The Role of Chemokine Receptor CXCR7 with EGFR in Breast Cancer Cell Proliferation

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UNIVERSITY OF MIAMI

THE ROLE OF CHEMOKINE RECEPTOR CXCR7 WITH EGFR IN BREAST CANCER CELL PROLIFERATION

By

Nicole Salazar

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2014
THE ROLE OF CHEMOKINE RECEPTOR CXCR7 WITH EGFR IN BREAST CANCER CELL PROLIFERATION

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Recent advances have revealed a significant contribution of chemokines and their receptors in tumor growth, survival after chemotherapy and organ-specific metastasis. The CXC chemokine receptor-7 (CXCR7) is the latest chemokine receptor implicated in cancer. Although over expressed in breast cancer cell lines and tumor tissues, its mechanism of action in breast cancer (BrCa) growth and metastasis was not clear. Studies in other cancers have implicated CXCR7 in cell proliferation, anti-apoptotic activity and cell-cell adhesion. The present study was initiated to examine the pattern of CXCR7 expression and its role in regulation of growth signaling in breast cancer.

The contribution of CXCR7 in BrCa cell proliferation was investigated in representative cell lines using real time quantitative PCR (q-PCR), proliferation assays, immunohistochemistry and immunoblotting. Phenotypic changes were examined after CXCR7 specific cDNA and siRNA transfection and expression levels were monitored by q-PCR. Further, the association of CXCR7 with epidermal growth factor receptor (EGFR) and modulation of its activity were investigated by Western blotting, immunofluorescence, and in-situ proximity ligation assays in human BrCa cells and tissues.
CXCR7 was expressed in both, estrogen receptor (ER) positive and negative BrCa cancer cell lines. We observed CXCR7 expression to a large extent in the cytoplasm of BrCa cells and the nucleus of the LNCaP prostate cancer cell line. In MCF7 cells we found that CXCR7 does not colocalize to membrane lipid rafts. We also show that in MCF7 and LNCaP cells, CXCR7 transcript is induced by the cytokines IL-8 and TNF-α. CXCR7 was also expressed unevenly in normal human breast tissues and to a higher extent in ER+ human cancer tissues.

Depletion of CXCR7 in MCF7 BrCa cells by RNAi decreased proliferation and caused cell cycle arrest. Further, proximity ligation assay (PLA) revealed colocalization of CXCR7 with EGFR in cancer cell lines and cancer tissues. CXCR7 depletion reduced levels of phospho-EGFR at tyrosine1110 after EGF-stimulation and also reduced phosphorylation of ERK1/2, indicating a potentially direct impact on mitogenic signaling in MCF7 cells. Using siRNA to knockdown β-arrestin2 in cells with EGFR over expression we were able to nearly deplete the CXCR7-EGFR colocalization events, suggesting that β-arrestin2 acts as a scaffold to enhance CXCR7 dependent activation of EGFR after EGF stimulation. Furthermore, our observations suggest that CXCR7 expression can affect β-arrestin2 distribution throughout the cell and its shuffling between nucleus and cytoplasm.

These results demonstrate coupling of CXCR7 with EGFR to regulate proliferation of BrCa cells and suggest an important ligand-independent role of CXCR7 in BrCa growth. Thus, the CXCR7-EGFR axis is a promising target for breast cancer therapy.
La realización de este pedregoso proceso educativo se la dedico a mi mamá, el alma más alegre que conozco, que siempre me puede mejorar los días.
ACKNOWLEDGEMENTS

I give thanks for this project, which has taught me so much about persistence, humility, and courage, to my research advisor, Dr. Bal Lokeshwar. Thank you for the opportunity to join your lab and for your incredible patience. Thank you for teaching me what it means to be a scientist and your unwavering support. And thanks for always seeing the best in me. I will always remember your kindness.

I thank my colleagues and my lab group for everything you helped me achieve: Mr. D. Muñoz, thank you for teaching me molecular biology and teaching me about how to pose and question arguments. Dr. Kallifatidis, thank you for teaching me how to write my first scientific research article. Thank you L. Zhang and J. Hoy for joining the lab and going through this process with me, and also M. Lopez for great illustrations. Thanks to all the members of the Lokeshwar lab throughout the years for creating this lab family.

I thank my committee members, for all their precious time and excitement for this project, indispensable feedback and support: Dr. Lopez, Dr. Robbins, Dr. Khan, and Dr. Jorda. Thank you to Dr. Gaines, Dr. Landgraf, Dr. Burnstein, Mr. S. Kennelly and Mr. J. Zerhusen for the key moments where you made an immense difference in my progress.

I thank my few dear friends near and far, for all the help and enthusiasm. And most importantly, I thank my greatest gift, my family, for their unconditional love and admiration: my mom, Belen, my constant source of inspiration and who made me everything I am; my dad, Gumercindo, who raised me like his own, and taught me what a father is; my brother Johan, the best big brother a girl could have; and finally, Belencho.
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<td>Antibody</td>
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<td>β-ARR2</td>
<td>Beta-arrestin-2</td>
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<td>BrCa</td>
<td>Breast cancer</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>CD31</td>
<td>Cluster of differentiation 31</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CXCL</td>
<td>C-X-C- chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C-chemokine receptor</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetate</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR/HER1</td>
<td>Human epidermal growth factor receptor 1</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER+</td>
<td>Estrogen receptor positive</td>
</tr>
<tr>
<td>ER-</td>
<td>Estrogen receptor negative</td>
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<tr>
<td>ERBB2</td>
<td>Erythroblastic leukemia viral oncogene (origin of name)</td>
</tr>
<tr>
<td>FD</td>
<td>Fold difference</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>IL8</td>
<td>Interleukin-8</td>
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<tr>
<td>ITAC</td>
<td>Interferon inducible T-cell alpha chemoattractant</td>
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<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PLA</td>
<td>Proximity ligation assay</td>
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<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
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<tr>
<td>qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
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<tr>
<td>SDF-1α/α</td>
<td>Stromal derived factor alpha</td>
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<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-tween</td>
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<tr>
<td>TNF-α/α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>4-IPP</td>
<td>4-iodo-6-phenylpyrimidine</td>
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Chapter 1: INTRODUCTION

1.1 Introduction to breast cancer: scope and impact

Breast cancer (BrCa) is the second most common cancer in women, regardless of race or ethnicity worldwide. This year, BrCa will cause nearly 40,000 women and 400 men in the United States and approximately 522,000 people to die, worldwide. A higher incidence of BrCa is common in developed countries; however, nearly 50% of BrCa cases and 58% of BrCa deaths occur in the less developed countries, accounting for approximately 25% of all cancer deaths. An estimated 232,670 people in the US and a whopping 1,655,000 people worldwide are expected to develop breast cancer by the end of 2014 [1-4]. Nevertheless, BrCa has one of the best survival odds if detected early and if adequate health care access is provided, which unfortunately restricts high survival rates mostly to developed countries. These greater survival odds are due to the persistence and innovation of scientific progress achieved throughout recent history.

1.2 Cancer therapy history

Advancements in surveillance of BrCa have decreased the overall BrCa death rates since the 1960s. This is mainly due to the prevalence of the mammogram. The mammogram has been extremely successful in detecting the incidence of cancer at an early stage. Its first implementations began in the 1920’s, however, it wasn’t until the 1960s that it became a well established technique used by physicians and was shown to successfully reduce breast cancer mortality [5]. Today, breast cancer testing is encouraged by the US Preventive Services Task Force every two years for women between the ages of 50-79, and earlier only if there is a family history. The mammogram screening method’s main limitation is that it is primarily an imaging test, therefore it
cannot determine whether the patient’s tumor is highly invasive, metastatic, highly malignant, or merely a fatty lump without further biopsy and full body scanning. Ergo, there is an essential need for improved screening methods to detect BrCa, and every other cancer for that matter.

In the last 25 years there have been improvements in diagnostic and treatment strategies of BrCa that tremendously changed the forecast of patients receiving a BrCa diagnosis today. The evolution of BrCa treatment has changed more in the last 25 years than ever, but this rapid advancement has been based on seminal works throughout the history of cancer therapy.

BrCa tumors can be palpable and visible. Therefore since ancient Greek and Roman times, physicians have tried to relieve the burden by surgically excising the tumors, even with the terrible consequences of surgery that only changed after major advances in aseptic and surgical techniques in the 19th and early 20th century. In the 19th century, in 1878, Thomas Beatson found that removing the ovaries from rabbits stopped milk production in their breasts. He found this powerful connection between the ovaries and breast tissues and then decided to test the effect of removal of the ovaries in advanced BrCa patients. He found that oophorectomy often resulted in improvement for BrCa patients. He had discovered the stimulating effect of the female ovarian hormone (estrogen) on breast cancer, even before the hormone itself was discovered. This work was the foundation for the modern use of hormone therapies, such as tamoxifen and aromatase inhibitors to treat or prevent breast cancer [6].

In 1896 Wilhelm Conrad Roentgen introduced the x-ray. Within 3 years x-rays were being used for diagnosis and radiation therapy, and they began being used for
cancer treatment [6]. Soon thereafter, in the 1920s, it was realized that radium, the initial source used for radiation therapy, also caused cancer due to exposure. Radium use was gradually abandoned in favor of Cobalt, which was later replaced by the linear accelerator (linac) for high energy x-rays in 1948 [7, 8]. These findings had improved cancer treatment primarily for superficial or accessible tumors, yet the more invasive or metastatic forms of cancer benefited little, or mainly as a palliative measure. In 1929, Ernest O. Lawrence invented the cyclotron, a particle accelerator that was re-invented in 1946 for proton therapy, which has now led to the state of the art of radiation therapy, using protons, Carbon and Helium beams. Importantly, in the 1970s, randomized research trials with BrCa patients showed that combining radiation therapy and conservative surgery provided the same overall survival as what a radical or mutilating surgery could [8-11]. These clinical trial results began to change clinical practice by considering quality of life an important consequence of cancer therapy strategies. These findings and concurrent research about radiation dosing formed the basis of modern radiation therapy, and the addition of computer technology has allowed it to be used in remarkable ways [6]. After over 100 years, the ultimate goal of radiation therapy however, remains the same, to deliver radiation to the tumor while sparing the affected organ. Despite this, radiation therapy has led to dramatic clinical benefit in patients with cancer and is still standard treatment for BrCa as well as most other forms of cancer.

Cancer therapy then evolved towards chemotherapy. During World War II, soldiers exposed to mustard gas were found to have decreased levels of white blood cells. Meanwhile, the US government was revealing the effect of a mustard gas-like compound called nitrogen mustard. This compound could kill cancer cells of the lymph nodes and
blood. Therefore it served as the model for compounds called alkylating agents that could specifically kill rapidly growing cancer cells by damaging their DNA [6]. In 1948 Sidney Farber demonstrated that aminopterin, a compound related to the vitamin folic acid, cured children with acute leukemia. Aminopterin blocked a critical chemical reaction needed for DNA replication. That drug was the predecessor of methotrexate, a cancer treatment drug used commonly today [12]. The first cured case of metastatic cancer happened in 1956 when methotrexate was used to treat a rare tumor called choriocarcinoma [6]. Continued research discovered other drugs that block functions in cell growth and replication and they began being used as chemotherapeutic drug combinations. A report in 1976 was the first evidence of chemotherapy’s role not just as a treatment, but also as adjuvant therapy. In this 1976 report, Bonadonna et al found a significant improved survival in BrCa patients that had undergone radical mastectomy and been treated with a chemotherapeutic drug combination as compared to the control group not treated with the chemo [9, 13]. These were the beginnings of chemotherapy. Surprisingly, not much has evolved and similar chemotherapeutic agents are still the standard of care to this day. Toxicity side effects associated with chemotherapies remain with terrible side effects on quality of life and are a significant burden for cancer patients and their caretakers.

Cancer therapy was also approached from a different perspective in the late 1800s, when it was noted that feverish infections in cancer patients or infected tumors would occasionally cause tumors to shrink. This observation led to the idea that the body's immune system could be harnessed and made to fight cancer [14, 15]. William Coley tested this hypothesis and by 1893 published a report of injected streptococcal cultures into cancer patients that resulted in tumor regression in some cases, yet his
methods did not uptake as a general treatment for cancer [16]. However, the bacille Calmette-Guérin (BCG) vaccine, which is used for tuberculosis and as the most effective therapy against superficial bladder cancer, was based on Coley’s toxin [15, 17, 18]. Moving forward, in the 1980s, scientists showed that the protein interferon alpha could cause tumors to shrink in patients with low-grade lymphoma. Interferon began being used to treat several different types of cancer. These findings were the beginning of non-specific cancer immunotherapy.

In the 1950s several important experiments showed that it was possible to immunize animals against their tumors. The experiments suggested there must be tumor-associated antigens--antigens associated with tumor cells that were recognized by the immune system [15, 19-22]. The concept of healthy immune cells getting rid of cancerous cells, a process named immunosurveillance, began to be accepted. Then, in 1975 Georges Köhler and César Milstein changed the face of immunotherapy with their discovery of how to make synthetic antibodies; these were the beginnings of targeted immune therapy [14]. The first therapeutic monoclonal antibodies, rituximab (Rituxan, anti-CD20 and trastuzumab (Herceptin, anti-HER2) were approved during the late 1990s to treat lymphoma and breast cancer, respectively [6]. Three forms of monoclonal antibodies targeting the cancer-associated protein Her2/neu are approved for BrCa treatment today [23].

A further refinement in cancer therapy began in the 1960s when Stanley Cohen and others recognized the role of growth factors and their receptors in growth and development of embryos and also in the growth and spread of cancer cells [24]. With the improvements of analytical molecular biology techniques, they were able to further
understand what kind of protein, for example a tyrosine kinase vs. a threonine kinase, the receptors were [24]. About the same time, in the 1960s, research was providing evidence that stimulation of vasoproliferation and tumor angiogenesis was mediated by diffusible factors produced specifically by tumor cells [25]. This was the foundation of Judah Folkman’s 1970 hypothesis that anti-angiogenesis treatment might be an effective anticancer strategy. Vascular endothelial growth factor (VEGF) was later identified in the 1980s as an essential regulator of normal and abnormal blood vessel growth and key in solid tumor blood supply [25, 26].

These were the beginnings of the targeted therapy approach, from which a long list of potential therapeutic targets has developed. The development of drugs that target specific genes or pathways has been one of the most studied areas of cancer biology since.

In 1972, Kerr, Wyllie, and Currie proposed that evasion of apoptosis is an important mechanism that contributes to cancer progression. This hypothesis was based on evidence dating back to 1842 cell death observations [27-29]. The field of apoptosis had begun, and by 1984, Yoshide Tsujimoto in Carlo Croce’s lab characterized BCL2, the first protein found to be involved in the regulation of apoptosis [29, 30]. Then in 1988, David Vaux found the ability of BCL2 to control apoptosis by demonstrating that BCL2 transfected cells did not proliferate in the absence of IL3 yet they did not die while the control cells did [31]. In the same year, Tsujimoto also showed that BCL2 could protect against various cell death inducing compounds, and BCL2 was thus established as an anti-apoptotic protein and classified as an oncogene. Surprisingly, low levels of BCL2 correlate with poor prognosis in patients with BrCa [29, 32]. During the 1990s, more
apoptotic family proteins were identified and signaling pathways began to elucidate how cancer cells become resistant to therapy. Improved targeted therapies combined with standard hormone therapy have received approval from the Food and Drug Administration and the European Medicines Agency as these combination therapies have demonstrated significant improvements for BrCa patients. This is the case for mTOR inhibitors, such as Everolimus, an mTOR inhibitor approved in 2012 for hormone receptor-positive, HER2-negative tumors in combination with hormone therapy for BrCa treatment [33].

Despite these substantial advances in cancer treatment throughout history, BrCa remains a major unresolved clinical and scientific challenge. Ultimately, advancements based on improved understanding of molecular mechanisms of disease progression are certainly necessary to find a cure. There are many state of the art strategies such as gene therapy and genomic analysis based approaches outlined to overcome this problem. However, one alternative approach has been to look at the success of other disease treatments.

A parallel field of biomedical research that focused on what was eventually known as G-protein coupled receptors similarly had groundbreaking findings that throughout the years have led to fruitful knowledge in treating heart disease and inflammation [34-37]. Because the G protein-coupled receptor super family has had so much success as drug targets, efforts are ongoing to test whether the inhibition of a group within this family, the chemokine receptor family, could potentially be exploited for the treatment of breast and other types of cancer [38-42].
1.3 Role of chemokine receptors in breast cancer

Chemokine receptors are members of the seven-transmembrane (7-TM) G-protein coupled receptor (GPCR) super family. Generally, upon binding to their chemokine ligands, chemokine receptors will transduce signals by coupling to guanine nucleotide-binding proteins (G-proteins). These receptors have three intracellular loops and an intracellular C-terminus where G-proteins may dock if they are activated. The second intracellular loop contains a DRYLAIV (Asp-Arg-Tyr-Leu-Ala-Ile-Val) motif, which is missing in the non-signaling receptors [43]. Chemokine receptors exist in cells of epithelial and hematopoietic lineage. Most chemokine receptors bind both specifically and promiscuously to their ligands, small peptides (8-12kDa) known as cytokines that have chemoattractant properties and are called chemokines. The chemokine sequence is CXC or CXXC or CXXC depending on the number of amino acids that separate the two conserved cysteines, one or two or three, respectively. Their receptors are hence known as CXCR, CXXR or CXXCR with the R emphasizing the receptor [43].

GPCRs, upon binding to their chemokine ligands, can also transduce signals by a non-classical way, through β-arrestins. β-arrestins normally dock onto the phosphorylated cytoplasmic tail of an activated GPCR, thus preventing further activation or downstream signaling, because it blocks the G proteins from docking onto the receptor. However, β-arrestins may play other roles in signal transduction by acting as scaffolds [44]. The arrestin scaffolds may serve as adapter molecules to assemble multi-protein complexes ultimately leading to receptor internalization, recycling back to the plasma membrane, and downstream signaling events, including ERK1/2 (extracellular signal-regulated
kinases) activation [36, 45, 46]. Arrestins may also shuttle between the cell nucleus and cytoplasm [47].

The expression of chemokine receptors may be altered by dysregulation of transcription factors or DNA. The chemokine receptor group plays important roles across many diseases including inflammatory and autoimmune disorders, allergies, and cancers [48]. Interestingly in cancers in particular, chemokine receptor levels can be disparate and studies have shown that they are key players in tumor metastasis and survival [48-51]. The aberrant expression of these receptors can give them particular functions that promote cancer [49, 52-54].

Particular attention has been paid to the SDF-1α-CXCR4 axis for its role in metastasis of breast cancer cells to common sites like bone and brain. The chemokine receptor CXCR4 is also considered a marker for both normal and cancer stem cells [55]. The role of CXCR4 has been found to be highly dependent on the gradient of its ligand SDF-1α [49]. SDF-1α, also known as, CXCL12 was believed to be a chemokine exclusive to CXCR4 until Burns et al. showed that CXCR4-/- E13 murine fetal liver cells were able to bind radiolabeled SDF-1α [56, 57]. The same effect was observed when molecular inhibitors of CXCR4 as well as another chemokine, CXCL11, affected CXCL12 binding in different cell lines not expressing CXCR4. They identified the binding site, an RDC1 (Receptor Dog cDNA 1) encoded protein and re-named it the seventh receptor identified to date for chemokines belonging to the CXC class, CXCR7 [57-59]. It is also known as atypical chemokine receptor 3, ACKR3.
1.4 The chemokine receptor CXCR7

CXCR7 is expressed in human breast, lung, brain, and prostate cancers among others [60-62]. CXCR7 is a seven-transmembrane protein that binds chemokines CXCL11 and CXCL12 and also dimerizes with CXCR4 [63, 64]. CXCR7 is considered an atypical chemokine receptor for several important reasons, explained later. CXCR7 is frequently expressed in a variety of tumor cell lines, activated endothelium and neovasculature associated with tumors but not on normal adult tissue [57, 60]. Furthermore, it seems to be a post-transcriptionally modified protein [65]. Most recently, CXCR7 was found to activate signaling through β-arrestin exclusively and not through G-protein activation proving that there is an alternative pathway aside from the classical G-protein coupled receptor pathway that has evolved to allow the signaling of this previously thought decoy receptor [66, 67].

CXCR7 is the newest member of the CXC-chemokine receptor family of plasma membrane receptors that binds CXCL11, CXCL-12 (SDF-1α), and MIF (macrophage migratory inhibitory factor) [57, 68]. CXCR7 binding to the chemokine SDF-1α induces a gradient shift critical for correct development and primordial germ cell migration which led to its reputation as a scavenger receptor [69, 70]. CXCR7 is considered an atypical chemokine receptor due to its modified amino acid motif (DRYSIT instead of DRYLAIV) at the second intracellular loop, which prevents it from coupling to G-proteins and inducing intracellular Ca$^{2+}$ mobilization [71, 72]. Therefore, CXCR7 does not signal through the classical G-protein coupled receptor (GPCR) mechanism of secondary messengers. Instead it has been shown to rather interact with β-arrestin2 (β-ARR2) as an accessory protein in a ligand dependent manner [66, 67].
The behavior of CXCR7 is tissue and context dependent. CXCR7 may also play an important role in development and the progression of cancer to the metastatic stage [62, 73, 74]. Increased expression of CXCR7 is attributed to IL-8 (Interleukin-8), inactivation of HIC1 (hypermethylated in cancer-1), activation of HIF-1α (hypoxia-inducible factor-1), and activation of NF-kB (nuclear factor kappa B) [75-81].

1.5 Breast cancer model

We use breast cancer for our model, as the role of chemokine receptors, more specifically the CXCR4 axis, is known to have a substantial effect on the metastasis, survival and proliferation of BrCa [82]. Because BrCa is a largely heterogeneous disease, this leads to much diversity in the current BrCa cell line models. Comprehensive gene expression profiling of large tumor sets have revealed five major molecular subtypes of BrCa based on gene expression difference, although most recently, even more subgroups have been identified [39, 83-85]. The best-established BrCa subtypes are: basal-like, luminal A, luminal B, HER2+/ER–, and normal breast-like [86-88]. Basal-like tumors have the worst prognosis while the luminal A-type tumors have the best prognosis [39, 89]. This innate heterogeneous complexity makes it clear that proliferation mechanisms change and may be altered during disease phases so that other players, such as chemokine receptors, may step in to support proliferation and other hallmarks of cancer.

The first goal of this study was to identify the expression of CXCR7 across representative breast cancer lines. The logic of this was to identify a phenotypic association between CXCR7 and well-established breast cancer lines and to find adequate models to use for this study. Previous work from our lab showed that CXCR7 promotes
prostate cancer growth and we asked whether the same mechanism of proliferation affects the other major endocrine related cancer, breast cancer.

In cancers, intracellular proteins that transduce growth signals can be overexpressed or activated by mutations, such as ras in the MAPK signaling pathway. With these self-sufficient mechanisms, cancer cells can be totally growth-independent of their environment. Our data shows that expression of CXCR7 affects growth of BrCa cells as demonstrated by marked cellular proliferation. At this inter-phase, the cell cycle is affected by critical kinases like Cdk4 and mitogen activated protein kinases (MAPK).

1.6 EGFR in BrCa

The MAPK cascade is activated by growth factors and receptors such as EGFR. EGFR is a protein-tyrosine kinase in which autophosphorylation of tyrosine residues results in downstream activation of proteins involved in cell proliferation [90]. The EGFR signaling pathway is one of the most important pathways to regulate normal cell proliferation [90]. Across many cancers, dysregulation of the EGFR pathways by overexpression or constitutive activation may promote angiogenesis and metastasis [91-94]. The EGFR family members are known to affect signaling via cross-talk amongst themselves and also with other receptor tyrosine kinases, as well as other families, such as the GPCR superfamily [36, 95]. Hypotheses that such alternative signaling pathways are linked to cancer growth and resistance to EGFR-targeted therapies have been posited [94].

The epidermal growth factor receptor or ErbB family is one of the most notable cancer molecular targets identified to date. The family includes: EGFR (also known as ErbB1 and HER1), HER2 (also known as HER2/neu and ErbB2), ErbB3 (also known as
HER3), and ErbB4 (also known as HER4). HER2, which is overexpressed in 20–25% of breast cancers, is the best-established therapeutic target in BrCa [96]. EGFR expression correlates with large tumor size, poor differentiation, and poor clinical outcomes in BrCa patients [97, 98]. EGFR overexpression is usually associated with triple negative breast cancer; however, its overexpression is observed in all subtypes of breast cancer [94, 99-101]. The role of EGFR in BrCa has not gone unnoticed, however, with clinical trials using EGFR therapies having been tested. Unfortunately for BrCa patients, the results for improvement using this therapy have been poor [94]. Better clinical study designs might improve these treatment strategies and results for future studies.

1.7 Hypothesis

Based on literature review and recent evidence about the importance of CXCR7, we asked whether the effect of CXCR7 on proliferation was due to its interaction with EGFR, a relevant growth factor in cancer biology.

We showed that CXCR7 is an important modulator of cell proliferation and cell cycle progression of CXCR7-expressing BrCa cells. We demonstrate that in BrCa, CXCR7 co-localizes with EGFR and CD31. We showed that down-regulation of CXCR7 affects the phosphorylation status of EGFR and partially decreases the phosphorylation of key mediators of the MAPK cascade leading to cell cycle arrest. We also show that CXCR7 interacts with EGFR in BrCa tissue and that in some BrCa cells this relationship may significantly contribute to BrCa proliferation in a ligand independent fashion in concert with β-arrestin2.

This CXCR7 control and association with EGFR is a novel finding in BrCa. Based on these observations, we propose that CXCR7 mediates breast cancer
proliferation by association with EGFR. Therefore, CXCR7 may play a critical role in conjunction with EGFR in BrCa progression. Furthermore, activation of the EGFR-mediated mitogenic pathway is due to a direct interaction of CXCR7 with EGFR and consequently, disruption of this interaction should decrease breast cancer growth.

The regulation of BrCa growth by chemokine receptor and growth factor receptor interaction is a relatively nascent area of research. This work could significantly improve our understanding of the mechanism of action cancer cells use to proliferate so effectively. To find and understand the relation of cancer proliferation with CXCR7 may provide the scientific community another critical tool to improve the design of directed-therapy approaches to attack CXCR7 expressing malignancies more selectively.

1.8 Impact

This project provides insight about BrCa proliferation through the molecular mechanism of CXCR7 and EGFR interaction. Modulating this interaction using small molecule inhibitors, RNAi, or specific antibodies, should potentially provide new avenues to control BrCa growth, invasion and metastasis. The assessment and disruption of this interaction provides a novel approach to control breast cancer aggression. The project should help in planning improved novel combination therapy drugs to combat BrCa.
Chapter 2: MATERIALS AND METHODS

2.1 Cell lines and cell culture: All cell lines were obtained from an authenticated source (ATCC, Manassas, VA) and used within six months of resuscitation of original cultures. The cell lines used in the study were also authenticated for their origin by Genetica DNA Laboratories Inc (Cincinnati, OH). Culture and maintenance of BrCa cell lines were performed by routine cell culture procedures. Cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum (FBS, 10%) and gentamicin (10 µg/ml). The normal breast epithelial cell line, MCF10A was maintained in MEBM medium supplemented with the additives obtained from Lonza/Clonetics Corporation as a kit: MEGM Kit Catalog No. CC-3150. The normal breast epithelial cell line, MCF12A was maintained in DMEM/F12 medium supplemented with the additives obtained from Lonza/Clonetics Corporation as a kit: MEGM Kit Catalog No. CC-3150 and 10% horse serum.

2.2 Gene knockdown with siRNA: Cells cultured for 24 h were transfected with gene-specific 21-mer siRNA sets (Smartpool siRNA, Dharmacon/Thermo Scientific Inc, Chicago, IL), using the Dharmafect-2 transfection protocol.

2.3 Stable silencing of CXCR7 expression by shRNA: MCF7 shCXCR7 and vector control cell lines were made as described before. Briefly, CXCR7 shRNA and scrambled sequence shRNA (Control shRNA) constructs were cloned into a pRS plasmid under the control of a U6 promoter for stable expression (HuSh-29mer, Origene Technology Inc. Baltimore, MD). Cells were transfected with shRNA-pRS using Lipofectamine 2000 (In Vitrogen Inc., Carlsbad, CA). Stable transfectants were selected from transfected cultures following two weeks of culture in puromycin selection medium (2.0 µg/ml). Emergent
cell colonies were evaluated for CXCR7 mRNA knockdown by q-PCR and immunoblotting.

![Figure 2.3 Map of plasmid used for stable shCXCR7 cell line.]

2.4 Gene over-expression with cDNA: Cells were transfected with a human full-length CXCR7 cDNA (pCMV6-Neo vector; OriGene, Rockville, MD) or CXCR7-green fluorescent protein (EGFP-N1 vector; BD Biosciences, San Jose, CA), a generous gift from K. Luker. β-arrestin2 cDNA was a generous gift from V.R. Jala. EGFR insert was cloned into a pLPCX plasmid under control of a CMV-IE promoter (Clontech, Mountainview, CA). Cells were transfected using Lipofectamine 2000 and analyzed for over expression by q-PCR and immunoblotting.
2.5 Cell proliferation assay and cell cycle phase analysis: Cell proliferation and cell viability were determined by cell counting and MTT reduction assays, respectively. Cell cycle phase-fractionation was conducted using preparations of propidium iodide labeled nuclear suspension analyzed on a Beckman-Coulter XCEL flow cytometer as described before. The MIF inhibitor used for cell proliferation was 4-IPP (Tocris Bioscience Cat. No. 3429).
2.6 Quantitative real time PCR (q-PCR): Total RNA isolated from cells 48 h later was subjected to cDNA synthesis and q-PCR using iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA) and the primers described in Table 2.6. The mRNA levels were normalized to that of the housekeeping protein, peptidylprolyl isomerase A (PPIA) based on the threshold cycle (Ct) of each sample in RT qPCR. Relative levels of mRNA expression were calculated from ΔCt where ΔCt = (test mRNA Ct - PPIA Ct). Values are shown as fold difference (FD), defined as FD = (2^{ΔCt})^{-1} x 100. Primers were designed in house using Primer3, PrimerBlast, or OligoExplorer. First the FASTA reference sequence, usually from Pubmed was accessed. Then the CDS or coding region section was used to design the primers within this region following a specific primer design protocol.

The sequences were then tested using the gene DNA folding website M-FOLD using a specific protocol.

<table>
<thead>
<tr>
<th>mRNA target</th>
<th>Forward Primer 5'--&gt;3'</th>
<th>Reverse Primer 5'--&gt;3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPIA</td>
<td>TCATCTGCACGTGCAAGACTG</td>
<td>CATGCCTTCTTTCACTTGGCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>CAACTGGGACGACATGGA</td>
<td>GTTGGCCCTTGGGGTCAG</td>
</tr>
<tr>
<td>CXCR7</td>
<td>ACAGGCTATGACACGACTG</td>
<td>ACGAGACTGACCACCCAGAC</td>
</tr>
<tr>
<td>CXCR4</td>
<td>TCACTCAAGCAAGGTGAG</td>
<td>GGCTCAAGGAAGCATAGA</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>AGAAGTCCAGCCCTAACTGC</td>
<td>TCGGTCCCTCAGGATGCA</td>
</tr>
<tr>
<td>CXCL11</td>
<td>TTCCACTGCCCCAAAAGGAGTC</td>
<td>CCGATGGTAACCAGCCTTTTC</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CACAGAAGGTCTCTGATTA</td>
<td>CATTGAAAAGCTGCAATCACA</td>
</tr>
<tr>
<td>EGFR</td>
<td>CTGTGAGCAAGGAGCACAAG</td>
<td>AGTGAAGGCCTTAAGCAGA</td>
</tr>
</tbody>
</table>

**Table 2.6: Primer Sequences used for quantitative real time PCR and procedure**

Using the CFX96 Thermocycler, the procedure programmed for all primers are as followed: 95°C for 30 seconds, 40 cycles of 95°C for 1 sec, and 60°C for 25 seconds. To generate a melt curve, a dissociation procedure was performed following the 40 cycles from 55°C to 95°C at 0.5°C increments, 10 seconds each.

### 2.7 Immunoblotting:

Expression of specific proteins in treated cells was analyzed by routine immune-blotting technique. Briefly, proteins were quantified using Micro BCA™ Protein Assay Kit (# 23235, Thermo Scientific, Lafayette, CO) and equal amounts of proteins were loaded and separated on 10% SDS acrylamide gels and blotted onto PVDF Immobilon-P Membrane (EMD Millipore, Billerica, MA). Nuclear and cytoplasmic protein fractions were extracted following the manufacturer’s protocol, using NE-PER Nuclear and Cytoplasmic Extraction Reagents, (#78833 Thermo Scientific, Pierce Biotechnology, Rockford, IL). Presence of specific proteins on the membranes was detected using specific antibodies and the ECL+ Kit (GE Health Science). Relative protein band intensities were quantified using densitometry (Gel Logic 2200, CareStream Instruments, Rochester, NY). Band densities were normalized to those of actin.

### 2.8 Antibodies

Antibodies used as described for cell cycle analysis and isotype controls; specific antibodies used were: CXCR7 Ab (GTX1000027, GeneTex, Irvine, CA) for confocal at 1:500; CXCR7 Ab (20423-1AP, Proteintech Group, Chicago, IL) 1:5000 for immunoblotting; EGFR Ab (E2760, Sigma Aldrich, St. Louis, MO) for confocal 1:500;
EGFR Ab (Cell Signaling 2646S, Beverly, MA) for western blotting at 1:3000; phospho-
EGFRY1110 Ab (2284-1, Epitomics, Cambridge, MA) for Western blotting 1:1000; β-
arrestin2 Ab (sc-13140, Santa Cruz, Dallas, TX) for Western blotting 1:500.

2.9 Immunofluorescence

Cells were plated 48 hrs before use, 10x10^3 cells in 250ul of media, in 8-chamber glass
slides. After the cells appeared firmly attached and healthy media was removed and cells
were washed PBS-T. The cells were then fixed with 4% paraformaldehyde solution in 1X
PBS for 15 min on ice. If permeabilized, cells were permeabilized with Triton X-100,
0.1% v/v, for 5 minutes, followed by PBS-T washes. A blocking solution of 10% goat
serum in PBS-T was used for 1hr at room temperature (250ul) per well. Cells were then
washed with PBS-T and primary antibody solutions were added, at a dilution of
1ug:250ul of antibody diluent volume. Antibody diluent was 10% goat serum in PBS-T.
Primary antibody solutions were incubated overnight at 4°C. AlexaFluor secondary
antibodies (rabbit and mouse, 1:500 v/v) were added after washing off primary antibody
solutions and incubated them 40 min at room temperature. Wells were then washed 3x
with PBS-T and stained with Vecta Shield DAPI and cover with a glass coverslide
followed by confocal microscopy analysis.

2.10 Chemotaxis and invasion assays

Transwell inserts are used by first adding 0.5 mL of medium and chemoattractant to the
bottom of the wells, then cells were suspended in media and added to the top insert
making sure no cells went to the bottom. Conditions were done in duplicates. Cells were
incubated 24hrs in CO2. And after 24 hrs cells where first checked to see if the cells were
on the membrane and on the bottom of the well with a microscope. Then cells were
analyzed with MTT (5mg/mL in PBS) assay. 8um pore transwells were used in 24-well plates. For invasion assays, the same protocol was followed, except adding a thin gel layer of basement membrane Matrigel to the top insert and allowing it to solidify before beginning the plating procedure. For invasion assays, 12um pore transwells were used in 24-well plates and analyzed in the same way as chemotaxis assays.

2.11 Tissue specimens
Resected human breast tissues were obtained with patient’s informed consent and were fixed in formalin, paraffin embedded and sectioned under an approved Institutional Review Board protocol at the University of Miami, FL. All specimens used in this study were blinded to protect patient identities as per HIPPA and IRB regulations. Xylene was used to remove all the paraffin from the tissues followed by washes with ethanol, and then a rehydration process of the tissues using graded washes of ethanol in water, ending in a final rinse in only water. As formaldehyde fixing forms methylene bridges between proteins and this is known to hinder epitope recognition by the primary antibodies, we used the heat-induced epitope retrieval method suing the Dako Target Retrieval Solution (10x) to unmask epitopes due to methylene crossbridges before blocking and applying antibodies of IHC or the PLA.

2.12 Proximity ligation assay: Proximity Ligation Assay (PLA), also called in-cell co-IP assay, by Olink Bioscience (Sweden), was used to identify specific colocalization events of CXCR7 and EGFR in fixed cells or tissue [102-104]. Prior to the PLA, cells were fixed in 4% paraformaldehyde at 4°C, 15 minutes, and human breast tissues were de-waxed. Cells and tissue were incubated with anti-CXCR7 rabbit IgG (1:500, GeneTex) and anti-EGFR mouse monoclonal antibody (1:500, Sigma) or normal rabbit IgG. Following incubation with primary antibody, cells were incubated with corresponding
secondary antibodies that were conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS). Then, ligation solution was added, consisting of ligase and two oligonucleotides that hybridize to the two PLA probes and form a closed circle if the PLA probes are in close enough proximity. Strand extension by rolling circle amplification (RCA) by T4-ligase and PCR amplification of double hybridized DNA was performed as described by the supplier [105]. Fluorescently labeled oligonucleotides were used for detection of the RCA product. The resulting signal (a red fluorescent signal of Texas-Red fluorescent tagged amplified DNA) occurs wherever the two molecules are colocalized. Quantification of the confocal images was performed using the Duolink Image Tool software.

2.13 Image acquisition and analyses

Cells and tissues were viewed under a Zeiss LSM 700 laser scanning confocal microscope. Images were acquired at 400x to 630x magnification using 40X or 63X oil-immersion objectives. Live cell imaging and time-lapse studies were performed using a Zeiss LSM 700 laser scanning confocal microscope. The procedure followed was described by Jala and Haribabu in a heated chamber for 30 minutes – 4 hr using an inverted fluorescence microscope [106]. The image acquisition was set every 10 seconds and kept monitoring for 30 minutes – 4 hr. Hardware control and acquisition of images was performed using the ZEN software.

2.14 Oncomine box plots

CXCR7 expression analysis was performed using Oncomine, a cancer microarray database and web-based data-mining database platform. We used subsequent databases based on mRNA expression collected via Affymetrix X3P Array. Filters were applied for
differential analysis comparing specific classifications of breast cancer to normal in breast tissues. Box plots represent CXCR7 expression changes in the Ma Breast 4 database as measured by Affymetrix Human X3P Array in Ma Breast 4 database.

2.15 Statistical analysis

All quantitative data shown, except the western blot quantification, were from three separate experiments each data point representing a mean of triplicate determination. Western blots have been repeated twice. Significance of data was analyzed with the Prism graph pad software. Immunohistochemistry data were independently evaluated by two investigators.
Chapter 3: CXCR7 EXPRESSION IN BREAST CANCER CELLS AND TISSUES

3.1 CXCR7 expression across breast cancer cell lines

We performed q-PCR to evaluate the expression level of CXCR7 in BrCa cell lines. Table 3.1 shows CXCR7 mRNA expression relative to the housekeeping gene PPIA. We also included CXCR4 mRNA expression for comparison of levels of CXCR4 vs CXCR7. The cell lines are categorized as previously described by Neve et al. [1]. The cell lines shown on Table 3.1, represent the heterogeneity of established cell lines and reflect the heterogeneity in breast cancers. The cell lines also exhibit different complexity of tumor formation and metastasis. The human immortalized non-tumorigenic MCF10A breast epithelial cell line will be the “normal” control line. The rationale is to find a phenotypic association between CXCR7 and BrCa lines.

CXCR7 is considered over-expressed (>2 fold difference) across established BrCa cell lines when compared to normal epithelial breast cell line MCF12A or MCF10A. CXCR7 transcript was highest in the ER+ luminal cells. CXCR7 was expressed by HER2 over-expressing BT474 cells, however, not expressed in HER2 over-expressing SkBr3 cells. Moreover, CXCR7 was expressed at a low level in the aggressive post-EMT cell line MDA-MB231. CXCR7 transcript was observed in both estrogen receptor positive (ER+) (MCF7, T47D, BT474 and HCC 202) and estrogen receptor negative (ER-) (HCC1954, HCC1569) cells (Figure 3.1.1 and Table 3.1). These findings are consistent with prostate cancer cell line CXCR7 expression, where both androgen receptor positive and negative cells express CXCR7 [2]. However, in BrCa as observed so far, only estrogen receptor positive (ER+) cells were shown to express CXCR7. This is explained by the findings of Boudot et al. that the CXCR7 axis is under the control of estrogen [3].
Western blot analysis was performed to validate expression of CXCR7 in breast cancer cells at the protein level in the most common cell lines (Figure 3.1.2). The breast cancer cell lines MCF7 and BT474 showed the highest CXCR7 expression at both mRNA and protein level compared to the normal epithelial cell line MCF12A. No CXCR7 protein expression was observed in T47D and SKBR3 cells. EGFR protein expression was also assessed to compare to CXCR7 protein expression. No EGFR protein was detected in T47D and SKBR3 cells and CXCR4 was expressed across all BrCa lines tested. Confocal microscopy was also used to verify protein expression of the most commonly used BrCa cells (Figure 3.1.3).

Figure 3.1.1 CXCR7, CXCR4, and EGFR relative mRNA transcript across breast cancer cell lines.
### Table 3.1. CXCR7 and CXCR4 relative to PPIA (FD values)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>Tumor type</th>
<th>Classification</th>
<th>CXCR7</th>
<th>CXCR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10A*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>F</td>
<td>Basal B</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>MCF12A*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>F</td>
<td>Basal B</td>
<td>0.01</td>
<td>0.47</td>
</tr>
<tr>
<td>MCF7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IDC</td>
<td>Luminal</td>
<td>0.53</td>
<td>0.03</td>
</tr>
<tr>
<td>T47D</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IDC</td>
<td>Luminal</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>BT474</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>IDC</td>
<td>Luminal</td>
<td>0.94</td>
<td>0.20</td>
</tr>
<tr>
<td>SKBR3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>AC</td>
<td>Luminal</td>
<td>0.00</td>
<td>0.19</td>
</tr>
<tr>
<td>MDAMB231</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>AC</td>
<td>Basal B</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>HCC202</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Duc Ca</td>
<td>Luminal</td>
<td>0.15</td>
<td>0.07</td>
</tr>
<tr>
<td>HCC 1569</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>MC</td>
<td>Basal A</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>HCC 1954</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Duc Ca</td>
<td>Basal A</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>DT 13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Primary</td>
<td></td>
<td>0.08</td>
<td>0.11</td>
</tr>
<tr>
<td>DT 28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Primary</td>
<td></td>
<td>0.04</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* immortalized normal epithelial cells

F = Fibrocystic disease
IDC = invasive ductal carcinoma
AC = adenocarcinoma
MC = metastatic carcinoma
Duc Ca = Ductal carcinoma

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**Figure 3.1.2** CXCR7 protein expression evaluated using Western blotting analysis. Panel A shows CXCR7, CXCR4, and EGFR expression of the most commonly used breast cancer cell lines. Panel B shows CXCR7 and EGFR expression for less commonly used breast cancer cell lines and includes an osteosarcoma line, HT1080, a primary cell line from an ER- breast cancer patient, DT13, and an MCF7 over-expressing EGFR, MCE.

CXCR7 is expressed across breast cancer cell lines. We observed that cell lines with higher expression of CXCR7 are both estrogen receptor positive (ER+) and of luminal cell origin. We also observed that CXCR7 mRNA expression was higher than CXCR4 mRNA expression. However, as protein expression does not always correlate with
mRNA level, we cannot assume that the protein level of CXCR7 is also higher than that of CXCR4 in tissues. Boudot et al. showed that the CXCR7 axis is under the control of the estrogen receptor and that CXCR7 expression is decreased upon addition of estrogen to MCF7 cells [3]. Surprisingly, we found CXCR7 expression also in ER- cells. The combination of these findings provides strong implications for the role of CXCR7 in metastasis. It is relevant to consider that many of the breast tumors that metastasize to bone are ER+ [4-6], thus, exploring CXCR7’s role (independently from CXCR4) in the context of metastasis will be highly relevant.

Moreover, EGFR expression correlated with CXCR7 expression across breast cancer cell lines and no EGFR expression was observed in the cell lines that lack CXCR7 protein expression. This may support the role of the heterotypic and unique CXCR7-EGFR interaction in breast cancer proliferation.

3.2 CXCR7 localization within cells

CXCR7 has been reported to be expressed intracellularly in T-cells at higher levels than in the cell membrane [7]. Using confocal microscopy we observed expression of CXCR7 at the cell surface, but also what appeared to be intracellularly, not localized to the cell membrane, although the cells were not initially permeabilized (Figure 3.2.1 A1-D-1). Therefore, to confirm whether the cells expressed CXCR7 intracellularly, we permeabilized BrCa cells as described in the methods. Our observations were consistent with previous findings identified using flow cytometry and confocal microscopy that CXCR7 is expressed intracellularly (Figure 3.2.1 A-2-D-2), however, here we show CXCR7 expression exclusively in cancer cell lines [8, 9]. We also used the prostate cancer cell line LNCaP, which is known to express a high level of CXCR7 protein, to see if the intracellular pattern of CXCR7 was also evident. We found that CXCR7 was also
expressed intracellularly in LNCaP as observed with the BrCa cells, however, it also appeared to show CXCR7 colocalizing with the nuclear DAPI stain (Figure 3.2.2). The same procedure was applied to MB231 cells, which express a low level of CXCR7 before permeabilization. When the cells were permeabilized, higher level of CXCR7 expression was observed (Figure 3.2.1 and enlarged Figure 3.3.2). Interestingly, only the LNCaP cells, while none of the BrCa cells we analyzed by confocal microscopy (Figure 3.1.3) showed a nuclear staining pattern. We did a Z-stack linescan analysis of images, which demonstrated that CXCR7 and DAPI colocalization at the nuclear level only applied to LNCaP cells, while not for MDA-MB-231 (Figure 3.3.1).

Figure 3.2.1 CXCR7 expression using confocal microscopy on BrCa cells. Top panels A-D1 indicate CXCR7 membrane expression on non-permeabilized BrCa cells. Middle panels A-D2 indicate cells were permeabilized using Triton X-100 before fixing them with PFA. Bottoms panels A-D3 indicate DAPI staining only showing general cell distribution.
Figure 3.2.2 LNCaP cells stained with α-CXCR7 antibody and labeled with anti-Rb-Alexa-fluor 488, green, and nuclei are labeled with DAPI, blue. A. non-permeabilized cells with RbIgG control primary antibody. B. shows surface expression of CXCR7 on fixed non-permeabilized LNCaP cells stained with α-CXCR7 antibody C. Permeabilized cells with RbIgG control primary antibody D. Intracellular and nuclear staining of CXCR7 on LNCaP cells permeabilized with Triton-X100 and then stained with α-CXCR7 antibody.
Figure 3.2.3 MB-231 cells stained with α-CXCR7 antibody and labeled with anti-Rb-Alexa-fluor 488, green, right side, and RbIgG control primary left side. Top panels show surface expression of CXCR7 on fixed MDA-MB231 cells stained with α-CXCR7 antibody and the bottom panels show surface and intracellular staining of CXCR7 on MDA-MB231 cells permeabilized with Triton-X100 and then stained with α-CXCR7 antibody. CXCR7 is labeled with Alexa-fluor 488, green and nuclei are labeled with DAPI, blue.
3.3 CXCR7 membrane localization

The chemokine receptor CXCR4 has been demonstrated to mediate its growth effects via lipid raft interaction with HER2 [10]. Lipid rafts are known to mediate important intermolecular encounters that lead to receptor cross-talk [11]. Therefore we asked whether CXCR7 localized to the lipid raft fraction of the cell membrane. Using lysates
treated for lipid raft fractionation (Dr. Renaud Sicard, U Miami), CXCR7 analysis was analyzed by Western blotting. Lipid raft fractionation of MCF7 cells, the parent line or (P group) and MCF cells with HER2 overexpression, called MCH2 cells, (H group) were done. MCH2 express higher levels of CXCR7 and also HER2, which served as a positive control for lipid raft presence in this experiment. Caveolin expression is considered positive in rafts found in lanes 2-4 normally, and these were used as the indicator of lipid raft positivity. CXCR7 antibody signal was not positive for the same fractions as caveolin, our indicator of lipid raft positivity (Figure 3.3). As CXCR7 expression did not co-localize with lipid rafts expression, this suggests that CXCR7 is in a functionally distinct area of the membrane not associated with lipid rafts.

**Figure 3.3** Lipid raft fractionation of MCF7 cells, P group and MCF cells with HER2 overexpression, MCH2 cells, H group. MCH2 express high levels of CXCR7 and also HER2, which served as a positive control for lipid raft presence in this experiment.

### 3.4 CXCR7 expression affects proliferation of breast cancer cells

Since CXCR7 was abundantly expressed in several ER+ BrCa cells, we investigated the role of CXCR7 in BrCa cell proliferation. We down regulated CXCR7 expression
using RNAi. Transient transfection of CXCR7 siRNA resulted in an 80 ± 5% decrease of mRNA levels as quantified by q-PCR, i.e. the Ct difference averaged 2.18 for MCF7 siRNA control (Ct 19.95) vs MCF7 siCXCR7 (Ct 22.13) in MCF7 cells. Protein expression was quantified using densitometry software. The stable shRNA CXCR7 levels averaged approximately 50% less CXCR7 protein expression than the shRNA vector control. CXCR7 down regulation resulted in a 67% decrease of clonogenic growth and decreased cell proliferation both in transient and stably transfected cell lines (Figure 3.4.1).

**Figure 3.4.1 CXCR7 affects MCF7 growth.** A. The clonogenic potential of CXCR7 depleted MCF7 cells was analyzed in colony forming assays with CXCR7 depleted stably shCXCR7 transfected cells (right well) compared to vector control transfected cells, control (left well). B. Quantification of the colonies. C. Effect of transient depletion of CXCR7 (72hrs). D. Cell proliferation of MCF7 cells with stable CXCR7 depletion (shCXCR7 polyclonal selection), compared to control cells (vector control).
Since MCF7 cells endogenously express high levels of CXCR7 we used them for down regulation experiments to identify exclusive and cooperative roles of CXCR7. The exclusive role of CXCR7 was identified by depletion of only CXCR7 whereas the cooperative role of CXCR7 with CXCR4 was determined by identifying phenotypic changes following down regulation of CXCR7, and inhibiting CXCR4 with siRNA. The MDA-MB-231 (MB231) BrCa cell line is a model for triple-negative, metastatic BrCa, and these cells have been used as a model of low CXCR7 receptor expression with high metastatic potential [12, 13]. Whether over-expressing CXCR7 in MB231 cells would augment or suppress the growth and metastatic potential of MB231 was observed. Following either transient or stable transfection of CXCR7 cDNA in MB231 cells, proliferation, survival, motility and invasive potential of transfected cells in vitro were assessed. MCF12A served as the normal negative control. It is important to consider that these phenotypic effects will be due to CXCR7 expression without stimulation of its ligands. That is presented later in the chapter.

In the immortalized epithelial cell line, MCF12A, which is considered a normal control, increased CXCR7 had a modest effect on cell proliferation however, the increase was not statistically significant, Figure 3.4.2 A. Based on literature review we expected that CXCR7 over expression in these normal cells would increase proliferation and behavior to become similar to cancer cells. However, because these cells are considered normal epithelium it is possible that they have mechanisms of control that prevent CXCR7 overexpression from causing increased proliferation and CXCR7 is not strong enough to cause oncogenic changes. In MDA-MB231 cells, overexpression of CXCR7
did increase the proliferation of the cells even when using different forms of the CXCR7 plasmid, as described in the methods section. For MDA-MB-231, already an aggressive and rapidly proliferating line, we did not expect increased proliferation necessarily, however, CXCR7 overexpression was able to induce statistically significant increases in cell proliferation, Figure 3.4.2 B.

We also used the cell line SKBr3 which is the only BrCa line we found has no endogenous CXCR7 transcript and found that in this cell line, overexpression of CXCR7 did not cause an increase in cell proliferation, Figure 3.4.2 C. As shown in the Western blots probing for CXCR7 and EGFR, we observed that only SKBr3 and T47D cells did not show neither CXCR7 nor EGFR protein expression (T47D does have CXCR7 transcript however). To see whether EGFR alone induced a proliferative effect, we overexpressed it in SKBr3 and MCF7 cells and found that when we overexpress EGFR in SKBr3, the proliferation of the cells was increased to statistically significant values. However, the same was not true for MCF7 cells in which overexpression of EGFR alone is not capable of inducing statistically significant increases in proliferation Figure 3.4.2 D. This suggest that the proliferation effects of CXCR7 are cell line dependent and may depend on endogenous levels of other receptors or factors. This mechanism of action will be elucidated in Chapter 4.

Previous studies have shown that elevated expression and function of the chemokine receptor CXCR4 has significant effects on survival, proliferation and metastasis of BrCa [14, 15]. Most of this was before identification of CXCR7 as a co-receptor for SDF-1α [16, 17]. CXCR7 heterodimerizes with CXCR4 [18]. CXCR4 depletion may result in decreased proliferative activity in MB231 cells; whether that is
compensated by overexpressing CXCR7 was examined. Previous and recent publications
suggest expression of CXCR7 and the release of its ligands by lymphocytes might
actively increase the endothelial migration, vasculogenesis etc., in early stages of cancer
and CXCR7 has also been directly linked to metastasis through its interaction with
CXCR4 [16, 19-22]. However, whether CXCR7 uniquely plays a solo role in the survival
and proliferation of these cells at the metastatic sites was not clear. These properties were
examined. To assess whether CXCR7 cooperates with its known co-receptor CXCR4 to
mediate proliferation we used siRNA to knockdown CXCR4 expression. We observed
significant decreased proliferation only after CXCR7 knockdown and increased
proliferation with CXCR7 over expression (Figure 3.4.3 A). Down regulation of CXCR4
had no significant decrease on proliferation compared to control. Down regulation of
CXCR7 in BT474 cells also resulted in decreased cell proliferation Figure 3.4.3 B. We
also over expressed CXCR7 in a cell line with low endogenous CXCR7, MDA-MB231,
where over expression of CXCR7 resulted in increased cell proliferation (Figure 3.4.3 C).

3.5 CXCR7 regulates the cell cycle machinery

To elucidate the inhibition of proliferation following CXCR7 down-regulation, we
performed cell cycle phase fractionation analysis of propidium iodide labeled cells using
flow cytometry. The results showed significant increase in the G0/G1 fraction (33%) and
a significant decrease in the S-phase fraction in siCXCR7 MCF7 cells compared to
control siRNA-transfected cells (Figure 3.5). Importantly, CXCR7 down regulation led to
marked changes in key proteins involved in regulating the cell cycle. CXCR7 depletion in
MCF7 cells affected the levels of proteins regulating the S-phase transition and
proliferation. The proliferative markers Cyclin B1 and Cdk4 decreased, while S-phase
inhibitory protein, p21 was dramatically increased in CXCR7 depleted cells (Figure 3.5 B). To test whether CXCR7 depletion has an effect on apoptosis, we used cell surface annexin-V labeling. Results of annexin-V binding assays on CXCR7 depleted cultures at 48 h and 72 h following siRNA transfection showed that down regulation of CXCR7 (CXCR7siRNA) did not induce apoptosis compared to cells transfected with control siRNA (Figure 3.5 C).

**Figure 3.4.2 CXCR7 growth of distinct BrCa cancer cells.** A. Proliferation effect of CXCR7 on different breast cancer cell lines assessed with MTT assay. Transient effect 72 hrs after transfection. A. Effect of CXCR7 overexpression in A. MCF12A cells. B. MDA-MB231 cells C. SKBr3 cells. In C. Different EGFR plasmids are also used to see proliferation effect in SKBr3 cells. D Proliferation of MCF7 cells with stable EGFR overexpression, compared to control MCF7 plasmid control. *p < 0.05.
Figure 3.4.3 CXCR7 affects growth independently from CXCR4. A. Effect of CXCR7- (siR7) and CXCR4- (siR4) down regulation and CXCR7 (R7) over expression on proliferation of MCF7 cells. B. Proliferation of CXCR7 depleted (siR7) BT474 cells and C. CXCR7 over expressing (R7) MB231 cells compared to corresponding controls (siC = control siRNA, C = empty plasmid control). *p < 0.05. ** p < 0.01.
Figure 3.5 CXCR7 affects cell cycle machinery. A. Cell cycle arrest of cells with down regulated CXCR7. B. Analysis of relevant cell cycle markers following siRNA mediated down regulation of CXCR7. C. CXCR7 depletion by siCXCR7 did not induce apoptosis as seen by negative Annexin-V staining.

3.6 CXCR7 effect on chemotaxis and invasion of BrCa cells

To identify how CXCR7 could be regulated to lead to the metastatic process, the effect of CXCR7 on BrCa cell motility and invasion was explored. IL-8 induces CXCR7 expression in prostate cancer [2]. Therefore, we wanted to know if this inflammatory
chemokine IL-8, could induce CXCR7 in BrCa cells as well. It has been demonstrated that IL-8 simulation of prostate cancer cells enhances NF-kB activity. CXCR7 has six NF-kB response elements in its promoter region and these are bound by activated NF-kB upon IL-8 stimulation, inducing CXCR7 transcription and expression [23]. IL-8 is also known to induce VEGF expression and together, IL-8 and VEGFR are important players in the angiogenic process. We used ELISA assays to determine if changes in VEGF secretion are affected by CXCR7 expression. We found that addition of IL-8 induced CXCR7 transcript levels similar to that observed by LNCaP (used as positive control (Figure 3.6.1). IL-8 treatment induced CXCR7 and CXCR4 transcript as early as 15 minutes and this CXCR7 upregulation was maintained throughout 60 minutes. The effect of TNF-α was similar to that observed by IL-8. IL-8 induction of CXCR7 was stronger in the prostate cancer cell line LNCaP, however, in this cell line the receptor transcript levels leveled off by the 60 minute time point, unlike in MCF7 cells. Furthermore, TNF-α had a similar effect in MCF7 cells as IL-8 on CXCR7 induction. Neither chemokine IL-8 nor TNF-α had an effect on MB231 cell expression of CXCR7 or CXCR4. Down regulation of CXCR7 using siRNA did not show a decrease in VEGF secretion levels. These data support the conclusion that IL-8 is capable of inducing CXCR7 up regulation in MCF7 cells in a similar manner as observed in LNCaP, via NF-kB.
Figure 3.6.1 Induction of CXCR7 in BrCa

A. qPCR relative FD values of cells treated with IL-8, or TNF-α at a concentration of 20ng/mL for the times specified in the table (three experiments done in duplicates). B. ELISA of VEGF expression after CXCR7 siRNA treatment of MCF7 cells (two experiments done in triplicate). VEGF ELISA not statistically significant values, p > 0.05.

To address if CXCR7 expression has an effect on invasive potential, we used the highly invasive MDA-MB-231 (MB231) (Figure 3.6.2). As mentioned above, for MDA-MB-231, I did not expect necessarily increased motility or invasiveness because these cells are already more aggressive than MCF7 cells, but rather we hypothesized that CXCR7 expression, if relevant in the metastatic process, should mediate changes in motility, invasive, and metastatic potential of the cells. Also, CXCR7 is a scavenger of SDF-1α, and thus creates a gradient of SDF-1α in tumor metastasis or in transwell migration. If CXCR7 levels are altered, this may also alter CXCR4 activity in the MB231 cells, so that we could expect motility and Matrigel invasion properties of the MB231 cells would change [21].

We observed that increased expression of CXCR7 in MB231 cells did not significantly alter their invasive potential, however, there was a modest increase in
invasion properties in our CXCR7 over expressing MB231 cells (Figure 3.6.2 B, D).

As CXCR7 is known to affect the invasive potential of CXCR4 expressing cells, we also used AMD3100, a specific inhibitor of CXCR4 to pre-treat the CXCR7 over expressing MB231 cells, which express CXCR4 endogenously and evaluated the effect of blocking CXCR4 on the invasiveness of the cells. The cells were treated to assess whether an effect could be seen due to CXCR7 exclusively and siCXCR4 was also used as an internal control (Figure 3.6.2C). There were no significant changes observed between the conditions. Would healing assays to determine cell migration were also done comparing MB231 cells to MB231-CXCR7 cells, scratched and grown in complete 10%
FBS media and stained for analysis 24 hours after scratch (Figure 3.6.2 D). There were no effects of increased migration in CXCR7 over-expressing cells. These observations are consistent with previous findings that suggest the role of CXCR7 is restricted to growth and enhanced invasion of CXCR4 expressing cells and does not modify chemotaxis of the cells [21, 24].

A noteworthy attribute of MCF-7 cells with stable depletion of CXCR7 was their resistance to detach from plastic following trypsinization. The same phenotype was observed with different shRNA clones (either T74 or T76 clones) of MCF-7 shCXCR7 cells. CXCR7 downregulation caused the MCF-7 cells to attach more strongly, suggesting CXCR7 may affect expression of adhesion molecules that regulate attachment (Figure 3.6.3). Another cell type that takes a long time to detach is MCF10A, a “normal” immortalized epithelial cell line. The phenotype of slowed growth and increased attachment of the cells with CXCR7 down regulation suggests that CXCR7 expression may affect the transition from normal to tumorigenic breast cancer cells.

Figure 3.6.3 CXCR7 expression affects MCF7 adhesion. MCF7 cells with decreased CXCR7 expression adhere more strongly to the plastic plate surface. A. An MCF7 cell line with stable depletion of CXCR7 (T76) detaches less from the plastic flask after a regular trypsinization procedure (3 minutes). B. MCF7 cells with stable depletion of CXCR7 (T74) compared to shRNA vector control, T303, treated with different conditioning medias and left in culture for 7 days. T74 cells attach longer to the plastic plate.
surface after treatment and cells appear much healthier than counterpart controls. The scratch (middle of wells) is therefore not necessarily attributed to migration, but rather the difference in growth of each cell kind.

3.7 Ligand independent effect of CXCR7

We hypothesized that CXCR7 induces proliferation in CXCR7 expressing cells due to ligand-mediated activation of the mitogenic pathway. To test this hypothesis, MCF7 cell cultures were starved of growth factors for 24 h and then stimulated with ITAC, SDF-1α, or EGF. Proliferation was evaluated 48 h later. As shown in Figure 3.7, no significant increase in cell numbers were observed between untreated and ITAC or SDF-1α treated cultures. ITAC stimulation only marginally decreased proliferation. However, a significant increase in growth was observed in cultures treated with positive controls, estrogen/estradiol (E2) or combination of estradiol with epidermal growth factor (E2 + EGF). MCF7 cells produce the CXCR7 ligand SDF-1α in an estrogen-dependent manner [3]. To verify whether endogenous SDF-1α induces CXCR7 mediated proliferation, we transfected the cells with siRNA against SDF-1α, incubated them in presence or absence of SDF-1α and then assessed proliferation. Down regulation of SDF-1α caused no significant changes in cell proliferation (Figure 3.7 B), neither did supplementation with SDF-1α cause increased growth. To determine if the non-canonical ligand MIF had a proliferation effect mediated through CXCR7, we added a specific inhibitor of MIF, 4-IPP, to BrCa cells with distinct CXCR7 levels [25, 26]. We found that use of 4-IPP did not have a specific proliferation effect on MCF7, or MCF7 shRNA vector control (shC) transfectant, compared to MCF7 shCXCR7 (shR7) transfectant or MB231 which are CXCR7 low (Figure 3.7 C). We performed proliferation experiments comparing the stable line MCF7 cells with vector control shRNA (shC) and shCXCR7
(shR7) and added EGF at the normal stimulation concentration (10ng/mL) for 72 hrs, then counted the cells. In the control cells there was a marginal increase in proliferation in EGF stimulated cells vs non stimulated cells, as seen in Figure 3.7 D. In cells with CXCR7 depletion, with CXCR7 shCXCR7 (shR7), there was no apparent or significant increase in EGF stimulated growth as compared to non stimulated shCXCR7 cells.

**Figure 3.7 CXCR7 ligand independent effect.** A. MCF7 cells were stimulated with CXCR7 ligands CXCL11 (ITAC), CXCL12 (SDF-1a) and proliferation was evaluated. A significant increase in growth was observed in cultures treated with positive controls, estrogen (E2) or combination of estrogen with epidermal growth factor (E2 + EGF). B. Effect of endogenous and external added SDF-1a on MCF7 cell proliferation. Rescue of potential SDF-1a mediated autocrine activation of CXCR7 using siRNA to down regulate endogenous SDF1a (siSDF-1) compared to control siRNA (siC) and addition of external SDF-1a to media (+SDF-1); ns: no statistical difference. C. Percent survival of cells based on quantification of optical density measurements of cells treated 4-IPP, a specific MIF inhibitor for 48 hrs. MTT assay was used for viability estimation. D. Proliferation experiments comparing the stable line MCF7 vector shRNA control (shC) and shCXCR7 (shR7) treated with EGF (10 ng/mL) for 72 hrs. Data presented from one representative experiment with triplicate determination of cell numbers.
3.8 CXCR7 is expressed in BrCa tissues and co-localizes with EGFR and CD-31

Since CXCR7 affects proliferation in BrCa cells independently of its ligands and co-receptor CXCR4, we wanted to validate the expression of CXCR7 in human breast tissues. We first evaluated expression of CXCR7 in normal vs. cancer breast tissue and found that CXCR7 showed a strong signal in cancer vs. normal tissues however, it was evident that CXCR7 expression was also present in the normal human tissues (Figure 3.8.1).

![CXCR7 expression on human breast tissues](image)

**Figure 3.8.1 CXCR7 expression on human breast tissues.** A-D CXCR7 expression (green) in representative normal human breast tissue. E-H, CXCR7 expression (green) in representative human ER+ breast cancer tissue. CD31 expression (red).

Next, we wanted to confirm that CXCR7 colocalized with the endothelium as reported in the literature for mouse tissues, and we found that CXCR7 also colocalized with CD-31, an endothelial cell marker, in BrCa tissues (Figure 3.8.1 G-H and 3.8.2).
Figure 3.8.2 CXCR7 co-localizes with CD-31 in breast tumor tissue. Expression of CXCR7 and endothelial cell marker CD31 in human breast tissue was analyzed by immunofluorescence. A. Nuclei of human breast tissue specimen counter stained with DAPI B. CXCR7 (green fluorescence) was mostly restricted to the luminal layer of breast ducts. C. CD31 (red fluorescence) marks endothelium tissue D. Overlay of B and C: CXCR7 (green fluorescence) colocalized with endothelial cell marker CD31 (red fluorescence) within the ER+ breast cancer tissue.

As CXCR7 is known to be expressed in endothelial cells and tumor associated vasculature of bladder and prostate cancers, we verified the expression of CXCR7 in the context of the endothelial cell marker CD31 in BrCa tissues. We used IF to evaluate expression and co-localization of endothelial cell marker CD31 with CXCR7 in human
breast tissue. Figure 3.8.2 shows that CXCR7 co-localizes with CD31, which is consistent with its reported expression in endothelium. Moreover, in order to assess whether CXCR7 expression is aberrant in the breast tumor microenvironment vs. normal tissue, we used the Oncomine data base to query. Our analysis using the Ma Breast 4 database [27] indicates that CXCR7 expression is higher in breast cancer tissue stroma compared to normal breast tissue stroma (Figure 3.8.3).

![Box plots of the Ma Breast 4 Study showing CXCR7 relative mRNA expression as measured by Affymetrix Human X3P Array in Ma Breast 4 database comparing CXCR7 A. In ductal breast carcinoma in situ stroma vs. normal tissue CXCR7 has an Over-expression Gene Rank: 59 (in top 1%), P-value: 1.10E-6, t-Test: 6.304 and Fold Change: 4.431 B. For invasive ductal breast carcinoma stroma vs. normal tissue CXCR7 has an Over-expression Gene Rank of 125 (in top 1%), P-value: 6.08E-5, t-Test: 5.148 and Fold Change: 4.610.]

Figure 3.8.3 CXCR7 in human breast tissue stroma. Box plots of the Ma Breast 4 Study showing CXCR7 relative mRNA expression as measured by Affymetrix Human X3P Array in Ma Breast 4 database comparing CXCR7 A. In ductal breast carcinoma in situ stroma vs. normal tissue CXCR7 has an Over-expression Gene Rank: 59 (in top 1%), P-value: 1.10E-6, t-Test: 6.304 and Fold Change: 4.431 B. For invasive ductal breast carcinoma stroma vs. normal tissue CXCR7 has an Over-expression Gene Rank of 125 (in top 1%), P-value: 6.08E-5, t-Test: 5.148 and Fold Change: 4.610.

Using immunofluorescence (IF), we also found that CXCR7 and EGFR co-localized strongly in BrCa tissue compared to normal breast tissue (Figure 3.8.4). We confirmed an separate expression in normal tissues and increased CXCR7-EGFR IHC co-localization in human ER+ breast cancer tissues compared to the normal breast tissues.
Figure 3.8.4 **CXCR7 and EGFR expression in human breast tissues.** CXCR7 unfortunately, is also expressed in normal breast tissues, as demonstrated when using our antibodies. In normal breast tissue, (left side) CXCR7 and EGFR are not usually seen in the same areas. In the breast cancer tissue however (right side), EGFR expression appears spread wildly and is more frequently observed near CXCR7 positive areas. Pictures are representative from triplicates of five normal breast tissues and five ER+ breast tumor tissues. CXCR7 is illustrated in green (Alexa fluor 488), EGFR is illustrated in red (Alexa fluor 555) and nuclei in blue (DAPI).

### 3.9 Discussion

Several reports have linked CXCR7 with other marker proteins including CD31 and EGFR [2, 22, 24]. Our results demonstrate for the first time that CXCR7 interacts with EGFR in human breast cancer. CXCR7 interacts with CD31 in human breast cancer tissues CXCR7 is known to be expressed in endothelial cells and tumor associated vasculature [19, 28]. Consistently, we show that CXCR7 co-localizes with the endothelial cell marker CD31 in breast tumor vessels. This is also in accordance with analysis of the Oncomine database which identifies CXCR7 as over expressed in breast tumor associated stroma when compared to normal tissue stroma (Figure 3.6.4). Expression of CXCR7 in the tumor associated vasculature and stroma suggests a role in endothelial cell proliferation and underlines the importance of CXCR7 for tumor growth and microenvironment.
Chapter 4: MECHANISM OF CXCR7 MEDIATED PROLIFERATION IN BREAST CANCER CELLS

4.1 Decreased CXCR7 lowers levels of p-ERK and p-EGFR in MCF7 cells

Data previously described in the literature and here in Chapter 3 demonstrates that expression of CXCR7 affects growth of BrCa cells. To assess whether down regulation of CXCR7 affected the mitogenic pathway, we assessed for changes in the MAPK/ERK kinase cascades. Since ERK1/2 is classically involved in cell proliferation and differentiation of many cell types, and is the last step in this cascade of events, we analyzed for its expression. The inhibition of CXCR7 by siRNA resulted in decreased ERK1/2 phosphorylation in EGF (10 ng/mL, 2 min) stimulated MCF7 cells (Figure 4.1), suggesting either this pathway is directly affected by CXCR7 or via its interaction with EGFR. In contrast to the existing paradigm that CXCR7 is a decoy receptor and its main activity is via CXCR4, these findings suggest that CXCR7 interacts through EGFR, independently of CXCR4 and CXCR7-EGFR coupling therefore plays a role in CXCR7 mediated cellular events. To understand the relationship between CXCR7 and EGFR, the role of CXCR7 in this signal transduction was determined. We also stimulated with SDF-1a, but were not able to observe ERK1/2 activation. Since EGFR is a known mitogenic driver of cell proliferation we next analyzed whether EGFR activation was also affected by CXCR7 depletion. Down regulation of CXCR7 decreased activation of EGFR at Tyrosine_1110 compared to control siRNA transfected cells (Figure 4.1).

EGFR is a protein-tyrosine kinase in which auto-phosphorylation of tyrosine residues results in downstream activation of proteins involved in cell proliferation (e.g. MAPKs) [1]. EGFR expression correlates with poor prognosis of breast cancer [2].
Chapter 3 data suggests a blockade in the G1/S interphase in CXCR7 depleted MCF7 cells, consequently, we reasoned this may be due to upstream inhibition of the mitogenic signaling cascade. CXCR7 depletion may lead to inhibition of EGF induced activation of EGFR that in turn blocks autophosphorylation of EGFR at various tyrosine residues, such as Tyrosine(Y)\textsubscript{1068}, Y\textsubscript{1110}, Y\textsubscript{1173}, etc. Furthermore, this inhibition may result in decreased MAP kinase activation, typically ERK1/2 phosphorylation. Figure 4.1 shows that depletion of CXCR7 in MCF7 cells leads to decreased pEGFR\textsubscript{Y1110}, and pERK1/2. The decrease was evident in MCF7 cultures stimulated with EGF (10 nM, 2 min).

Figure 4.1 Down regulation of CXCR7 affects EGFR phosphorylation. Levels of pY1110EGFR, total EGFR, pERK1/2 and total ERK in MCF7 cells transfected with siCXCR7 or control siRNA. The protein levels were determined by Western blotting of cell lysates prepared from serum-starved cells stimulated with EGF (10 ng/mL) for 0, 2, and 5 minutes.
4.2 β-arrestin2 enhances CXCR7 mediated phosphorylation of EGFR and ERK.

CXCR7 is known to signal through β-arrestin2 in a variety of cell types including rat vascular smooth muscle cells, mouse medial ganglionic eminence interneurons, HEK cells, and normal human epidermal melanocytes [3-6]. To evaluate the role of the CXCR7/β-arrestin2 axis in the EGFR mediated mitogenic pathway, we over expressed β-arrestin2 in MCF7 cells. We over expressed β-arrestin2 in the presence or absence of CXCR7 siRNA and analyzed phosphorylation levels of ERK and EGFR. Our studies show increased phosphorylation of ERK1/2 and EGFR at Y1110 in β-arrestin2 overexpressing cells. However, with CXCR7 down regulation, we observed a significant decrease in phosphorylation of both ERK1/2 and EGFRY1110 even after β-arrestin2 overexpression (Figure 4.2).
Figure 4.2 β-arrestin2 plays a role in CXCR7 mediated phosphorylation of EGFR and ERK. Cells were seeded in cell culture plates and 24 h later the cells transfected with the combinations annotated. 48 hrs later the cells were stimulated with EGF (10 ng/mL) and were then were lysed, proteins were collected and quantified, then ran for pY1110EGFR, total EGFR, pERK1/2, and total ERK1/2 expression was analyzed by Western blot analysis as described in Material and Methods.

Figure 4.2.1 Quantification of β-ARR2 phosphorylation effect. A. The ratio of p-ERK1/2 over total ERK1/2 in western blot was quantified from analysis of MCF7 cell lysates that were transfected with control plasmid (pcEGFP), β-arrestin2 or β-arrestin2 in combination with siCXR7 and stimulated with EGF. B. The ratio of p-EGFRY1110 over total EGFR was quantified in cells transfected with control plasmid (pcEGFP), β-arrestin2, or β-arrestin2 in combination with siCXR7 and stimulated with EGF. The western blot experiments were repeated twice and one representative experiment is shown.

4.3 EGF-mediated movement of CXCR7 and β-arrestin2

β-arrestin2 has been established as the preferential way in which CXCR7 induces signaling events after binding SDF-1α. Therefore it was necessary to determine whether CXCR7 affects the mitogenic pathway via EGFR directly or indirectly. β-arrestins can interact directly with component kinases of the extracellular signal regulated kinase ERK/MAPK cascades leading to metastable β-arrestin complexes with ERK1/2 [7, 8]. Therefore, we explored whether the phosphorylation effects we observed on EGFR and ERK1/2 after CXCR7 down regulation could be mediated through β-arrestin2. This
would clarify whether the CXCR7-EGFR interaction is direct or indirectly being mediated by this key third player. To test if β-arrestin2 mediates CXCR7-EGFR interaction or induced activation of downstream signaling pathways, we used GFP tagged β-arrestin2 [9, 10] with or without siCXCR7 to visualize this interaction using confocal microscopy and Western blotting. The phosphorylation studies show increased phosphorylation of ERK1/2 and EGFR at Y1110 after over expressing β-arrestin2. However, with CXCR7 down regulation, there was significant phosphorylation decreases at both ERK1/2 and EGFR Y1110 even after β-arrestin2-GFP over expression.

Confocal microscopy images comparing over expression of β-arrestin2-GFP with or without siCXCR7 showed distinct behaviors (of β-arrestin2 re-arrangement) for each condition. Time-lapse images demonstrate the distribution changes of β-arrestin2-GFP in CXCR7 depleted vs. CXCR7 wildtype cells. SDF-1α addition to MCF7 cells over expressing β-arrestin2-GFP showed minimal movement in the 45 min window (Figure 4.3). The experiment results were the same after a 4 h window. This lack of SDF-1α mediated activation (change in β-arrestin2 re-arrangement) was consistent with the lack of SDF-1α mediated proliferation or ERK1/2 activation in MCF7 cells noted earlier. EGF, on the other hand, induced a shift of β-arrestin2-GFP rearrangement throughout the cell, when compared to SDF-1α and PBS negative control stimulation (Figure 4.3). In the control cells (β-arrestin2-GFP + siCntrl) the β-arrestin2-GFP was re-distributed throughout the cell after EGF stimulation, with a tendency to distribute towards the cell center (nucleus). By comparison to the control (β-arrestin2-GFP + siCntrl), the test cells (β-arrestin2-siCXCR7) had barely detectable β-arrestin2 redistribution, and what does re-distribute, does so with a tendency to come outward (cytoplasm) and rearrange itself
outside the nucleus (Figure 4.3.1). Therefore, β-arrestin2 appears to have a distinct and relevant role in the CXCR7/EGFR relationship. We next questioned whether CXCR7 could be affecting nuclear distribution events taking place in this system.

**Figure 4.3 β-arrestin2 distribution after SDF1α of EGF stimulation.** A-D Left. Cells were seeded in 35mm glass bottom culture plates and 24 h later the cells transfected with the combinations indicated. 48 hrs later the cells were stimulated with SDF-1 (100 ng/mL) and were then were imaged by confocal microscopy at 200x magnification, on a 37°C stage as described in Material and Methods. A-D Right. Cells were seeded in 35mm glass bottom culture plates and 24 h later the cells transfected with the combinations indicated. 48 hrs later the cells were stimulated with EGF (10 ng/mL) and were then were imaged by confocal microscopy at 200x magnification, on a 37°C stage as described in Material and Methods.

**Figure 4.3.1 β-arrestin2 re-distribution after EGF stimulation.** Cells were seeded in 35mm glass bottom culture plates and 24 h later the cells transfected with the combinations indicated. 48 hrs later the cells were
stimulated with EGF (10 ng/mL) and were then were imaged by confocal microscopy at 630x magnification on a 37°C stage as described in Material and Methods.

β-arrestin2 nuclear translocation has been observed before in T-cells stimulated with SDF-1α, and β-arrestin1 nuclear translocation has been observed in spermatozoa [11]. To verify if β-arrestin2 was indeed moving or shuffling between the cytoplasm and the nucleus, we extracted the nuclear and cytoplasmic portions of cell lysates from cells with β-arrestin2 over expression and/or CXCR7 siRNA down regulation. The effect of CXCR7 expression on arrestin shuffling could then be assessed by Western blotting (Figure 4.3.2). We observed that after over expression of β-arrestin2, there was increased β-arrestin2 found in the nuclear fraction of lysates. We also found that cells with β-arrestin2 and siCXCR7 showed an enhanced level of β-arrestin2 expression in the cytoplasmic fraction compared to the β-arrestin2 only or pcDNA control lysates. These findings coupled with our confocal microscopy observations suggest that CXCR7 expression can affect the β-arrestin2 distribution throughout the cell, between nucleus and cytoplasm. Furthermore, the nuclear and cytoplasmic protein lysate Western blot quantification indicates that the effect of CXCR7 expression on β-arrestin2 does not depend on EGF addition. This is because addition EGF does not appear to enhance the localization changes seen in β-arrestin2, whereas CXCR7 depletion does. However, it is important to note that our protein lysate studies were limited to a 5 minute window, whereas the confocal experiments (presented here) where an effect of EGF addition is evident, stretch out to a 45 min window.
Figure 4.3.2 CXCR7 affects β-arrestin2 cytoplasmic distribution. Cells were seeded in cell culture plates and 24 h later the cells were transfected with the combinations annotated. 48 hrs later the cells were stimulated with EGF (10 ng/mL) and were then lysed, and the proteins were collected and fractionated, nuclear and cytoplasmic, then quantified, then ran for pY1110EGFR, total EGFR, pERK1/2, and total ERK1/2 expression was analyzed by Western blot analysis as described in Material and Methods.
4.4 CXCR7-EGFR colocalization in MCF7 cells and tissues

To corroborate our results and evaluate their clinical significance, we analyzed human breast samples for colocalization of CXCR7 and EGFR. We evaluated co-localization/interaction of CXCR7 and EGFR in the breast cancer cell line MCF7. We performed a specific immunofluorescence approach, an in-cell co-immunoprecipitation assay, also known as a proximity ligation assay (PLA). Figure 4.4 demonstrates co-localization of CXCR7 with EGFR in MCF7 cells. Figure 4.4B shows co-localization of CXCR7 and EGFR in the absence of 10 ng/ml EGF stimulation. The CXCR7-EGFR co-localization was increased upon stimulation with EGF for 2-5 minutes (Figure 4.4C). Furthermore, we used an EGFR plasmid to minimally over express EGFR as MCF7 cells express a relatively low level of EGFR and observed a further increase of CXCR7-EGFR co-localization compared to negative controls (Figure 4.4D). Using immunofluorescence (IF), we also found that CXCR7 and EGFR co-localized strongly in breast cancer tissue compared to normal breast tissue (Figure 3.8.4 and 4.4.1 A-B). We went further to analyze for CXCR7-EGFR co-localization events in human breast tissue using the PLA assay. We confirmed an increased CXCR7-EGFR interaction in human ER+ breast cancer tissues compared to normal breast tissues (Figure 4.4.1 C-D).
Figure 4.4 CXCR7 co-localizes with EGFR. A-D. After fixation of MCF7 cells, PLA (in situ co-immunoprecipitation) was performed with specific CXCR7 [GTX100027, (1:500), GeneTex, Irvine, CA] and EGFR antibodies [E2760, (1:500), Sigma Aldrich, St. Louis, MO] to visualize heterodimerization of CXCR7 with EGFR. Colocalization is shown as a red fluorescent PLA signal (CX7/EGFR), and nuclei were counterstained with DAPI (blue) (630x magnification). As the PLA technique requires two specific antibodies to give a red fluorescent signal we used a single primary antibody as a negative signal control. A. Negative controls experiment using EGFR antibody alone. B. Basal level of CXCR7-EGFR colocalization in non-stimulated MCF7 cells. C. CXCR7-EGFR colocalization in MCF7 cells stimulated for 2 min with EGF (10 ng/mL). D. CXCR7-EGFR colocalization in EGFR low over expression in MCF7 cells following 2 min EGF (10 ng/mL) stimulation.
Figure 4.4.1 CXCR7 co-localizes with EGFR in human breast tissue. A. IF of normal human breast tissue for detection of CXCR7 (green fluorescence) and EGFR (red fluorescence) (400× magnification). B. Immunofluorescence of ER+ breast cancer tissue stained as explained in B. C. PLA of normal human breast tissue using CXCR7 and EGFR antibodies to visualize colocalization of CXCR7 with EGFR (CX7/EGFR), nuclei were counterstained with DAPI; 400× magnification. D. PLA of ER+ human breast tumor tissue stained as explained in G.

Figure 4.4.2 Quantification of colocalization signals from Duolink assay of five ER+ breast tumor tissues and four normal breast cancer tissues.
4.5 Using MCE cells to explore CXCR7-EGFR interaction

It was important to use EGFR over expression as EGFR is generally expressed at low levels in MCF7 cells. It was also important to study the effect of CXCR7 in the context of EGFR over expression in order to determine the relationship between CXCR7 and EGFR. To avoid confusion, proper controls with unstimulated and starved cells were used. For this purpose we obtained a well-established MCF7 line with EGFR overexpression, from Dr. El-Ashry (U of Miami). It is possible that over expression of EGFR in cells may lead to mis-sorting which could lead to interactions that are synthetic due to forced EGFR dimerization, however, this cell line is well established.

MCE cells are MCF7 cells with EGFR stable over expression that introduces an over activated MAPK cascade. We wanted to see if CXCR7-EGFR colocalization behaved similar in this cell line to what we observed in MCF7 cells. We verified even more widely spread distribution of colocalization events in these cells. The observations identified with the MCE cells confirm that CXCR7 participates with EGFR in the activation of the MAPK pathway upon stimulation by EGF. Also it suggests this colocalization is dependent on EGFR activation by EGF and EGFR expression.

Interestingly, we found that CXCR7 co-localized with EGFR in MCE cells only after EGF stimulation. Our findings in MCF7 cells however, show that CXCR7 and EGFR are already colocalized, although at a low level, even before EGF stimulation. Ultimately the best indicator of whether this heterodimeric interaction between CXCR7 and EGFR occurs independently of EGF ligand may be our tissue data which indicates colocalization is present, albeit at low levels in normal (non tumor) tissues.
Figure 4.5 MCE cell PLA assay. A-D. After fixation of the MCE cells, PLA (in situ co-immunoprecipitation) was performed with specific CXCR7 [GTX100027, (1:500), GeneTex, Irvine, CA] and EGFR antibodies [E2760, (1:500), Sigma Aldrich, St. Louis, MO] to visualize heterodimerization of CXCR7 with EGFR. Colocalization is shown as a red fluorescent PLA signal (CX7/EGFR) and nuclei were counterstained with DAPI (blue) (400x magnification). A. In this condition, the cells were starved overnight (0.1% FBS) and then fixed for Duolink CXCR7-EGFR assay. B. CXCR7-EGFR colocalization in starved MCE cells stimulated for 2 min with EGF (10 ng/mL). C. CXCR7-EGFR colocalization in starved MCE cells stimulated for 5 min with EGF (10 ng/mL). D. Basal CXCR7-EGFR colocalization level in non-starved MCE cells cultured in regular complete media (10% FBS).

4.6 β-arrestin2 plays a role in CXCR7 mediated phosphorylation of EGFR and ERK

In order to visualize whether β-arrestin2 is involved in the colocalization of CXCR7-EGFR, we used siRNA to down regulate β-arrestin2, followed by EGF
stimulation and PLA to assess the level of CXCR7-EGFR heterodimerization. As MCF7 cells express relatively low levels of EGFR, we concomitantly employed a pLPCX-vector construct to over express EGFR. Non-specific isotype control antibodies were used as negative control (Figure 4.6A). EGFR over expressing cells showed an increase of CXCR7-EGFR co-localization compared to non-transfected cells (Figure 4.6B). However, siRNA mediated knockdown of β-arrestin2 in EGFR over expressing MCF7 cells resulted in an almost complete loss of CXCR7-EGFR colocalization events (Figure 4.6C). Similarly, down regulation of CXCR7 via siRNA as a control, abrogated the CXCR7-EGFR interaction (Figure 4.6D).
**Figure 4.6 β-arrestin2 plays a role in CXCR7 mediated phosphorylation of EGFR and ERK.** A. Incubation with non-specific isotype antibody (negative control). B. PLA signal indicating CXCR7/EGFR dimers in non-stimulated MCF7 cells. C. PLA signal indicating CXCR7/EGFR dimers in MCF7 cells transfected with EGFR-WT plasmid and siRNA against β-arrestin2. D. CXCR7/EGFR dimers in MCF7 cells transfected with EGFR-WT plasmid and siRNA against CXCR7 (630x).

**4.7 Discussion**

*Down regulation of CXCR7 decreases ERK1/2 and EGFR activation.* Seven transmembrane receptors have been shown to heterodimerize with ERBB family members and studies have demonstrated that the CXCR7 co-receptor CXCR4 interacts with EGFR in other types of cancer, like tumors of the prostate and bladder [12, 13]. EGFR is also known to be transactivated by seven transmembrane receptors to regulate events such as ERK signaling and proliferation [14]. We show that down regulation of CXCR7 decreased the level of activation of phospho-EGFR and phoshpo-ERK1/2, which suggests a strong association of CXCR7 with EGFR in breast cancer in accordance with our in situ co-IP data and confirms a role of CXCR7 in EGFR mediated ERK signaling. Additionally, our studies using RNAi indicate that disruption of the CXCR7-EGFR interaction following down regulation of CXCR7 not only affects the phosphorylation activity of EGFR and ERK1/2 but leads to cell cycle arrest and decreased cell proliferation (Chapter 3).

*CXCR7 affects phosphorylation of EGFR and ERK via β-arrestin2.* Following binding of SDF-1α, CXCR7 preferentially induces signaling events via interaction with β-arrestin2 [3, 15]. β-arrestins can interact directly with component kinases of the extracellular signal regulated kinase ERK/MAPK cascades leading to metastable β-arrestin complexes with ERK1/2 [7, 8]. β-arrestins are also relevant in BrCa progression, particularly β-arrestin2, which is expressed across both, luminal and basal breast cell lines [16]. However, it had not been reported whether β-arrestins can also mediate ligand
independent CXCR7 signaling. Therefore it was necessary to determine whether CXCR7 affects the mitogenic pathway via interaction with EGFR directly or indirectly through β-arrestin2. Our phosphorylation studies show an increased phosphorylation of ERK1/2 and EGFR at Y1110 in β-arrestin2 over expressing cells. Importantly, CXCR7 down regulation resulted in a significant decrease of phospho ERK1/2 and EGFR Y1110 even after β-arrestin2 over expression. Furthermore, we found that decreasing β-arrestin2 expression substantially decreased the colocalization of CXCR7-EGFR and thus plays a significant role in their association. These results suggest that the CXCR7/β-arrestin2 partnership is necessary to play a key role in activation of EGFR and ERK and the CXCR7-EGFR crosstalk to mediate breast tumor proliferation.
Chapter 5: OUTLOOK

5.1 Summary

This was an investigation of the interaction of CXCR7 with EGFR and helps determine the interaction’s relative contribution and consequence in BrCa cell proliferation. Taken together, these data demonstrate a significant heterotypic molecular interaction between two cell surface receptors with high potential clinical significance. We elucidate how CXCR7, via the accessory protein β-arrestin2, and EGFR promote proliferation through ERK1/2. Interestingly, we found that CXCR7 ligands are not necessary to see the significant proliferation effect CXCR7 expression has on BrCa cell growth.

Initially we explored the relative expression level of CXCR7 across a panel of BrCa cells to identify its expression pattern in this disease. BrCa is a heterogeneous disease and as such, it is important to address the context of expression patterns in order to rationalize our research approach for improved targeted therapies. We identified several important features of CXCR7 expression. The first one is that CXCR7 protein was expressed across a variety of BrCa cell lines and normal immortalized breast epithelial cells as well. Another important observation is that CXCR7 expression (using antibodies) was evident throughout the cell and not only in the cell membrane, which is the expected final localization for a surface protein. Furthermore, CXCR7 was not localized to the lipid rafts of MCF7 cell membrane. We also show evidence for CXCR7 expression in the nucleus of prostate cancer LNCaP cells, however, not in any of the breast cancer cells analyzed in this work. These findings open up many questions about
the regulation of this chemokine receptor, which may clarify why it has such important phenotypes throughout development and disease.

We questioned the role CXCR7 expression has in BrCa proliferation, to understand not only whether it affected growth but also asking how it could implement its growth effects. We identified significant changes in growth inhibition based uniquely on CXCR7 expression. We confirmed literature data that suggests that CXCR7 is important in growth. We enhanced the literature by demonstrating that CXCR7 expression alone, independently of its co-receptor CXCR4 and its ligand CXCL12, affects the proliferation of different types of BrCa cell lines. We show this phenomenon in BrCa cell lines with varying levels of CXCR7 expression, MCF7, BT474, MDA-MB231, and normal epithelial breast cells MFC12A. We also looked at other properties of cancer progression to determine if CXCR7 has a relevant role in them. We found modest changes that increasing CXCR7 expression had in MDA-MB231 cell invasion; however, we found no significant expression changes overall in migration due to CXCR7 over expression. The attachment of cells to a plastic surface was stronger in MCF7 cells with CXCR7 depletion than normal MCF7 cells (high CXCR7 expression), even when treated with different media conditions. These observations suggest that CXCR7 has a relevant role in BrCa tumor growth and likely in the metastatic process by affecting the attachment and therefore invasive processes of cells to metastatic sites.

To assess whether down regulation of CXCR7 affected the mitogenic pathway, we assessed for changes in the MAPK kinase cascade. Since ERK1/2 is classically involved in cell proliferation and differentiation of many cell types, and is the last step in this cascade of events, we analyzed for its expression. The inhibition of CXCR7 by
siRNA resulted in decreased ERK1/2 phosphorylation in EGF (10 ng/mL, 2 min) stimulated MCF7 cells. Since EGFR is a known mitogenic driver of cell proliferation and we demonstrated co-localization between CXCR7 and EGFR earlier, we next analyzed whether EGFR activation was also affected by CXCR7 depletion. Down regulation of CXCR7 decreased activation of EGFR at Tyrosine1110 compared to control siRNA transfected cells. Furthermore, we confirmed these results in EGFR over expressing cells. CXCR7 depletion decreased phosphorylation at the Y1110 site in EGFR over expressing MCF7 cells, compared to cells over expressing EGFR alone, demonstrating a significant role of CXCR7 in EGFR activation.

Finally, we validated the expression of CXCR7 in breast tissues derived from patients undergoing a tumor removal and lateral non-affected tissue extraction as a control. CXCR7 expression was clearly evident in the tumor tissues compared to their normal tissue counterparts. We show that CXCR7 is more strongly expressed in the luminal layer of breast tissue architecture. This observation correlates well with the higher CXCR7 transcript level identified in BrCa cells of luminal origin vs those of basal origin (Chapter 3). In agreement with CXCR7 literature, we also did find CXCR7 expression co-localized with expression of the epithelial marker CD-31. As another novel finding about CXCR7, we show that CXCR7 colocalizes with EGFR not only in MCF7 cells, but also in human breast tumor tissues.

5.2 Conclusion

In the present work we demonstrate that CXCR7 is an important modulator of cell proliferation through regulation of cell cycle progression and mitogenic activation of CXCR7-expressing BrCa cells. Interestingly, we found a ligand independent role of
CXCR7 mediated cell proliferation. We investigated the interaction of CXCR7 with EGFR and its role in BrCa cell proliferation and progression. We demonstrate that CXCR7 employs ERK1/2 mediated proliferation via the scaffold protein β-arrestin2 as its aid to interact with EGFR and offer a model for this interaction (Figure 5.2). Our results demonstrate for the first time that CXCR7 colocalizes with EGFR in human breast cancer cell lines and tissues and suggests that CXCR7 may regulate EGFR activation. New therapeutic strategies should employ relevant CXCR7 C-terminus inhibitors, not just the currently available ligand-like inhibitors to disrupt the CXCR7 mediated cancer phenotype. Therefore, exploring the role of CXCR7 in BrCa progression is clinically relevant. Targeting the CXCR7-EGFR interaction might be a first line of therapy for patients presenting not only with primary tumors with CXCR7 over expression, but based on our literature view and findings, this interaction may be of significance even at the metastatic stage of disease.

**Figure 5.2 A proposed model of CXCR7-EGFR Interaction.** CXCR7 signals through β-arrestin2, which by shuffling between them mediates interaction of CXCR7 with EGFR and activation of EGFR mediated signaling. In some instances CXCR7 might activate mitogenic signaling independently from EGFR (and vice versa). This EGFR transactivation, which occurs in a CXCR7-ligand independent fashion, induces proliferation effects in breast cancer cells.
5.3 Future directions

These findings can be applied to other cancer models where expression of CXCR7 and EGFR is higher than the normal tissue counterparts. This work is only part of the beginning of many significant applications of this basic research knowledge to further study CXCR7 in the fields of metastasis, angiogenesis, inflammation, developmental biology, and drug development [1-4].

In breast cancer specifically, it is important to return to what is known about the disease to better understand the context of the problem. As mentioned in Chapter 1, we now organize BrCa by categories based on gene expression profiles. These genetic profiles are based on multiple comprehensive and independent studies that have used state of the art technology to determine how best to categorize BrCa. A next step is to identify the interaction of CXCR7 with EGFR in each particular group or category of breast cancer and identify which subgroup is likely best suited for targeting this particular interaction. For example, even if we were to find that only the ER+ subtype has a significantly higher number of colocalization events than the other subtypes, then it would be interesting to target the CXCR7-EGFR interaction with anti CXCR7 or anti EGFR inhibitors in combination with hormone therapies such as tamoxifen in animal studies. These types of studies would significantly advance the translational application of our targets.
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