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Expression of the C-C Chemokine Receptor 7 Mediates Metastasis of Breast Cancer to the Lymph Nodes in Mice[1]

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Abstract

C-C chemokine receptor 7 (CCR7) controls lymphocyte migration to secondary lymphoid organs. Although CCR7 has been implicated in targeting the metastasis of cancers to lymph nodes, the role of CCR7 in the metastasis of breast cancer, along with the molecular mechanisms that are controlled by CCR7 that target breast cancer metastasis to the lymph nodes, has yet to be defined. To explore the cellular and molecular mechanisms of breast cancer cell migration to the lymph nodes, we used the mouse MMTV-PyVmT mammary tumor cells (PyVmT) transfected with CCR7 and the human CCR7-expressing MCF10A and MCF7 mammary cell lines. We found that the CCR7 ligands CCL19 and CCL21, controlled cell migration using the β_1-integrin heterodimeric adhesion molecules. To define a physiological significance for CCR7 regulation of migration, we used the FVB syngeneic mouse model of metastatic breast cancer. When CCR7-negative PyVmT cells transfected with control vector were orthotopically transferred to the mammary fat pad of FVB mice, tumors metastasized to the lungs (10/10 mice) but not to the lymph nodes (0/10). In contrast, CCR7-expressing PyVmT (CCR7-PyVmT) cells metastasized to the lymph nodes (6/10 mice) and had a reduced rate of metastasis to the lungs (4/10 mice). CCR7-PyVmT tumors grew significantly faster than PyVmT tumors, which mirrored the growth *in vitro*, of CCR7-PyVmT, MCF7, and MCF10A mammospheres. This model provides tools for studying lymph node metastasis, CCR7 regulation of tumor cell growth, and targeting of breast cancer cells to the lymph nodes.

Translational Oncology (2010) 3, 354–361

Introduction

When a breast tumor has metastasized to more than four lymph nodes, a woman's chance of surviving breast cancer is significantly reduced. Consequently, clinical staging of breast cancer involves determining whether breast tumors have metastasized to the lymph nodes. Therefore, it is puzzling that there is a subset of women with metastasis to the lymph nodes that are long-term breast cancer survivors. Whereas few molecular mechanisms that target breast tumor metastasis to the lymph nodes have been defined, recently, it was found that C-C chemokine receptor 7 (CCR7), which responds to small (8-12 kDa) chemotactic cytokines, is expressed in breast tumor metastases [1]. In these patients, CCR7-expressing metastases were more likely to be found in surgically removable lymphoid tissue than in visceral tissues [2]. In contrast, co-expression of the epidermal growth factor receptor (EGFR) with CCR7

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with breast cancer has been linked with a poor prognosis [1]. Because an animal model of CCR7-expressing tumors, which mirrors what has been observed clinically, has yet to be developed, the roles of CCR7 in disease progression and in breast cancer metastasis to the lymphoid tissues and other organs remain controversial.

CCR7 is activated by binding to two chemokines, CCL19 and CCL21 [3,4]. Under normal circumstances, naive T cells use CCL21 to enter lymphoid tissues from the blood through high endothelial venules and into the T-cell zones before returning to circulation. T cells are activated by CCL19, which is expressed by mature dendritic cells [5]. Consequently, CCL19 and CCL21 are important for the coordination of the adaptive immune responses between dendritic cells, B cells, and T cells, and these ligands function as critical mediators of the inflammatory response. Therefore, it is likely that chemotaxis of CCR7-expressing tumors to CCL19 or CCL21 may contribute to tumor cell lymph node localization.

It is thought that metastasis results from nonrandom events where tumor cells target organs in response to unique factors found within the targeted organ [6]. These factors are thought to contribute to tumor cell adhesion, migration, organ invasion, and growth. During metastasis, a tumor cell must detach from the primary tumor, rearrange shape, and extravasate through the microvessel walls to migrate to the target tissue. CCR7 regulates T-cell migration, which makes it important to understand its role during breast tumor metastasis.

CCR7 is upregulated in certain breast cancers [7]. To understand the role of CCR7 up-regulation, we initially examined the levels of CCR7 expressed in fresh human breast cancer tissues. We adapted a mouse mammary tumor virus (MMTV)–polyoma virus middle T antigen (PyVmT) model of metastatic breast cancer to express low levels of CCR7 similar to those found in humans and to study the role of CCR7 in breast cancer progression and metastasis. In this model, PyVmT cells orthotopically transplanted to the mammary fat pads preferentially metastasize to the lungs [8]. We expressed CCR7 in PyVmT cells to allow us to determine the role for CCR7 in breast cancer progression and metastasis. *In vivo*, we found that CCR7 directs the migration of metastasis to the lymph nodes, in preference to the lung, the site where CCR7 (–) PyVmT cells metastasize. To define the mechanisms used by tumors to metastasize, we used *in vitro* studies. *In vitro*, we examined the ability of PyVmT tumor cells to grow in the presence of lung and lymph node tissues using mammosphere cultures. We observed that whereas CCR7 (–) PyVmT mammospheres grow at equivalent rates in the presence of lung and lymph nodes, CCR7-expressing PyVmT mammospheres grow preferentially in the presence of lymph node tissues. To identify the mechanisms used by CCR7-expressing PyVmT to grow in the lymph nodes, we examined the role of CCR7 ligands in controlling cell spread and proliferation, the essential steps in cell migration and survival. We found that similar to its role in lymphocytes, CCR7 promotes cell spread and migration of PyVmT cells transfected with CCR7 as well as in CCR7(+) human breast cancer cell lines. In addition, we found that, in both *in vivo* and in three-dimensional *in vitro* culture, CCR7-expressing tumors grow more rapidly in the presence of CCL19 or CCL21 compared with untreated controls. Taken together, these studies define a new model that can be used to study lymph node metastasis and describe chemokine receptor–regulated mechanisms used by breast cancer cells to successfully metastasize to the lymph nodes.

Materials and Methods

Chemicals and Reagents

Chemicals and reagents were purchased unless otherwise specified. The following antibodies were purchased: M2 (anti-FLAG; Sigma, St Louis, MO), fluorescein-conjugated antimouse (Jackson Immunoresearch, West Grove, PA), phycoerythrin-conjugated anti–β_1-integrin (R&D Systems, Minneapolis, MN), and fluorescein isothiocyanate–conjugated anti-CCR7 (R&D Systems). The murine CCR7 complementary DNA (cDNA) was a generous gift from Dr James Campbell (Harvard Medical School, Boston, MA), and the human CCR7 cDNA was from Dr Robert Hromas (University of New Mexico [UNM] Cancer Center, Albuquerque, NM). pcDNA3.1 (Invitrogen, Carlsbad, CA) or pEGFP-N-1 (Clontech, Mountain View) control vectors, fluorescein-conjugated donkey antimouse (Jackson Immunoresearch), and fibronectin (Sigma) were purchased.

Human Breast Cancer Tissues

Human breast cancer tissues were collected and analyzed at the UNM with informed consent. Briefly, primary tumors were isolated, from excess nondiagnostic tissue after either lumpectomy or mastectomy. The tissue was resected under the supervision of UNM physician, Donald Morris, MD, and separated into normal breast or malignant tissue under the supervision of UNM pathologist, Nancy Joste, MD. The tissues were prepared as described previously [9]. Briefly, minced tissues were incubated for 16 hours at 37°C, in 0.05% rat tail collagenase A, and large cell clumps were removed by allowing the cells to settle. Selection of both normal epithelial and malignant epithelial cells was by a Miltenyi (Miltenyi Biotech, Gladbach, Germany) magnetic separator using the anti–epithelial membrane antigen (Clone 29; Miltenyi Biotech) antibody as the selection marker. Cells were divided into two groups, stained with PE-conjugated anti-CCR7 (R&D Systems) or isotype control (R&D Systems), and analyzed by flow cytometry.

Cell Lines, Mice, and In Vivo *Manipulations*

PyVmT cells were generated as described [8]. MCF10A and MCF7 were purchased from ATCC (Manassas, VA). FVB/N mice were purchased from Jackson Laboratories (Bar Harbor, ME). PyVmT cells were transiently transfected using Lipofectamine Plus (Invitrogen) as per the manufacturer's instructions, with the following modifications: For adhesion assays and *in vivo* injections, cells were plated at 80% density and transfected 16 to 24 hours later with twice the suggested amount of DNA in serum-free Dulbecco modified Eagle medium (DMEM). Cells were incubated in the presence of DNA/Lipofectamine complexes for 4 hours. Complexes were removed, cells were rinsed in prewarmed PBS, and medium was adjusted to 10% fetal bovine serum, and the cells were allowed to recover overnight. In the morning, fresh medium was added. Cells were transfected each evening on three consecutive days. After the final transfection, cells were trypsinized and plated on Petri dishes overnight at 37°C in a 5% CO_2 humidified atmosphere in complete DMEM (10% FBS/90% DMEM/2 mM L-glutamine). Cells were isolated with light trypsin, neutralized with cDMEM, and injected into the left thoracic mammary fat pad of FVB/N mice (Jackson Laboratories). Animals were palpated for tumors starting on day 21, and length, width, and height were measured with calipers every other day until the tumors reached 18 mm in any measurement. At that point, the animals were killed, and the tumors and lungs were collected and processed for histology or polymerase chain reaction (PCR).

Flow Cytometry

Transfected PyVmT cells were dissociated from tissue culture with cell dissociation solution (Sigma), rinsed three times with PBS, and labeled with anti-FLAG (M2) antibody (1:1000) for 1 hour on ice in 1% BSA/PBS. Cells were rinsed three times in 1% BSA/PBS and labeled

with fluorescein-conjugated donkey antimouse (1:200) for 1 hour on ice 1% BSA/PBS. Cells were rinsed twice in 1% BSA/PBS, resuspended in 200 μl of 1% BSA/PBS, and assayed immediately by flow cytometry (FACSCalibur; BD Bioscience, San Jose, CA).

Detection of PyVmT Expression in Lungs and Lymph Nodes

Histologic slides were blinded, examined, and scored for the presence of metastasis by two individuals. After physical examination and isolation of the lymph nodes, the DNA was extracted using a Qiagen Tissue Isolation Kit. DNA was quantified; equal amounts were used to amplify PyVmT DNA as described [8].

Chemotaxis Assay

MCF10A, MCF7, and transfected PyVmT cells were dissociated from tissue culture with cell dissociation solution, rinsed with PBS, and counted. Fifty thousand cells were resuspended in serum-free medium added in 50 μl to the top well of a chemotaxis chamber (Neuroprobe, Gaithersburg, MD) that had been preloaded with a 100-ng/ml fibronectin-coated 5-μm membrane and ligand in each lower well or serum-free medium as the control. Cells were allowed to migrate for 3 hours. At the end of the assay, the medium in the top wells was removed, the chamber was disassembled, and the cells in the lower wells were counted on a hemocytometer.

Cell Spreading Assay

Cell spreading assay was as described [10]. Briefly, PyVmT cells were transfected 72 hours before assay with Lipofectamine Plus. Cells were seeded in six-well dishes and transfected with either FLAG-muCCR7, pEGFP (control to monitor transfection efficiency), or pcDNA3.1 at 2 μg of DNA per well in serum-free DMEM. At 3 hours, the medium was adjusted to 10% FBS, and the cells were incubated overnight. The following day, cells were rinsed in 1× Ca^{+2}/Mg^{+2} free PBS and allowed to grow for 24 hours. For MCF10A, or MCF7 cells were passaged at least 3 days before assay and were not allowed to grow to greater than 80% confluency. On the day of the assay, the medium was aspirated, and the cells were rinsed in Ca^{+2}/Mg^{+2} free PBS, incubated in a cell dissociation solution (Sigma), and once the cells started to detach, the flask was rapped, and the cells were removed by pipetting. The cells were rinsed in Ca^{+2}/Mg^{+2} free PBS three times and resuspended in serum-free DMEM + 200 mM L-glutamine. A total of 3×10^4 cells were centrifuged at 25°C at 3000 rpm onto 13-mm coverslips that had been preincubated with 10 μg/ml fibronectin/0.05% BSA and allowed to spread for 4.5 hours. Cells were then fixed in 1% paraformaldehyde/PBS. Cells were imaged using Metamorph (Molecular Devices, Dowington, PA), and cell spread was measured. A minimum of 100 cells was counted per condition.

Mammosphere Cultures

Mammosphere cultures were generated as described [11]. Six million exponentially growing PyVmT cells were isolated by trypsinization, neutralized with complete media, and rinsed twice in PBS and electroporated using a Bio-Rad electroporator according to the manufacturer's protocol (Bio-Rad Bulletin 1349; Bio-Rad, Hercules, CA) and allowed to recover overnight. In some cases, cells were transfected with LipoD293 (Signajen, Ijamsville, MD). Transfected cells were isolated by trypsinization and rinsed twice in PBS to remove all traces of serum. For cocultures with primary cells, we used PyVmT-GFP that had been stably transduced with a GFP-expressing lentivirus, pGIPZ (Thermo Scientific, Huntsville, AL). PyVmT-GFP were then transfected with CCR7 (PyVmT-GFP-CCR7) or vector (PyVmT-GFP-pcDNA3.1) and mixed at a 1:1 ratio with primary subinguinal lymph node or lung cells, which had been isolated from FVB mice. Lungs were minced with fine scissors. Minced lung tissues and lymph nodes were dissociated by passing through a wire mesh. Red blood cells were lysed with ACK buffer (155 mM NH_4Cl/7.2 mM $KHCO_3$/4.8 mM EDTA) for 8 minutes at room temperature. Cells were rinsed twice with PBS and counted. Single cells were plated on ultra low-attachment six-well plates (Corning, Inc, Corning, NY) at a density of 30,000 viable cells per well. Cells were grown in serum-free mammary epithelial basal medium (Lonza, Walkersville, MD) supplemented with 20 ng/ml EGF, 5 μg/ml insulin, 1 μg/ml hydrocortisone (Lonza), 20 ng/ml basic fibroblast growth factor, B27 (Invitrogen), 4 μg/ml heparin (MP Biomedicals, Irvine, CA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Manassas, VA). Cells were fed every 3 days by addition of one-third volume of fresh medium. Seven days after the initial plating, primary mammospheres were measured using Metamorph. Significance of difference between PyVmT-CCR7 and PyVmT-vector control cells in the presence or absence of ligand was determined using an unpaired *t* test with the Welch correction. To assess integrin involvement, cells were grown in the presence of 2 μg/ml anti–β_1-integrin or isotype control antibodies.

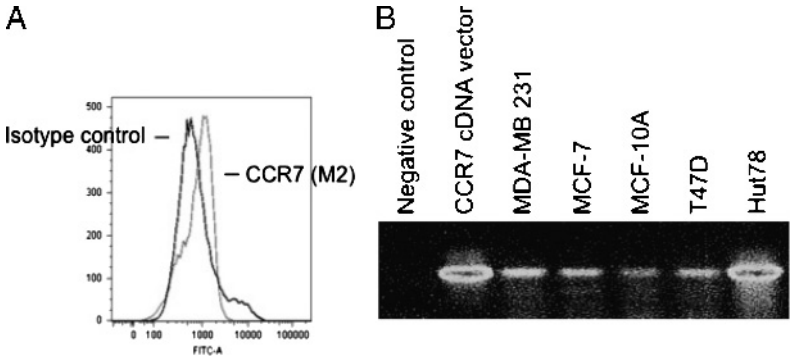

Figure 1. Transient transfection of PyVmT with N-FLAG-CCR7 results in low level of CCR7 expression. (A) PyVmT were transiently transfected with an N-terminally tagged murine CCR7, dissociated from culture, stained for expression of FLAG with an M2 antibody, counterstained with fluorescein isothiocyanate–conjugated donkey antimouse, and assayed on a FACSCalibur. (B) Human CCR7 cDNA and cDNA isolated from MDA-MB-231, MCF7, MCF10A, and T47, and human breast tumor cells and Hut78 human T cells were generated and amplified by RT-PCR.

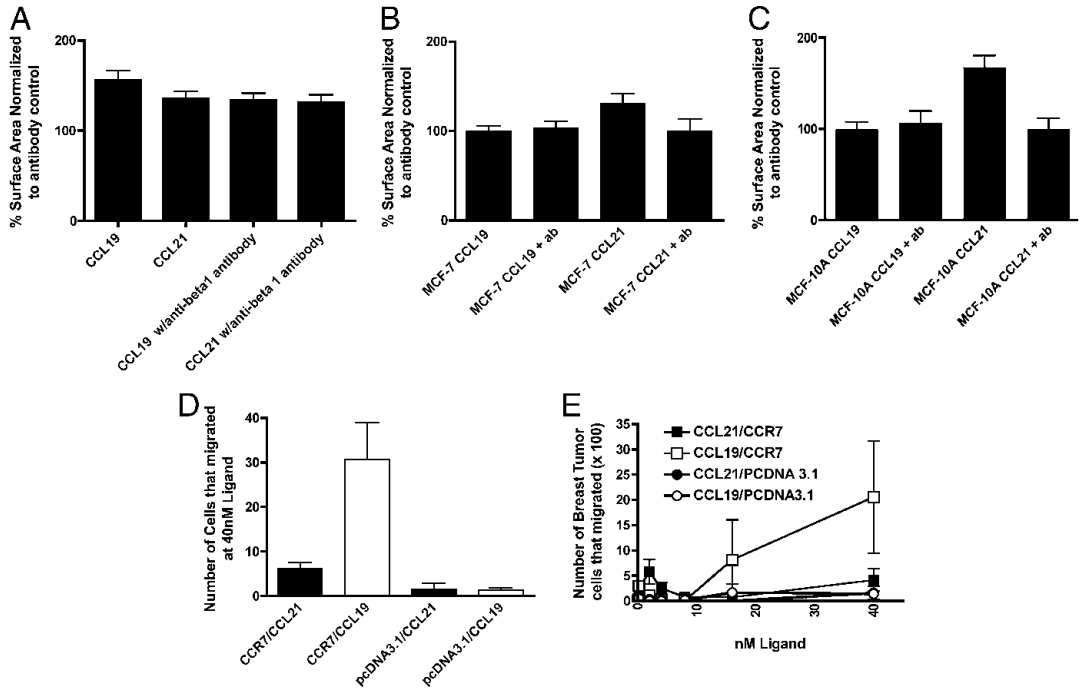

Figure 2. CCR7 promotes cell spreading and migration of tumor cell lines. Glass coverslips were coated with 10 μg/ml 0.05% BSA fibronectin overnight. Then, 400 nM of CCL21 or CCL19 was layered on coverslips in 100 μl of serum-free media. A total of 5×10^4 (A) PyVmT-CCR7, (B) MCF7, or (C) MCF10A cells in 100 μl of serum-free medium were plated on each coverslip and allowed to spread for 4.5 hours. Cells were fixed, and surface areas were measured using Metamorph. Surface areas were normalized to the area of cells that had been blocked with anti–β_1-integrin antibodies and were therefore round. (D) PyVmT transfectants were allowed to migrate on fibronectin-coated 8-μm pore membranes (10 μg/ml) for 3 hours to gradients of 40 nM CCL19 or CCL21. After 3 hours, migrated cells were counted ($n = 3$). (E) PyVmT transfectants were allowed to migrate to 2, 20, 40, 80, 160, and 400 nM ligand for 3 hours through 8-μm pores, through fibronectin (10 μg/ml)-coated membranes. After 3 hours, cells that had migrated to the bottom well of the chemotaxis chamber were counted ($n = 3$).

Results

CCR7 Is Expressed at Low Levels in Primary Human Breast Cancers

CCR7 expression has been detected in fixed tumors [2,7,12–14]. To examine the levels of CCR7 expression on the surface of fresh human breast cancer cells, we first isolated epithelial cells from infiltrating ductal carcinoma human breast tumor biopsy samples or from the adjacent normal tissue. We labeled the isolated cells with anti-CCR7 antibodies (n = 6) and measured levels of CCR7 by flow cytometry. To this end, primary epithelial cells, dissociated with collagenase were isolated by positive selection using magnetic beads that target epithelial cell markers. We found CCR7 elevated in three of the six patients. Overall, there was a 46% ± 24% increase in the level of the CCR7 receptor expressed in malignant breast epithelia compared with that in the normal tissue. Although this is a relatively low level of expression, this increase in receptor expression levels can profoundly affect the cellular response to ligand binding [15].

We hypothesized that low levels of CCR7 can be used by breast cancers to metastasize to the lymph nodes to regulate cell spread, a cellular function that mediates extravasation and migration. Using reverse transcription (RT)–PCR, we determined that CCR7 is not expressed on PyVmT cells (data not shown). Therefore, to examine the roles of CCR7 in metastasis, we expressed CCR7 in PyVmT cells [8]. We used a FLAG-tagged murine CCR7 to generate N-FLAG-muCCR7 expressing PyVmT (PyVmT-CCR7) transient transfectants. These cells expressed a modest level of CCR7 that was similar to levels that we observed in fresh tumor isolates from human breast cancer patients (Figure 1). We used RT-PCR to confirm the expression of CCR7 in a number of cultured human breast tumor cell lines (Figure 1).

CCR7 Regulation of β_1-Integrins

Chemokines control integrin-mediated adhesion and cell spreading [10]. Whereas adhesion promotes attachment, cell spreading is thought to be an early step in migration. Adhesion of tumor cells through β_1-integrins promotes tumor metastasis [16]. To determine if CCR7 expression affects cell spreading through β_1-integrins, we used *in vitro* assays. Cover slips were coated with the β_1-integrin ligand fibronectin at 10 μg/ml, and cells were allowed to adhere and spread for an hour. To confirm specificity for the β_1-integrins, we used a function blocking β_1-integrin antibody. Activation of CCR7 on PyVmT-CCR7 promoted a significant increase in β_1-integrin–specific cell spreading (Figure 2*A*) to either CCL19 or CCL21. We have used flow cytometry to confirm that MCF10A and MCF7 cells express human CCR7 ([7] and data not shown). To determine whether CCR7 regulated cell spreading in human cells as well, we used the MCF10A mammary cell line along with the MCF7 human breast cancer cell line. We observed that CCR7 activation

Table 1. Average Growth of Tumors *In Vivo*.

No. Cells Implanted	Mean Days to Develop Tumor	No. Animals with Tumors
1,000,000	24	6/6
100,000	24	6/6
10,000	29	2/6

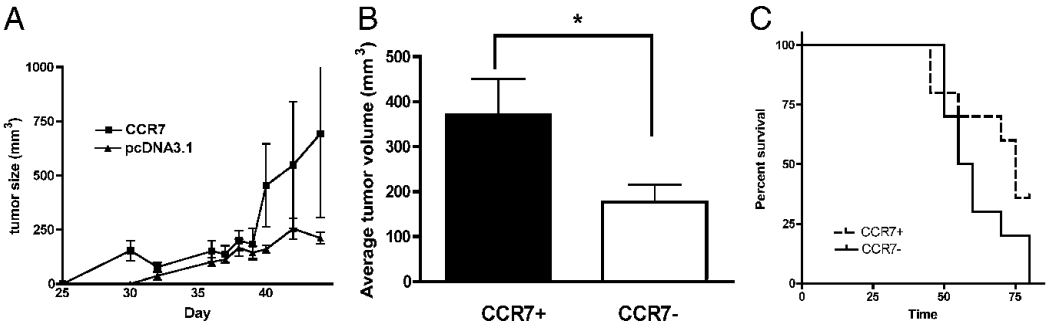

Figure 3. PyVmT-CCR7 tumor volumes are significantly larger than tumors derived from PyVmT-vector control cells. (A) A total of 10^5 cells were injected into the fifth mammary fat pad of FVB mice. Palpable tumors were detected as early as day 25 in both groups. Tumors growth as measured with calipers (length × width × height). (B) At day 48, the average tumor volume of PyVmT-CCR7 tumors (259.9 ± 71.22) was significantly larger than the PyVmT-vector controls (119.1 ± 27.30), Student *t* test, $P = .002$. (C) Survival curve for mice injected with PyVmT-CCR7 tumors (CCR7+) *versus* PyVmT-vector controls.

promoted cell spreading in human cell lines as well (Figure 2, *B* and *C*) when compared with cells treated with antibodies against the β_1-integrin.

After detachment from the primary tumor, an essential step in metastasis is migration of cells to a second site. To determine whether CCR7 can promote migration, we used the PyVmT-CCR7, which was used in our mouse model in an *in vitro* transwell chemotaxis assay on β_1-integrin–coated surfaces. In these assays, cells were detached from culture with a nonenzymatic cell dissociation solution and allowed to migrate for 3 hours to CCL19 or CCL21 through fibronectin-coated polycarbonate membranes. We found that CCR7 promoted migration to CCL19 and CCL21 in PyVmT-CCR7 (Figure 2, *D* and *E*). From these results, we conclude that CCR7 can promote directed migration of breast cancer cells to lymphoid organ chemokines.

Development of a Mouse Model

To examine the physiological significance of CCR7 in cell spreading and migration of breast cancer to CCR7 ligands, we used the PyVmT model [8] that had been selected for metastasis preferentially to the lungs. Previous studies, which examined the role of a different chemokine receptor, CXCR4, in metastatic behavior of tumor cells, revealed that differences were observed between CXCR4-expressing tumors and vector controls only when low levels of tumor cells were seeded [17]. Therefore, to determine the lowest number of PyVmT cells that could be used to generate tumors in the syngeneic FVB mice, we compared the numbers of tumors generated after the introduction of 10^6, 10^5, and 10^4 tumor cells. In our studies, 100% (6/6) of the animals with 10^6 tumor cells developed palpable tumors within 24 days, 100% (6/6) of the animals with 10^5 tumor cells developed tumors within 50 days, and 33% (2/6) of the mice developed tumors when 10^4 cells were implanted (Table 1).

We injected 10^5 PyVmT-CCR7 or 10^5 PyVmT-vector (CCR7 (–)) tumor cells into the fifth mammary fat pad of FVB mice. To obtain statistically significant data, we used 10 mice per group (Figure 3). Volumes of the PyVmT-CCR7 tumors were significantly greater than the PyVmT-vector controls during the study (Figure 3). On day 48 after implantation, tumors in two of the PyVmT-CCR7 mice grew to sizes greater than 18 mm in a single dimension, our criterion for euthanasia, and were immediately removed from the study. There was no significant difference in the mean survival ($P = .0842$) of the mice injected with PyVmT-CCR7 cells (75 days) when compared with the mice injected with PyVmT-vector cells (58 days; Figure 3).

To determine whether CCR7 altered the metastatic behavior of the tumors, we used PCR of isolated DNA to determine whether PyVmT cells were present in the lymph nodes. To this end, we isolated the draining lymph node and used half of the lymph node to isolate DNA for PCR and used the other half for histologic preparations. By PCR, we found that 60% of the PyVmT-CCR7 animals had PyVmT DNA in the draining lymph nodes, contrasting with 0% of the PyVmT-vector controls. In addition, we found that while 4 of the 10 PyVmT-CCR7 animals developed metastasis in their lungs, 100% (10/10) of the PyVmT-vector control mice had between one and four metastases in their lungs (Table 2). We found that there were significantly more tumors per lung in the mice that had been injected with PyVmT-vector control cells when compared with animals injected with PyVmT-CCR7 cells (*t* test, $P = .0366$). Histologic sections were made, and there were no significant differences in the appearance of the lymph nodes between the two groups of animals (Figure 4).

To determine the contribution of CCL19 and CCL21 to differences in growth between PyVmT-CCR7 and PyVmT-vector control animals, we used measured growth in tissue culture. We found that, when cells were grown in tissue culture, there was no statistically significant difference in the rates of proliferation of CCR7 expressing cells (Figure 5). Breast cancer cells can grow in three dimensions as mammospheres [18]. To determine whether CCR7 promoted growth under conditions that allow three-dimensional proliferation of PyVmT, we used a mammosphere assay. To this end, we grew PyVmT-CCR7, PyVmT-vector, ± (CCL21), or (CCL19) in three dimensions [11]. Although we observed a dose-dependent response on the size of the mammospheres grown in the presence of ligand when compared with the PyVmT-vector controls, which mirrored the increased growth rate of the PyVmT-CCR7 that we observed in our FVB mice *in vivo* (Figure 5 and Table 2), we found no significant difference in the number of mammospheres generated. Because growth in the presence of CCL19 was greater than with CCL21, we examined the growth of MCF7 and MCF10A cells in response to increasing concentrations of CCL19. We observed that CCL19 promoted

Table 2. Metastatic Targets of PyVmT Tumors *In Vivo*.

Observed Differences in Mice Injected Orthotopically with PyVmT-CCR7 or PyVmT-Vector Control Cells		
Cell Line	Animals with Tumors in Lung (Average Number of Tumors)	Presence of PyVmT in Lymph Node by PCR
PyVmT-CCR7	4/10	6/10
PyVmT-vector	10/10	0/10

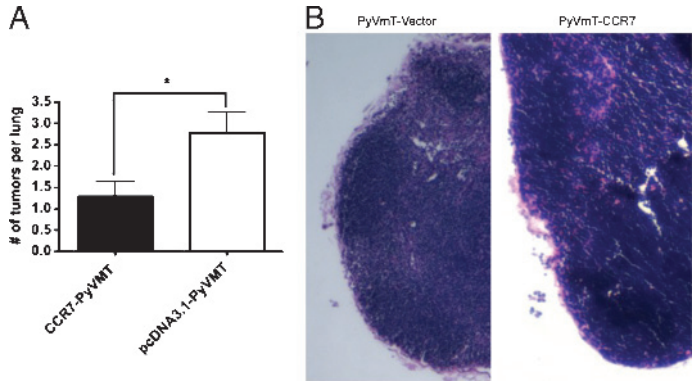

Figure 4. Significantly more PyVmT tumors grew within the lungs when compared with PyVmT-CCR7, but there was no difference in lymph node morphology. (A) A total of 10^5 cells were injected into the fifth mammary fat pad of FVB mice. Once tumors reached 18 mm in any dimension, the animals were killed, the lungs were fixed in buffered formalin, and tumors were enumerated. (B) Draining lymph nodes were removed, fixed in buffered formalin, and cut in half and used to generate DNA for PCR or stained with hematoxylin and eosin.

growth of all three cell lines. From these studies, we conclude that CCR7 can regulate the growth of tumor cells.

CCR7-expressing cells may show preferential migration to or preferential proliferation within lymph nodes. Although PyVmT cells migrate to CCR7 ligands, it was important to determine whether PyVmT-CCR7 cells grow more efficiently in the presence of lymph node cells than lung cells. To this end, we used mammosphere assays. To be able to discriminate between PyVmT cells and primary lung or lymph node tissues, we used PyVmT that had been stably transduced with GFP (Figure 6). PyVmT-CCR7 or PyVmT-vector controls were grown in the presence of equal ratios (1:1) of lymph node or lung cells. The presence of primary cells from either the lung or the lymph nodes had no effect on the proliferation of PyVmT-vector cells (Figure 7). In contrast, whereas PyVmT-CCR7 were able to grow in the presence of lymph node tissues, there was a significant (P = .0036) reduction in the average size of PyVmT-CCR7 mammospheres formed in the presence of lung tissue. We conclude that when PyVmT express CCR7, the cells grow less efficiently in the presence of lung tissues, where CCR7 ligands are scarce.

Because CCR7 promotes activation of β_1-integrins and integrins are important ligands in mediating entry into lymph nodes, we examined the effects of inhibiting β_1-integrins on three-dimensional culture proliferation (Figure 8). We observed that in the presence of β_1-integrin function–blocking antibodies and CCL19, cells proliferated at the same rate as the controls that were not grown in the presence of CCL19. There was no significant reduction in the growth rate of three-dimensional cultures in the presence of isotype antibodies, when compared with untreated controls. We conclude that PyVmT-CCR7 grows more efficiently when allowed to adhere and grow in the presence of CCR7 ligands.

Discussion

We have established a CCR7-expressing mouse model of breast cancer metastasis that allowed us to examine the roles of CCR7 in breast cancer progression and metastasis both *in vivo* and *in vitro*. Similar to what has been observed in cancer patients, CCR7 can control targeting of tumor metastases *in vivo*. In addition, we found that CCR7 functions through β_1-integrins to induce cell spread and migration in both human and murine breast cancers. In this way, CCR7 directs PyVmT-CCR7 cells to the lymph nodes *in vivo*, whereas CCR7-expressing tumors are inhibited in generating lung metastasis.

In clinical studies, the spread of CCR7-expressing breast tumors and bone metastasis was restricted to the surgically removable lymphoid

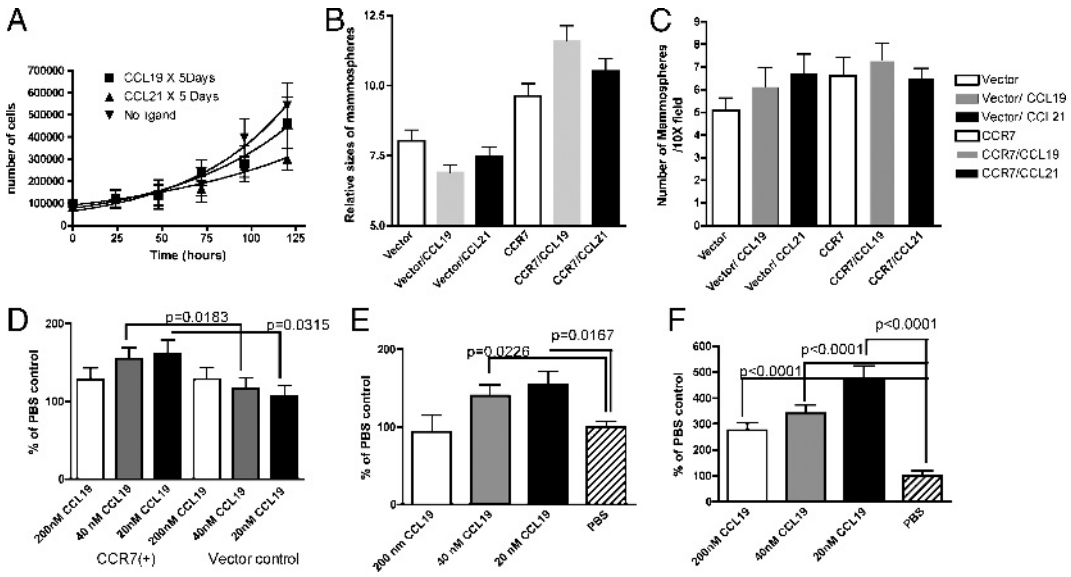

Figure 5. Stimulation of CCR7 does not affect proliferation of PyVmT tumors in two-dimensional tissue but controls growth in three-dimensional tissue. (A) A total of 10^5 PyVmT-CCR7 cells were allowed to grow ± 40 nM ligand on tissue culture plates for the indicated periods. Cells were trypsinized and counted in duplicate. Results are means ± SD (n = 3). (B) Mean size of PyVmT mammospheres in 40 nM ligand. (C) Mean number of PyVmT mammospheres in 40 nM ligand per 10× field. (D) PyVmT, (E) MCF10A, or (F) MCF7 volume of mammospheres relative to PBS controls at the indicated concentrations of CCL19 ligand. Mammospheres were imaged by Metamorph, and sizes were measured or numbers of mammospheres were counted (n = 5).

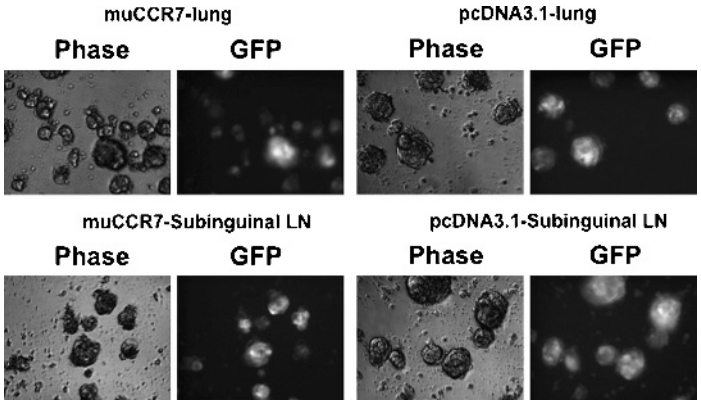

Figure 6. PyVmT-GFP-CCR7 mammospheres grow at different rates in the presence of primary lung and lymph node cells. PyVmT-GFP cells were transiently transfected with CCR7 or pcDNA3.1 and mixed at a 1:1 ratio with subinguinal lymph node or lung cells. Cells were allowed to form mammospheres on low adhesion plates for 7 days, fixed in 1% paraformaldehyde, and then imaged and measured using Metamorph.

organs [14]. Without an animal model, it has remained difficult to identify the cellular mechanisms that result in targeting metastasis from the mammary fat pad to the lymph nodes. From our studies, we find that CCR7 expression leads to increased β_1-integrin–mediated tumor growth and promotes tumor migration to the lymph nodes. We found that both PyVmT-CCR7 and the CCR7-expressing breast cancer cell line MCF7 grew significantly larger mammospheres compared with vector controls. Proliferation of MCF7 and MCF10A increased in a dose-dependent manner in the presence of CCL19. Importantly, we found that in the lung, PyVmT grew more efficiently in the absence of CCR7. It is unclear to what extent CCR7 prevents cell death or promotes proliferation, although recently, it has been shown that CCR7 prevents anoikis of MDA-MB-231, MDA-MB-361, and MDA-MB-453 cancer cells; mediates activation of mitogen-activated protein kinases [19,20]; promotes [21] proliferation of B-cell lymphocytic leukemias; and blocks apoptosis in hematopoietic cells [22–24]. Whereas CCR7 had been shown to prevent apoptosis before our study [24], it had not been implicated in enhancing breast tumor growth or regulating breast tumor proliferation in a tissue-specific manner. Although we found that PyVmT-CCR7 mammospheres grew at different rates than vector controls, it will be important to use these mammospheres in future biochemical assays to further our understanding of the mechanisms used by CCR7 in breast cancer cells to control the extent of tumor growth in different tissues in the body.

Activation of β_1-integrins in breast tumor cells is linked to increased proliferation [16]. Although CCR7 has been found to regulate lymphocyte-specific integrins such as LFA-1 in T cells because breast cancer tumor cells do not express the LFA-1, it was unclear to what extent CCR7 could regulate the β_1-integrins. We found that in CCR7-expressing PyVmT, CCL19 and CCL21 promote β_1-integrin–mediated cell spread, migration, and proliferation in both human and murine cell cultures. However, human and murine tumors migrate differently on fibronectin-coated membranes to the two CCR7 ligands. Whereas PyVmT-CCR7 migrates preferentially to CCL19, MCF10A and MCF7 migrate to CCL21. We and others have shown that T cells differentially regulate desensitization of CCR7 [25,26]. It is unclear if breast tumor cells respond differently to regulate signaling from CCL19 and CCL21, and our future studies will focus on understanding mechanisms used by human and mouse tumor cells to desensitize signaling through CCR7 in response to CCL21 and CCL19.

Because the PyVmT cell line used in this study metastasizes primarily to the lung [8], we hypothesized that expression of CCR7 would increase migration of breast cancer cells to the lymph nodes. Because

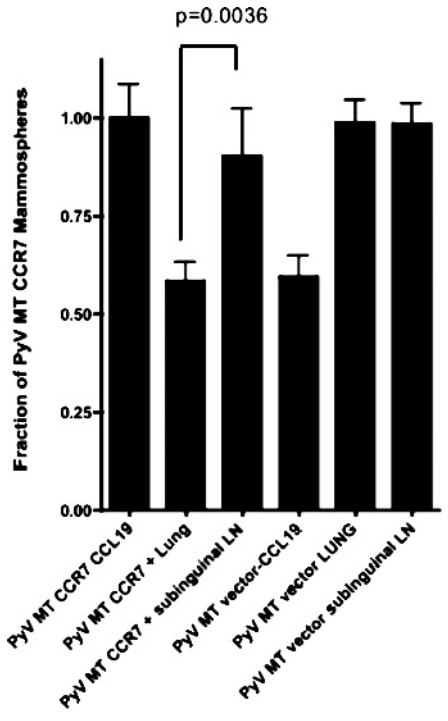

Figure 7. PyVmT-CCR7 mammospheres grow more efficiently in the presence of lymph node tissues than in the presence of lung tissues. PyVmT-GFP cells were transiently transfected with CCR7 or pcDNA3.1 and mixed at a 1:1 ratio with subinguinal lymph node or lung cells. Cells were allowed to form mammospheres on low-adhesion plates for 7 days, fixed in 1% paraformaldehyde, and then imaged and measured using Metamorph.

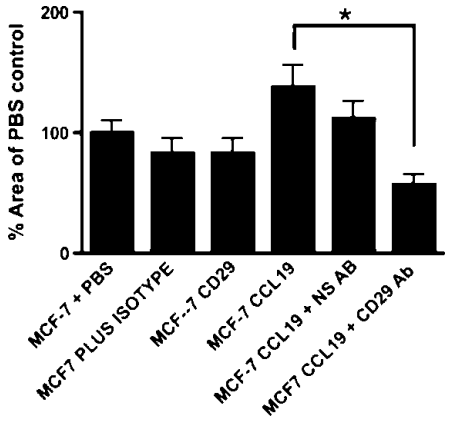

Figure 8. Adhesion through the β_1-integrins mediates proliferation of three-dimensional cultures. MCF7 were allowed to grow in low-adhesion plates at 40 nM CCL19 ligand in the presence of function-blocking β_1-integrin antibodies or isotype control. Mammospheres were imaged by Metamorph, and sizes were measured or numbers of mammospheres were counted ($n = 3$).

the presence of lymph node metastasis correlates with a poor prognosis, we expected that increased migration of breast cancer cells to lymph nodes would lead to increased migration of PyVmT-CCR7 tumors in the lungs. However, it is important to bear in mind that the function that lymph nodes play in the development of cancer is unknown, and we have considered several possibilities. If the lymph nodes serve as filters to trap escaped tumor cells, we would expect to find lymph node metastasis in both PyVmT-CCR7 and PyVmT-vector control mice. We found lymph node metastasis only in the PyVmT-CCR7 mice, which implicates CCR7 in actively directing metastasis to the lymph nodes. We also found that CCR7-expressing PyVmT cells did not grow well in the presence of lung tissue, when compared with lymph node tissue co-culture (Figures 6 and 7). In addition, we found significantly more tumors per mouse in the lungs of animals injected with PyVmT-vector (Figure 4) when compared with the animals injected with PyVmT-CCR7. We did observe that blocking β_1-integrin adhesion reduced proliferation of the human MCF7 breast cancer mammospheres (Figure 8). That CCR7 actively targets migration to and promotes proliferation of PyVmT within the lymph nodes and less efficiently to the lungs in both humans and mice will allow us to use our model to better understand the development of metastatic breast cancer to different sites in the body.

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