor growth in vivo. Furthermore, increased MMP-1 and CXCR4 enhanced the invasive and migratory behavior of HMFs, verifying the functional contributions of these cells to tumor progression (Figs. 4 and 5). Finally, we validated these findings by demonstrating that mammary CAF isolates from patients with breast cancer have amplified basal MMP-1 and CXCR4 levels, which augment their invasive and migratory potential, respectively (Figs. 6 and 7). In vivo, this phenotype may permit CAFs to degrade matrix as they migrate within the tumor microenvironment. Our findings emphasize the possible clinical relevance of MMP-1 and CXCR4 induction in CAFs, and show that normal HMFs exposed to soluble breast cancer factors acquire phenotypic characteristics shared with CAF specimens (Figs. 6 and 7), indicating that breast cancer cells may initiate the conversion of resident HMFs to CAFs.

The process of matrix invasion by malignant cells requires both degradation of the matrix and cellular migration (62). Studies conducted previously in our laboratory showed that MMP-1 derived from breast cancer cells is necessary for tumor growth and invasion at the primary site (32, 50). Because MMP-1 is secreted, it is reasonable to propose that stromal-derived MMP-1 functions similarly to that produced by the breast cancer cells. Furthermore, not all breast cancer cells produce MMP-1 (63), increasing the importance of CAF-derived MMP-1 in tumor progression.

CXCR4 up-regulation is most commonly linked to breast cancer epithelial cells (36); on the other hand, the ligand for CXCR4, SDF-1, has been classified as CAF-related (8). In this report, we have identified CXCR4 as a novel CAF-associated gene. This finding, coupled with the discovery that CAFs express increased levels of SDF-1 (8), implies that an autocrine feedback loop may exist (64-67). It is possible that SDF-1 produced by CAFs binds to and signals through, CAF CXCR4, potentially causing important downstream effects. Specifically, the SDF-1/CXCR4 signaling axis results in a multitude of
cellular functions: migration, homing, adhesion, proliferation, differentiation, and the induction of MMPs (68–71). For example, SDF-1 signaling through CXCR4 on the activated fibroblasts in patients with rheumatoid arthritis results in their activation, migration, and proliferation (37, 72). Several breast cancer cell lines also secrete SDF-1 (65, 73); thus, it is feasible that the SDF-1/CXCR4 axis functions not only in an autocrine manner, but also in a paracrine fashion within the tumor microenvironment, in which SDF-1 produced by tumor cells binds to CXCR4 on surrounding CAFs and vice versa.

Breast cancer cells overexpressing CXCR4 home to organs constitutively expressing SDF-1, such as the lungs, liver, and bone (36), yet the in vivo function of CXCR4 on CAFs is currently unknown. Nonetheless, the in vivo function of CXCR4 on fibroblasts in other inflammatory diseases has been well documented (39, 61) and cancers have been likened to a wound that does not heal (14). For example, in patients with idiopathic pulmonary fibrosis, fibroblast precursor cells expressing CXCR4 have been shown to home to the lungs, which express elevated levels of SDF-1 (39, 61), thereby indicating that activated fibroblasts have the ability to migrate in vivo. Because we show that CXCR4 on CAFs is functional and that these cells are endowed with the ability to migrate towards SDF-1, we speculate that CAFs may migrate throughout the microenvironment of the primary tumor, degrading collagen through the secretion of MMP-1.

A comprehensive list of CAF marker designations has not been definitively established. Instead, these cells have been associated with a variety of markers. Because CAFs may arise from various sources, it is not surprising that these cells could express different markers, and as a result, contribute to carcinoma progression differently. We did not observe increased levels of FAP, vimentin, and αSMA in HMFs exposed to soluble breast cancer factors. However, we document that normal HMFs increase several other CAF-related genes (see Fig. 2A), including IL-8, SDF-1, MMP-3, and MMP-1. In addition, we showed increased CXCR4 in CAFs and in HMFs in response to factors secreted by 102 breast cancer cells, a factor which has not previously been associated with CAFs. Because MMP-1 and CXCR4 are elevated in CAF isolates from the clinic, we suggest that the induction of specific genes by 102CM may represent an early transition phase of an HMF into a CAF and that these gene products facilitate breast cancer cell invasion and dissemination. Additionally, perhaps the high constitutive levels of MMP-1 and CXCR4 seen in the CAFs taken from patients have resulted from genetic alterations made within these cells (74).

Despite reports documenting that normal HMFs or CAFs enhance tumor growth when injected simultaneously with mammary carcinoma cells into nude mice (8, 44), it has also been proposed that normal HMFs can suppress breast tumor formation in vivo (8). Indeed, then, it would be reasonable to propose that early in tumor formation, normal, resident mammary fibroblasts suppress tumor growth, but that following the breast cancer cell–induced transition of HMFs to CAFs, these cells enhance tumor pathogenesis. Alternatively, it is possible that HMFs require prolonged treatment with breast cancer cell conditioned medium. This concept coincides with data from Mishra and colleagues, who showed that fibroblast precursor cells become fully CAF-like (as determined by SDF-1, αSMA, and FAP expression) after 30 days of exposure to breast cancer cell conditioned medium (18).

![FIGURE 7](image)

**FIGURE 7.** CAF isolates from clinical specimens have increased MMP-1 and CXCR4 and, as a result, are more invasive and migratory than CPFs and HMFs. A. Primary cells were embedded in a collagen matrix for 48 h in the presence or absence of anti-FLAG antibody control (1 μg/ml) or an MMP-1 neutralizing antibody (1 μg/ml). Media were then weighed to quantify matrix degradation (see Materials and Methods). The negative values on the Y-axis result from evaporation of the medium. Columns, mean; bars, SD (*, P < 0.05; **, P < 0.01). B. Primary cells were serum-starved for 24 h. FBS, 0.1% BSA (negative control for SDF-1), or SDF-1 (100 ng/ml) was then added to bottom chamber and cells were allowed to migrate for 18 h. Migrated cells were stained with Calcein AM and counted in six fields of view (×20) per condition to quantify migration. Columns, mean; bars, SD (***, P < 0.001). C. CAFs were plated in transwells and serum-starved for 24 h. Cells that received AMD3100 (5 μg/ml) were treated for 30 min prior to the addition of chemottractants FBS (positive control), BSA (negative vehicle control), or SDF-1 to bottom chambers. Migrated cells were stained with Calcein AM and cells were counted in six fields of view (×40) per condition to quantify migration. Columns, mean; bars, SD (***, P < 0.001). Results are representative of individual experiments, using CAFs from different patients.
In conclusion, we show that CAFs display amplified MMP-1 and CXCR4 and that normal HMFs acquire enhanced MMP-1 and CXCR4 levels in response to soluble breast cancer agents. Our data suggest that breast cancer cells may initiate the conversion of normal, resident HMFs to CAFs.

Materials and Methods

Cell Lines and Cell Culture

SUM102 breast cancer cells (Asterand) were cultured in Ham’s F-12 medium supplemented with 2% FBS, 5 μg/mL of insulin, 10 ng/mL of epidermal growth factor, 1 μg/mL of hydrocortisone, HEPES buffer, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. MDA-MB-231 breast cancer cells (American Type Culture Collection) and their lung metastatic derivative (LM2, a kind gift from Dr. Massague at Memorial Sloan-Kettering Cancer Center) were maintained in DMEM 50/50 F-12 (Life Technologies, Inc.) supplemented with 10% FBS (HyClone), penicillin 100 units/mL, streptomycin 100 μg/mL, and l-glutamine. Primary HMFs were isolated from women who had undergone reduction mammoplasty surgery (Dartmouth Hitchcock Medical Center) and were cultured in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and l-glutamine. All patients were enrolled in an Institutional Review Board–approved protocol. Primary CAFs and their respective CPFs (Asterand) were cultured in DMEM supplemented with 5% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and l-glutamine. All cells were cultured at 37°C and in 5% CO₂. All primary cells were used between passages two and six.

Breast Tumorigenesis In vivo

Cells were suspended in 100 μL of Matrigel (BD Biosciences), and female mice (strain nu/nu, ~49 days old; Charles River) were injected orthotopically (fourth mammary fat pad) with 2 × 10⁶ SUM102 cells, 2 × 10⁶ HMFs, or 1 × 10⁶ of each cell line simultaneously. Eight mice were injected concomitantly with both cell lines, whereas six mice were injected with each cell line alone. Tumors were measured biweekly with calipers, and mice were sacrificed when tumor diameter exceeded 12 mm, or at the termination of the study (6 mo). The Dartmouth College Institutional Animal Care and Use Committee approved animal studies.

Generation of Conditioned Medium

To condition medium, cells were plated at 5 × 10⁶ cells (102 breast cancer cells) or 1 × 10⁶ (HMFs) per 150 mm plate (~85% confluence) and left to seed for 24 h in serum-containing medium. The cells were then rinsed thrice with Hank’s buffered saline solution (HBSS) and 15 mL of serum-free Ham’s F12 medium, containing 0.2% lactalbumin hydrolysate, were added per 150 mm culture plate and conditioned for 24 h at 37°C. After incubation, media were transferred to a conical tube, centrifuged at 1,500 rpm for 5 min to pellet cellular debris, and then decanted into a new conical tube. Conditioned media were stored at −80°C until needed for experiments, at which time, the media were thawed and added directly to cells.

Quantitative Real-time Reverse Transcription-PCR

Total cellular RNA was purified using Qiagen’s RNeasy kit (Qiagen) as described by the manufacturer. Total cellular RNA was reverse transcribed using the TaqMan Reverse Transcriptase reagent kit (Applied Biosystems), as directed by the manufacturer. Real-time PCR reactions were done using 250 ng of input RNA per reaction, as described (30, 32, 50, 75). Data were averaged and normalized to the housekeeping gene, hypoxanthine-phosphoribosyl transferase (HPRT). mRNA values were calculated using 2⁻ΔΔCt (76) and are presented as fold change compared with control conditions. Primer sequences were CXCR4, 5′-TGGGTTGTTGTTGTTGATTTTT-3′ (forward) and 5′-ATGCAATAAGCAGAAGATGATG-3′ (reverse); HPRT, 5′-AGCTTGCTGTTGTAAGAGGAC-3′ (forward) and 5′-CCAGATGTTCACAACCTCAATTG-3′ (reverse); IL-8, 5′-CTCTGGACGGTCTTTGATT-3′ (forward) and 5′-TATGCACTGACATCTAAAAGTTGTC-3′ (reverse); MMP-1, 5′-AGCTAGCTCAAGTAGACATTGTG-3′ (forward) and 5′-GCCGCGGTTGGTCCACAG-3′ (reverse); MMP-3, 5′-TTCGCGCTGTCCTAAGATGATAT-3′ (forward) and 5′-AAAGACGACGGAGATACATTG-3′ (reverse); SDF-1, 5′-CACGCTTGGGACCTTTGTTAAGGCG-3′ (forward) and 5′-ACGCAGGCAAGGTCGTTGCTG-3′ (reverse). cDNAs were assayed in triplicate.

In vitro Collagen Degradation Assay

This assay was carried out as previously described (32, 50). Briefly, neutralized collagen was mixed with equivalent volumes of cells to yield 5 × 10⁶ cells/mL and 500 μL of the collagen/cell mixture was added to each well of a 24-well plate. After the collagen solidified at 37°C (~15 min), 0.5 mL of control HMFCM or 102CM containing ~0.00003% trypsin to activate latent MMPs was added carefully to each well in the presence or absence of anti-FLAG antibody control (1 μg/mL; Sigma), an MMP-1 neutralizing antibody (1 μg/mL; Chemicon) or Iлом мастат (25 μmol/L; Chemicon). After 48 h at 37°C, the overlying medium was removed and weighed. The medium released from a collagen gel was calculated by weighing the total medium collected minus the original medium added (0.5 mL = 0.5 g). Samples were analyzed in triplicate and experiments were repeated at least thrice. Assays stained for MMP-1 using immunohistochemistry were terminated at 6 h, so that gels embedded with HMFs and treated with 102CM were not entirely degraded. Collagen gels were removed from wells with a rubber spatula, fixed in 1090 neutral buffered formaldehyde and processed for histologic staining in the Pathology Department at Dartmouth Hitchcock Medical Center. Goat MMP-1 antibody (R&D Systems) was diluted to 1:100.

Immunoblotting and Antibodies

Proteins from HMFs and 102 breast cancer cells were analyzed by SDS-PAGE (32, 50, 75). MMP-1 protein was precipitated with trichloroacetic acid from 1 mL of medium, whereas CXCR4 was analyzed in whole cell lysates, which were suspended in 2× Laemmli lysis buffer (Sigma). Blots were probed with an MMP-1 antibody (Chemicon) diluted 1:2,000, a CXCR4 antibody (Chemicon) diluted 1:2,000, phosphospecific antibodies (Cell Signaling Technology, Inc.) diluted 1:1,000, or actin antibodies (Calbiochem) diluted 1:10,000. Secondary antibodies were diluted 1:2,000 in the appropriate blocking buffer.

SDF-1/CXCR4 Signaling

HMFS were plated at 2.5 × 10⁵ per 60 mm culture plate and allowed to seed overnight. After 24 h, the cells were rinsed with...
HBs and 102CM was added to all plates for 8 h, after which time SDF-1α (100 ng/mL; R&D Systems) was added for 0, 0.5, 1, 3, or 5 min and were lysed in 150 μL of Laemmli buffer and prepared for immunoblotting for p44/42. Samples treated with AMD3100 (5 μg/mL; Sigma Aldrich), a small molecule inhibitor of CXCR4, were pretreated for 30 min, and then either vehicle control (0.1% bovine serum albumin in 1× PBS) or SDF-1α (100 ng/mL) was added for 5 min.

Migration Assays
HMFs (5 × 10^4) were plated with medium containing serum FluoroBlok inserts (6.5 mm, 8.0 μm pore; BD Falcon) as described previously (77), and allowed to seed overnight. The following morning, cells were rinsed with HBSS and treated with either HMFCM or 102CM. After 8 h, DMEM containing 10% FBS (positive control), 0.1% bovine serum albumin in PBS (negative vehicle control), or SDF-1α (100 ng/mL) was added to the bottom chambers. Samples treated with AMD3100 (5 μg/mL) were pretreated 30 min prior to adding attractants. Cells were permitted to migrate for ~18 h, after which time the transwell inserts were exposed to the fluorescent dye, Calcein AM (4 μg/mL in HBSS; BD Bioscience), to visualize and count only cells that had migrated through the membrane. Migration assays with CAFs were conducted similarly, but rather than using FluoroBlok inserts, standard transwell inserts (6.5 mm, 8.0 μm pore; Corning) were used. To analyze migration with this method, transwell inserts were exposed to Calcein AM for 30 min, and then cells on the bottom were detached by incubating inserts in prewarmed trypsin with agitation for 15 min at 37°C. Medium containing 10% FBS was added to neutralize trypsin and pipetted to disperse cells evenly off the membrane. Cells were incubated for 3 h and then were visualized by green fluorescent protein fluorescence using excitation from a 100 W mercury lamp on an Olympus 1×50 inverted phase contrast microscope and counted to quantify cell migration.

Flow Cytometry
HMFs or CAFs were grown to confluence in 150 mm plates and serum-starved overnight prior to being harvested for analysis of surface CXCR4 by flow cytometry. On the day of the harvest, cells were rinsed with 1× PBS and incubated in acidic glycine buffer [0.05 mol/L glycine HCl buffer (pH 3), with 0.1 mol/L NaCl] for 1 min to ensure that receptors were stabilized, and then rinsed twice in 1× PBS. Prewarmed Cell Stripper (a nonenzymatic cell dissociation solution from Sigma), together with gentle scraping, removed cells from the plates, and cells were immediately added to cold PBS with 10% FBS and 0.1% sodium azide and centrifuged. The supernatant was decanted and cells were either left unstained or stained with an antihuman CXCR4 PE–conjugated antibody (diluted 1:50; R&D Systems) for 30 min at 4°C and in the dark. After staining cells, they were fixed in 4% paraformaldehyde for 20 min and subsequently washed thrice in cold PBS with 10% FBS. Cells were then resuspended in PBS supplemented with 10% FBS and 0.1% sodium azide, and were analyzed using FACScan.

Statistical Analysis
Student’s t test determined statistical significance. All samples were prepared in triplicate and experiments were conducted at least thrice. Statistical power at P < 0.05 was considered significant.

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

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CXCR4 and Matrix Metalloproteinase-1 Are Elevated in Breast Carcinoma–Associated Fibroblasts and in Normal Mammary Fibroblasts Exposed to Factors Secreted by Breast Cancer Cells

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