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Secreted CXCL12 (SDF-1) Forms Dimers under Physiologic Conditions

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Abstract

Chemokine CXCL12 signaling through receptors CXCR4 and CXCR7 has essential functions in development and underlies diseases including cancer, atherosclerosis, and autoimmunity. Chemokines may form homodimers that regulate receptor binding and signaling, but previous studies with synthetic CXCL12 have produced conflicting evidence for homodimerization. We used bioluminescence imaging with *Gaussia* luciferase fusions to investigate dimerization of CXCL12 secreted from mammalian cells. By column chromatography and *Gaussia* luciferase complementation, we established that CXCL12 was secreted from mammalian cells as both monomers and dimers. Secreted CXCL12 also formed homodimers in the extracellular space. Monomeric CXCL12 preferentially activated CXCR4 signaling through Gαi and AKT, while dimeric CXCL12 more effectively promoted recruitment of β-arrestin 2 to CXCR4 and chemotaxis of CXCR4-expressing breast cancer cells. We also showed that CXCR7 preferentially sequestered monomeric CXCL12 from the extracellular space and had minimal effects on dimeric CXCL12 in cell-based assays and an orthotopic tumor xenograft model of human breast cancer. These studies establish that CXCL12 secreted from mammalian cells forms homodimers under physiologic conditions. Since monomeric and dimeric CXCL12 have distinct effects on cell signaling and function, our results have important implications for ongoing efforts to target CXCL12 pathways for therapy.

Keywords

chemokine; chemokine receptor; protein fragment complementation; bioluminescence; luciferase; breast cancer

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Author contributions

PR, SAL, LAM, and SCL-P performed experiments. ST, KEL, and GDL provided new reagents. PR, SAL, LAM, KEL, and GDL analyzed data. PR and GDL wrote the paper. KEL and GDL supervised the project.

Introduction

Chemokine CXCL12 (SDF-1) originally was identified as a growth factor for B lymphocytes and a chemoattractant molecule for T lymphocytes and monocytes [1]. In addition to effects on proliferation and trafficking of immune cells, CXCL12 has numerous other functions in development and normal physiology. Mice lacking CXCL12 die *in utero* with multiple abnormalities, including deficient vascularization of the gastrointestinal tract, heart defects, impaired myelopoiesis, and perturbed migration of neurons in the central nervous system [2, 3]. CXCL12 also is essential for normal development of alveoli in the lung [4]. This chemokine is required for homing of hematopoietic stem cells to bone marrow, and inhibition of CXCL12 signaling through receptor CXCR4 is used to mobilize stem cells for bone marrow transplant [5]. Effects of CXCL12 on multiple organs and tissues are mediated through its receptors CXCR4 and CXCR7, which independently or collectively regulate chemotaxis and invasion of cells, increase cell adhesion, and activate intracellular signaling pathways that control cell proliferation and survival.

Beyond critical functions in normal development and physiology, CXCL12 and its signaling pathways appear to underlie pathogenesis of numerous diseases that are challenging to treat with current therapies. CXCL12 has been implicated in growth and organ-specific metastasis of more than 20 different human cancers, including lung, breast, prostate, and ovarian [6]. Elevated levels of CXCL12 and its receptors are associated with poor prognosis and overall survival in many of these malignancies [7, 8]. CXCL12 regulates progression of atherosclerosis, and this molecule recruits stem and progenitor cell populations to sites of ischemic or infarcted tissue in sites including heart and brain [9, 10]. CXCL12 also is associated with pathophysiology and progression of autoimmune diseases including rheumatoid arthritis and multiple sclerosis [11, 12]. These studies highlight the rationale for developing CXCL12-targeted therapies and emphasize the need to understand the biology of CXCL12 to optimally utilize new drugs regulating this chemokine pathway.

Several chemokines form homodimers and heterodimers that activate signaling pathways distinct from monomeric proteins *in vitro* and *in vivo* [13, 14]. However, data about homodimerization of CXCL12 are unclear. Crystal structures show CXCL12 as dimers, but NMR studies detect monomers at concentrations less than 5 mM in solution [15–17]. The monomer-dimer equilibrium of CXCL12 is regulated by pH, phosphate, and oligosaccharides with heparan sulfate and similar proteoglycans present on cell membranes and the extracellular space promoting dimerization [18–20]. In the presence of heparin oligosaccharides, CXCL12 forms dimers at low micromolar concentrations, which are substantially less than concentrations required for dimerization of pure protein [21].

Studies using recombinant mutants of CXCL12 that favor dimers or monomers have produced inconsistent results for signaling and function. Using a monocytic leukemia cell line, Veldkamp et al concluded that monomeric CXCL12 was the active form, while a dimeric mutant was a partial agonist that opposed chemotaxis [22]. This research group also determined that only monomeric CXCL12 protected the heart from ischemic damage in an *ex vivo* model [23]. However, a mutant of CXCL12 deficient in oligosaccharide binding and dimerization was less effective than wild-type chemokine as a chemoattractant for hepatoma

cells, suggesting that dimeric CXCL12 increased migration of these cells [21]. Although these studies had different conclusions about activities of monomers versus dimers, the data support homodimerization of CXCL12 under physiologic conditions and indicate that monomers and dimers have distinct effects on CXCL12-dependent signaling and function.

We used bioluminescence imaging strategies to investigate dimerization of CXCL12 secreted from mammalian cells. Using a *Gaussia* luciferase fusion to CXCL12 and *Gaussia* luciferase complementation, we established that secreted CXCL12 forms dimers under physiologic conditions. Monomeric and dimeric CXCL12 activated downstream signaling pathways and cell migration to differing extents in cell-based assays. CXCL12 monomers also were preferentially scavenged by receptor CXCR7. These results advance understanding of CXCL12 structure and function and inform ongoing efforts to target and utilize this chemokine for therapy.

Experimental Procedures

Plasmids

pGloSensor-20F firefly luciferase reporter plasmid for cAMP was from Promega. The reporter was excised with HindIII and BamHI and transferred to the XbaI site of lentiviral vector FUGW by blunt end ligation.

Cells

HEK 293T and MDA-MB-231 breast cancer cells were obtained from the ATCC. 293T cells stably expressing unfused *Gaussia* luciferase (GL) or CXCL12 fused to *Gaussia* luciferase (CXCL12-GL) and MDA-MB-231 cells stably transduced with CXCR4, CXCR7, or vector control have been described previously [24]. 293T and 231 cells were stably transduced with lentiviruses expressing CXCL12-NG, CXCL12-CG, unfused secreted CG, or both CXCL12-NG and CXCL12-CG complementation reporters [25]. 231 cells expressing both CXCL12-NG and CXCL12-CG were transduced with constitutively-expressed eqFP650, a far red fluorescent protein [26]. We also generated 231 cells expressing CXCL12-GL and pGlo Sensor 20F. Cells were cultured in DMEM (Invitrogen), 10% fetal bovine serum, 1% glutamine, and 0.1% penicillin/streptomycin/gentamicin. Cells were grown in a 37° incubator with 5% CO_2.

Column chromatography and analysis of *Gaussia* luciferase activity in recovered fractions

293T-CXCL12-GL or 293T-GL cells were cultured overnight in phenol red free DMEM (Invitrogen). Supernatants were filtered through a PVDF membrane (Corning) and concentrated using an Amicon filter unit with 10 kDa cutoff. One ml of concentrated supernatants was applied to a 50 cm, 1.5 cm inner diameter Kontes Flex column (Fisher Scientific) packed with Superdex 75 (Sigma) and equilibrated with column buffer [50 mM Tris (pH 7.5) and 100 mM KCl]. Size standards for elution were albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12 kDa). The void volume of the column was determined using dextran blue (200 kDa) (Sigma). The column flow rate was 800 μl/min. *Gaussia* luciferase activity in column fractions was measured by bioluminescence imaging with coelenterazine as described [24]. Fractions with monomeric and dimeric CXCL12-GL

were determined based on protein standards. Fractionation was performed at 4 °C, and recovered fractions were kept on ice until used in assays that began 3–4 hours later.

Spheroid culture and imaging

Three dimensional spheroids were cultured in a custom polystyrene hanging drop array plate (HDAP) coated with an amphiphilic solution (0.1%, Pluronic F108, BASF Co., Ludwigshafen, Germany) [27]. Plates were sterilized with UV. Hanging drops for culturing spheroids were formed by pipetting 5×10^4 cells in 15 μl of complete medium from the top side of the access holes, allowing the sample liquid to form a hanging droplet on the bottom surface of the HDAP. Co-cultures were prepared with 2.5×10^4 cells of each type. Cells were cultured for 48 hours before imaging.

Spheroids were washed four times in stepwise fashion with phenol red free DMEM with 0.2% BSA 8 hours prior to imaging. To quantify bioluminescence from secreted CXCL12 fusion proteins, 6 μl of supernatant from each hanging drop were collected before imaging spheroids. GL activity in spheroids and supernatants was measured on an IVIS 100 system (Caliper) immediately after adding 1 μg/ml coelenterazine. Data were quantified as photon flux [24].

Transwell migration assay

Cell migration was assayed in a 96 well MultiScreen-Mic (Millipore) transwell cell culture system. 231-CXCR4 cells were starved overnight, and 5×10^4 cells were plated in the top chamber in 100 μl DMEM with 0.2% BSA. Isolated dimeric or monomeric CXCL12-GL from 293T-CXCL12 cells was added to the lower chamber of each transwell. A fraction with no CXCL12-GL bioluminescence or medium with 5% serum was added to lower wells as negative and positive controls, respectively. Assays were terminated after 8 hours of migration, and cells in the top chamber were removed gently with cotton swabs. Cells that migrated to the lower surface of the membrane were fixed and stained with 0.2% crystal violet in methanol. Images were taken using an inverted microscope (Olympus 1X70), and data for per cent membrane surface occupied by transmigrated cells were quantified using Image J.

Western blotting

Cells were cultured in serum-free medium overnight and then stimulated for 10 minutes with equal amounts of bioluminescence from dimer and monomer fractions of CXCL12. Cell lysates were blotted for phosphorylated AKT (Cell Signaling) as described previously [24]. Blots were stripped and re-probed for total AKT as a loading control. Relative intensities of bands were quantified with Image J and expressed as the ratio of phosphorylated to total AKT.

Cell-based imaging for cAMP

231 cells stably expressing the cAMP reporter plasmid pGloSensor-20F were plated at 2×10^4 cells per well in black wall 96 well plates 1 day before assays. We incubated cells for 10 minutes with equal amounts of monomeric or dimeric CXCL12-GL fractions based on bioluminescence. Column fractions were diluted in phenol red free DMEM. Immediately

before imaging firefly luciferase bioluminescence, we added 5 μM forskolin (Sigma) and 15 mg/ml luciferin (Promega). We acquired bioluminescence images for 4 minutes with large binning on an IVIS 100 (Caliper).

Recruitment of β-arrestin 2 to CXCR4 or CXCR7

We quantified interaction of CXCR4 or CXCR7 with β-arrestin 2 by firefly luciferase complementation as described previously [28, 29]. Cells were incubated with equal amounts of monomeric or dimeric CXCL12-GL fractions based on bioluminescence for 20 minutes (CXCR4) or 2 hours (CXCR7) before quantifying firefly luciferase activity.

Cell based assays for accumulation of bioluminescent CXCL12 dimers or monomers

231-CXCR7 or 231-control cells were plated at 2×10^4 cells per well in 96 well black wall plates one day before assays. Cells were incubated for 1 hour with monomeric or dimeric column fractions of CXCL12-GL. Monomeric and dimeric inputs were normalized to equal total amounts of CXCL12 based on bioluminescence. As a control, cells were incubated with a column fraction containing no CXCL12-GL. Bioluminescence from *Gaussia* luciferase was measured as described previously and normalized to total protein per well quantified by sulforhodamine B staining [24].

For co-culture experiments, 1×10^5 231-CXCR7 or 231-control cells were plated with equal numbers of 231 cells stably expressing CXCL12-GL or CXCL12-NG/CXCL12-CG. The following day, cells were washed with PBS and then incubated in phenol red free DMEM. At various times, 10 μl samples of culture media were collected and assayed for *Gaussia* luciferase bioluminescence as described previously [24].

Animal studies

All animal procedures were approved by the University of Michigan Committee for the Use and Care of Animals. 5×10^5 231-CXCL12-NG/CXCL12-CG cells were implanted with equal numbers of either 231-CXCR7 or 231-control cells into 4th inguinal mammary fat pads of 6 week old female NOD/SCID *IL2rγ*$^{-/-}$ mice (Taconic). 20 mu;l blood samples were collected by retroorbital puncture using heparinized capillary tubes. Blood was kept in these tubes for ≈ 10 seconds before transfer to microfuge tubes. Blood recovered from capillary tubes clotted within 30 seconds of transfer to standard microfuge tubes. Samples from mice without tumors were used as negative controls. Collected blood was centrifuged in a microfuge at $1 \times 10^4 \times g$ for 10 minutes, and bioluminescence was quantified in 10 mu;l serum samples [30, 31]. Bioluminescence in negative control samples was subtracted from values obtained from tumor-bearing mice.

Animal imaging

Bioluminescence imaging was performed on an IVIS Spectrum (Caliper). For *Gaussia* luciferase imaging, mice were injected intravenously via tail vein with 4 mg/kg coelenterazine [30]. Mice were imaged immediately after injection using 3 minute acquisition and large binning. Fluorescence imaging for eqFP650 was performed as described previously [26]. Data were quantified as photon flux (bioluminescence) or radiant efficiency (fluorescence) with Living Image software (Caliper).

Statistics

Graphs and statistical analyses were prepared with GraphPad Prism. Data were plotted as mean values with standard error of the mean (SEM). Pairs of data were analyzed by Mann-Whitney U test to determine statistically significant differences.

Results

Secreted CXCL12 forms monomers and dimers

Studies analyzing CXCL12 as monomers or dimers have used recombinant or synthetic fragments or full-length forms of chemokine. These studies show that dimers of CXCL12 only form at concentrations greater than amounts measured in cell culture supernatants or serum [15, 16, 19]. However, local concentrations of CXCL12 in the secretory pathway in mammalian cells may be higher than concentrations of chemokine in the extracellular space, which may promote dimerization. Binding of CXCL12 to heparan sulfate proteoglycans or other glycosaminoglycans on the surface of cells or released into solution also may increase local concentrations of chemokine to promote dimerization under physiologic conditions [11, 21, 32].

To investigate monomers and dimers in secreted CXCL12, we used HEK 293T cells expressing CXCL12-α fused to *Gaussia* luciferase (CXCL12-GL). We previously demonstrated that CXCL12-GL is secreted from cells and activates signaling through CXCR4 to the same extent as synthetic CXCL12 [24]. We collected supernatants containing CXCL12-GL from 293T cells and separated proteins based on size by gel filtration chromatography. CXCL12-GL produced two peaks of bioluminescence in fractions at ≈ 58 and 29 kDa, respectively (Fig 1A, Fig S1). These molecular weights correspond with predicted sizes of dimers and monomers of CXCL12-GL, respectively. There was approximately 10-fold more bioluminescence in the monomeric fraction, indicating that CXCL12 secreted into the extracellular space predominantly is a monomer (Table 1). To exclude the possibility that GL promoted dimerization of CXCL12-GL, we also separated secreted GL by gel filtration. Based on bioluminescence, GL produced a single peak at the expected size (≈ 20 kDa) for a monomer, showing that GL alone does not form dimers (Fig 1B). We note that recovered bioluminescence from GL was higher than combined values for both dimer and monomer fractions of CXCL12-GL because fusing another protein to this luciferase decreases its activity [33].

We measured stability of isolated monomer and dimer fractions of CXCL12 in solution. After collecting the monomeric fraction of CXCL12-GL, we incubated this fraction for either 1 or 3 hours at 37° C before repeating separation of this chemokine. The monomer fraction of CXCL12-GL was very stable within these time periods. Following 1 hour at 37° C, all recovered bioluminescence was in the monomer fraction, while the ratio of luminescence for dimer to monomer fractions was ≈ 1:7 after 3 hours (Table 1). Since we observed a small conversion of monomer to dimer CXCL12-GL after 3 hours at 37°C, we extended the incubation period to 3 days at either 37° or 4° C before repeating the fractionation procedure. For samples incubated 3 days at 37° C, substantially more monomer CXCL12-GL was isolated as dimer upon repeat fractionation. Under these conditions, the

ratio of dimer to monomer was ≈ 3.5:1. Conversion of monomer to dimer CXCL12-GL was substantially less in samples maintained for 3 days at 4° C with a dimer to monomer ratio of 1:1.3. By comparison, the isolated dimer fraction did not produce any monomer bioluminescence following incubation under these same conditions (data not shown). Stability of the dimer fraction may be because CXCL12-GL molecules remain associated with glycosaminoglycans released from 293T cells secreting this chemokine. Collectively, these results demonstrate that CXCL12 secreted from mammalian cells under physiologic conditions exists as monomer and dimers.

CXCL12 forms dimers in the extracellular space

We used *Gaussia* luciferase (GL) protein fragment complementation to further investigate formation of dimers by CXCL12. GL complementation is based on dividing this enzyme into inactive N-terminal and C-terminal fragments (NG and CG) that do not associate spontaneously [34]. NG and CG fragments are fused to potential interacting proteins of interest. When brought together by specific protein-protein interactions, NG and CG reconstitute an active enzyme and produce bioluminescence. Complementation between NG and CG fragments is completely reversible, so bioluminescence ceases when proteins dissociate. Since bioluminescence is produced only when separate NG and CG fragments are brought together by interacting proteins, GL complementation provides a quantitative assay for protein association and dissociation.

To use GL complementation to analyze CXCL12 dimers, we fused CXCL12-α to either NG or CG fragments of GL (CXCL12-NG and CXCL12-CG, respectively). As a control, we also used unfused, secreted CG [25]. We generated stable populations of 293T cells that express CXCL12-NG, CXCL12-CG, CG, or both CXCL12-NG and CXCL12-CG. Cells expressing both CXCL12-NG and CXCL12-CG were referred to as co-transduced. We initially co-cultured 293T-CXCL12-NG cells with equal numbers of cells expressing either CXCL12-CG or CG in 96 well plates. While we readily detected bioluminescence from co-transduced cells, we could not detect bioluminescence above background levels from two dimensional co-cultures of CXCL12-NG and CXCL12-CG cells (data not shown).

We hypothesized that the relatively large volume of extracellular medium present in two-dimensional cultures lowered local concentrations of secreted CXCL12 and prevented dimerization. To reduce the extracellular volume and simulate compact, three-dimensional intercellular relationships present in tumors and other sites *in vivo*, we used hanging drop cultures to generate spheroids from various combinations of these cells. We quantified GL bioluminescence in spheroids and cell supernatants after 2 days in culture.

Cells co-transduced with CXCL12-NG and CXCL12-CG produced the greatest bioluminescence in spheroids and supernatants, showing that complementation occurred between CXCL12-NG and CXCL12-CG. Co-expression of both fusion proteins in the same cell likely generates high signal because dimers of CXCL12 fusion proteins form in the secretory pathway prior to release into the extracellular space (Fig 2A, B; Fig S2). By comparison, cells co-expressing CXCL12-NG and unfused CG did not produce detectable bioluminescence above background, showing specificity of the complementation signal (data not shown). We also detected GL complementation in spheroids formed from co-

cultures of cells expressing CXCL12-NG or CXCL12-CG, albeit at lower levels than cells co-transduced with both constructs. Supernatants from spheroids combining CXCL12-NG with CXCL12-CG cells also produced GL bioluminescence (Fig 2B). GL signal in co-cultures only is produced by dimerization of secreted CXCL12-NG and CXCL12-CG proteins in the extracellular space, so bioluminescence selectively measures steady state levels of CXCL12 dimers. These dimers remain associated with spheroids or are released into culture supernatants. By comparison, combinations of cells expressing CXCL12-NG and unfused CG had no detectable bioluminescence in spheroids or supernatants, confirming that non-specific association of NG and CG enzyme fragments does not produce bioluminescence. Spheroids with only CXCL12-NG or CXCL12-CG cells also had no GL signal associated with cells or supernatants. These data establish that secreted CXCL12 forms dimers in the extracellular environment under physiologic conditions.

Differential effects of monomeric and dimeric CXCL12 on CXCR4-dependent signaling and function

CXCL12 binding to CXCR4 activates downstream signaling pathways including inhibition of cAMP through Gαi, recruitment of the cytosolic adapter protein β-arrestin 2, and phosphorylation of AKT. We analyzed effects of dimeric and monomeric CXCL12 on these signaling events in breast cancer cells. We used MDA-MB-231 human breast cancer cells stably transduced with a firefly luciferase biosensor for cAMP. We incubated cells with equal amounts of dimeric or monomeric CXCL12-GL based on bioluminescence or vehicle only for 10 minutes before treatment with forskolin to elevate intracellular cAMP. Relative to control cells, monomeric CXCL12-GL suppressed intracellular cAMP to a significantly greater extent than dimeric CXCL12-GL following treatment with forskolin ($p < 0.05$) (Fig 3A).

We have developed a firefly luciferase complementation assay to quantify recruitment of β-arrestin 2 to CXCR4 in response to ligand binding [28]. We treated 231 breast cancer cells stably expressing the CXCR4 and β-arrestin 2 complementation pair with monomeric or dimeric fractions of CXCL12-GL for 20 minutes before quantifying bioluminescence. Both monomeric and dimeric fractions of CXCL12-GL increased association of CXCR4 with β-arrestin 2 as compared with control cells (Fig 3B). Effects of dimeric CXCL12-GL were significantly greater than the monomeric fraction of this chemokine ($p < 0.05$). We also analyzed CXCL12-dependent activation of AKT, an established downstream effector of CXCR4 signaling through G proteins. Following overnight culture in serum-free medium, cells were treated for 10 minutes with either monomeric or dimeric fractions of CXCL12-GL. While both monomeric and dimeric CXCL12-GL activated AKT above control, the monomeric fraction produced substantially greater activation (Fig 3C).

CXCL12 signaling through CXCR4 and/or CXCR7 promotes chemotaxis of multiple cell types. To determine to what extent chemotaxis is affected by dimerization of CXCL12, we tested effects of monomeric and dimeric CXCL12 on transwell migration of MDA-MB-231 breast cancer cells stably transduced with CXCR4 (231-CXCR4) [24]. We isolated monomer and dimer fractions of CXCL12-GL and added equal amounts of light for each fraction to lower wells of a transwell migration system. This strategy adds equivalent

numbers of molecules of CXCL12 to each well since the chemokine is fused directly to GL. As negative and positive controls, we added a column fraction containing no CXCL12-GL bioluminescence or medium containing 5% serum, respectively. 231-CXCR4 cells showed robust migration toward the fraction with dimeric CXCL12-GL, which was essentially the same as migration toward 5% serum. However, migration of 231-CXCR4 cells toward monomeric CXCL12-GL was only marginally greater than a fraction with no CXCL12-GL activity. Collectively, these studies show that dimeric and monomeric CXCL12 secreted from mammalian cells preferentially activate distinct aspects of CXCR4 signaling and function in breast cancer.

CXCR7 preferentially sequesters monomeric CXCL12

In addition to CXCR4, CXCL12 also binds to chemokine receptor CXCR7 [35, 36]. CXCR7 functions at least in part to sequester CXCL12 from the extracellular space and degrade it, thereby controlling levels and gradients of this chemokine available for signaling [37, 38]. We used two complementary approaches to investigate uptake of dimeric and monomeric CXCL12 by CXCR7. First, we separated CXCL12 into dimer and monomer fractions by column chromatography and incubated 231 cells expressing CXCR7 (231-CXCR7) or vector control (231-control) with equal amounts of chemokine by bioluminescence [38]. We measured cell-associated GL bioluminescence after 1 hour and determined that 231-CXCR7 cells accumulated significantly more monomeric than dimeric CXCL12-GL ($p < 0.05$) (Fig 5A). Uptake of dimeric CXCL12-GL did not differ between 231-CXCR7 and 231-control cells, and total amounts of cell associated bioluminescence were at background levels quantified with the control fraction containing no CXCL12. Although accumulation of monomeric CXCL12-GL was slightly higher in 231-control cells, 231-CXCR7 cells had 2.2-fold more monomeric CXCL12-GL than control cells, while uptake of dimeric CXCL12-GL did not differ significantly from control.

As a second approach, we used co-cultures of 231-CXCR7 or 231-control cells with 231 cells co-transduced with either CXCL12-NG/CXCL12-CG or CXCL12-GL. The co-culture format models human breast tumors that contain cells expressing CXCR7 and/or secreting CXCL12 in the tumor microenvironment [39, 40]. Co-cultures using 231 cells co-transduced with CXCL12-NG/CXCL12-CG allowed us to selectively quantify uptake of CXCL12 dimers, since GL complementation occurs only when these fusion proteins interact. We quantified bioluminescence from CXCL12-NG/CXCL12-CG dimers in supernatants at various times through 6 hours. Bioluminescence increased progressively over time and did not differ between co-cultures with 231-CXCR7 or 231-control cells (Fig 5B). However, in co-cultures using 231 cells expressing CXCL12-GL, there was significantly less chemokine in the extracellular space when these cells were combined with 231-CXCR7 cells (Fig 5C). Based on data with column chromatography, secreted CXCL12-GL predominantly exists as a monomer, confirming data for preferential uptake of monomeric CXCL12 by CXCR7.

Chemokine binding to CXCR7 recruits β-arrestin 2, which can be quantified by firefly luciferase and other complementation strategies [29, 41]. We treated 231 cells stably expressing luciferase complementation reporters for CXCR7 and β-arrestin 2 for two hours with equal amounts of monomeric and dimeric CXCL12-GL based on bioluminescence.

While both monomeric and dimeric fractions of CXCL12-GL increased association of CXCR7 with β-arrestin 2, monomeric CXCL12-GL produced a significantly greater effect ($p < 0.05$) (Fig 5D). The magnitude of difference between monomeric and dimeric CXCL12-GL is greater for CXCR7-dependent accumulation of monomeric chemokine than recruitment of β-arrestin 2. We previously have shown that accumulation of CXCL12 by CXCR7 only is partially dependent on β-arrestin 2 [29]. These data suggest that β-arrestin 2-independent mechanisms for chemokine uptake by CXCR7 also favor monomeric CXCL12-GL. Collectively, these data establish that CXCR7 preferentially interacts with monomeric CXCL12-GL to remove this chemokine from the extracellular space.

CXCR7 has minimal effect on CXCL12 dimers in breast tumors

We recently demonstrated that 231-CXCR7 cells scavenge CXCL12-GL in orthotopic breast cancer xenografts, reducing amounts of chemokine detectable in primary tumors and serum [42]. To investigate effects of CXCR7 on amounts of dimeric CXCL12 in breast tumors, we implanted either 231-CXCR7 or 231-control cells with 231 cells co-transduced with CXCL12-NG/CXCL12-CG into mammary fat pads of mice. 231 cells with CXCL12-NG/CXCL12-CG also were transduced with a far red fluorescent protein, eqFP650, to monitor relative numbers of these cells in each tumor [26]. After tumors reached ≈ 8 mm diameter, we imaged bioluminescence from CXCL12-NG/CXCL12-CG in primary tumors and quantified amounts of dimeric chemokine released into sera of tumor-bearing mice.

231-CXCR7 cells did not significantly lower amounts of bioluminescence from CXCL12-NG/CXCL12-CG in primary tumors relative to tumors with 231-control cells (Fig 6A, B). 231-CXCR7 cells in primary tumors also did not alter amounts of CXCL12-NG/CXCL12-CG released into sera obtained from these mice (Fig 6C). Although blood samples were collected in heparizined capillary tubes, blood was in these tubes for only ≈ 10 seconds and clotted rapidly when transferred out of the capillary tube. Continued clotting suggests very minimal transfer of heparin from the tube to blood. By comparison to results with dimeric CXCL12-NG/CXCL12-CG, we recently established that 231-CXCR7 cells decrease amounts of CXCL12-GL in the tumor microenvironment and secreted into sera of tumor-bearing mice [42]. These results provide further evidence that CXCR7 is less effective at scavenging dimeric CXCL12 rather than the monomeric form of this chemokine.

Discussion

CXCL12 has key functions in pathogenesis of multiple diseases, making it a promising therapeutic target. For diseases such as cancer, treatments are focused on blocking functions of CXCL12 and its receptors CXCR4 and CXCR7. In pre-clinical models, inhibitors of CXCL12 signaling limit tumor growth and metastasis when administered as single agent therapy, and antagonists of CXCL12-CXCR4 improve efficacy of standard chemotherapeutic drugs [36, 43] [44]. CXCL12 also promotes chemotaxis of stem and progenitor cells, so the chemokine is being investigated as a possible therapeutic agent to increase stem cell trafficking for tissue regeneration and repair in settings including myocardial infarction and ischemic vascular disease [45, 46]. To enable development and

optimal utilization of CXCL12-targeted treatments, it is essential to identify biologically active forms of this chemokine for specific signaling pathways and cellular functions.

We established that CXCL12 secreted from mammalian cells forms dimers under physiologic conditions. Using *Gaussia* luciferase and *Gaussia* luciferase complementation to detect and quantify interactions between CXCL12 molecules in cell-based assays and living mice, we showed that stable dimers of this chemokine were secreted from cells. We also demonstrated that CXCL12 dimers formed in the extracellular space when cells expressing either CXCL12-NG or CXCL12-CG fragments were co-cultured in spheroids. Since spheroid cultures model the compact, three-dimensional intercellular interactions that occur in normal organs and tumors, these data suggest that CXCL12 forms dimers *in vivo*. While we established that GL does not artificially promote dimerization of CXCL12, fusing GL to CXCL12 possibly could increase formation of monomers, in which case our results would underestimate the relative abundance of dimeric CXCL12.

We previously have shown that secreted CXCL12-GL and CXCL12 *Gaussia* luciferase complementation proteins are present at 15 – 30 ng/ml in cell culture supernatants [24, 25]. These concentrations are lower than values reported for dimerization of pure synthetic CXCL12 proteins in solution or crystals. Previous studies have shown that basic amino acids in CXCL12 confer binding to glycosaminoglycans and substantially reduce concentrations of synthetic chemokine required for dimerization in solution [47, 48]. It is likely that dimerization of secreted CXCL12 is increased by binding to glycans and glycosaminoglycans on cell membranes and released into the extracellular space, which increases local concentrations of chemokine. Dimerization of CXCL12 also may be enhanced because the chemokine is concentrated in endosomes in the secretory pathway. While this manuscript was under review, Drury et al showed that CXCL12 purified from bacteria also existed as monomers and dimers, although dimers did not form with less than 0.5 μg purified protein [49]. Overall, these studies support the conclusion that dimerization of CXCL12 occurs physiologically.

Monomeric and dimeric CXCL12 have distinct profiles for receptor interaction, signaling, and cell function. Monomeric CXCL12 more effectively signaled through CXCR4 to suppress cellular cAMP and activate AKT, both of which are dependent upon G protein pathways. By comparison, dimeric CXCL12 produced greater recruitment of β-arrestin 2 and was a more potent chemoattractant molecule for migration of breast cancer cells expressing CXCR4. Since CXCR4 signaling through β-arrestin 2 is required for chemotaxis, our data show that dimeric CXCL12 preferentially activates this component of CXCR4 signal transduction and function [50]. Our results support prior work by Fermas et al, who showed reduced chemotaxis of hepatoma cells toward a synthetic mutant of CXCL12 with minimal binding to oligosaccharides (CXCL12 3/6) and normal affinity for CXCR4 [21]. These data suggested that dimerization of CXCL12 mediated by glycan molecules was essential for cell migration. By comparison, Veldkamp et al concluded that only monomeric CXCL12 promoted chemotaxis, while dimeric chemokine had no effect [22]. Potentially, these differences may be due to use of recombinant, purified CXCL12 versus chemokine secreted from mammalian cells in the context of other secreted molecules. Discordant results among these studies also suggest cell-type specific differences for biologic effects of

monomeric versus dimeric CXCL12. Further studies are needed to establish effects of monomeric and dimeric CXCL12 on signal transduction and resultant functions of cells *in vivo*.

In addition to differences in signaling and chemotaxis, we also determined that receptor CXCR7 preferentially sequesters monomeric CXCL12 both in cell culture assays and in a mouse model of breast cancer. CXCR7 functions as a scavenger receptor to remove CXCL12 from the extracellular space and degrade it in lysosomes [37, 38]. By controlling levels of CXCL12 in the extracellular space, the scavenger function of CXCR7 is proposed to maintain and regulate CXCL12 signaling through CXCR4 [51, 52]. Greater scavenging of monomeric versus dimeric CXCL12 by CXCR7 may preferentially promote selective CXCL12-CXCR4 dependent signaling pathways, such as chemotaxis, in normal development and disease.

Our results provide evidence that dimerization of CXCL12 occurs under physiologic conditions, and effects of CXCL12 dimers in cell signaling and function are distinct from monomeric CXCL12. Factors that regulate the monomer-dimer equilibrium for CXCL12, including pH and glycosaminoglycans on cell membranes, are known to change among normal and diseased cells in various anatomic sites. Shifts in proportions of monomeric and dimeric CXCL12 likely will substantially alter types and/or extent of pathways activated in response to this chemokine. The *Gaussia* luciferase fusion proteins used in this work provide new approaches for detecting and quantifying dimerization of CXCL12 or other chemokines in cell-based assays and mouse models. This strategy and complementary methods will further advance understanding of CXCL12 monomers and dimers in normal physiology and disease conditions, which will facilitate ongoing efforts to target CXCL12 for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cAMP	cyclic adenosine monophosphate
CG	unfused secreted carboxy fragment of *Gaussia* luciferase
CXCL12-NG and CXCL12-CG	CXCL12 fused to amino and carboxy fragments of *Gaussia* luciferase
CXCL12-GL	CXCL12 fused to *Gaussia* luciferase
GL	*Gaussia* luciferase
HDAP	hanging drop array plate

NG	amino fragment of *Gaussia* luciferase

References

1. Tashiro K, Tada H, Heilker R, Shirozu M, Nakano T, Honjo T. Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. Science. 1993; 261:600–603. [PubMed: 8342023]
2. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature. 1996; 382:635–638. [PubMed: 8757135]
3. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature. 1998; 393:591–594. [PubMed: 9634237]
4. Chen W, Tzeng Y, Li H, Tien W, Tsai Y. Lung defects in neonatal and adult stromal-derived factor-1 conditional knockout mice. Cell Tissue Res. 2010; 342:75–85. [PubMed: 20824284]
5. Pusic I, DiPersio J. Update on clinical experience with AMD3100, an SDF-1/CXCL12-CXCR4 inhibitor, in mobilization of hematopoietic stem and progenitor cells. Curr Opin Hematol. 2010; 17:319–326. [PubMed: 20473162]
6. Duda D, Kozin S, Kirkpatrick N, Xu L, Fukumura D, Jain R. CXCL12 (SDF1{alpha})-CXCR4/CXCR7 Pathway Inhibition: An Emerging Sensitizer for Anticancer Therapies? Clin Cancer Res. 2011; 17:2074–2080. [PubMed: 21349998]
7. Jiang Y, Wu X, Shi B, Wu W, Yin G. Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. Gynecol Oncol. 2006; 103:226–233. [PubMed: 16631235]
8. Li Y, Pan Y, Wei Y, Cheng X, Zhou B, Tan M, Zhou X, Xia W, Hortobagyi G, Yu D, Hung M. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell. 2004; 6:459–469. [PubMed: 15542430]
9. Zernecke A, Bidhzhkov K, Noels H, Shaqdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh M, Lutgens E, Wang S, Olson E, Schrober A, Weber C. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal. 2009; 2:ra81. [PubMed: 19996457]
10. Bogoslovsky T, Spatz M, Chaudhry A, Maric D, Luby M, Frank J, Warach S. Investigators NNHoS. Stromal-derived factor-1[alpha] correlates with circulating endothelial progenitor cells and with acute lesion volume in stroke patients. Stroke. 2011; 42:618–625. [PubMed: 21257825]
11. Santiago B, Baleux F, Palao G, Gutierrez-Canas I, Ramirez J, Arenzana-Seisdedos F, Pablos J. CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. Arthritis Res Ther. 2006; 8:R43. [PubMed: 16507142]
12. Cruz-Orengo L, Holman D, Dorsey D, Zhou L, Zhang P, Wright M, McCandless E, Patel J, Luker G, Littman D, Russell J, Klein R. CXCR7 influences leukocyte entry into the CNS parenchyma by controlling abluminal CXCL12 abundance during autoimmunity. J Exp Med. 2011; 208:327–329. [PubMed: 21300915]
13. Nesmelova I, Sham Y, Gao J, Mayo K. CXC and CC chemokines form mixed heterodimers: assocation free energies from molecular dynamics simulations and experimental correlations. J Biol Chem. 2008; 283:24155–24166. [PubMed: 18550532]
14. Nesmelova I, Sham Y, Dudek A, van Eijk L, Wu G, Slungaard A, Mortari F, Griffioen A, Mayo K. Platelet factor 4 and interleukin-8 CXC chemokine heterodimer formation modulates function at the quaternary structural level. J Biol Chem. 2005; 280:4948–4958. [PubMed: 15531763]
15. Dealwis C, Fernandez E, Thompson D, Simon R, Siani M, Lolis E. Crystal structure of chemically synthesized [N33A] stromal cell-derived factor 1alpha, a potent ligand for the HIV-1 “fusin” coreceptor. Proc Natl Acad Sci USA. 1998; 95:6941–6946. [PubMed: 9618518]
16. Ohnishi Y, Senda T, Nandhagopal N, Sugimoto K, Shioda T, Nagal Y, Mitsui Y. Crystal structure of recombinant native SDF-1alpha with additional mutagenesis studies: an attempt at a more

comprehensive interpretation of accumulated structure-activity relationship data. J Interferon Cytokine Res. 2000; 20:691–700. [PubMed: 10954912]

17. Baryshnikova O, Sykes B. Backbone dynamics of SDF-1alpha determined by NMR: interpretation in the presence of monomer-dimer equilibrium. Protein Sci. 2006; 15:2568–2578. [PubMed: 17075134]
18. Kjellen L, Pettersson I, Hook M. Cell-surface heparan sulfate: An intercalated membrane proteoglycan. Proc Natl Acad Sci USA. 1981; 78:5371–5375. [PubMed: 6458040]
19. Veldkamp C, Peterson F, Pelzek A, Volkman B. The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. Protein Sci. 2005; 14:1071–1081. [PubMed: 15741341]
20. Murphy J, Cho Y, Sachpatzidis A, Fan C, Hodsdon M, Lolis E. Structural and functional basis of CXCL12 (stromal cell-derived factor-1 alpha) binding to heparin. J Biol Chem. 2007; 282:10018–10027. [PubMed: 17264079]
21. Fermas S, Gonnet F, Sutton A, Charnaux N, Mulloy B, Du Y, Baleux F, Daniel R. Sulfated oligosaccharides (heparin and fucoidan) binding and dimerization of stromal cell-derived factor-1 (SDF-1/CXCL 12) are coupled as evidenced by affinity CE-MS analysis. Glycobiology. 2008; 18:1054–1064. [PubMed: 18796646]
22. Veldkamp C, Seibert C, Peterson F, De la Cruz N, Haugner, Basnet H, Sakmar T, Volkman B. Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. Sci Signal. 2008; 16:ra4. [PubMed: 18799424]
23. Veldkamp C, Ziarek J, Su J, Basnet H, Lennertz R, Weiner J, Peterson F, Baker J, Volkman B. Monomeric structure of the cardioprotective chemokine SDF-1/CXCL12. Protein Sci. 2009; 18:1359–1369. [PubMed: 19551879]
24. Luker K, Gupta M, Luker G. Bioluminescent CXCL12 fusion protein for cellular studies of CXCR4 and CXCR7. Biotechniques. 2009; 47:625–632. [PubMed: 19594447]
25. Luker K, Mihalko L, Schmidt B, Lewin S, Ray P, Shcherbo D, Chudakov D, Luker G. *In vivo* imaging of ligand receptor binding with *Gaussia* luciferase complementation. Nat Med. 2011 Dec 4. [Epub ahead of print]. 10.1038/nm.2590
26. Shcherbo D, Shemiakina I, Ryabova A, Luker K, Schmidt B, Souslova E, Gorodnicheva T, Strukova L, Shidlovskiy K, Britanova O, Zaraysky A, Lukyanov K, Loschenov V, Luker G, Chudakov D. Near infrared fluorescent proteins. Nat Methods. 2010; 7:827–829. [PubMed: 20818379]
27. Tung Y, Hsiao A, Allen S, Torisawa Y, Ho M, Takayama S. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. Analyst. 2011; 136:473–478. [PubMed: 20967331]
28. Luker K, Gupta M, Luker G. Imaging CXCR4 signaling with firefly luciferase complementation. Anal Chem. 2008; 80:5565–5573. [PubMed: 18533683]
29. Luker K, Gupta M, Steele J, Foerster B, Luker G. Imaging Ligand-dependent Activation of CXCR7. Neoplasia. 2009; 11:1022–1035. [PubMed: 19794961]
30. Tannous B, Kim D, Fernandez J, Weissleder R, Breakefield X. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. Mol Ther. 2005; 11:435–443. [PubMed: 15727940]
31. Wurdinger T, Badr C, Pike L, de Kleine R, Weissleder R, Breakefield X, Tannous B. A secreted luciferase for ex-vivo monitoring of *in vivo* processes. Nat Methods. 2008; 5:171–173. [PubMed: 18204457]
32. Wang A, Miralem T, Templeton D. Heparan sulfate chains with antimitogenic properties arise from mesangial cell-surface proteoglycans. Metabolism. 1999; 48:1220–1229. [PubMed: 10535382]
33. Suzuki T, Kondo C, Kanamori T, Inouye S. Video-rate bioluminescence imaging of matrix metalloproteinase-2 secreted from a migrating cell. PLoS One. 2011; 6:e25243. [PubMed: 21969874]
34. Remy I, Michnick S. A highly sensitive protein-protein interaction assay based on Gaussia luciferase. Nat Methods. 2006; 3:977–979. [PubMed: 17099704]

35. Balabanian K, Lagane B, Infantino S, Chow K, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, Bachelerie F. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J Biol Chem. 2005; 280:35760–35766. [PubMed: 16107333]
36. Burns J, Summers B, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold M, Sunshine M, Littman D, Kuo C, Wei K, McMaster B, Wright K, Howard M, Schall T. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med. 2006; 203:2201–2213. [PubMed: 16940167]
37. Boldajipour B, Mahabaleshwar S, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, Raz E. Control of chemokine-guided cell migration by ligand sequestration. Cell. 2008; 132:463–473. [PubMed: 18267076]
38. Luker K, Steele J, Mihalko L, Luker G. Constitutive and chemokine-dependent internalization and recycling of CXCR7 in breast cancer cells to degrade chemokine ligands. Oncogene. 2010; 29:4599–4610. [PubMed: 20531309]
39. Hall J, Korach K. Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. Mol Endocrinol. 2003; 17:792–803. [PubMed: 12586845]
40. Miao Z, Luker K, Summers B, Berahovich R, Bhojani M, Rehemtulla A, Kleer C, Essner J, Nasevicius A, Luker G, Howard M, Schall T. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. Proc Natl Acad Sci U S A. 2007; 104:15735–15740. [PubMed: 17898181]
41. Zabel B, Wang Y, Lewen S, Berahovich R, Penfold M, Zhang P, Powers J, Summers B, Miao Z, Zhao N, Jalili A, Janowaska-Wieczorek A, Jaen J, Schall T. Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. J Immunol. 2009; 183:3204–3211. [PubMed: 19641136]
42. Luker K, Lewin S, Mihalko L, Schmidt B, Winkler J, Coggins N, Thomas D, Luker G. Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells. Oncogene. 2012 in press.
43. Parameswaran R, Yu M, Lim M, Groffen J, Heisterkamp N. Combination of drug therapy in acute lymphoblastic leukemia with a CXCR4 antagonist. Leukemia. 2011; 25:1314–1323. [PubMed: 21483439]
44. Tamamura H, Hori A, Kanzaki N, Hiramatsu K, Mizumoto M, Nakashima H, Yamamoto N, Otaka A, Fujii N. T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer. FEBS Lett. 2003; 550:79–83. [PubMed: 12935890]
45. Atluri P, Woo Y. Pro-angiogenic cytokines as cardiovascular therapeutics: assessing the potential. BioDrugs. 2008; 22:209–222. [PubMed: 18611064]
46. Ghadge S, Muhlstedt S, Ozcelik C, Bader M. SDF-1α as a therapeutic stem cell homing factor in myocardial infarction. Pharmacol Ther. 2011; 129:97–108. [PubMed: 20965212]
47. Mbemba E, Gluckman J, Gettegno L. Glycan and glycosaminoglycan binding properties of stromal cell-derived factor (SDF)-1alpha. Glycobiology. 2000; 10:21–29. [PubMed: 10570220]
48. Hamon M, Mbemba E, Charnaux N, Slimani H, Brule S, Saffar L, Vassy R, Prost C, Lievre N, Starzec N, Gattegno L. A syndecan-4/CXCR4 complex expressed on human primary lymphocytes and macrophages and HeLa cell line binds the CXC chemokine stromal cell-derived factor-1 (SDF-1). Glycobiology. 2004; 14:311–323. [PubMed: 15033938]
49. Drury L, Ziarek J, Gravel S, Veldkamp C, Takekoshi T, Hwang S, Heveker N, Volkman B, Dwinell M. Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. Proc Natl Acad Sci U S A. 2011 Oct 11. Epub ahead of print.
50. Fong A, Premont R, Richarson R, Yu Y, Lefkowitz R, Patel D. Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci U S A. 2002; 99:7478–7483. [PubMed: 12032308]
51. Sanchez-Alcaniz J, Haege S, Mueller W, Pla R, Mackay F, Schulz S, Lopez-Bendito G, Stumm R, Marin O. Cxcr7 controls neuronal migration by regulating chemokine responsiveness. Neuron. 2011; 69:77–90. [PubMed: 21220100]

52. Wang Y, Li G, Stanco A, Long J, Crawford D, Potter G, Pleasure S, Behrens T, Rubenstein J. CXCR4 and CXCR7 have distinct functions in regulating interneuron migration. Neuron. 2011; 69:61–76. [PubMed: 21220099]

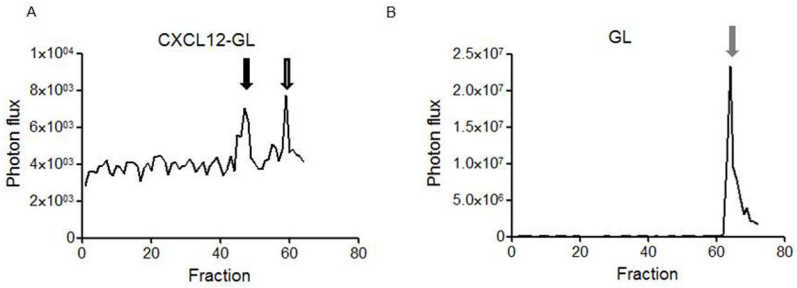

Figure 1. Secreted CXCL12-GL fractionates as dimers and monomers

A) Supernatants from 293T cells expressing CXCL12 fused to *Gaussia* luciferase (CXCL12-GL) were fractionated by column chromatography. Graph shows bioluminescence values measured in each sample. Black and open arrows denote fractions with bioluminescence from dimeric and monomeric CXCL12-GL at ≈ 58 and 29 kDa, respectively. B) Bioluminescence in fractions from unfused GL secreted from 293T cells. Gray arrow shows bioluminescence from monomeric GL at ≈ 20 kDa.

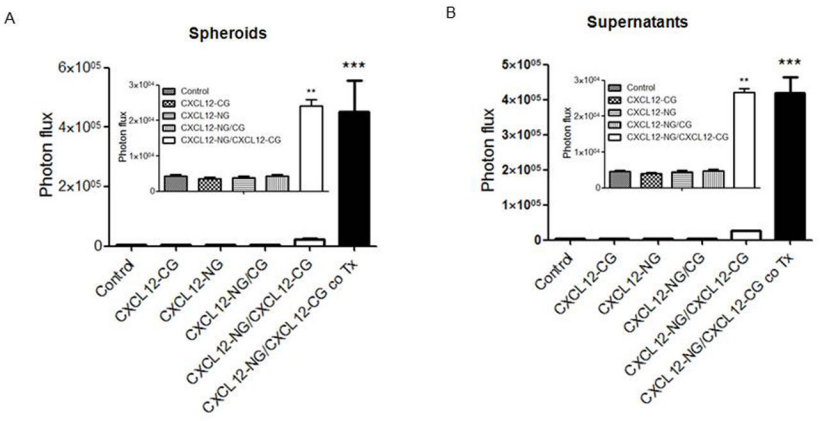

Figure 2. *Gaussia* luciferase complementation shows dimerization of secreted CXCL12 in spheroids and culture supernatants

A) Spheroids were prepared from control 293T cells, cells stably expressing CXCL12-NG only, CXCL12-CG only, both CXCL12-NG and CXCL12-CG (Co-Tx), mixtures of cells with CXCL12-NG or unfused CG, or mixtures of cells expressing CXCL12-NG or CXCL12-CG. Graph shows mean values + SEM for GL bioluminescence in spheroids after 2 days in culture (n = 8 per condition). Inset shows data for all cell combinations except co-transduced spheroids that express both CXCL12-NG and CXCL12-CG. B) Supernatants from spheroids in panel A were assayed for bioluminescence from GL complementation for CXCL12. Data were graphed as mean values + SEM. Inset shows values for all conditions except cells co-transduced with both CXCL12-NG and CXCL12-CG. **, $p < 0.01$; ***, $p < 0.005$.

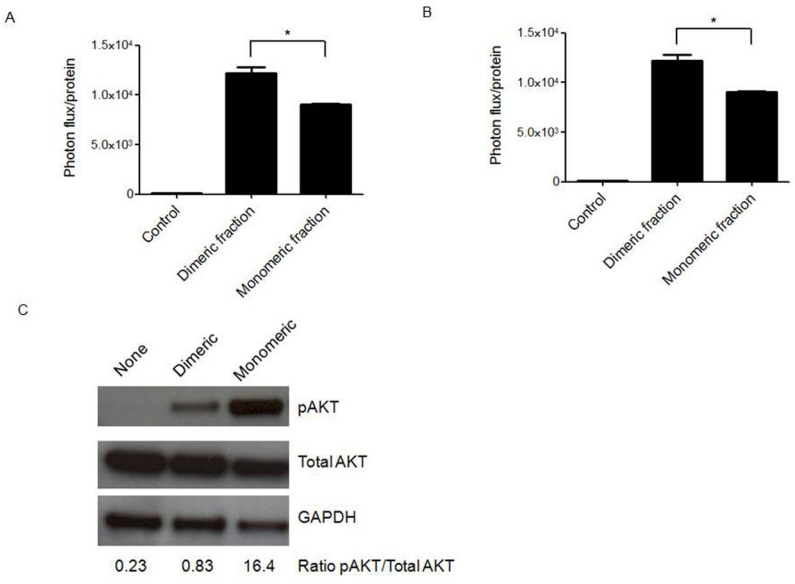

Figure 3. Differential regulation of CXCR4 signaling by monomeric and dimeric CXCL12

A) 231-CXCR4 cells expressing a firefly luciferase reporter for cAMP were incubated with monomeric or dimeric CXCL12-GL fractions or medium alone for 10 minutes before treatment with forskolin. Graph shows mean values + SEM for bioluminescence from the cAMP reporter. B) 231 cells expressing luciferase complementation reporters for CXCR4 interaction with β-arrestin 2 were incubated for 20 minutes with monomeric or dimeric fractions of CXCL12-GL. Graph shows mean values + SEM for bioluminescence from firefly luciferase complementation. C) 231-CXCR4 cells were serum-starved overnight and then treated for 10 minutes with medium alone or equal amounts of monomeric or dimeric CXCL12-GL based on bioluminescence. Western blotting was used to detect phosphorylated (active) AKT. The blot then was stripped and analyzed for total AKT and then GADPH as loading controls. Numbers are quantified data for intensities of bands expressed as phosphorylated AKT divided by total AKT. *, $p < 0.05$.

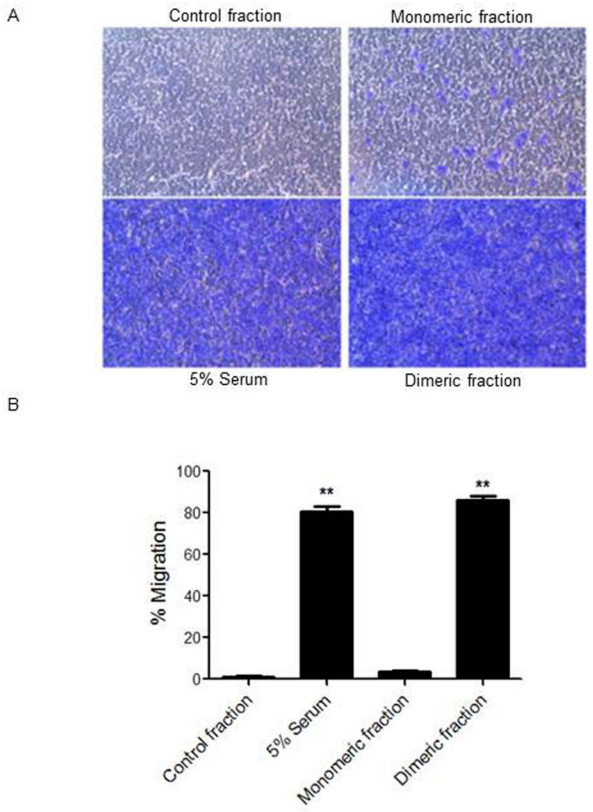

Figure 4. Dimeric CXCL12 promotes chemotaxis of breast cancer cells expressing CXCR4

A) Monomeric and dimeric fractions of CXCL12-GL were isolated, and equal amounts of bioluminescence from each fraction were added to the bottom chamber of transwells. Additional wells had a fraction with no CXCL12-GL bioluminescence or a fraction with 5% serum added. MDA-MB-231 breast cancer cells stably transduced with CXCR4 (231-CXCR4) were added to the top chamber. Cells that migrated through the filter were stained with crystal violet. Photograph shows representative staining of breast cancer cells that migrated through the transwell membrane. B) Data for per cent membrane area occupied by transmigrated breast cancer cells graphed as mean values + SEM. **, $p < 0.01$.

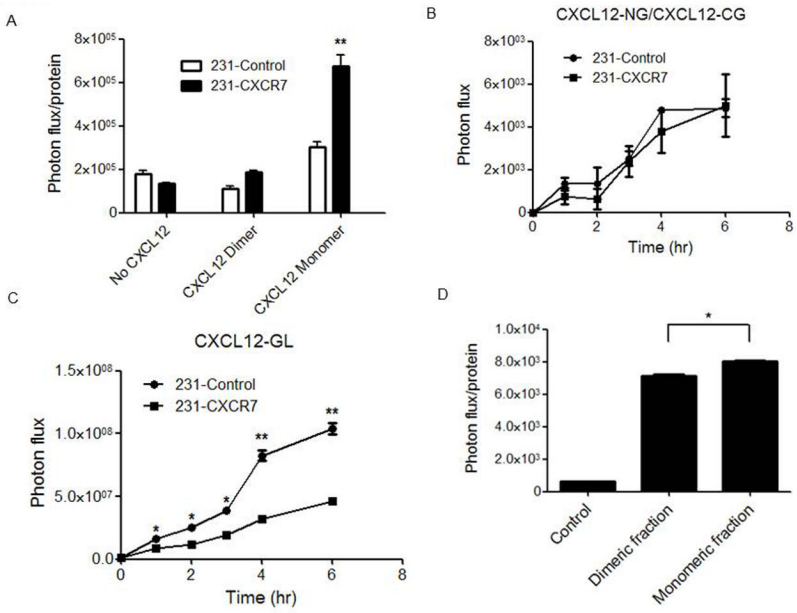

Figure 5. CXCR7 preferentially interacts with monomeric CXCL12

A) MDA-MB-231 breast cancer cells stably expressing CXCR7 (231-CXCR7) or vector control (231-control) were incubated with equal amounts of input light from dimeric and monomeric fractions of CXCL12-GL. Cells were incubated with CXCL12-GL fractions for 1 hour and then washed with an acidic solution to remove extracellular chemokine prior to imaging GL activity. Data were graphed as mean values + SEM for each condition (n = 4). B) 231-CXCR7 or 231-control cells were co-cultured with 231 cells stably transduced with CXCL12-NG and CXCL12-CG. Bioluminescence from dimeric CXCL12-NG/CXCL12-CG in cell culture media was quantified at times up to 6 hours and graphed as mean values ± SEM (n = 4 per time point). C) Co-cultures of 231-CXCR7 or 231-control cells with 231 cells secreting CXCL12-GL were incubated as described in panel B. Bioluminescence from CXCL12-GL in the extracellular space was quantified at various times. Data were presented as mean values ± SEM (n = 4 per time point). D) 231 cells expressing luciferase complementation reporters for association of CXCR7 with β-arrestin 2 were incubated for 2 hours with monomeric or dimeric fractions of CXCL12-GL. Graph shows mean values + SEM. *, $p < 0.05$; **, $p < 0.01$.

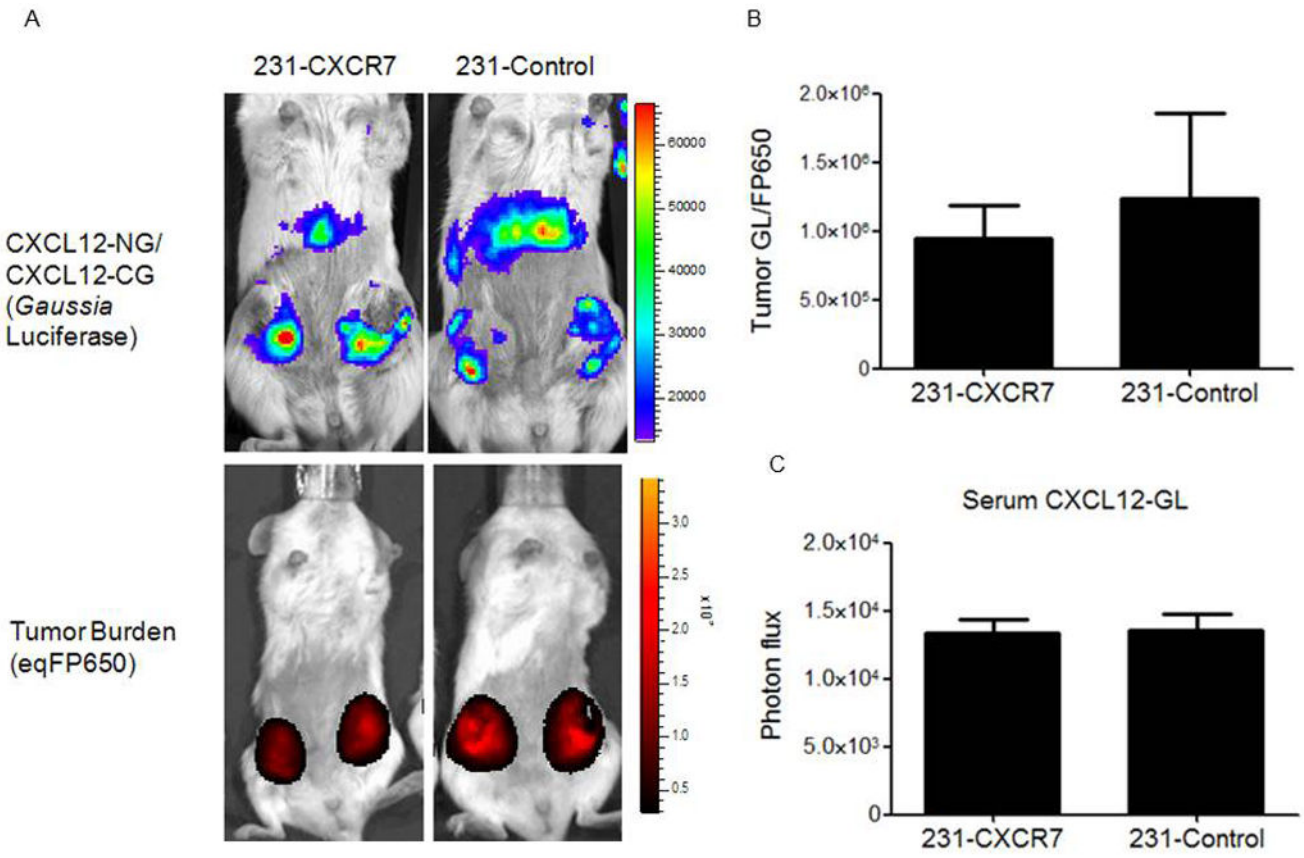

Figure 6. CXCR7 minimally alters levels of CXCL12 dimers *in vivo*

A) MDA-MD-231 breast cancer cells expressing both CXCL12-NG and CXCL12-CG were implanted orthotopically into mammary fat pads with either 231-CXCR7 or 231-control cells, respectively. Representative images are shown for GL bioluminescence produced by CXCL12-NG/CXCL12-CG dimers in primary tumors. Fluorescence image of the same mice shows eqFP650 in 231-CXCL12-NG/CXCL12-CG cells. Scale bars depict range of pseudocolors used to display bioluminescence and fluorescence imaging data. B) Quantified data for dimeric CXCL12-NG/CXCL12-CG bioluminescence in primary tumors normalized to fluorescence from eqFP650 in each tumor (n = 5 mice per group). Data were graphed as mean values +SEM. C) Blood samples were collected from mice in each group, and bioluminescence from CXCL12-NG/CXCL12-CG dimers was quantified in serum (n = 5 per group). Graph displays mean values + SEM for GL bioluminescence corrected for background values from mice with no tumors.

Table 1

Column chromatography for CXCL12-GL

		% of input bioluminescence		
Input material	**Incubation conditions before re-fractionation**	**Dimer CXCL12-GL**	**Monomer CXCL12-GL**	**Ratio (dimer to monomer)**
Cell culture supernatant	NA	0.8	7.6	1 : 9.5
Monomer fraction	37° C for 1 hour	0	8.6	0
Monomer fraction	37° C for 3 hours	0.7	4.9	1 : 7
Monomer fraction	37° C for 3 days	8.5	2.4	3.5 : 1
Monomer fraction	4° C for 3 days	4.1	5.2	1 : 1.3