Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis

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Summary

The receptor tyrosine kinase HER2 enhances tumor metastasis; however, its role in homing to metastatic organs is poorly understood. The chemokine receptor CXCR4 has recently been shown to mediate the movement of malignant cancer cells to specific organs. Here, we show that HER2 enhances the expression of CXCR4, which is required for HER2-mediated invasion in vitro and lung metastasis in vivo. HER2 also inhibits ligand-induced CXCR4 degradation. Finally, a significant correlation between HER2 and CXCR4 expression was observed in human breast tumor tissues, and CXCR4 expression correlated with a poor overall survival rate in patients with breast cancer. These results provide a plausible mechanism for HER2-mediated breast tumor metastasis and establish a functional link between HER2 and CXCR4 signaling pathways.

Introduction

The aberrant activation of the human epithelial growth factor receptor (HER, ErbB) family of receptor tyrosine kinases (RTKs), which includes HER1 (ErbB1, epithelial growth factor [EGFR]), HER2 (ErbB2, neu), HER3, and HER4, has been implicated in tumor growth and progression (Yarden and Sliwkowski, 2001). Of the four HER family members, HER2 is the most potent oncoprotein. *HER2* is amplified or overexpressed in about 30% of breast cancers and other cancers and is associated with a poor clinical outcome, including a positive correlation with metastasis (Slamon et al., 1989; Yu and Hung, 2000). In addition, HER2 increases the metastatic potential in murine and human cancer cell lines (Tan et al., 1997; Yarden and Sliwkowski, 2001; Yu and Hung, 1991, 2000; Yu et al., 1994) and induces mammary tumors and lung metastases in transgenic animal models (Guy et al., 1992; Muller et al., 1988). It has already been used as a target for cancer therapies such as trastuzumab (Herceptin), an anti-HER2 antibody that has shown a good clinical benefit in patients with HER2-driven metastatic breast cancer (Slamon et al., 2001). Although the functionality of HER2 in breast cancer has been extensively studied (Yarden and Sliwkowski, 2001; Zhou and Hung, 2003), its role in tumor progression is still far from being completely understood, especially its role in targeted metastasis, such as homing to the lung.

Metastasis, the major cause of morbidity and mortality in most cancers, is a complex pathophysiological process that is highly organ selective and involves numerous interactions between the cancer cells and the host (Fidler and Hart, 1982; Steeg, 2003; Yeatman and Nicolson, 1993). Although many molecules have been implicated in cancer metastasis, the detailed mechanism of organ-specific tumor metastasis is still not completely understood. Recently, it was suggested that chemokine stromal cell-derived factor-1α (SDF-1α, also known as CXC chemokine ligand 12) and its receptor, CXCR4, are involved in breast cancer metastasis (Liotta, 2001; Muller et al., 2001). Chemokines are a superfamily of small, cytokine-like peptides, which are divided into subclasses according to the motifs on their first two cysteine residues in the N terminus (Proudfoot, 2002; Rossi and Zlotnik, 2000). Through interaction with chemokine receptors, chemokines induce cytoskeletal rearrangement of hematopoietic cells, increase their adhesion, and direct them to migrate to a home-specific organ. Chemokine receptors are G protein-coupled seven-transmembrane receptors (GPCR), and

SIGNIFICANCE

Metastasis is the main cause of morbidity and mortality in most cancers. Recently, CXC chemokine receptor 4 (CXCR4) was found to play a very important role in the targeted metastasis of breast cancer; the CXCR4 is expressed in malignant breast cancer cells, while the natural ligand stromal cell-derived factor-1α (SDF-1α) is released by certain metastatic organs. Overexpression of HER2 enhances the metastatic potential and correlates with poor prognosis. Here, we show that HER2 upregulates CXCR4 expression, and the inhibition of CXCR4 expression suppresses HER2-induced malignancy in vitro and in vivo lung metastasis. Our data reveal the mystery for HER2-mediated homing to metastatic organs and provide a crucial link between the chemokine receptor CXCR4 and the receptor tyrosine kinase HER2 in tumor progression and metastasis.

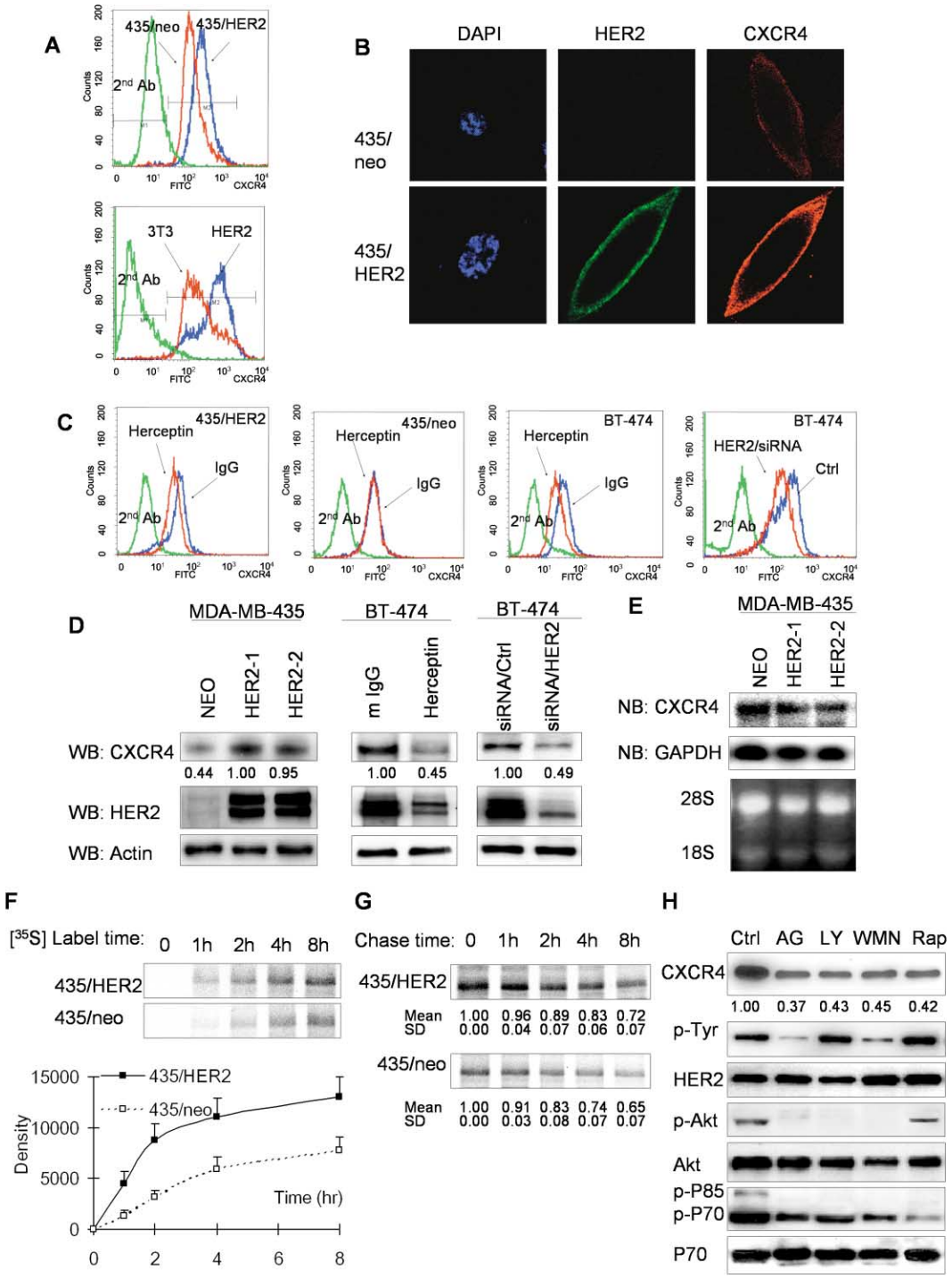

Figure 1. HER2 enhances the chemokine receptor CXCR4 expression

A: MDA-MB-435 cells and their HER2 stable transfectants were stained for the CXCR4 antibody (MAB 172; R&D) and analyzed using FACS. The upper panel shows CXCR4 expression in HER2/435 (blue) and 435/neo (red) vector control cell lines compared with an isotype control preparation (green). The lower panel shows similar staining for NIH 3T3 (red) and its HER2 transfectant (blue).

B: HER2 enhances CXCR4 expression demonstrated by immunofluorescent staining. 435/neo and 435/HER2 cells were stained with anti-HER2 (green) and anti-CXCR4 (red) antibodies and examined under a confocal microscope (Olympus).

C: Herceptin reduces CXCR4 level in HER2-overexpressing cells. 435/HER2, 435/neo, and BT-474 cells were treated with Herceptin 15 μg/ml for 36 hr (red); BT-474 cells were treated with HER2 siRNA and subjected for FACS analysis.

D: Western blot supporting CXCR4 (top panel) expression and HER2 status (second panel) of MDA-MB-435 cells, two HER2 stable transfectants, and BT-474 cells used in FACS. The human actin was used as a loading control for the Western blot (bottom). Band intensities were quantitated, and the relative ratios of CXCR4 intensity among the different treatments were shown below the first lane.

E: Analysis of CXCR4 mRNA expression. 435/neo cells and two 435/HER2 stable transfectants were harvested, and total RNA was prepared for Northern blotting using human CXCR4 (top) cDNA probes. The human GAPDH and total 18S and 28S ribosomal RNA were used as a loading control (second and bottom panel).

CXCR4 is one of the best-characterized chemokine receptors. In addition to inducing leukocyte trafficking (Hernandez et al., 2003), CXCR4 is essential for B cell lymphopoiesis and myelopoiesis (Nagasawa et al., 1996), gastrointestinal tract vascularization (Tachibana et al., 1998), neuronal and germ cell migration (Knaut et al., 2003; Kunwar and Lehmann, 2003; Zou et al., 1998), and HIV invasion of host cells (Connor et al., 1997; Scarlatti et al., 1997). Moreover, CXCR4 and its ligand, SDF-1α, play an important role in the targeted metastasis of breast cancer (Liotta, 2001; Muller et al., 2001). Chemokines such as SDF-1α are released in high amounts by certain organs, such as lung, bone, and liver. Malignant breast cancer cells, which express the chemokine receptor CXCR4, invade the extracellular matrix and circulate in the blood and lymphatic vessels. The attraction between SDF-1α and CXCR4 causes breast cancer cells to leave the circulation and migrate into organs with large amounts of chemokines, where the cancer cells proliferate, induce angiogenesis, and form metastatic tumors. CXCR4 is also involved in the metastasis of prostate cancer to the bone marrow (Taichman et al., 2002) and of colon cancer to the liver (Zeelenberg et al., 2003).

In an attempt to understand how HER2 overexpression increases metastatic potential and, in particular, homing to its metastatic organs, which cause the majority of cancer patient deaths (Hortobagyi, 1998; Landis et al., 1999; Slamon et al., 2001), we found that HER2 enhances the expression of CXCR4. CXCR4 expression was also shown to predict poor patient survival. Furthermore, we found that CXCR4 is required for HER2-mediated metastatic potential in vitro and in vivo. Our data establish a molecular mechanism whereby HER2-overexpressing cancer cells home to specific organs and provide crucial evidence of a functional link between the HER2 and CXCR4 signaling pathways.

Results

HER2 enhances the chemokine receptor CXCR-4 expression

It is well known that HER2 enhances cancer invasion and lung metastasis (Tan et al., 1997; Yarden and Sliwkowski, 2001; Yu and Hung, 2000), and the chemokine receptor CXCR4 is involved in the metastasis of breast cancer to the lung (Muller et al., 2001). We hypothesized that CXCR4 plays a role in HER2-mediated metastasis. To test this hypothesis, we examined CXCR4 expression by fluorescence-activated cell sorting (FACS) analysis and found that CXCR4 expression was 2.8 $\pm$ 0.6-fold higher in the HER2 transfectants of MDA-MB-435 breast cancer cells (435/HER2) than that in the vector control cells (435/neo) (Figure 1A). In addition, this phenomenon was supported in two independent HER2 stable transfectants of MDA-MB-435 breast cancer cells by Western blot (Figure 1D, left panel) and also observed in the NIH 3T3 cell and its HER2 stable transfectant (Figure 1A). The increase in CXCR4 expression by HER2 was further supported by fluorescence confocal microscopy (Figure 1B). The 435/HER2 cells expressed much higher levels of CXCR4 than did the vector control cells (435/neo) (red) (Figure 1B); the HER2 status was confirmed using an anti-HER2 antibody staining (green). To further examine whether HER2 is required for the enhanced CXCR4 expression in HER2-overexpressing cancer cells, we used a HER2-specific monoclonal antibody, Herceptin, that is known to downregulate HER2 (Carter et al., 1992; Klos et al., 2003; Pietras et al., 1998) and found that Herceptin decreases the CXCR4 expression in 435/HER2 cells, compared to control IgG. However, Herceptin does not affect the CXCR4 expression in 435/neo cells (Figure 1C). Downregulating CXCR4 by Herceptin was also observed in endogenous HER2 overexpressing BT-474 cells by FACS (Figure 1C) and Western blot (Figure 1D, middle panel). Similar results were also observed using RNA interference to deplete HER2 expression (Figures 1C and 1D, right panel). Therefore, these results indicate that HER2 is able to increase the expression of the chemokine receptor CXCR4.

We further investigated the molecular mechanism behind HER2-induced CXCR4 expression. HER2 does not enhance *CXCR4* mRNA level, as shown by Northern blot analysis (Figure 1E). Therefore, it is likely that HER2 regulates CXCR4 at the protein level. We used metabolic labeling assays to detect the protein synthesis and degradation of CXCR4. Pulse-labeling studies demonstrated that HER2 increases the protein synthesis rate of the CXCR4 receptor, which is 2.5-fold faster than the control before the first 2 hr (Figure 1F). Pulse-chase experiments (Figure 1G) indicated that HER2 only slightly lowers the basal protein degradation rate of CXCR4 (please note that this is different from the ligand-dependent degradation that is described in Figure 3B); however, considering the standard deviation from three independent experiments, there is no statistical difference between them. Thus, the increased CXCR4 protein level in the HER2-expressing cells is primarily caused by enhanced translational rate (Figure 1F) and is less influenced by the basal degradation rate (Figure 1G). Since HER2 can activate Akt (Zhou et al., 2000), which is known to stimulate the mammalian target of rapamycin (mTOR) to enhance protein synthesis (Gingras et al., 2001), we next examined whether the enhancement of CXCR4 protein synthesis by HER2 might occur through the PI-3K/Akt/mTOR pathway. If mTOR is involved in the HER2-enhanced CXCR4 translation, blockage of the mTOR activity and its upstream signals such as PI-3 kinase and HER2 tyrosine kinase should inhibit HER2-induced CXCR4 expression. Con-

F: Metabolic pulse-labeling experiment. [^{35}S]Met-Cys was used to measure the protein synthesis rate of CXCR4 in 435/neo and 435/HER2 cells (upper panel), and the relative synthesis rates of CXCR4 protein and standard deviation (SD) were plotted in the bottom panel. The experiments were repeated three times.

G: Pulse-chase assay to measure the protein degradation rate. Serum-starved 435/neo and 435/HER2 cells were pretreated with [^{35}S]Met-Cys, and then the cells were rinsed and incubated in medium containing unlabeled Met-Cys for the indicated time prior to preparation of cell lysates and immunoprecipitation of CXCR4. All the experiments were repeated three times. Mean and SD are shown below the lanes.

H: Effect of HER2/PI-3K/Akt/mTOR pathway on CXCR4 expression. After overnight serum starvation, in the presence of 10% serum stimulation, different inhibitors of HER2 (AG825, 100 μM [AG]), PI-3K (Wortmannin, 100 μM [WMN], and LY294002, 50 mM [LY]), and mTOR (rapamycin, 100 μM [Rap]) were used to treat MDA-MB-435 HER2 stable transfectants for 6 hr. The effects of all these inhibitors on the activity of HER2, PI-3K/Akt, and FRAP were relatively assessed by phosphorylation of tyrosine residue of HER2, phosphorylation of Akt at residue serine 473, and phosphorylation of p70 S6k at residue threonine 389.

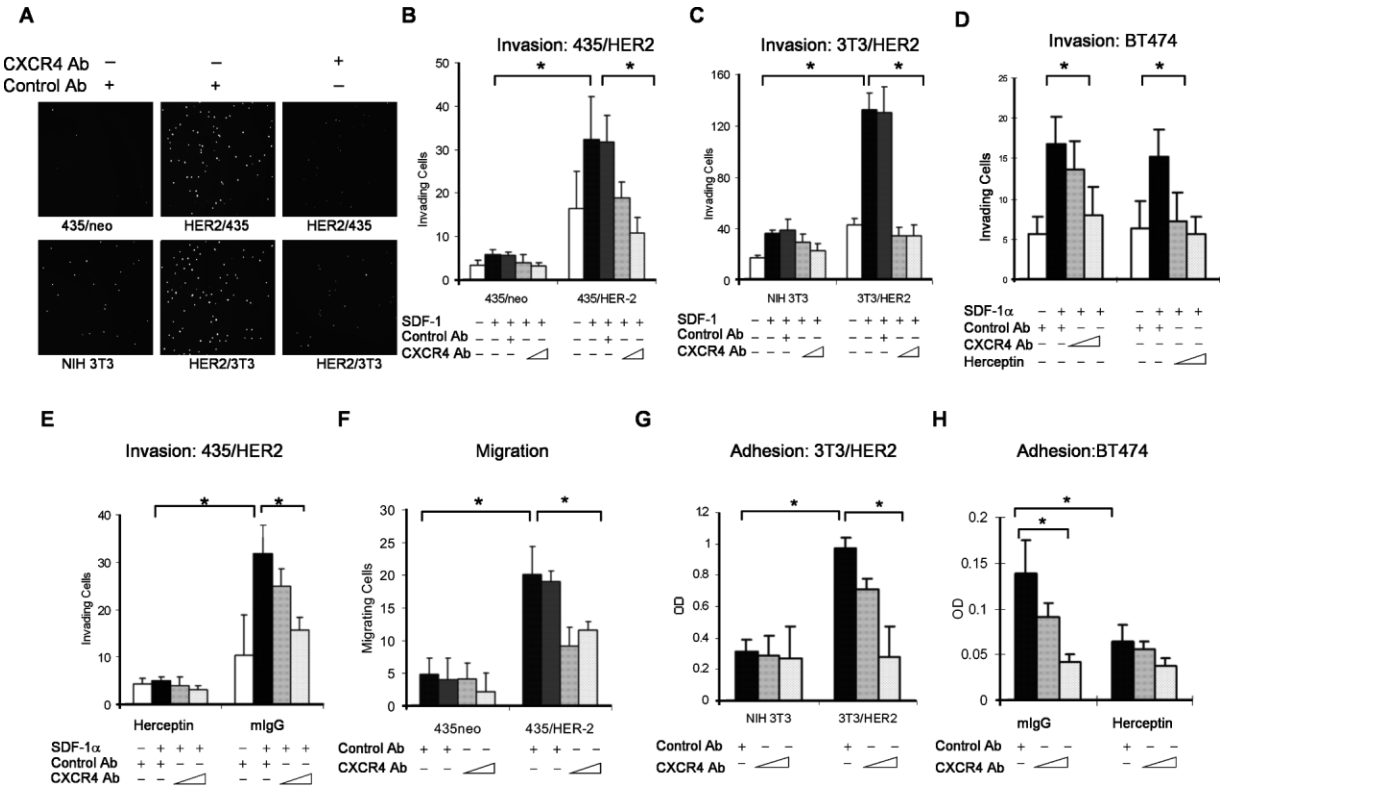

Figure 2. CXCR4 is required for HER2-enhanced invasion, migration, and adhesion in vitro

A: Invasive assays were performed using the Boyden Chamber assay coated with Matrigel, which mimics basement membrane composition, and SDF-1α, a ligand for CXCR4, as a chemoattractive agent in the lower chamber. For the blocking assay, either CXCR4 blocking antibody (1–10 μg/ml) or Herceptin (5 μg/ml; 15 μg/ml) was added into cell culture. Cells were counted in triplicate wells and in five identical experiments.

B–E: Quantification of invasion assays. *$p < 0.05$.

F: The migration assay was performed in a manner similar to the invasive assay, except in the absence of Matrigel. Quantification of migration is shown. *$p < 0.05$.

G and H: HER2 significantly enhances adhesion to endothelial cells. Cell-to-cell adhesion was quantified using a fluorescent plate reader. Data are presented as mean raw fluorescent counts per well of the mean. *$p < 0.05$.

sistent with this notion, treatment of different inhibitors, including the inhibitors for HER2 tyrosine kinase (AG825), PI-3 kinase (Wortmannin and LY294002), and mTOR (rapamycin) indeed inhibited CXCR4 expression in 435/HER2 cells. The phosphorylation of tyrosine residue of HER2, phosphorylation of Akt at residue serine 473, and phosphorylation of p70S6k at residue threonine 389 were also examined as controls for proper effects of these inhibitors (Figure 1H). The result suggests that PI-3K/Akt/mTOR pathway may attribute to the HER2-induced CXCR4 expression.

CXCR4 is crucial for HER2-induced invasion, migration, and adhesion activities in vitro

Because HER2 induces CXCR4 expression, and both HER2 and interaction between CXCR4 and SDF-1α play an important role in metastasis, we examined whether SDF-1α/CXCR4 interaction contributes to HER2-driven invasive activities. Invasion assays were performed using the Boyden Chamber coated with Matrigel. It was clear that SDF-1α stimulated the in vitro invasive activity in 435/HER2 cells but not in the 435/neo control cells. Moreover, the SDF-1α-induced invasive activity was inhibited in a concentration-dependent manner by a CXCR4-blocking antibody (Figures 2A and 2B), but not by a control IgG antibody. Similar results were observed for 3T3 and 3T3/HER2 cells (Figures 2A and 2C) and endogenous HER2-overexpressing BT-474 cells (Figure 2D). Since Herceptin downregulated CXCR4 expression (Figures 1C and 1D), the SDF-1α-induced in vitro invasive activity was also inhibited by Herceptin in two HER2-overexpressing cell lines, 435/HER2 and BT-474 (Figures 2D and 2E). Thus, these results indicate that upregulation of CXCR4 by HER2 is required for in vitro invasion induced by SDF-1α.

Cancer cell invasion involves a sequential series of critical steps, including adhesion to endothelial cells and migration toward chemotactic factors. To better understand which steps of HER2-induced invasion CXCR4 may contribute to, we examined the migration and adhesion activities of HER2 stable transfectants and their parental cells in response to SDF-1α stimulation. Migration was inhibited by a CXCR4-blocking antibody but not by the mouse IgG antibody (Figure 2F), indicating that CXCR4 is required for HER2-enhanced migration. The phenomenon was observed in 435/neo and 435/HER2 cells (Figure 2F) and in 3T3 and 3T3/HER2 cells (data not shown). The adhesion assay, which measures adherence to cultured endothelial cells, also indicated that the CXCR4-blocking antibody inhibited HER2-induced adhesion activity in BT-474 and 3T3/HER2 cells (Figures 2G and 2H). Taken together, these results demonstrate that HER2-induced

CXCR4 expression plays a critical role in increasing the migration of cancer cells and their adhesion to endothelial cells in responding to SDF-1α stimulation. Since SDF-1α is highly expressed in metastatic organs such as lung, liver, and bone, this finding provides in vitro support for a plausible mechanism for HER2-enhanced metastasis and homing to metastatic organs (please see the in vivo study later).

It should be mentioned that other mechanisms, in addition to increased CXCR4 expression, might contribute to SDF-1α-enhanced adhesion activity. We have previously shown that the basal level of adhesion activity in MDA-MB-435 is not increased by enforcing HER2 expression in the 435/HER2 cells (Tan et al., 1997). Consistent with this notion, we noticed that SDF-1α did not stimulate adhesion activity in both 435 and 435/HER2 cells; accordingly, the CXCR4-blocking antibody did not affect the adhesion activity of 435/neo and 435/HER2 cells (data not shown). Thus, other mechanisms must be involved in enhancing the basal level of adhesion activity in MDA-MB-435 cells. Further investigation is required to determine whether this mechanism is through a CXCR4-independent pathway or downstream signaling of CXCR4.

HER2 protects CXCR4 from ligand-induced protein degradation

Although CXCR4 guides malignant cancer cells to metastatic organs in which SDF-1α is enriched (Muller et al., 2001), stimulation of SDF-1α can degrade CXCR4 (Marchese and Benovic, 2001). Therefore, we would expect that once the malignant cancer cells reached the SDF-1α-enriched organs, partial CXCR4 would be degraded, which would reduce the ability to contribute to other steps in metastasis, such as adhering to the endothelium and invading the basement membrane. However, the above results (Figures 2A–2H) suggest that CXCR4 may have a role in invasion, migration, and adhesion activity in the SDF-1α-enriched organs. Thus, we investigated whether HER2 overexpression might prevent CXCR4 from SDF-1α-induced degradation. We examined the CXCR4 downregulation rate following SDF-1α stimulation by FACS and found that the CXCR4 level quickly decreases; after about 1 hr of SDF-1α stimulation, the CXCR4 level in MDA-MB-435 cells was 60% of that in untreated cells, consistent with a previous report (Marchese and Benovic, 2001). However, the CXCR4 level remained almost the same in the 435/HER2 cells after ligand stimulation for 1 hr (Figure 3A). We further used the pulse-chase assay to detect the degradation rate of CXCR4 following SDF-1α stimulation and found that HER2 significantly lowers the ligand-induced protein degradation rate of CXCR4 (Figure 3B). The data indicated that HER2 protects CXCR4 from ligand-induced downregulation. Thus, in HER2-overexpressing cancer cells, CXCR4 may not only play a role in homing to the metastatic organs, but also contribute to the invasive processes including enhanced migration and adhesion activity in the SDF-1α-enriched organs.

CXCR4 degradation correlates with its ubiquitination status (Marchese and Benovic, 2001), and ubiquitination of CXCR4 serves as a targeting signal for lysosomal degradation. To further address the molecular mechanism of HER2 protection of CXCR4 from ligand-induced degradation, we next investigated the role of HER2 on ubiquitination of CXCR4. In the presence of SDF-1α, HER2-expressing cells exhibit a reduced level of ubiquinated CXCR4 (Figure 3C), suggesting that HER2 inhibits CXCR4 monoubiquitination. To further support this notion, myc-tagged CXCR4 was cotransfected with HA-tagged ubiquitin and either active HER2 or kinase-dead HER2 into HEK293 cells; CXCR4 was then immunoprecipitated, and the precipitate was immunoblotted with anti-HA antibody to detect HA-ubiquitin. The level of the ubiquitinated receptor was significantly lower in the lysate from active HER2 transfection than that from kinase-dead form transfection (Figure 3D). This result further supported the idea that HER2 inhibits CXCR4 ubiquitination and that HER2 kinase activity is required for this action. Recently, it has been shown that the Nedd4-like E3 ubiquitin ligase AIP4 mediates agonist-promoted ubiquitination of CXCR4 (Marchese et al., 2003). To further investigate whether AIP4 might be involved in HER2-mediated CXCR4 upregulation, we cotransfected HA-tagged CXCR4, HER2, and either AIP4 or its catalytically inactive mutant C830A at the ratio of 1:2:4, then measured CXCR4 expression using an anti-HA antibody (Figure 3E). As expected, HER2 enhanced CXCR4 expression (lanes 1 and 2), which was completely suppressed in the presence of AIP4 E3 ligase (lanes 3 and 4). However, C830A did not suppress HER2-induced CXCR4 expression (lanes 5 and 6). These results indicated that AIP4 inhibits HER2-induced CXCR4 expression. Taken together, HER2 inhibits ligand-induced CXCR4 ubiquitination and then prevents CXCR4 from ligand-induced degradation.

CXCR4 is required for HER2-induced lung metastasis in vivo

To gather further support for the idea that CXCR4 is required for HER2-induced in vitro invasion and to determine whether CXCR4 is also required for HER2-induced metastasis in vivo, we designed a retroviral RNAi vector (pSR) to inhibit CXCR4 expression (Brummelkamp et al., 2002) (Figure 4). The retrovirus expressing small interfering RNA (siRNA) against CXCR4 was used to infect 435/HER2 transfectants, and the puromycine-resistant stable colonies were pooled together and named 435HER2/SR-iCXCR4. A similar control pool infected with the retrovirus containing an empty vector was selected and named 435HER2/SR-Ctrl. As expected, CXCR4 expression was reduced in the 435HER2/SR-iCXCR4 cells (Figure 4A), and their invasive activity was significantly decreased to a level comparable to that of the negative control 435/neo cells (Figure 4B). The positive control 435HER2/SR-Ctrl cells maintained a high level of invasive activity (Figure 4B). Hence, the siRNA approach further supports our findings that CXCR4 is required for HER2-induced invasive activity in responding to SDF-1α stimulation.

We next determined the in vivo metastatic potential of *CXCR4* siRNA stable and control transfectants in 435/HER2 and 435/neo cell lines by using the experimental metastasis assay. The mice were euthanized 120 days after injection with one of the above cell lines, and pulmonary and extrapulmonary metastases were examined. While the HER2 significantly increased lung metastasis, as reported previously (Tan et al., 1997), the number of metastatic lung nodules in the mice injected with 435HER2/SR-iCXCR4 was significantly lower than that in the control mice ($p < 0.01$) (Figures 4C and 4D). In addition, only two of the ten mice in the 435HER2/SR-iCXCR4 groups developed lung metastases, compared with eight of ten mice in the control 435HER2/SR-Ctrl group (Table 1). Both the size of the lung metastases and the total lung weight were significantly lower in the 435HER2/SR-iCXCR4 group (Table 1 and Figure 4D). All the metastatic tumors were confirmed by pathological examination (data not shown). Taken together,

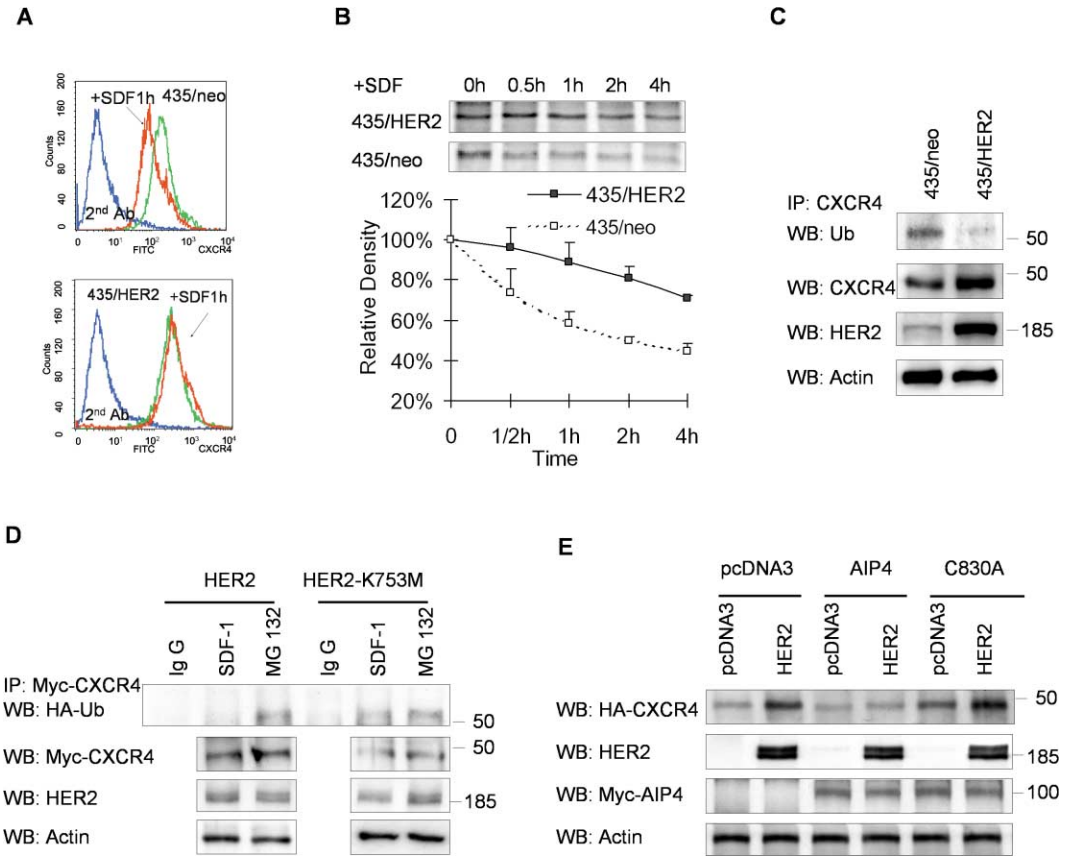

Figure 3. HER2 protects SDF-1α-induced downregulation of CXCR4

A: HER2 prevents SDF-1α-induced downregulation of CXCR4. After cycloheximide treatment (30 min), SDF-1α was added, the endogenous expression of cell surface CXCR4 was analyzed by FACS at different time points, and the mean percentages of CXCR4 fluorescence were plotted. Representative FACS assay results are shown to compare CXCR4 expression before SDF-1α stimulation (green) and 1 hr poststimulation (red).

B: Pulse-chase assay to measure the protein degradation rate under the SDF-1α stimulation: the means of the relative density were plotted in the bottom panel. All the experiments were repeated three times.

C and D: HER2 inhibits SDF-1α-induced ubiquitination of CXCR4. **C**: Serum-starved 435/neo and 435/HER2 cells were pretreated with the cycloheximide treatment for 30 min and then treated with SDF-1α for 1 hr, and the endogenous expression of cell surface CXCR4 was immunoprecipitated and blotted with monoclonal anti-ubiquitin antibody. **D:** HEK293 cells were transfected with Myc-tagged CXCR4 and HA-tagged ubiquitin plus either HER2 or kinase-dead form K753M. Cells were incubated in the absence or presence of 100 nM SDF-1α for 1 hr at 37°C. Proteosome inhibitor MG 132 was added with SDF-1α serving as positive control. Receptors were immunoprecipitated, and membranes were blotted with an anti-HA antibody to detect the incorporation of epitope-tagged ubiquitin. Under these conditions, ubiquitinated receptor was slightly detectable in cells expressing HER2 but was increased and easily detected in cells expressing kinase-dead form K753M. Total cell lysates were also subjected to immunoblotting to detect the expression of CXCR4 and HER2.

E: Effect of AIP4 and its catalytically inactive mutant C830A on HER2 mediated the upregulation of CXCR4. We cotransfected HA-tagged CXCR4, HER2, and either AIP4 or its catalytically inactive mutant C830A at the ratio of 1:2:4, then measured CXCR4 expression using an anti-HA antibody.

these results clearly show that the inhibition of CXCR4 by stable siRNA reduces HER2-induced lung metastasis and that CXCR4 is required for HER2-mediated lung metastasis in vivo.

CXCR4 is upregulated in HER2-overexpressed primary breast tumor tissues and is correlated with poor patient survival

To determine whether HER2-enhanced CXCR4 expression is also observed in primary tumor tissues, we performed immunohistochemical staining and scoring analysis (Camp et al., 1999; Zhou et al., 2001). Immunohistochemical scoring (H score) was determined by multiplying the staining intensity by the percentage of positive tumor cells (Camp et al., 1999). According to bimodal H score distribution, tissues with scores <130 were designated as CXCR4 low, and scores >130 were designated as CXCR4 high. We found a significantly positive correlation between HER2 and CXCR4 expression (Figure 5A and Table 2). In human breast tumor tissues in which HER2 expression was positive, CXCR4 expression was also positive (case 1 in Figure 5A), whereas in HER2-negative tumor tissues, CXCR4 expression was also undetectable (case 2 in Figure 5A). It should be mentioned that the two images (top left and right, or bottom left and right) were derived from nearby sections of the same tumor tissue. Among the 219 breast cancer tumor tissue samples that we examined, the correlation between HER2 and CXCR4 was statistically significant ($p < 0.001$; Table 2), supporting the conclusion that overexpression of HER2 upregulates CXCR4 expression in vivo. Importantly, CXCR4 expression was also found to be correlated with poor patient overall survival ($p < 0.05$) (Figure 5B). These results further support the idea

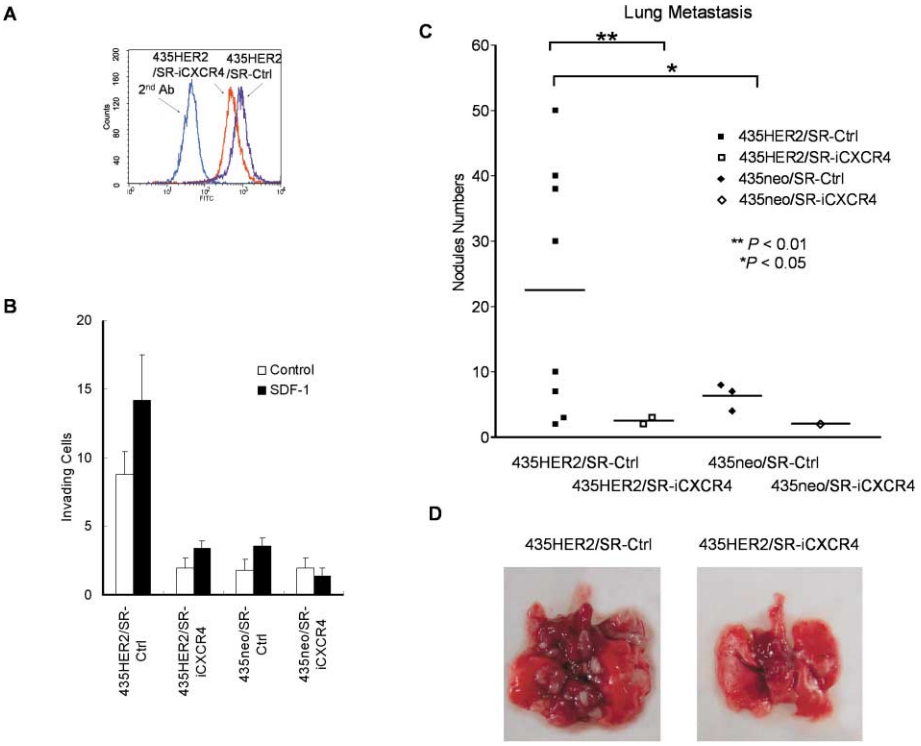

Figure 4. CXCR4 is required for HER2-mediated lung metastasis in vivo

A: FACS analysis of retroviral CXCR4 siRNA stably transfected HER2/435 cells, 435HER2/SR-iCXCR4 (red), and its vector control cells (435HER2/SR-Ctrl) (blue) after puromycine selection.

B: Invasion analysis of the retroviral CXCR4 siRNA transfected HER2/435 (435HER2/SR-iCXCR4) cells and the control cells (435HER2/SR-Ctrl).

C: In vivo lung metastasis driven by HER2 is reduced by CXCR4 inhibition. The mice were euthanized 120 days after injection, and the number of lung metastasis nodules larger than 0.5 mm in diameter in each group was examined. *$p < 0.05$; **$p < 0.01$.

D: A representative set of gross lungs with metastatic nodules.

that HER2 overexpression enhances CXCR4 expression and indicate that CXCR4, like HER2 (Slamon et al., 1989), also serves as a predictor for poor overall patient survival.

Discussion

The RTK HER2 and the GPCR CXCR4 are two structurally unrelated receptors, but the current study demonstrates that HER2 enhances CXCR4 expression and that CXCR4 is required for HER2-induced lung metastasis, therefore resolving a longstanding puzzle of how HER2 overexpression guides cancer cells to home to their metastatic organs. CXCR4 expression was recently found to be correlated with lymph node metastasis in a 79-case cohort of patients with invasive ductal carcinoma (Kato et al., 2003). In our 219-case breast cancer study, CXCR4 was further found to be associated with poorer patient survival, and a significant correlation between HER2 and CXCR4 expression was also observed ($p < 0.001$). In view of the fact that HER2 overexpression is a known marker of poor prognosis in breast cancer, our observations add strong clinical support for the identified mechanism, namely HER2 enhancement of CXCR4 expression.

Cancer metastasis results from several highly organized sequential steps involving numerous interactions between the cancer cells and host, but the detailed molecular mechanism is still not completely understood (Steeg, 2003; Yeatman and Nicolson, 1993). CXCR4 is involved in breast cancer metastasis to specific organs (Liotta, 2001; Muller et al., 2001); however, the detailed mechanism of CXCR4 upregulation in malignant cancer remains poorly understood. Recently, CXCR4 was found to be transactivated by hypoxia-induced factor-1α (HIF-1α) at the transcriptional level in von Hippel-Lindau mutated renal cell carcinoma (Bernards, 2003; Staller et al., 2003), which serves as one plausible mechanism for CXCR4 upregulation. In the current study, we identified two distinct mechanisms for CXCR4 upregulation by HER2 overexpression, enhancement of CXCR4 protein synthesis and inhibition of ligand-induced degradation.

Table 1. Inhibition of in vivo metastatic potential by CXCR4 siRNA

	Frequency (metastasis/total)	No. of nodules (mean [range])	Size of nodules (range in mm)	Lung weight (g)
435HER2/SR-Ctrl	8/10	26 (0–50)	0.5–8.9	7.8 ± 3.4
435HER2/SR-iCXCR4	2/10**	2.5 (0–3)**	0.8–1.0	3.0 ± 0.7**
435neo/SR-Ctrl	3/7*	6.3 (0–8)*	0.5–4.0	3.1 ± 0.6*
435neo/SR-iCXCR4	1/7	2 (0–2)	0.5–1.0	2.8 ± 0.6

An experimental metastasis assay was used to determinate in vivo lung metastatic potential among MDA-MB-435cells, HER2 stable transfectants, and CXCR4 siRNA stable transfectants. The mice were euthanized 120 days after tail vein injection, and the frequency, number, and size of lung metastases and the whole lung weight were measured. Lung nodules >0.5 mm in diameter were counted. Student's t test was used to compare differences between each group. *Compared with the 435HER2/SR-Ctrl, $p < 0.05$. **Compared with the 435HER2/SR-Ctrl, $p < 0.01$.

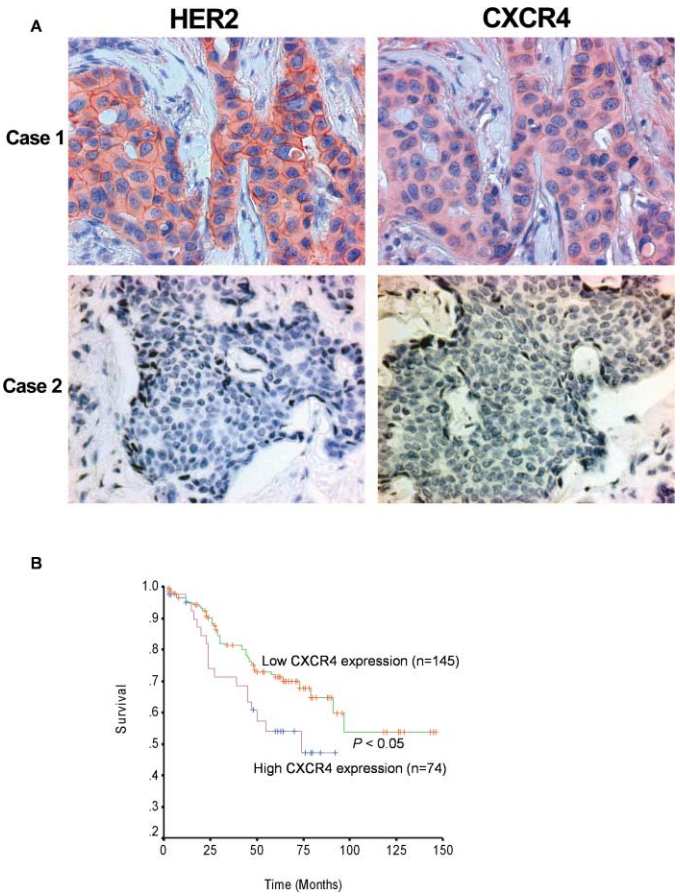

Figure 5. Correlation between CXCR4 and HER2 expression in human breast tumor tissues

A: Representative human primary breast tumor tissue samples with immunohistochemical staining. Case 1: HER2-positive staining (top left) and CXCR4 expression-positive staining (top right). Case 2: HER2-negative staining (bottom left) and CXCR4-negative staining (bottom right). The two samples came from nearby tumor sections. Original magnification, 400×.

B: Kaplan-Meier analysis of overall survival in patients with breast cancer carcinoma according to their CXCR4 expression levels ($p < 0.05$).

Table 2. Summary of HER2 and CXCR4 immunohistochemical staining in tumor tissue sample from 219 cases primary breast cancer

		CXCR4		Total
		−	+	
HER2/neu	−	116 (81.7%)	26 (18.3%)	142
	+	29 (37.7%)	48 (62.3%)	77
Total		145	74	219

The 219 surgical specimens of breast tumor were stained with anti-HER2 and anti-CXCR4 antibody as shown in Figure 5. Expression patterns in all samples are summarized here. The Pearson chi-square was used to analyze for significance of the relationship between CXCR4 expression and HER2 expression ($p < 0.001$).

Presently, the detailed molecular mechanisms for receptor endocytosis, endosome sorting, and degradation of GPCRs, including CXCR4, are not completely understood (Shenoy et al., 2001). Recently, it has been suggested that the mechanisms involved in CXCR4 ubiquitination and sorting may share some common features with EGFR endocytosis and degradation processes, although they are structurally unrelated membrane receptors (Hendriks et al., 2003; Lloyd et al., 2002; Marchese et al., 2003; Polo et al., 2002; Worthylake et al., 1999). One mechanism to degrade CXCR4 is internalization of CXCR4 through early endosomes, and then sorting into late endosomes and lysosomes. HER2 may inhibit CXCR4 ubiquitination and abrogate subsequent sorting steps and therefore prevent its degradation. Taken together, CXCR4 expression can be regulated through different mechanisms. It would be interesting to explore when and where each mechanism is used to regulate CXCR4 expression.

The CXCR4 ligand SDF-1α is a locally secreted small cytokine and is expressed constitutively in some tissues, including bone marrow and lung, which are major homing organs for metastatic breast cancer (Muller et al., 2001; Nagasawa et al., 1996). SDF-1α is present in serum at low concentrations, but in an inactive form (Villalba et al., 2003). Thus, serum SDF-1α may not affect homing of CXCR4-expressing cancer cells. In addition to attracting CXCR4-expressing malignant cancer cells to certain organs (Muller et al., 2001), SDF-1α can also induce CXCR4 degradation (Marchese and Benovic, 2001). This raises the interesting question of whether CXCR4 could be involved in multiple-step metastatic processes, such as invasion through the basement membrane and adherence to endothelial cells, which are critical in the extravasation process for cancer cells to leave the circulation and migrate into normal organs. Our results (Figure 2) clearly indicate that CXCR4 is required for invasion, migration, and adhesion activities under SDF-1α-stimulated conditions, suggesting that CXCR4 may play a role in these steps toward malignant metastases. Blockage of SDF-1α-induced CXCR4 downregulation by HER2 (Figure 3) provides a plausible mechanism to allow continuing CXCR4 expression, which suggests that CXCR4 not only plays a role in homing to the metastatic organs, but also contributes to invasive processes, such as enhanced migration and adhesion activity, in SDF-1α-enriched organs for HER2-overexpressing cancer cells. It is not yet clear whether malignant cancer cells in which HER2 is not overexpressed acquire a similar mechanism to prevent CXCR4 degradation by SDF-1α or if they activate other mechanisms to contribute to invasion and adhesion activities in the extravasation process. The MDA-MB-435 metastatic breast cancer cell line, which does not overexpress HER2, has acquired a strong adhesion activity through other mechanisms, as HER2 does not enhance its ability to adhere to endothelial cells (Tan et al., 1997) and a CXCR4-blocking antibody does not affect its adhesion activity (data not shown).

In summary, HER2 upregulates the expression of CXCR4, which is required for HER2-enhanced invasion, migration, adhesion, and metastasis to the lung. CXCR4 expression is correlated with overall patient survival in breast cancer. Similar to HER2, this observation will have important clinical implications. The study of linkage between these two structurally unrelated membrane receptors, HER2 and CXCR4, both of which play critical roles in cancer metastasis, has advanced our knowledge of how HER2-overexpressing cancer cells home to their meta-

static organs. It may also help us to better understand molecular mechanisms of cancer metastasis.

Experimental procedures

Cell lines, DNA constructs, and antibodies

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum. Cell line NIH3T3 subtype and its HER2 stable transfectant and human breast cancer cell line MDA-MB-435 and its two HER2 stable transfectants have been described previously (Tan et al., 1997, 2002; Yu and Hung, 2000; Zhou et al., 2001). HA-CXCR4 and FLAG-ubiquitin constructs were kindly provided by Dr. Benovic (Marchese et al., 2003); wild-type and catalytically inactive AIP4 plasmids were kindly provided by Dr. Tony Pawson. The anti-myc and anti-HA polyclonal antibodies were purchased from Sigma-Aldrich. siRNA directed against human HER2 was from Dharmacon Research. SDF-1α and CXCR4 antibodies (MAB170, MAB172) were from R&D systems. Other CXCR4 antibodies were from Abcam and Lab Vision/Neomarkers. The monoclonal anti-ubiquitin antibody (6C1) was from Sigma.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described previously (Li et al., 2003), with 1 μg of antibody against CXCR4 or normal control IgG in 1.0 mg whole lysate protein.

FACS and immunofluorescent staining

For FACS analysis, cells were treated with mouse anti-CXCR4 antibody (MAB172; R&D systems, Minneapolis, MN) or normal IgG overnight at 4°C and then stained with FITC-conjugated IgG at room temperature for 30 min and analyzed with a Becton Dickinson flow cytometer (Franklin Lakes, NJ). For the ligand-induced receptor degradation, serum-starved cells were pretreated with 20 μg/ml cycloheximide for 30 min, and then SDF-1α (R&D Systems) was added to the final concentration of 100 ng/ml for 1 to 4 hr. Immunofluorescent staining was performed as described previously (Zhou et al., 2001).

Northern blot analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). RNA (10 μg) was separated and then transferred to blot membranes. The RNA was crosslinked, prehybridized, and then hybridized overnight with ^{32}P-labeled probes. The hybridized membranes were quantified using a Phosphoimager running ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Metabolic labeling assays

A total of 2×10^6 cells were plated and pretreated for 1 hr in methionine-free DMEM. Protein synthesis was measured by pulse-labeling experiment: [^{35}S]Met-Cys was added to a final concentration of 0.3 mCi/ml, and the cells were pulse labeled for 1, 2, 4, and 8 hr and then harvested. Protein degradation was measured by pulse-chase assay: the cells were rinsed after 2 hr pulse labeling, incubated in medium containing nonradioactive Met-Cys and 20 μg/ml cycloheximide for 1, 2, 4, and 8 hr, and then harvested. Immunoprecipitation and SDS-PAGE were then performed as described previously (Li et al., 2003).

Invasion and migration (chemotaxis) assay

Cell invasion was examined as described before with modification (Tan et al., 1997). SDF-1α (600 μl; 100 ng/ml) was added to the lower chamber, and 1–10 μg/ml CXCR4 blocking antibody (MAB170/12G5; R&D Systems) or 5–15 μg/ml Herceptin (Genotech, CA) was added to the top chamber to block the CXCR4 receptor binding. Invading cells were stained using DAPI and counted under fluorescence microscopy at medium-power fields (200× or 100×). Cell migration was assayed using a similar approach without Matrigel coating; 3×10^4 cells were added to the upper chamber and incubated from 6 hr to overnight.

Adhesion assay

The cells were labeled with 3′-O-acetyl-2′,7′-bis(carboxyethyl)-4-5-carboxyfluorescein ester for 30 min. Labeled cells (3×10^4) were deposited onto human umbilical vein endothelial cells or simian virus-40 transformated murine lymphoid endothelial cell monolayer. Pretreatment was performed by incubating the cells with 100 ng/ml SDF-1α for 1 hr at 37°C. Cell-to-cell adhesion then proceeded for 30 min at 37°C. OD was measured at 490 nm.

Retroviral siRNA stable cell lines

Retroviral siRNA vector (pSUPER.RETRO) was purchased from OligoEngine (Seattle, WA) (Brummelkamp et al., 2002). The CXCR4 RNAi primers were 5′-GATCCCCTGGATTGGTCATCCTGGTCTTCAAGAGAGACCAGGATGACCAATCCATTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAATGGATTGGTCATCCTGGTCTCTCTTGAAGACCAGGATGACCAATCCAGGG. Control primers were 5′-GATCCCCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGG-3′. The retrovirus was produced using the Phoenix Retroviral System (Orbigen, San Diego, CA). Cells were infected with retroviral supernatant and selected in puromycine medium. CXCR4 expression was confirmed by FACS.

Experimental metastasis assay

Eight-week-old female nude mice (Charles River, Wilmington, MA) were used in the experimental metastasis assay described previously (Tan et al., 1997). The mice were euthanized 120 days after the injection of cancer cells. The animal experiments were approved and in compliance with the institution's guidelines.

Immunohistochemical staining and analysis

Human breast cancer tissue samples were collected and stained, as described before (Deng et al., 2002; Zhou et al., 2001) with anti-human CXCR4 monoclonal Ab (MAB170; 1:400 dilution) and anti-human HER2 Ab (DAKO; 1:100). CXCR4 positivity was assessed semiquantitatively using staining intensity and percentage by two independent pathological investigators. H score was determined by multiplying the staining intensity by the percentage of positive tumor cells (Camp et al., 1999). Chi-square analysis was used to analyze the relationship between CXCR4 and HER2 expression. The Kaplan-Meier method was used to analyze breast cancer patient overall survival. Statistical significance was defined as $p < 0.05$.

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References

Bernards, R. (2003). Cancer: cues for migration. Nature *425*, 247–248.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell *2*, 243–247.

Camp, R.L., Rimm, E.B., and Rimm, D.L. (1999). Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer *86*, 2259–2265.

Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., and Shepard, H.M. (1992). Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA *89*, 4285–4289.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., and Landau, N.R. (1997). Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. J. Exp. Med. *185*, 621–628.

Deng, J., Miller, S.A., Wang, H.Y., Xia, W., Wen, Y., Zhou, B.P., Li, Y., Lin, S.Y., and Hung, M.C. (2002). β-catenin interacts with and inhibits NF-κB in human colon and breast cancer. Cancer Cell *2*, 323–334.

Fidler, I.J., and Hart, I.R. (1982). Biological diversity in metastatic neoplasms: origins and implications. Science *217*, 998–1003.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D., and Muller, W.J. (1992). Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA *89*, 10578–10582.

Hendriks, B.S., Wiley, H.S., and Lauffenburger, D. (2003). HER2-mediated effects on EGFR endosomal sorting: analysis of biophysical mechanisms. Biophys. J. *85*, 2732–2745.

Hernandez, P.A., Gorlin, R.J., Lukens, J.N., Taniuchi, S., Bohinjec, J., Francois, F., Klotman, M.E., and Diaz, G.A. (2003). Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. Nat. Genet. *34*, 70–74.

Hortobagyi, G.N. (1998). Treatment of breast cancer. N. Engl. J. Med. *339*, 974–984.

Kato, M., Kitayama, J., Kazama, S., and Nagawa, H. (2003). Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. Breast Cancer Res. *5*, R144–R150.

Klos, K.S., Zhou, X., Lee, S., Zhang, L., Yang, W., Nagata, Y., and Yu, D. (2003). Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone. Cancer *98*, 1377–1385.

Knaut, H., Werz, C., Geisler, R., and Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature *421*, 279–282.

Kunwar, P.S., and Lehmann, R. (2003). Developmental biology: Germ-cell attraction. Nature *421*, 226–227.

Landis, S.H., Murray, T., Bolden, S., and Wingo, P.A. (1999). Cancer statistics, 1999. CA Cancer J. Clin. *49*, 8–31.

Li, Y.M., Wen, Y., Zhou, B.P., Kuo, H.-P., Ding, Q., and Hung, M.-C. (2003). Enhancement of Bik antitumor effect by Bik mutants. Cancer Res. *63*, 7630–7633.

Liotta, L.A. (2001). An attractive force in metastasis. Nature *410*, 24–25.

Lloyd, T.E., Atkinson, R., Wu, M.N., Zhou, Y., Pennetta, G., and Bellen, H.J. (2002). Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in *Drosophila*. Cell *108*, 261–269.

Marchese, A., and Benovic, J.L. (2001). Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. J. Biol. Chem. *276*, 45509–45512.

Marchese, A., Raiborg, C., Santini, F., Keen, J.H., Stenmark, H., and Benovic, J.L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. Dev. Cell *5*, 709–722.

Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P. (1988). Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell *54*, 105–115.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature *410*, 50–56.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635–638.

Pietras, R.J., Pegram, M.D., Finn, R.S., Maneval, D.A., and Slamon, D.J. (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. Oncogene *17*, 2235–2249.

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M.R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P.P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature *416*, 451–455.

Proudfoot, A.E. (2002). Chemokine receptors: multifaceted therapeutic targets. Nat. Rev. Immunol. *2*, 106–115.

Rossi, D., and Zlotnik, A. (2000). The biology of chemokines and their receptors. Annu. Rev. Immunol. *18*, 217–242.

Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H.K., Malnati, M.S., Plebani, A., Siccardi, A.G., Littman, D.R., et al. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat. Med. *3*, 1259–1265.

Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated β2-adrenergic receptor and β-arrestin. Science *294*, 1307–1313.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707–712.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. *344*, 783–792.

Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E.J., and Krek, W. (2003). Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature *425*, 307–311.

Steeg, P.S. (2003). Metastasis suppressors alter the signal transduction of cancer cells. Nat. Rev. Cancer *3*, 55–63.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., et al. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature *393*, 591–594.

Taichman, R.S., Cooper, C., Keller, E.T., Pienta, K.J., Taichman, N.S., and McCauley, L.K. (2002). Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res. *62*, 1832–1837.

Tan, M., Yao, J., and Yu, D. (1997). Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. Cancer Res. *57*, 1199–1205.

Tan, M., Jing, T., Lan, K.H., Neal, C.L., Li, P., Lee, S., Fang, D., Nagata, Y., Liu, J., Arlinghaus, R., et al. (2002). Phosphorylation on tyrosine-15 of p34(Cdc2) by ErbB2 inhibits p34(Cdc2) activation and is involved in resistance to taxol-induced apoptosis. Mol. Cell *9*, 993–1004.

Villalba, S., Salvucci, O., Aoki, Y., De La Luz Sierra, M., Gupta, G., Davis, D., Wyvill, K., Little, R., Yarchoan, R., and Tosato, G. (2003). Serum inactivation contributes to the failure of stromal-derived factor-1 to block HIV-I infection in vivo. J. Leukoc. Biol. *74*, 880–888.

Worthylake, R., Opresko, L.K., and Wiley, H.S. (1999). ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. J. Biol. Chem. *274*, 8865–8874.

Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. Nat. Rev. Mol. Cell Biol. *2*, 127–137.

Yeatman, T.J., and Nicolson, G.L. (1993). Molecular basis of tumor progression: mechanisms of organ-specific tumor metastasis. Semin. Surg. Oncol. *9*, 256–263.

Yu, D.H., and Hung, M.C. (1991). Expression of activated rat neu oncogene is sufficient to induce experimental metastasis in 3T3 cells. Oncogene *6*, 1991–1996.

Yu, D., and Hung, M.C. (2000). Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene *19*, 6115–6121.

Yu, D., Wang, S.S., Dulski, K.M., Tsai, C.M., Nicolson, G.L., and Hung, M.C. (1994). c-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. Cancer Res. *54*, 3260–3266.

Zeelenberg, I.S., Ruuls-Van Stalle, L., and Roos, E. (2003). The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res. *63*, 3833–3839.

Zhou, B.P., and Hung, M.C. (2003). Dysregulation of cellular signaling by HER2/neu in breast cancer. Semin. Oncol. *30*, 38–48.

Zhou, B.P., Hu, M.C., Miller, S.A., Yu, Z., Xia, W., Lin, S.Y., and Hung, M.C. (2000). HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J. Biol. Chem. *275*, 8027–8031.

Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M.C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat. Cell Biol. *3*, 973–982.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595–599.