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PRINCIPAL INVESTIGATOR: Ingrid Schraufstatter, Ph.D.

CONTRACTING ORGANIZATION: La Jolla Institute for Molecular Medicine
San Diego, CA 92121

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Ingrid Schraufstatter, Ph.D.

E-Mail: ingrid@ljimm.org

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14. ABSTRACT

The chemokine receptor CXCR4 is functionally expressed on the cell surface of various cancer cells, and plays a role in cell proliferation and migration of these cells. Specifically, in breast cancer cells the CXCR4/CXCL12 axis has been implicated in cell migration in vitro and in metastasis in vivo, but the underlying signaling mechanisms are incompletely understood. The xenograft-derived MDA-MB-231 breast cancer cell line (231mfp), which was shown previously to grow more aggressively than the parent cells, showed increased CXCR4 expression at the mRNA, total protein and cell surface expression level. This correlated with an enhanced response to CXCL12, specifically in augmented and prolonged Akt activation in a Gi, Src family kinase and PI-3 kinase dependent fashion. 231mfp cells migrated towards CXCL12—in contrast to the parent cell line—and this chemotaxis was blocked by inhibition of Gi, Src family kinases, PI-3 kinase and interestingly, Akt itself, as could be shown with two pharmacological inhibitors, a dominant negative Akt construct and with Akt shRNA. Collectively, we have demonstrated that prolonged Akt activation is an important signaling pathway for breast cancer cells expressing CXCR4 and is necessary for CXCL12-dependent cell migration.

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INTRODUCTION

Background: Early metastasis and resistance to chemotherapy remain problems in breast cancer therapy, and a subgroup of breast cancers is associated with poor clinical outcome even with early detection. Expression of CXCR4, the receptor for the chemokine SDF-1, and of the receptor tyrosine kinase HER2 are both poor prognostic indicators in breast cancer, which appear to be up-regulated concomitantly (1). HER2-dependent Akt activation has been shown to increase CXCR4 expression in breast cancer cells (2), and SDF-1 can transactivate HER2 (3). Our preliminary results indicate that cross-talk between the two receptors also occurs in reverse, i.e. an inhibitor of HER2 (AG825) attenuated SDF-1 mediated Akt phosphorylation in 231 mfp cells. This subline was derived from a tumor formed by MDA-MB-231 cells (ATCC) in the mammary fat pad of immune-compromised mice. Gene arrays indicated that 231 mfp cells expressed 8 x as much CXCR4 as the parent cell-line, and FACS analysis confirmed that >85% of these cells showed surface expression of CXCR4. This correlated with prolonged (up to 2 hr) Akt phosphorylation following stimulation with SDF-1, a pathway which protects cancer cells from apoptosis. These cells do not express SDF-1 themselves, but high SDF-1 expression was seen in the tumor stroma. Furthermore, results in small cell lung cancer cells indicate that SDF-1 expressed by co-cultured M2-10B4 bone-marrow stromal cells (ATCC) protected from cell killing by chemotherapeutic agents. This was prevented with a CXCR4 inhibitor, or almost as efficiently with anti-β_1- or α_5-integrin antibody (4). Protection from cell killing was only observed, if SDF-1 was presented on a surface (M2-10B4 cells or fibronectin-coated culture dish), indicating the importance of integrins down-stream of CXCR4 activation. Possible links between CXCR4 and integrins include GSK-3β, - a downstream target of Akt which increases the recycling rate of integrins including $\alpha_5\beta_1$, thus enhancing their activity (5), - and integrin-linked kinase (ILK). ILK activation has been associated with prolonged Akt activation in breast cancer cells, but not in normal epithelial cells, and HER2 over-expression leads to ILK over-expression. The scheme shows a hypothetical synopsis of these pathways: Increased activity is shown in red, increased expression in black and inhibition in blue. In summary, the intertwined CXCR4/integrin/HER2 pathways are expected to cause Akt-mediated resistance to chemotherapeutic agents in addition to increased metastasis.

Hypothesis: Since SDF-1 induces prolonged Akt activation in 231 mfp cells, a pathway which activates multiple anti-apoptotic pathways, it is hypothesized that CXCR4 expression renders cells resistant to chemotherapy in the presence of the SDF-1. Inhibition of SDF-1/CXCR4 axis is expected to overcome the resistance to chemotherapeutic agents. In addition we will determine the mechanism which leads to prolonged Akt activation in the presence of SDF-1.

Objectives: Specific Aim 1: To determine the role of surface-presented SDF-1 in chemo-resistance of CXCR4-expressing breast cancer cells, recovery of response to chemotherapeutic drugs in the presence of CXCR4 inhibitors. **Specific Aim 2:** To determine the relationship between CXCR4, HER2 and α_5, β_1 integrin expression and Akt activation in order to gain a mechanistic understanding of the behavior observed in aim 1.

Methods: Aim 1: Breast cancer cell lines expressing CXCR4 (231 mfp and BT-474) will be exposed to surface-presented SDF-1 and cell killing with chemotherapeutic drugs (doxorubicin, cyclophosphamide and paclitaxel) will be determined +/- inhibitors (small molecular weight inhibitor and siRNA), which block CXCR4 or Akt activation. Aim 2: 231 mfp cells will be stimulated with SDF-1, and Akt, HER2 and GSK-3β phosphorylation will be determined by Western blots. In parallel, surface expression of CXCR4, HER2 and α_5 and β_1 integrins will be detected by FACS. Inhibitors and antibodies (against CXCR4, HER2, Akt, GSK and ILK and α_5 and β_1 integrins) will be used to screen signaling pathways. If inhibitors show an effect, the validity of the results will be verified with siRNA or dominant negative constructs.

Relevance: If it is true that CXCR4 activation mediates resistance to chemotherapy, inhibition of the SDF-1/CXCR4 pathway will increase the efficacy of chemotherapy in cancers that express CXCR4. CXCR4 inhibitors have been developed for use in HIV infection. While the drugs proved safe in phase II clinical trials, they showed limited effect for protection from virus infection. However, these inhibitors would be available for use as 'sensitizers' during chemotherapy.

BODY

CXCR4 expression by MDA-MB-231 and 231mfp breast cancer cells

To explore CXCR4-dependent signaling pathways which mediate breast cancer cell chemotaxis, we chose the invasive breast cancer cell line MDA-MB-231 as a model, which had been used by other investigators to determine CXCL12 mediated cellular responses (6). When these cells failed to be chemo-attracted by CXCL12 in our hands, CXCR4 expression was checked at the mRNA and protein level. Initially CXCR4 mRNA expression was analyzed by standard reverse transcription-PCR (RT-PCR), which indicated that MDA-MB-231 cells expressed barely detectable levels of CXCR4 mRNA. In contrast, 231mfp cells, isolated from an MDA-MB-231 xenograft tumor growing in the mouse mammary fat pad (mfp) and passaged for up to six passages (7), showed increased CXCR4 mRNA (Fig. 1A). Real-time PCR results indicated a fourfold increase of CXCR4 expression in 231mfp cells (Fig. 1B). Subsequently, Western blots showed that 231mfp cells also expressed increased CXCR4 protein (Fig. 1C), which is quantified in Fig. 1D. The most dramatic difference was seen, however, when cell surface expression of CXCR4 was detected by flow cytometric analysis (Fig. 1E). Nearly 50% of 231mfp cells showed cell surface staining for CXCR4 compared to 2% positive cells in the parental MDA-MB-231 cells, which indicated that the 231mfp subline was a good model to study CXCR4 signaling in breast cancer cells. In contrast in the parent cell line, much of the CXCR4 protein present did not appear to be amenable to ligand activation, as most of the receptor appeared to reside intracellularly. To verify this conclusion CXCR4-immunostaining was performed in permeabilized cells. Confocal microscopy of these cells indicated that much of the CXCR4 staining in the parent MDA-MB-231 cells was intracellular with only very weak staining in the plasma membrane, while 231mfp staining showed prominent receptor staining in the plasma membrane (Fig. 1F), but also, interestingly, strong staining of the nucleus, presumably of the nuclear membrane (Fig. 1F).

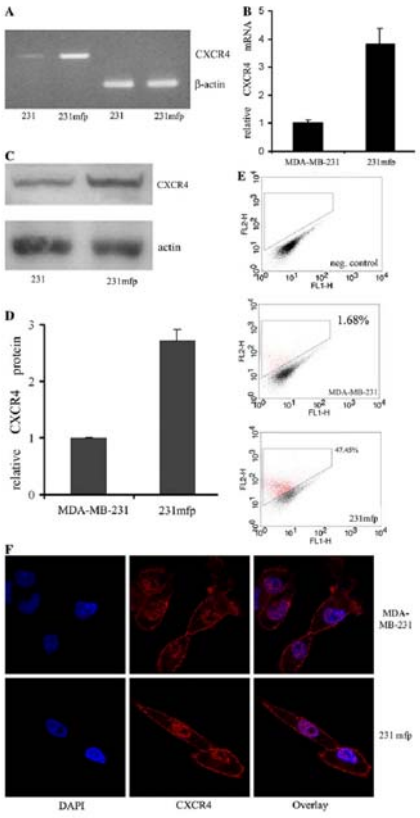

Fig. 1: Comparison of CXCR4 expression in 231mfp cells and in the parental MDA-MB-231(231) cell line. (A) CXCR4 mRNA expression was determined by standard RT-PCR. Total RNA was isolated from an equal number of cells, and an equal amount of RNA was used in the RT-PCR assay. PCR products were run on an agarose gel stained with ethidium bromide. β-actin served as a control. (B) CXCR4 mRNA expression was further quantified by real-time PCR, and normalized to GAPDH expression (mean ± SD, n = 3). (C) Detection of total CXCR4 expression at the protein level by Western blot. (D) Quantification of CXCR4 protein expression. Pixel intensities of immunoblots as shown in Fig. 1B were quantified using Scion Imaging software and normalized to actin (n = 3). (E) Flow cytometric analysis of CXCR4 expression on the cell surface. MDA-MB-231 and 231mfp cells were stained with PE-conjugated anti-CXCR4 antibody. Cell surface expression of CXCR4 is indicated as the percentage of CXCR4-positive cells (1 experiment representative of 4). (F) Immunostaining of CXCR4 in permeabilized MDA-MB-231 and 231mfp cells. Note that apart from the cell surface expression of CXCR4, there is also prominent CXCR4 expression in the nucleus.

The MDA-MB-231 parent cell line showed practically no chemotaxis toward SDF-1, while EGF used as a positive control indicated that the cells were capable to migrate, when given the right stimulus (Fig. 3A). In contrast, 231 mfp cells clearly migrated towards SDF-1 (Fig. 3B). Akt appeared to play an important role in SDF-1 mediated migration of mfp 231 cells. Two inhibitors of Akt (API-2 and Akt inhibitor IV), an inhibitor of PI-3 kinase (LY294002) upstream of Akt, an inhibitor of Src (PP1) and dominant negative (DN) PI-3K as well as DN Akt all blocked SDF-1 mediated chemotaxis (Fig. 3C-E). While the parent MDA-MB-231 cell line showed barely detectable cell surface CXCR4 expression, it still showed an —albeit weaker—response to CXCL12 (Fig. 2A) indicating that a small number of receptors was sufficient to invoke a response, but that this response was enhanced with increased receptor expression. To confirm that increased CXCR4 expression could augment Akt activation, 231mfp cells expressing endogenous CXCR4 were infected with retrovirus from packing cells LinX-A transfected with CXCR4 encoding plasmid. The selected stable line showed increased CXCR4 cell surface expression, and further increased Akt phosphorylation (Fig. 2B). The immortalized normal breast epithelial cell line MCF-10A was found to express barely detectable levels of CXCR4 on the cell surface (results not shown), and showed a weak and transient Akt response to CCL12 (Fig. 2C). Another breast cancer

cell line, SKBR-3 did not express CXCR4, as assessed by FACS, and showed no Akt activation in response to CXCL12 (Fig. 2D). These results indicated that the expression level of endogenous cell surface CXCR4 was related to the magnitude of the downstream Akt signaling.

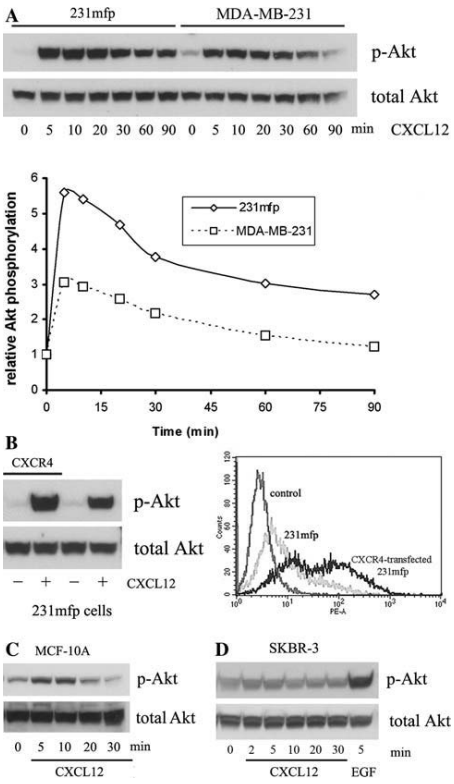

Fig. 2: Comparison of CXCL12-mediated Akt phosphorylation in breast cancer cell lines expressing different levels of CXCR4. Breast cancer cells were serum-starved overnight, and stimulated with 50 nM CXCL12 for the indicated times. Akt activation was detected by immunoblotting with anti-phospho-Akt (Ser473) antibody, and equal loading was confirmed by re-developing the blot with anti-Akt antibody. (A) Time course of Akt phosphorylation in MDA-MB-231 and 231mfp cells. The blot was quantified by densitometry and normalized to Akt expression. (B) 231mfp cells were infected with retrovirus from packing cells LinX-A transfected with CXCR4 encoding plasmid, and the selected cells stably expressing CXCR4 were used in the assay. Cells were stimulated with CXCL12 for 5 min, and Akt phosphorylation was detected as above, and compared with non-transfected 231mfp cells. CXCR4 cell surface expression was assessed by FACS analysis as described in Methods section in Fig. 1E. (C) and (D) Akt activation was detected in SKBR-3 cells and MCF-10A cells stimulated with CXCL12. Statistically significant differences are indicated by asterisk for $p < 0.05$ and double asterisks for $p < 0.01$.

CXCL12 induced chemotaxis: CXCL12 induced chemotaxis of MDA-MB-231 cells was first reported by Mueller et al. (6). However, MDAMB-231 cells differ in various laboratories, and in our hands using MDA-MB-231 cells obtained from ATTC and cultured for no more than ten passages, we could not detect any CXCL12-mediated chemotaxis in the parental line (Fig. 3A), although these cells responded well to EGF used as a positive control in parallel experiments (Fig. 3A). In contrast, CXCL12 served as a chemotactic factor for 231mfp cells (Fig. 3B), indicating that a threshold level of CXCR4 expression was needed for cell migration.

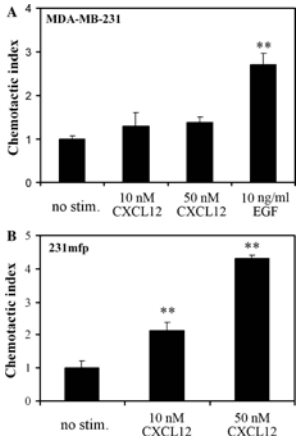

Fig. 3: CXCL12 induced chemotaxis of MDA-MB-231 (A)or 231mfp (B) breast cancer cells. Cells were brought into suspension and added to the upper chambers of the chemotaxis Transwells. A dose range of CXCL12 or EGF were used in the lower wells of the Transwells as indicated, and the assay was conducted at 37C for 4 h. Results are presented as the chemotactic index, which is the ratio of the number of cells migrating to stimulus over that of cells migrating to medium alone. The figure is representative of at least three separate experiments with similar results.

Akt signaling pathways in 231mfp cells: To detect the possible involvement of signaling intermediates in Akt activation, we screened an array of pharmacological inhibitors in 231mfp cells stimulated with CXCL12. PTX blocked CXCR4-mediated Akt phosphorylation completely, indicating that Gi was the only G-protein necessary for Akt activation (Fig. 4A). Genistein, a pan-protein tyrosine kinase inhibitor also inhibited Akt activation suggesting the involvement of protein tyrosine kinases in this process (Fig. 4A). More specifically, pretreatment of cells with the Src family kinase inhibitor PP1 significantly blocked Akt activation in 231mfp cell stimulated with CXCL12 (Fig. 4A) suggesting that Src family kinases play a role upstream of Akt activation. As expected, the PI3K inhibitor LY294002 abolished Akt phosphorylation (Fig. 4B). Two novel Akt inhibitors, Akt inhibitor IV and API-2 blocked Akt activation downstream of PI3K (Fig. 4B), but—in contrast to LY294002—had no effect on PI3K activity (results not shown). To ascertain the inhibitor results with specific genetic means, dominant-negative constructs of Src, PI3K and Akt were transfected into 231mfp cells and shown to block CXCL12 induced Akt activation effectively (Fig. 4 B), although the inhibitory effect of dominant-negative Src was less complete than that of dominant-negative PI3K or Akt.

To determine the relationship between Src activation and PI3K activation—both upstream of Akt phosphorylation— the PI3K regulatory subunit p85 was immunoprecipitated in CXCL12 stimulated 231mfp

cells, and its phosphorylation was detected using a phosphotyrosine antibody. The result showed that CXCL12 increased the level of phospho-p85 in a time-dependent manner, and this phosphorylation could be prevented by PP1 (Fig. 4C), again suggesting that Src family kinases play a role upstream of PI3K.

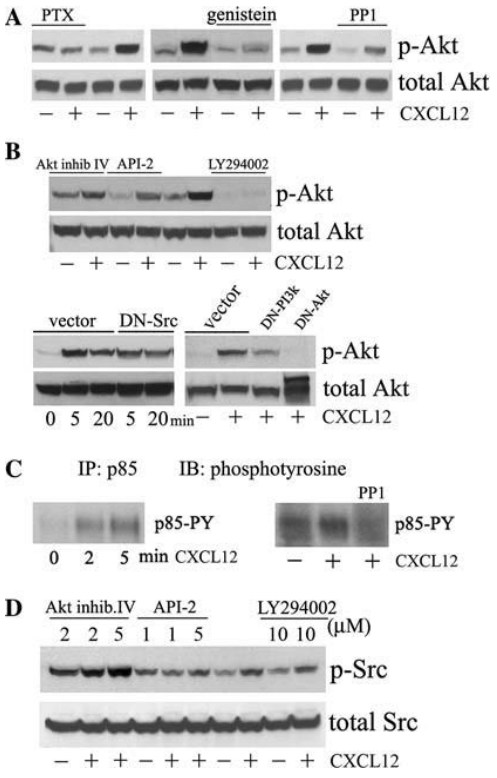

Fig. 4: Effect of inhibitors on Akt phosphorylation in 231mfp cells. (A) and (B) 231mfp cells were cultured in 60 mm dishes and serum-starved overnight. In some experiments cells were transiently transfected with DN-Src, DN-PI3K, DN-Akt, or vector DNA as indicated. Cells were pretreated with various inhibitors for 30 min (100ng/ml pertussis toxin/PTX, 100 μM genistein, 10 μM PP1, 5 lM Akt inhibitor IV and 5 μM API-2, 10 μM LY294002) prior to the addition of CXCL12 for 5 min. Whole cell lysates were separated on SDS gels, transferred to nitrocellulose and immunostained with anti-phospho-Akt antibody to detect Akt activation, and re-probed with anti-Akt antibody to assure equal loading. The figure is representative of at least three separate experiments. (C) 231mfp cells were serum-starved and treated with CXCL12 as in (A), and p85 immunoprecipitates were separated and detected with-phospho-tyrosine (PY) antibody. (D) Effect of Akt inhibition on Src activity. Cells were serum-starved overnight, pre-incubated with various inhibitors for 30 min, and treated with CXCL12 for 5 min. Src activation was evaluated by immunoblotting with anti-phospho-Src antibody. The blot was re-probed with anti-Src antibody to assure that an equal amount of protein was loaded in each lane. The figure is representative of three separate experiments.

Since the inhibitor studies suggested involvement of Src in Akt activation in 231mfp cells stimulated with CXCL12, we next investigated if CXCR4 stimulation could increase Src phosphorylation. Src phosphorylation was slightly increased in breast cancer cells stimulated with CXCL12 as assessed using anti-phospho-Src antibody and Western blotting to detect phospho-Src (Fig. 4D). Akt inhibitors had no effect on Src activity, and high concentrations of them would even augment basal Src phosphorylation (Fig. 4D), perhaps due to feed back regulation or inhibition of an unknown phosphatase.

Chemotaxis of breast cancer cells to CXCL12: To detect which signaling molecules were involved in CXCR4-mediated chemotaxis, the same inhibitors as used above were added to cells in cell migration assays. The Src family kinase inhibitor PP1, the PI3K inhibitor LY294002 and the two Akt-specific inhibitors (Akt inhibitor IV and API-2) could inhibit 231mfp cells migration to CXCL12 (Fig. 5A, B), suggesting that Src, and the PI3K/Akt pathways were essential for CXCR4-mediated chemotaxis. Unlike LY294002, the two Akt inhibitors block Akt activation downstream of PI3K, allowing to dissect the specific role of Akt from that of PI3K.

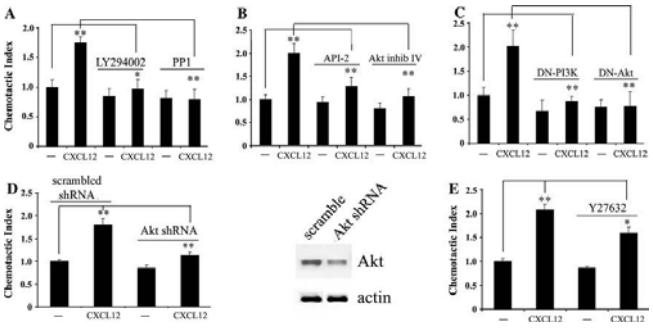

Fig. 5 Effect of inhibitors on chemotaxis of 231mfp cells to CXCL12. The figure represents the mean and standard deviation of 2–3 separate experiments in triplicate for each condition. In (A), (B) and (E) 231mfp cells were harvested and pre-incubated with different inhibitors as indicated in the figure for 30–45 min prior to initiation of the chemotaxis assay. (C) 231mfp cells were transfected with dominant-negative DNA constructs or control DNA, and chemotaxis was carried out as above. (D) 231mfp cells stably expressing Akt shRNA or scrambled shRNA were used in chemotaxis assays (left panel). The Akt knock-down result was confirmed by Western blotting (right panel). Statistically significant differences are indicated by asterisk for $p < 0.05$ and double asterisk for $p < 0.01$.

To confirm the pharmacological data, dominant-negative forms of PI3K and Akt were used to inhibit Akt activation (Fig. 4B). This approach similarly inhibited chemotaxis of 231mfp cells to CXCL12 (Fig. 5C), which was further evidence that the PI3K/Akt pathway is required for CXCR4-mediated chemotaxis. We also utilized

RNA interference to knock down Akt expression in 231mfp cells. When 231mfp cells stably expressing Akt shRNA (described in (8)) were used in cell migration assays, there was a significant reduction in chemotaxis assays towards CXCL12 compared with cells expressing a scrambled shRNA sequence (Fig. 5D). Inhibitors of additional pathways showed only a partial effect: Although RhoA and Rho kinase (ROCK) play a pivotal role in lymphocyte chemotaxis to CXCL12 (9), the ROCK inhibitor Y27632 (5 μM) had only a partial, but statistically significant effect on CXCL12-mediated breast cancer cell chemotaxis (Fig. 5E), and may constitute one of several pathways downstream of Akt activation (10). Ilk, another downstream target of Akt, did not appear to be important for chemotaxis as shown by the lack of any effect a dominant negative Ilk construct.
The experiments presented up to this point have been published (11) and are included in the appendix.
Further signaling experiments intended to show cross activation between the Akt and HER2 pathways were not successful, as expression of HER2 on 231 mfp cells was unexpectedly low, which made it unfeasible to determine HER2 transactivation.

Akt activation protects from apoptosis: 231 mfp cells were very resistant to killing by doxorubicin and paclitaxel (Fig. 5A), and concentrations necessary for cell killing were almost an order of magnitude higher than in the parent MDA-MB-231 cells. Inhibition of Akt induced a slight toxic effect on its own and considerably enhanced doxorubicin-mediated cell killing suggesting a role for Akt in protection from apoptosis (Fig. 5B). Soluble SDF-1 showed only a minor effect on doxorubicin mediated cell killing, while SDF-1, which was presented bound to fibronectin, - for which it shows specific high affinity binding(12), partially protected from doxorubicin-mediated cell killing (Fig. 5C) suggesting that fibronectin-binding integrins may be involved in SDF-1 mediated protection from chemotherapeutic drugs.

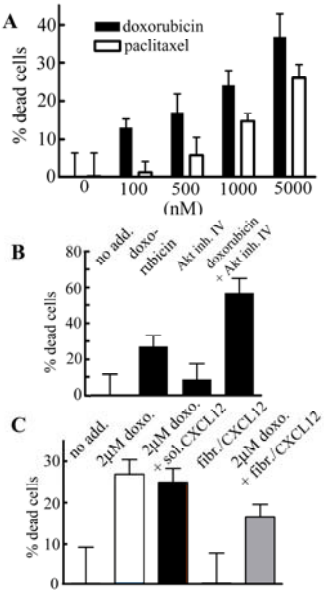

Fig. 5: Effect of doxorubicin and paclitaxel on cell death in 231 mfp cells. Fig. 5A: Dose-response to doxorubicin and paclitaxel. 231 mfp cells (2x 10^3cells/well) were plated in 200μl full media in 96 well plates in the presence of increasing concentrations of doxorubicin or paclitaxel as indicated. Forty-eight hours later, cell viability was determined by MTT assay (13)(mean +/- S.D. of quadruplicate samples). Fig. 5B: Effect of Akt inhibition on doxorubicin mediated cell killing. The assay was performed as in Fig. 5A. 231 mfp cells were cultured without any addition, in the presence of 2μM doxorubicin, 2μM Akt inh. IV, or both as indicated. Fig. 5C: Effect of SDF-1 on cell killing in the presence of doxorubicin. Cells were grown without any addition, with 2μM doxorubicin, 2μM doxorubicin + 100nM soluble SDF-1, or wells were coated with 10μg/ml fibronectin followed by 100 nM SDF-1 and washing with 0.2% BSA in PBS. Cells were then plated and presented with no drug or with doxorubicin as indicated. Mean +/- S.D. of quadruplicate samples.

KEY RESEARCH ACCOMPLISHMENTS

- Definition of Akt activation as a key factor in CXCL12/SDF-1 mediated cell migration of breast cancer cells expressing the CXCR4
- Detection that Akt activation by CXCL12 renders mfp231 breast cancer cells still more chemotherapy resistant
- Observation of nuclear CXCR4 expression in mfp231 cells

REPORTABLE OUTCOMES

Zhao M., Mueller B.M., Discipio R.G., and Schraufstatter I.U. 2008. Akt plays an important role in breast cancer cell chemotaxis to CXCL12. *Breast Cancer Res Treat. 110:211-222*
(appendix)

CONCLUSION:

Akt inhibition during chemotherapy of CXCR4 positive breast cancers could potentially increase the chemotherapy success, since Akt is 1) likely to play a role in CXCL12-mediated metastasis, for which cell migration is a prerequisite and 2) since it contributes to chemoresistance.

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PRECLINICAL STUDY

Akt plays an important role in breast cancer cell chemotaxis to CXCL12

Ming Zhao · Barbara M. Mueller · Richard G. DiScipio · Ingrid U. Schraufstatter

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Abstract The chemokine receptor CXCR4 is functionally expressed on the cell surface of various cancer cells, and plays a role in cell proliferation and migration of these cells. Specifically, in breast cancer cells the CXCR4/CXCL12 axis has been implicated in cell migration in vitro and in metastasis in vivo, but the underlying signaling mechanisms are incompletely understood. The xenograft-derived MDA-MB-231 breast cancer cell line (231mfp), which was shown previously to grow more aggressively than the parent cells, showed increased CXCR4 expression at the mRNA, total protein and cell surface expression level. This correlated with an enhanced response to CXCL12, specifically in augmented and prolonged Akt activation in a G_i, Src family kinase and PI-3 kinase dependent fashion. 231mfp cells migrated towards CXCL12—in contrast to the parent cell line—and this chemotaxis was blocked by inhibition of G_i, Src family kinases, PI-3 kinase and interestingly, Akt itself, as could be shown with two pharmacological inhibitors, a dominant negative Akt construct and with Akt shRNA. Collectively, we have demonstrated that prolonged Akt activation is an important signaling pathway for breast cancer cells expressing CXCR4 and is necessary for CXCL12-dependent cell migration.

M. Zhao (✉) · B. M. Mueller · R. G. DiScipio · I. U. Schraufstatter
Division of Cancer Biology, La Jolla Institute for Molecular Medicine, 4570 Executive Drive, San Diego, CA 92121, USA
e-mail: mzhao@ljimm.org

B. M. Mueller
e-mail: bmueller@ljimm.org

R. G. DiScipio
e-mail: richard@ljimm.org

I. U. Schraufstatter
e-mail: ingrid@ljimm.org

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Abbreviations

PI3K	PI-3 kinase
MMP	Matrix metalloproteinase
FCS	Fetal calf serum
PTX	Pertussis toxin

Introduction

It has been shown that the chemokine receptor, CXCR4 and its ligand, CXCL12/stromal-derived factor 1 (SDF-1), are among the palette of critical factors in breast cancer metastasis [1]. Since CXCL12 is a chemotactic factor, it has been suggested that cells which express CXCR4 migrate toward areas in which CXCL12 is expressed in the highest concentrations, i.e., to lymph nodes, bone marrow, lung, and liver. Several lines of evidence indicate that inhibition of CXCR4 on breast cancer cells attenuates metastasis in mouse models. These include the use of anti-CXCR4 antibodies [1], CXCL12 inhibitory peptides [2, 3] or siRNA directed against CXCR4 [4]. Finally, high expression levels of CXCR4 on human breast cancers have been associated with aggressive tumors, poor prognosis [5, 6], and early lymph node metastasis [7].

In spite of these results, the underlying mechanisms of CXCR4-dependent breast cancer metastasis are insufficiently understood, since cancer metastasis is a complex behavior in which invasive tumor cells detach from the primary tumor, gain access to blood or lymphatic vessels, lodge in distant sites, escape from immune-surveillance

and are able to survive and proliferate in the new environment. In this scenario the increased migratory potential of CXCR4-expressing cells appears insufficient for the support of tumor growth at secondary sites. Furthermore, in spite of the mounting in vivo evidence for a role for CXCR4 in breast cancer metastasis, expression of CXCR4 in cultured breast cancer cells is often minimal. Apparently, conditions in the host environment contribute to CXCR4 expression, which are not fully mimicked in tissue culture [8]. In vivo, CXCR4 expression was increased following lung metastasis, which was suggested to be due to clonal selection [9] but may also be due to up-regulation by local conditions and factors. In breast cancer cells, autocrine VEGF promoted CXCR4 expression and was required for chemotaxis to CXCL12 [10]. Similarly, EGF could promote CXCR4 expression in a hypoxic environment in non-small cell lung cancer cells, and this was dependent on the PI-3 kinase (PI3K)/Akt-mTOR pathway [11]. Crosstalk also appears to operate in the opposite direction: CXCL12 stimulated ovarian cancer cell growth was dependent on transactivation of the EGF-receptor [12], and HER2 was shown to be transactivated by CXCL12 in breast cancer cells [13]. HER2-mediated breast cancer metastasis was reported to depend on CXCR4 expression [8], and HER2 can enhance CXCR4 expression and prevent its degradation, thus promoting increased CXCR4 signaling in cancer cells [8].

CXCR4 and its ligand CXCL12 are widely expressed, and—apart from cancer metastasis—play a fundamental role in embryonic development, hematopoiesis, in particular in hematopoietic stem cell/progenitor homing, and inflammation. All these processes are dependent on cell migration. Various signaling molecules have been implicated in CXCR4-mediated cell migration, including PI3K, Pyk2, Cbl, SHP2, Rho, Ca^{2+}, ERK, p38, the Src family kinase Lck, and β-arrestin [9, 14–18], suggesting either redundancy or that the signaling pathways may differ for different cell types.

The serine/threonine kinase Akt is a direct downstream effector of PI3K. The principal role of Akt is to facilitate growth factor-mediated cell survival and to suppress apoptosis induced by various stimuli. Akt is highly expressed in cancer cells and represents a potential target for the development of novel anti-cancer drugs [19]. Akt is activated when tumor suppressors such as $p27^{Kip1}$ and PTEN lose their function, and such constitutive Akt activation correlates with poor prognosis [20]. Specifically Akt activation is associated with poor prognosis in breast cancer patients, particularly those with HER2-overexpressing tumors [21]. Due to its anti-apoptotic effects, increased Akt activity appears to be a reason for chemoresistance in a variety of cancers including breast and small cell lung cancer [15]. Akt has been associated with cell migration under specific circumstances [22, 23], but it has not found much attention in this respect in contrast to its up-stream activator PI3K [24]. This distinction is, however, important as PI3K causes activation of additional down-stream pathways—such as PKCζ—with the potential of enhanced toxicity of PI3K inhibitors compared to Akt inhibitors.

In the present study, we demonstrate CXCL12-induced prolonged Akt activation in breast cancer cells—which was considerably longer than in non-malignant cells such as lymphocytes. This prolonged Akt activation was required for CXCR4-mediated chemotaxis of breast cancer cells. To our knowledge, this is the first report indicating that Akt activity itself—rather than its upstream activator PI3K and its multiple possible downstream effectors—plays a role in CXCR4-mediated chemotaxis of cancer cells, thus contributing to the understanding of the mechanism of CXCR4 signaling in breast cancer cells.

Materials and methods

Materials

Pertussis toxin (PTX), PD98059, AG1478, Y27632, LY294002, Akt inhibitor IV and V (API-2) were purchased from Calbiochem, San Diego, CA. Genistein and PP1 were from Biomol, Plymouth Meeting, PA. Anti-phospho-tyrosine, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-Src family (Tyr416) antibodies were from Cell Signaling, Beverly, MA; anti-Src monoclonal antibody was from Upstate Biotechnology, Lake Placid, NY; PE conjugated anti-CXCR4 antibody was purchased from R&D Systems, Minneapolis, MN; p85 PI3K and goat anti-CXCR4 antibody used in Western blots were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The CXCR4 construct has been described previously [25]. Dominant-negative Src was provided by Dr D. Yu (MD Anderson Cancer Center, Houston, TX). Akt shRNA, which knocks down both Akt 1 and Akt 2, and scrambled shRNA constructs were the generous gift of Dr J. Bolanos [26]. CXCL12 was expressed and purified from transformed *Escherichia coli* cells as described [27]. DNA encoding human CXCL12-α was cloned into the expression vector pET45b, and the plasmid was transformed into E. coli strain Rosetta-gami DE3 from Novagen/EMB Biosciences, San Diego, CA. During early growth phase the recombinant protein was induced with 0.1 mM IPTG. Bacteria were harvested, lysed, and the debris removed by centrifugation. The supernatant was applied to a column of Tris(carboxymethyl)ethylenediamine-Sepharose charged with Ni^{2+} [28]. After elution in 2 M guanidine HCl in 40 mM sodium acetate pH 4.3, the chimeric protein was renatured by dialysis against 10 mM Mops pH 7.3/0.15 M

NaCl. After excising the chemokine from the chimera and purification on CM-Sephadex, CXCL12 was observed as a single band after SDS-PAGE. Lymphocyte chemotaxis used as an activity assay showed comparable dose-responses for CXCL12 purified in this fashion and commercial CXCL12.

Cell lines, Cell culture, transfection, and infection

The breast cancer cell lines SKBR-3, MDA-MB-231, and the normal breast epithelial cell line MCF-10A were obtained from ATCC, Rockville, MD, and used within the first ten passages. The xenograft-derived tumor subline (231mfp) has been described [29]. SKBR-3 cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS and 2 mM glutamine. MCF10-A cells were cultured in DMEM/F-12 medium (Invitrogen) containing 5% heat-inactivated horse serum, 10 μg/ml insulin (Sigma, St. Louis, MO), 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 100 ng/ml cholera toxin (Calbiochem), and 2 mM glutamine. MDA-MB-231 and 231mfp cells [29] were grown in L-15 medium (Invitrogen) containing 10% FCS (Tissue Culture Biologicals, Tulare, CA), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Where indicated, cells were serum-starved overnight prior to the stimulation with CXCL12. Transient transfection was performed by using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction, and the transfection efficiency was generally higher than 70% as determined by co-transfected plasmid encoding green fluorescent protein. Retrovirus-based infection of 231mfp cells was carried out as described previously using an amphotropic packaging cell line (LinX-A) [30].

Flow cytometry

Cells were harvested, washed once with FACS buffer (PBS containing 2% FCS), and incubated with PE-conjugated anti-CXCR4 polyclonal antibody at room temperature for 40 min, then washed 3× with FACS buffer. Fluorescence intensity of antibody-stained cells was detected on a Becton Dickinson FACSCAN or LSR II, Mountain View, CA, with dead cells excluded by a gate on forward and side scatter.

Immunofluorescence

Breast cancer cells grown on coverslips were washed once with PBS before fixation with 4% paraformaldehyde for 30 min, and permeabilization with 0.2% Triton X-100 for 10 min. After blocking with 2% BSA for 1 h at room temperature, cells were incubated with anti-CXCR4 for 1 h, followed by staining with Alexa Fluor 595 anti-rabbit antibody (Invitrogen) in 1% BSA for 1 h at room temperature. Cells were then stained with DAPI (Sigma-Aldrich) for 5 min and washed twice with PBS before mounting with AntiFade (Invitrogen). Images were taken on an Olympus FV1000 confocal microscope.

Western blotting

Monolayer cells were serum-starved overnight and stimulated as described in the figure legends, then lysed with modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 10% glycerol, 1% NP-40, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium vanadate, and 10 mM NaF) and clarified by centrifugation. The clarified cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, blocked with 3% dry milk in TBS-Tween, and exposed to specific primary antibodies as described for each experiment. Antibody binding was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies and enhanced chemi-luminescence (ECL, Amersham Bioscience, Piscataway, NJ, USA). Phosphoblots were re-probed with a second antibody as indicated, e.g., anti-Akt antibody, to assure equal loading.

Immunoprecipitation

Cells were serum-starved and stimulated as described in the figure legends, then lysed with modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 10% glycerol, 1% NP-40, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium vanadate, and 10 mM NaF) and clarified by centrifugation. The supernatants were incubated with primary antibodies for 2 h at 4°C, followed by capture of the immunocomplexes with protein A beads for 1 h at 4°C. The immunoprecipitates were washed twice with lysis buffer and once with PBS to remove non-specifically bound proteins. The bound proteins were analyzed with immunoblotting.

Chemotaxis assay

Breast cancer cell migration was assessed in 24-well Transwell chambers using inserts with 8 μM pore

diameter membranes (Corning Costar, Acton, MA). Transwells were pretreated with 10 μg/ml collagen and washed with PBS. Cells were harvested and were either untreated or pretreated with various inhibitors at 37°C for 30 min in chemotaxis buffer (DMEM containing 0.1% BSA). Various concentrations of CXCL12 (0–50 nM) were added to the lower wells, 150 μl of cells (5×10^4 cells/ml) were added to the upper chambers and the assembled chambers were incubated for 4 h in a tissue culture incubator. After this incubation, non-migrated cells were removed by wiping with a cotton swab, the Transwell inserts were fixed with paraformaldehyde, and stained with crystal violet. The total number of transmigrated cells was counted under a microscope. The chemotaxis result are presented as chemotactic index, which is the ratio of the number of cells migrating to CXCL12 over that of migrating cells to medium. Each assay was performed in triplicate and the representative results from at least three independent experiments are shown.

RT-PCR and real-time quantitative PCR

Total RNA was isolated from breast cancer cells by using the RNeasy kit according to the manufacturer's instruction (Qiagen, Santa Clarita, CA). First strand cDNA synthesis was performed using the Ominiscript RT kit (Qiagen), and followed by 30 cycles of a regular PCR with Taq PCR Master Mix kit (Qiagen). Real-time PCR was carried out using SYBR green PCR master mix (Bio-Rad, Foster City, CA) on a Bio-Rad iQ5 cycler as described [31]. The following primers were used: CXCR4 forward primer, 5′-AATCTTCCTGCCCACCATCT-3′; CXCR4 reverse primer, 5′-GACGCCAACATAGACCACCT-3′ [32]; GAPDH forward primer, 5′-CCACCCAGAAGACTGTGGAT-3′; and GAPDH reverse primer, 5′-TTCAGCTCAGGGATGACCTT-3′ [31]. The reaction conditions were 95°C for 10 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. After each real-time PCR run, a melting curve was performed to ensure that only a single amplicon was generated. CXCR4 message expression was normalized to GAPDH expression.

Statistical analysis

Statistical significance was determined by Student's *t*-test or if more than two conditions were compared by one-way ANOVA test followed by Tukey post-test using GraphPad Prism software Version 4 (Graph Pad, San Diego, CA).

Results

CXCR4 expression by MDA-MB-231 and 231mfp breast cancer cells

To explore CXCR4-dependent signaling pathways which mediate breast cancer cell chemotaxis, we chose the invasive breast cancer cell line MDA-MB-231 as a model, which had been used by other investigators to determine CXCL12 mediated cellular responses [1]. When these cells failed to be chemo-attracted by CXCL12 in our hands, CXCR4 expression was checked at the mRNA and protein level. Initially CXCR4 mRNA expression was analyzed by standard reverse transcription-PCR (RT-PCR), which indicated that MDA-MB-231 cells expressed barely detectable levels of CXCR4 mRNA. In contrast, 231mfp cells, isolated from an MDA-MB-231 xenograft tumor growing in the mouse mammary fat pad (mfp) and passaged for up to six passages [29], showed increased CXCR4 mRNA (Fig. 1A). Real-time PCR results indicated a fourfold increase of CXCR4 expression in 231mfp cells (Fig. 1B). Subsequently, Western blots showed that 231mfp cells also expressed increased CXCR4 protein (Fig. 1C), which is quantified in Fig. 1D. The most dramatic difference was seen, however, when cell surface expression of CXCR4 was detected by flow cytometric analysis (Fig. 1E). Nearly 50% of 231mfp cells showed cell surface staining for CXCR4 compared to 2% positive cells in the parental MDA-MB-231 cells, which indicated that the 231mfp subline was a good model to study CXCR4 signaling in breast cancer cells. In contrast in the parent cell line, much of the CXCR4 protein present did not appear to be amenable to ligand activation, as most of the receptor appeared to reside intracellularly. To verify this conclusion CXCR4-immunostaining was performed in permeabilized cells. Confocal microscopy of these cells indicated that much of the CXCR4 staining in the parent MDA-MB-231 cells was intracellular with only very weak staining in the plasma membrane, while 231mfp staining showed prominent receptor staining in the plasma membrane (Fig. 1F), but also, interestingly, strong staining of the nucleus, presumably of the nuclear membrane (Fig. 1F).

Response of 231mfp cells to CXCL12

During the exploration of signaling molecules activated by CXCR4 stimulation in 231mfp cells, it was detected that Akt phosphorylation was significantly enhanced as determined by immunoblotting with a phospho-Akt-specific antibody. This activation was prolonged to at least 90 min after the addition of CXCL12 (Fig. 2A). Although the

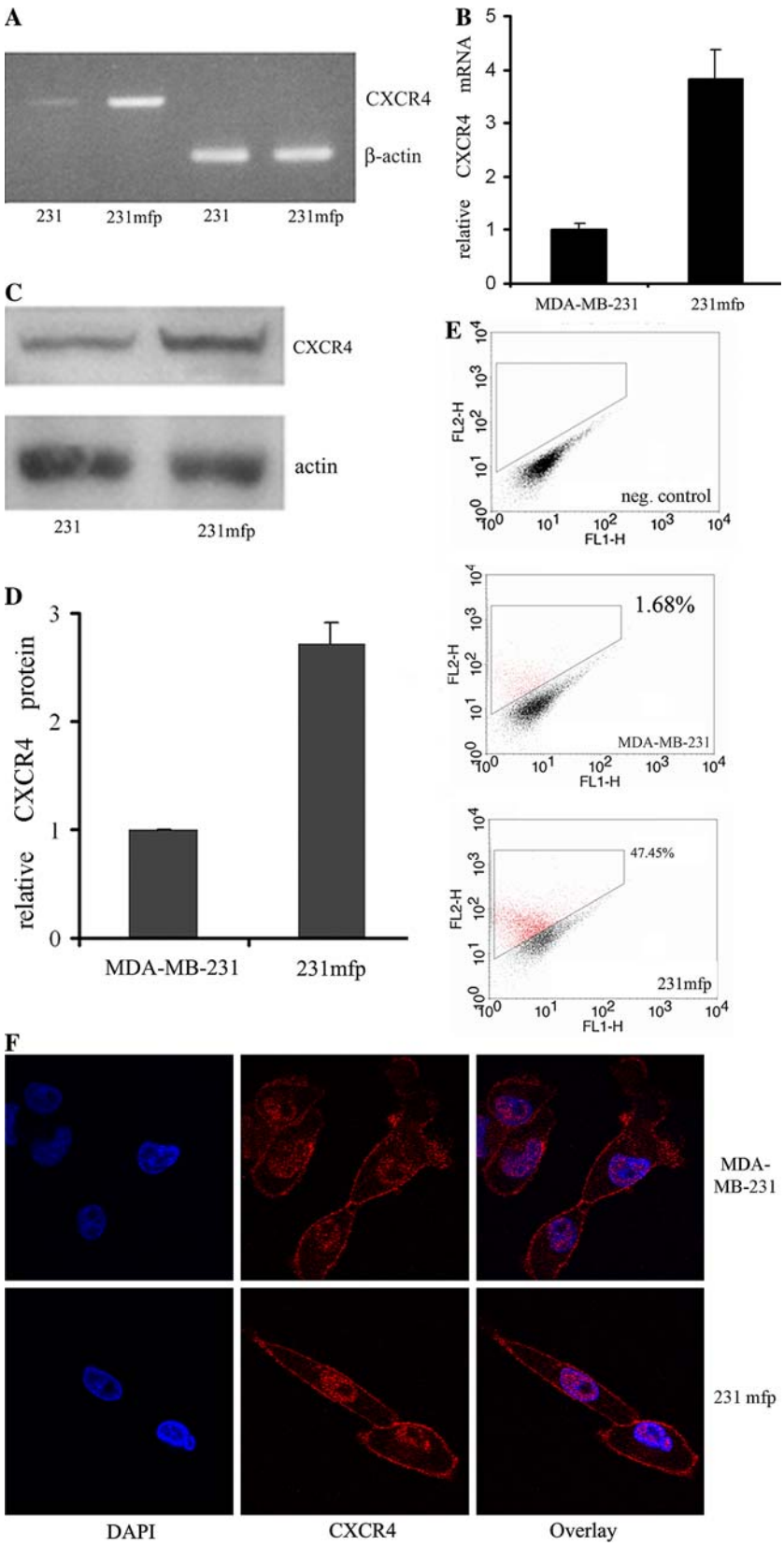

Fig. 1 Comparison of CXCR4 expression in 231mfp cells and in the parental MDA-MB-231 (231) cell line. (**A**) CXCR4 mRNA expression was determined by standard RT-PCR. Total RNA was isolated from an equal number of cells, and an equal amount of RNA was used in the RT-PCR assay. PCR products were run on an agarose gel stained with ethidium bromide. β-actin served as a control. (**B**) CXCR4 mRNA expression was further quantified by real-time PCR, and normalized to GAPDH expression (mean ± SD, $n = 3$). (**C**) Detection of total CXCR4 expression at the protein level by Western blot. Whole cell lysates were prepared, separated and detected as described in Methods, actin was used to assess equal loading (1 experiment representative of 3). (**D**) Quantification of CXCR4 protein expression. Pixel intensities of immunoblots as shown in Fig. 1B were quantified using Scion Imaging software and normalized to actin ($n = 3$). (**E**) Flow cytometric analysis of CXCR4 expression on the cell surface. MDA-MB-231 and 231mfp cells were stained with PE-conjugated anti-CXCR4 antibody. Cell surface expression of CXCR4 is indicated as the percentage of CXCR4-positive cells (1 experiment representative of 4). (**F**) Immunostaining of CXCR4 in permeabilized MDA-MB-231 and 231mfp cells

parent MDA-MB-231 cell line showed barely detectable cell surface CXCR4 expression, it still showed an—albeit weaker—response to CXCL12 (Fig. 2A) indicating that a small number of receptors was sufficient to invoke a response, but that this response was enhanced with increased receptor expression. To confirm that increased CXCR4 expression could augment Akt activation, 231mfp cells expressing endogenous CXCR4 were infected with retrovirus from packing cells LinX-A transfected with CXCR4 encoding plasmid. The selected stably line showed increased CXCR4 cell surface expression, and further increased Akt phosphorylation (Fig. 2B). The immortalized normal breast epithelial cell line MCF-10A was found to express barely detectable levels of CXCR4 on the cell surface (results not shown), and showed a weak and transient Akt response to CCL12 (Fig. 2C). Another breast cancer cell line, SKBR-3 did not express CXCR4, as assessed by FACS, and showed no Akt activation in response to CXCL12 (Fig. 2D). These results indicated that the expression level of endogenous cell surface CXCR4 was related to the magnitude of the downstream Akt signaling.

CXCL12 induced chemotaxis of MDA-MB-231 cells was first reported by Mueller et al. [1]. However, MDA-MB-231 cells differ in various laboratories, and in our hands using MDA-MB-231 cells obtained from ATTC and cultured for no more than ten passages, we could not detect any CXCL12-mediated chemotaxis in the parental line (Fig. 3A), although these cells responded well to EGF used as a positive control in parallel experiments (Fig. 3A). In contrast, CXCL12 served as a chemotactic factor for 231mfp cells (Fig. 3B), indicating that a threshold level of CXCR4 expression was needed for cell migration. At least 10% of the cell input migrated on all occasions in response to CXCL12 in the 231mfp cells and in response to EGF in the parent cell line with comparable cell migration seen for in the unstimulated wells.

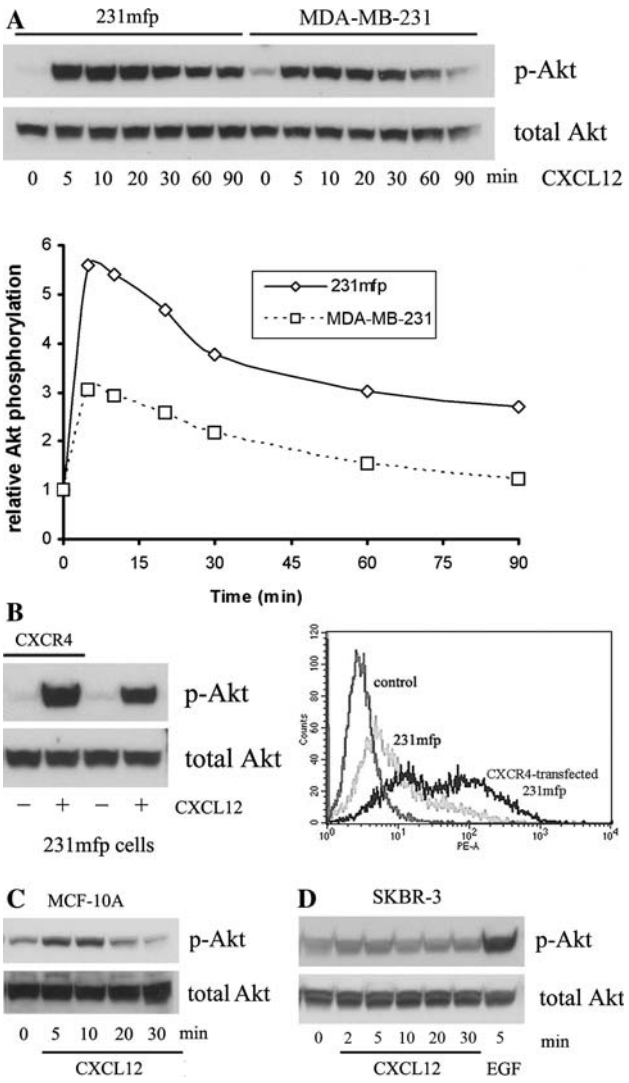

Fig. 2 Comparison of CXCL12-mediated Akt phosphorylation in breast cancer cell lines expressing different levels of CXCR4. Breast cancer cells were serum-starved overnight, and stimulated with 50 nM CXCL12 for the indicated times. Akt activation was detected by immunoblotting with anti-phospho-Akt (Ser473) antibody, and equal loading was confirmed by re-developing the blot with anti-Akt antibody. (**A**) Time course of Akt phosphorylation in MDA-MB-231 and 231mfp cells. The blot was quantified by densitometry and normalized to Akt expression. (**B**) 231mfp cells were infected with retrovirus from packing cells LinX-A transfected with CXCR4 encoding plasmid, and the selected cells stably expressing CXCR4 were used in the assay. Cells were stimulated with CXCL12 for 5 min, and Akt phosphorylation was detected as above, and compared with non-transfected 231mfp cells. CXCR4 cell surface expression was assessed by FACS analysis as described in Methods section in Fig. 1E. (**C**) and (**D**) Akt activation was detected in SKBR-3 cells and MCF-10A cells stimulated with CXCL12. Statistically significant differences are indicated by *asterisk* for $p < 0.05$ and *double asterisks* for $p < 0.01$

Akt signaling pathways in 231mfp cells

To detect the possible involvement of signaling intermediates in Akt activation, we screened an array of pharmacological inhibitors in 231mfp cells stimulated with CXCL12. PTX blocked CXCR4-mediated Akt phosphorylation completely, indicating that Gi was the only G-protein necessary for Akt activation (Fig. 4A). Genistein, a pan-protein tyrosine kinase inhibitor also inhibited Akt activation suggesting the involvement of protein tyrosine kinases in this process (Fig. 4A). More specifically, pretreatment of cells with the Src family kinase inhibitor PP1 significantly blocked Akt activation in 231mfp cell stimulated with CXCL12 (Fig. 4A) suggesting that Src family kinases play a role upstream of Akt activation. As expected, the PI3K inhibitor LY294002 abolished Akt phosphorylation (Fig. 4B). Two novel Akt

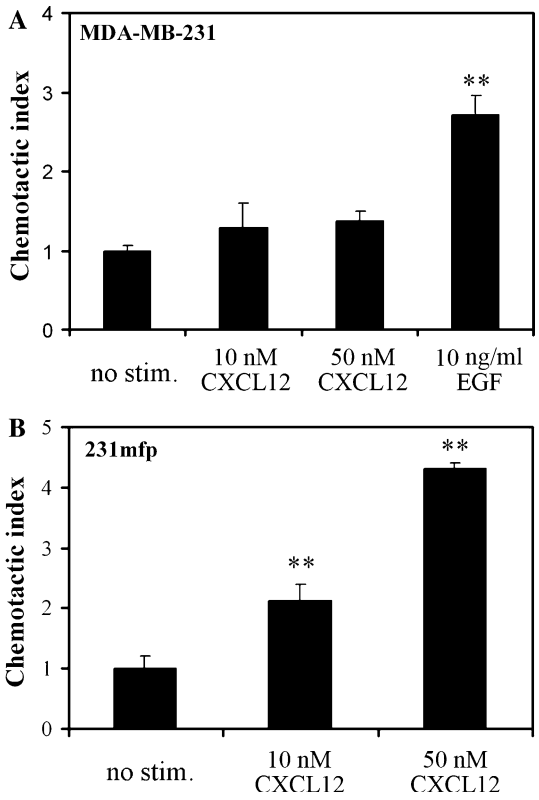

Fig. 3 CXCL12 induced chemotaxis of MDA-MB-231 (**A**) or 231mfp (**B**) breast cancer cells. Cells were brought into suspension and added to the upper chambers of the chemotaxis Transwells. A dose range of CXCL12 or EGF were used in the lower wells of the Transwells as indicated, and the assay was conducted at 37°C for 4 h. Results are presented as the chemotactic index, which is the ratio of the number of cells migrating to stimulus over that of cells migrating to medium alone. The figure is representative of at least three separate experiments with similar results

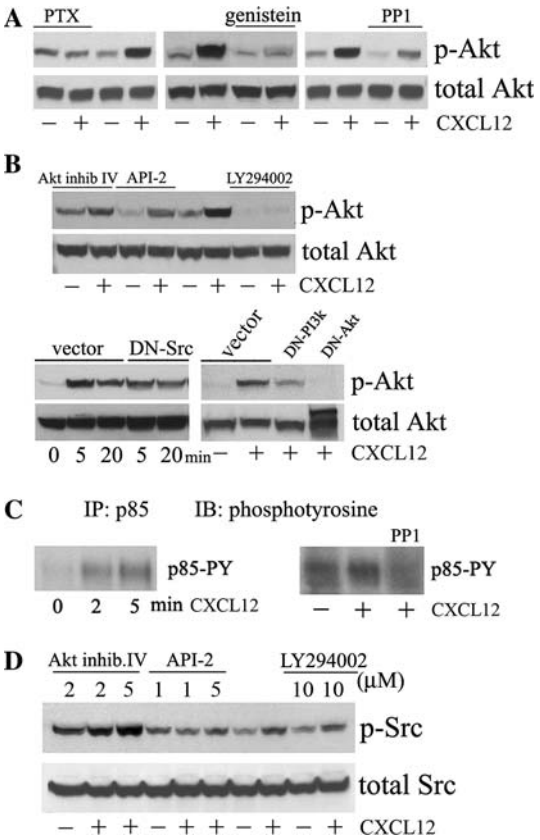

Fig. 4 Effect of inhibitors on Akt phosphorylation in 231mfp cells. (**A**) and (**B**) 231mfp cells were cultured in 60 mm dishes and serum-starved overnight. In some experiments cells were transiently transfected with DN-Src, DN-PI3K, DN-Akt, or vector DNA as indicated. Cells were pretreated with various inhibitors for 30 min (100 ng/ml PTX, 100 μM genistein, 10 μM PP1, 5 μM Akt inhibitor IV and 5 μM API-2, 10 μM LY294002) prior to the addition of CXCL12 for 5 min. Whole cell lysates were separated on SDS gels, transferred to nitrocellulose and immunostained with anti-phospho-Akt antibody to detect Akt activation, and re-probed with anti-Akt antibody to assure equal loading. The figure is representative of at least three separate experiments. (**C**) 231mfp cells were serum-starved and treated with CXCL12 as in (**A**), and p85 immunoprecipitates were separated and detected with-phospho-tyrosine (*PY*) antibody. (**D**) Effect of Akt inhibition on Src activity. Cells were serum-starved overnight, pre-incubated with various inhibitors for 30 min, and treated with CXCL12 for 5 min. Src activation was evaluated by immunoblotting with anti-phospho-Src antibody. The blot was re-probed with anti-Src antibody to assure that an equal amount of protein was loaded in each lane. The figure is representative of three separate experiments

inhibitors, Akt inhibitor IV and API-2 blocked Akt activation downstream of PI3K (Fig. 4B), but—in contrast to LY294002—had no effect on PI3K activity (results not shown). To ascertain the inhibitor results with specific genetic means, dominant-negative constructs of Src, PI3K and Akt were transfected into 231mfp cells and shown to block CXCL12 induced Akt activation effectively (Fig. 4 B), although the inhibitory effect of dominant-negative Src was less complete than that of dominant-negative PI3K or Akt.

To determine the relationship between Src activation and PI3K activation—both upstream of Akt phosphorylation—the PI3K regulatory subunit p85 was immunoprecipitated in CXCL12 stimulated 231mfp cells, and its phosphorylation was detected using a phosphotyrosine antibody. The result showed that CXCL12 increased the level of phospho-p85 in a time-dependent manner, and this phosphorylation could be prevented by PP1 (Fig. 4C), again suggesting that Src family kinases play a role upstream of PI3K.

Since the inhibitor studies suggested involvement of Src in Akt activation in 231mfp cells stimulated with CXCL12, we next investigated if CXCR4 stimulation could increase Src phosphorylation. Src phosphorylation was slightly increased in breast cancer cells stimulated with CXCL12 as assessed using anti-phospho-Src antibody and Western blotting to detect phospho-Src (Fig. 4D). Akt inhibitors had no effect on Src activity, and high concentrations of them

would even augment basal Src phosphorylation (Fig. 4D), perhaps due to feed back regulation or inhibition of an unknown phosphatase.

Chemotaxis of breast cancer cells to CXCL12

To detect which signaling molecules were involved in CXCR4-mediated chemotaxis, the same inhibitors as used above were added to cells in cell migration assays. The Src family kinase inhibitor PP1, the PI3K inhibitor LY294002 and the two Akt-specific inhibitors (Akt inhibitor IV and API-2) could inhibit 231mfp cells migration to CXCL12 (Fig. 5A, B), suggesting that Src, and the PI3K/Akt pathways were essential for CXCR4-mediated chemotaxis. Unlike LY294002, the two Akt inhibitors block Akt activation downstream of PI3K, allowing to dissect the specific role of Akt from that of PI3K. To confirm the pharmacological data, dominant-negative forms of PI3K and Akt were used to inhibit Akt activation (Fig. 4B). This approach similarly inhibited chemotaxis of 231mfp cells to CXCL12 (Fig. 5C), which was further evidence that the PI3K/Akt pathway is required for CXCR4-mediated chemotaxis. We also utilized RNA interference to knock down Akt expression in 231mfp cells. When 231mfp cells stably expressing Akt shRNA (described in [26]) were used in cell migration assays, there was a significant reduction in chemotaxis assays towards CXCL12 compared with cells expressing a scrambled shRNA sequence (Fig. 5D).

Inhibitors of additional pathways showed only a partial effect: Although RhoA and Rho kinase (ROCK) play a pivotal role in lymphocyte chemotaxis to CXCL12 [33], the ROCK inhibitor Y27632 (5 μM) had only a partial, but statistically significant effect on CXCL12-mediated breast cancer cell chemotaxis (Fig. 5E), and may constitute one of several pathways downstream of Akt activation [34].

Discussion

Accumulating evidence suggests an important role for CXCR4 signaling in breast cancer growth and metastasis [35], and there appears to be a correlation between increased CXCR4 expression and aggressive growth in several cancers [5, 36, 37]. This clinical observation is consistent with our finding that the more aggressive and metastatic 231mfp cells [29] expressed more CXCR4 than the parent MDA-MB-231 cells.

CXCR4 expression is enhanced by hypoxia, EGF, HER2, VEGF, TNF-α, and even by its own ligand CXCL12 [8, 10, 11, 38–40]. This up-regulation often involves the PI3K/Akt pathway and HIF-1 [40]. Although it is not clear, whether these pathways were instrumental in

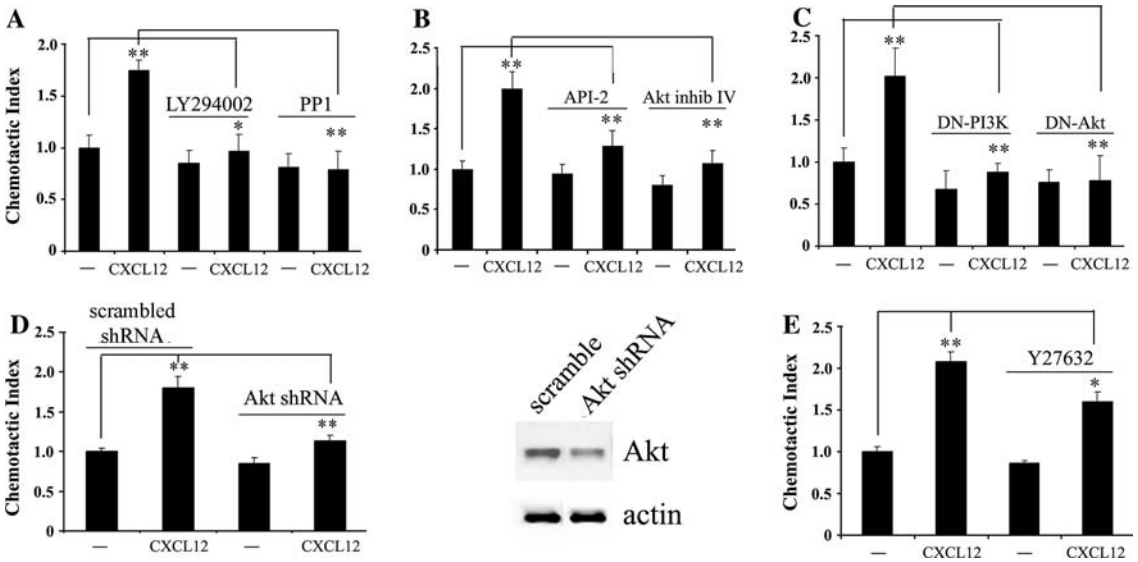

Fig. 5 Effect of inhibitors on chemotaxis of 231mfp cells to CXCL12. The figure represents the mean and standard deviation of 2–3 separate experiments in triplicate for each condition. In (**A**), (**B**) and (**E**) 231mfp cells were harvested and pre-incubated with different inhibitors as indicated in the figure for 30–45 min prior to initiation of the chemotaxis assay. (**C**) 231mfp cells were transfected with dominant-negative DNA constructs or control DNA, and chemotaxis was carried out as above. (**D**) 231mfp cells stably expressing Akt shRNA or scrambled shRNA were used in chemotaxis assays (left panel). The Akt knock-down result was confirmed by Western blotting (right panel). Statistically significant differences are indicated by *asterisk* for $p < 0.05$ and *double asterisk* for $p < 0.01$

the up-regulation of CXCR4, which occurred following implantation of the parent cell line into the mouse mammary fat pad, several of these conditions would have been present in vivo: CXCL12 is produced by fibroblasts infiltrating mammary carcinomas [41], but not by the cancer cells themselves [10, 41], and VEGF and hypoxia are also expected to have been present, but would not be produced during in vitro culture of isolated breast cancer cells. However, CXCR4 expression in 231mfp cells remained elevated during culture in vitro for at least 6–8 passages, and only gradually decreased following continued passaging. This fairly stable expression of the CXCR4 suggests that the in vivo conditions selected for a sub-population of cells with greater tumorigenicity, which are characterized by higher CXCR4 expression.

Interestingly, there was nuclear CXCR4 staining in 231mfp and to a lesser degree in MDA-MB-231 cells. While such staining has been documented in a large breast cancer pathology study [6], the possible consequences and signaling mechanisms have not been addressed. This is an area, which deserves further investigation, since nuclear GPCRs have effects on gene transcription, e.g., of COX-2 expression [42, 43], which are not seen, when the same receptors are expressed in the plasma membrane.

Numerous signaling molecules have been suggested to be involved in CXCR4-mediated chemotaxis in different cell types, including G_i, GRK6 [44], PI3K, Src family kinases [45], CD45 [46], PTEN [47], the tyrosine phosphatase SHP2 and the adaptor protein Cbl [14], MMP-2 [48], PAK [49], p38 [16, 18], PKC-ζ [50], and β-arrestin [16, 18]. Although some of these pathways may be part of consecutive signaling cascades, this plethora of different signaling mechanisms may reflect the possibility that certain signaling pathways are cell-type specific. While Akt played a clear role in the cell migration of breast cancer cells as shown here, it only showed minimal involvement in lymphocyte chemotaxis, perhaps due to the transient nature of Akt activation in those cells.

In our study, Src family kinases appear to be involved upstream of PI3K since the Src family kinase inhibitor PP1 could block p85 phosphorylation induced by CXCL12. Src kinases have been reported to regulate the PI3K/Akt axis through PTEN [51]. PTEN, a tumor suppressor, dephosphorylates membrane phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol-3,4-bisphosphate, but activated Src can inhibit this effect of PTEN [51], thus prolonging the phosphatidylinositol 3,4,5-trisphosphate-dependent activation of Akt. If PTEN concentrations are rate-limiting, such a mechanism may explain the prolonged effect of CXCL12 on Akt activation in 231mfp cells. The prolonged Akt activation (>90 min) seen in 231mfp cells is not typical for CXCL12-induced Akt activation, which is usually short-lived. For instance, CXCL12-induced Akt phosphorylation in lymphocytes is maximal by 30 s and barely lasts for 10 min [52].

Compared to its critical role in cell survival, Akt is rarely reported in the context of chemotaxis, although there is some precedence for a role for it, e.g., in response to sphingosine-1-phosphate [22], and perhaps only prolonged activation of Akt is associated with cell migration. Furthermore there has been a recent report which indicates that Akt 1 and Akt 2 play opposite roles in cell migration with Akt 1 promoting it, while Akt 2 inhibits it [23]. It stands to be seen which of the two isoforms is dominant in 231mfp cells. The underlying mechanism of Akt mediated chemotaxis of breast cancer cells to CXCL12 is not entirely understood. Indeed, the substrates for Akt that are relevant to many of its actions still require identification. Interestingly, p122RhoGAP has recently been described as a substrate for activated Akt [34], which leads to activation of the RhoA/Rho kinase pathway. Consistent with this, inhibition of Rho kinase by Y27632 attenuated CXCL12-dependent migration of 231mfp cells (Fig. 5E), although further unknown pathways may also contribute. Several downstream targets of Akt, including GSK-3β, PAK, and NF-κB [53, 54], have been shown to be involved in the regulation of cell chemotaxis for other ligands. In sphingosine1-phosphate stimulated cells, activated Akt phosphorylated the EDG-1 receptor, and this trans-phosphorylation was necessary for Rac activation and cell migration [22], but it remains to be seen whether a similar mechanism applies to other G-protein coupled receptors such as CXCR4.

The PI3K inhibitor LY294002 is often used to evaluate the role of Akt in signal transduction pathways, which is not entirely correct, since PI3K has numerous additional downstream effectors including p70S6-kinase, PKCζ, Rac and Tec family kinases [55]. Akt activation was implied to be required for Hela cells chemotaxis to CXCL12, but this conclusion was based on the use of the PI3K inhibitor, LY294002, which also blocks all these other pathways [56]. Similarly, involvement of PI3K has been shown in breast cancer cells migrating towards CXCL12 [14]. This report also showed FAK phosphorylation. While we could detect increased FAK phosphorylation (results not shown), we think it is a parallel signaling pathway downstream of Src, which does not play a role in cell migration, since FRNK, a non-signaling FAK competitor failed to inhibit CXCL12-mediated chemotaxis. In our study, specific Akt inhibitors, a dominant-negative Akt construct and Akt shRNA were used to inactivate Akt without any effect on PI3K, to establish the role of Akt in CXCR4-mediated chemotaxis in breast cancer cells.

Since p38 has been suggested to be involved in CXCL12 mediated chemotaxis [16, 18], the Akt inhibitors used in this study were tested for off-target effects on p38, but did

not show any effect on p38 activity, which excluded non-specific inhibition effects through p38 (results not shown).

Although this study concentrated on the role of Akt in cell migration, the prolonged Akt activation seen following stimulation with CXCL12 would be expected to have additional negative effects in cancer cells such as increased resistance to chemotherapy due to the anti-apoptotic effects of Akt, and Akt activation in breast cancer cells has been associated with tamoxiphen resistance due to activation of ERα [57] and to transcriptional up-regulation of both the ERα and the ERβ [58]. More generally, high concentrations of CXCL12—present for instance in bone marrow—may augment the survival chance of CXCR4-expressing breast cancer cells through activation of Akt. This may play a role in the primary formation of metastasis as well as in poor response to chemotherapy.

In summary, CXCR4 activation in breast cancer cells caused prolonged Akt activation, which was a signaling pathway important for chemotaxis of these cells, a pathway which has not found much attention in this respect. Inhibition of this pathway may constitute a novel target in breast cancer chemotherapy.

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