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Targeting CXCR1/2 Significantly Reduces Breast Cancer Stem Cell Activity and Increases the Efficacy of Inhibiting HER2 via HER2-Dependent and -Independent Mechanisms

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Abstract

Purpose: Breast cancer stem-like cells (CSC) are an important therapeutic target as they are predicted to be responsible for tumor initiation, maintenance, and metastases. Interleukin (IL)-8 is upregulated in breast cancer and is associated with poor prognosis. Breast cancer cell line studies indicate that IL-8 via its cognate receptors, CXCR1 and CXCR2, is important in regulating breast CSC activity. We investigated the role of IL-8 in the regulation of CSC activity using patient-derived breast cancers and determined the potential benefit of combining CXCR1/2 inhibition with HER2-targeted therapy.

Experimental Design: CSC activity of metastatic and invasive human breast cancers ($n = 19$) was assessed *ex vivo* using the mammosphere colony-forming assay.

Results: Metastatic fluid IL-8 level correlated directly with mammosphere formation ($r = 0.652$; $P < 0.05$; $n = 10$). Recombinant IL-8 directly increased mammosphere formation/self-renewal in metastatic and invasive breast cancers ($n = 17$). IL-8 induced activation of EGFR/HER2 and downstream signaling pathways and effects were abrogated by inhibition of SRC, EGFR/HER2, phosphoinositide 3-kinase (PI3K), or MEK. Furthermore, lapatinib, which targets EGFR/HER2, inhibited the mammosphere-promoting effect of IL-8 in both HER2-positive and negative patient-derived cancers. CXCR1/2 inhibition also blocked the effect of IL-8 on mammosphere formation and added to the efficacy of lapatinib in HER2-positive cancers.

Conclusions: These studies establish a role for IL-8 in the regulation of patient-derived breast CSC activity and show that IL-8/CXCR1/2 signaling is partly mediated via a novel SRC and EGFR/HER2-dependent pathway. Combining CXCR1/2 inhibitors with current HER2-targeted therapies has potential as an effective therapeutic strategy to reduce CSC activity in breast cancer and improve the survival of HER2-positive patients. *Clin Cancer Res; 19(3); 643–56. ©2012 AACR.*

Introduction

Breast cancer stem-like cells (CSC) are responsible for tumor initiation, maintenance, and metastasis. They are defined functionally by their capacity to initiate a tumor in immunocompromised mice, to self-renew giving rise to a new tumor when passaged, and their ability to differentiate into non–self-renewing cells which constitute the bulk of the tumor (1). By evading the effects of radiotherapy, chemotherapy, and endocrine therapy, breast CSCs are predicted to be responsible for disease recurrence (2–5).

Primary human breast cancers and breast cancer cell lines contain a subpopulation of cells characterized by their capacity to survive anoikis in nonadherent conditions and form floating colonies known as mammospheres (6–8). By showing enhanced tumor forming capacity *in vivo*, we have shown that anoikis resistant cells represent a breast CSC–enriched population (9). Hence, the mammosphere culture system can be used to investigate factors, which regulate CSC activity and assess the efficacy of novel therapeutic agents.

Up to 25% of breast cancers overexpress human EGF receptor 2 (HER2), which confers a higher rate of recurrence and mortality (10). Studies suggest that HER2 overexpression increases CSC self-renewal and invasion (11). Trastuzumab (Herceptin) has improved the survival of HER2-positive patients, possibly due to its ability to reduce the CSC population (11–13). Despite the success of trastuzumab,

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Translational Relevance

Breast cancer stem-like cells (CSC) are an important cellular target as they are responsible for tumor initiation, maintenance, and metastases. Interleukin (IL)-8 has been reported to be upregulated in breast cancer, associated with poor prognosis, and CSCs in breast cell lines are regulated by its receptors, CXCR1/2.

Our research article establishes that IL-8 levels in metastatic fluids correlate directly to breast CSC activity measured *ex vivo* using patient-derived breast cancer samples. IL-8 stimulation and CXCR1/2 inhibition affects CSC activity in patient-derived samples and we show mechanistically that CXCR1/2 regulates breast stem cell activity via HER2-dependent and independent pathways. Furthermore, we show that inhibiting CXCR1/2 is useful in combination therapies targeting CSC activity in HER2-positive breast cancer. Overall, we establish that activation of HER2 via CXCR1/2 has important biologic and clinical implications and is likely to be especially relevant in patients expressing high levels of IL-8 and other CXCR1/2-activating ligands.

some HER2-positive breast cancers are resistant to treatment resulting in incurable metastatic disease (14, 15). This has led to the development of agents such as lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor. Neoadjuvant lapatinib has been shown to inhibit breast CSC activity in HER2-positive tumors, and lapatinib in combination with chemotherapy reduces time to progression of trastuzumab-resistant patients (3, 16).

Although HER2 is an important regulator of CSC self-renewal, factors within the tumor microenvironment are also important and consequently represent novel therapeutic targets. Targeting these factors in combination with HER2 could reduce disease recurrence and improve survival. Inflammation is an established hallmark of cancer and multiple cytokine networks may be important in regulating breast CSCs activity through paracrine and autocrine routes. Interleukin (IL)-8, which is known to be upregulated in breast cancer and associated with poor prognosis, has been shown to increase CSC self-renewal in cell line models *in vitro* (17–19). IL-8 signals via 2 cell surface G-protein–coupled receptors, CXCR1 and CXCR2. Inhibition of these receptors was recently shown to reduce the CSC population, self-renewal, and increase the efficacy of docetaxel in reducing tumor size in xenografts (20).

We determined the role of IL-8 in the regulation of breast CSC activity using patient-derived breast cancer cells isolated directly from metastatic ascites, pleural effusions, and primary invasive cancers. IL-8 concentrations in metastatic fluid directly correlated with mammosphere formation and IL-8 activation of CXCR1/2 increased patient-derived mammosphere formation and self-renewal *ex vivo*. CXCR1/2 inhibition decreased mammosphere formation and aldefluor positivity and added to the efficacy of inhibiting HER2 in HER2-positive cancers. A novel pathway involving transactivation of EGFR/HER2 was mechanistically responsible for the effect of CXCR1/2 activation on CSC activity. Given the importance of HER2 in the regulation of breast CSC activity (11, 12), a pathway driving the activation of this receptor via CXCR1/2 may have important biologic and therapeutic implications, especially in tumors which express high levels of IL-8 and other CXCR1/2-activating ligands.

Materials and Methods

Patient-derived breast cancers

Nineteen patient-derived breast cancer samples are summarized in Table 1. Ethical approval was granted by the Central Office for Research Ethics Committee (study numbers: 05/Q1402/25 and 05/Q1403/159) and patients gave written informed consent.

Metastatic fluid samples. Fifteen metastatic fluid samples were obtained from 12 patients undergoing palliative drainage of symptomatic ascites or pleural effusions at The Christie, Manchester. Of these, 2 patients provided 2 and 3 samples, respectively. Where more than one fluid sample was obtained from the same patient, these were collected on different days. Estrogen, progesterone, and HER2 receptor status of the primary tumors were reported by the Department of Pathology at The Christie according to established criteria (21, 22). HER2 receptor status was unknown for two patients as they presented before the implementation of routine HER2 testing. Eleven patients were treated with multiple cycles of chemotherapy, with or without endocrine therapy and trastuzumab depending on receptor status. One patient presented with advanced metastatic disease and had not received any prior treatment. Thirteen metastatic samples were obtained from patients with estrogen receptor (ER)-positive primary invasive cancers and 3 samples were obtained from a patient with a HER2-positive primary invasive cancer.

Invasive breast cancer samples. Four invasive breast cancer, samples were obtained from patients undergoing surgery for invasive ductal carcinoma. Three tumors were HER2-positive; these patients were all treated with neoadjuvant trastuzumab and chemotherapy before surgery due to locally advanced disease.

Isolation of breast cancer epithelial cells

Metastatic breast cancer cells were harvested as previously described with minor modifications (9). Invasive breast cancer tissue (1–2 cm^3) was collected, dissected into 1–2 mm^3 cubes, and digested in media comprising Dulbecco's Modified Eagle's Media (DMEM), 15 mmol/L HEPES, 50 μg/mL penicillin/streptomycin (Invitrogen), and 10% collagenase/hyaluronidase (Stem Cell Technologies) at 37°C for 16 hours. Digested tissue was filtered sequentially through 100, 70, and 40 μm sieves. Red blood cells were removed using Lymphoprep (Axis-Shield), and leukocytes were removed with CD45-negative magnetic sorting according to the manufacturer's instructions (Miltenyi Biotech).

Table 1. Summary of metastatic and invasive patient-derived cancers

Sample	Sample origin	Tumor histology	Grade	ER status	PR status	HER2 status	Sites of metastases	Medical treatment	MFE, %	Metastatic fluid IL-8 level, pg/mL
BB3RC36-1	PE	IDC	2	Pos	Pos	Neg	Li and Lu	ET	1.73	UA
BB3RC36-2	PE	IDC	2	Pos	Pos	Neg	Li and Lu	ET	1.43	UA
BB3RC38	Asc	IDC	3	Neg	Neg	Neg	B, Li, and Lu	CT	0.47	42.6
BB3RC39	PE	IDC	3	Neg	Neg	Neg	B, Br, and Li	CT	1.95	201.1
BB3RC40	Asc	IDC	3	Pos	Pos	Neg	Li	CT, ET	0.25	UA
BB3RC41	Asc	IDC	3	Pos	Pos	Neg	Li	CT, ET	1.20	135.9
BB3RC42	PE	IDC	3	Pos	Neg	Neg	Lu	CT, ET	0.27	25.2
BB3RC43-1	Asc	IDC	3	Pos	Pos	Pos	Li and Lu	CT, ET, T	0.98	106.8
BB3RC43-2	Asc	IDC	3	Pos	Pos	Pos	Li and Lu	CT, ET, T	0.81	UA
BB3RC43-3	Asc	IDC	3	Pos	Pos	Pos	Li	CT, ET, T	0.57	UA
BB3RC47	Asc	IDC	3	Pos	Neg	Neg	Li	CT, ET	0.45	54.0
BB3RC48	Asc	ILC	2	Pos	Pos	Neg	Li and Lu	ET	1.32	38.2
BB3RC51	PE	IDC	2	Pos	Pos	Neg	Panc and duod	CT, ET	1.50	94.3
BB3RC53	Asc	IDC	2	Pos	Pos	NK	B, Li	CT, ET	1.0	50.1
BB3RC54	PE	IDC	3	Pos	Pos	NK	B, Li, Lu	CT, ET	1.51	45.1
BB2RC12	IDC	IDC	3	Pos	Neg	Pos	None	Neo CT and T	0.98	NA
BB2RC13	IDC	IDC	3	Neg	Neg	Neg	None	None	2.71	NA
BB2RC14	IDC	IDC	3	Neg	Neg	Pos	None	Neo CT and T	0.30	NA
BB2RC15	IDC	IDC	3	Neg	Neg	Pos	None	Neo CT and T	1.95	NA

NOTE: Nineteen patient-derived breast cancer samples were used in this study. Primary invasive cancer samples are highlighted in gray. Tumor histology and grade for metastatic samples (Asc and PE) relates to the primary cancer. Samples with hyphenated numbers (e.g., BB3RC46-1 and BB3RC46-2) were obtained from the same patient at different time points. Metastatic fluid IL-8 levels were measured following removal of cellular matter using an ELISA.

Abbreviations: Asc, ascites; B, bone; Br, brain; CT, chemotherapy; duod, duodenum; ET, endocrine therapy; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Li, liver; Lu, lung; NA, not applicable; Neg, negative; Neo, neoadjuvant; NK, not known; Panc, pancreas; PE, pleural effusion; Pos, positive; PR, progesterone receptor; T trastuzumab; UA, unavailable.

Mammosphere culture and self-renewal assay

Mammosphere culture was conducted as previously described (9). A detailed description of the mammosphere assay protocol for the quantification of breast stem cell activity is described in our recent publication (23). Single cells were plated at 500 cells/cm^2 and the following treatments were added to the culture media: 100 ng/mL recombinant IL-8 (R&D Systems); 100 nmol/L SCH563705 (CXCR1/2 inhibitor), or 1 μmol/L lapatinib (GSK). Control cells were treated with 0.01% bovine serum albumin [BSA (Fisher Scientific)] in PBS w/v (for IL-8 treatment) and 0.01% dimethyl sulfoxide (DMSO; for SCH563705 and lapatinib treatments). Mammosphere-forming efficiency (MFE) was calculated by dividing the number of mammospheres (colonies > 60 μm in diameter) formed by the number of cells plated and expressed as a percentage. Each experiment was carried out in triplicate. To assess self-renewal, mammospheres were counted on day 7, centrifuged (115 × *g*), and dissociated into a single cell suspension by incubation for 2 minutes at 37°C in trypsin EDTA 0.125% (Sigma), followed by mechanical dissociation as described previously (6). Single cells were replated at 500 cells/cm^2 and the number of secondary mammospheres counted after 7 days. Mammosphere self-renewal was calculated by dividing the number of secondary mammospheres formed by the number of primary mammospheres formed. To test clonality, single cells were plated in individual wells and secondary or tertiary mammosphere colonies photographed at 7 (MCF7/HER2-18; Supplementary Fig. S1A and S1B) or 10 days (patient-derived samples; Fig. 1E and F).

Immunohistochemistry

Patient-derived mammospheres were collected after 7 days, centrifuged at 800 × *g* for 2 minutes, and fixed in 4% formaldehyde for 10 minutes at room temperature. Mammospheres were washed in PBS and resuspended in 1% high melting point agarose (37°C), and paraffin-embedded. Formalin-fixed, paraffin-embedded (FFPE) patient-derived mammospheres were then cut into 3-μm sections as described previously (6). CXCR1 expression was determined using human CXCR1/IL-8 RA antibody (R and D systems, MAB330, clone number 42705) and aldehyde dehydrogenase 1 (ALDH1) expression was assessed using human ALDH1 antibody (BD Biosciences, 611195, clone number 44/ALDH). Antibody binding was detected using Dako Envision Detection System Peroxidase/DAB, Rabbit/Mouse (K5007).

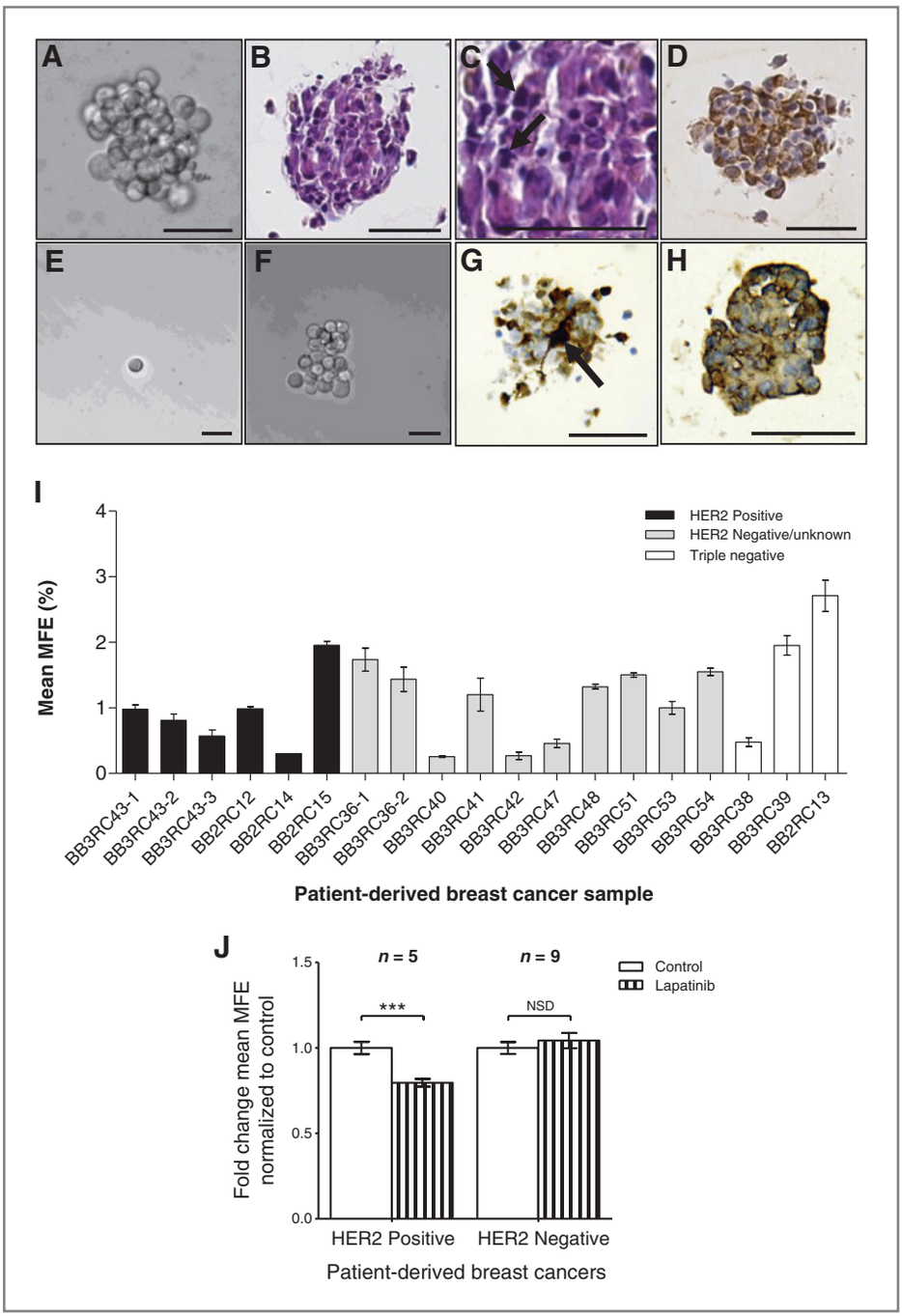


Figure 1. Mammosphere formation from patient-derived breast cancers and effects of EGFR/HER2 inhibition. A, typical bright-field photomicrograph of a mammosphere (>60 μm in diameter) grown from single cells plated at 500 cells/cm^2 after 7 days in nonadherent culture. B–D, photomicrographs of 3-μm sections of an FFPE patient-derived mammosphere showing (B) H&E staining, (C) pleomorphic nuclei (arrows), and (D) pancytokeratin expression (brown). Mammospheres generated after 7 days were dissociated into single cells and replated at 1 cell per well. E, bright-field photomicrograph of a single cell replated in a 96-well plate. F, a mammosphere regenerated from a single cell plated per well after 10 days in nonadherent culture. G and H, photomicrographs of 3-μm sections of an FFPE patient-derived mammosphere showing (G) ALDH1 expression (brown staining), arrow shows intense expression in a single cell and (H) CXCR1 expression (brown staining). A–H, scale bars, 50 μm. I, MFE of 19 independent patient-derived breast cancers. There was no significant difference in MFE between HER2-positive, HER2-negative, and triple-negative cancers. Columns, mean; bars, SEM of triplicate observations. J, MFE of HER2-positive ($n = 5$) and HER2-negative ($n = 9$) cancers after 7 days in nonadherent culture with/without lapatinib (1 μmol/L). Controls were treated with vehicle (0.01% DMSO). Columns, fold change in MFE normalized to control; bars, SEM. ***, $P < 0.001$; NSD, no significant difference.

IL-8 quantification using ELISA

Metastatic fluid was centrifuged at 1,000 × *g* for 10 minutes at 4°C; the supernatant was collected and IL-8 level determined using a human IL-8 ELISA set (BD Biosciences, 555244) according to manufacturer's instructions.

Cell line monolayer culture and treatment

The MCF7/HER2-18 cell line was a gift from Professor CC Benz, University of California San Francisco (San Francisco, CA) and generated by stable overexpression of HER2 in the parental MCF-7 cell line (24). Cells were maintained in monolayer in complete medium [DMEM, 10% fetal calf serum (FCS), and L-glutamine] as previously described (25) and regularly tested for mycoplasma to verify their negative status.

Mammosphere assay. Subconfluent monolayer cells were enzymatically (trypsin-EDTA 0.125%) and manually (25-G needle) dissociated into a single cell suspension.

Cells were plated out (300 cells/cm^2) in mammosphere culture conditions and the following treatments were added to the culture media: 100 ng/mL recombinant IL-8; 100 nmol/L SCH563705, 10 μmol/L lapatinib, 1 to 100 μmol/L LY294002 (PI3K inhibitor, Cell Signalling Technologies, 9901), 1 to 100 μmol/L PD98059 (MEK1 inhibitor, Cell Signalling Technologies, 9900), or 10 μmol/L 4-amino-3-(4-chlorophenyl)-1-(*t*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (PP2, inhibitor of SRC family tyrosine kinases, Sigma-Aldrich, P0042). Control cells were treated with 0.01% BSA in PBS w/v (for IL-8 treatment) and 0.01% DMSO (for SCH563705, lapatinib, LY294002, PD98059, and PP2 treatments). Mammospheres (colonies >60 μm in diameter) were counted after 4 days.

CXCR1/2-activated cell signaling. Cells were plated out (2.5×10^4 cells/cm^2) in adherent culture conditions, left for 24 hours, and then serum-starved for 48 hours. Media were changed 1 hour before treatment to remove any stimulating factors which may have accumulated. Cells were pretreated with vehicle control (DMSO 0.01%), lapatinib (10 μmol/L), SCH563705 (100 nmol/L), or PP2 (10 μmol/L) and then stimulated with IL-8 (100 ng/mL) for 2, 5, 10, 30, and 60 minutes. After the specified time, media were removed, cells were rinsed in ice-cold PBS, and scraped into lysis buffer. Immunoblotting was conducted as previously described (26, 27). Membranes were probed for HER2 (Cell Signalling 2242), phospho-HER2$^{Tyr1221/1222}$ (Cell Signalling 2249), EGFR (Cell Signalling 2232), phospho-EGFR^{1148} (Cell Signalling 4404), AKT (Cell Signalling 9272), phospho-AKT^{Ser473} (Cell Signalling 4051), ERK1/2 (Cell Signalling 4695), phospho-ERK1/2$^{Thr202/Tyr204}$ (Cell Signalling 9101), and β-Actin (Sigma A1978) according to the suppliers' recommendations.

siRNA knockdown of HER2. Two predesigned FlexiTube siRNAs to HER2 (siHER2-14 and siHER2-15) and a non-targeting scramble control (All Stars Negative Control) were purchased from Qiagen. MCF7/HER2-18 cells were plated out at 2×10^5 cells per well (6 well plate) in complete medium and transfected with 30 ng siRNA according to the manufacturer's instructions. After 48 and 72 hours, media were removed, cells were rinsed in ice-cold PBS, and lysates were collected as described above to determine the extent of protein knockdown by imunoblotting. The effect of HER2 knockdown on CSC activity was determined using the mammosphere assay. Monolayer cells were enzymatically and mechanically dissociated at 48 hours posttransfection and plated out in mammosphere culture conditions as above. Cultures were treated with IL-8 (100 ng/mL) or vehicle (0.01% BSA in PBS) and MFE was assessed as above. A minimum of 3 independent experiments each with three technical replicates per experiment was carried out for each construct.

Statistical analysis

Throughout this article, data are represented as mean ± SEM taken over a minimum of 3 independent experiments with 3 technical replicates per experiment unless otherwise stated. Statistical differences in MFE between control and treatment conditions were determined using Whitney *U* test. Kruskal–Wallis test was used to determine statistical differences between conditions and Conover–Inman *post hoc* test used was used to make multiple pairwise comparisons. Statistical differences in mammosphere self-renewal was assessed using Wilcoxon matched pairs test. Differences were considered statistically significant if the 2-tailed probability value (P) was ≤ 0.05. Pearson correlation coefficient (r) was used to measure the correlation between metastatic fluid IL-8 level and mean MFE; a 2-tailed $P \leq 0.05$ was considered statistically significant. Statistical analysis was conducted using StatsDirect statistical software Version 2.7.2.

Results

Mammosphere formation from patient-derived breast cancers and effects of EGFR/HER2 inhibition

To assess breast CSC activity in patient-derived breast cancers, we used the mammosphere assay as previously described (6, 9). Mammospheres formed after 7 days and a typical bright-field image is shown in Fig. 1A. To confirm that the mammospheres were composed of malignant epithelial cells their cellular and nuclear composition was examined histologically. Hematoxylin and eosin (H&E) staining showed that mammospheres were composed of cells with pleomorphic nuclei characterized by large, irregular nuclei and prominent nucleoli, which was consistent with the reported nuclear grade of the original tumors (Fig. 1B and C), confirmed by a clinical breast cancer pathologist (G. Landberg). Pancytokeratin immunostaining confirmed the epithelial origin of the constituent cells in the mammospheres as shown in Fig. 1D. Plating of single mammosphere cells confirmed that mammospheres are clonal in origin (Fig. 1E and F). Immunostaining for the stem cell marker ALDH1 showed strong expression in a single cell (Fig. 1G), whereas the cytokine receptor CXCR1 was expressed on the membrane of the majority of mammosphere cells (Fig. 1H).

MFE of individual cancers ranged from 0.3% to 2.7% (Table 1 and Fig. 1I). There was no statistical difference in MFE between HER2-positive, HER2-negative, and triple-negative patient-derived breast cancers. Lapatinib (1 μmol/L) treatment resulted in a 20.4% ± 2.3% reduction in MFE in HER2-positive cancers compared with control ($P < 0.001$) but had no significant effect on MFE in HER2-negative cancers ($P = 0.605$; Fig. 1J). These data show that the mammosphere colony formation/self-renewal assay can be used in patient-derived invasive and metastatic breast cancers, and despite prior treatment, HER2-positive mammosphere colonies remain responsive to lapatinib.

CXCR1/2 signaling regulates patient-derived mammosphere formation/self-renewal activity

To investigate the involvement of IL-8 in the regulation of mammosphere formation/self-renewal, we analyzed metastatic ascites and pleural effusion fluid for IL-8 protein level. IL-8 was detected in all metastatic fluid samples tested ($n = 10$) with a mean IL-8 concentration of 79.2 pg/mL (range,

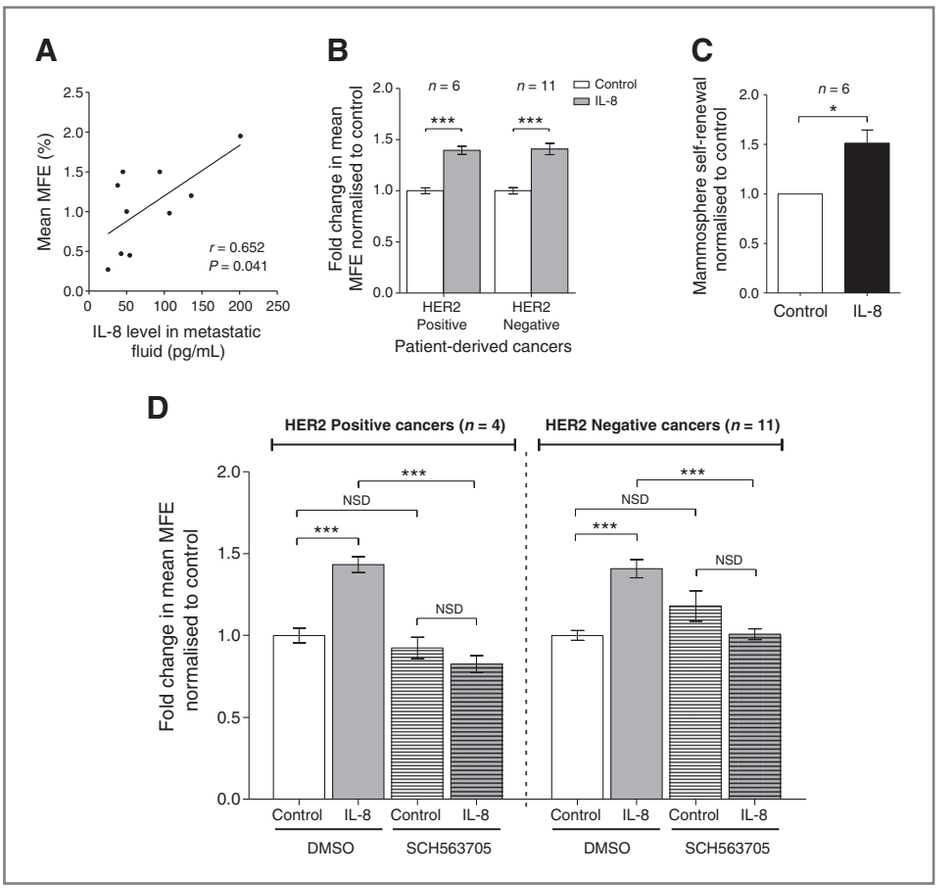


Figure 2. CXCR1/2 signaling regulates patient-derived mammosphere formation/self-renewal activity. A, correlation between metastatic fluid IL-8 level and MFE, $n = 10$ cancers (Pearson correlation coefficient, $r = 0.652$; $P < 0.05$). Metastatic fluid IL-8 level was determined following removal of cellular matter using an ELISA. Details of MFE and metastatic fluid IL-8 level for each cancer are summarized in Table 1. B, MFE of HER2-positive ($n = 6$) and HER2-negative ($n = 11$) patient-derived breast cancers after 7 days in nonadherent culture with/without IL-8 (100 ng/mL). Controls were treated with vehicle. C, secondary mammosphere formation, that is, self-renewal of patient-derived breast cancers (HER2-positive, $n = 2$; HER2-negative, $n = 4$) treated with IL-8 (100 ng/mL) in the first generation. Columns, fold change in mammosphere self-renewal normalized to control; bars, SEM. *, $P < 0.05$. D, MFE of HER2-positive ($n = 4$) and HER2-negative ($n = 11$) cancers treated with either IL-8 (100 ng/mL, gray bars), SCH563705 (100 nmol/L, white stripped bars), or SCH563705 and IL-8 (gray stripped bars) at the stated respective doses. Controls (white bars) were treated with vehicle. B and D, columns, fold change in MFE normalized to control; bars, SEM. ***, $P < 0.001$; NSD, no significant difference.

25.2–201.1 pg/mL), see Table 1. There was a significant direct correlation between metastatic fluid IL-8 level and MFE ($r = 0.652$; $P = 0.041$) as shown in Fig. 2A. These data establish that patients with higher levels of IL-8 in their metastatic fluid have greater *ex vivo* CSC activity.

We therefore investigated the effect of IL-8 on patient-derived mammosphere formation/self-renewal. Mammosphere cultures treated with recombinant IL-8 (100 ng/mL) resulted in a significant increase in MFE in both HER2-positive (39.6% ± 4.0% increase compared with control, $P < 0.001$, $n = 6$ cancers) and HER2-negative cancers (40.8% ± 5.5% increase compared with control, $P < 0.001$, n = 11 cancers), see Fig. 2B. Secondary mammosphere formation of patient-derived breast cancer cells (HER2-positive, $n = 2$; HER2-negative, $n = 4$) treated with IL-8 (100 ng/mL) in the first generation was used to assess self-renewal (51.3% ± 13.2% increase compared with control, $P < 0.05$, $n = 6$ cancers), see Fig. 2C.

The effect of CXCR1/2 inhibition on patient-derived mammosphere formation was subsequently determined using SCH563705, a small-molecule CXCR1/2 antagonist (28, 29). SCH563705 (100 nmol/L) alone had no significant effect on MFE in HER2-positive ($P = 0.454$, $n = 4$) or HER2-negative cancers ($P = 0.236$, $n = 11$), Fig. 2D. However, SCH563705 abrogated the effect of IL-8 in both HER2-positive and HER2-negative cancers, Fig. 2D. These data showed that IL-8 can directly regulate patient-derived mammosphere formation/self-renewal and this can be blocked by inhibiting CXCR1/2.

CXCR1/2 inhibition adds to the efficacy of EGFR/HER2 inhibition in HER2-positive patient-derived mammospheres

Having showed that HER2 and IL-8 independently regulate patient-derived mammosphere formation activi-

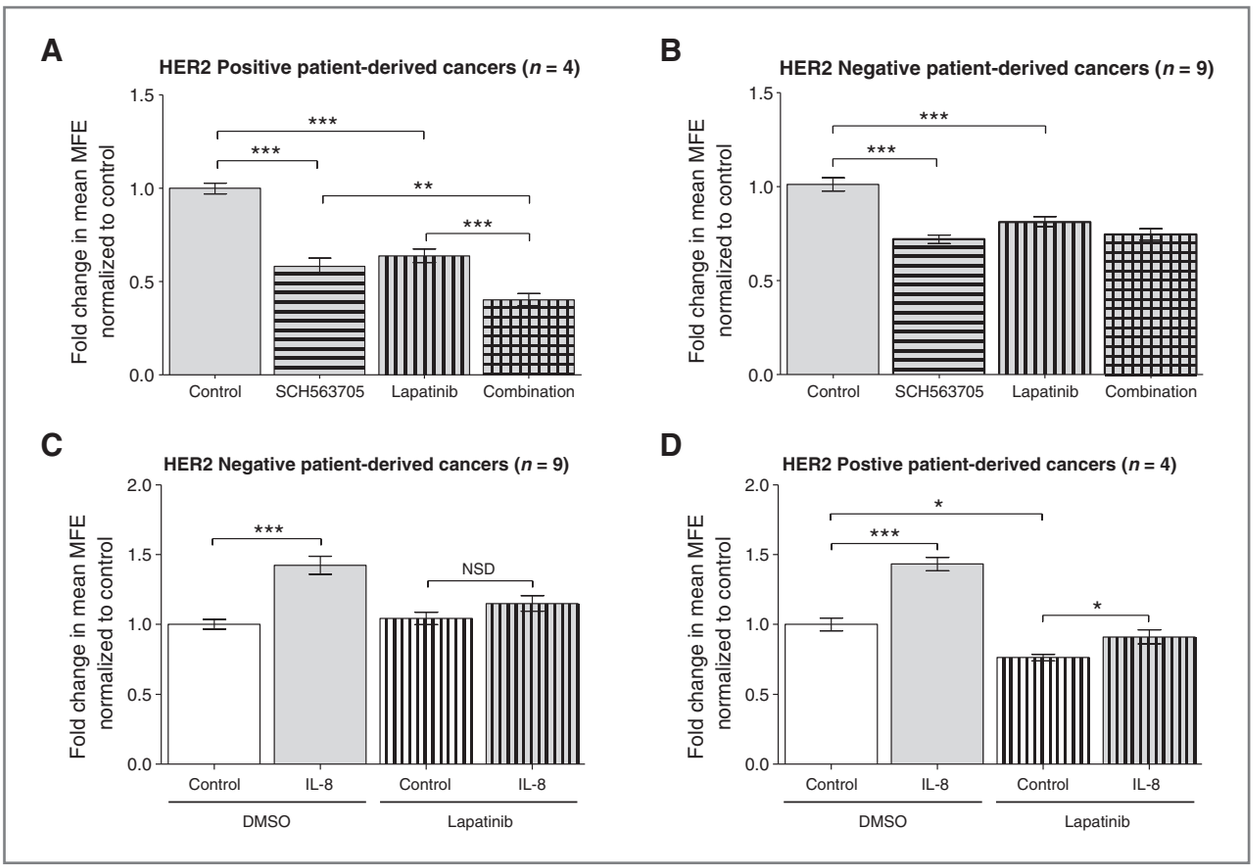


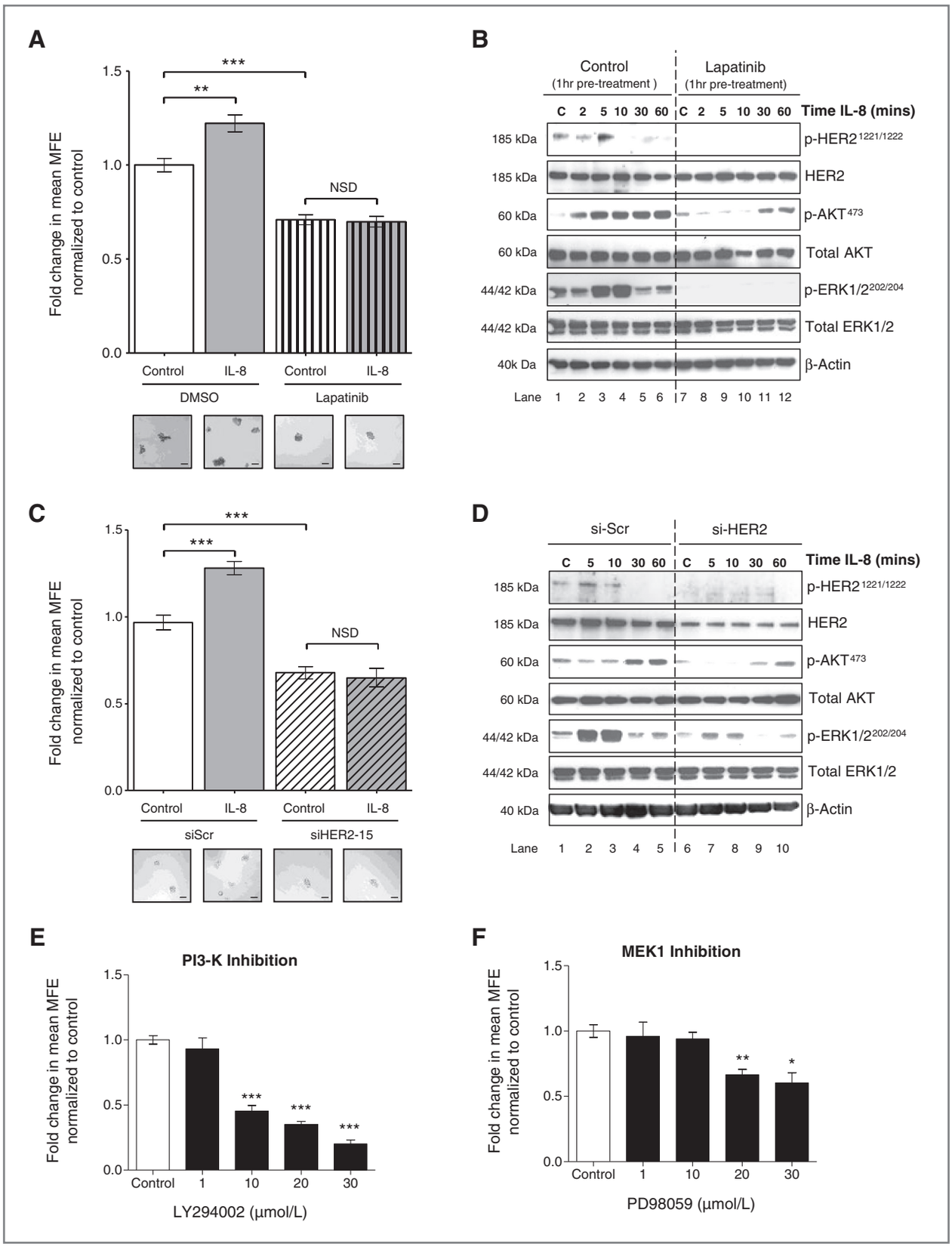
Figure 3. Interaction between CXCR1/2 and HER2 signaling in mammosphere formation. HER2-positive ($n = 4$) and HER2-negative ($n = 9$) patient-derived breast cancer cells were plated out in nonadherent culture conditions. A and B, the effect of SCH563705 (100 nmol/L), lapatinib (1 μmol/L) or a combination of lapatinib and SCH563705 at the stated respective doses on MFE in (A) HER2-positive and (B) HER2-negative breast cancers. All conditions, including control, were supplemented with recombinant IL-8 (100 ng/mL). C and D, the effect of IL-8 (100 ng/mL), lapatinib (1 μmol/L), or lapatinib and IL-8 at the stated respective doses on MFE in (C) HER2-negative and (D) HER2-positive breast cancers. Controls were treated with vehicle. A–D, columns, fold change in MFE normalized to control; bars, SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NSD, no significant difference.

ty, we next determined the effect of inhibiting these signaling pathways in combination. As the inhibitory effect of SCH563705 was dependent on exogenous IL-8, all conditions including control were supplemented with recombinant IL-8 (100 ng/mL). As above, SCH563705 (100 nmol/L) significantly inhibited MFE in HER2-positive and HER2-negative cancers, Fig. 3A and B. Lapatinib significantly inhibited MFE in HER2-positive cancers, but also significantly inhibited MFE in HER2-negative cancers, suggesting that CXCR1/2 signaling is at least partly dependent on EGFR/HER2 receptor activity, see Fig. 3A and B.

Combination treatment of SCH563705 and lapatinib resulted in a significantly greater reduction in MFE compared with either treatment alone in the HER2-positive cancers (combination vs. SCH563705, $P < 0.01$; combination vs. lapatinib, $P < 0.001$) as shown in Fig. 3A. However, combination treatment was no more effective than either treatment alone in the HER2-negative cancers (combination vs. SCH563705, $P = 0.923$; combination vs. lapatinib, $P = 0.053$), see Fig. 3B.

IL-8–mediated effects on patient-derived mammosphere formation are EGFR/HER2-dependent

We have shown that in HER2-negative cancers, lapatinib significantly inhibited mammosphere formation under IL-8 supplemented conditions, whereas no significant effect was observed in the absence of IL-8 (Figs. 3B and 1J). This suggests that the functional effect of IL-8 was dependent on EGFR/HER2 activity. Indeed, in HER2-negative cancers, lapatinib abolished the effect of IL-8 on mammosphere formation as shown in Fig. 3C. A similar effect was observed in the HER2-positive cancers; under lapatinib-treated conditions, the effect of IL-8 was significantly inhibited compared with control conditions (18.7% ± 6.2% increase in MFE compared with 43.3% ± 4.8% increase; $P < 0.05$), see Fig. 3D. These data establish that the effect of CXCR1/2 activation on patient-derived mammosphere formation activity involves an EGFR/HER2-dependent pathway. The data also suggest that downstream of CXCR1/2 in HER2-negative cancers, EGFR is activated and/or HER2 is expressed and activated even though the *HER2* gene is not amplified. Further inhibition of patient-derived mammosphere



formation activity with SCH563705 in combination with lapatinib in HER2-positive cancers indicates that CXCR1/2 signaling is also mediated via an EGFR/HER2-independent pathway.

Transactivation of EGFR/HER2 by CXCR1/2

We next used the HER2-positive breast cancer cell lines, MCF7/HER2-18 (24, 25) and SKBR3, to further explore the role of EGFR/HER2 in CXCR1/2-mediated signaling. Subconfluent cells grown in monolayer were detached and single cells were plated out (300 cells/cm^2) in mammosphere culture conditions. Treatment with IL-8 (100 ng/mL) significantly increased MFE and self-renewal (Fig. 4A and C and Supplementary Fig. S1C, S1E, and S1F). SCH563705 and lapatinib each decreased MFE similarly to the patient-derived cancers and abrogated the effect of IL-8 (Fig. 4A and Supplementary Fig. S1C, S1G, S1I, and S1J). SCH563705 also significantly reduced Aldefluor positivity, further supporting its role in regulating CSC activity (Supplementary Fig. S1H).

Next, we investigated the role of EGFR/HER2 in mediating downstream signaling events induced by CXCR1/2 activation by Western blotting for EGFR, HER2, AKT, and ERK1/2 and their phosphorylated, activated isoforms. MCF7/HER2-18 or SKBR3 cells were incubated with lapatinib (10 μmol/L MCF7/HER2-18; 1.5 μmol/L SKBR3) or vehicle control (0.01% DMSO) for 1 hour before stimulation with IL-8 (100 ng/mL) for up to 60 minutes. Under control conditions, a rapid, transient increase in phosphorylation of HER2$^{1221/1222}$ (MCF7/HER2-18) or EGFR^{1148} (SKBR3) was observed within 5 minutes of stimulation with IL-8 (Fig. 4B and Supplementary Fig. S2A). Importantly, inhibiting CXCR1/2 was shown to prevent HER2 phosphorylation (Supplementary Fig. S1D). IL-8 also markedly increased phosphorylation of AKT (p-AKT^{473}) and ERK1/2 (p-ERK1/2$^{202/204}$) within 10 minutes (Fig. 4B and Supplementary Fig. S2A).

One hour pretreatment with lapatinib markedly reduced basal levels of p-HER2$^{1221/1222}$ and p-ERK1/2$^{202/204}$ in MCF7/HER2-18 cells and p-AKT^{473} and pERK1/2$^{202/204}$ in SKBR3 cells (Fig. 4B and Supplementary Fig. S2B). Importantly, lapatinib pretreatment completely blocked the effect of IL-8 on p-HER2$^{1221/1222}$ and p-ERK1/2$^{202/204}$ and partially inhibited the effect of IL-8 on p-AKT^{473} in MCF7/HER2-18 cells (Fig. 4B) and inhibited p-ERK1/2$^{202/204}$ and p-AKT^{473} in SKBR3 cells (Supplementary Fig. S2B). These studies show that activation of AKT and ERK1/2 signaling pathways by CXCR1/2 is mainly mediated via transactivation of EGFR/HER2 which can be inhibited by lapatinib. The importance of AKT and ERK1/2 signaling pathways was confirmed by targeting the upstream kinases, phosphoinositide 3-kinase (PI3K) and MEK1, using the inhibitors LY294002 and PD98059, respectively. They were both effective in reducing mammosphere formation in MCF7/HER2-18 cells (Fig. 4E and F) and the PI3K inhibitor significantly reduced mammosphere formation activity in SKBR3 cells (Supplementary Fig. S2C and S2D).

To validate the role of HER2 in mediating the signaling events induced by CXCR1/2, we targeted HER2 expression using siRNA. First, the efficacy of 2 siRNA constructs targeting HER2 (siHER2-14 and siHER2-15) was assessed by Western blotting. A nontargeting scramble sequence was used as a control (siScr). Successful knockdown of HER2 protein level was achieved with siHER2-14 and siHER2-15 at 48 and 72 hours posttransfection compared with scramble control siScr (Supplementary Fig. S3A). Neither construct had any effect on EGFR gene expression (Supplementary Fig. S3B). Mammosphere formation was assessed 48 hours posttransfection and a 30.2% ± 3.3% and 34.3% ± 2.5% reduction in MFE was observed with siHER2-14 and siHER2-15 respectively compared with siScr (Supplementary Fig. S3C).

Next, the effect of HER2 knockdown on mammosphere formation induced by IL-8 was assessed. IL-8 (100 ng/mL) increased MFE in cells transfected with siScr (28.2% ± 3.8% compared with vehicle control, $P < 0.001$), Fig. 4C. However, IL-8 had no effect on cells transfected with siHER2-15 as shown in Fig. 4C. A similar effect was observed with siHER2-14 construct. These findings validate experiments with lapatinib show that HER2 function is critical in mediating the effect of CXCR1/2 activation on breast CSC activity.

To corroborate the above functional studies, the effect of IL-8 on HER2 activation and downstream signaling pathways following HER2 knockdown was investigated. MCF7/HER2-18 cells transfected with either siScr or siHER2 (siHER2-15) were stimulated with IL-8 (100 ng/mL) for 5, 10, 30, and 60 minutes. Under siScr conditions, IL-8

Figure 4. CXCR1/2-mediated effects on mammosphere formation and downstream signaling pathways are dependent on HER2 phosphorylation. A, MCF7/HER2-18 cells were plated out in nonadherent culture conditions and treated with either IL-8 (100 ng/mL), lapatinib (10 μmol/L), or lapatinib and IL-8 at the stated respective doses. Controls were treated with vehicle. Graph shows the effect of treatments on MFE. Inserts show representative bright-field photomicrographs of mammospheres cultured under each condition. Scale bars, 100 μm. B, MCF7/HER2-18 cells were plated out in adherent culture conditions and pretreated with either vehicle control (lanes 1–6) or lapatinib (10 μmol/L, lanes 7–12) for 1 hour before stimulation with IL-8 (100 ng/mL) for 2, 5, 10, 30, and 60 minutes. Controls (C) were treated with vehicle for 10 minutes. Immunoblots show the effect of IL-8 on phosphorylation of HER2, AKT, and ERK1/2. C, MCF7/HER2-18 cells transfected with either scramble control siRNA (siScr) or siRNA to HER2 (siHER2-15) were plated out in nonadherent culture conditions and treated with either IL-8 (100 ng/mL) or vehicle control. Graph shows the effect of these treatments on MFE. Inserts show representative bright-field photomicrographs of mammospheres cultured under each condition. Scale bars, 100 μm. D, MCF7/HER2-18 cells were transfected with either siScr (lanes 1–5) or siHER2-15 (lanes 6–10). After 24 hours, cells were serum starved for 48 hours and then stimulated with IL-8 (100 ng/mL) for 5, 10, 30, and 60 minutes. Controls (C) were treated with vehicle for 10 minutes. Immunoblots show the effect of IL-8 on phosphorylation of HER2, AKT, and ERK1/2. E and F, MCF7/HER2-18 cells were plated out in nonadherent culture conditions and treated with increasing doses of LY294002, a PI3K inhibitor (E) and PD98059, a MEK1 inhibitor (F). Graphs show the effect of treatments on MFE. A, C, E, and F, columns, fold change in MFE normalized to control; bars, SEM, $n = 3$ independent experiments. **, $P < 0.01$; ***, $P < 0.001$; NSD, no significant difference. B and D, blots are representative of 3 independent experiments. β-Actin was used as a loading control; molecular weight for each protein is shown.

increased p-HER2$^{1221/1222}$, p-AKT^{473}, and p-ERK1/2$^{202/204}$ in a time-dependent manner, Fig. 4D. However, under HER2 knockdown conditions, phosphorylation of HER2 by IL-8 was no longer detectable, and the increase in p-AKT^{473} and p-ERK1/2$^{202/204}$ was reduced compared with scramble control conditions as shown in Fig. 4D. These effects were similar to those observed with lapatinib (Fig. 4B), providing further experimental evidence that activation of AKT and ERK1/2 signaling pathways by CXCR1/2 is mainly mediated via transactivation of HER2. Remaining ERK1/2 phosphorylation where HER2 is knocked down (Fig. 4D) compared with lapatinib treatment (Fig. 4B) is hypothesized to be because of either incomplete loss of HER2 protein or the continued activity of EGFR.

Next, we investigated the mechanism by which HER2 becomes transactivated by CXCR1/2. HER2 is a member of the EGFR family of receptors and while few studies have shown transactivation of HER2 by G-protein–coupled receptors, a well-described mechanism of EGFR transactivation by G-protein–coupled receptors involves ligand-dependent activation. Agonist stimulation of G-protein–coupled receptors is reported to activate various metalloproteases, such as members of the disintegrin and metalloprotease (ADAM) family, which results in the cleavage and release of membrane-bound EGF-like ligands. These soluble ligands can subsequently activate EGFR via a paracrine and/or autocrine manner (30–32).

Unlike EGFR, HER2 is devoid of its own activating ligand but becomes activated by heterodimerizing with EGFR. We therefore hypothesized that HER2 may become activated by heterodimerizing with EGFR following ligand-dependent transactivation of EGFR by CXCR1/2 which are both G-protein–coupled receptors. This was investigated using an EGFR blocking antibody which prevents EGF-like ligands binding to the ligand-binding domain of the receptor. Following dose optimization, the effect of an EGFR blocking antibody on HER2 activation and downstream signaling pathways induced by CXCR1/2 activation was determined. Adherent MCF7/HER2-18 cells were serum-starved for 48 hours and then pretreated with either IgG_1 control antibody (5 μg/mL) or EGFR ligand blocking antibody (5 μg/mL) for 1 hour before stimulation with IL-8 (100 ng/mL). Phosphorylation of HER2 and ERK1/2 induced by IL-8 was not inhibited by the EGFR blocking antibody as shown in Fig. 5A. These findings indicate that transactivation of HER2 by CXCR1/2 is independent of ligand activation of EGFR and therefore likely to be mediated via an intracellular mechanism. We hypothesized that SRC kinase could be involved, as it is known to transduce intracellular signals downstream of cytokine receptors and to interact with EGFR/HER2 receptors. We therefore tested the SRC family kinase inhibitor PP2 for its effects on IL-8–induced signaling and established that it effectively blocks HER2, AKT, and ERK1/2 phosphorylation in MCF7/HER2-18 cells (Fig. 5B). In addition, PP2 abrogates IL-8–induced mammosphere formation activity (Fig. 5C). These data indicate that SRC family kinases mediate an intracellular mechanism downstream of CXCR1/2 that transactivates HER2, AKT, and ERK1/2 signaling pathways and contribute to mammosphere formation activity (Fig. 5D).

Discussion

We investigated the role of IL-8 in regulating activity of breast CSCs isolated directly from patients and determined the benefit of combining CXCR1/2 inhibition with HER2-targeted therapy. IL-8 concentration measured in fluid from metastatic ascites and pleural effusions directly correlated with mammosphere formation activity *ex vivo*. Using patient-derived breast cancers, we report for the first time that IL-8 is important in regulating mammosphere formation and self-renewal activity. Mechanistically, we showed that IL-8/CXCR1/2–mediated effects are critically dependent on HER2 function and inhibition of CXCR1/2 signaling added to the efficacy of inhibiting HER2 on HER2-positive mammosphere formation activity.

Like normal mammary stem cells, breast CSCs have the capacity to survive in nonadherent culture conditions and form floating colonies known as mammospheres (6, 8, 33). This culture system provides a valuable method of assessing CSC activity *in vitro* and has been used extensively in breast cancer cell lines. We and others have used this technique to investigate factors affecting CSC activity and the efficacy of novel inhibitors in primary pre-invasive, invasive, and metastatic breast cancers *ex vivo* (3, 6, 9). Previous studies have suggested that HER2-positive preinvasive and invasive breast cancers have a greater proportion of CSCs, as determined by their enhanced capacity to form mammospheres and greater $CD24^-/CD44^+$ cell fraction respectively, compared with HER2-negative cancers (3, 6). In contrast, we found no statistical difference in MFE in samples mostly derived from metastatic fluid between HER2-positive and HER2-negative cancers. However, it is possible that our sample size was too limited to observe statistically significant differences between tumor sub-types.

Metastatic fluid contains inflammation-related proteins and diverse cell types including mesothelial cells, macrophages, neutrophils, and erythrocytes. We measured IL-8 in ascites and pleural effusion fluid from patients with metastatic breast cancer and found a significant positive correlation between metastatic fluid IL-8 level and MFE of tumor cells derived from these fluids cultured *ex vivo*. Whether increased mammosphere formation was a consequence of metastatic fluid IL-8 level in the patient is unknown, as is whether the source was the tumor cells, other cells in the fluid or relates to systemic IL-8 levels.

SCH563705, a small-molecule CXCR1/2 inhibitor, prevented the effect of recombinant IL-8 on mammosphere formation. However when used alone, the compound had no effect on mammosphere formation. These data suggest that while CSCs respond to IL-8, they do not secrete it in an autocrine loop. Thus, IL-8 probably acts via a paracrine route, secreted by stromal cells and inflammatory cells which comprise the tumor microenvironment (34, 35).

These observations have important clinical implications as there is strong evidence that CSC activity is related to prognostic factors. We and others have previously shown an

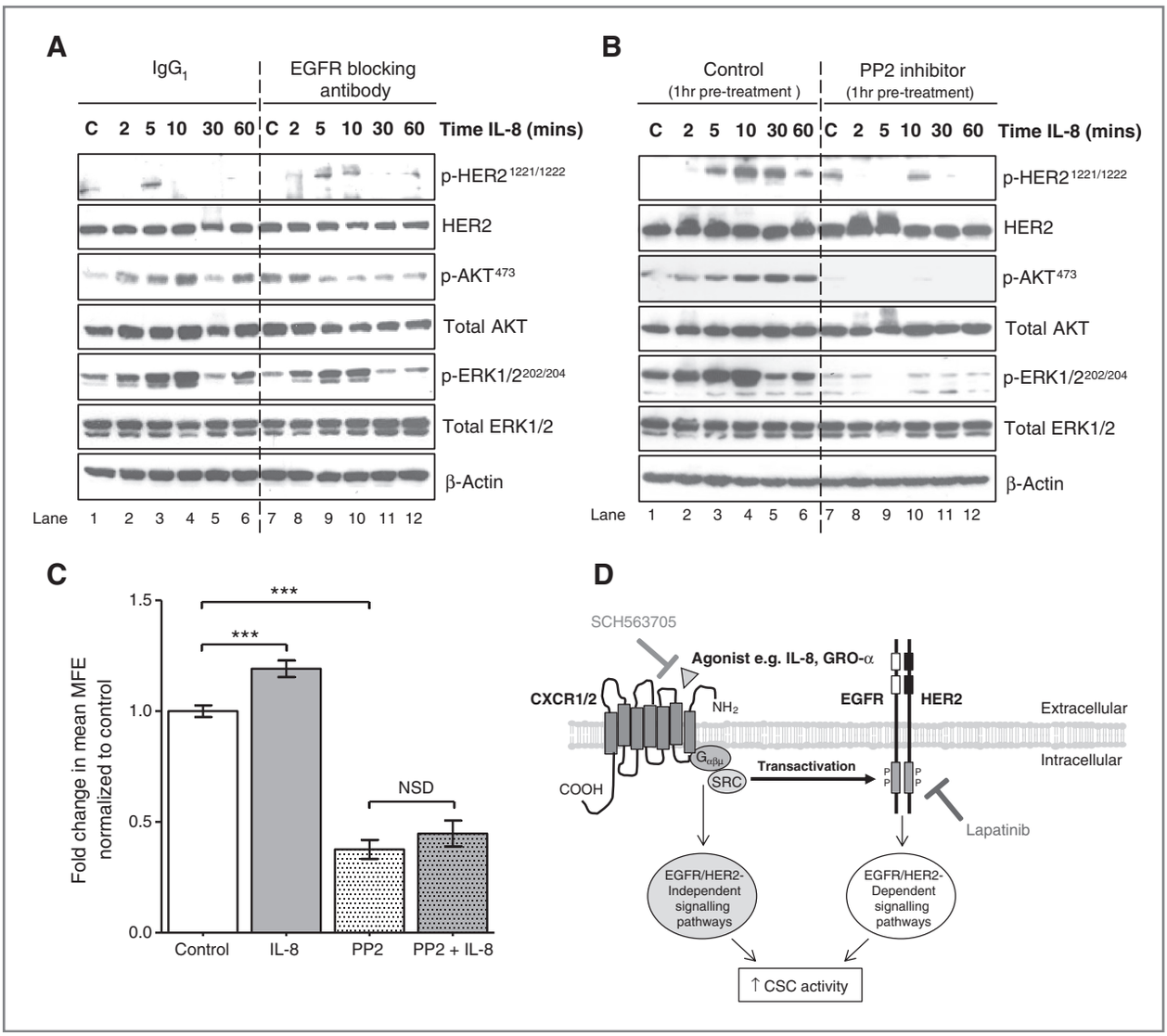


Figure 5. Transactivation of HER2 by CXCR1/2 is independent of ligand activation of EGFR but dependent on SRC kinase activity. A, MCF7/HER2-18 cells were plated out in adherent culture conditions and pretreated with either IgG_1 control antibody (5 μg/mL, lanes 1–6) or EGFR ligand blocking antibody (5 μg/mL, lanes 7–12) for 1 hour before stimulation with IL-8 (100 ng/mL) for 2, 5, 10, 30, and 60 minutes. Controls (C) were treated with vehicle for 10 minutes. Immunoblots show the effect of IL-8 on phosphorylation of HER2, AKT, and ERK1/2. B, MCF7/HER2-18 cells were plated out in adherent culture conditions and pretreated with either vehicle control (lanes 1–6) or PP2 (10 μmol/L, lanes 7–12) for 1 hour before stimulation with IL-8 (100 ng/mL) for 2, 5, 10, 30, and 60 minutes. Controls (C) were treated with vehicle for 10 minutes. Immunoblots show the effect of IL-8 on phosphorylation of HER2, AKT, and ERK1/2. C, MCF7/HER2-18 cells were plated out in mammosphere culture conditions and treated with either IL-8 (100 ng/mL), PP2 (10 μmol/L), or PP2 and IL-8 at the stated respective doses. Graph shows the effect of these treatments on MFE. Columns, fold change in MFE normalized to control; bars, SEM, $n = 3$ independent experiments. ***, $P < 0.001$; NSD, no significant difference. D, putative model of the interaction of CXCR1/2 and HER2 in the regulation of HER2-positive breast CSC activity. Agonist stimulation of CXCR1/2 causes transactivation of HER2 which increases CSC activity via HER2-dependent signaling pathways. Activation of HER2-independent signaling pathways by CXCR1/2 also promotes CSC activity. HER2 inhibition with lapatinib prevents CXCR1/2-mediated transactivation of EGFR/HER2 resulting in a decrease in CSC activity. Blockade of CXCR1/2 with SCH563705 causes a further reduction in CSC activity through inhibition of HER2-independent signaling pathways.

enrichment of breast CSCs with an increase in tumor grade, and high-grade tumors are known to be associated with poorer prognosis (6, 36).

Although there is evidence that CXCR1/2 can transactivate EGFR in other tumor types (32, 37–39), there are no previous reports that CXCR1/2 causes transactivation of ErbB family receptors in breast cancer. Our data show, for the first time, that CXCR1/2 regulates mammosphere formation via an EGFR/HER2-dependent pathway. In both HER2-positive and HER2-negative cancers, the mammosphere-promoting effect of IL-8 was inhibited by lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor. siRNA knockdown of HER2 in MCF7/HER2-18 cells blocked the effect of IL-8 on mammosphere formation. Together these findings

establish that HER2 function is essential in mediating the effect of CXCR1/2 activation on breast CSC activity. There may also be a role for HER3 and HER4 in modulating EGFR/HER2 effects downstream of CXCR1/2 but we have yet to address this possibility. A very recent publication reports that HER2/HER3 activity leads to overexpression of IL-8, which would potentially increase auto-activation of the HER2 pathway (40).

Mechanistically, we discovered that IL-8 induced transient phosphorylation of EGFR/HER2 and downstream AKT and ERK1/2 signaling pathways. Detectable HER2 phosphorylation varied in its timing from 2 to 30 minutes (compare Figs. 4B and D, 5A and 5B and Supplementary Fig. S1D) but consistently stimulated downstream AKT and ERK1/2 phosphorylation. These signaling events were dependent on HER2 function as both pharmacologic inhibition with lapatinib and genetic knockdown of HER2 inhibited the effect of IL-8 on phosphorylation of HER2, AKT, and ERK1/2. These studies indicate that activation of AKT and ERK1/2 signaling pathways by CXCR1/2 occurs via transactivation of HER2. PI3K and/or MEK1 inhibition upstream of AKT and ERK1/2 markedly reduced mammosphere formation activity in our study. This is important as these signaling pathways are critical in regulating breast CSC activity (41).

Given the importance of HER2 in regulating breast CSC activity (11, 12), a pathway driving the activation of this receptor via CXCR1/2 will have important biologic consequences. This will be very relevant in ER-negative and HER2-positive breast cancers which are reported to have higher levels of IL-8 and this may contribute to their aggressive phenotype (42, 43).

Using an EGFR blocking antibody, we found that phosphorylation of HER2 and ERK1/2 by IL-8/CXCR1/2 was independent of ligand binding to EGFR. As recently showed for CXCR4, another cytokine receptor, we found transactivation of HER2 by CXCR1/2 involves a ligand-independent intracellular signaling mechanism involving SRC kinases (44).

Inhibiting CXCR1/2 added to the efficacy of inhibiting HER2 in our assays, resulting in a greater reduction in mammosphere formation in HER2-positive breast cancers. As the effect of IL-8 was only partially inhibited by blocking HER2 in HER2-positive cancers, we propose that the additional benefit of targeting CXCR1/2 was derived from inhibition of HER2-independent signaling pathways. A putative model of the interaction of CXCR1/2, SRC, and HER2 in the regulation of breast CSC activity is shown in Fig. 5D. CXCR1/2 signaling is known to involve many signal transduction pathways and some of these, such as focal adhesion kinase, have been implicated in breast CSC maintenance (45). Thus, CXCR1/2 inhibition likely prevented the activation of these additional HER2-independent signaling pathways.

In summary, our findings indicate that combining CXCR1/2 inhibition with HER2 inhibition is an effective strategy to target HER2-positive mammosphere formation activity. Interestingly, we also observed that EGFR/HER2 inhibition can prevent CXCR1/2-induced mammosphere formation activity in HER2-negative tumor subtypes. Validation that this would be effective in targeting CSCs requires *in vivo* studies, although it has already been shown that inhibition of HER2 or CXCR1/2 alone decreases tumor growth, metastases, and CSC self-renewal in xenograft mouse models (12, 20). Phase I clinical trials indicate that repertaxin, a CXCR1/2 inhibitor, is well tolerated, and trials are under development to determine its efficacy in breast cancer (46, 47). Given the emerging importance of IL-8 as a key environmental regulator of breast CSC activity, combining CXCR1/2 inhibitors with current HER2-targeted therapies has the potential as an effective therapeutic strategy to decrease CSC activity and improve the survival of patients with HER2-positive breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.K. Singh, N.J. Bundred, B.M. Simões, A. Shergill, G. Landberg
Writing, review, and/or revision of the manuscript: J.K. Singh, G. Farnie, N.J. Bundred, A. Shergill, S. Howell, R.B. Clarke
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.K. Singh
Study supervision: N.J. Bundred, G. Landberg, R.B. Clarke

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