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Mesenchymal Stem Cells promote mammary cancer cell migration *in vitro* via the CXCR2 receptor

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Abstract

Bone metastasis is a common event during breast cancer progression. Recently, mesenchymal stem cells (MSCs) have been implicated in the metastasis of primary mammary cancer. Given that bone is the native environment for MSCs, we hypothesized MSCs facilitate the homing of circulating mammary cancer cells to the bone. To test this hypothesis, we examined *in vitro* whether bone derived MSCs from FVB mice could influence the migration of syngeneic murine mammary cancer cell lines derived from the polyoma virus middle-T (PyMT) model of mammary gland tumorigenesis. Our data show that conditioned media derived from MSCs significantly enhanced the migration of PyMT mammary cancer cell lines. Analysis of conditioned media using a cytokine array revealed the presence of numerous cytokines in the MSC conditioned media, most notably, the murine orthologs of CXCL1 and CXCL5 that are cognate ligands of the CXCR2 receptor. Further investigation identified that; 1) CXCL1, CXCL5 and CXCR2 mRNA and protein were expressed by the MSCs and PyMT cell lines and; 2) neutralizing antibodies to CXCL1, CXCL5 and CXCR2 or a CXCR2 small molecule inhibitor (SB265610) significantly abrogated the migratory effect of the MSC conditioned media on the PyMT cells. Therefore, *in vitro* evidence demonstrates that bone derived MSCs play a role in the migration of mammary cancer cells, a conclusion that has potential implications for breast to bone metastasis *in vivo*.

Keywords

Breast to bone metastasis; chemokine; CXCL; CXCR; mesenchymal stem cell; migration

1. Introduction

Approximately 30% of women diagnosed with breast cancer will develop bone metastases and in 60% of those patients, debilitating pathologic fractures will occur due to the typically osteolytic nature of the lesions [1]. Pathologic fractures result in pain, disability,

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Conflicts of Interest Statement

None Declared

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hospitalization, and potentially require major surgical procedures designed to restore mobility and provide palliation. Much emphasis has been placed on interrogating and therapeutically targeting the established metastases but by comparison, a paucity of information concerning the mechanisms that facilitate cancer cell homing to the bone exists.

Previous studies have identified chemokines as important mediators of tumor progression and metastasis [2]. The chemokine superfamily is comprised of approximately 45 members that are categorized into 4 main groups based on the position of conserved cysteine amino acids in the N-terminal region (CXC, CC, CX₃C and C). Chemokines mediate their effect by binding to G-coupled protein receptors (GPCRs). There are 17 chemokine receptors and some heterogeneity exists with respect to ligand-receptor specificity [2]. Chemokines that bind to CXCR2 (CXCL1, 2, 3, 5, 6, 7 and 8) mediate biological processes such as leukocyte migration and angiogenesis [2]. More recently chemokines have been implicated in the progression and metastasis of several cancers [2; 3]. For example, CXCR4 interaction with CXCL12 has been well characterized in facilitating the metastasis and homing cancers to secondary sites such as bone [4; 5]. Recently, MSCs recruited to mammary gland tumors have been shown to facilitate the expansion of cancer stem cells and ultimately metastasis via the secretion of chemokines such as CXCL7 and CCL5 respectively [6; 7].

MSCs reside in the bone marrow microenvironment and are pluripotent cells that, when given the correct signaling cues, give rise to chondrocyte, muscle, adipose and osteoblast cell types [8; 9]. MSCs express several chemokines and chemokine receptors that permit their infiltration into damaged/inflamed tissues [10; 11; 12]. While MSCs are implicated in the metastasis of cancer cells from primary to secondary sites [7], little is known as to whether MSCs in their native bone marrow microenvironment can influence the homing/recruitment of circulating tumor cells to the bone.

In the current study, we investigated whether bone derived MSCs influenced the migration of murine mammary cancer cell lines that have metastatic ability. Our data identifies that MSCs secrete a number of chemokines but demonstrate that CXCL1 and CXCL5 play a significant role in the migration of the mammary cancer cell lines via CXCR2 leading us to posit the potential importance of this signaling axis in the homing of circulating tumor cells to the bone.

2. Materials and Methods

2.1. Cell Lines

Mesenchymal stem cells were isolated from the pooled bone marrow of FVB mice (n=3), and verified for stem cell properties as previously described [13; 14; 15]. The isolated cells were maintained at low passage number and maintained in low glucose Dulbecco's minimum essential media containing 10% Hyclone FBS (Mediatech Inc, Mansassas, VA), 250ng/ml fungizone (Invitrogen, Carlsbad, CA), 5units/5µg/ml Pen/Strep (Invitrogen) and 50ng/ml platelet derived growth factor (PDGF) (R&D Systems, Minneapolis, MN). Two independent syngeneic FVB mammary tumor cell lines, PyMT-Luc and 17L3C-Luc, were isolated from the polyoma virus middle T antigen (PyMT) model of mammary gland tumorigenesis. The cell lines had previously been transduced with a luciferase (Luc) reporter construct for the analysis of tumor progression *in vivo* [16; 17; 18]. While the expression of luciferase was not essential for the experiments herein, future *in vivo* studies will benefit from the inclusion of the luciferase reporter system. The PyMT cell lines were maintained in 10% serum containing DMEM (Invitrogen).

2.2. Conditioned Media

For the collection of conditioned media (CM), 5×10^5 cells were seeded into 100mm dishes and the cells were grown to sub-confluence. The cells were carefully rinsed in sterile phosphate buffered saline (PBS) and pre-incubated in serum free DMEM for 2 hours prior to rinsing and replenishing with 5ml of serum free media per plate. The media was allowed to condition for 24 hours. The protein concentration of the CM was calculated using a bicinchoninic assay (BCA) (Thermo Scientific, Rockford, IL) and aliquots were stored at 4°C for no more than 2 weeks.

2.3. Migration Assay

Migration assays utilized a modified Boyden chamber assay with 8µm pore insert. For co-culture migration assays, 1×10^5 MSC cells were seeded into 24 well plates and allowed to grow to sub-confluency. The cells were rinsed carefully with PBS and then incubated in 650µl of serum free media for 24 hours. Subsequently, 1×10^5 PyMT-Luc or 17L3C-Luc cells in 250µl of serum free media were added to the upper compartment of the insert. For migration assays to conditioned media, a similar approach was taken with 650µl of MSC-CM added to the lower chamber and the same number of PyMT-Luc or 17L3C-Luc added to the upper chamber of the insert. For migration assays utilizing neutralizing antibodies, the antibodies; CXCL1 (10µg/ml, AF-453 R&D systems); CXCL5 (10µg/ml, MAB433 R&D Systems) and; CXCR2 (50µg/ml, MAB2164 R&D Systems) were added to the 250µl aliquot of the tumor cells prior to being added to the upper chamber of the insert. Neutralization dosages were selected based on activity information provided by manufacturer. The appropriate IgG isotype control was added at the same concentration in control experiments. For studies involving small molecule inhibition of CXCR2 signaling, SB265610 (Tocris, Ellisville, MO), was added at a final concentration of 1µM to the migration assay with the appropriate concentration of the carrier (EtOH) added to controls.

For all migration assays, the cells were allowed to migrate for a period of 4 hours. Afterwards, the inserts were isolated and adhered cells on the upper surface of the insert were removed using a cotton tipped applicator soaked in 1x PBS. The upper surface of the insert was swabbed three times with rinses of PBS between washes and then fixed in ice-cold methanol for 5 minutes at -20°C. The inserts were stained with hematoxylin (Sigma-Aldrich, St. Louis, MO) and eosin (Sigma-Aldrich) prior to dehydration in 70% ethanol. The membranes containing the migrated cells were carefully excised from the insert housing using a scalpel and subsequently aqueously mounted on glass slides. All migration experiments were performed in quadruplicate. Multiple 20x bright field microphotographs were captured per experiment, the images were printed and then manually counted. The migration data is presented as number of migrating cells/field. Importantly, counts were performed in a blinded manner in that the identities of the fields were not known until the graphing of the data.

2.4. RT-PCR

Total RNA was isolated from cell lines using TRIzol (Invitrogen) according to the manufacturer's instructions. For reverse transcription, 1µg of total RNA was primed with oligoDT (0.5µg/µl, Applied Biosystems, Carlsbad, CA) for 10 minutes at 70°C prior to the generation of cDNA via the addition of a master mix of reverse transcriptase 200 units MMLV-RT (Promega, Madison, WI), 1mM dNTP (Promega) and 10mM dithiothreitol per reaction for 1 hour at 37°C. The cDNA was then utilized for PCR using standard PCR procedures with the following primers for CXCL1; sense 5'-CACCCAAACCGAAGTCATAG-3' and anti-sense 5'-AAGCCAGCGTTCACCAGA-3' (annealing temperature of 55°C), CXCL5; sense 5'-GGTCCACAGTGCCCTACG-3' and anti-sense 5'-GCGAGTGCATTCCGCTTA-3' (annealing temperature of 55°C), CXCR2;

sense 5'-GCTGTCGTCCTTGTCTTC C-3' (forward) and anti-sense 5'-GCCTTGTCATGTCATCGC-3' (annealing temperature of 60°C) and GAPDH; sense 5'-ACCACAGTCCATGCCATCAC-3' and anti-sense 5'-TCCACCACCCTGTTGCTGTA-3'. The expected product sizes were, CXCL1; 168 base pairs (bp), CXCL5; 146bp; CXCR2; 130bp and GAPDH; 452bp. Products were separated on 1.5% agarose gel containing ethidium bromide. Bands were visualized and images recorded via ultra violet light (Cell Biosciences, Santa Clara, CA). Lewis lung carcinoma (LLC) and total RNA isolated from CXCR2 null mice were also used as controls [19].

2.5. Immunoblot, cytokine array and ELISA

Cell lysates were collected on ice using a standard protein lysis buffer (0.1 % sodium dodecyl sulfate, 0.5 % sodium deoxycholate, 1 % triton X100, 10 mM Tris pH 7.5, 150 mM NaCl). For experiments involving analysis of phosphorylated proteins a complete proteinase inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (Sigma-Aldrich) were added as per the manufacturer's instructions. Protein concentration in isolated cell lysates and in conditioned media was quantitated using a BCA assay (Thermo Scientific-Pierce). Equal concentrations of total protein (10µg per sample) were loaded on to a denaturing 8% SDS-PAGE gel and subsequently transferred to nitrocellulose membrane. Blots were blocked for one hour at room temperature in a solution of 5% milk in 1xTris buffered saline (TBS; 50mM tris and 150mM NaCl at pH 7.6). The blots were then panned with antibodies directed to phosphorylated (pCXCR2; ab61100, Abcam, Cambridge, MA) or native CXCR2 (NB300-696 Novus Biologicals, Littleton, CO). Antibodies were diluted 1:1,000 in 5% milk/1XTBST (TBS with 0.05% Tween 20) overnight with rocking at 4°C. The following day, blots were washed extensively with 1xTBST prior to the addition of a secondary infra-red labeled antibody (1: 5,000 dilution in 1xTBST, Rockland Immunochemicals Inc, Gilbertsville, PA) for 1 hour at room temperature with rocking, in lightproof conditions. After washing in 1xTBST, blots were developed and bands of interest were quantitated using the Odyssey system (LI-COR Biosciences, Lincoln, NB).

Cytokine array (Mouse Cytokine Array 3-Cat# AAM-CYT-G3-8, RayBiotech, Norcross, GA) and ELISA (CXCL1, MKC00B, R&D systems; CXCL5, DY443, R&D Systems) were performed as per manufacturer's instructions using samples normalized for total protein.

2.6. Immunofluorescence

For CXCR2 localization by immunofluorescence, PyMT-Luc and 17L3C-Luc cells were seeded into 8 well chamber slides at a concentration of 1×10^4 cells per chamber (Thermo Scientific, Nunc-LabTek). After overnight incubation in normal serum containing media, the cells were rinsed in 1xPBS prior to fixation for 5 minutes in ice cold methanol at -20°C. The cells were washed in 1xPBS and then blocked at room temperature for 30 minutes in 1xPBS containing 4% milk. Antibodies to phosphorylated CXCR2 (ab61100, Abcam) or IgG controls diluted 1:500 in blocking solution were then added to cells for 1 hour at room temperature with shaking. After washing in 1xPBS (three washes of 5 minutes each), primary antibodies were detected with a fluorescently tagged species specific secondary antibody (Anti Rabbit-AlexaFluor568, Invitrogen) diluted 1:1,000 in blocking solution for 30 minutes at room temperature under light proof conditions. Cells were washed extensively prior to mounting in aqueous media containing 4',6'-diamidino-2-phenylindole (DAPI) at a final concentration of 1nM. Cells were visualized using an Axiophot upright microscope (Carl Zeiss, Thornwood, NY).

2.7. MTT assay

The effect of CXCL1, CXCL5 (10ng/ml, 433-MC, R&D Systems) or a neutralizing antibody to CXCR2 (MAB2164, R&D Systems) on the growth of the PyMT-Luc and 17L3C-Luc

cells was assessed by tetrazolium-based colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega). Tumor cells were plated in 96-well plates at a density of 1000 cells/well and 24 h after seeding, cells were treated with 100 μ l of serum free media containing 10ng/ml CXCL1 or CXCL5 (453-KC, 433-MC, R&D Systems) or normal growth media containing 1 μ M SB265610 (Tocris). After 24h of treatment, 20 μ l of MTS was added to each well, and the reactions were allowed to incubate for 3 h at 37°C. The absorbance of each sample was measured at 490 nm using microplate reader (Perkin Elmer, Waltham, MA). Experiments were performed in quadruplicate.

2.8. Statistics

Statistical analysis was performed with Graph Pad Prism (GraphPad Software Inc., La Jolla, CA). T-tests and one way Anova analyses were performed where appropriate. A p-value of <0.05 was considered statistically significant. All experiments were repeated on at least three independent occasions with similar results recorded.

3. Results

3.1. MSCs promote the migration of mammary cancer cell lines

To test if MSCs isolated from the bone could impact mammary cancer cell migration, we initially performed migration/co-culture assays in which the MSCs were seeded into the bottom of a modified Boyden chamber system. Our results show that the effect of the MSCs on PyMT-Luc cell migration was significantly higher than serum free control conditions and that the effect was similar to migration induced by 10% serum (Fig. 1a, b). The MSC CM also significantly promoted the migration of the PyMT-Luc cells suggesting that soluble factors produced by the MSCs were responsible for the effect (Fig. 1a, b). The impact of the conditioned media on mammary cancer cell line migration was also validated with an independent cell line 17L3C-Luc (Fig. 1b). Furthermore, analysis of the parental cell lines from which the luciferase expressing clones were derived, yielded similar results (data not shown).

While the migration to MSC-CM was not as robust as the migration observed when the MSCs were grown in co-culture, i.e. present in the bottom chamber, we predict that the difference may be potentially due to the induction of cytokines in the MSCs by the cancer cell lines that in turn promote an even stronger migratory effect. However, our hypothesis was to identify the factors expressed by MSCs that would stimulate the initial migration of the cancer cells and not the subsequent reciprocal interactions between the two populations. Therefore, we focused on identifying factors present in the MSC-CM that could mediate the noted migratory effect of the cancer cell lines being studied.

3.2. The CXCR2 ligands, CXCL1 and CXCL5 are expressed by MSCs

In a bid to identify the factors in the MSC-CM that were promoting the migration of the mammary cancer cell lines, we performed a cytokine array. Surprisingly, CXCL12 and a number of interleukins were not detected by the array but cytokines such as monocyte chemoattractant protein-1 (MCP-1) and chemokine ligand 5 (CCL5) that are known to stimulate cell migration, were (Fig. 2a). Our results also identified that KC and LPS induced chemokine (LIX), the murine orthologs for CXCL1 and CXCL5 were present in the MSC-CM and both of these cytokines mediate their effect via the CXCR2 receptor [2]. MIP-2, another CXCR2 ligand was also detected but to a lesser extent compared to CXCL1 and CXCL5. Our preliminary studies identified that recombinant MCP-1 did not impact the migration of the mammary cancer cell lines (data not shown). Since a role for MSC derived CCL5/RANTES has been described in mediating metastasis [7], we focused our efforts on determining whether CXCL1 and CXCL5 could impact the migration of mammary cancer

cell lines. Initially, the expression of CXCL1 and CXCL5 in the mammary cancer cell lines and in the MSCs was examined at the mRNA and protein level. RT-PCR analysis revealed all of the cell lines being investigated expressed CXCL1 and CXCL5 (Fig. 2b). ELISA analysis also confirmed the presence of the cytokines in the conditioned media derived from PyMT-Luc and the MSCs (Fig. 2c) and surprisingly demonstrated that PyMT-Luc cells had significantly higher levels of CXCL1 but significantly lower levels of CXCL5 compared to the MSCs (Fig. 2c).

3.3. CXCR2 is expressed by the PyMT mammary cancer cell lines

Next we examined whether CXCR2, the cognate receptor for CXCL1 and CXCL5, was expressed by the PyMT derived cancer cell lines. Our data show that CXCR2 is expressed at the mRNA level by the PyMT-Luc and 17L3C-Luc cell lines (Fig. 3a). Interestingly, our data also show that the receptor is expressed at the protein level by both the PyMT-Luc, 17L3C-Luc and MSC cells (Fig. 3b and data not shown). Immunofluorescence revealed that the phosphorylated species of CXCR2 was present in the mammary cancer cell lines (Fig. 3c). These data demonstrate that the CXCR2 receptor is expressed by the mammary cancer cell lines and therefore, based on these findings we hypothesized that the CXCR2-CXCL1/CXCL5 interaction may mediate the migration of the mammary cancer cell lines to the MSCs.

3.4. CXCL1 and CXCL5 promote mammary cancer cell migration to MSC-CM

Next, we examined whether CXCL1 or CXCL5 impacted the migration of the PyMT derived mammary cancer cells. Using neutralizing antibodies specific to either ligand, we found that the migration of the PyMT-Luc cell line to the MSC-CM was significantly abrogated compared to the MSC-CM containing the same concentration of isotype control antibody (Fig. 4a, b). We also examined whether the addition of recombinant ligand would impact the growth of the cancer cell lines and found that 24 hour incubation with either ligand (CXCL1 or CXCL5) had no significant impact on the growth of the cells compared to control serum free conditions as measured by MTT assay (Fig 4c). These data indicate that CXCL1 and CXCL5 promoted the migration of the PyMT-Luc cell lines but do not appear to stimulate the growth of the cell lines *in vitro*.

3.5. CXCR2 mediates mammary cancer cell migration to MSCs

In order to determine whether CXCR2 mediated the migration of the mammary cancer cell lines tested, we utilized a neutralizing antibody approach. Our results show that the migration of the PyMT-Luc and 17L3C-Luc cell lines to the MSC-CM was significantly lower, approximately 50%, in the presence of the neutralizing antibody compared to IgG control treated cell lines (Fig. 5a, b). Furthermore, treatment with a commercially available CXCR2 antagonist, SB625610, complemented our findings obtained with the CXCR2 neutralizing antibody (Fig. 6a, b). Next, we examined whether treatment with the antibody or small molecule inhibitor affected the growth of the cancer cell lines and found by MTT assay that neither the neutralizing antibody or SB625610 when added to normal growth media impacted the growth of the cancer cell lines over a 24 hour period compared to controls (Fig. 6c and data not shown). The effect of SB625610 on CXCR2 activity was also tested and we observed that treatment with the inhibitor reduced the amount of CXCR2 phosphorylation, implying that the small molecule inhibitor was mediating migration inhibition, in part, via antagonizing CXCR2 receptor activity and preventing downstream signaling (Fig. 6d).

4. Discussion

Breast to bone metastasis is a common event during breast cancer progression and defining the mechanisms that govern the homing of the circulating tumor cells to the bone offers a therapeutic opportunity to prevent this debilitating disease. In the current study, we identified that MSCs derived from the bone marrow express chemokines that promote the migration of CXCR2 positive cancer cells and hypothesize that *in vivo*, MSCs may promote homing and establishment of circulating tumor cells to the bone via the secretion of chemokine ligands such as CXCL1 and CXCL5.

MSCs have previously been implicated in mediating the metastasis of cancer cells from the primary tumor via chemokine expression and chemokine receptors [7]. The MSC-secreted chemokine CCL5 (RANTES) was found to increase the motility, invasion, and lung metastasis of breast cancer cell lines by binding to CCR5 on cancer cells and initiating reversible phenotypic changes [7]. Interestingly, although MSCs enter the circulation and are recruited to damaged and inflamed tissues systemically, they originate within the bone marrow and whether they could potentially control the recruitment and establishment of newly arriving cancer cells has not been investigated thus far [9]. Our studies document that *in vitro*, MSCs contribute to cancer cell migration, a phenomenon that is in part mediated by CXCR2. While we have not explored the signaling mechanism in full, based on evidence in the literature we posit that CXCR2 mediates the cancer cell migratory effect via mitogen activated protein kinase (MAPK) and nuclear kappa B (NFkB) signaling [2; 20; 21]. Reciprocal effects of CXCR2 activity in cancer cells on the MSCs, for example, the potential effect of CXCR2 targeted gene products on the behavior of MSCs, will require further examination.

Undoubtedly, our model system used here has limitations in that it utilizes independent cell lines derived from the same tumor type, i.e. those generated by the PyMT model of mammary tumorigenesis. Further, spontaneous bone metastasis has not been documented in the transgenic model. This may be due to the fact that the animals succumb to lung metastasis burden relatively quickly. However, we have shown extensively, the cell lines derived from the model can metastasize to bone via the hematogenous route and grow in the bone microenvironment [16; 18]. Therefore, the use of the cell lines in subsequent studies allows us to examine the role of CXCR2 in bone metastasis using syngeneic immunocompetent mice and intracardiac inoculation with luciferase activity as a read out for skeletal related events in future *in vivo* studies.

CXCR2 is expressed by several breast cancer cell lines and polymorphisms in the receptor have been correlated with disease aggressiveness [22; 23]. CXCR2 has been implicated in the growth of prostate cancer and melanoma and a recent study identifies a role for the CXCR2 ligand, CXCL7 in stimulating breast cancer growth in an orthotopic murine model via the expansion of cancer stem cells [6; 24; 25]. Our data did not reveal a role for CXCR2 in mediating the growth of the PyMT cancer cell lines tested, albeit over a 24 hour period *in vitro*. Interestingly, the PyMT cancer cell lines express CXCL1 and CXCL5 but neutralizing antibodies targeted to these factors had no impact on growth. Recent studies have implicated a role for MSCs in mediating breast cancer growth via the CXCR2 receptor but this growth effect appears to be limited to breast cancer stem cells [6]. Therefore, our PyMT model system may not be optimized for examining the role of CXCR2 in breast cancer growth. However, the PyMT cell lines do migrate to MSC derived factors and more information in the literature exists as to a role for CXCR2 in the process of metastasis. For example, CXCL5/CXCR2 plays an important role in mammary gland tumor metastasis to the lung although the incidence of bone metastasis was not examined/reported [26]. Our studies show that CXCL1 and CXCL5 are expressed by the mammary cancer cell lines and so the

question arises as to why the cancer cells would migrate to CXCL1/CXCL5 produced by the MSCs. Our ELISA data show that CXCL5 is expressed at a higher level by the MSCs compared to the PyMT mammary cancer cell lines while the converse is true for CXCL1 (Fig. 2c). Therefore, the establishment of gradients of these ligands may be important in initiating a migratory response. Furthermore, we also hypothesize *in vivo* that chemokine factors generated endogenously by the circulating cancer cells may be diluted in the blood stream thus making sources or tissues that have high concentrations of CXCR2 ligands more “visible”. Given that bone is a repository for MSCs, it is therefore plausible that MSCs resident in the bone marrow that have not been mobilized to the primary tumor, may influence the recruitment and establishment of CXCR2 positive circulating metastatic cells by establishing gradients of CXCL1 and CXCL5. Our PYMT cell lines express a luciferase reporter gene that will allow us to test our hypotheses *in vivo* using intracardiac inoculation in an immunocompetent model in follow up studies.

Previous reports have implicated important roles for CXCL12 in mediating breast and prostate to bone metastasis to bone via the CXCR4 receptor [4; 5]. The CXCR4-CXCL12 signaling axis mediates lung metastasis in the PyMT model and our original hypothesis was that this would play an important role in the MSC mediated cell migration [26]. However, CXCL12 was not detected in the conditioned media of the MSCs by cytokine array and preliminary experiments using varying concentrations of recombinant CXCL12 did not promote the migration of the PyMT cancer cells (data not shown). Our *in vitro* data does not negate the important role for CXCR4 in bone metastasis but we hypothesize, based on our findings, that the homing of circulating tumor cells to the bone microenvironment may involve more than a single chemokine signaling axis.

Other mechanisms may also be involved in the migration of the PyMT cell lines since our *in vitro* data show that neutralization with a CXCR2 antibody or small molecule inhibitor results in an approximately 50% reduction in the number of migrated cells. While this may be due to the efficiency of the antibody in terms of neutralization, it may also be due to the fact that other factors present in the MSC-CM as detected by cytokine array (or present in the MSC CM but not detected by the cytokine array) contribute to migration in an individual or collective manner. CCL5 is an obvious candidate given recent reports, but other chemokines may also play a role [7]. For example, macrophage colony stimulating factor (M-CSF) and macrophage inflammatory protein-2 (MIP-2) mediate leukocyte migration and therefore may similarly impact cancer cell migration [27; 28]. Furthermore, while our study indicates that MSCs promote the migration of the mammary cancer cell lines tested, it should be noted that the population of these cells in the bone marrow microenvironment is considered to be relatively low [8]. Therefore, it will be interesting to test *in vivo* whether the MSCs in the busy signaling microenvironment of the bone marrow can set up a sufficient chemokine gradient concentration to recruit CXCR2 positive circulating tumor cells.

In conclusion, our data have identified that bone derived MSCs express the CXCR2 ligands, CXCL1 and CXCL5, and that these ligands mediate mammary cancer cell line migration in a CXCR2 dependent fashion, a process that can be inhibited via small molecule CXCR2 inhibition. Our data combined with other reports collectively suggest that multiple targets exist for the development of therapeutics that will inhibit breast to bone metastasis.

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Fig. 1.

MSCs stimulate the migration of murine mammary cancer cell lines. (A) Representative low power objective (10x) hematoxylin and eosin stained filters illustrating the presence of migrated PyMT-Luc cells. MSC indicates experiments in which MSCs were grown in the lower chamber of the migration assay while MSC-CM indicates experiments where only conditioned media derived from the MSCs was utilized. (B) Number of migrated PyMT-Luc cells (each data point indicates the number of cells per individual field) in each experimental condition. An unrelated mammary cancer cell line, 17L3C-Luc, was also tested. These data are delineated from PyMT-Luc by dashed line. Asterisk denotes statistical significance of individual group compared to serum free media control group with $p<0.05$.

Fig. 2.

The CXCR2 cognate ligands, CXCL1 and CXCL5 are expressed by MSCs. (A) Cytokine array analysis of factors present in the MSC conditioned media. Controls and antibodies to chemokines are immobilized to the array in duplicate. Numbers highlight areas of positivity and key chemokines present in the MSC CM are identified on table in lower panel. (B) RT-PCR analysis for the expression of CXCL1 and CXCL5 in the MSCs, and the mammary cancer cell lines, PyMT-Luc and 17L3C-Luc. The murine Lewis Lung Carcinoma cell line (LLC) was used as a positive control. GAPDH was used to control for loading. (C) ELISA for CXCL1 and CXCL5 protein concentration in the PyMT-Luc and MSC conditioned media. Asterisk denotes statistical significance with $p < 0.05$.

Fig. 3. Mammary cancer cell lines express CXCR2. (A) RT-PCR analysis of CXCR2 mRNA expression. Total RNA isolated from a CXCR2 null mouse (CXCR2^{-/-}) was used as a negative control while LLC was used as a positive control. (B) Immunoblot analysis of CXCR2 in cell lysates. (C) Immunofluorescent localization of phosphorylated CXCR2 in PyMT-Luc cells. Dashed line represents area of magnification. Scale bars are 50 μm.

Fig. 4. CXCL1 and CXCL5 promote the migration of mammary cancer cell lines. (A, B) Analysis of PyMT-Luc migration in the presence of IgG control (MSC-CM) or a neutralizing antibody against CXCL1 (α -CXCL1; 10 μ g/ml) (A) or CXCL5 (α -CXCL5; 10 μ g/ml) (B). Asterisk denotes statistical significance with $p < 0.05$. (C) MTT assay of PyMT-Luc growth in control (serum free), CXCL1 (10ng/ml) or CXCL5 (10ng/ml) containing serum free media. N.S. denotes that p values did not reach statistical significance.

Fig. 5. CXCR2 mediates the migration of mammary cancer cell lines. (A, B) Representative photomicrographs of PyMT-Luc (A) and 17L3C-Luc (B) cell migration to MSC-CM in the presence or absence of a CXCR-2 neutralizing antibody (50µg/ml). (C, D) Analysis of PyMT-Luc (C) or 17L3C-Luc (D) cell migration in the presence or absence of a neutralizing antibody against CXCR2 (α-CXCR2; 50µg/ml). Isotype IgG (50µg/ml) was used in control experiments. Asterisk denotes statistical significance with $p<0.05$.

Fig. 6. A CXCR2 antagonist SB625610 significantly abrogates mammary cancer cell line migration. (A) Representative photomicrographs of PyMT-Luc cell migration to MSC-CM in the presence or absence of SB625610 (1 μ M). (B) Analysis of PyMT-Luc migration in the presence of SB625610. SB625610 diluent (100%) ethanol was used in control experiments. Asterisk denotes statistical significance with $p < 0.05$. (C) Effect of SB625610 (1 μ M) treatment on growth of PyMT-Luc cells using MTT assay. N.S. denotes non-significant p-value. (D) Phospho CXCR2 (pCXCR2; arrow head) levels in PyMT-Luc cells treated with SB625610. β -actin was used a loading control.