Pharmacological targeting of CXCL12/CXCR4 signaling in prostate cancer bone metastasis

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Abstract

Background: The CXCL12/CXCR4 axis transactivates HER2 and promotes intraosseous tumor growth. To further explore the transactivation of HER2 by CXCL12, we investigated the role of small GTP protein Gαi2 in Src and HER2 phosphorylation in lipid raft membrane microdomains and the significance of CXCR4 in prostate cancer bone tumor growth.

Methods: We used a variety of methods such as lipid raft isolation, invasion assays, an in vivo model of intratibial tumor growth, bone histomorphometry, and immunohistochemistry to determine the role of CXCR4 signaling in lipid raft membrane microdomains and effects of targeting of CXCR4 for bone tumor growth.

Results: We determined that (a) CXCL12/CXCR4 transactivation of EGFR and HER2 is confined to lipid raft membrane microdomains, (b) CXCL12 activation of HER2 and Src is mediated by small GTP proteins in lipid rafts, (c) inhibition of the CXCL12/CXCR4 axis through plerixafor abrogates the initial establishment of tumor growth without affecting the growth of established bone tumors, and (d) inhibition of EGFR signaling through gefitinib leads to inhibition of established bone tumor growth.

Conclusions: These data suggest that lipid raft membrane microdomains are key sites for CXCL12/CXCR4 transactivation of HER2 via small GTP binding protein Gαi2 and Src kinase. The initial establishment of prostate cancer is supported by the endosteal niche, and blocking the CXCL12/CXCR4 axis of this niche along with its downstream signaling severely compromises initial establishment of tumors in the bone microenvironment, whereas expanding bone tumors are sensitive only to the members of growth factor receptor inhibition.

Keywords: CXCL12, CXCR4, Src, EGFR, HER2, Plerixafor, Gefitinib, Bone metastasis

Background

Prostate cancer (PC) is the most commonly diagnosed solid malignancy in men, with over 192,000 new cases yearly, and the second leading cause of cancer-related death in men in the US, with over 27,000 deaths each year. PC has a specific propensity to metastasize to bone; in fact, in the majority of cases, bone metastases develop long before metastatic growth is apparent in soft viscera.

Bone metastases cause pain, compression fractures, spinal cord compromise and other complications. Bone metastasized cancer cells induce bone turnover by recruiting bone resident osteoclasts and osteoblasts, and the resultant bone turnover enhances tumor growth by creating a vicious cycle [1]. Cancer cells use similar mechanisms as hematopoietic stem cells in homing to bone by competing for the occupation of osteoblastic niches in bone tissue [2], where chemokine signaling plays a key role in attracting cells to the bone microenvironment [2–4].

The CXCL12/CXCR4 axis has been involved in homing of breast [5] and prostate [4, 6] cancer cells to bone where cancer cells have aberrant expression of CXCR4, the receptor for the CXCL12 chemokine [7–9]. Transcriptional
regulation of the CXCR4 gene is a key determinant of net cell surface expression of CXCR4 and its subsequent function in transformed epithelial cells and cancer cells. We showed that TMPRSS2-ERG fusions regulate CXCR4 expression in prostate tumors; thus, androgen induced ERG expression transcriptionally regulates CXCR4 expression in PC cells [7, 9]. In addition, several factors and organ microenvironments have been shown to regulate CXCR4 expression in tumor cells [10–17]. The mammary fat pad and bone microenvironment have been shown to induce CXCR4 gene expression in cancer cells [16, 18]. At the cellular level, osteoblasts, stromal cells and endothelial cells all express CXCL12 [4, 6, 10, 19] and contribute to bone metastasis of PC cells [4, 6].

CXCR4 expression increases during progression of PC; localized prostate carcinoma and bone metastasis tissue express significantly higher levels than benign prostate tissue [20, 21]. Among PC patients, higher expression of CXCR4 was documented in prostate tumor tissues from African Americans [22], suggesting aggressive phenotypes often associated with higher CXCR4 expression. CXCR4 expression is associated with shorter progression free survival in cancers [23], and in prostate cancers its expression is significantly associated with metastatic disease and poor survival [24, 25]. The chemokine CXCL12 is also over-expressed in PC metastatic tissue compared to normal tissue [20]. The CXCL12/CXCR4 axis has been shown to play an important role in PC cell proliferation, migration and invasion [4, 6, 20, 26–30].

Previously, we showed that activation of the CXCL12/CXCR4 axis transactivates HER2 [3, 6] and promotes intraosseous tumor growth [3]. To further explore the transactivation of HER2 by CXCL12, we investigated the role of the small GTP protein Gαi2 in Src and HER2 phosphorylation in lipid raft membrane microdomains and the significance of CXCR4 inhibition by plerixafor, a bone cell mobilizer, in prostate cancer bone tumor growth. Given the important role of CXCL12/CXCR4 signaling in PC bone metastases, our data suggest that CXCL12/CXCR4 inhibition may impact the development of bone metastasis.

Methods
Cell culture
Cell lines were cultured in a humidified incubator with 5% CO2 at 37 °C. All media were supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Inc., Carlsbad, CA). PC3 cells maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), PC-3 M-luc2 cells in EMEM medium supplemented with 10% FBS, and C4-2B cells, in T-Medium supplemented with 10% FBS. All cell lines were authenticated with STR analysis (Genomics core at Michigan State University, East Lansing, MI) and shown to have markers respective for each cell line as established by ATCC and also tested for mycoplasma contamination prior to use with Venor-GeM mycoplasma detection kit (Sigma Biochemicals, St. Louis, MO).

Western blot analysis
Cells were washed with PBS, and total cellular proteins were extracted with buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 1 mM PMSE, and 1X Protease inhibitor cocktail (Roche, Indianapolis, IN). Protein content was quantified with a BCA protein assay (Pierce Biotechnology, Rockford, IL), and equal amounts of protein were resolved by 10% SDSPAGE. Immunoblot was performed with antibodies against pHER2 (Y1248) (Catalog # A00318-100, GenScript, Piscataway, NJ), total HER2 (Catalog #, SC-284, Santa Cruz Biotechnology, Dallas, TX) pEGFR (Y1173) (Catalog # 4407 s, Cell Signaling, Beverly, MA), total EGFR (Catalog # 4267 s, Cell Signaling, Beverly, MA), Flotillin (Catalog # 610383, BD Biosciences, San Jose, CA), β-tubulin (Catalog # SC-5274, Santa Cruz Biotechnology, Dallas, TX), pSrc (Catalog # 2101 s, Cell Signaling Technologies, Beverly, MA), total Src (Catalog # 2109 s, Cell Signaling Technologies, Beverly, MA), Gαi2 (Catalog # SC-7276, Santa Cruz Biotechnology, Dallas, TX), pAkt (S473) (Catalog # 9271 s, Cell Signaling, Beverly, MA), Akt (Catalog # 2938 s, Cell Signaling, Beverly, MA), and GAPDH (Catalog # SC-25778, Santa Cruz Biotechnology, Santa Cruz, CA). The band intensities were determined by quantitation of pixel intensities using ImageJ software (version 10.2; National Institutes of Health, Bethesda, MD).

Cell fractionation
A successive detergent solubilization method for isolating lipid rafts was previously described [3] and detergent free preparation of cell lysate and density gradient centrifugation was followed as per Macdonald et al. [31].

Invasion assay
For cells to be treated with Dasatinib (Catalog # D3307, LC Laboratories, Woburn, MA), C4-2B cells were plated on the upper chamber of Matrigel-coated transwell filters (2 × 105 cells/filter) in growth media, supplemented with 1% FBS, containing 0.5 μM Dasatinib or vehicle control. For Pertussis toxin (PTX) (Catalog # 516561, Calbiochem, La Jolla, CA) studies, cells were pretreated with 200 ng/ml PTX for 3 h prior to cell invasion studies. For cells to be treated with Src siRNA, C4-2B cells were transfected with scrambled or Src siRNA using Lipofectamine 2000 (Invitrogen) 24 h prior to seeding 2 × 105 cells in serum free medium on Matrigel coated inserts. For both Dasatinib and siRNA conditions, cells were allowed to invade for 24 h in the presence or absence of 200 ng/mL CXCL12 added to the bottom chamber. Cotton swabs were used to remove non-migrated cells from the upper chamber, and inserts
were stained with 0.9 % crystal violet. Total number of migrated cells was counted under 10X magnification in five fields. Assays were performed in triplicate. *: p < 0.05; **: p < 0.005. For protein analysis, cells were treated with Dasatinib or Src siRNA as performed for the invasion assays, in the presence or absence of CXCL12. Cell lysate was collected after 24 h and analyzed by Western blot.

In vivo studies and tumor tissue analyses
PC-3 M-luc2 cells were injected intratibially on Day 0 and saline control or plerixafor treatment was started via an osmotic pump (Alzet, Cupertino, CA). Plerixafor is obtained from Genzyme Corporation and administered in the animal model using an osmotic pump at the rate of 0.5 μl per hour at 20 mg/ml concentration. After 21 or 23 days, tumor growth was determined by in vivo luciferase imaging. For treating of established tumors, mice were sacrificed and ex vivo x-ray imaging of tumor-bearing tibiae was performed at 22 or 24 days post-injection. For treating established tumors, tumors were imaged at day 17, and, based on luciferase signals, tumors were randomly divided into two groups: plerixafor vs saline (control); plerixafor or saline pumps were then implanted at 18 days post tumor cell implantation in tibiae. Further luciferase imaging was performed at day 21 to monitor tumor growth. Mice were sacrificed and ex vivo x-ray imaging of tumor-bearing tibiae was performed at 26 days post-injection. C4-2B cells were infected with lentiviruses expressing luciferase to generate C4-2B-luc cells and stable cells were selected with puromycin treatment. For gefitinib study animals imaged at 15 days post tumor cell injection were randomized as control and treatment groups. Gefitinib (Catalog # G-4408, LC Laboratories, Woburn, MA) is formulated in 0.5 % Tween 80 and administered through oral gavage at 200 mg/kg body weight. Animals were imaged at 23, 29 and 40 days and x-rays, obtained at the 40th day. Luciferase imaging was performed with either Kodak in vivo imager or Carestream in vivo Xtreme imager (Bruker, Bellerica, MA).

Immunohistochemistry
Formalin-fixed, paraffin-embedded serial tissue sections from control or plerixafor treated bone tumors were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubating in 3 % H₂O₂ for 20 min; antigen retrieval was performed with proteinase K (Sigma-Aldrich, St. Louis, MO). Slides were then blocked with Blocking Serum from ABC Vectastain Kit (Vector Labs, Burlingame, CA). Slides were incubated at 4 °C overnight in a humidified atmosphere in primary antibody. Slides were then washed with PBS and incubated with biotinylated secondary antibody. After washing, sections were incubated with ABC Vectastain Kit, according to manufacturer’s protocol, followed by incubation with 3, 3-diaminobenzidine tetrahydrochloride (Vector Laboratories, Inc., Burlingame, CA). Nuclei were counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO).

Statistical analyses
Data were analyzed using GraphPad Prism software and Microsoft Excel 2008. All data are presented as mean ± SE. The in vivo luciferase imaging was performed with two different machines (Kodak in vivo imaging (old) and Bruker in vivo Xtream (new) equipment) in case of Figs. 4b, c and 5b, c. Therefore, the expression levels of photons generated by the old machine were normalized by those by the new machine using their geometric means as follows. Suppose there are n and m photon expression levels generated by old and new machines, respectively (i.e., X_1, X_2, ..., X_n, Y_1, Y_2, ..., Y_m). Then the i th expression level x_i for the old machine will be normalized by Y_i = X_i / (\prod_{k=1}^{m} Y_k)^(\frac{1}{m}) / (\prod_{j=1}^{n} X_j)^(\frac{1}{n}) where i = 1, 2, ..., m.

Statistical comparisons were performed using Wilcoxon rank sum test and a p-value < 0.05 was considered statistically significant.

Results
CXCL12/CXCR4 axis transactivates EGFR members in lipid raft membrane microdomains
Previously, we have shown that CXCL12 signaling is capable of transactivating HER2 in the lipid rafts domains in PC3 cells [3]. In an effort to determine if this transactivation is confined to lipid raft membrane microdomains or occurs elsewhere in the cell and if this transactivation was limited to HER2, we performed experiments with C4-2B and PC3 cells and investigated CXCL12/CXCR4 induced phosphorylation of HER2 and EGFR. Western blot analysis demonstrated that treatment with CXCL12 did not significantly alter phosphorylation of HER2 or EGFR (Fig. 1a). Furthermore, immunoprecipitation of HER2 and EGFR also did not show changes in phosphorylation of either HER2 or EGFR upon treatment with CXCL12 in either C4-2B or PC3 cells (Fig. 1b). On the contrary, a successive detergent solubilization method of lipid raft isolation showed that CXCL12 had indeed induced phosphorylation of both HER2 and EGFR in PC3 and C4-2B cells (Fig. 1c). Our previous studies used detergents to solubilize cells for lipid raft preparation by sucrose density gradient centrifugation, and to avoid detergent induced artifacts in cellular signaling in lipid raft preparation [32], we avoided detergents for cell lysis and prepared lipid rafts
using sucrose density gradients. However, upon fractionation, increased levels of pHER2 and pSrc were still found, in the lipid raft fraction of both control and CXCL12 treated PC3 cells (Fig. 1d). These data demonstrate that CXCL12/CXCR4 transactivates both HER2 and EGFR, that this transactivation occurs exclusively in lipid raft microdomains, confirming our previous results, and that detergents in raft preparation do not significantly affect the growth factor receptor transactivation.

**G**α**i** proteins mediate CXCL12/CXCR4 induced Src and HER2 phosphorylation

Members of small G-proteins were shown to promote prostate cancer cell invasion [33–36]. To determine the requirement of heterotrimeric Gαi proteins in CXCL12/CXCR4 induced HER2 phosphorylation and cellular invasion, we treated C4-2B cells with pertussis toxin (PTX) for 3 h to inhibit Gαi proteins (Fig. 2) and this treatment did not affect the cell viability. Pertussis toxin treatment leads to inhibition of basal phosphorylation of both HER2 and Src in lipid raft membrane microdomains (Fig. 2a). To determine the effect of PTX on CXCL12 dependent HER2 and Src phosphorylation, cells were pre-treated with PTX followed by CXCL12 activation. PTX treatment inhibited CXCL12 activated HER2 and Src phosphorylation in a dose dependent manner in lipid raft membrane microdomains, without significant changes in their counterparts from the cellular membranes and cytosol fractions (Fig. 2a).

To further investigate the role of Gαi proteins in this activation, C4-2B cells were transfected with wild type Gαi (WT-Gαi2), constitutively active Gαi2^Q205L, or plasmid...
**Fig. 2** CXCL12 activation of HER2 and Src is mediated by $G_{\alpha i}$ GTP proteins in lipid raft membrane microdomains and this activation induces cell invasion. **a** C4-2B cells were cultured in the presence or absence of increasing concentrations of pertussis toxin (PTX) for 3 h, followed by treatment with CXCL12 for 15 min. Cell lysate was collected, and protein expression of phosphorylated and total HER2 and Src was determined by Western blot analysis. Fliotillin was utilized as a loading control for the lipid raft fraction; $\beta$-Tubulin was utilized as a control for the membranes and cytosol fractions. Bottom panel shows the quantitation of phosphorylated HER2 and Src. **b** C4-2B cells were transfected with wild type $G_{\alpha i}$ (WT-G$\alpha i2$), constitutively active G$\alpha i$ (Q205L), or plasmid control (pcDNA3.1). Cells were then cultured in the presence or absence of CXCL12 for 15 min, and cell lysate was collected. Protein expression of phosphorylated and total HER2 and Src as well as G$\alpha i2$ were determined by Western blot analysis. **c** C4-2B cells were transfected with plasmid control or constitutively active G$\alpha i$ (Q205L) and cultured in the presence or absence of CXCL12. Lipid raft fractions were collected and immunoblotted with phospho HER2 and Src antibodies. **d** C4-2B cells were either treated or not, with PTX (200 ng/ml) and a chemoinvasion assay was performed in the presence or absence of 200 ng/ml CXCL12. **e** C4-2B cells were transfected with pcDNA3.1 or pcDNA3.1 G$\alpha i2^{Q205L}$ plasmids and a chemoinvasion assay was performed in the presence or absence of 200 ng/ml CXCL12. Bottom panels show the quantitation of invaded cells. Experiment was performed in triplicates; a representative field of invading cells are shown (**d** and **e**). Number of invading cells was quantitated in three independent experiments and statistical analyses were performed using ANOVA; significance was calculated using Tukey Posttest analysis using GraphPad Prism software. $P$ value <0.05 is shown as a *
control (pcDNA3.1) (Fig. 2b). Overexpression of G\textsubscript{α\textsubscript{i2}} increased basal levels of pHER2 and pSrc relative to pcDNA3.1 transfected control; these levels were further increased by CXCL12 treatment. Mutation of the activating residue of G\textsubscript{α\textsubscript{i}} subunit (Q205L) results in the inability of the G\textsubscript{α\textsubscript{i}} subunit to dissociate from GTP and is thus constitutively active. G\textsubscript{α\textsubscript{Q205L}} transfection resulted in high levels of pHER2 and pSrc, which were further increased by CXCL12 treatment (Fig. 2b). Overexpression of G\textsubscript{α\textsubscript{2}} also activated HER2 and Src phosphorylation in lipid raft membrane microdomains (Fig. 2c). These results show that activation of G\textsubscript{α\textsubscript{i}} is sufficient for HER2 and Src phosphorylation, and that, in cancer cells, activation of small G proteins can activate downstream pathways including HER2 and Src. To determine the impact of G\textsubscript{α\textsubscript{i}} protein activation of HER2 and Src on cellular invasion, C4-2B cells were treated with PTX and matrigel invasion assays were performed. CXCL12 induced cellular invasion of C4-2B cells due to the activation of Src and HER2 signaling. Further, PTX pretreatment inhibited both basal and CXCL12 invasion due to the inhibition of trimeric G-protein activation and subsequent suppression of Src and HER2 activation (Fig. 2d). Expression of G\textsubscript{α\textsubscript{Q205L}} enhanced basal invasion and CXCL12 treatment further enhanced cellular invasion (Fig. 2e). Together, these data demonstrate that G protein signaling mediates HER2 and Src kinase activation, and this activation may contribute to cellular invasion downstream of the CXCL12/CXCR4 axis.

**Src inhibition decreases CXCL12-mediated invasion**

The previous results implicate G proteins in the activation of Src. To determine the downstream effects of this activation, the role of Src in CXCL12-mediated invasion was next investigated. To this end, dasatinib was used to inhibit Src as a pharmacological approach and Src siRNA was used as a genetic approach. Dasatinib treatment of C4-2B cells leads to inhibition of basal and CXCL12 induced matrigel invasion (Fig. 3a). Western blot analysis confirmed the inhibition of Src phosphorylation by dasatinib (Fig. 3a). To confirm the role of Src in CXCL12-mediated invasion, Src expression in C4-2B cells was downregulated using siRNA 24 h prior to plating on Matrigel-coated inserts. As shown in Fig. 3c, Src siRNA resulted in a decrease in the CXCL12-mediated invasion. Western blot analysis confirmed the downregulation of Src by siRNA (Fig. 3d). These results confirm that, upon transactivation of Src through CXCL12/CXCR4, Src is capable of mediating downstream effects such as invasion.

**Inhibition of CXCR4 decreases initial establishment of bone tumors without affecting the expansion of existing bone tumors**

To determine the biological significance of the CXCL12/CXCR4 axis in bone tumor growth, an intratibial model was employed. In this model system, CXCR4 inhibition was obtained by use of plerixafor. Plerixafor is a hematopoietic stem cell mobilizer that has been shown to block the binding of CXCL12 to CXCR4. To this end, PC-3 M-luc2 cells were used to test the inhibition of CXCR4 signaling with plerixafor. Western blot analysis showed that PC-3 M-luc2 cells express higher CXCR4, but similar levels of EGFR, compared to PC-3 cells (Additional file 1: Figure S1). PC-3 M-luc2 cells were injected intratibially and saline control or plerixafor treatment was started via an osmotic pump on day 0 (Fig. 4a). After 21 or 23 days, in vivo luciferase imaging was performed. Twenty-two or twenty-four days post-injection of tumor cells and initiation of treatment, mice were sacrificed and ex vivo x-ray imaging was performed on the harvested tibiae. Treatment of the mice with plerixafor resulted in decreased tumor growth and decreased bone destruction relative to the saline control, as shown by luciferase and x-ray imaging (Fig. 4b-d). Bone histomorphometry performed on bone tissue sections revealed that plerixafor treatment resulted in decreased tumor volume and a decrease in bone destruction (Fig. 4e, f). Immunohistochemical analysis demonstrated reduced expression of CXCR4 in the plerixafor treated mice (Fig. 4g). Additionally, treatment with plerixafor resulted in decreased proliferation, as determined by Ki67 staining (Fig. 4h). Plerixafor also inhibited tumor growth and tumor induced bone destruction in a C4-2B-luc2 model (Additional file 1: Figure S2). These results demonstrate that inhibition of CXCR4 at the time of prostate tumor implantation in the bone results in decreased tumor growth, which is associated with decreased CXCR4 expression, decreased tumor induced bone osteolysis, and decreased proliferation.

To determine if inhibition of CXCR4 would have similar effects on tumor growth with established tumors, mice were again injected intratibially with PC-3 M-luc2 cells. After allowing tumor growth for 18 days, mice were divided into two groups with relatively equal luciferase signal. Osmotic pumps were implanted the next day with either plerixafor or saline. Tumor growth was monitored by in vivo imaging at 17 and 21 days post-injection of the cells. Mice were sacrificed 26 days after tumor cell injection, and ex vivo imaging of the tibiae was performed (Fig. 5a). As shown by luciferase imaging as well as by x-ray scans and histology, treatment with plerixafor at this stage did not affect tumor growth relative to the saline control (Fig. 5b-e). Immunohistochemical analysis of bone sections did not reveal a change in CXCR4 expression upon plerixafor treatment (Fig. 5f). Additionally, plerixafor treatment did not affect proliferation, as determined by Ki67 (Fig. 5g). These results demonstrate that inhibition of CXCR4 subsequent to prostate tumor development in the bone...
does not affect tumor growth. Taken together with the results of Fig. 4, it is likely that CXCL12/CXCR4 signaling plays a key role in the initial establishment of prostate tumors in the bone; however, the importance of CXCL12/CXCR4 appears to be taken over by other signaling mechanisms during the enlargement phase of bone tumors.

EGFR and HER2 inhibition blocked the expansion of bone tumor

Our data suggest that CXCR4 induces growth factor receptor activation through EGFR and HER2 phosphorylation (Fig. 1) and subsequent expression of proteases and cellular invasion [3]. Growth factor ligands activate EGFR by direct binding, whereas HER2 is inactive in direct ligand binding but heterodimerization with ligand bound EGFR potently amplify its signaling [37]. Gefitinib is a broad EGFR family member inhibitor which has been shown to inhibit EGFR signaling and HER2 signaling through heterodimerization with EGFR [38]. Since CXCR4 transactivates EGFR and HER2, we tested whether growth factor receptor signaling contributes to bone tumor growth. Mice having established bone tumors were randomized and treated with either gefitinib or Tween 20 (formulation used for dissolving gefitinib). Tumor growth was monitored at 23, 29 and 40 days by in vivo imaging and bioptic x-rays were performed at the 40th day of post tumor cell implantation (Fig. 6a). Gefitinib treatment inhibited bone tumor growth starting from as early as 8 days after treatment and persisted till the 25th day. X-rays showed less osteolysis in gefinitib treated animals (Fig. 6b). This data suggest that EGFR and HER2 signaling is actively involved in bone tumor growth in the PC-3 M-luc2 bone tumor model.

Discussion

The CXCL12/CXCR4 axis has been shown to be involved in metastasis of several types of cancers, including prostate cancers. In support of CXCL12/CXCR4 function in tumor metastasis, herein, we show that: i) CXCL12/CXCR4 transactivates members of growth factor receptors, EGFR and HER2 and that this transactivation is largely confined to lipid raft membrane microdomains in PC cells; ii) \( \alpha_i \) protein activation is required for downstream signaling involving HER2 and Src, and constitutively active \( \alpha_i \) proteins can activate HER2 and Src signaling, independent of CXCR4 activation; iii) Src activation mediates CXCL12/CXCR4 induced cell invasion; iv) plerixafor is effective in inhibition of tumor growth, when given at the time of tumor implantation, but not effective against established tumors, suggesting the CXCL12/CXCR4 axis is crucial for initial interaction with bone microenvironment and that this interaction dictates subsequent growth of bone tumors; and v) the pan EGFR family member inhibitor gefitinib inhibited growth of established bone tumors, suggesting that growth factor signaling is critical for the expansion/enlargement of bone metastases.
Treatment with plerixafor results in decreased intratibial tumor growth when administered concurrently with tumor cell injection. **a** Diagram of experiment timeline. On Day 0, PC-3 M-luc2 cells were injected intratibially and saline control or plerixafor treatment was started via an osmotic pump. **b** After 21 or 23 days, in vivo luciferase imaging was performed. Images shown are taken at either 21 or 23 days. **c** Tumor growth at 21 or 23 days post injection of cells was determined by in vivo luciferase imaging ($n = 6$). * Represents $p < 0.05$. **d** Ex vivo x-ray imaging of media injected (left) and tumor bearing (right) tibiae was performed at 22 and 24 days post-injection. **e** H&E was performed on tissue sections from PC-3 M-luc2 intratibial tumors treated with saline control or plerixafor. Representative images were taken at 5X and digitally merged. **f** H&E staining was used for bone histomorphometric analysis to determine the ratio of cortical bone (left), trabecular bone (middle), and tumor cells (right) in each tissue section. **g** Tissue sections from PC-3 M-luc2 intratibial tumors were immunostained with anti-CXCR4 antibody. Images were taken at 20X and 40X (insert). **h** Proliferation of PC-3 M-luc2 tumors was analyzed by Ki67 staining; an average number of Ki67+ cells from five 20X fields was determined.
Lipid rafts are specialized entities in plasma membrane, enriched with tightly packed saturated lipids and cholesterol which gives liquid ordered states. These raft microdomains are known to segregate different constituents on the membrane thereby facilitating signal transduction. Previous studies show that CXCR4 signaling localizes to lipid raft membrane microdomains and that disruption of lipid rafts leads to inhibition of basal and CXCL12 signaling [3, 6]. The association of CXCR4 with lipid rafts in hematopoietic stem cells was shown to promote bone marrow retention and regulate homing as well as mobilization of hematopoietic stem cells [39]. Previous studies used detergents to isolate lipid rafts based on the fact that these rafts were insoluble in non-ionic detergents, but the use of detergents was implicated in artificial coalescence of signaling proteins. To address this issue we used detergent-free cell lysate preparations for lipid raft isolation using the sucrose density buoyant centrifugation

![Fig. 5](image-url) Fig. 5 Treatment with plerixafor does not alter intratibial tumor growth when administered subsequent to tumor formation. a Diagram of experiment timeline. On Day 0, PC-3 M-luc2 cells were injected intratibially. After allowing tumors to grow for 18 days, saline control or plerixafor treatment was started via an osmotic pump. b After 21 days post-injection of tumor cells, in vivo luciferase imaging was performed. c Tumor growth at 21 days post injection of cells was determined by in vivo luciferase imaging (n = 10). NS represents no significant difference between groups. d Ex vivo x-ray imaging of media injected (left) and tumor bearing (right) tibiae was performed at 26 days post-injection. e H&E was performed on tissue sections from PC-3 M-luc2 intratibial tumors treated with saline control or plerixafor. Representative images were taken at 5X and digitally merged. f Tissue sections from PC-3 M-luc2 intratibial tumors were immunostained with anti-CXCR4 antibody. g Proliferation of PC-3 M-luc2 tumors was analyzed by Ki67 staining; the average number of Ki67+ cells from five 20X fields was calculated.
method and show that the lipid raft marker flotillin co-
sediments with CXCR4 and phosphorylated HER2 and 
Src kinases (Fig. 1d). Previous studies also show that 
EGFR family members localize to lipid raft preparations 
in detergent-free conditions [33, 35]. These observa-
tions suggest that CXCR4 and its signaling partners 
localize to lipid rafts and initiate signaling events lead-
ing to cell invasion.

G-proteins are key regulators of G-protein coupled 
receptor (GPCR) signaling and were shown to promote 
oncogenic signaling. Previous studies show that activated 
forms of G12 and G13 promote PC cell invasion, and 
Gαi proteins were also shown to promote cell migration. 
Our data show that PTX abrogated both HER2 and Src 
phosphorylation and cellular invasion, suggesting that 
Gαi proteins are indispensable for CXCR4 induced cellular 
invasion. Moreover, we show that constitutively activated 
Gαi proteins are sufficient to mediate HER2 and Src phosphorylation and this phosphorylation may con-
tribute to cellular invasion of PC cells, suggesting that 
CXCR4 activated Gαi proteins are sufficient for PC cell 
invansion. Recent studies support the role of Gαi proteins 
in Src induced formation of invadosomes, which are 
cellular protrusions exhibited in migrating/invading cells, 
where a transient activation of Cdc42 is implicated down-
stream of GPCR activation [40]; thus, we cannot rule out 
the role of Cdc42 downstream of CXCR4 activated Src ac-
tivation in PC cells.

CXCL12/CXCR4 signaling regulates hematopoietic stem 
cell (HSC) movement in and out of bone marrow. HSCs 
home to the CXCL12 rich endosteal niche in bone marrow, 
where upon entering the bone, they anchor to the niche 
through CXCR4 activation of α4β1 integrins with vascular 
cell adhesion molecule 1 (VCAM1) expressed in the niche. 
These chemokine (CXCL12/CXCR4) and cell adhesion 
molecule (VCAM1/α4β1) interactions restrain the HSCs in 
the bone marrow niche. Plerixafor is a stem cell mobilizer, 
where it competitively inhibits CXCL12 binding to CXCR4, 
thereby disrupting the HSC interaction with bone marrow 
niches, leading to exit of cells from bone marrow. Here we 
show that inhibition of CXCR4 with plerixafor is effective 
against bone tumor growth when given initially during 
tumor implantation. Similar results were previously 
documented for neutralization of CXCR4 with anti-CXCR4 
antibodies in an intracardiac model of metastases where 
anti-CXCR4 antibodies reduced total metastatic burden, in-
cluding that of long bone [29]. Recent studies suggest that 
initial arrival of tumor cells in the bone microenvironment 
leads to competition of tumor cells with HSCs for occupa-
tion at osteoblastic niches [2]. Since both HSCs and PC cells 
use the CXCL12/CXCR4 axis for occupying the osteoblastic 
niche, plerixafor inhibited CXCL12/CXCR4 interactions, 
leading to reduced tumor growth when given at the time of 
tumor implantation. Reduction in tumor growth was also ac-
companied by reduced tumor induced bone destruction. 
This suggests that plerixafor not only mediated egress of 
HSCs from bone but also inhibited initial interaction of 
tumor cells with the osteoblastic niche, leading to inhibition 
of tumor growth. In support of our observation, while this 
manuscript was in preparation, Gravina et al. showed that 
plerixafor inhibited intratibial tumor growth when adminis-
tered right after tumor cell implantation [41]. As the putative 
PC stem cells use the CXCL12/CXCR4 axis for metastasis and 
chemotherapies often are effective in eliminating these cell 
populations [42], plerixafor mediated inhibition of CXCR4 
may inhibit bone metastasis by these cell populations.

Plerixafor administration to established tumors did not 
significantly impact tumor growth, suggesting that, at high 
tumor burden, plerixafor mediated egress of tumor cells
from bone, as suggested by previous studies [2], is overcome by the growth signals in tumors. CXCL12/CXCR4 transactivates members of growth factor receptor in PC cells [3, 6], and expression of HER2 in PC patients correlates with tumor cell proliferation [43] and activates androgen receptor signaling in advanced disease [44]. To address the potential role of growth factor receptor activation contributing to the bone tumor growth, we treated established bone tumors with gefitinib and found our data (Fig. 6) support the notion that inhibition of growth factor receptor mediated signals with gefitinib led to reduction in tumor burden and tumor induced osteolysis in bone tumors. It remains to be determined that combination of the two therapies have an added effect over individual therapies, but based on the mechanism of target inhibition combination therapy may provide a more efficacious inhibition of bone tumor growth.

Taken together, CXCL12/CXCR4 signaling has a dual impact in bone metastasis: newly arrived cancer cells in bone use CXCL12/CXCR4 and integrin interactions to localize to the endosteal niche, and established bone tumors use CXCL12/CXCR4 transactivated growth factor receptor signaling for expansion of bone tumors.

Conclusions

CXCL12/CXCR4 transactivation of members of growth factor receptors exclusively occurs in lipid raft membrane microdomains. $G_{\text{oi}}$ protein activation is required for downstream signaling involving EGFR, HER2 and Src in lipid raft membrane microdomains. Plerixafor, a competitive CXCR4 inhibitor and a stem cell mobilizer, is effective in inhibiting initial establishment of tumor cells into the bone microenvironment, whereas the same drug is ineffective in containing the expansion of pre-existing bone metastasis. Interestingly, in our model system, the growth factor receptor inhibitor gefitinib is highly effective against expanding bone tumors. Based on our preclinical observations, plerixafor may be a candidate drug for the patient populations which are high risk for developing metastasis, with low metastasis burden, and who are prone to relapse, whereas gefitinib may be a candidate for patients with metastatic disease with rising PSA. Further experiments are in progress to determine the efficacy of different drug regimens (sequence of gefitinib and plerixafor) with or without chemotherapy combination for treating established bone tumors.

Additional file

Additional file 1: Figure S1. CXCR4 and EGFR expressed in PC-3 M-Luc2 cells. Western blot analysis of EGFR and CXCR4 shows that PC-3 M-Luc2 cells have higher CXCR4 expression compared to PC3 and C4-2B. No significant change in EGFR expression was observed in these cells. Figure S2. Plerixafor treatment inhibited intratibial tumor growth in C4-2B-luc cells. A) Diagram of experimental timeline. On Day 0, C4-2B-luc cells were injected intratibially and saline control or Plerixafor treatment was started via an osmotic pump. B) After 12, 19, 26 and 33 days, in vivo luciferase imaging was performed. Images shown are taken at 33 days. Ex vivo x-ray imaging of media injected (left) and tumor bearing (right) tibiae was performed at 23 days post-injection. C) Tumor growth at 12, 19, 26 and 33 days post injection of cells was determined by in vivo luciferase imaging. (PTX 659 kb)

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Availability of data and materials
Not applicable.

Authors’ contributions
SRC: Study conception and design, acquisition of data, analysis and interpretation of data and manuscript writing. MKC-L: Study design, acquisition of data, analysis and interpretation of data, and manuscript writing. HIE: Study design and manuscript preparation. MLC: Study design and manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable. No human subjects are involved in the study.

Ethics approval

The animal studies were performed in facilities monitored by division of laboratory animal resources (DLAR) at Wayne State University. The DLAR facility’s PHS Animal Welfare Assurance number is A3310-01, approved on February 28, 2014 and USDA registration number is 34-R-0014. The University’s AAALAC file number is 393. No human subjects were involved in the study.

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Abbreviations

Akt kinase: Autologous killer t-cell kinase; ATCC: American type culture collection; BCA: Bicinchoninic acid; CXCL12: A prostate cancer cell line derived from LNCaP cells; Cdc42: Cell division control protein -42 homolog; CXCL12: Chemokine ligand 12 containing CXC motif; CXCR4: Chemokine receptor 4, whose ligand is CXCL12; eGFP: Epidermal Growth Factor Receptor; ERG: A member of Ets transcription family; ETOP: Ethanol; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde phosphate dehydrogenase; GTP: Guanine nucleotide triphosphate; $G_{\text{oi}}$: Inhibitory trimeric G-protein alpha 2 isofrom; HER2: Heregulin; EGFR: Epidermal growth factor receptor 2; HSC: Hematopoietic stem cell; Ki67: A cellular marker for proliferation; Luc: Luciferase; NFκB: Nuclear factor kappa B; PC: Prostate cancer; PC3: A prostate cancer cell line; PTX: Pertussis toxin; RPMI: A cell culture media developed at Roswell Park Memorial Institute; Scc: Scrambled; SE: Standard error; siRNA: Small inhibitory RNA; Src: Non-receptor tyrosine kinase (Src is an abbreviation for sarcoma); STR: Short tandem repeat; TMRPSS2: Transmembrane serine proteinase 2; VCAM: Vascular cell adhesion molecule; VCaP: A prostate cancer cell line; $\alpha$: An integrin protein consisting of alpha 4 and beta 1 polypeptide chains; $\mu$: Microgram

Additional file

Additional file 1: Figure S1. CXCR4 and EGFR expressed in PC-3 M-Luc2 cells. Western blot analysis of EGFR and CXCR4 shows that PC-3 M-Luc2 cells have higher CXCR4 expression compared to PC3 and C4-2B. No significant change in EGFR expression was observed in these cells. Figure S2. Plerixafor treatment inhibited intratibial tumor growth in C4-2B-luc cells. A) Diagram of experimental timeline. On Day 0, C4-2B-luc cells were injected intratibially and saline control or Plerixafor treatment was started via an osmotic pump. B) After 12, 19, 26 and 33 days, in vivo luciferase imaging was performed. Images shown are taken at 33 days. Ex vivo x-ray imaging of media injected (left) and tumor bearing (right) tibiae was performed at 23 days post-injection. C) Tumor growth at 12, 19, 26 and 33 days post injection of cells was determined by in vivo luciferase imaging. (PTX 659 kb)
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