Involvement of chemokine receptors in breast cancer metastasis

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Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver. Tumour cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors. Here we report that the chemokine receptors CXCR4 and CCR7 are highly expressed in human breast cancer cells, malignant breast tumours and metastases. Their respective ligands CXCL12/SDF-1α and CCL21/6Ckine exhibit peak levels of expression in organs representing the first destinations of breast cancer metastasis. In breast cancer cells, signalling through CXCR4 or CCR7 mediates actin polymerization and pseudopodia formation, and subsequently induces chemotactic and invasive responses. *In vivo*, neutralizing the interactions of CXCL12/CXCR4 significantly impairs metastasis of breast cancer cells to regional lymph nodes and lung. Malignant melanoma, which has a similar metastatic pattern as breast cancer but also a high incidence of skin metastases, shows high expression levels of CCR10 in addition to CXCR4 and CCR7. Our findings indicate that chemokines and their receptors have a critical role in determining the metastatic destination of tumour cells.

Metastasis is the result of several sequential steps and represents a highly organized, non-random and organ-selective process[1]. Although a number of molecules have been implicated in the metastasis of breast cancer, the precise mechanisms determining the directional migration and invasion of tumour cells into specific organs remain to be established[1–3].

Chemokines are a superfamily of small, cytokine-like proteins that induce, through their interaction with G-protein-coupled receptors, cytoskeletal rearrangement, firm adhesion to endothelial cells and directional migration[4–6]. These secreted proteins act in a coordinated fashion with cell-surface proteins, including integrins, to direct the specific homing of various subsets of haematopoietic cells to specific anatomical sites[4–10].

We therefore thought that tumour cells may use chemokine-mediated mechanisms such as those regulating leukocyte trafficking during the process of metastasis. Here we report that tumour cells express a distinct, non-random pattern of functionally active chemokine receptors. Signalling through CXCR4 or CCR7 mediates actin polymerization and pseudopodia formation in breast cancer cells, and induces chemotactic and invasive responses. In addition, we find that organs representing the main sites of breast cancer metastasis are the most abundant sources of ligands for these tumour-associated receptors. *In vivo,* neutralizing the interactions of CXCL12/CXCR4 leads to a significant inhibition of lymph-node and lung metastasis.

Expression of chemokine receptors in tumour cells

To determine whether chemokine/chemokine receptor interactions are involved in the metastatic process, we performed a comprehensive, quantitative analysis of the expression of all known chemokine receptors (CCR1–CCR10, CXCR1–CXCR5, XCR1 and CX_3CR1) in seven human breast cancer cell lines, and compared expression in normal primary mammary epithelial cells. Absolute messenger RNA levels determined using real-time quantitative polymerase chain reaction (PCR) showed that breast cancer cells express chemokine receptors in a defined rather than a random manner (Fig. 1a).

Three different patterns of receptor expression were observed. First, the chemokine receptor was expressed in normal mammary epithelial cells, but uniformly downregulated by breast cancer cells (for example, CXCR2). Second, both normal mammary epithelial cells and malignant cells expressed significant levels of receptor mRNA; this pattern was detected for CCR7 and CCR8, with CCR7 showing the most consistent upregulation in breast cancer cells. Third, receptor expression was undetectable in normal mammary epithelial cells but markedly upregulated in breast cancer cells (for example, CXCR4).

Flow cytometric analyses confirmed strong cell-surface expression of CXCR4 on breast cancer cell lines (data not shown), and on primary breast cancer cells (82.33–97.98% CXCR4-positive malignant cells) recovered from patients with malignant pleural effusion ($n = 5$) (see Supplementary Information).

We quantitatively measured CCR7 and CXCR4 mRNA expression in primary tumours of human invasive lobular or ductal breast carcinoma ($n = 12$), and in normal mammary gland ($n = 5$) and its cellular constituents (Fig. 2a, b). According to the TNM classification[11], six tumours were classified as T_2, four as T_3 and two as T_4, with evidence of axillary nodal metastasis in five cases. Figure 2a and b shows that mRNA of both CCR7 and CXCR4 was significantly upregulated in primary breast tumours compared with normal mammary gland tissue ($P < 0.05$, $P < 0.005$, respectively). Normal primary mammary epithelial and stromal cells derived from three different donors did not express detectable levels of CXCR4 mRNA. There was no significant correlation between the TNM status of the 12 patients studied and absolute levels of CCR7 and CXCR4 mRNA expression in primary tumours.

We confirmed *in vivo* protein expression of CXCR4 by immunohistochemistry (Fig. 2c–n). Stained tissue sections from normal mammary gland ($n = 4$) (Fig. 2c–e) or invasive ductal carcinoma ($n = 8$) (Fig. 2f–k) indicated that breast cancer cells express high levels of CXCR4, but normal mammary ductal cells do not express this receptor. In addition to primary tumours, axillary lymph-node metastases ($n = 5$) (Fig. 2l–n) and distant metastases of lung and liver ($n = 8$) (data not shown) exhibited strong CXCR4 expression

in tumour cells.

Although CXCL12/SDF-1 mRNA has been reported to be expressed in many different tissues[12], quantitative analysis of CXCL12 expression in many human organs revealed that CXCL12 mRNA is expressed preferentially in lymph nodes, lung, liver and bone marrow, and shows markedly lower expression in small intestine, kidney, skin, brain and skeletal muscle (Fig. 2o). Organs exhibiting the highest CXCL12 expression represent the most common sites of metastasis in breast cancer[13].

In addition, both CCL21/6Ckine and CCL19/MIP-3β had showed most abundant expression in lymph nodes; however, CCL21 was expressed at higher levels, supporting its critical role in the homing of cells into lymph nodes[14,15] (Fig. 2p; and see Supplementary Information). Comparing CXCL12 and CCL21 expression in primary breast tumours and breast cancer cell lines with that in normal human organs showed that tumour cells express low or undetectable amounts of mRNA of these particular chemokines (see Supplementary Information).

To determine whether the distinct expression of chemokine receptors is unique to breast cancer, we also analysed malignant melanoma, which, like breast cancer, preferentially develops lymph-node, lung, liver and bone-marrow metastases, but also has a high frequency of skin metastases. In addition to CXCR4 and CCR7, malignant melanoma cells expressed high levels of CCR10 mRNA as compared with normal primary melanocytes (Fig. 1b). Notably, the CCR10 ligand CCL27/CTACK represents a skin-specific homeostatic chemokine (Fig. 2q) that has been associated with the homing of memory T cells into the skin[7,8].

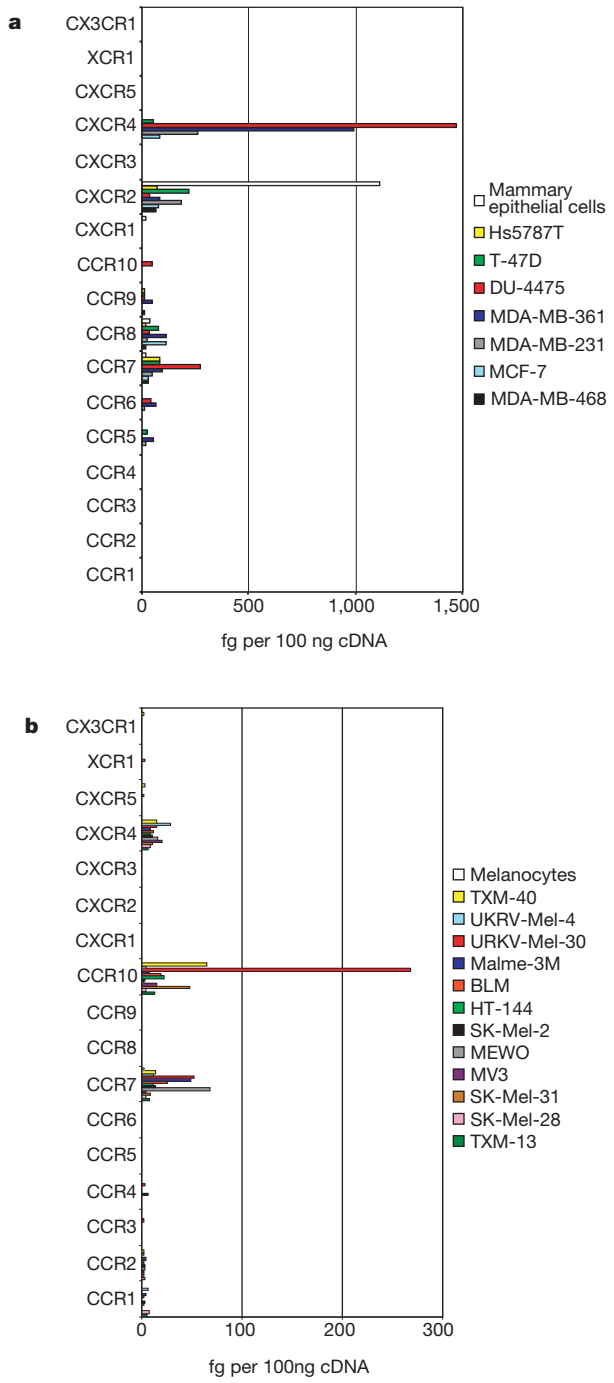

Figure 1 Expression of chemokine receptors in human tumour cells. Quantitative RT–PCR analyses of all known chemokine receptors in seven human breast cancer cell lines compared with normal primary mammary epithelial cells (**a**), and in 12 human malignant melanoma cell lines compared with normal primary melanocytes (**b**). Values are expressed as femtograms of target gene in 100 ng of total cDNA.

Breast cancer cells express active CXCR4 and CCR7

In vitro, chemokine ligand–receptor interactions trigger intracellular actin polymerization in leukocytes, a process that is prerequisite for cell motility and migration[16,17]. Consistent with findings in leukocytes[16], CXCL12 (100 nM) and CCL21 (100 nM) induced, respectively, a transient 2.2- and 1.6-fold increase in intracellular filamentous actin (F-actin) in human breast cancer cells within 20 s (Fig. 3a). Conversely, the chemokine CX_3CL1/fractalkine, whose receptor CX_3CR1/V28 was not detected on breast cancer cells (Fig. 1a), did not induce actin polymerization (Fig. 3a).

In tumour cells, high levels of actin polymerization are required for the formation of pseudopodia, which in turn are needed for the invasion of malignant cells into tissues and for efficient metastases formation[18]. Confocal laser scan microscopy of breast cancer cells stimulated in suspension with either CXCL12 or CCL21 revealed intense F-actin staining in the periphery of the cells and a redistribution of F-actin towards a leading edge (Fig. 3b–e). In adherent breast cancer cells, distinct pseudopodia formation was observed after 20 min of stimulation with either CCL21 or CXCL12 (Fig. 3f, g).

In agreement with these findings, both CXCL12 and CCL21 induced directional migration of breast cancer cells (Fig. 4a–d) and directional invasion through a reconstituted basement membrane (Fig. 4e, f) in a dose-dependent manner. Optimal migratory/invasive responses to CXCL12 (Fig. 4a, c, e) or CCL21 (Fig. 4b, d, f) were observed at concentrations of 100 nM, or 100 and 200 nM, respectively, reminiscent of observations made with leukocytes[16,19,20]. Compared with breast cancer cells of well-characterized cell lines (MDA-MB-231, MDA-MB-361), primary tumour cells derived from a patient with malignant pleural effusion exhibited significant chemotactic responses to both CXCL12 and CCL21 (Fig. 4c, d). CXCL12- and CCL21-mediated chemotaxis and invasion could be blocked by neutralizing anti-CXCR4 or anti-CCL21 antibodies, respectively, confirming the specificity of the chemotactic response induced by these chemokines (data not shown).

Migratory response to organ-derived proteins

To determine the biological relevance of CXCL12-mediated chemotaxis in the context of all chemotactic factors present in the extracellular matrix of a particular organ, protein extracts of normal human lung, liver, skin and muscle, and conditioned media from human primary bone-marrow and lymph-node stromal cells, were tested for their chemotactic activity on breast cancer cells (Fig. 5a–f). Protein extracts of lung and liver induced chemotactic responses in breast cancer cells, indicating that extracts derived from these organs contain factors with chemotactic properties (data not shown). Blocking the CXCL12/CXCR4 interactions with neutralizing anti-CXCR4 (Fig. 5a, b) or anti-CXCL12 antibodies (see Supplementary Information) significantly impaired

these migratory responses by 63–76% and 60–62%, respectively, indicating that CXCL12 is one of the main chemotactic factors for breast cancer cells in the extracellular matrix of these organs.

Furthermore, migration of breast cancer cells in response to conditioned medium from human bone-marrow (Fig. 5c) or lymph-node (Fig. 5d) stromal cells was significantly reduced (53–61% and 51–54%, respectively) after CXCR4 blocking. In contrast, protein extracts from organs that represent rare targets of breast cancer metastasis, such as skin (Fig. 5e) or muscle (Fig. 5f), exhibited weak overall chemoattractive properties for breast cancer cells, compared with protein extracts from lung and liver. Notably, these migratory responses were not affected by CXCR4 neutralization (Fig. 5e, f).

CXCR4-neutralization inhibits metastasis *in vivo*

Having established that breast cancer cells express functionally active CXCR4, we evaluated the contribution of CXCL12/CXCR4 interactions on metastasis *in vivo* by using experimental and spontaneous metastasis models of breast cancer. The CXCR4-positive, human breast carcinoma cell line MDA-MB-231 was injected either intravenously (i.v.) into the tail vein or orthotopically into the mammary fat pad of severe combined immunodeficient (SCID) mice.

In vitro, chemotaxis and invasion assays confirmed that MDA-MB-231 cells respond to murine CXCL12 (data not shown). Real-time quantitative PCR analyses showed that there was significant expression of human CXCR4 mRNA in both primary tumours and metastases-infiltrated lungs of SCID mice that had been injected with MDA-MB-231 cells (Fig. 6a). Immunohistochemical staining for human CXCR4 depicted strong protein expression by tumour cells of primary tumours (Fig. 6c, d) and lung metastases (Fig. 6e, f), indicating that MDA-MB-231 cells maintained high levels of CXCR4 expression *in vivo*. In agreement with CXCL12 mRNA expression in normal human organs (Fig. 2o), quantitative analysis

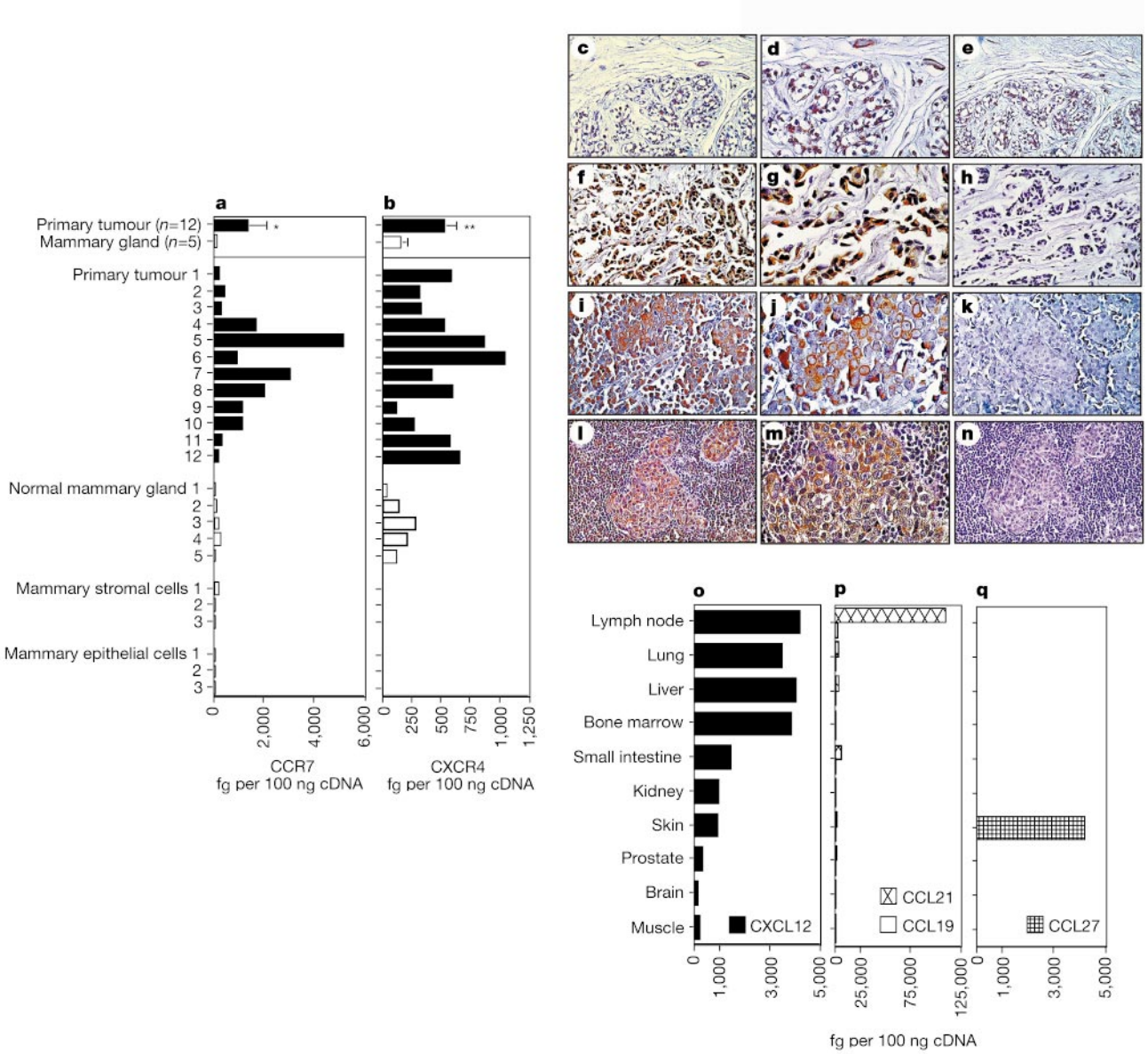

Figure 2 Expression of CCR7 and CXCR4 in breast cancer. **a**, **b**, Quantitative RT–PCR analyses of CCR7 (**a**) and CXCR4 (**b**) mRNA expression in primary breast carcinoma ($n = 12$), normal mammary gland tissue ($n = 5$), primary mammary epithelial ($n = 3$) and stromal ($n = 3$) cells. Mean ± s.e.m. (Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.005$). **c**–**n**, Immunohistochemical evaluation of CXCR4 expression. **c**–**e**, Section of normal mammary gland tissue. **f**–**h, i**–**k,** Invasive ductal carcinoma of two different patients. **l**–**n**, Axillary lymph-node metastasis. Original magnification, ×200 (**c**, **f**, **i**, **l**, anti-CXCR4; **e**, **h**, **k**, **n**, isotype); ×400 (**d**, **g**, **j**, **m**, anti-CXCR4). Quantitative RT–PCR analyses of CXCL12/SDF-1 (**o**), CCL21/6Ckine and CCL19/MIP-3β (**p**) or CCL27/CTACK (**q**) mRNA expression in various normal human organs. Values are expressed as femtograms of target gene in 100 ng of total cDNA.

of murine CXCL12 mRNA in normal organs of SCID mice showed peak levels of expression in mouse lung, lymph nodes, bone marrow and liver (data not shown).

Twenty-eight days after i.v. injection of MDA-MB-231 cells and the twice weekly treatment with either neutralizing anti-human CXCR4 monoclonal antibody or isotype control, a significant decrease in lung metastasis was observed in anti-CXCR4-treated mice (relative suppression, 61–68%; $P < 0.001$) (Fig. 7a–c, e). Furthermore, spontaneous metastasis to the lung 44 days after orthotopic injection of MDA-MB-231 cells was impaired significantly by treatment with a neutralizing anti-CXCR4 monoclonal antibody (relative suppression, 73–82%; $P < 0.001$) (Fig. 7d, f).

In addition to the histological evaluation of lung infiltrates, the individual tumour burden per lung was measured by quantitative PCR using primers specific for the human housekeeping gene *HPRT*. Expression of human *HPRT* was detectable in cultured human MDA-MB-231 breast carcinoma cells, primary tumours and tumour-infiltrated lungs of untreated SCID mice, but undetectable in normal mouse lungs (Fig. 6b). The human *HPRT* mRNA content correlated directly with the tumour load of each sample. Subsequent quantitative PCR analyses of human *HPRT* in lungs of either isotype- or anti-CXCR4-treated mice indicated a significant reduction in lung-infiltrating tumour cells after anti-CXCR4 treatment in both experimental (i.v.) and spontaneous metastasis models (relative suppression, 61–73%; $P < 0.05$) (Fig. 7e, f). Furthermore, levels of human CXCR4 mRNA in the lungs of CXCR4-treated mice showed a relative suppression, ranging from 67 to 89%, as compared with isotype-treated mice (Fig. 7g, h). Thus, quantitative PCR analyses confirmed the histological quantification.

Clinical evaluation of draining lymph nodes 44 days after orthotopic injection of MDA-MB-231 cells showed that anti-CXCR4 treatment inhibited metastasis to inguinal and axillary lymph nodes

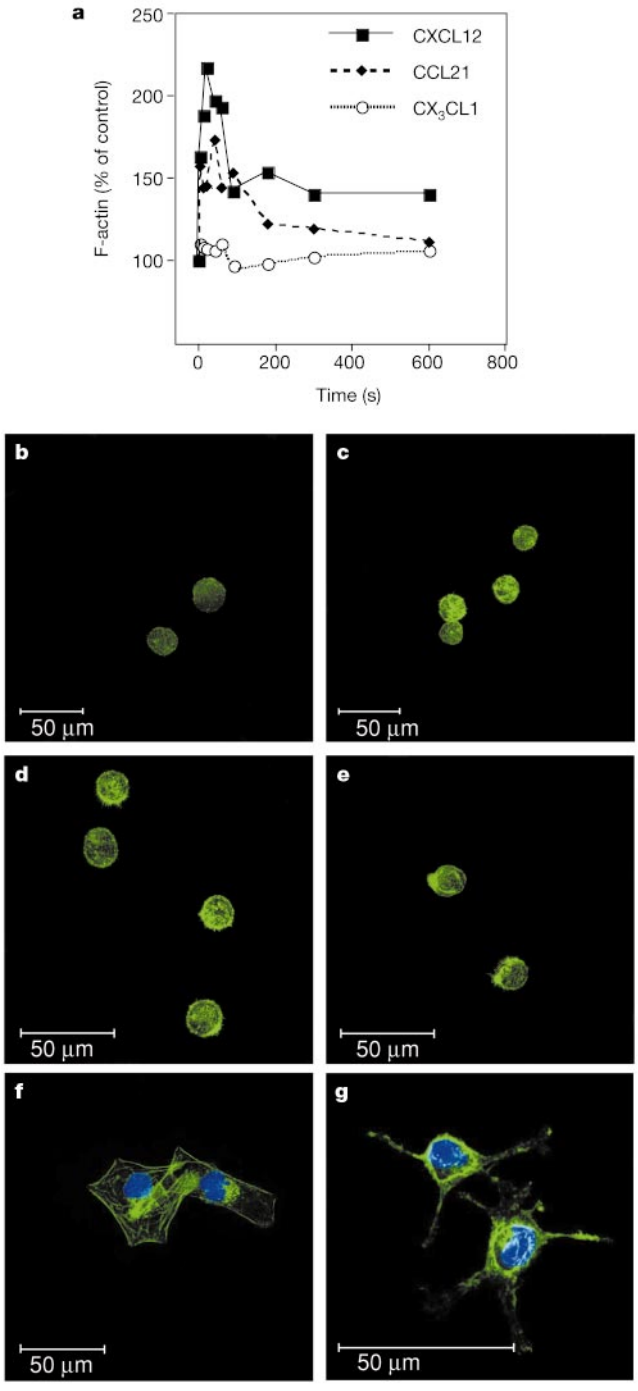

Figure 3 F-actin polymerization in human breast carcinoma cells. **a**, Intracellular F-actin content measured by flow cytometry in MDA-MB-231 cells after stimulation with CXCL12/SDF-1α (100 nM), CCL21/6Ckine (100 nM) or CX_3CL1/fraktalkine (100 nM). Data points are plotted relative to the mean fluorescence before the addition of chemoattractant. **b**–**e**, Confocal microscopy series of breast carcinoma cells after CXCL12 (100 nM) stimulation in suspension. MDA-MB-231 cells unstimulated (**b**) or stimulated for 20 s (**c**), 3 min (**d**) or 20 min (**e**). **f**, **g**, Adherent MDA-MB-231 cells unstimulated (**f**) or stimulated (**g**) with CXCL12 (100 nM) for 20 min. Scale bar, 50 µm.

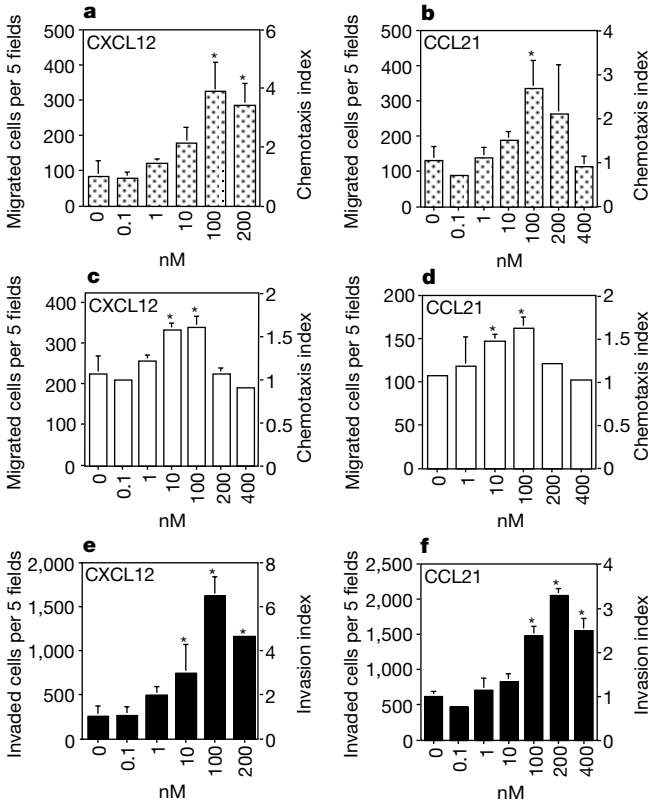

Figure 4 Chemokine-mediated migration and invasion of breast carcinoma cells. Chemotactic response of MDA-MB-231 cells to different concentrations of CXCL12/SDF-1α (**a**) or CCL21/6Ckine (**b**). Directional migration of primary breast carcinoma cells towards CXCL12 (**c**) or CCL21 (**d**) gradients. Effects of various concentrations of CXCL12 (**e**) or CCL21 (**f**) on the invasion of MDA-MB-361 cells. Results are expressed as the mean number of migrating cells per well. Chemotaxis indices were calculated as ratio of cells migrated toward a chemokine gradient to cells migrated in the negative control. Mean ± s.d. (Student's *t*-test; $^*P < 0.005$).

(Table 1). All isotype-treated mice developed ipsi- and contralateral inguinal, and axillary lymph-node metastases at the site of injection (ipsilateral) with diameters exceeding 3 mm. In contrast, only 38% of anti-CXCR4-treated mice presented inguinal lymph-node metastases at the site of injection (diameter < 3 mm) (Table 1).

Discussion

It has been proposed that molecules regulating the metastatic dissemination of tumour cells to specific anatomical sites need to fulfil the following criteria[1,2]. First, they have to be constitutively expressed at principal sites of metastasis. Second, adhesion of target cells to the endothelium and transendothelial migration need to be promoted. Third, these molecules must be capable of mediating the invasion of cells into tissues that provide supportive microenvironments. Last, this process requires the expression of a distinct receptor repertoire by the target cells, depending on their metastatic profile.

Given their well-established roles in leukocyte trafficking and homeostasis, chemokines are perfectly positioned to fulfil these criteria[4,5,7–10,21,22]. We have shown that, out of all known chemokine receptors, breast cancer cells specifically express functionally active CXCR4 and CCR7, which trigger actin polymerization, pseudopodia formation, and the directional migration and invasion of breast cancer cells. Together with the distinct tissue distribution of their ligands CXCL12 and CCL21 in organs representing the main sites of breast cancer metastasis and the significant inhibition of breast cancer metastasis observed *in vivo*, this finding indicates that chemokine ligand–receptor interactions may be crucial for the metastatis of breast cancer.

Although chemokines have been implicated in tumour cell growth[2,23], angiogenesis[24] and the host immune response against malignant cells[2,25], our study provides evidence that chemokines may promote metastasis by acting directly on tumour cell migration and invasion. Both CXCR4 and CCR7 are critical for cell trafficking and tissue homeostasis[4,9,10,26–29]. CXCL12 is the only known ligand for CXCR4 (refs 26–29). This chemokine ligand–receptor pair

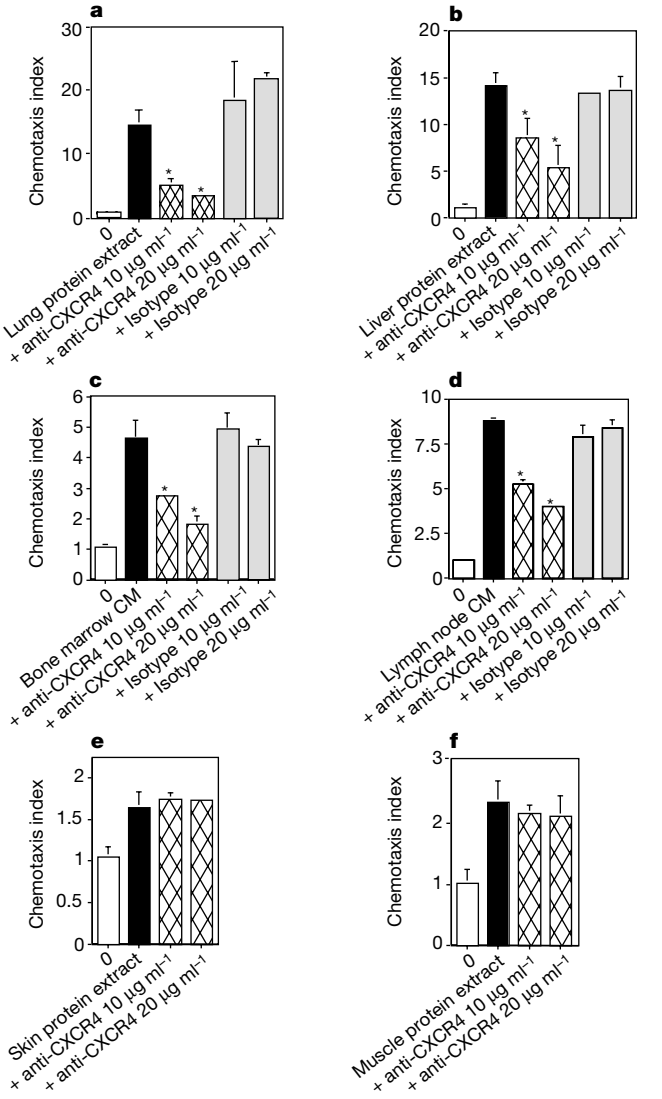

Figure 5 Migration of breast carcinoma cells in response to organ-derived proteins. Chemotaxis of MDA-MB-231 breast carcinoma cells in response to protein extracts of human lung (**a**) or liver (**b**), conditioned medium (CM) from human primary bone marrow (**c**) or lymph-node stromal cells (**d**) and protein extracts from human skin (**e**) or muscle (**f**) in the presence of a neutralizing anti-CXCR4 monoclonal antibody or isotype control. Chemotaxis indices were calculated as ratio of cells migrated toward a protein gradient to cells migrated in the negative control. Mean ± s.d. (Student's *t*-test; $*P < 0.001$).

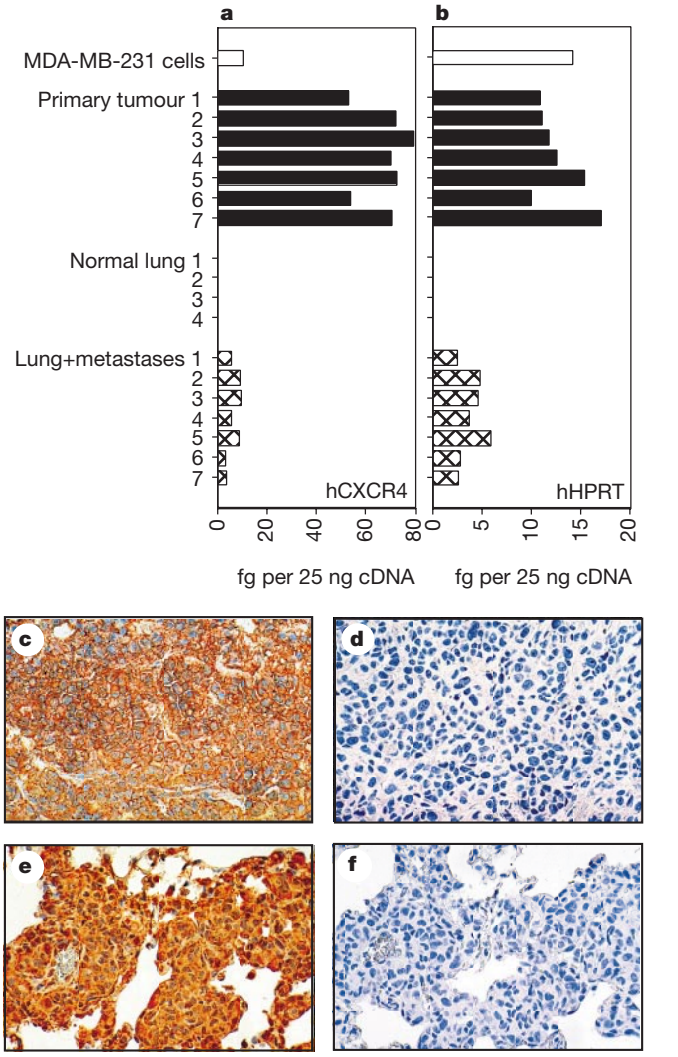

Figure 6 Expression of human CXCR4 and human HPRT in the MDA-MB-231 breast cancer metastasis model. Quantitative real-time RT–PCR (TaqMan) analyses of hCXCR4 (**a**) and hHPRT (**b**) mRNA expression in primary tumours ($n = 7$), metastases-infiltrated lungs ($n = 7$), and normal lungs of SCID mice. Values are expressed as femtograms of target gene in 25 ng of total cDNA. Immunohistochemistry of human CXCR4 in primary tumours (**c**, **d**) and lung metastases (**e**, **f**). Representative staining in tissue samples from one out of six mice. Original magnification, ×400 (**c**, **e**, anti-CXCR4; **d**, **f**, isotype).

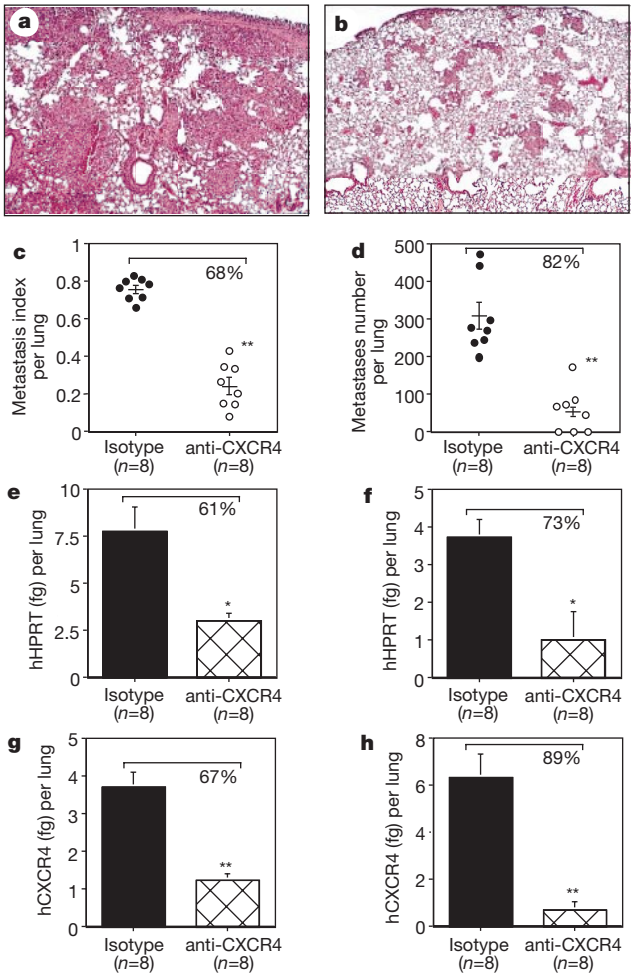

Figure 7 Effect of CXCR4-neutralization on metastasis *in vivo*. **a**, **b**, Haematoxylin and eosin staining of lungs from SCID mice injected with human MDA-MB-231 breast carcinoma cells and treated with either isotype control (**a**) or anti-hCXCR4 monoclonal antibody (**b**). Original magnification, ×100. **c**, Lung colony formation after i.v. injection of MDA-MB-231 cells in isotype- or anti-CXCR4-treated mice. Representative data of one out of two experiments. **d**, Spontaneous lung metastasis after orthotopic injection of MDA-MB-231 cells. **e**, **f**, Metastases quantification by quantitative RT–PCR analyses in contralateral lungs. Expression of hHPRT mRNA after i.v. (**e**) or orthotopic (**f**) injection. Expression of hCXCR4 mRNA after i.v. (**g**) or orthotopic (**h**) injection. Values are expressed as femtograms of target gene in 25 ng of total cDNA. Mean ± s.e.m. (Student's *t*-test; $*P < 0.05$, $**P < 0.001$). Percentages indicate relative suppression compared with isotype.

Table 1 Frequency of lymph-node metastases

Mice*	Ipsilateral inguinal LN	Contralateral inguinal LN	Axillary LN
Isotype (n = 8)	8/8 (>3 mm)	8/8 (>3 mm)	8/8 (>3 mm)
Anti-CXCR4 (n = 8)	3/8 (<3 mm)	0/8	0/8

*MDA-MB-231 breast carcinoma cells (10^7) were injected into the inguinal mammary fat pad of SCID mice, which were treated with anti-CXCR4 monoclonal antibody or isotype control. Regional lymph-node (LN) metastasis was evaluated on day 44.

exhibits unparalleled chemotactic efficacy for leukocytes *in vitro* and is a highly potent chemoattractant *in vivo*[16,19,26–29]. Both CXCR4- and CXCL12-deficient mice display perinatal lethality owing to profound defects in embryonic development of the haematopoietic, cardiovascular and nervous systems[26–29]. These phenotypic changes are mediated by the disrupted migration of embryonic progenitor cells into appropriate microenvironments, suggesting that CXCL12/CXCR4 interactions are vital for the migration of haematopoietic and non-haematopoietic cells *in vivo*.

Furthermore, *in vivo* studies using neutralizing antibodies to CXCR4 implicate this receptor in the homing and repopulation of human stem cells into the bone marrow of SCID mice[9]. As expression of CXCR4 by breast cancer cells is related to efficient, CXCL12-induced migration and invasion of these cells both *in vitro* and *in vivo*, it is conceivable that CXCL12/CXCR4 interactions may contribute to bone-marrow infiltration by breast cancer cells. The preferential expression of CXCL12 in lung, liver and lymph node also suggests a role for this chemokine in the tropism of breast cancer cells for these anatomical sites.

Data point to the crucial role of CCL21 and its receptor CCR7 in the homing of lymphocytes into secondary lymphoid organs. A natural mutation in mice, designated *plt* (for paucity of lymph-node T cells) that results in the loss of one of the forms of mCCL21 (refs 14, 15, 30), and targeted disruption of the CCR7 gene[10] cause impaired homing of naive T cells to secondary lymphoid organs. Thus, the abundant expression of the homeostatic chemokine CCL21 in lymph nodes makes it a likely candidate to attract CCR7-positive tumour cells. In fact, CXCL12 and CCL21 may have synergistic effects, as both chemokines are highly expressed in lymph nodes and mediate chemotactic and invasive responses of breast cancer cells *in vitro*.

Our findings are probably not unique to breast cancer. Other tumour entities of haematopoietic and non-haematopoietic origin, including acute myeloid and lymphoblastic leukaemia[31], chronic lymphocytic leukaemia[17,32], non-Hodgkin B-cell lymphoma[33] and pancreatic cancer[34], express functionally active chemokine receptors that mediate tumour cell migration *in vitro*. Our results in breast cancer and malignant melanoma suggest that malignant cells, in general, express distinct and non-random patterns of chemokine receptors. Furthermore, the association of CCR10 expression by malignant melanoma cells with the skin-specific expression of its ligand CCL27/CTACK[7,8] and the high incidence of skin metastases in this malignant disease support the involvement of chemokine receptors in metastasis.

Currently, intense efforts are underway to identify small-molecule antagonists for many chemokine receptors[35]. We propose that small molecule antagonists of chemokine receptors, such as CXCR4, may be useful to interfere with tumour progression and metastasis in tumour patients. □

Methods

Cell lines

The following human breast cancer and melanoma cell lines were obtained from the ATCC: DU-4475, MCF-7, T-47D, Hs578T, MDA-MB-468, MDA-MB-361, MDA-MB-231, SK-Mel-2, SK-Mel-28, SK-Mel-31, HT-144 and Malme-3M. MV3 melanoma cells were a gift from D. J. Ruiter, Nijmegen; TXM-14 and TXM-30 cells were obtained from I. J. Fidler, Houston; UKRV-Mel-4 and UKRV-Mel-30 melanoma cells were a gift from D. Schadendorf, Mannheim. Human primary mammary epithelial and stromal cells, and human primary melanocytes and bone-marrow stromal cells were obtained from Clonetics (San Diego, CA). Human primary lymph-node stromal cells were cultured from normal human lymph-node tissue as described[33].

Real-time quantitative PCR

Total RNA from cells or homogenized tissue samples was extracted and reverse transcribed as described[8]. Complementary DNA was quantitatively analysed for the expression of human chemokines and chemokine receptors by the fluorogenic 5′-nuclease PCR assay[36] as reported[8]. Specific primers and probes were obtained from Applied Biosystems. Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) during 40 cycles. For metastasis quantification, specific primers for human *HPRT* which do not cross-react with its mouse counterpart were designed (forward, 5′-TTCCTTGGTCAGGCAGTATAATCC-3′; reverse, 5′-AGTCTGGCTTATATCCAACACTTCG-3′). 18S ribosomal RNA was used for normalization.

Tissue samples and immunohistochemistry

Tissue samples of primary tumours from untreated patients (n = 12) with invasive ductal

(n = 9) or lobular (n = 3) breast carcinoma were taken, with informed consent, from either diagnostic biopsies or after lumpectomy/mastectomy. Histopathological diagnosis was confirmed for each specimen. Tissue samples of normal human organs were obtained from the National Disease Research Interchange. RNA of various normal human organs was obtained from Clontech. The study was approved by the local ethics committee.

Primary tumours and metastases were routinely fixed with formalin and embedded in paraffin. Antigen retrieval was performed and sections were stained with either anti-human CXCR4 monoclonal antibody (12G5, IgG_{2a}) or IgG_{2a} isotype (R&D Systems) using a standard indirect avidin–biotin horseradish peroxidase method. Colour was developed with diaminobenzidine (DAB) and sections were counterstained with haematoxylin. For staining human CXCR4 in paraffin-embedded or cryo-preserved tissue samples of tumour-bearing SCID mice, 3-amino-9-ethylcarbazole (AEC) was used for colour development.

Actin polymerization assay and microscopy

Actin polymerization was tested as described[17,37]. Breast cancer cells were incubated either with CXCL12/SDF-1α or CCL21/6Ckine (R&D Systems) at concentrations shown to be optimal in chemotaxis experiments, or with CX_3CL1/fractalkine as negative control. At the indicated time points, cells were fixed, permeabilized and stained in a solution containing paraformaldehyde, lysophosphatidylcholine and fluorescein isothiocyanate (FITC)-labelled phalloidin. Fixed cells were subjected to flow cytometry or analysed by confocal microscopy (Leica DMR). For experiments with adherent cells, breast cancer cells were pre-seeded and incubated with CXCL12, CCL21 or assay buffer (DMEM/0.1% bovine serum albumin (BSA)/ 12 mM HEPES) for 20 min. Cells were stained with phalloidin and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes).

Chemotaxis and chemoinvasion assays

Migration and invasion was assayed in 24-well cell-culture chambers using inserts with 8-μm pore membranes as described[38]. Membranes were pre-coated with fibronectin (2.5–7.5 $\mu g\,ml^{-1}$) for chemotaxis or Matrigel (28 μg per insert) and fibronectin for invasion studies. Breast cancer cells were resuspended in chemotaxis buffer (DMEM/ 0.1% BSA/ 12 mM HEPES) at 2 or 4×10^4 cells per ml. After incubation for 6 or 24 h for chemotaxis or chemoinvasion assays, respectively, cells on the lower surface of the membrane were stained and counted under a light microscope in at least five different fields (original magnification, ×200). Assays were performed in triplicates. Chemokinesis was tested in checkerboard assays and was uniformly negative for both CXCL12 and CCL21. Proteins from homogenized normal human lung, liver, skin and muscle were extracted in Tris-HCl and protease inhibitor as described[39]. Conditioned media from human primary bone-marrow or lymph-node stromal cells were generated as described[16,17,33].

For neutralization studies, cells were pre-incubated with various concentrations of either anti-human CXCR4 monoclonal antibody (44717.111, IgG_{2b}), anti-human CXCL12 polyclonal antibody (goat IgG) or anti-human CCL21 polyclonal antibody (goat IgG) (all R&D Systems).

In vivo metastasis studies

CB-17 SCID mice (Taconic Farms, Germantown, NY) were injected with MDA-MB-231 breast carcinoma cells either i.v. (10^6 cells) into the tail vein, orthotopically into the inguinal mammary fat pad (10^7 cells). Mice were treated with intraperitoneal injections of a neutralizing anti-human CXCR4 antibody (44717.111, IgG_{2b}) or control IgG_{2b} twice weekly (1 mg per injection). No cytotoxicity for either the anti-human CXCR4 antibody or control IgG_{2b} could be detected in *in vitro* assays. For studies of experimental metastasis, lungs were collected on day 28 and fixed or snap frozen for immunohistochemistry and RNA extraction.

Micro-metastasis was quantified by counting the total tissue area per lung section (D1) and micro-metastasis present in the same area (D2) using a 21-mm^2 reference grid. The metastatic index was calculated by the ratio D2/D1. For spontaneous metastasis experiments, primary tumours and organs were collected on day 44 after injection. The number of lung colonies was counted by light microscopy (magnification, ×40). The anti-human CXCR4 monoclonal antibody showed no cross-reactivity with murine CXCR4.

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