Chemokine (C-C Motif) Ligand 2 Mediates the Prometastatic Effect of Dysadherin in Human Breast Cancer Cells

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Abstract

Dysadherin, a cancer-associated membrane glycoprotein, down-regulates E-cadherin and promotes cancer metastasis. This study examined the role of dysadherin in breast cancer progression. Expression of dysadherin was found to be highest in breast cancer cell lines and tumors that lacked the estrogen receptor (ER). Knockdown of dysadherin caused increased association of E-cadherin with the actin cytoskeleton in breast cancer cell lines that expressed E-cadherin. However, knockdown of dysadherin could still suppress cell invasiveness in cells that had no functional E-cadherin, suggesting the existence of a novel mechanism of action. Global gene expression analysis identified chemokine (C-C motif) ligand 2 (CCL2) as the transcript most affected by dysadherin knockdown in MDA-MB-231 cells, and dysadherin was shown to regulate CCL2 expression in part through activation of the nuclear factor-κB pathway. The ability of dysadherin to promote tumor cell invasion in vitro was dependent on the establishment of a CCL2 autocrine loop, and CCL2 secreted by dysadherin-positive tumor cells also promoted endothelial cell migration in a paracrine fashion. Finally, experimental suppression of CCL2 in MDA-MB-231 cells reduced their ability to metastasize in vivo. This study shows that dysadherin has prometastatic effects that are independent of E-cadherin expression and that CCL2 could play an important role in mediating the prometastatic effect of dysadherin in ER-negative breast cancer. (Cancer Res 2006; 66(14): 7176-84)

Introduction

Dysadherin (also known as FXYD5, or Related to Ion Channel) was recently cloned as a cancer-associated membrane glycoprotein (1). It is a member of the FXYD family of single-span type I membrane proteins, which interact with and modulate properties of the Na^+K^+ ATPase (2, 3). The dysadherin gene is up-regulated in cells transformed by several oncogenes, including E2a-Pbx1, v-Ras, Neu, and v-Src (4), and dysadherin is expressed to various extents in many different types of tumors, such as stomach, colon, pancreatic, and breast tumors (1). In contrast, only a limited number of normal cell types, including lymphocytes, endothelial cells, and basal cells of stratified squamous epithelium, show dysadherin expression (1). Collectively, the data suggest that overexpression of dysadherin might contribute to tumor progression and could constitute a novel molecular target for the development of cancer therapeutics.

In support of this hypothesis, it was shown that transfection of a liver cancer cell line with the cDNA of dysadherin resulted in reduced cell-cell adhesiveness and down-regulation of E-cadherin protein (1). Furthermore, on injection into mouse spleens, dysadherin transfectants formed a significantly larger number of intrahepatic metastatic nodules compared with the mock transfectants, suggesting a capacity of dysadherin to promote metastasis. Experimental overexpression of dysadherin in a pancreatic cancer cell line also promoted metastasis in an orthotopic mouse model (5). Clinically, increased expression of dysadherin is significantly correlated with distant metastasis and poor prognosis in human pancreatic, colorectal, thyroid, gastric, and tongue cancers (6–10).

Thus, both clinical and experimental data suggest that dysadherin may play a particularly important role in cancer cell invasion and metastasis, and that dysadherin expression could be a useful biological predictor of tumor aggressiveness and poor prognosis in human cancers (11). However, the molecular mechanisms of dysadherin effects on cancer progression are still poorly understood. Because dysadherin expression was recently shown to correlate with poor survival in a small cohort of breast cancer patients (1), here we have investigated further the possible functional involvement of dysadherin in breast cancer progression. We find that dysadherin is particularly highly expressed in estrogen receptor (ER)–negative breast cancer and show that dysadherin may promote breast cancer metastasis by a novel E-cadherin-independent mechanism that involves the up-regulation of chemokine (C-C motif) ligand 2 (CCL2).

Materials and Methods

Cell culture and reagents. The human breast cancer cell lines BT-474, MCF-7, ZR-75B, T-47D, MDA-MB-468, SK-BR-3, MDA-MB-231, and HS578T and human umbilical cord vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (Manassas, VA). MDA-MB-435 and MDA-MB-435LV/Br were kindly provided by Dr. Janet Price (University of Texas M. D. Anderson Cancer Center, Houston, TX). The human breast cancer cell lines were maintained in DMEM (Invitrogen, Grand Island, NY) containing 5% CO2. HUVEC were cultured as described previously (12). In support of this hypothesis, it was shown that transfection of a liver cancer cell line with the cDNA of dysadherin resulted in reduced cell-cell adhesiveness and down-regulation of E-cadherin protein (1). Furthermore, on injection into mouse spleens, dysadherin transfectants formed a significantly larger number of intrahepatic metastatic nodules compared with the mock transfectants, suggesting a capacity of dysadherin to promote metastasis. Experimental overexpression of dysadherin in a pancreatic cancer cell line also promoted metastasis in an orthotopic mouse model (5). Clinically, increased expression of dysadherin is significantly correlated with distant metastasis and poor prognosis in human pancreatic, colorectal, thyroid, gastric, and tongue cancers (6–10).

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
InSolution nuclear factor-κB (NF-κB) activation inhibitor [6-amino-4-(4-phenoxypyphenethylamino)quinazoline] was purchased from Calbiochem (La Jolla, CA).

Reverse transcription-PCR. Total RNA from human cancer cells was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription-PCR (RT-PCR) was then done using the SuperScript One-Step RT-PCR System (Invitrogen). The primer sets for amplification of human glyceraldehyde-3-phosphate dehydrogenase were 5'-AGGAAGAGGAGAC-CCTCAGTGC-3' (forward) and 5'-ATGACAGTGGCGGTCCT-3' (reverse). The primer sets for amplification of human chemokine (C-C motif) receptor 2 (CCR2) were purchased from Superarray (Fredrick, MD). PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunoblotting. The cell lysates were subjected to 10% SDS-PAGE, and separated proteins in the gel were electrophoblotted to polyvinylidene difluoride membrane (Millipore, Canton, MA). Anti-dysadherin monoclonal antibody (mAb; NCC-M53, 1:500 dilution; ref. 6), E-cadherin mAb (1:500 dilution; ref. 15), ER-α mAb (1:250 dilution; Cell Signaling Technology, Beverly, MA), and β-actin mAb (1:5,000 dilution; Sigma-Aldrich, St. Louis, MO) were used as described previously (16). Triton X-100-soluble and Triton X-100-insoluble (cytoskeleton) fractions were prepared essentially as described previously (17).

RNA interference. The small interfering RNA (siRNA) sequence chosen to target dysadherin was at positions 141 to 162 in the nucleotide sequence of dysadherin (Genbank accession no. AB072911) as described previously (5). Dysadherin siRNA was purchased from Dharmacon (Lafayette, CO). Nonspecific control I siRNA (Dharmacon) was used as a negative control. siRNA transfection was done using Oligofectamine (Invitrogen) according to the manufacturer's instructions. For most experiments, cells were treated for 48 hours with siRNA at a final concentration of 50 nmol/L. Dysadherin expression was determined by immunoblotting.

Transient transfection of dysadherin in T-47D cells. The expression vector for dysadherin, pcDNA-L3HSV, has been previously described (1). Transfection was done with LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Matrigel invasion assay. Breast cancer cell invasion was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm; BD Biosciences Discovery Labware, Bedford, MA) according to the manufacturer's protocol. Briefly, the top chamber was seeded with 1 × 10^5 viable tumor cells in a serum-free medium (AIM-V medium; Invitrogen). The bottom chamber was filled with DMEM supplemented with 10% FBS as a chemoattractant. CCL2-blocking antibody (MAB679; R&D Systems, Minneapolis, MN) or isotype-matched IgG antibody (MAB0041; R&D Systems) was added to the top chamber to a final concentration of 50 ng/mL. After 12 to 24 hours of incubation, cell lysates were harvested for assay using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luminescence was measured by VICTOR^2 (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

Immunofluorescence. A total of 1 × 10^5 cells per well were grown on glass coverslips in 12-well-flat-bottomed plates for 24 hours, and siRNA transfection was done as described above. The cells were fixed with 4% formaldehyde followed by 100% ethanol at −20°C. Permeabilization was done with 0.1% Triton X-100, and nonspecific binding was blocked with 2% normal swine serum. Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Eugene, OR) was used to visualize F-actin. The samples were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and examined by multiphoton fluorescence microscopy (Bio-Rad Laboratories). Three visual fields per coverslip were randomly selected, avoiding any overlapping for the morphologic analysis. The experiments were done in triplicate.

Endothelial cell migration assay. HUVEC chemotaxis was done using micro-Böden chambers as described previously (12). Briefly, polycarbonate filters of 8 μm pore size (BD Biosciences Discovery Labware) were coated with fibrinogen (10 μg/mL; Sigma-Aldrich) overnight at 4°C. Conditioned medium from MDA-MB-231 cells transfected with control siRNA or dysadherin siRNA was placed in the lower compartment of the chamber, and 1% FBS/DMEM was used as the medium control. HUVEC (5 × 10^5 cells/mL) resuspended in 1% bovine serum albumin/DMEM were then added to the upper compartment. Where indicated, CCL2 (15 ng/mL final concentration) was added with conditioned medium in the lower compartment of the chamber. The chambers were incubated for 15 hours at 37°C. After the filters were removed, the upper surface was scraped, fixed with methanol, and stained with leukostat (Fisher Scientific, Pittsburgh, PA). The number of cells was expressed as the number of migrated cells per five fields at >100 magnification. Each sample was tested in triplicate.

In vivo experimental metastasis study. All animals were maintained according to the NIH Animal Care and Use Committee guidelines under approved animal study protocols. To determine metastatic potential, 7-week-old female severe combined immunodeficient (SCID) BALB/c mice (National Cancer Institute-Frederick, Frederick, MD) were injected i.v. with 1 × 10^6 cells in 0.1 mL PBS in the tail vein. Mice were euthanized and lungs were collected on day 42, inflated, and fixed with 10% buffered formalin. The relative lung weight was calculated using the formula: lung weight / body weight.
weight × 100 (%). Macroscopic quantitation of metastases was done by counting the number of nodules on the surface of the lung. The presence of metastases was confirmed by pathologic examination of H&E-stained sections.

**In silico analysis of clinical microarray data.** In silico analysis of published clinical microarray data was done using the database and analysis tools at http://www.oncomine.org.

Statistical analysis. Unpaired parametric Student’s t test and nonparametric Mann-Whitney U test were used to analyze the data unless otherwise indicated in the text.

**Results**

Dysadherin expression is elevated in more aggressive, ER-negative breast cancer cell lines and tumors. To investigate the possible involvement of dysadherin in breast cancer progression, we examined dysadherin expression in a panel of related human breast cell lines that represent different stages of breast carcinogenesis. MCF10A is a spontaneously immortal human mammary epithelial cell line; MCF10AT1k is Ha-ras transfectant of MCF10A that forms premalignant lesions on xenografting; MCF10Ca1h and MCF10Ca1a are tumorigenic variants of the MCF10AT1k cells, forming well-differentiated, slow-growing tumors and poorly differentiated, rapidly growing tumors, respectively. Notably, only MCF10Ca1a can metastasize (13, 19). Immunoblot analysis indicated that dysadherin protein was strongly increased in the high-grade metastatic cell line (MCF10Ca1a) and not in low-grade tumorigenic or premalignant lines (MCF10A, MCF10AT1k, and MCF10Ca1h). E-cadherin was also somewhat decreased in MCF10Ca1a cells (Fig. 1A). In silico analysis of results from a large clinical microarray study (20) also showed a significant trend toward increased dysadherin mRNA expression with increasing tumor grade in human breast cancers (Fig. 1B).

![Figure 1. Expression of E-cadherin, dysadherin, and ER-α in human breast cancer cell lines and tumors.](image)

A, immunoblot analysis of E-cadherin and dysadherin in cell lines representing different stages in breast cancer progression. β-Actin was used as a loading control. B, dysadherin mRNA expression in human breast tumors as a function of tumor stage [extracted from van’t Veer et al. (20)]. C, immunoblot analysis of E-cadherin, dysadherin, and ER-α in human breast cancer cell lines. S30 is a subclone of MDA-MB-231 that has been stably transfected with ER-α. 10A is a control clone derived from the parental ER-negative MDA-MB-231 line. β-Actin was used as a loading control. D, dysadherin mRNA expression in human breast tumors as a function of ER status [extracted from Wang et al. (23) and van de Vijver et al. (24)].
Fig. 2. Effect of dysadherin knockdown on E-cadherin expression and invasion in vitro. A, immunoblot analysis of dysadherin in breast cancer cell lysates of parental cells (CON; Oligofectamine reagent alone) and cells transfected with nonspecific control I siRNA (NC) or dysadherin siRNA (siD). β-Actin was used as a loading control. B, immunoblot analysis of the expression and distribution of E-cadherin and dysadherin in lysates of breast cancer cells transfected with nonspecific control and dysadherin siRNA. Total, total protein extract; TS, Triton X-100-soluble fraction; TI, Triton X-100-insoluble fraction. C, effect of dysadherin knockdown on invasion of breast cancer cells through Matrigel. Columns, mean (n = 3 independent replicates); bars, SD. *, P < 0.001. D, immunoblot analysis of E-cadherin in the breast cancer cell lines used in the invasion assay.

ER-negative breast cancer is generally more aggressive and has a poorer prognosis than ER-positive breast cancer (21, 22). We therefore examined the correlation between dysadherin and ER-α expression in breast cancer cell lines. Immunoblot analysis showed that expression of dysadherin protein is more prevalent in ER-α-negative than in dysadherin-positive breast cancer cell lines (Fig. 1C). Moreover, in silico analysis of results from two large clinical microarray studies (23, 24) showed that dysadherin mRNA levels correlated negatively with ER status in breast cancer (Fig. 1D).

Restoration of ER to the ER-negative cell line MDA-MB-231 resulted in a reduction in dysadherin expression (Fig. 1C). Because dysadherin has been shown previously to down-regulate E-cadherin (11), we also confirmed that E-cadherin expression was more prevalent in dysadherin-negative breast cancer cell lines than in dysadherin-positive cell lines (Fig. 1C).

Dysadherin can promote invasion in vitro in the absence of functional E-cadherin. To investigate further the functional role of dysadherin in breast cancer progression, we used siRNA technology to knockdown dysadherin in breast cancer cells. Expression of dysadherin protein was efficiently suppressed in MCF10Ca1a and T-47D cells compared with control counterparts (Fig. 2A). Immunoblot analysis of total protein extracts showed that dysadherin siRNA did not change total E-cadherin expression in E-cadherin-positive cell lines (MCF10Ca1a and T-47D cells) compared with control counterparts (Fig. 2B). However, analysis of the distribution of E-cadherin showed that knockdown of dysadherin causes an increase in functional (Triton X-100-insoluble, cytoskeleton-associated) E-cadherin. These results suggest that dysadherin may not influence expression levels of total E-cadherin but rather may influence interactions of E-cadherin with the actin cytoskeleton in breast cancer cell lines. In agreement with this finding, knockdown of dysadherin did not cause any recovery of E-cadherin protein expression in the E-cadherin-negative breast cancer cell lines.

To understand the biological functions that dysadherin may affect during cancer progression, we examined whether knockdown of dysadherin was able to suppress invasiveness of breast cancer cells in vitro. Unexpectedly, we found that knockdown of dysadherin significantly reduced invasion through Matrigel in both E-cadherin-positive (MCF10Ca1a) and E-cadherin-negative cell lines (MDA-MB-231 and MDA-MB-435LV/Br; Fig. 2C and D). These results led us to hypothesize that dysadherin can promote cellular invasion by a novel mechanism that is independent of E-cadherin expression.

Identification of CCL2 as a dysadherin target gene. To investigate possible molecular mechanisms underlying the E-cadherin-independent effects of dysadherin, we compared gene expression patterns of MDA-MB-231 cells transfected with siRNA against dysadherin or nonspecific control siRNA. Seven hundred twenty-eight genes differed at the P < 0.001 level between dysadherin siRNA-transfected and nonspecific control-transfected cells. We filtered for genes that showed a 4-fold change in the average difference between two groups, which gave a set of 26 genes, 3 of which were up-regulated and 23 down-regulated by knockdown of dysadherin (Supplementary Table S1).

The gene most highly affected by knockdown of dysadherin was CCL2. We focused on this gene, because chemokines have been
implicated previously in metastasis (25). First, we analyzed the expression level of CCL2 mRNA by real-time quantitative RT-PCR (Fig. 3A) and confirmed a >10-fold reduction of CCL2 mRNA level in MDA-MB-231 cells transfected with dysadherin siRNA compared with control counterpart. Using an ELISA assay, we then showed that dysadherin siRNA caused a ~7-fold decrease in the levels of CCL2 protein secreted into cell culture supernatants, confirming that decreased mRNA expression results in reduced protein expression (Fig. 3A). Conversely, we investigated whether overexpression of dysadherin could enhance CCL2 expression. T-47D cells, which express relatively low levels of endogenous dysadherin, were transiently transfected with a dysadherin expression vector. The dysadherin-overexpressing cells showed a ~2-fold increase of CCL2 secretion compared with mock-transfected cells (Fig. 3B).

We next examined the correlation between dysadherin and CCL2 expression in additional breast cancer cell lines. Quantitative RT-PCR analysis showed that expression of dysadherin mRNA was generally associated with elevated CCL2 mRNA (Fig. 3C). These data agree with those of Mestdagt et al. (30), who showed an inverse relationship between E-cadherin and CCL2 expression in a subset of these cell lines. As we have found with dysadherin, CCL2 mRNA was also elevated in ER-negative compared with ER-positive human breast tumors in two large clinical microarray studies (Fig. 3D; refs. 23, 24). The data suggest that dysadherin is an important determinant of CCL2 expression in multiple breast cancer cell lines and clinical samples.

**Autocrine CCL2 mediates dysadherin effects on invasion and actin organization in vitro.** To determine whether CCL2 plays an important role in dysadherin-driven biological responses, we tested the invasiveness of breast cancer cells through Matrigel following experimental manipulation of CCL2 expression. Exogenous addition of CCL2 had no effect on the basal invasiveness of MDA-MB-231 cells. However, knockdown of dysadherin caused a ~3-fold reduction in invasiveness of these cells (Fig. 4A), which was largely restored by addition of recombinant CCL2 (Fig. 4A). Moreover, the basal invasive activity of three breast cancer cells (MDA-MB231, MDA-MB-468, and Hs578T) was significantly inhibited by CCL2-blocking antibody (Fig. 4B). CCL2 therefore seems to be a positive autocrine regulator of invasion in multiple breast cancer cell lines. The biological effects of CCL2 are primarily mediated through binding to its receptor CCR2 (26, 27), and we confirmed that CCR2 mRNA was expressed in both T-47D and MDA-MB-231 cells (Supplementary Fig. S1).

The actin cytoskeleton is thought to be an important mediator of cell migration in the process of cancer cell invasion and metastasis (28). It was shown previously that knockdown of dysadherin could increase transverse actin stress fibers, reduce cell motility, and suppress the metastatic potential of human pancreatic cancer cells (5). Using confocal microscopy, we found that MDA-MB-231 cells transfected with dysadherin siRNA tended to have a larger, more spread-out shape and increased number of transverse actin stress fibers when compared with cells transfected with nonspecific control. Adding recombinant CCL2 to dysadherin siRNA-transfected cells decreased cell spreading and formation of filamentous transverse actin stress fibers (Fig. 4C). This result suggests that CCL2 expression may contribute to dysadherin-mediated invasion.
by causing reorganization of the actin cytoskeleton in an E-cadherin-independent fashion.

**Dysadherin could promote angiogenesis through paracrine effects of CCL2 on endothelial cell migration.** CCL2 has been shown previously to act as a direct mediator of angiogenesis (12). Given our results that dysadherin can regulate the level of CCL2 secreted by cancer cells, we tested whether breast cancer cell-conditioned medium could influence *in vitro* endothelial cell migration. The migration of HUVEC induced by conditioned medium from dysadherin siRNA-transfected MDA-MB-231 cells was 4-fold less than that induced by conditioned medium from the control cells (Fig. 4D). Addition of CCL2 to the conditioned medium of dysadherin siRNA-transfected cells largely restored the migration response, indicating that dysadherin-mediated control of CCL2 levels may influence angiogenesis by modulating endothelial cell migration.

**Dysadherin up-regulates CCL2 expression in part through activation of the NF-κB signaling pathway.** The CCL2 promoter contains sites for regulation by CAAT/enhancer-binding protein, NF-κB, c-ETS, and β-catenin/TCF4 (29–31). We found no activation of the β-catenin pathway in the MDA-MB-231 cells as indicated by the absence of Top-Flash reporter activity (data not shown). However, we did find that dysadherin overexpression significantly up-regulated transcription from a NF-κB promoter-reporter construct in T-47D cells, whereas knockdown of dysadherin significantly down-regulated transcription from the NF-κB promoter in MDA-MB-231 cells (Fig. 5A). Using quantitative RT-PCR, we showed that a NF-κB inhibitor significantly decreased CCL2 expression in MDA-MB-231 cells (Fig. 5B). The data suggest that dysadherin enhances activation of the NF-κB pathway and that this leads to increased CCL2 expression.

**Knockdown of CCL2 significantly reduces invasion *in vitro* and metastasis *in vivo*.** To determine whether CCL2 plays an important role in cancer invasion and metastasis, we stably knocked down CCL2 in MDA-MB-231 cells using shRNA (Supplementary Fig. S2). To minimize the effect of clonal variation, we used two pooled populations of three CCL2 shRNA-transfected clones (8, 9, 11) and three nonsilencing shRNA-transfected clones (1–3), respectively. In *in vitro* invasion assays, the pooled cells transfected with CCL2 shRNA showed a ~3-fold reduction in ability to invade
through Matrigel when compared with parent MDA-MB-231 cells and the pool transfected with the nonsilencing shRNA (Fig. 6B); this is comparable with the reduction in invasion that was induced by dysadherin siRNA (Fig. 4A). The decreased invasion is not due to a decreased proliferation rate, as CCL2 shRNA and nonsilencing shRNA-transfected clone pools showed growth curves similar to that of parental MDA-MB-231 (data not shown).

We then examined the effect of CCL2 knockdown in an experimental metastasis assay. We recognize that measurement of lung colonization following i.v. injection of tumor cells does not recapitulate the complete spectrum of events that are required for successful metastasis of a cell from a primary tumor. However, we wished to be able to distinguish direct effects of CCL2 knockdown specifically on steps involved in metastasis from effects that might be secondary consequences of CCL2 knockdown on primary tumor growth or phenotype.

Parent MDA-MB-231, the nonsilencing shRNA-transfected clone pool, and the CCL2 shRNA-transfected clone pool were injected into the tail vein of SCID mice. The mice were euthanized 42 days after injection of cells, and lung weight and metastases were examined. The relative lung weight was significantly lower in the CCL2 shRNA group compared with control counterparts, suggesting a lower metastatic burden (Fig. 6C). Indeed, the number of metastatic lung nodules in the mice injected with CCL2 shRNA-transfected clone pool was significantly lower (~3-fold reduction) than those in the control counterparts (Fig. 6D). The presence of metastases was confirmed by pathologic examination (data not shown). Taken together, these results clearly show that the knockdown of CCL2 by stable shRNA reduced lung colonization in vivo following i.v. injection of the breast cancer cells. Thus, CCL2 can increase the efficiency of one or more steps in the metastatic process that lie downstream of invasion of the primary tumor through the basement membrane and subsequent intravasation. Candidate steps that might be affected would include tumor cell extravasation into the lung, and expansion of extravasated cells into macroscopically visible colonies, dependent on successful angiogenesis.

**Discussion**

Metastasis is the most devastating consequence of cancer progression, and much remains to be discovered about the biological and molecular mechanisms that control the metastatic process. Dysadherin has recently been implicated as a molecular player in this process, and the prometastatic activity of dysadherin has been attributed to its ability to down-regulate E-cadherin (11). In the present study, we have investigated the role of dysadherin specifically in breast carcinogenesis. We showed that dysadherin is highly expressed in ER-negative breast cancer cell lines and tumors, a subclass of breast tumors that has a generally poor prognosis. Unsurprisingly, we found that dysadherin could promote invasion of breast cancer cells that did not express E-cadherin, suggesting a novel mechanism of action in this cell type. We identified the chemokine CCL2 as a key mediator of dysadherin, with autocrine stimulatory effects on tumor cell invasion and paracrine stimulatory effects on endothelial cell migration. The central importance of CCL2 was confirmed by the demonstration that CCL2 knockdown significantly reduced metastasis of a dysadherin-positive breast cancer cell line in vivo.

Chemokines are emerging as important players in carcinogenic progression. Many tumors express one or more chemokines that may attract inflammatory cells, such as macrophages, which can promote progression (25). Breast cancer cells have been shown previously to express the chemokine receptors CXCR4 and CCR7, which increase the efficiency of tumor cell homing to metastatic target sites, such as the lung, where the cognate ligand CXCL12 is highly expressed (32). Tumor-associated myoepithelial and myofibroblast cells also overexpress CXCL12 and CXCL14, which promote proliferation, migration, and invasion of breast cancer cells in a paracrine fashion (33). In some cases, a tumor cell may express both chemokine and cognate receptor allowing establishment of a tumor-promoting autocrine loop as has been shown for CCL5 (RANTES) in prostate cancer, CXCL8 [interleukin (IL)-8] in melanoma, and CXCL12 (SDF-1) in breast cancer (34–36). Here, we have shown that expression of dysadherin establishes a stimulatory autocrine loop involving CCL2, which directly promotes invasion of the tumor cell in vitro, without any effects on proliferation. To the best of our knowledge, this is the first demonstration of an autocrine effect of CCL2 on a carcinoma cell, although an autocrine CCL2 loop has been implicated in the promotion of transendothelial migration of myeloma cells (37).

CCL2 (also known as MCAF or monocyte chemotactant protein-1) is a CC chemokine that is chemotactic for monocytes, memory T cells, and natural killer cells (38). It is expressed by a wide variety of cancer types, and although CCL2 has been shown to display an antitumoral effect in some models (39-40), a large number of studies showed that CCL2 generally facilitates tumor progression (41–43). Metastasis is a multistep process, and CCL2 could affect several of these steps, including chemotaxis of tumor-promoting leukocytes, tumor cell migration, and angiogenesis (12, 44). Here, we have shown that the CCL2 that is secreted by the tumor cell in response to dysadherin expression can promote tumor cell invasion in an autocrine manner and can also exert paracrine effects on endothelial cell recruitment that could enhance angiogenesis. Relevant to the autocrine invasion activity, CCL2

![Figure 5](cancerres.aacrjournals.org)
knockdown technology, it is not clear to what extent gene expression is changed as a direct result of signaling from dysadherin or whether many of the changes are secondary to dysadherin-induced effects on cell shape. In rabbit synovial fibroblasts, cell shape change and reorganization of the actin cytoskeleton is associated with Rac activation and the production of reactive oxygen species, leading to activation of the NF-κB pathway (49). Conceivably, dysadherin effects on cell shape might initiate a similar cascade of events in breast cancer cells. Because the NF-κB inhibitor did not fully block the dysadherin-induced up-regulation of CCL2, dysadherin almost certainly modulates other signal transduction pathways in addition to NF-κB to bring about the observed gene expression changes. Dysadherin is a member of the FYXD family that regulates Na⁺,K⁺ ATPase (3, 50). The Na⁺,K⁺ ATPase has been shown to act as a signal transducer, in addition to being an ion pump (51, 52), so dysadherin might also contribute to cancer metastasis through mechanisms involving changes in ion flux leading to effects on the protein kinase C and extracellular signal-regulated kinase 1/2 pathways. These possibilities are under active investigation.

In summary, we have shown that dysadherin may play an important role in ER-negative breast cancer by promoting invasion and metastasis through a mechanism that involves enhanced signaling through the NF-κB pathway, leading to increased production of the tumor-promoting chemokine, CCL2. Because antagonists of both the NF-κB pathway and of CCL2/CCR2 are under active development for the treatment of chronic inflammatory disorders (48, 53), these mechanistic insights suggest new approaches to the treatment of this particularly aggressive subclass of breast cancer.

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