

CCR5 Antagonist Blocks Metastasis of Basal Breast Cancer Cells

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Abstract

The roles of the chemokine CCL5 and its receptor CCR5 in breast cancer progression remain unclear. Here, we conducted microarray analysis on 2,254 human breast cancer specimens and found increased expression of CCL5 and its receptor CCR5, but not CCR3, in the basal and HER-2 genetic subtypes. The subpopulation of human breast cancer cell lines found to express CCR5 displayed a functional response to CCL5. In addition, oncogene transformation induced CCR5 expression, and the subpopulation of cells that expressed functional CCR5 also displayed increased invasiveness. The CCR5 antagonists maraviroc or vicriviroc, developed to block CCR5 HIV coreceptor function, reduced *in vitro* invasion of basal breast cancer cells without affecting cell proliferation or viability, and maraviroc decreased pulmonary metastasis in a preclinical mouse model of breast cancer. Taken together, our findings provide evidence for the key role of CCL5/CCR5 in the invasiveness of basal breast cancer cells and suggest that CCR5 antagonists may be used as an adjuvant therapy to reduce the risk of metastasis in patients with the basal breast cancer subtype. *Cancer Res*; 72(15); 3839–50. ©2012 AACR.

Introduction

Breast cancer causes the death of 40,000 women in the United States and 410,000 women in the world annually (1). Despite advances in the treatment of the disease, 20% to 30% of patients with early breast cancers will experience relapse with distant metastatic disease (2). In those patients, metastasis is the main cause of death. Patients with basal tumors have increased risk of metastasis and lower survival rate (3, 4). Kennecke and colleagues studied 3,726 patients with breast cancer and reported that the basal tumors have higher frequencies of metastases and reduced time from identification of metastases to death than that of patients with luminal A or B tumors (4). The absence of androgen receptor, estrogen receptor (ER), and HER-2 commonly found in basal breast tumors (5) means that they are unlikely to respond to hormone therapies or HER-2–targeted therapies. Currently, chemotherapy, radiation, and surgery are the only choices for patients with basal

breast cancers, but all show poor outcomes (6). The need for a specific targeted therapy for basal breast cancer remains urgent.

Cancer metastasis is regulated by chemokines in the microenvironment (7). Chemokines bind to cell surface receptors that belong to the G-protein–coupled receptor family (GPCR), controlling diverse biologic and pathologic processes from immunosurveillance, inflammation, and cancer. Previous studies of human breast cancer and breast cancer cell lines showed that the chemokine receptors CXCR4 and CCR7 are expressed in breast cancer cells, malignant breast tumors, and metastasis. Their related ligands, CXCL12 (SDF1) and CCL21, are also expressed at the site of metastasis (8). Subsequent studies identified altered expression of CCL5 (RANTES) in patients with breast cancer, correlating with disease progression (9–11).

CCL5 can be expressed and secreted either by breast cancer cells (9–12) or by nonmalignant stromal cells at the primary or metastatic sites (13). However, the roles of CCL5 and its receptors in breast cancer are not fully understood. CCL5 facilitates disease progression by recruiting and modulating the activity of inflammatory cells, which subsequently remodel the tumor microenvironment (14, 15). Accordingly, inhibition of CCR5 by a peptide antagonist reduced leukocyte infiltration and reduced tumor growth after subcutaneous injection of 410.4 cells into immunocompetent mice (16). Studies analyzing the role of CCR5 in breast cancer cell proliferation have used mainly luminal MCF-7 cells and suggest a role for p53. Manes and colleagues reported that abrogation of cell surface CCR5 expression enhanced MCF-7 cell proliferation *in vivo* in the presence of p53 but did not affect proliferation in xenografts encoding a p53 mutation (17). Murooka and colleagues reported that in CCR5-transfected MCF-7 cells, CCL5

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promotes proliferation and survival in an mTOR-dependent manner (18). Autocrine secretion of CCL5 controls migration and invasiveness of human breast cancer cells *in vitro* (12, 19). Forced expression of CCL5 increased tumor metastasis approximately 1.8-fold in one study of MDA-MB-231 cells but had no effect in the 168 breast cancer cell line (13, 20). Furthermore, other authors have shown that shRNA inhibition of CCL5 expression in murine mammary cancer cells does not affect growth rate or metastatic frequency *in vivo* (20).

Discrepancies in the reported effects of CCL5 and CCR5 in breast cancer may be due to the different models used. To study whether CCL5/CCR5 signaling is active in specific genetic subtypes of breast cancer, we analyzed the combined expression of CCL5 and CCR5 using a microarray database comprising 2,254 human breast cancer samples from 27 independent studies (21). This report is the first to show that CCL5/CCR5 signaling is preferentially active in the basal and HER-2 subtypes. Using basal-like breast cancer cell lines, we showed that only a subpopulation of cells express CCR5 and respond to CCL5. Importantly, CCR5⁺ cells displayed increased invasiveness, indicating that CCR5 contributes to the metastatic phenotype of basal breast cancer cells. Therefore, we examined the possibility that CCR5 inhibition may block the invasion and metastasis of basal-like breast cancer cells. We used drugs originally developed to prevent the interaction of CCR5 with the gp120 from HIV-1. The CCR5 antagonist maraviroc (Selzentry) received full U.S. Food and Drug Administration (FDA) approval for use in treatment-naïve adults with CCR5-trophic HIV. Vicriviroc (SCH 417690), another CCR5 antagonist, has shown good tolerance and partial therapeutic success in phase II clinical trials for HIV (22). CCR5 inhibition blocked breast cancer cell invasiveness *in vitro* and efficiently reduced metastatic colonization *in vivo*. The antimetastatic effect produced by CCR5 inhibition was associated with reduced arrival of breast cancer cells to the target tissue but was independent of changes in cell proliferation or tumor growth. Our findings suggest that CCR5 antagonists may be used to reduce the risk of metastasis in patients with the basal subtype of breast cancer.

Materials and Methods

Breast cancer patients data set and statistical analysis

A microarray data set that was previously compiled (21) from the public repositories Gene Expression Omnibus (23) and ArrayExpress (24) was used to evaluate CCR5 and CCL5 expression in the context of clinical samples. Samples in this data set were assigned to 5 canonical breast cancer subtypes, including luminal A, luminal B, normal-like, basal, and HER-2-overexpressing disease. The classification of microarray samples among these 5 subtypes was achieved by computing their correlation against an expression profile centroid representative of each subtype and assigning samples to the subtype with the highest corresponding correlation coefficient (25). Samples with a maximum correlation coefficient below 0.3 were considered unclassified. Analysis of CCL5 and CCR5 transcript was then conducted specifically among the luminal A, luminal B, basal, normal-like, and

HER-2 subtypes. Differential expression of the averaged gene signature magnitude among these sample subsets was evaluated using 2-tailed Student *t* test. Kaplan–Meier analysis was used to evaluate survival trends within the sample subsets. Scatter plots of CCL5 versus CCR5 samples were also generated to observe coregulation patterns specific to each subtype. For these scatter plots, gene profiles were median-centered and scaled to unitary SD.

Cell lines and cell culture

MDA-MB-231, MCF-7, and Hs578T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. SUM-159 cells (kindly provided by Dr. Stephen Ethier, Wayne State University, Detroit, MI) were maintained in Ham's F-12 supplemented with 4 µg/mL of insulin, 1 µg/mL of hydrocortisone, and 5% FBS. Oncogene-transformed derivatives of MCF-10A cells (MCF10A-NeuT, MCF10A-Src, and MCF10A-Ras; ref. 26) were maintained in DMEM:Ham's F-12 (50 of 50) supplemented with 4 mg/mL of insulin, 10 ng/mL of EGF, and 1 mg/mL of hydrocortisone. A total of 100 µg/mL of each penicillin and streptomycin were included in all media. Cells were cultured in 5% CO₂ at 37°C. For *in vitro* treatments, maraviroc was dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium. The final concentration of DMSO in treated and control cultures was 0.5%. Vicriviroc was dissolved in culture medium.

Fluorescence-activated cell-sorting analysis

Cell labeling and fluorescence-activated cell-sorting (FACS) analysis for CCR5 were based on prior publications (27) with minor modifications. Before labeling, the cells were blocked with normal mouse IgG (1 of 100) and purified rat anti-mouse Fcγ III/II receptor antibody (1 of 100; Pharmingen) for 30 minutes and then incubated with allophycocyanin (APC)-labeled CCR5 antibody (R&D Systems). All experiments were conducted at 4°C. Sample analysis was conducted on FACS-Calibur flow cytometer (BD Biosciences). These data were analyzed with FlowJo software (Tree Star, Inc.).

Invasion assay

The 3-dimensional invasion assay was conducted as previously reported (12). Briefly, 100 µL of 1.67 mg/mL Rat Tail collagen type I (BD Biosciences) was pipetted into the top chamber of a 24-well 8-µm pore Transwell (Corning). The Transwell was incubated at 37°C overnight to allow the collagen to solidify. A total of 30,000 cells were then seeded on the bottom of the Transwell membrane and allowed to attach. Serum-free growth medium was placed into the bottom chamber, whereas 15 ng/mL CCL5 or 5% FBS was used as a chemoattractant in the medium of the upper chamber. The cells were then chemoattracted across the filter through the collagen above for 3 days. Cells were fixed in 4% formaldehyde, permeabilized with 0.2% Triton-X in PBS, and then stained with 40 µg/mL propidium iodide (PI) for 2 hours. Fluorescence was analyzed by confocal *z*-sections (one section every 20 µm) at ×10 magnification from the bottom of the filter using a Zeiss LSM 510 Meta inverted confocal microscope at the Kimmel Cancer Center Bioimaging Facility.

Intracellular calcium assay

Calcium responses induced either by CCL5 or FBS in human cancer cell lines were monitored under fluorescence confocal microscope as previously reported (28). Briefly, breast cancer cells were seeded in 4-well labtek chambers (Nunc) at 10^4 cells/cm² and incubated for 1 day. After 12-hour starvation, cells were labeled by incubating them with 2 mmol/L Fluo-4-AM (Molecular Probes) in HBSS for 30 minutes, washed twice, and incubated for additional 30 minutes before imaging under the microscope. Time-lapse images were collected using a Zeiss LSM 510 Meta inverted confocal microscope with the incubator at 37°C. Relative intracellular Ca²⁺ concentration was determined by the changes in fluorescent intensity (FI) of Fluo-4-AM upon the addition of CCL5 (60 ng/mL) or FBS (5%) and was calculated as $(FI_t - FI_0)/FI_0$.

MTT assay

The effects of CCR5 antagonists on cell viability and proliferation rate were estimated using the soluble tetrazolium salt MTT assay (29). MTT is reduced by the mitochondria of viable cells, and the amount of reduced formazan is proportional to the number of viable cells. After 72 hours of exposure to the drugs, cells were incubated with 1 mg/mL of MTT for 90 minutes. Then, the reduced (insoluble and colored) formazan was dissolved in DMSO and measured spectrophotometrically at 570 nm. The effect of CCR5 overexpression in breast cancer cell proliferation was studied in MDA-MB-231 cells transfected with full-length human CCR5 subcloned into pcDNA3.1+/Zeo⁺ vector (kindly provided by Dr. Eleanor Fish, University of Toronto, Toronto, ON, Canada) and selected with Zeocin (200 µg/mL) as previously described (18). MTT assays were conducted in sextuplicate using 96-well microplates.

Viral cell transduction

A lentiviral vector encoding firefly luciferase 2 (Luc2)-eGFP fusion protein was a generous gift from Dr. Sanjiv S. Gambhir (School of Medicine, Stanford University, Stanford, CA; ref. 30). Lentivirus propagation was conducted following the protocol described by Zahler and colleagues (31). Breast cancer cell lines were transduced at a multiplicity of infection of 20 in the presence of 8 mg/mL polybrene (Sigma) for 24 hours (30, 31).

Experimental metastasis assay and bioluminescence imaging

MB-MDA-231 cells expressing Luc2-eGFP (called MDA.pFLUG for the rest of the article) were detached with a nonenzymatic cell dissociation buffer (4 mmol/L EDTA in Ca²⁺ and Mg²⁺-free PBS), resuspended in Dulbecco's PBS without Ca²⁺ and Mg²⁺ and immediately injected into the tail vein of 8-week-old, female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (NCI, Bethesda MD). Each mouse received 10^6 cells. Mice were treated by oral gavage with maraviroc (8 mg/kg every 12 hours) or vehicle (5% DMSO in acidified water; ref. 32). Treatment was started immediately after injection or 10 days later for the experiments analyzing the proliferation of established metastasis. For *in vivo* bioluminescence imaging (BLI), mice were given an intraperitoneal (i.p.) injection with 200 µL of D-luciferin (30 mg/mL). Mice

were anesthetized with isoflurane (2% in 1 L/min oxygen), and bioluminescence images were acquired 10 to 15 minutes after D-luciferin injection using the IVIS XR system (Caliper Life Sciences). Acquisition times ranged from 10 seconds (for later time points) to 5 minutes (for early time points). Data are expressed as total photon flux and were analyzed using Living Image 3.0 software (Caliper Life Sciences). For *ex vivo* BLI, D-luciferin was diluted in PBS to a final concentration of 300 µg/mL and used to soak freshly isolated lungs for 2 to 3 minutes before imaging. Some lungs were stained with India ink, as previously reported (33), or processed and stained with hematoxylin and eosin to corroborate the presence of pulmonary tumors. For homing assays, mice were euthanized 24 hours after the intravenous injection of MDA.pFLUG cells. Lungs were perfused with PBS, fixed with freshly prepared formaldehyde (4% in PBS), and frozen in optimum cutting temperature (Sakura Finetek). Cryosections (10 µm) were counterstained with 4',6-diamidino-2-phenylindole analyzed by confocal microscopy. Animal experiments were approved by the Thomas Jefferson University's Institutional Animal Care and Use Committee.

Reagents and antibodies

CCL5 (catalog no. 278-RN) and anti-CCR5 APC antibody (catalog no. FAB1802A) were purchased from R&D Systems. A rabbit anti-human CCR5 polyclonal antibody (GenScript; catalog no. A00979) was used for immunohistochemical staining. Rat tail collagen type I was purchased from BD Biosciences. Vicriviroc and maraviroc were obtained from Selleck Chemicals. Luciferin was obtained from Gold Biotechnology.

Results

Active CCL5/CCR5 signaling in basal breast cancer

To examine the relative abundance of CCL5 and its receptor CCR5 by genetic subtype, interrogation was conducted within a combined microarray database comprising 2,254 human breast cancer samples from 27 independent studies (21). The relative abundances of CCL5 and CCR5 were significantly increased in the basal and HER-2 subtypes compared with the normal-like, luminal A and luminal B subtypes (Fig. 1A). The increased expression of CCL5 and CCR5 correlated positively in individual breast cancer samples and the correlation was highly significant in the basal and HER-2 subtypes (Fig. 1B). The proportion of patients with a CCL5/CCR5-positive signature was more than 58% in the basal and HER-2 subtypes (Fig. 1C). In agreement with previous reports, clinical information of the cases in this database showed that the probability to develop metastasis is increased in the basal, luminal B, and HER-2 subtypes (Fig. 1D).

To determine the gene expression signaling pathway associated with enrichment of CCR5 and CCL5, GSEA analysis using KEGG and GO was conducted of these tumor samples (Supplementary Fig. S1A). These studies showed enrichment for gene expression of pathways including lymphocyte activation, Janus-activated kinase (JAK)-STAT signaling, and Toll-like receptor activation (Supplementary Fig. S1A). The receptors for CCL5 include CCR1 and CCR3. Increased expression of CCL5 associated with increased CCR1, but not CCR3, in the

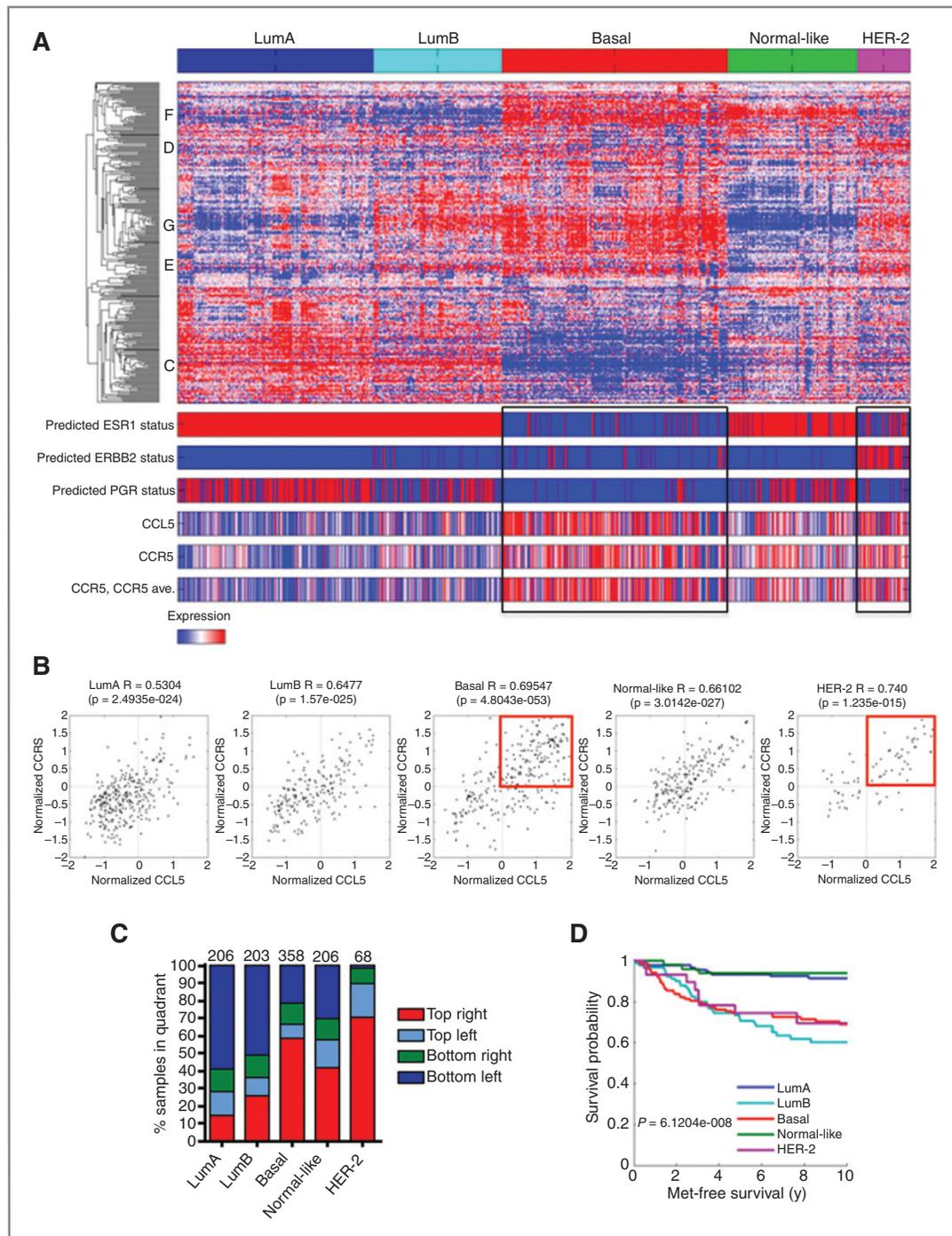
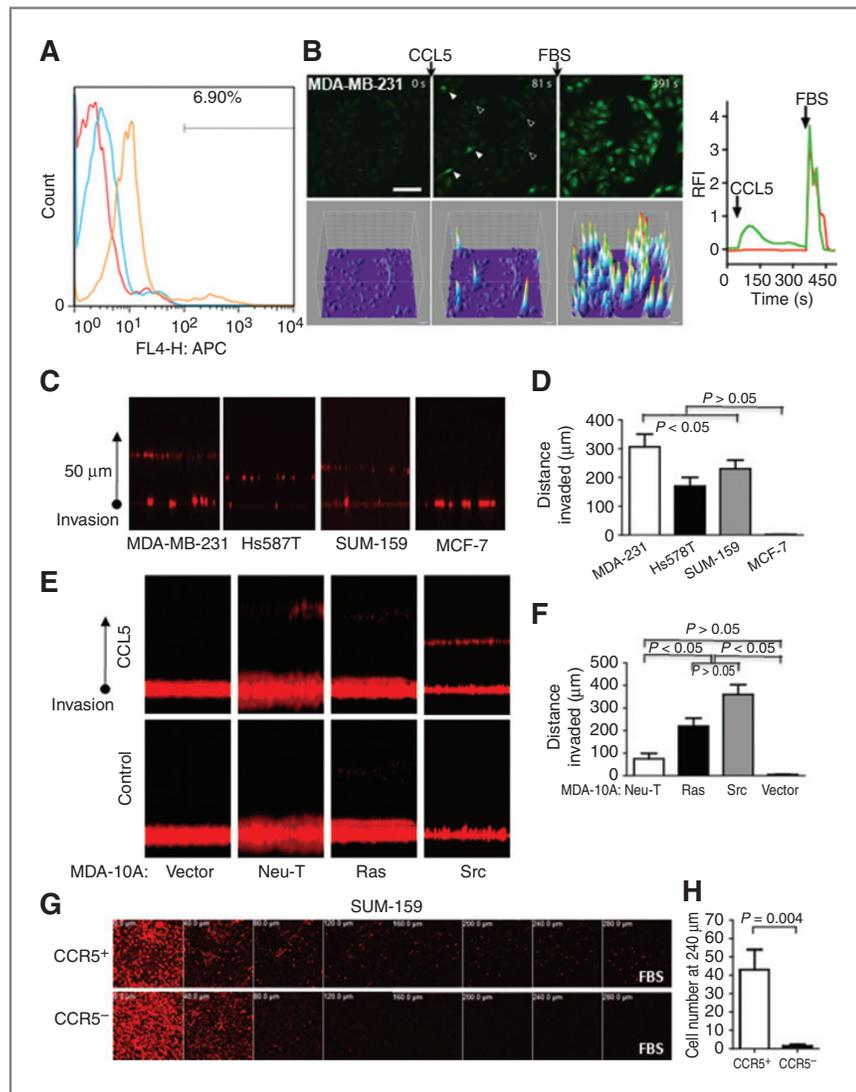


Figure 1. CCL5 and CCR5 are overexpressed in the basal breast cancer subtype. **A**, heatmap of the expression of CCL5 and its receptor CCR5 in samples from patients with breast cancer divided by genetic subtype based on their gene expression pattern. The relative abundances of CCL5 and CCR5 are increased in patients with the basal and HER-2 subtypes. **B**, scatter plots and correlation analysis (Student *t* test) of the expression of CCL5 and CCR5 among the breast cancer molecular subtypes. **C**, quantification of the proportions of the samples overexpressing CCL5 and CCR5 (red fraction of the bar) displayed in **B**. The number of samples in each subtype is indicated at the top of the bar. **D**, metastasis-free Kaplan–Meier plots and log-rank analysis for the different genetic subtypes in the analyzed database (described in Materials and Methods). As reported elsewhere, patients with the basal or HER-2 subtypes display increased probability to form metastasis.

Figure 2. Human breast cancer cell lines that express CCR5 respond to CCL5. **A**, flow cytometric histograms of the CCR5 expression in MDA-MB-231 breast cancer cells identified a subpopulation of CCR5⁺ cells. **B**, induction of calcium signaling in cells loaded with Fluo-4-AM before the sequential addition of CCL5 (60 μg/mL) and FBS (5%). A fraction of cells responded to CCL5 (closed arrowheads in the middle of micrographs) whereas the rest did not (open arrowheads). The average changes in fluorescence on 5 responsive (green line) and 5 nonresponsive (red line) cells are represented in the far right graphs. Data shown are representative of 3 to 5 independent experiments for each cell line. Bar, 100 μm. **C**, 3D invasion into collagen gels by breast cancer cell lines, using CCL5 (15 μg/mL) as chemoattractant. **D**, mean distances of invasion ± SEM from 3 independent experiments. **E**, 3D invasion assays, and their corresponding quantification (**F**, mean ± SEM, *n* = 3), for MCF-10A cells and MCF-10A-NeuT, -Ras, and -Src derivatives showing that CCL5-induced invasion is activated by oncogenic transformation. **G**, CCR5⁺ cells display increased invasiveness. CCR5⁺ and CCR5⁻ subpopulations from SUM-159 cell line were isolated by FACS and invasion into collagen gels was evaluated using FBS as chemoattractant. Quantification is shown in **H** as mean ± SEM of 2 independent experiments. Statistical analysis was conducted using the Student *t* test.



basal and HER-2 genetic tumor type (Supplementary Fig. S1B and S1C). In ER-negative patients receiving chemotherapy, there was an insignificant trend toward reduced metastasis-free survival and relapse-free survival in the increased CCR5 population, compared with the population with reduced CCR5 expression (Supplementary Fig. S1D). A comparison of expression levels for CCL5 versus CCR5, CCR1, and CCR3, comparing normal breast with breast cancer showed increased correlation between receptor and ligand expression levels in tumors compared with healthy breast tissue (Supplementary Fig. S2).

CCL5 promotes breast cancer Ca²⁺ signaling and cellular invasion

We chose to use as models in our studies 3 human breast cancer cell lines with a basal phenotype and molecular

signature: MDA-MB-231, Hs578T, and SUM-159 (34–37). Analysis of CCR5 expression by FACS showed that a small subpopulation of cells were positive for the receptor in all 3 cell lines (Fig. 2A for MDA-MB-231 and Supplementary Fig. S3A and S3C for Hs578T and SUM-159). Because CCR5 activation induces calcium flux (38, 39), we assessed the activation of calcium signaling by CCL5. Addition of CCL5 to the cultures induced immediate calcium fluxes in a subpopulation of cells (Fig. 2B for MDA-MB-231 and Supplementary Fig. S3B and S3D for Hs578T and SUM-159), providing evidence that CCR5 is functional in basal breast cancer cells. As a positive control, the same cultures were exposed to 5% FBS (40). Calcium flux, assessed by relative fluorescence intensity, increased in more than 95% of the cells after FBS addition (Fig. 2B and Supplementary Fig. S3B and S3D). To further distinguish CCL5-dependent signaling, SUM159

cells were stably transduced with a CCR5 expression vector and the Ca^{+2} response to CCL5 versus FBS was conducted (Supplementary Figs. S4C and D vs. S4E and F). CCR5 induced Ca^{+2} signaling in the CCR5-overexpressing cells, whereas both lines responded similarly to FBS induced Ca^{+2} activation (Supplementary Fig. S4).

We next assessed the effect of CCR5 activation on breast cancer cell invasion using 3D migration assays. CCL5 induced invasion of the basal MDA-MB-231, Hs578T, SUM-159 but not the luminal MCF-7 cells (Fig. 2C and D). CCL5 promoted invasion of MCF-10A cells engineered to express either NeuT, H-Ras, or c-Src oncogenes, compared with MCF10A vector-transduced cells (Fig. 2E and F), suggesting that CCL5 responsiveness may be acquired during transformation and requires specific cooperative oncogenic signals. The finding that CCL5 induced cellular invasion led us to examine the migratory capacity of CCR5⁺ cells versus that of CCR5⁻ cells. Within the same SUM-159 breast cancer cell line, CCR5⁺ cells showed an approximately 40-fold greater cellular invasiveness (Fig. 2G and H), indicating that the expression of CCR5 correlates with a proinvasive phenotype.

CCR5 antagonists block breast cancer calcium signaling and cell invasion

The importance of CCR5 in HIV infection led to the development of different drugs that target this receptor. We therefore examined whether the CCR5 antagonists maraviroc and vicriviroc were capable of blocking the CCL5/CCR5 signaling in basal breast cancer cells. Both CCR5 antagonists blocked CCL5-induced calcium mobilization. In MDA-MB-231 cells, maraviroc and vicriviroc inhibited calcium responses by 65% and 90%, respectively (Fig. 3A and B). Similar observations were made with both drugs in Hs578T cells (Fig. 3C and D), indicating that CCR5 expressed in different basal breast cancer cells is sensitive to pharmacologic inhibition.

To evaluate the functional relevance of CCR5 in cellular migration and invasion, we tested the effects of maraviroc and vicriviroc in 3D invasion assays. Using 2 different cell lines, we found that both CCR5 antagonists inhibited FBS-induced breast cancer cell invasion at the clinically relevant concentration of 100 nmol/L (Fig. 4A–D). Thus, the proinvasive effect of CCR5 can be abrogated by using specific antagonists.

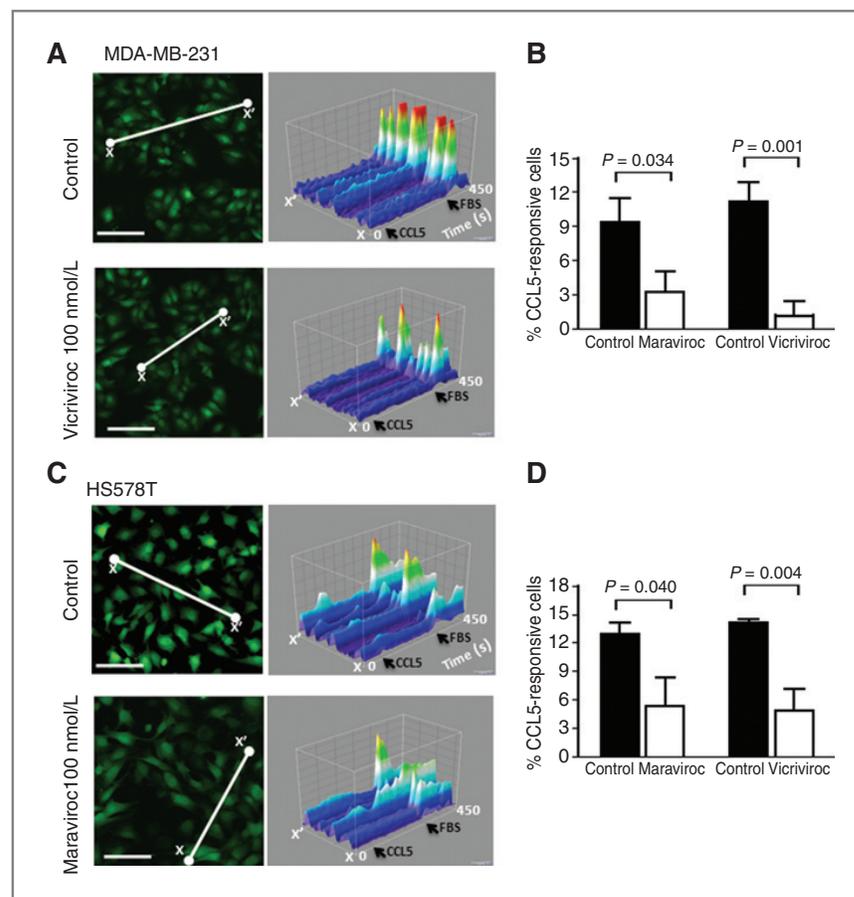
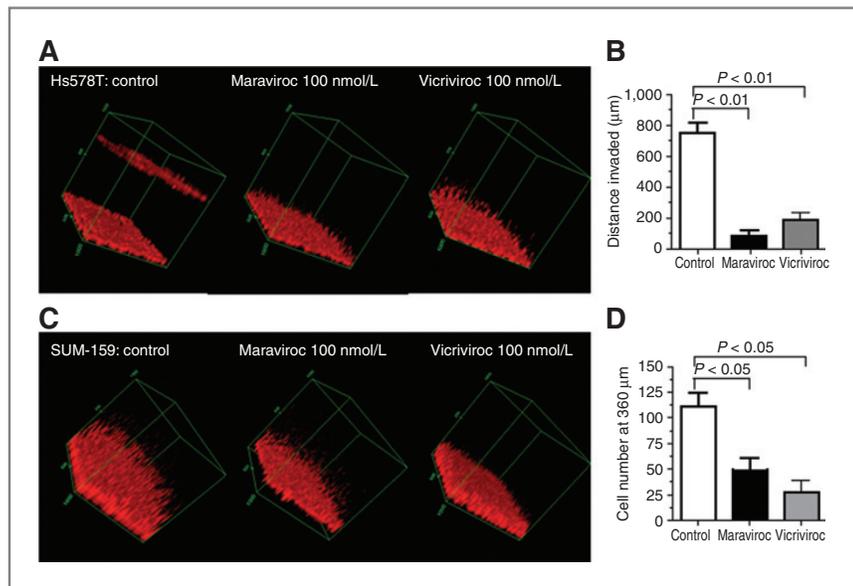


Figure 3. CCR5 antagonists block CCL5-induced calcium signaling. A, intensity versus time analysis of Fluo-4 AM-loaded MDA-MB-231 cells treated with the CCR5 antagonists maraviroc or vicriviroc (100 nmol/L) for 30 minutes before the addition of CCL5 (60 $\mu\text{g}/\text{mL}$). Micrographs illustrate the axis ($x-x'$) of the pseudocolor scan plot. Those axes were used to construct the adjacent intensity versus time plots. B, comparison of the fraction of cells with increased fluorescence intensity upon addition of CCL5. C, CCL5-induced calcium signaling was also blocked by CCR5 antagonists in Hs578T cells. The corresponding quantification is shown in D. Data in B and D are mean \pm SEM of 3 to 4 independent experiments. Statistical analysis was conducted using the Student *t* test.

Figure 4. CCR5 antagonists block FBS-induced breast cancer cell invasion. 3D reconstruction of FBS-induced invasion into collagen gels by Hs578T (A) or SUM-159 (C) breast cancer cells in presence of CCR5 antagonists (100 nmol/L). The corresponding quantifications (mean \pm SEM, $n = 3$) and analysis (Bonferroni t test) are displayed in B and D.



CCR5 inhibition blocks breast cancer metastasis *in vivo*

In view of the finding that CCR5 inhibition by CCR5 antagonists reduced calcium signaling and cell invasion, we determined the *in vivo* effect of maraviroc on lung metastasis. We used MDA-MB-231 cells transduced within the Luc2-eGFP lentiviral vector (MDA.pFULG cells) in an experimental metastasis model. The *Luc2* gene is a codon-optimized version of *Luc* and cells expressing this reporter were 10 to 100 times brighter than the unmodified *Luc* gene (30). After injection of MDA.pFULG cells into the tail vein of mice, noninvasive BLI enabled the early detection of breast cancer metastasis (41). Weekly BLI was conducted for 5 weeks and the radiance antemortem was used as a surrogate measurement of tumor burden. Mice treated with maraviroc (8 mg/kg twice daily) showed a significant reduction in both the number and the size of pulmonary metastases compared with vehicle-treated mice (Fig. 5A and B, Supplementary Fig. S5). To avoid the possibility that metastases were missed because of inappropriate imaging, we conducted *ex vivo* imaging, India ink staining (Fig. 5C), and histology (Fig. 5E) of the lungs. Histologic analysis corroborated that tumor burden corresponds to bioluminescence, as previously shown (30). Metastatic tumors were still detectable in 50% of the maraviroc-treated mice, but their mean size was reduced by 65% (Fig. 5D and F). Interestingly, analysis of CCR5 expression in lungs from control mice showed an 8-fold enrichment of the CCR5⁺ fraction (Supplementary Fig. S6). Collectively, these results provide evidence that CCR5 antagonists reduce breast cancer metastasis *in vivo*.

CCR5 antagonist impairs lung colonization but not cell proliferation or tumor growth

We determined whether the reduction in metastatic tumors by maraviroc involved changes in cellular proliferation and/or target organ colonization. We analyzed the

effect of CCR5 inhibition on cell viability and proliferation both *in vitro* and *in vivo*. Maraviroc or vicriviroc treatment of MDA-MB-231 cells for 48 hours did not affect the MTT reduction, which was used as a surrogate measurement of cancer cell number (Fig. 6A). In agreement, overexpression of CCR5 in MDA-MB-231 cells did not modify their proliferation rate compared with cells transfected with the empty vector (Fig. 6B and C). Finally, maraviroc treatment of mice with established pulmonary metastasis did not modify tumor growth (Fig. 6D and E), indicating that CCR5 activation does not promote the proliferation of basal breast cancer cells *in vitro* nor in the pulmonary microenvironment of immunocompromised mice.

On a different *in vivo* experiment, we examined the effect of maraviroc on breast cancer cell homing to lungs. To reach a steady-state concentration in plasma and tissues, mice were given 10 administrations of maraviroc (twice a day for 5 days) before the intravenous injection of MDA.pFULG cells (Fig. 6F). Inoculation of equal numbers of MDA.pFULG cells in control and treated groups was corroborated by BLI immediately after injection. Maraviroc reduced the number of eGFP⁺ cells in the lungs by 40% (Fig. 6G and H), suggesting that the *in vivo* antimetastatic effect of maraviroc is caused by a reduction in the number of cancer cells that colonize the target organ from the circulation.

Discussion

The current studies show for the first time that: (i) enrichment of CCL5/CCR5 expression occurs in patients with basal and Her2 positive genetic subtypes of breast cancer; (ii) oncogenic transformation of immortalized human breast cells by distinct oncogenes induces CCL5 responsiveness; and (iii) maraviroc, an FDA-approved drug

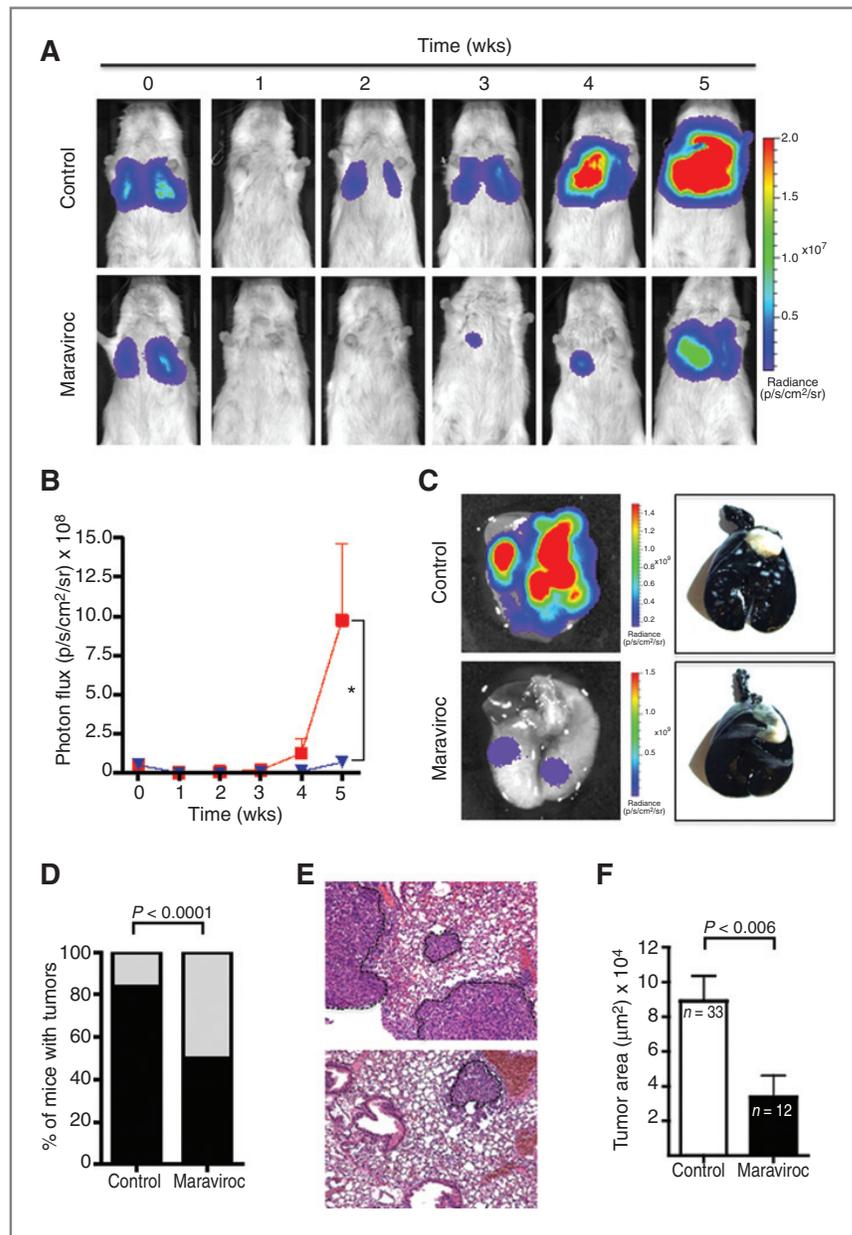


Figure 5. The CCR5 antagonist maraviroc inhibits lung metastases *in vivo*. **A**, MDA-MB-231 cells transduced with Luc2-eGFP fusion protein were injected into the tail vein of NOD/SCID mice and the *in vivo* bioluminescent signal was quantified weekly. Representative *in vivo* images of vehicle- or maraviroc-treated (8 mg/kg every 12 hours) mice are shown in **A**. Quantification (mean \pm SEM, $n = 6$) of BLI in the control (red line) and treated groups (blue line) are portrayed in **B**. Statistical comparison (*, $P = 0.048$) was carried out using Student *t* test with Welch correction for heterogeneous variances. **C**, the presence of pulmonary tumors and the differences between treatments were corroborated by *ex vivo* imaging (left) and India ink staining (right). **D**, the fraction of mice with metastatic tumors was significantly larger in the control group ($P < 0.0001$, Fisher exact test). **E**, histologic analysis (hematoxylin and eosin staining, $\times 100$) and the corresponding quantification (**F**) of the area covered by metastatic tumors in lung slides. Tumor area was quantified with the Nikon Elements BR 3.0 software analyzing at low magnification ($\times 40$) 2 random fields of 2 different histologic sections (separated 600 μm from each other) per mouse. Statistical analysis was conducted using the Student *t* test with Welch correction for heterogeneous variances ($n = 33$ and 12 for control and treated groups, respectively).

for the treatment of CCR5-trophic HIV infection, reduce metastatic tumor burden *in vivo*.

Previous studies showed that CCL5 levels are elevated in breast primary and metastatic tumors (9–11), suggesting a role of CCL5 in the acquisition of malignancy. We report here that increased expression of CCL5 and CCR5 are associated and that CCL5/CCR5 expression levels are different among the different genetic subtypes of breast cancer. Increased expression of CCL5 and CCR5 is found in the basal and HER-2 subtypes. In agreement, increased CCL5 expression has been

found predominantly in ER-negative patients (42). Increased CCL5 also correlated with increased CCR1 in basal and Her2 genetic subtypes of breast cancer. A trend toward reduced metastasis-free survival and relapse-free survival was observed among the CCR5-overexpressing tumors in patients who received chemotherapy.

Given the aggressive clinical behavior of basal breast cancer and the lack of targeted therapies for it, we evaluated the importance of the CCL5/CCR5 axis in invasion and metastasis in the human breast cancer cell lines MDA-MB-231, Hs578T

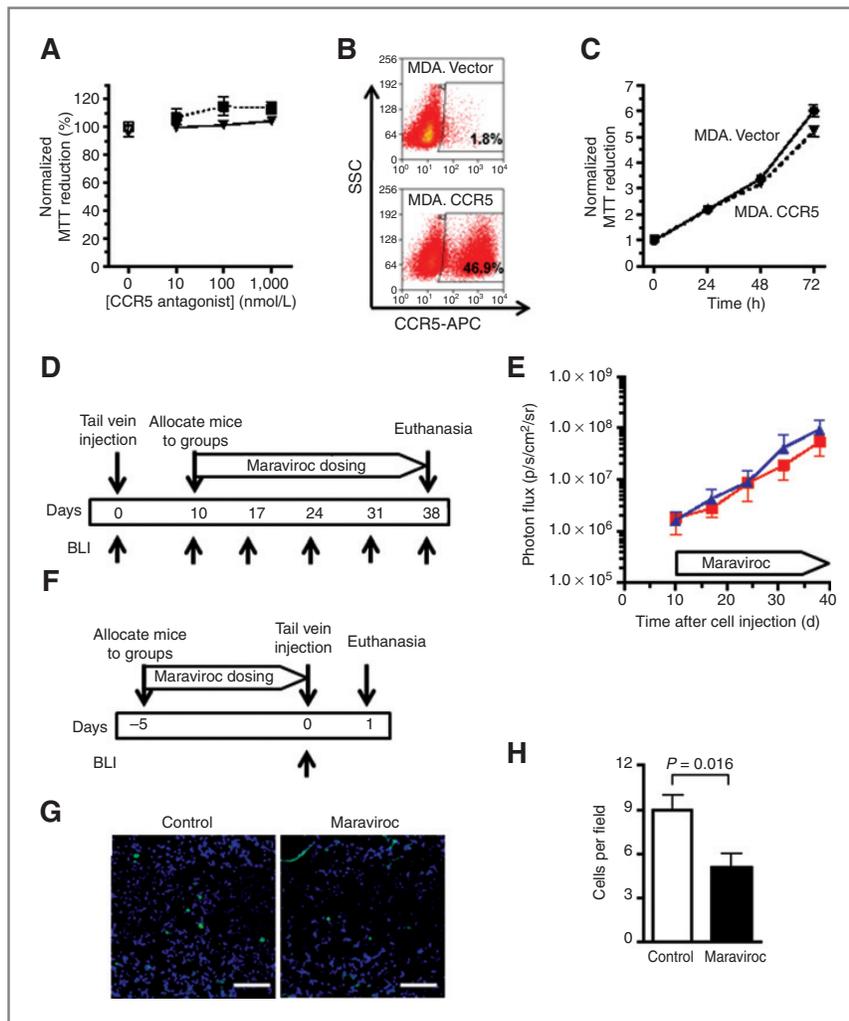


Figure 6. Maraviroc reduces lung colonization but does not modify cell proliferation. **A**, effect of CCR5 antagonist on breast cancer cell viability. MDA-MB-231 cells were exposed to increasing concentrations of maraviroc (inverted triangles) or vicriviroc (squares) for 48 hours and the cell viability was evaluated by MTT assay. Graph is from a representative experiment carried out by sextuplicate. No statistical differences were found (ANOVA) in 3 independent experiments. **B**, CCR5 expression in MDA-MB-231 cells stably transfected with pcDNA3.1⁺/Zeo⁺ (MDA.Vector) or human CCR5 cloned into pcDNA3.1⁺/Zeo⁺ (MDA.CCR5). **C**, comparison of *in vitro* proliferation rates of MDA.Vector versus MDA.CCR5 showed no differences (ANOVA). Representative experiment from 2 carried out by sextuplicate. **D**, to evaluate the *in vivo* effect of maraviroc on growth of established metastasis, treatment of mice was initiated 10 days after injection of MDA.pFULG cells as illustrated. **E**, quantification (mean \pm SEM, $n = 5$) of *in vivo* BLI in the control (red) and treated groups (blue) showed no differences in the growth rate. **F**, schema of the experimental design used to evaluate CCR5 role in lung colonization. **G**, representative confocal images and quantification (**H**) of the number of eGFP⁺ cells in lungs 24 hours after injection of MDA.pFULG cells. Cells expressing eGFP were counted in 3 random fields of 2 different histologic sections (separated 700 μ m from each other) per mouse ($n = 5$ mice per group). Statistical analysis was conducted using Student *t* test. Bar in micrographs, 100 μ m.

and SUM-159. These cell lines reflect the clinicopathologic features of the basal subtype of breast cancer (including the lack of HER-2, ER, and progesterone receptor), a basal-like molecular signature, the activation of specific signaling pathways (e.g., hypoxic or EGF receptor responses) and overexpression of epithelial-mesenchymal transition proteins (FN, VIM, and matrix metalloproteinase 2; refs. 34–37). Only a small fraction of cells within the cell lines used in this study expressed CCR5 as evaluated by FACS analysis. Our findings

are consistent with studies by Müller and colleagues who showed CCR5 expression in MDA-MB-231 by quantitative real-time PCR (8). Our studies confirmed the expression of CCR5 in MDA-MB-23 cells by reverse transcriptase PCR and showed the presence of the CCR5 protein by FACS analysis (Supplementary Fig. S7), and showed that CCR5 immunohistochemical staining was localized primarily to the breast cancer epithelial cell, compared with normal breast tissue (Supplementary Fig. S8).

We showed that CCL5 activates calcium flux in basal-like human breast cancer cells, as previously described in cells of the immune system (39, 43) and CCR5-transfected cells (27, 44, 45). By using the selective CCR5-antagonists maraviroc and vicriviroc (both with IC_{50} below 30 nmol/L; refs. 44, 45), we showed that CCL5-activated signaling is mediated by CCR5. However, the fraction of CCL5-responsive cells (10% and 12% for MDA-MB-231 and Hs578T cells, respectively) is higher than the percentage of CCR5-expressing cells determined by FACS. This may be due to the greater sensitivity of the Ca^{2+} activation assays compared with the sensitivity of analysis by FACS. In addition, CCL5-induced calcium redistribution is not completely blocked by CCR5 antagonists. This may be caused by the expression of other receptors to CCL5, namely CCR1 and CCR3. CCR5 has been identified as the main CCL5 receptor in MDA-MB-231 cells (13) and CCR1 and CCR3 transcripts are absent in both MDA-MB-231 or Hs578T cell lines (8) and breast tumor samples (11). We were able to detect CCR1 and CCR3 by FACS (Supplementary Fig. S7), suggesting a possible mechanism for the incomplete response to the CCR5 antagonist.

We observed that the subpopulation of CCR5⁺ cells displayed increased invasiveness, indicating that CCR5 favors cell migration and invasion in basal-like breast cancer cells. The failure of luminal-like MCF-7 cells to respond to CCL5 is in agreement with previous publications (12). Our studies also showed that CCR5 inhibition with either maraviroc or vicriviroc reduced *in vitro* FBS-induced breast cancer cellular invasion without affecting cellular viability. The finding that CCR5 antagonists block FBS-induced invasion is novel and suggested that CCR5 activation contribute to the production of metastasis *in vivo* where different chemotactic and growth signals are present. The mechanisms involved in CCR5 regulation of FBS-activated invasiveness are uncharacterized but they may include heterodimerization and ligand affinity regulation of other GPCRs (46), or the transactivation of growth factor receptor- (47) or integrin-mediated signaling (48), as described in noncancerous cells.

The *in vivo* antimetastatic effect of maraviroc was shown by injecting MDA.pFULG cells into the circulation of immunodeficient mice and treating them with clinically relevant doses of the drug. In humans, oral doses of 300 mg produce an average C_{max} of 1,200 nmol/L (49), whereas in mice 16 mg/kg produce an average C_{max} of 1,045 nmol/L (32). Because the drug is taken twice a day in the clinical setting, we administered 16 mg/kg/d divided into 2 doses during our experiments. Maraviroc significantly reduced the pulmonary tumor burden. Although it has been proposed that pharmacologic CCR5 inhibition may be beneficial for patients with breast cancer, to our knowledge this is the first study showing that systemic administration of a CCR5 antagonist reduces metastatic colonization of basal breast cancer cells.

The antimetastatic effect of maraviroc is not caused by alterations in growth of established metastasis. CCR5 activation by CCL5 drives proliferation in CCR5-transfected MCF-7 breast cancer cells (18) and prostate cancer cells (50), but this study and others (13) showed that the CCL5/CCR5 axis does not play a role in cell proliferation or survival in the basal-like MDA-MB-231 cells. Furthermore, inhibition of

CCR5 surface expression through a dominant-negative form of CCR5 (CCR5Δ32) in MDA-MB-231 cells does not change *in vivo* proliferation or apoptotic response (17). On the other hand, we found that maraviroc reduces lung colonization by MDA.pFULG cancer cells. This result is consistent with previous studies in which inhibition of CCR5 expression within breast cancer cells or administration of anti-CCL5 neutralizing antibody to tumor-bearing mice reduced the enhanced metastatic capability induced by coinjection of mesenchymal stem cells (MSC; ref. 13). The authors identified cancer cell extravasation as the crucial metastatic step affected by CCL5/CCR5 inhibition (13). Together, these data support a role for CCR5 antagonists in blocking the ability of basal breast cancer cells to reach the metastatic sites instead of inhibiting their proliferation or survival after arrival. Blocking the homing of cancer cells to metastatic sites is a desirable characteristic in a true antimetastatic drug (51). Therefore, CCR5 antagonists may be useful as adjuvant therapy for breast basal tumors with CCR5 overexpression or other tumor types where CCR5 promotes metastasis, such as prostate cancer (50) or gastric cancer (52). Our preclinical studies will expedite the path toward the clinical use of CCR5 antagonists as new treatments for dissemination of basal breast cancer.

Disclosure of Potential Conflicts of Interest

R.G. Pestell has ownership interest (including patents) in Prostagene, LLC. No potential conflicts of interest were disclosed by the other authors. The Department specifically disclaims responsibility for any analysis, interpretations, or conclusions.

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CCR5 Antagonist Blocks Metastasis of Basal Breast Cancer Cells

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