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**ROLE OF KLF8-CXCR4 SIGNALING IN BREAST CANCER
METASTASIS**

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
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ABSTRACT

Krüppel-like factor 8 (KLF8) has been strongly implicated in breast cancer metastasis. However, the underlying mechanisms remain largely unknown. In this study we report a novel signaling from KLF8 to C-X-C cytokine receptor type 4 (CXCR4) in breast cancer. Overexpression of KLF8 in MCF-10A cells induced CXCR4 expression at both mRNA and protein levels. This induction was well correlated with increased Boyden chamber migration, matrigel invasion and transendothelial migration (TEM) of the cells towards the ligand CXCL12. On the other hand, knockdown of KLF8 in MDA-MB-231 cells reduced CXCR4 expression associated with decreased cell migration, invasion and TEM towards CXCL12. Histological and database mining analyses of independent cohorts of patient tissue microarrays revealed a correlation of aberrant co-elevation of KLF8 and CXCR4 with metastatic potential. Promoter analysis indicated that KLF8 directly binds and activates the human CXCR4 gene promoter. Furthermore, CXCR4-CXCL12 engagement downstream of KLF8 leads to the feed-forward activation of FAK. Interestingly, KLF8 expression, through CXCR4 engagement, triggered the formation of filopodium-like protrusions (FLP) and thereby enhanced the proliferation rate of breast cancer cells in 3D Matrigel-on-Top culture, under prolonged treatment with CXCL12. This indicates that KLF8 plays a major role in promoting aggressive colonization of tumor cells in a CXCL12-enriched foreign tissue microenvironment, thereby aiding in secondary macrometastasis formation. Xenograft studies showed that overexpression of CXCR4, but not a dominant-negative mutant of it, in the MDA-MB-231 cells prevented the invasive growth of primary tumor and lung metastasis from inhibition by knockdown of KLF8. Apart from lung, KLF8 overexpression also induced spontaneous secondary metastasis to other CXCL12-rich

organs through CXCR4 signaling. These results collectively suggest a critical role for KLF8 and the CXCR4-CXCL12 pathway in promoting breast cancer metastasis and shed new light on potentially more effective anti-cancer strategies.

Dedicated to

My maternal grandfather Late Dr. P. N Chatterjee and my paternal grandmother Late Mrs.
Sadhana Mukherjee,

and to my parents, Mr. Bhaskar Mukherjee and Mrs. Helen Mukherjee, who walked with me and
showed me the way.

You inspire me everyday.

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LIST OF ABBREVIATIONS

| | |
|---------------|---|
| <i>BLI</i> | <i>Bioluminescent Imaging</i> |
| <i>BOP</i> | <i>Biotinylated oligonucleotide precipitation</i> |
| <i>ChIP</i> | <i>Chromatin immunoprecipitation</i> |
| <i>CTC</i> | <i>Circulating Tumor Cells</i> |
| <i>CXCL12</i> | <i>C-X-C motif chemokine ligand 12</i> |
| <i>CXCR4</i> | <i>(C-X-C) motif receptor 4</i> |
| <i>ECM</i> | <i>Extracellular Matrix</i> |
| <i>EMT</i> | <i>Epithelial- To-Mesenchymal</i> |
| <i>FAK</i> | <i>Focal adhesion kinase</i> |
| <i>FLP</i> | <i>Filopodia-like Protrusions</i> |
| <i>HA</i> | <i>Hemagglutinin</i> |
| <i>HUVEC</i> | <i>Human umbilical vein endothelial cells</i> |
| <i>IP</i> | <i>intraperitoneal</i> |
| <i>IV</i> | <i>Intravenous</i> |
| <i>KLF8</i> | <i>Krüppel-like Factor 8</i> |
| <i>mAb</i> | <i>monoclonal antibody</i> |
| <i>MMP14</i> | <i>matrix metalloproteinase 14</i> |
| <i>MMP9</i> | <i>matrix metalloproteinase 9</i> |
| <i>MoT</i> | <i>Matrigel-on-Top</i> |
| <i>PARP</i> | <i>Poly (ADP-ribose) polymerase</i> |
| <i>PCR</i> | <i>polymerase chain reactions</i> |

| | |
|--------------|---|
| <i>PI3K</i> | <i>Phosphatidylinositol-4,5-bisphosphate 3-kinase</i> |
| <i>qRT</i> | <i>quantitative real time</i> |
| <i>SDF-1</i> | <i>Stromal-derived factor 1</i> |
| <i>TEM</i> | <i>transendothelial migration</i> |
| <i>TNF</i> | <i>tumor necrosis factor</i> |
| <i>VEGF</i> | <i>vascular endothelial growth factor</i> |

CHAPTER 1: BACKGROUND

Breast Cancer: The disease

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-associated mortality among women. According to the American Cancer Society, in 2015 alone, an estimated 292,130 women were diagnosed with breast cancer in the United States. Typically, breast cancers arise out of tissues that make up the lobules or ducts of the breast. The glands which produce milk in the breast are termed lobules, while ducts connect the lobules to the nipple. Like all other cancers, uncontrolled proliferation of malignant cells is a hallmark of breast cancer. As such, breast cancers can be detected in the form of a lump or tumor on the breast. In recent years, 80% of breast cancer cases have been identified on detecting lump on the breast. Breast cancer usually produces almost no symptoms when the tumor size is small and well-confined. At this early stage, it is largely curable through adjuvant therapies or surgical resection. At a later stage, however, tumor cells can proliferate rapidly to form invasive tumors which fail to remain confined at the primary site and subsequently spread to distant organs, thereby giving rise to metastatic disease. At this stage, breast cancer is rendered largely incurable due to the resistance of invasive metastatic cells to current therapeutics. Indeed, breast cancer metastasis causes >90% of breast-cancer associated deaths [1]. This underscores the importance of regular screening procedures like clinical or self-breast exam and mammography to achieve early detection of breast tumors in order to successfully treat the disease.

The Normal Breast

In order to understand the potential site of origin of breast cancer, it is important to study the structure and development of the normal breast. The breast serves as the mammary gland which secretes milk post childbirth to feed the new-born. Morphologically, the breast is a conical structure largely consists of adipose tissue and fibrous connective tissue, covering much of the chest area with its base at the chest wall and the apex at the nipple. The normal breast is made up of 15-20 lobes which consist of smaller lobules. The lobules are the structural units of the breast and are further made up of smaller alveoli. The alveoli are the milk-producing sub-units in the breast. The milk ducts transport milk from the alveoli of the lobules to gradually converge to constitute major ducts that terminate at the nipple. The breast structure also contains both blood vessels and lymphatic vessels [2].

Prior to puberty, the human breast structure consists of a simple system of ducts that remains almost identical in males and females. On reaching puberty, elevated levels of estrogen, in conjunction with growth hormone stimulates the growth and development of the female breast. Under the influence of estrogen, the rudimentary tubules develop into the mature ducts which form intricate ductules around which alveolar structures are formed. During lactation, milk is drained from the alveoli by these fine ductules which gradually join each other to form a single large duct called the lactiferous duct. Thus, milk is transported from each lobe by a single lactiferous duct. In addition to its role in mediating ductal development in the breast, estrogen also promotes the accumulation of adipose tissue in the breast, resulting in the increase in breast size post puberty [3].

During pregnancy, the dramatic increase in the levels of estrogen and progesterone hormones results in further development of the mature ductal system in the breast. In addition, estrogen, progesterone and other hormones like prolactin, growth hormone, insulin-like growth factor 1/2 contribute together to mediate the completion of the lobuloalveolar development in preparation of lactation and breastfeeding [4-7].

Most cases of breast cancers originate in the cells that line the ducts (ductal cancers), while a smaller number of cases have been found to begin in the cells lining the lobules (lobular cancers). Certain rare forms of breast cancers may also arise in the other tissues of the breast. At later stages of cancer progression, dissemination of malignant cells from the primary tumor occurs through the blood vessels or lymphatic system present in the breast.

Risk Factors

- Genetic predisposition

Inherited mutation in two autosomal tumor suppressor genes, BRCA1 and BRCA2, account for most of the diagnosed cases of familial breast cancer among women worldwide. The BRCA1 and BRCA2 proteins repair double-strand breaks in the DNA helix. Inability to repair these breaks contributes to loss of genetic stability and failure to control cellular proliferation. In the general population, a woman possesses a 7% chance of developing breast cancer by 70 years of age. In contrast, an individual carrying the lethal BRCA mutation possesses a 60% to 80% chance of being diagnosed with breast cancer at some stage in their life. The risk of carrying the BRCA gene mutation is two-fold higher in women who have a first-degree family member diagnosed with cancer, as opposed to women who have no family history of the disease. [8]

Apart from BRCA1 and BRCA2 mutations, several other genetic mutations have been identified to be critical contributing factors to the development of the disease. Mutations in the DNA damage response genes ATM and Chk have been found to dramatically increase the risk of breast cancer in women [9]. Loss of p53 occurs in almost 50% of all cancers, including breast cancer. PTEN is a tumor suppressor gene that suppresses cell division and regulates apoptosis. PTEN mutations have been identified in a number of breast cancer patients [10].

- Prior history of cancer

Patients who have a prior history of breast, ovarian or uterine cancer are at a higher risk of developing breast cancer in future. The risk is even higher if the diagnosis was done at an early stage. Indeed, women who develop early-onset breast cancer below the age of 40 are found to be 2.5 times more likely to develop subsequent breast cancer. [11]

- Gender and Age

Women are at a much higher risk of developing breast cancer than men owing to possessing significantly more breast tissue than men that is anatomically distinct and consistently exposed to growth-stimulating hormones such as progesterone and estrogen during child bearing years. Age is another important factor in breast cancer, whereby older post-menopausal women are at a significantly higher risk than younger women. Indeed, only 1 in 8 of the invasive breast cancers diagnosed are found in women under 45, while 2 out of 3 develop in women over 55.

- Breast tissue structure

Women with denser breast tissue, i.e., having more glandular and less fatty tissues, are at an increased risk of developing breast cancer.

- Endogenous hormone levels

Post-menopausal women who have a greater level of endogenous sex hormones are at an elevated risk of developing breast cancer. Additionally, higher the number of menstrual cycles that a woman undergoes, the longer is her lifetime exposure to reproductive hormones. This significantly increases the risk of developing breast cancer at a later stage in life. [12]

- Lifestyle choices

Early pregnancy is related to decreased propensity towards breast cancer. Reproducing later in life or not reproducing at all increases the overall risk of developing breast cancer. Studies have demonstrated that breastfeeding for a year or more slightly reduces breast cancer risk. However, breast feeding for a longer duration (three years or more) is associated with higher risk of breast cancer. “Suggestive but not sufficient” evidence has been uncovered which indicates that smoking may increase the risk of breast cancer among women. Obesity is another factor that significantly increases a woman’s overall risk of developing breast cancer. Numerous studies have suggested that the risk is about 1.5 times higher in overweight and about 2 times higher in obese women than in lean women. In addition, regular physical activity among women reduces the risk of breast cancer by 10%-25%. [13-15]

- Radiation exposure

Sustained exposure to radiation is a crucial factor in increasing the risk of breast cancer. The relationship between exposure to radiation and breast cancer has been reported in atomic bomb survivors and women who received high-dose radiation therapy to the chest. Studies have demonstrated that, girls and women between 10-30 years of age, who were treated with high-

dose radiation to the chest as a treatment for Hodgkin lymphoma mostly developed breast cancer later in life [16, 17].

Classification of Breast Cancer

Breast cancers are routinely categorized based on different schemes in order to accurately characterize the tumor and assess prognosis. Each category serves a distinct purpose that is crucial for formulating the appropriate therapy for the individual patient. The major aspects accounting for breast cancer classification include histopathological type, the tumor grade, stage of the tumor and the receptor status of the tumor.

- Histopathological type

Most breast cancers are derived from epithelial cells lining the walls of the duct or lobules of the breast. This type of cancer originating in the epithelium is called carcinoma. Based on the histopathological features of the tumor tissue, breast cancers may be classified into the following subtypes.

Ductal carcinoma in situ

Ductal carcinoma in situ (DCIS) is a pre-cancerous or non-invasive cancerous lesion characterized by the presence of abnormal cells lining the milk ducts present in the breast. “In situ” literally means “in place”, i.e., in DCIS, abnormal cells remain contained within the mammary duct and do not breach the duct wall into the surrounding breast tissue. DCIS is considered to be precancerous since the tumor cells have the potential to adopt an invasive phenotype. However, current treatment options for evaluating the potential of such precancerous lesions to develop into aggressive invasive cancers remain fairly limited. Currently, around 20%-

30% of women who do not receive adequate treatment for DCIS develop breast cancer at a later stage [18].

Lobular carcinoma in situ

Lobular carcinoma in situ (LCIS) is a fairly uncommon condition, whereby abnormal, indolent cells are formed in the milk-producing lobules of the breast. Individuals diagnosed with LCIS are at a significantly higher risk of developing invasive breast cancer [19].

Invasive ductal carcinoma

Invasive ductal carcinoma (IDC) is the most common type of breast cancer, accounting for 70%-80% of invasive breast cancer cases. In IDC, abnormal cells lining the milk ducts successfully breach the duct wall and penetrate through the basement membrane which separates the ductal epithelial layer from the underlying tissue. The invasive tumor cells infiltrate into the surrounding breast tissue and can subsequently metastasize to secondary organs through the blood vessels or lymphatic system in the breast [20].

Invasive lobular carcinoma

This type of invasive cancer is relatively uncommon, accounting for about 5%-15% of the total cases metastatic disease. It originates in the lobules of the breast and can penetrate into the surrounding stromal tissue. Studies have demonstrated that the prognosis for ILC tends to be more favorable than IDC in the short term, especially since ILC metastasizes later than ductal carcinoma [21].

- Tumor Grade

The grading of tumor is based on its morphological similarity to normal breast tissue. Normal cells of any organ are highly differentiated based on the specific function performed by the cell in the organ. An important hallmark of cancer progression is the loss of cellular differentiation. As such, tumor cells lose the ability to control proliferation and divide rapidly to form disordered structures, thereby disrupting the organized tissue architecture previously present in the breast. As cancer cells progressively lose features seen in normal cells, pathologists categorize the tumors as well-differentiated (low grade, Grade 1), moderately differentiated (intermediate grade, Grade 2) and poorly differentiated (high grade, Grade 3). Grade 1 tumors represent the best prognosis while grade 3 tumors are associated with very poor survival rate and warrant the use of aggressive medication and therapeutic options [22].

- Staging of the tumor

A staging system is the standardized way to determine the extent of cancer in the patient. The stage of the tumor provides important information, including the invasiveness, size and metastatic status of the tumor. It is one of the most important factors in determining prognosis and therapeutic options.

In the clinical setting, pathologists may use a staging system called the TNM system to categorize breast cancer tumors. The TNM classification uses information on the tumor size and the level of infiltration within the breast tissue (T), the extent of local lymph node metastasis (N) and the presence of distant metastasis (M). On determining the T, N and M a specific stage is assigned to the tumor which then goes on to form the basis of the treatment ,

Stage 0 is used to describe non-invasive breast cancers, such as DCIS, which have shown no evidence of infiltration into the surrounding tissue

Stage I describes 2 cm or less invasive tumors which have breached the ductal wall and may or may not be associated with micrometastasis in 1-3 axillary lymph nodes.

Stage II: At this stage, the tumor is larger than 2 cm but less than 5 cm across. Usually metastatic growth can be seen in internal mammary lymph nodes at this stage. However no distant metastasis can be seen in Stage II

Stage III: At this stage the tumor is larger than 5cm and can metastasize to 10 or more axillary lymph nodes and enlarge the internal mammary lymph nodes. However, no distant metastatic dissemination can be seen at this stage.

Stage IV: At the final stage, the primary tumor can be of any size characterized by detectable macrometastasis in distant organs. The most common secondary sites for breast cancers include bone, liver and lung.

- Receptor Status

Breast cancer tumors may be categorized into four main molecular subtypes based on the presence or absence of hormone receptors and excess levels of HER2

Luminal A

74% of all breast cancers are of the luminal A subtype. This subtype is characterized by the expression of the estrogen receptor (ER+) and/or the progesterone receptor (PR+) but not HER2 (HER2-). These cancers are less aggressive than other subtypes. Among the four main subtypes, Luminal A tumors represent the most favorable prognosis, since hormone therapies can be used to treat such tumors due to the presence of hormone receptors [23].

Triple-negative

Approximately 12% of all breast cancers are triple-negative, i.e., they are ER-, PR- as well as HER2-. Most triple negative cancers are basal-like. This type of breast cancer is common in individuals with the BRCA1 gene mutation. Currently, triple-negative breast cancers are associated with poor prognosis. This is partially because targeted therapy against this type of breast cancer is currently unavailable [24].

Luminal B

About 10% of all breast cancers are of the luminal B sub-type. This type of breast cancers are ER+ and/or PR+ and are further characterized by being HER2+. These tumors are also highly positive for the proliferation marker Ki67, indicative of a large proportion of rapidly dividing cells. Luminal B breast cancers are usually more aggressive than Luminal A tumors but are responsive to hormone-therapy [23].

HER-2 enriched

This type of breast cancer is relatively rare, whereby about 4% of all breast cancers are HER-2 enriched, i.e. they express HER-2 receptors but not hormone-receptors. These tumors tend to be less aggressive and grow more slowly than the other tumors. Prognosis is usually poorer for this type of cancers compared to ER+ tumors. However, recent progress in developing targeted therapies against HER2+ cancers has significantly improved the prognosis for this sub-type of breast cancer [25].

Current treatment options

- Local treatment

This kind of treatment is used on the tumor without affecting the rest of the body. This treatment option is usually for localized less advanced cancer.

Surgery

Surgical resection of the primary tumor and surrounding tumor stroma is the first step and the most common treatment option against breast cancer. Depending on the extent or number of tumors in the breast, complete surgical removal of one or both breasts (mastectomy) may be necessary. Though initially successful, a relapse in the primary tumor site is not uncommon.

Radiation therapy

Radiation therapy is used as adjuvant therapy in order to minimize the risk of local recurrence for patients who have undergone surgery to remove the primary tumor. It involves the use of high-energy X-rays or gamma rays targeting the primary tumor or the post-surgery tumor site. Radiation therapy is essential for killing the cancer cells that may remain in the tumor site post-surgery. The main drawback of using targeted radiation therapy is that along with the malignant cells, it also affects normal cells of the affected region. This often results in painful side-effects in patients receiving radiation treatment for an extended period.

- Systemic treatment

Systemic therapy refers to the removal of breast cancer cells that have disseminated throughout the body. Usually, systemic treatment is used as an adjuvant treatment along with surgical resection in advanced cases of breast cancer.

Chemotherapy

Breast cancer chemotherapy is associated with the use of cytotoxic drugs against breast cancer. Since it is a systemic therapy, the drugs travel throughout the body through the bloodstream in order to kill cancer cells at both the primary sites and the metastatic sites. It is usually used in conjunction with other forms of therapy, including hormonal and targeted therapy. It is also an important pre-surgery procedure that ensures shrinking of the tumor before surgical resection. The most common drugs used as part of chemotherapeutic treatment programs include The most common chemotherapy drugs used for breast cancer is doxorubicin and paclitaxel. These drugs are sometimes used in conjunction with other drugs, like fluorouracil (5-FU) and cyclophosphamide.

Hormonal therapy

Hormonal therapy is used as an adjuvant therapy post-surgery to minimize the risk of breast cancer relapse. This form of treatment has been significantly successful in treating hormone receptor positive cancers. As discussed before, about 60% -80% of diagnosed breast cancer cases are ER+ and/or PR+. Estrogen acts as growth signal for hormone –receptor positive cancer cells. As such, hormone therapy relies on the ability to either shut down estrogen production or blocking the receptors from binding to extracellular estrogen. Tamoxifen is a widely used drug that blocks estrogen receptors in breast cancer cells and thereby shuts down the estrogen induced cell proliferation pathways in the cell. It is used for treating both primary and metastatic breast cancer. Aromatase inhibitors (AIs) inhibit estrogen production in postmenopausal women and have been approved as a potent therapeutic option for hormone –

receptor positive cancers. The main limitation of this therapy is that it is ineffective in the case of hormone-receptor negative breast cancers, including triple-negative breast cancer cases [26-28].

Targeted therapy

Targeted cancer therapies target specific characteristics of cancer cells, such as a protein expressing in the cancer cell that promotes the uncontrolled proliferation of the host cell. For example, in case of HER-2 enriched breast cancers, trastuzumab (Herceptin) is used as a blocker of HER2 activity in breast cancer cells, thereby slowing tumor progression. In case of advanced cancers, trastuzumab is usually used as an adjuvant treatment along with chemotherapy to inhibit cancer growth and ensure patient survival. The main advantage of targeted therapy over chemotherapy is that this treatment program is less detrimental to normal cells, thereby minimizing painful side-effects in breast cancer patients. In recent years, several other types of targeted therapies involving angiogenesis blockers and signal transduction inhibitors are being researched extensively to improve the success rate in fighting breast cancer. A large proportion of drugs related to targeted therapy are being currently tested in clinical trials [29].

Breast Cancer Metastasis: Progression of the Invasion-Metastasis Cascade

Metastasis represents the end point of a sequence of complex cell-biological events beginning with the detachment of an invasive cancer cell from the primary tumor mass and ending with the dissemination and subsequent adaptation of malignant cells to a secondary metastatic site at a distant organ. As discussed above, metastasis causes more than 90% of breast-cancer associated deaths.[30] This underscores the importance of understanding the cell-biological details of metastasis in order to successfully treat metastatic disease. Indeed, our

potential to eradicate breast cancer rests largely on our ability to successfully inhibit the process of metastasis.

The process of metastasis progresses through a succession of cell-biological processes termed as the invasion metastatic cascade, whereby the invasive cells in the primary tumors i) dissociate from the tumor, ii) locally invade into the surrounding stroma, iii) intravasate into the lamina of blood vessels to travel with the blood as circulating tumor cells (CTCs), iv) extravasate out into the foreign tissue parenchyma, v) initially survive and sustain in the foreign microenvironment to form dormant microenvironment, and finally, vi) successfully reinitiate proliferation to form clinically detectable macrometastatic lesions at the secondary sites (metastatic colonization)[31]. Successful metastatic outgrowth at the secondary site is dependent on the effective completion of each event of the metastatic cascade.

Local invasion

This is the key event involving the dissociation of invasive tumor cells from the primary tumor into the surrounding stromal tissue by breaching the basement membrane. Most types of carcinomas can either invade into the extracellular matrix (ECM) collectively or as individual tumor cells. In case of the latter, two distinct invasion programs are followed: a) “mesenchymal invasion” program or the b) “amoeboid invasion” program [32]. It is important to note that the tumor cell can interchange between various invasion strategies according to the changing conditions encountered in the microenvironment. The main mechanism that drives invasion through the basement membrane and ECM components is active proteolysis, mainly affected by matrix metalloproteinases (MMPs). MMP-bearing invasive cancer cells can degrade the BM and other ECM components to invade through the stromal tissue, while also liberating growth factors

sequestered in the stroma which subsequently fosters further tumor growth and invasion. Recently, the interaction between the tumor stroma and the tumor cell itself has garnered widespread interest. Several studies have shown irrefutable evidence that tumor-associated stromal cells nurture the growth and development as well as invasiveness of primary tumor cells.

Intravasation

The transmigration of locally invasive tumor cells into the lumina of blood or lymphatic vessels is called intravasation. This process is largely influenced by the structural features of tumor associated blood vessels. Neangiogenesis driven by vascular endothelial growth factors (VEGF) in the tumor microenvironment is a crucial factor in driving both tumor growth and intravasation. Unlike normal blood vessels, tumor-associated vasculature is tortuous and features weak interaction between adjacent endothelial cells, thereby promoting the invasion of tumor cells across the endothelial barrier into the lumina of blood vessels [33].

Survival in the Circulation

On successfully intravasating into the lumina of blood vessels, tumor cells must survive in the blood in order to be disseminated to secondary metastatic sites via the circulating bloodstream. Recently, the detection of circulating tumor cells or CTCs in the blood is being used as a diagnostic tool to identify cancer cases [34]. Survival in the blood poses a formidable challenge for circulating tumor cells especially since they are deprived of integrin-dependent anchorage to the extracellular matrix. In addition to matrix detachment, tumor cells face a myriad of other stresses in circulation, including predatory cells of the innate immune system as well as

the hemodynamic shear forces in the blood stream. As such, it has been theorized that only a small subset of initially intravasated cells are able to persist in circulation.

Extravasation

Extravasation of breast cancer tumor cells involves the arrest of the CTCs at the microvasculature of distant organs and subsequent transmigration of the cells through the endothelial barrier and into the foreign tissue parenchyma. It has been long noted that instead of disseminating to a wide range of different organs, breast cancer cells preferentially metastasize in specific target organs, mainly in the lung, liver and bone marrow. The process of extravasation is widely thought as the reverse of intravasation. However, recent studies have revealed that these processes may be distinct from one another. This is owing to the fact that unlike in intravasation, supporting cells like tumor-associated macrophage are unavailable to foster extravasation. In addition, the microvasculature in distant organs is highly functional and likely to be less permeable, unlike the neovasculature in the primary tumor. In order to overcome this obstacle, primary tumor cells are capable of secreting factors like Angptl4, Angpt2, MMP-1, COX-2 and MMP-2 that can disrupt the distant microenvironments and promote hyper permeability in the microvasculature at the metastatic site. Studies have indicated that extravasation causes the attrition of about 20% of initially disseminated tumor cells [35].

Micrometastasis formation

Disseminated carcinoma cells must initially persist in the novel microenvironment post extravasation in order to be able to subsequently form macroscopic metastasis at these secondary sites. Persisting in these foreign conditions poses a considerable challenge for tumor cells since

the microenvironment at the metastatic locus is vastly different from the native primary tumor site. Important differences include types of available growth factors and cytokines, types of stromal cells and surrounding ECM components. Indeed, the microarchitecture of the secondary tissue itself may differ greatly from that at the primary tumor site. Thus, tumor cells are initially poorly adapted to their newfound environment. In order to persist in the foreign tissue sites, cancer cells must take advantage of cell-autonomous programs to meet the demands imposed by the harsh climate at the metastatic site. Detailed studies in experimental models have revealed that micrometastasis formation is preceded by a very high attrition rate of tumor cells [35]. About 96% of successfully extravasated cells failed to generate micrometastasis, further underscoring the importance of successfully adapting to the foreign microenvironment in order to ultimately generate metastatic lesions.

Metastatic colonization

The disseminated tumor cells that successfully survive in the foreign microenvironment usually exist at a quiescent stage, whereby their proliferative programs at the metastatic sites are greatly impaired. This non-proliferative dormant state can be largely attributed to the inability of breast cancer cells to engage FAK, integrin $\beta 1$ and trigger Src pathways due to the inherent incompatibility with the novel microenvironment that surround the cells [36, 37]. The ability to reestablish proliferative programs on foreign soil is largely dependent on cell-nonautonomous mechanisms that may transform the foreign microenvironment into hospitable niches for growth and survival. Firstly, micrometastatic cells must establish productive interactions with the parenchymal ECM and secondly, they must adapt to the unfamiliar growth factor and cytokine environments to garner mitogenic signals to trigger proliferation. [38]As such, this final step of

the invasion-metastatic cascade is highly inefficient and represents the dominant rate-limiting step, whereby about 99.3% of dormant micrometastatic cells lodged in the foreign tissue parenchyma fail to generate macrometastatic lesions [1]. A number of studies have documented that certain organs may be more suitably hospitable than others for the proliferation of disseminated mammary carcinoma cells. It has been postulated that the organs that represent an intrinsically favorable environment for reestablishing proliferation of indolent breast cancer are the most common sites for secondary metastasis. This organ-specific metastasis further underscores the importance of this final rate-limiting step towards establishing clinically detectable macroscopic metastasis.

Krüppel-like factor 8

Introduction

Krüppel-like Factor 8 or KLF8 is a member of the KLF family of proteins which are identified as transcriptional activators and repressors that play a vital role in regulating gene expression to ultimately affect the progression of various diseases. All KLF proteins bear three highly conserved Cys2His2 zinc finger DNA-binding domains at the C-terminus which interacts with the GT box region on the target promoter [39]. KLF8 represses beta-globin expression and regulates various cellular processes, including cell proliferation, epithelial to mesenchymal transition (EMT), oncogenic transformation, tumor invasion and metastasis. KLF8 has been found to be barely detectable in normal cells, whereas it expresses at elevated levels in cancer cells [40]. Indeed, in recent years, KLF8 has emerged as a crucial determinant of cancer progression.

Structure of KLF8

The human KLF8 gene is located at the X chromosome [41]. The full protein consists of 359 amino acid residues. Like other transcription factors, KLF8 possesses a nuclear localization signal for nuclear transport, a DNA binding domain to bind target promoter sequence and a regulatory domain to control gene expression. Highly conserved zinc finger domains make up the DNA binding domain and can also mediate protein-protein interaction. At the N-terminal of the full-length KLF8 protein, a Pro-Val-Asp-Leu-Ser (PVDLS) motif binds the transcriptional repressor C-terminal binding protein (CtBP) thereby mediating the transcriptional repressor activity of KLF8 [42]. KLF8 has been recognized as a dual transcription factor, i.e., it is capable of both transcriptional repression and activation [43, 44]. The transcriptional activation function of KLF8 is mediated by the glutamine residues Q118 and Q248 which are critical elements of the transcriptional activation domain of KLF8 [45]. The zinc finger domains 1 and 2 interact with importin-beta, which is an important regulator of nuclear import of cytosolic proteins. The serine 165 (S165) and lysine 171 (K171) residues also appear to be critical for the nuclear localization of KLF8. Although the precise role of the S165 and K171 residues remains to be investigated, it has been postulated that PKC phosphorylates KLF8 at the S165 residue to facilitate the nuclear localization. A mutated form of the KLF8 protein without the S165 and K171 residues failed to activate cyclin D1 expression, thereby implicating these sites in the cell proliferation function of KLF8 [46]. Recent reports have confirmed that PARP-1 binds to KLF8 via Zn finger motifs 1 and 2 to stabilize the KLF8 protein in the nucleus and inhibit KLF8 export into the cytoplasm. In addition, studies emerging from our laboratory have confirmed that the KLF8-PARP1 interaction

and PARylation in the nucleus helps to maintain the transcriptional activity and overall function of the KLF8 protein [47].

Function of KLF8 as a dual transcription factor

KLF8 acts as a transcriptional repressor by binding to CtBP through the PVDLS domain or the repression domain. Elegant promoter-reporter assays have confirmed that the KLF8- CtBP complex can trigger transcriptional repression of the beta-globin gene [48]. However, it is interesting to note that disruption of the PVDLS only partially inhibited KLF8 repressor activity. This suggests that the repression of gene expression by KLF8 can take place through both CtBP-dependent and independent mechanisms [42]. Cyclin D1 has been shown to be a target of KLF8's role as a transcriptional activator. It binds directly on the GT box sequence present on the cyclin D1 promoter to trigger its activation [43]. Knockdown of KLF8 in invasive cancer cells dramatically reduced cyclin D1 expression and cell proliferation. Furthermore, KLF8 can recruit transcriptional co-activators like P300 and PCAF to its activation domain in order to further enhance its transcriptional activity [45]. Mutating the Q118 and Q248 residues in the activation domain successfully abolished KLF8's role as a transcriptional activator. Through its role as a dual transcription factor, KLF8 can influence tumor malignancy by directly or indirectly affecting cellular pathways associated with tumor progression. KLF8 can transcriptionally repress KLF4 expression to enhance proliferation [44]. KLF8's role in E-cadherin transcriptional repression has been well-documented [49]. Loss of E-cadherin expression is a hallmark of epithelial-to-mesenchymal activity, whereby it leads to the disruption of cell-to cell contact and subsequent abolishment of organized architecture of the epithelial phenotype. KLF8 promotes local invasion of cancer cells by positively regulating the expression of matrix

metalloproteinases MMP-9 and MMP-14 [50, 51]. These are protease enzymes which degrade the extracellular matrix components at the primary tumor site in order to promote local invasion through the tumor microenvironment. In addition, recently uncovered that KLF8 can directly activate the transcription of *Epsti1* and upregulate EGFR to promote tumor invasion and metastasis [52, 53]. Taken together, KLF8's role as a transcriptional activator as well as repressor induces an overall effect favoring tumor progression.

KLF8 in cancer

Several studies have demonstrated the aberrant overexpression of KLF8 in various types of human cancer including breast [49, 50], ovarian [54, 55], renal [56, 57], liver [58], gastric [59] and brain [60] cancer. The role of KLF8 in cancer was initially investigated by studying its role in inducing oncogenic transformation of NIH3T3 cells. Our group demonstrated that inducible overexpression of KLF8 induced oncogenic transformations featuring loss of contact inhibition and serum independent growth [56]. Furthermore, several reports by our group and others have confirmed KLF8's role in enhancing cell proliferation through the activation of cyclinD1 expression and repression of KLF4 transcription. The role of KLF8 as a critical EMT inducer has been well documented. It directly regulates the transcriptional repression of E-cadherin expression, thereby triggering EMT and human mammary epithelial cell line MCF-10A. Importantly, KLF8 can act as an EMT inducer independent of other known EMT inducers like Snail, Twist, etc. KLF8 has also been found to promote local invasiveness of tumor cells by activating the transcription of proteolytic enzymes such as MMP-9 and MMP-14.[49, 50] Importantly, KLF8 knockdown in highly metastatic breast cancer cell line significantly decreases metastatic dissemination in the lung in nude mouse [50]. Our group has also demonstrated

previously FAK to PI3K to AKT to SP1 signaling leads to downstream KLF8 upregulation in ovarian cancer cells, thereby leading to downstream cyclin D1 expression and enhanced cell proliferation [54].

CXCR4 in Breast Cancer

Introduction

CXCR4 is a seven transmembrane heterotrimeric G-protein coupled receptor that binds to the chemokine CXCL12. Chemokines are essentially chemoattractant cytokines that are implicated in cellular trafficking. The CXCL12-CXCR4 interaction was initially studied due to its implication in HIV infection of T lymphocytes [61, 62], and subsequently in lymphocyte trafficking, hematopoietic stem cell homing and maintenance of secondary lymphoid structure [63]. The lung, liver, bone marrow and lymph nodes represent the organs in which CXCL12 expression is the highest [64]. The CXCR4-CXCL12 interaction is crucial in metastatic spread of breast cancer as well as most other types of cancer. Interestingly, the organs which have the highest secretion of CXCL12 also represent the sites in which breast cancer cells preferentially metastasize. Inhibition of the CXCR4-CXCL12 interaction severely reduced this organ-specific metastasis. As such, several studies have indicated that CXCR4-high breast cancer cells have “hijacked” the CXCL12-guided cell homing mechanism to establish secondary metastasis [65].

CXCL12-CXCR4 signaling pathway

CXCL12-CXCR4 engagement leads to several downstream signaling pathways that lead to various downstream effects including cell migration and survival. CXCR4 is a G-protein-coupled seven-transmembrane receptor having with seven helical regions connected by six

extramembrane loops. The intracellular region of the seven transmembrane structure contains the $G\alpha$, $G\beta$ and $G\gamma$ subunits which remain bound to GDP at the inactive state. On ligand binding, the receptor is activated, whereby; GDP is replaced by GTP causing the subsequent separation of the G subunits into GTP-bound monomeric $G\alpha$ and a $G\beta\gamma$ dimer. $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ are the four forms in which $G\alpha$ can exist. Although it was initially believed that CXCR4 is primarily associated with $G\alpha_i$, recent reports suggest that CXCR4 may be associated with other $G\alpha$ subunits as well [66]. The four $G\alpha$ subunits can relay CXCR4-CXCL12 signaling through different routes. After activating the relevant downstream pathways, GTP is converted to GDP, thereby resulting in reassembly of the CXCR4 receptor and its associated G-proteins. The $G\alpha_s$ subunit activates adenylyl cyclase to produce cyclic AMP (cAMP) to stimulate Protein kinase A (PKA) in order to activate Cyclic AMP-responsive element-binding protein or CREB downstream. CREB is a transcriptional factor that regulates gene expression of Glutathione S-transferase protein 1 (GSTP1) [67] thereby directly causing tumor growth. The $G\alpha_i$ subunit is pertussis toxin sensitive and activates PLC to produce diacylglycerol (DAG) and inositol triphosphate (IP3) to subsequently release intracellular calcium, thereby inducing chemotactic movement. The protein kinase C family can also be stimulated by DAG. $PKC\alpha$ phosphorylates $I\kappa\beta$ and activates it, after which it can translocate to the nucleus and aid in NF κ B-mediated transcription of genes associated with cancer progression [68]. The activation of MAPK pathways through $G\alpha_i$ and $G\alpha_q$ subunit has been implicated in cellular chemotaxis. PI3K activation by both the $G\beta\gamma$ dimer and $G\alpha$ monomer has also been demonstrated to promote CXCL12-dependant chemotaxis and migration. PI3K activation leads to the activation of FAK thereby stimulating downstream signaling pathways involving cellular migration.

CXCR4 receptor phosphorylation by GPCR kinases plays a pivotal role in regulating CXCR4 downstream signaling. CXCL12-dependent CXCR4 phosphorylation promotes receptor desensitization and promotes G α independent signaling, arrestin recruitment, thereby leading receptor endocytosis [69]. GRK2, GRK3 and GRK6 kinases induce CXCL12-dependant phosphorylation of seven serine residues in the C-terminal region of the CXCR4 receptor. Downstream ERK1/2 activation is facilitated by GRK3- and GRK6- mediated phosphorylation, while ERK activation as well as calcium mobilization is suppressed on GRK2- mediated CXCR4 phosphorylation. Thus, the serine-threonine rich cytoplasmic tail of CXCR4 is highly essential in mediating downstream signaling independent of G-protein mediated pathways. Recent reports suggest that the cytoplasmic tail may also be essential in acting as a conduit of G protein-dependent signaling.

Involvement of CXCR4 in primary tumor dissemination and metastasis

Aberrant overexpression of CXCR4 in breast cancer is tightly related to significantly elevated invasive potential. In 2011, Yagi et al demonstrated that CXCR4 is essential for local invasion of tumor cells and subsequently for transendothelial migration across the endothelial barrier. This study showed that CXCL12-CXCR4 enhances intravasation through the downstream activation of the small GTPase Rho [70]. Hypoxic conditions induce CXCR4 expression in tumor cells along with concomitant endothelial CXCL12 expression in the tumor microenvironment to facilitate invasion and transendothelial migration. Therefore CXCR4 is critical in the initial phases of metastatic dissemination. A breakthrough study in 2001 demonstrated that tumor cells travel around the body in response to chemokine signals. This study showed, for the very first time, the critical nature of CXCL12-CXCR4 engagement in

promoting metastasis in lung and other CXCL12-rich secondary sites [64]. Subsequent studies have demonstrated that disrupting the CXCR4-CXCL12 interaction or silencing of the CXCR4 receptor dramatically reduces breast cancer metastasis [71, 72]

Therapeutic implications and challenges

Successful disruption of CXCL12-CXCR4 engagement and downstream processes in cancer cells is pivotal to our ability to interdict, or even reverse the metastatic process. It is interesting to note that certain anti-cancer therapy like chemotherapy and radiation therapy promotes hypoxia which directly results in CXCR4 expression. This further demonstrates that disruption of CXCR4 or inhibiting CXCR4 overexpression in cancer cells is critical for effective treatment. The CXCR4 specific antagonist AMD3100 has been demonstrated to successfully inhibit metastasis in animal models and has also been tested in clinical trials along with other CXCR4 antagonists (ClinicalTrials. Gov identifier: NCT01120457). However, there are several potential drawbacks to using CXCR4 antagonists as an adjuvant therapeutic tool to curb metastatic spread in human patients. Owing to the fact that the CXCR4-CXCL12 interaction is an intrinsic part of the immune system and mediates a myriad of immune responses, long-term treatment of CXCR4 antagonists causes potentially lethal side effects on the immune system. In addition, CXCR4 antagonists severely disrupt the process of homing of hematopoietic stem cells (HSCs) in the bone marrow. Therefore, it is imperative to inhibit abnormal upregulation of CXCR4 expression in tumor cells specifically, without global CXCR4 inhibition. Therefore, identifying genes upstream of CXCR4 which can induce its expression in tumor cells specifically is crucial and can serve as potential therapeutic targets to curb aberrant CXCR4-CXCL12 signaling in the metastatic progression of breast cancer.

CHAPTER 2: KRÜPPEL-LIKE FACTOR 8 ACTIVATES THE TRANSCRIPTION OF C-X-C CYTOKINE RECEPTOR TYPE 4 TO PROMOTE BREAST CANCER CELL INVASION, TRANSENDOTHELIAL MIGRATION AND METASTASIS

Abstract

Krüppel-like factor 8 (KLF8) has been strongly implicated in breast cancer metastasis. However, the underlying mechanisms remain largely unknown. Here we report a novel signaling from KLF8 to C-X-C cytokine receptor type 4 (CXCR4) in breast cancer. Overexpression of KLF8 in MCF-10A cells induced CXCR4 expression at both mRNA and protein levels, as determined by quantitative real-time PCR and immunoblotting. This induction was well correlated with increased Boyden chamber migration, matrigel invasion and transendothelial migration (TEM) of the cells towards the ligand CXCL12. On the other hand, knockdown of KLF8 in MDA-MB-231 cells reduced CXCR4 expression associated with decreased cell migration, invasion and TEM towards CXCL12. Histological and database mining analyses of independent cohorts of patient tissue microarrays revealed a correlation of aberrant co-elevation of KLF8 and CXCR4 with metastatic potential. Promoter analysis indicated that KLF8 directly binds and activates the human CXCR4 gene promoter. Interestingly, a CXCR4-dependent activation of focal adhesion kinase (FAK), a known upregulator of KLF8, was highly induced by CXCL12 treatment in KLF8-overexpressing, but not KLF8 deficient cells. This activation of FAK in turn induced a further increase in KLF8 expression. Xenograft studies showed that overexpression of CXCR4, but not a dominant-negative mutant of it, in the MDA-MB-231 cells prevented the invasive growth of primary tumor and lung metastasis from inhibition by knockdown of KLF8. These results collectively suggest a critical role for a previously

unidentified feed-forward signaling wheel made of KLF8, CXCR4 and FAK in promoting breast cancer metastasis and shed new light on potentially more effective anti-cancer strategies.

Introduction

Breast cancer is one of the leading causes of cancer-related deaths among women [73, 74]. Current mainstream strategies including surgical resection and adjuvant therapies are effective for well-confined primary tumors only. However, metastatic breast cancer remains largely incurable and responsible for 90% of the patient deaths [1, 30]. Metastasis is an event of sequential steps including initial dissociation of some cells from the primary tumor, invasion through the stroma to access vascular vessels, penetration through the vessel wall via TEM to enter the blood or intravasation, traveling with blood, leaving the circulation via TEM once again or extravasation to land on distant organs/tissues, initial secondary growth to form a dormant micrometastasis, and finally re-initiation of a massive growth to form metastasis or colonization [75]. Yet, the precise mechanisms underlying these steps remain poorly understood. A proper elucidation of these mechanisms is critical for better diagnostic and therapeutic strategies.

KLF8 is a dual transcription factor known to express at marginally detectable levels in most normal tissue types and aberrantly overexpress in a number of human cancer types including breast cancer [40]. Cell culture studies have demonstrated a critical role of KLF8 in promoting cell cycle progression [40, 43, 54], transformation [56, 76], epithelial-to-mesenchymal transition (EMT) [40, 49, 77, 78], cancer stem cell induction [55] and DNA-damage response [79]. Patient tissue analyses have revealed a strong correlation of KLF8 expression with metastatic potential [50, 79]. Xenograft studies have pointed to the role of KLF8

in promoting invasive growth and metastasis of breast cancer [50-53]. Despite the discovery of an array of target genes and microRNAs of KLF8 that are associated with cancer [40, 55, 76] and mechanisms regulating KLF8 at protein and subcellular levels [40, 44-47, 79-81], research on the molecular mechanisms by which KLF8 promotes metastasis remains in its infancy.

CXCR4 is a receptor for chemokine CXCL12 and has recently been linked to breast cancer metastasis [63, 82, 83]. The receptor-ligand interaction was initially implicated in HIV infection of T lymphocytes [62, 84-86], chemotaxis and homing of CXCR4-high leucocytes and hematopoietic stem cells to CXCL12-rich inflammatory sites, lymphoid organs and bone marrow [63]. Recent studies have drawn much attention to CXCR4-high cancer cell metastasis to CXCL12-rich tissues suggesting that such cancer cells have hijacked the CXCL12-guided mechanism of cell homing to establish metastasis [63, 64, 82, 87-89]. Indeed, breast cancer cells metastasize preferentially to CXCL12-rich tissues such as the lungs and bone marrow [1, 63-65, 82, 87, 88]. Inhibition of CXCR4-CXCL12 interaction has led to reduction in experimental metastasis of breast cancer [71, 72, 90]. Aberrant CXCR4 expression have been found in patient tissues of breast cancer [64, 88, 91, 92].

In this report, we show strong evidence that KLF8 directly activates the CXCR4 gene promoter. This activation causes an increased CXCR4 expression and subsequent feed-forward activation of FAK, resulting in CXCR4/CXCL12-dependent increase in migration, invasion, TEM and establishment of secondary metastasis in breast cancer cells. These novel findings may guide development of more effective and less toxic, targeted therapeutic strategies.

Materials & Methods

Antibodies and reagents

Antibodies include mouse monoclonal for HA-probe (F-7) (sc-7392), β -actin (C4) (sc-47778), and GFP (sc-101525) and rabbit polyclonal for FAK (sc-557) (Santa Cruz Biotechnology Inc, Dallas, TX, USA), CXCR4 rabbit polyclonal Ab (Ab-2074) (Abcam, Cambridge, MA, USA), and pY397-FAK rabbit monoclonal Ab (Invitrogen, 44625G, Carlsbad, CA, USA), and vimentin mouse monoclonal Ab (550513) (BD Pharmingen, San Jose, CA, USA). Anti-KLF8 rabbit polyclonal Ab was described previously [51, 52]. Peroxidase substrate kit (DAB) (SK-4100) was purchased from Vector laboratories Inc. (Burlingame, CA, USA). The CXCR4 inhibitor AMD3100 (or octahydrochloride hydrate) (A5602) and the FAK inhibitor PF573228 (PZ0117) were purchased from Sigma (St. Louis, MO, USA). Recombinant human CXCL12 (300-28A) was purchased from Peprotech (Rocky Hill, CT, USA).

Plasmid construction, cell line generation, cell culture and transfection

The mammalian expression plasmids pKH3, pKH3-KLF8 and pKH3-mKLF8 were previously described [45, 50]. The human CXCR4 gene promoter (-2088 base pairs) was cloned by PCR from template genomic DNA isolated from MDA-MB-231 cells using the Wizard Genomic DNA purification Kit (A1120) from Promega (Madison, WI, USA). The PCR product was cut with Kpn1 and Nhe 1 and ligated to the respective sites in the pGL3basic vector (Promega, Madison, WI, USA) to form the pGL3b-CXCR4p promoter reporter plasmid. The promoter truncation mutant plasmids were constructed by PCR deletion of desired promoter fragment from the pGL3b-CXCR4p plasmid. The GT-box specific mutations were created by site-directed mutagenesis overlapping PCR [93-95] using the pGL3b-CXCR4p vector as the

template. The CXCR4 cDNA was PCR-synthesized from mRNA isolated from MDA-MB-231 cells and cloned into the pKH3 vector between the SmaI and EcoRI sites to form the pKH3-CXCR4 plasmid. The amino terminal 20 amino acids of CXCR4 in the plasmid were removed by deletion PCR to obtain the dominant-negative mutant plasmid pKH3-CXCR4-dN20. To construct lentiviral vectors pLVZP-CXCR4 and pLVZP-CXCR4-dN20, we sub-cloned HA-CXCR4 and HA-CXCR4-dN20 from the pKH3-CXCR4 and pKH3-CXCR4-dN20 plasmids respectively, into the lentiviral vector pLVPZ between the PstI and NotI sites [51] by PCR. All the primers are listed in Supplementary Information. All the constructs were verified by DNA sequencing. The 231-K8ikd cell line expressing ectopic CXCR4 or its dN20 mutant was generated by infecting the 231-K8ikd cells with the lentivirus of pLVZP-CXCR4 or pLVZP-CXCR4-dN20 followed by puromycin selection. The HEK293, NIH3T3, MCF-10A, MDA-MB-231, the MCF-10A that expresses doxycycline-inducible KLF8 (10A-iK8), the MDA-MB-231 that expresses doxycycline-inducible shRNA against KLF8 (231-K8ikd) were described previously [50]. These cells were maintained in Dulbecco's modified Eagle's medium/F-12 with 5% horse serum or Dulbecco's modified Eagle's medium with 10% fetal bovine serum. According to experimental conditions, the inducible cell lines were in the absence (U) or presence (I) of doxycycline. Doxycycline hydrochloride was purchased from Sigma (D3072) (St. Louis, MO, USA). Plasmid DNA transfections were performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA).

Quantitative real time-PCR (qRT-PCR) and western blotting

Cell lines and antibodies used have been described above. qRT-PCR was performed as previously described [49, 50, 52, 56, 76]. Western blotting for CXCR4 was conducted as previously described by A. Marchese [96, 97].

Bioinformatics

Breast cancer microarray data of GEO DataSet (GSE9014) was downloaded from GEO website ([http:// www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) (78). RNA sequencing data of 817 breast cancer samples was downloaded from TCGA Data portal (<https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>) (79). Pearson's correlation was analyzed between KLF8 and CXCR4.

Promoter reporter, chromatin immunoprecipitation (ChIP) and biotinylated oligonucleotide precipitation (BOP) assays

These assays were performed essentially as previously described [50, 52]. For reporter assays, cells were co-transfected with each of the pGL3b-CXCR4p reporter or mutant plasmids and pKH3-KLF8, pKH3-mKLF8 or pKH3 vector alongside a Renilla luciferase reporter internal control for 16-18 hours followed by quantification of luciferase activity. ChIP assays were performed by transfecting HEK293 cells with pKH3-KLF8 or pKH3 control vector followed by anti-HA co-precipitation of promoter fragments. HA antibody-conjugated agarose beads were purchased from Sigma (St. Louis, MO, USA). The control IgG was purchased from Jackson ImmunoResearch, Inc. (West Grove, PA, USA). For the BOP assays, the lysates of HEK293 cells transfected with pKH3-KLF8 were processed for analysis. (See Supplementary Information for ChIP primers and BOP oligonucleotides).

Boyden chamber migration and matrigel invasion assays

The chambers were purchased from BD Biosciences (San Jose, CA, USA) and the assays were performed as instructed by the manufacturer with necessary modifications as previously described [49-53]. Cells were incubated inside the upper inserts in serum-free conditions. In some specified cases, the cells were pre-treated with AMD3100 (35 ng/ml) or PF573228 (1 μ M) for three hours prior to seeding into the insert. After allowed for 20 hours to migrate or invade towards recombinant CXCL12 (200 ng/ml) in the bottom chamber, the cells on the undersurface of the insert were stained with 0.2% Crystal Violet in 10% methanol and processed for quantitative analysis.

Transendothelial migration (TEM) assay

TEM assay was performed using the CytoSelect Tumor TEM assay kit (CBA-216, Cell Biolabs, Inc. San Diego, CA, and USA). Briefly, HUVEC cells (25,000 – 50,000) (CRL-2922, ATCC, Manassas, VA, USA) were allowed up to 72 hours to form a monolayer on the upper surface of the membrane inside the insert and then activated by overnight serum starvation or TNF α treatment. The 10A-iK8 or 231-K8ikd cells (50,000) were incubated with the Cytotracker dye for 1 hour prior to further incubation in the insert on top of the HUVEC monolayer in the presence or absence of doxycycline. Depending upon specific experimental need, AMD3100 (35 ng/ml) was included to inhibit CXCR4 prior to and during the 22-hour assay time. The cells that migrated through the HUVEC monolayer were lysed and quantified using a Fluorescence multi-plate reader (PolarStar Omega, BMG LabTech, Cary, NC, USA).

Bioluminescent imaging (BLI) analysis of tumor growth and metastasis

Female Balb/c nude mice (NCI, Frederick, MD, USA) of 4-6 weeks old (six per group) were used for testing orthotopic tumor growth and lung metastasis by the 231-K8ikd cells with or without the stable overexpression of CXCR4 or its dN20 mutant. The cells were implanted prior to induction of KLF8 knockdown in the mice. The mammary fat pad and tail vein injection of the cells, the Dox Diet feeding, and the weekly BLI monitoring of the tumor growth or lung metastasis were performed as previously described [50-53]. The mice were maintained at the UCF vivarium under conditions approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. The animal experiments were guided by the university-approved IACUC protocol with thorough consideration of humane care of the mice.

Hematoxylin and eosin (H/E) and immunohistochemical (IHC) staining

The collection and preparation of the primary and metastatic tumor tissues, the human breast cancer tissue array used, and the histological analysis procedures were previously described (9, 16-19). The antibodies for KLF8, CXCR4, GFP and vimentin were described above.

Statistical analysis

Mean +/- standard deviation is presented with a minimum of three observations per group. Student's t-test, unpaired, paired or single sample, with the Bonferroni correction for the multiple comparisons was applied as appropriate. The two by two tables were analyzed by

Fisher's exact test. The alpha level of 0.05 was used to determine difference with statistical significance.

Supplementary Materials

The PCR primers used to clone the human CXCR4 gene promoter:

CXCR4p-F, 5'- ACG CGG TAC CGT GCA GCT TAC GGT C -3'

CXCR4p-R, 5'- TGT CCG CTA GCA TGG TAA CCG CTG GTT C-3'.

Primers used to generate the truncation mutants of the human CXCR4 gene promoter:

The CXCR4p-R primer described above was used for constructing all the truncations. All the forward primers listed below contained a KpnI restriction site.

-1520CXCR4p-F, 5'-AAC TGG TAC CGG AAC ACT CCA GAC TG -3'

-1372CXCR4p-F, 5' - TCG GTG GTA CCT ATC TCC GCA GCG-3'

-1230CXCR4p-F, 5'-TGC AAG GTA CCG CCA GTC TGA GAT CG -3'

-397CXCR4p-F, 5'- ACG CGG TAC CGA GAG ACG CGT TCC -3'

-217CXCR4p-F, 5'- ACC TGG TAC CTC CTT CCT CGC GTC TGC - 3'

-150CXCR4p-F, 5'- ATG CGG TAC CCT CGG AGC GTG T - 3'

Primers used to construct the GT-box specific mutants of the human CXCR4 gene promoter:

CXCR4p-mGT1-F, 5'- TCC CCT CTC ACG CCT TCT CCC TC -3'

CXCR4p-mGT1-R, 5'- AAG GCG TGA GAG GGG AGA GGG GCA -3'

CXCR4p-mGT4-F, 5'- CGC CCT CTC AAG TCT CCG CGC CGG AG - 3'

CXCR4p-mGT4-R, 5'- AGA CTT GAG AGG GCG GGG GAG CGA GTG -3'

CXCR4p-mGT5-F, 5'- AGC GGC TCT CAC GCC TGC GGA C- 3'

CXCR4p-mGT5-R, 5'- AGG CGT GAG AGC CGC TGC GGA GAT A- 3'

CXCR4p-mGT7-F, 5'- AGC CAC TCT CAT CCA AGA ACC TGG- 3'

CXCR4p-mGT7-R, 5' - TTG GAT GAG AGT GGC TGG AAG AGG- 3'

These mutant primer sets for each of the mutant GT-box (mGT) were used along with the CXCR4p-F/CXCR4p-R primer set described above.

Primers used to clone the cDNA of the human CXCR4 and the dN20 mutant:

hCXCR4-Sma-F, 5'- TCC CCC GGG CGT GGA GGG GAT CAG TAT ATA -3'

hCXCR4-dN20-Sma-F, 5'- TCC CCC GGG CTA TGA CTC CAT GAA GGA AC -3'

hCXCR4-RI-R, 5'- AGG AAT TCT TAG CTG GAG TGA AAA CTT G -3'

Primers used to construct the pLVZP-CXCR4 and pLVZP-CXCR4-dN20:

pKH3-F, 5'- CCC AAG CTT CTG CAG GTC G - 3'

pLVPZ-CXCR4-Not1-R, 5' - ATA AGA ATG CGG CCG CTT AGC GAA AAC -3'

Results

KLF8 upregulates CXCR4 expression in invasive breast cancer

Aberrant CXCR4 overexpression has been implicated in breast tumor growth and metastasis [64, 82, 91, 98]. Our previous study showed that KLF8 expression is undetectable in breast epithelial cells like MCF-10A, while it is aberrantly overexpressed in invasive breast cancer cells such as MDA-MB-231[49]. Furthermore, CXCR4 is highly expressed in MDA-MB-231 cell-line and shows very low expression in MCF-10A cells. Our previous cDNA microarray analysis showed that CXCR4 mRNA expression is significantly upregulated upon overexpression of KLF8 [99]. To verify this result, we examined the changes in CXCR4 expression in response to doxycycline-induced overexpression or knockdown of KLF8 in our MCF-10A cell line expressing inducible KLF8 (or 10A-iK8) and MDA-MB-231 cell line expressing inducible shRNA against KLF8, respectively [50-53] using quantitative real-time PCR (qRT-PCR) (Figure 1A, top panel), semi-quantitative reverse-transcription PCR (Figure 1A, middle panel) , and western blotting (Figure 1A, bottom panel). The expression of CXCR4 was markedly increased at both mRNA and protein levels when KLF8 expression was induced in the CXCR4-low 10A-iK8. Conversely, knockdown of KLF8 in the CXCR4-high 231-K8ikd cells [70, 100-102] led to significant CXCR4 reduction.

It is known that both KLF8 and CXCR4 are aberrantly overexpressed in breast cancer [63, 82, 89]. To test if KLF8 and CXCR4 are co-overexpressed in patient tumors, we performed immunohistochemical (IHC) staining for CXCR4 in a human breast cancer tissue microarray in which KLF8 expression was determined previously [50-53] (Figure 1B, images). We found that

89.28% of the KLF8-positive tumors also express CXCR4. By contrast, among the KLF8-negative samples, only 29.78% express CXCR4 (Figure 1B, top-right table). We also found that their co-expression is positively correlated with the invasive potential (Figure 1B, bottom-right table). This result was further demonstrated by OncoPrint analysis of an independent cohort of patient tissues (Figure 1C top). Furthermore, microarray data profiling of invasive human breast cancer tissues downloaded from GEO and TCGA database was applied to examine the co-expression of KLF8 and CXCR4. The Pearson's correlation analysis identified the positive correlation of KLF8 and CXCR4 in invasive breast cancers (Figure 1C bottom and Figure 1D), specifically in invasive ductal carcinoma (IDC) (Figure 1D).

These results strongly suggest that CXCR4 expression could be aberrantly upregulated by KLF8 in breast cancer cells particularly in invasive tumors.

KLF8 directly targets CXCR4 gene promoter for transcriptional activation

KLF8 regulates target gene promoters by binding to a CACCC (or GGGTG) or GT-box site [42, 45]. Since our results indicated that KLF8 can induce CXCR4 expression at both mRNA and protein levels, we tested whether KLF8 regulates the transcription of CXCR4. We cloned the human CXCR4 gene promoter (CXCR4p) that contains seven GT-boxes (Figure 2A). We first tested if the activity of the CXCR4p can be regulated by KLF8. The promoter reporter assay showed that co-expression of KLF8 in NIH3T3 (as well as HEK293. Not shown) cells caused >10-fold increase in the promoter activity (Figure 2B). By contrast, the activation domain-

deficient mutant of KLF8 (mKLF8) [45] failed to do so. This result suggests that KLF8 regulates CXCR4 expression by transcriptional activation.

We then attempted to map the KLF8 responding region in CXCR4p by step-wise truncation of CXCR4p. We found that truncation through -217 bp position did not significantly prevent the promoter from responding to KLF8 until the deletion to the -150 bp position (Figure 2C). This result suggests that the promoter region between -217 bp and -150 bp is the most critical for the activation of CXCR4p by KLF8. And this region contains GT-box 1.

We then tried to determine if the GT-box 1 plays a role for the activation of CXCR4p by KLF8 by disabling the GT-box 1 in the full-length CXCR4p. Subsequent reporter assays showed that activation of CXCR4p by KLF8 was completely prevented by mutation of the GT-box 1, but none of the other GT-boxes indicated (Figure 2D). This result suggests that the GT-box 1 site is required for KLF8-mediated activation of CXCR4p.

To determine if KLF8 directly binds GT-box 1, we performed chromatin immunoprecipitation (ChIP) assays using HEK293 cells overexpressing HA-KLF8. A fragment of endogenous CXCR4p that spans the GT-box 1 was specifically co-precipitated by the anti-HA antibody but not by the control IgG (Figure 2E). This was further corroborated by biotinylated-oligonucleotide precipitation (BOP) assay showing that KLF8 can bind the CXCR4 promoter region in a GT-box 1-dependent manner given that the binding was abolished by GT-box 1 disruption (mGT1) (Figure 2F).

These results demonstrate that KLF8 directly activates the transcription of CXCR4 likely through binding CXCR4p at GT-box 1.

KLF8 promotes CXCL12-stimulated cell migration and invasion

CXCR4 mediates cell trafficking along a chemotactic gradient CXCL12 [63, 82, 89]. To test if KLF8 can positively regulate cell migration and invasion towards CXCL12, we performed Boyden chamber migration and matrigel invasion assays, respectively. The rates of both the cell migration and invasion were significantly increased when overexpression of KLF8 was induced in the 10A-iK8 cells (Figure 3A and 3B). Conversely, the 231K8-ikd cells migrated and invaded at a much slower rate upon knockdown of KLF8 (Figure 3C and 3D). In the absence of CXCL12 as a chemoattractant, migration and invasion rate of 231K8-ikd cells remains unchanged upon KLF8 knockdown [50]. Importantly, the knockdown-induced decrease in invasion was fully recovered by stable overexpression of CXCR4, but not its ligand binding-defective mutant dN20 [103] (Figure 3D).

These results strongly suggest that KLF8 promotes CXCL12-dependant migration and invasion by upregulating the expression of CXCR4 in the cell.

KLF8 promotes CXCR4-dependent transendothelial migration (TEM) towards CXCL12

Metastatic tumor cell disseminates by invading the stromal matrix, followed by crossing the endothelial barrier via TEM to enter the blood stream. TEM is further implicated in the exit of circulating tumor cells from blood stream to land on a distant site [104]. Previous reports suggested that the CXCR4/CXCL12 signaling-mediated chemotaxis may be involved in

migration of CXCR4⁺ cancer cells towards an increasing gradient of CXCL12 across vascular wall [104, 105]. We thus tested if KLF8 promotes CXCR4-dependent TEM towards CXCL12 (Figure 4A). We found that induction of KLF8 overexpression in the 10A-iK8 cells led to a > 2-fold increase in TEM rate which was blocked by the CXCR4-specific antagonist AMD3100 [89] (Figure 4B). Conversely, TEM rate of the 231-K8ikd cells was markedly reduced upon knockdown of KLF8 or treatment with AMD3100 (Figure 4B). Overexpression of CXCR4, but not its dN20 mutant, protected the TEM capability of the cells from inhibition by knockdown of KLF8 (Figure 4D). These results strongly indicate that KLF8 plays a critical role in promoting TEM requiring CXCR4 engagement during intravasation and/or extravasation.

Upregulation of CXCR4 by KLF8 leads to a CXCL12-dependent feed-forward activation of FAK upstream of KLF8

Previous reports suggested that engagement of CXCR4 by CXCL12 causes the activation of FAK that contributes to CXCL12-dependant chemotaxis in breast cancer cells [106, 107]. To test if FAK can be activated by CXCL12 stimulation and whether KLF8 plays a role, we performed western blotting for the phosphorylation of FAK at tyrosine 397 (or pFAK) [108, 109] in the 10A-iK8 cells (Figure 5A). When the overexpression of KLF8 was not induced, CXCL12 treatment could not activate FAK (Figure 5A, left panel). By contrast, when the overexpression of KLF8 was induced, CXCL12 stimulated a rapid activation of FAK without affecting the overall expression of FAK (Figure 5A, middle panel), which was prevented by treatment with AMD3100 (Figure 5A, right panel). These results suggest that the induction of CXCR4 expression by KLF8 and subsequent ligand engagement lead to the activation of FAK.

Interestingly, FAK was initially identified as the upstream inducer of KLF8 expression [43]. We thus tested if the CXCL12-stimulated FAK activation increases KLF8 expression. We found that CXCL12 exposure led to a further increase in KLF8 mRNA level in the 10A-iK8 cells when the overexpression of the ectopic KLF8 was induced, which was prevented by pre-inactivation of either CXCR4 or FAK (Figure 5B).

Taken together, these results point to a potentially important feed-forward signaling wheel consisting of KLF8, CXCR4 and FAK and CXCL12 is a critical driving force for the cycling of the wheel in breast cancer cells (Figure 5C).

KLF8 promotes CXCR4-dependent invasive growth of the primary tumor

To determine the extent to which CXCR4 contributes to primary breast tumor progression downstream of KLF8, we injected the 231-K8ikd cells, the 231-K8ikd cells with stable overexpression of CXCR4 or its dN20 mutant into the mammary fat pad, induced the knockdown of KLF8 *in vivo* and examined the growth and invasion of the orthotopic tumor. The 231-K8ikd cell line stably expresses a GFP and luciferase fusion protein which was utilized to track the tumor growth and invasiveness[50]. As expected, knockdown of KLF8 (I) significantly slowed down the tumor growth (Figure 6A and 6B, compare I with U). However, this reduction was completely prevented by overexpression of CXCR4, but not its dN20 mutant (Figure 6 A and 6B, compare I+CXCR4 or I+dN20 with I). Histological analyses revealed that the dramatic inhibition of the tumor invasion into the surrounding tissues by knockdown of KLF8 (Figure 6C,

compare I with U) was also well prevented by overexpression of CXCR4, but not its dN20 mutant (Figure 6C, compare I+CXCR4 or I+dN20 with I).

These results suggest that CXCR4 plays a critical role downstream of KLF8 in mediating the primary tumor growth and invasion where interaction with CXCL12 is essential.

KLF8 promotes CXCR4-dependent lung metastasis

We then examined whether CXCR4 is needed downstream of KLF8 for metastasis. We injected the above-described 231-K8ikd cell lines into the tail veins, induced the knockdown of KLF8 in vivo and examined their lung metastasis. Knockdown of KLF8 caused a dramatic decrease in the lung metastatic rate as determined by bioluminescent imaging (BLI) and whole mount lung observation (Figure 7A and 7B, compare I with U). This decrease was again well prevented by overexpression of CXCR4, but not its dN20 mutant (Figure 7A and 7B, compare I+CXCR4 or I+dN20 with I). These results were subsequently verified by histological analyses using hematoxylin and eosin (H/E) staining and immunohistochemical (IHC) staining for the human tumor cell-specific expression of GFP and vimentin (Figure 7C).

Taken together, our results support a critical role of CXCR4 engagement by CXCL12 downstream of KLF8 for breast cancer metastasis.

Figures

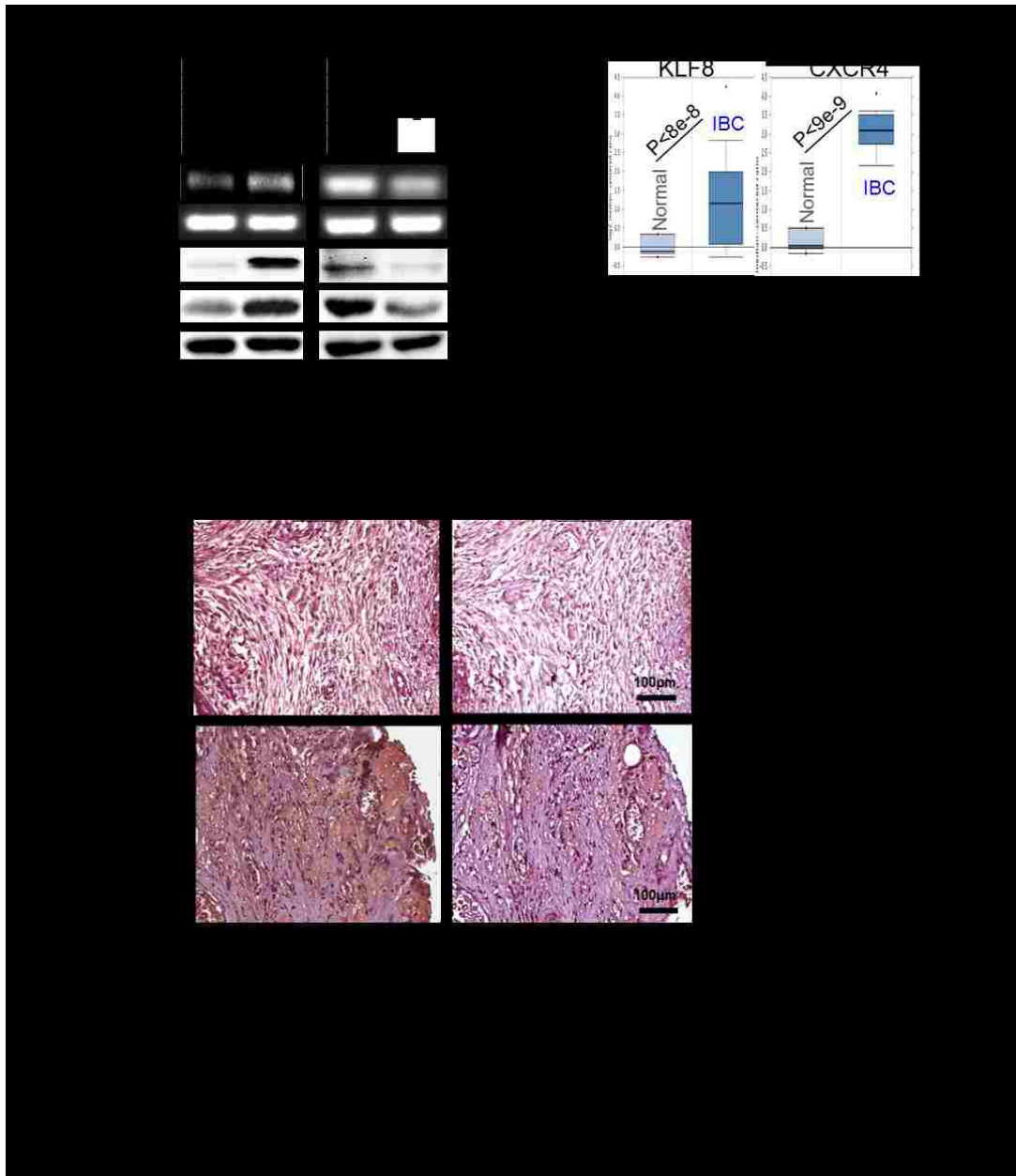


Figure 1. KLF8 upregulates CXCR4 associated with invasive potential.

(a) Overexpression and knockdown of KLF8 induces and reduces CXCR4 expression in the 10A-iK8 and 231-K8ikd cells, respectively. Total RNA and protein lysate were prepared sub-confluent cells grown under doxycycline-uninduced (U) and induced (I) conditions. The level of CXCR4 was determined by qRT-PCR (top graph), semi-quantitative RT-PCR (middle panel) or western blotting (bottom panel). Overexpression of HA-KLF8 and knockdown of KLF8 were examined by western blot. **(b)** Aberrant co-elevation of KLF8 and CXCR4 protein in patient invasive breast carcinoma (IBC) tumors. IHC staining of KLF8 or CXCR4 (brown) in the human breast cancer tissue array containing specimens in duplicate

from 75 patient tumors or normal tissues was performed. Images representing a benign sample negative for both KLF8 and CXCR4 (case 1) and an IBC sample positive for both KLF8 and CXCR4 (case 2) are shown. Correlation of CXCR4 and KLF8 expression is outlined in the tables. (c) Aberrant co-elevation of KLF8 and CXCR4 mRNA in IBC tumors. Oncomine analysis was performed on an independent cohort of Finak dataset containing 6 normal and 53 IBC samples. Pearson correlation of KLF8 and CXCR4 was applied to 53 IBC samples in Finak dataset downloaded from GEO (GSE9014). (d) The expression of KLF8 and CXCR4 is correlated in invasive breast cancers. RNA sequencing data was downloaded from TCGA breast cancer (2015)

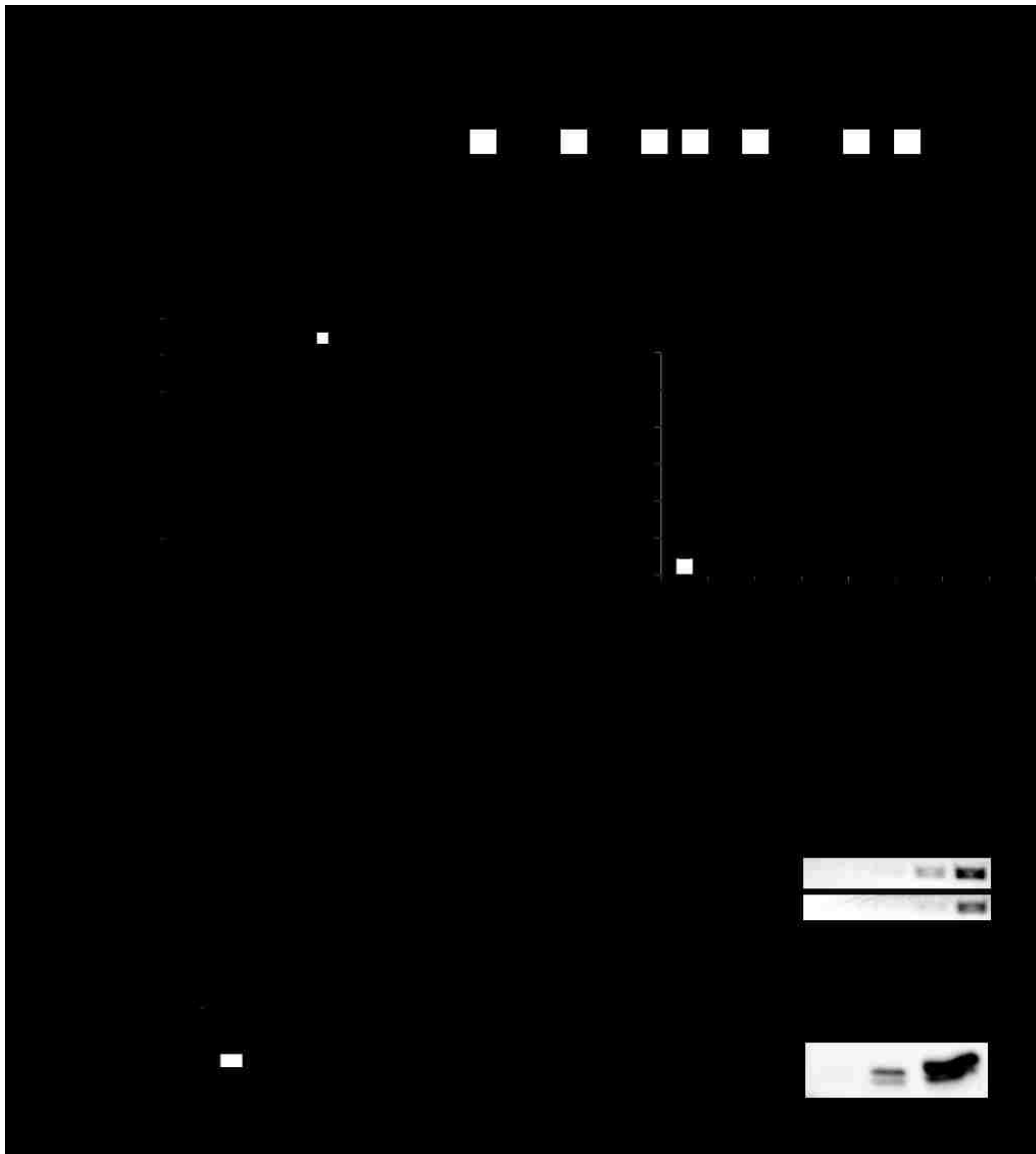


Figure 2. KLF8 upregulates CXCR4 transcriptions

(a) Schematic diagrams of CXCR4p. Potential KLF8-binding sites (GT boxes) are shown. The CXCR4p was isolated from MDA-MB-231 genomic DNA and inserted in the pGL3basic luciferase reporter vector.

(b) KLF8 activates the CXCR4p. The CXCR4p or control vector was con-transfected with KLF8 or activation domain-defective mutant (mKLF8) into NIH3T3 cells. Reporter activity was performed as described in Materials and Methods. **(c)** KLF8 responsive site is located between -150 and 217 bp of the CXCR4p. Serial CXCR4 truncation mutants were constructed and tested for changes in the promoter activation by KLF8. **(d)** The GT-box 1 is required for KLF8 to activate the CXCR4p. Indicated GT boxes were mutated (mGT) and tested for changes in the promoter activation by KLF8. **(e, f)** KLF8 directly binds CXCR4p at the GT-box 1 site. HA-KLF8 was overexpressed in 293 HEK cells. The cells were processed for either ChIP assay using primers spanning the GT-box 1 (e) or BOP assay using oligos spanning the wild type GT1 (WT) or its mutant (mGT1) (f) as described in as described Materials and Methods

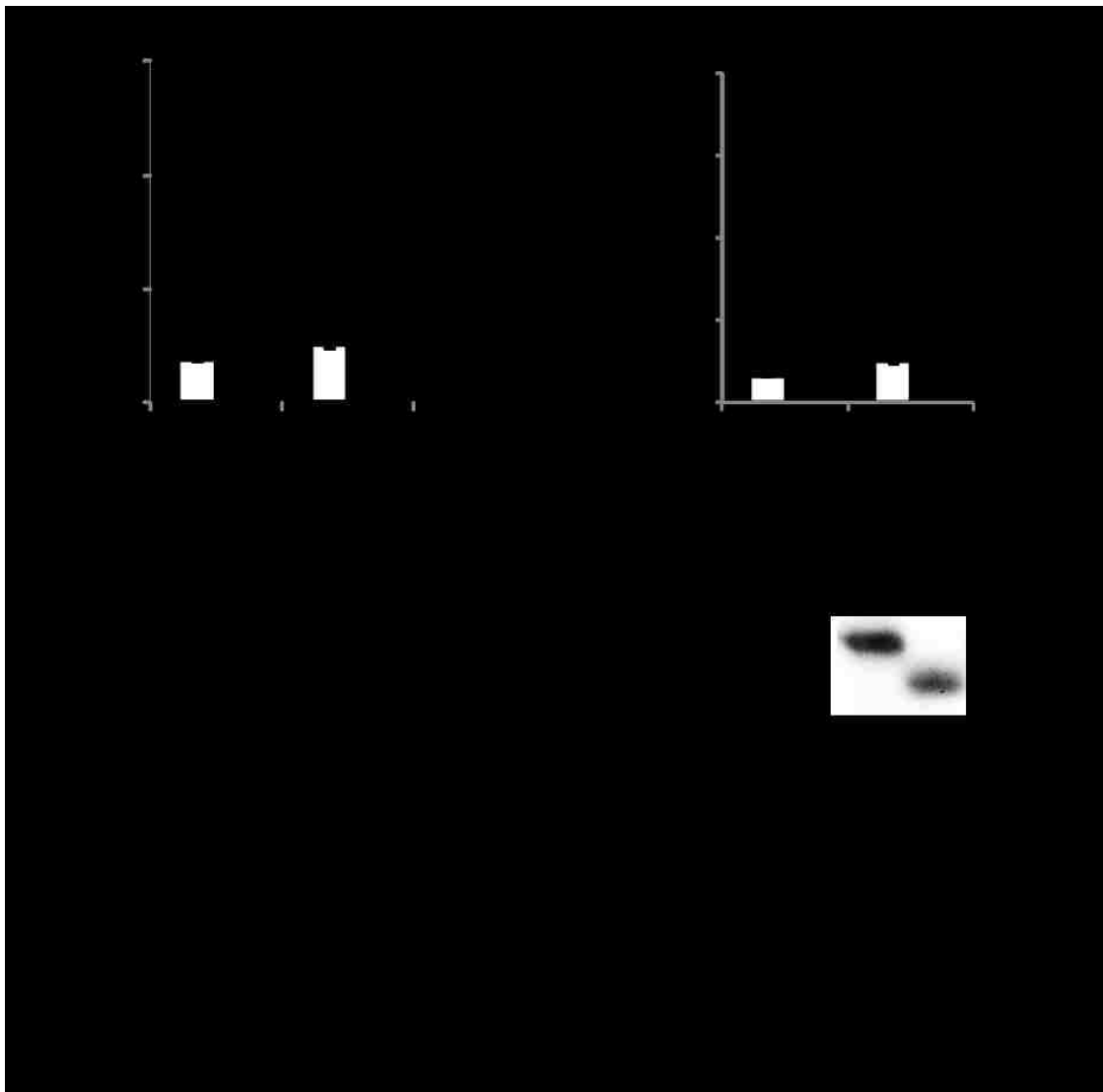


Figure 3. KLF8 promotes cell migration and invasion towards CXCL12.

(a, b) Overexpression of KLF8 increases migration and invasion towards CXCL12. The 10A-ik8 cells grown under U or I conditions were subject to Boyden chamber migration Assay (a) or Matrigel invasion

assay (b). **(c, d)** Knockdown of KLF8 inhibits CXCR4-dependent migration and invasion towards CXCL12. The 231-K8ikd cells grown under U or I conditions with or without overexpression of CXCR4 (WT) or its ligand-binding defective mutant (dN20) were subject to Boyden chamber migration Assay (c) or Matrigel invasion assay (d). Inset, ectopic expression of CXCR4 and dN20 mutant were verified by anti-HA western blotting.

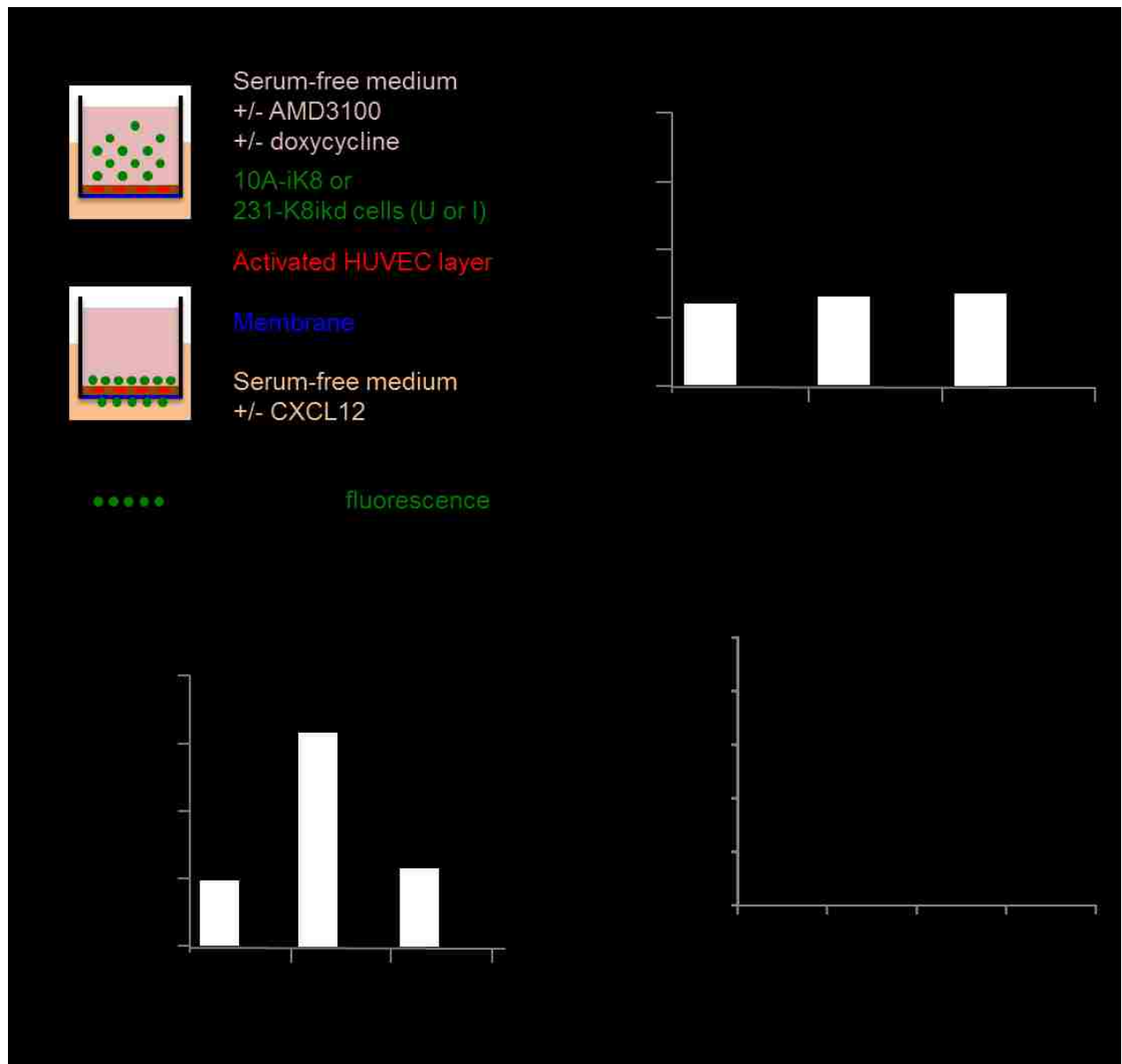


Figure 4. KLF8 promotes CXCR4-dependent TEM towards CXCL12.

(a) Illustration of TEM assay procedure. **(b)** Overexpression of KLF8 is sufficient to promote CXCR4-dependent TEM of 10A-iK8 cells towards CXCL12. **(c)** Knockdown of KLF8 inhibits CXCR4-dependent

TEM of 231-K8ikd cells towards CXCL12. **(d)** Overexpression CXCR4 but not the dN20 mutant prevents inhibition of TEM of 231-K8ikd cells towards CXCL12 by KLF8 knockdown.

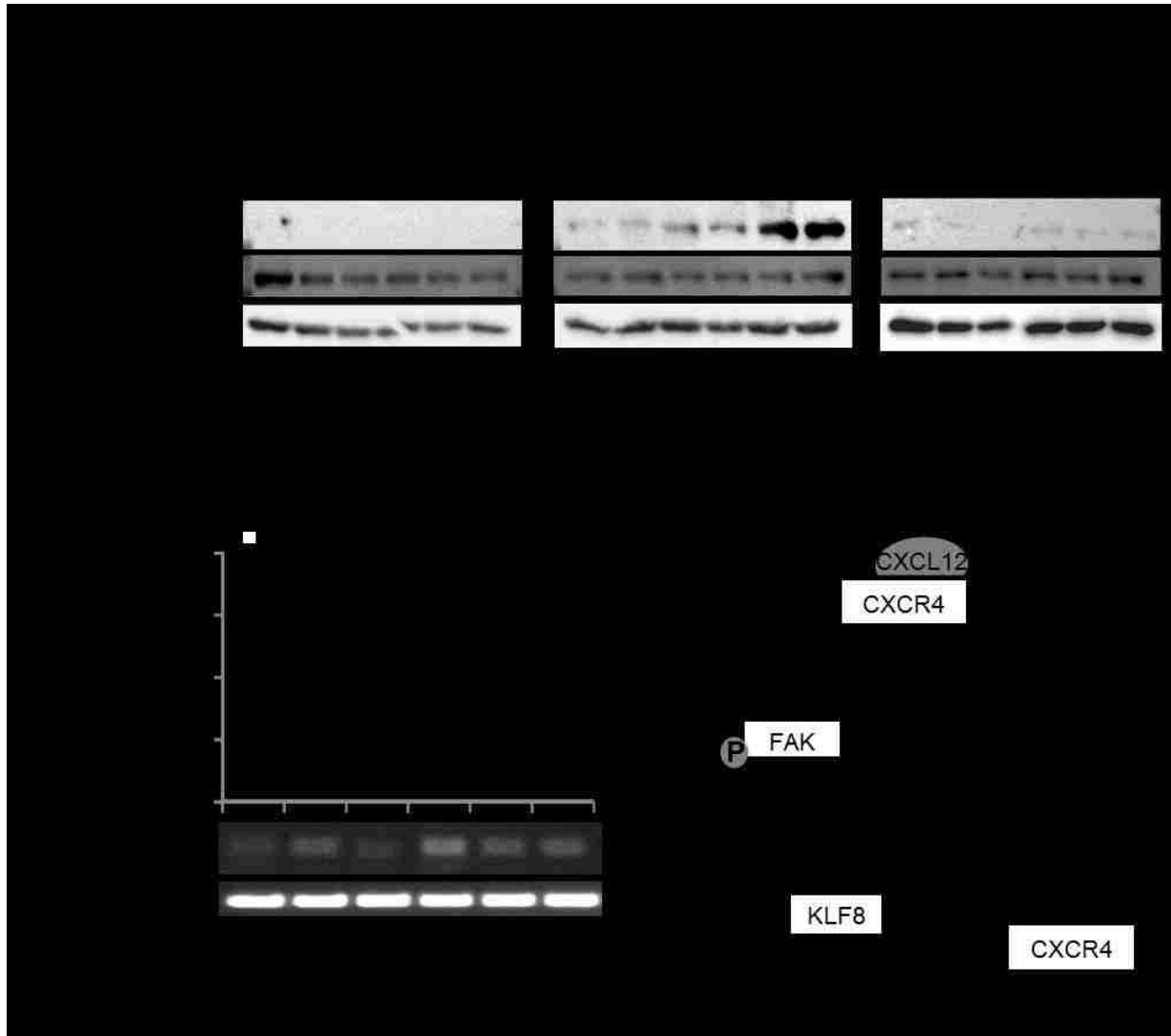


Figure 5. KLF8 upregulation of CXCR4 leads to a feed-forward activation of FAK upstream of KLF8

(a) Overexpression of KLF8 causes CXCL12/CXCR4-dependent activation of FAK. Doxycycline-uninduced (U) and induced (I) 10A-iK8 cells were serum starved for 24 hours followed by 3 hour AMD3100 (35 ng/ml) or mock treatment hours prior to CXCL12 (100ng/ml) stimulation. Whole cell lysates were prepared for western blotting for FAK phosphorylation at Y397 (pFAK) and total FAK. **(b)** Overexpression of KLF8 induces CXCL12/CXCR4-dependent expression of endogenous KLF8. The 10A-iK8 cells were grown and treated similarly as in A except for inclusion of PF223, a FAK-specific inhibitor. Total RNA was prepared for qRT-PCR (top panel) and semi-quantitative RT-PCR (bottom panel). **(c)** Hypothetic model of KLF8 to CXCR4/CXCL12 to pFAK to KLF8 feed-forward signaling loop.

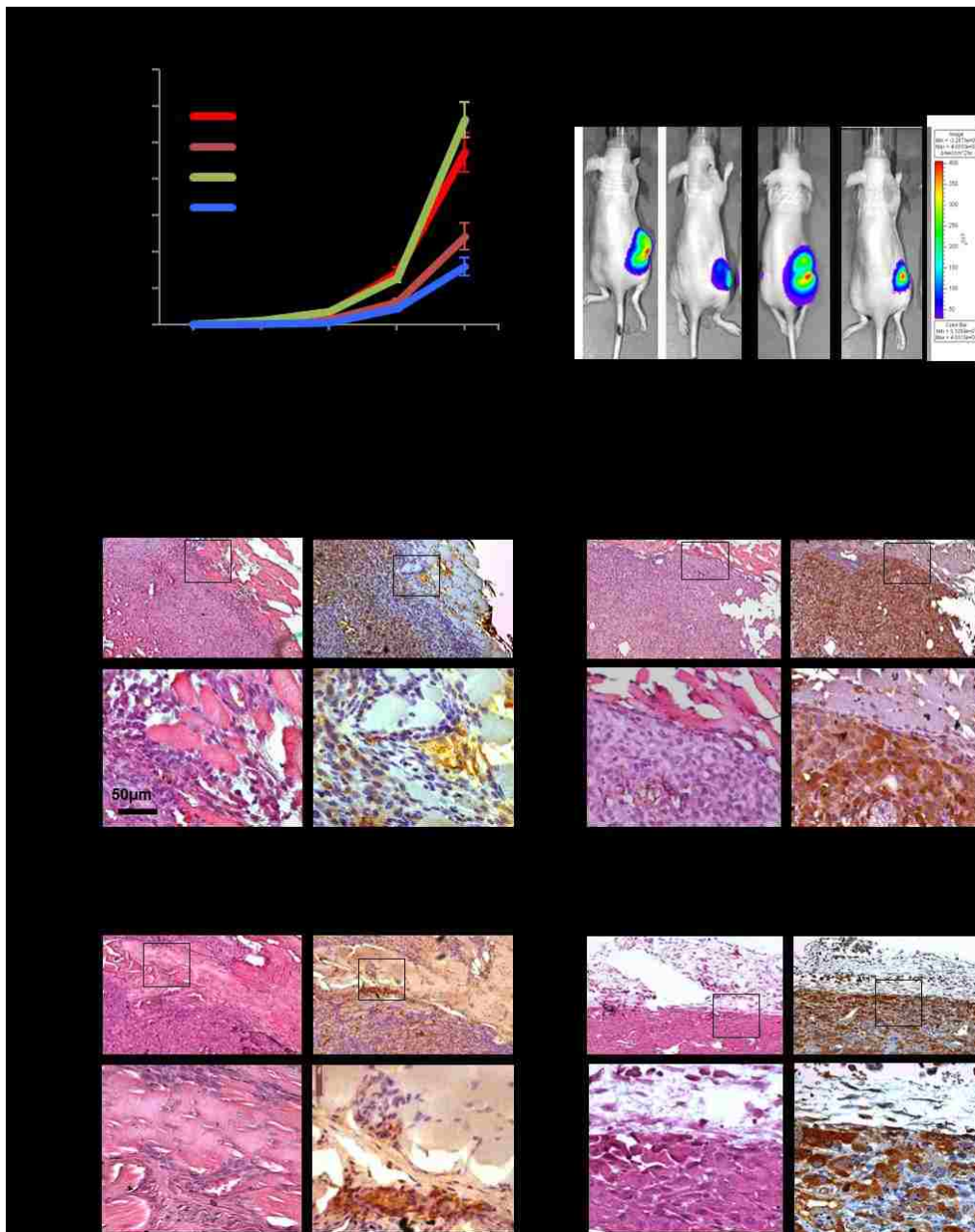


Figure 6. KLF8 to CXCR4 signaling is required for invasive growth of orthotopic mammary tumors.

(a, b) Overexpression of CXCR4 but not the dN20 mutant prevents inhibition of the mammary tumor growth by KLF8 knockdown. The 231-K8ikd, 231-K8ikd/CXCR4 and 231-K8ikd/dN20 cells were injected into the mammary fat pad of mice. The mice were fed with Dox-diet (I, KLF8 knockdown induction) or control diet (U). The tumor growth rate was followed up for 5 weeks by BLI analysis (a) between U and I groups as well as I + CXCR4 and I + dN20 groups. Representative BLI images in the

end of week 5 are shown (b). (c) Overexpression of CXCR4 but not the dN20 mutant prevents inhibition of the tumor invasion by KLF8 knockdown. The above described tumors were processed for Hematoxylin and eosin (H/E) and IHC staining with GFP antibody. *P<0.05

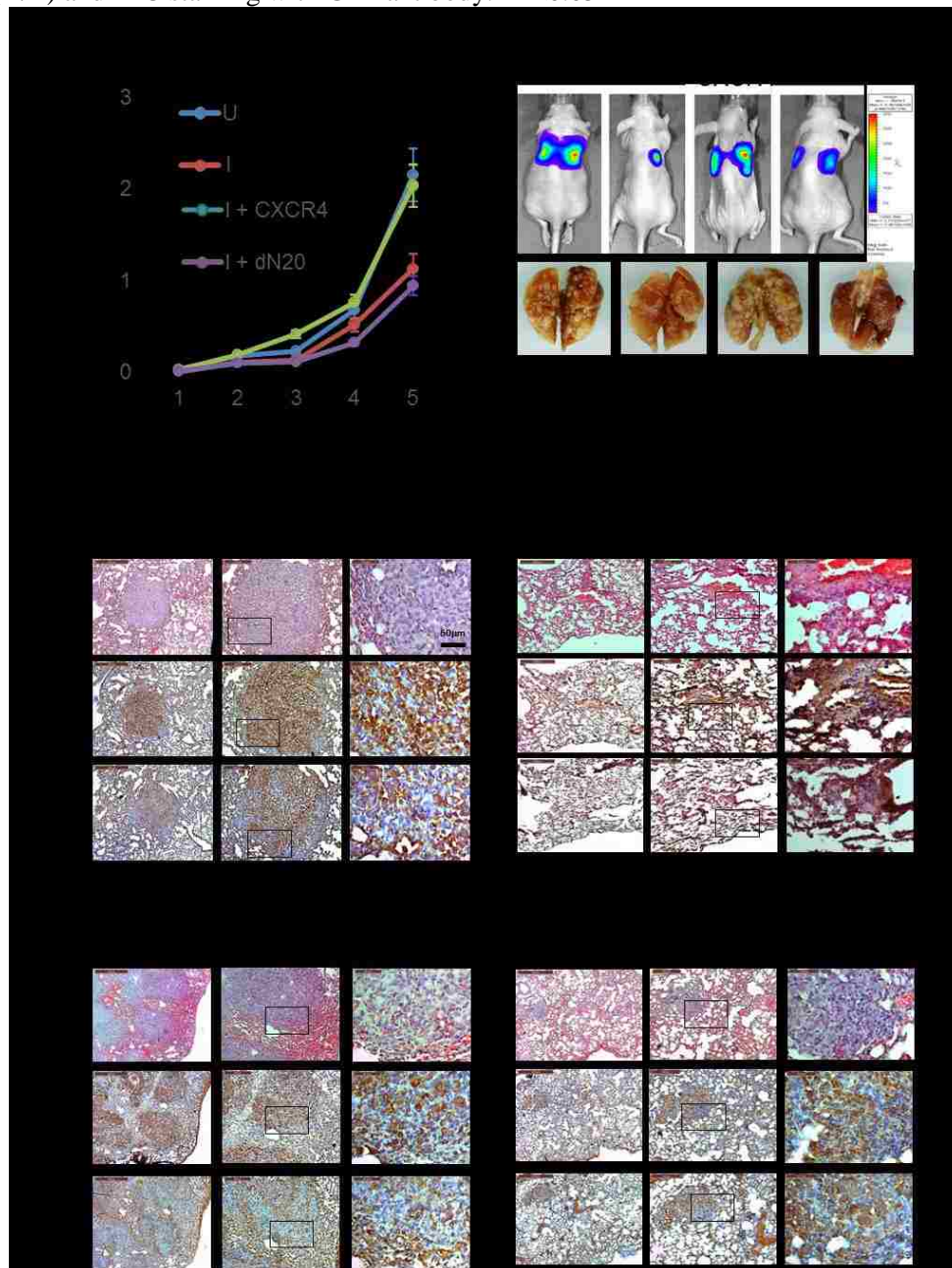


Figure 7. KLF8 to CXCR4 signaling is required for lung metastasis.

(a, b) Overexpression of CXCR4 but not the dN20 mutant prevents inhibition of lung metastasis by KLF8 knockdown. The 231-K8ikd, 231-K8ikd/CXCR4 and 231-K8ikd/dN20 cells were injected into the tail vein of mice. The mice were fed with Dox-diet (I, KLF8 knockdown induction) or control diet (U). The lung metastatic rate was followed up for 5 weeks by BLI analysis (a) between U and I groups as well

as I + CXCR4 and I + dN20 groups. Representative BLI images, metastatic nodules on the whole mount lungs and statistic values in the end of week 5 are shown (b). (c) Overexpression of CXCR4 but not the dN20 mutant prevents inhibition of lung metastasis by KLF8 knockdown. The above described lungs were processed for Hematoxylin and eosin (H/E) and IHC staining with antibodies against GFP and human vimentin. *P<0.05

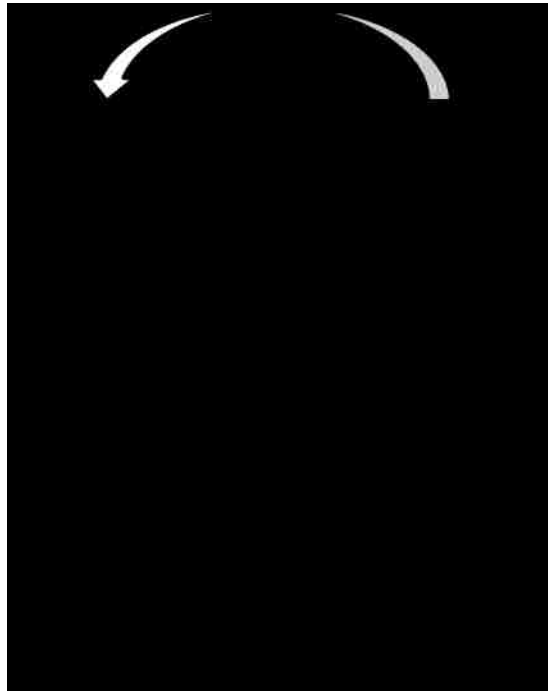


Figure 8. A model of the role of the feed-forward signaling loop involving KLF8/CXCR4/pFAK in driving metastatic progression

Discussion

This study identified CXCR4 as a novel direct target of transcriptional activation by KLF8 and a key mediator of KLF8's role in promoting CXCL12-dependant breast cancer cell migration and invasion required for the invasive growth of the primary tumor as well as TEM essential for the lung metastasis involving a feed-forward activation of FAK (Figure 8).

As shown in Figure 2, KLF8 directly interacts with the CXCR4p GT-box to activate the promoter. Mutation of this GT-box completely abolishes the activation. This result strongly suggests that the GT-box 1 site plays an indispensable role for the promoter activation by KLF8. We noticed that promoter deletion upstream of this GT-box also slightly reduces the promoter activation by KLF8 (Figure 2C), suggesting that those deleted regions, particularly that between the -1230 bp and -1372 bp and that between the -1520 bp and -2088 bp, also play a role in mediating the promoter activation by KLF8. This mode of activation is likely indirect through unknown KLF8 target transcription factors given that KLF8 binds the promoter at the GT-box 1 site (Figure 2E and 2F) and mutation of the other GT-boxes individually did not change the promoter responsiveness to KLF8 (Figure 2D). Several transcription factors have been implicated in activation of the CXCR4 gene promoter [82]. It will be interesting to test if they are regulated by KLF8 and mediate the promoter activation by KLF8 in the cells.

Consistent with our results, it was reported that FAK was activated by CXCL12 stimulation in hematopoietic precursor cells [110, 111], which enhances cell migration towards CXCL12 [110, 112]. In those cells, it appears that activation of PI-3 kinase by $G\alpha_i$ is important in mediating activation of FAK in response to engagement of CXCR4 by CXCL12, although

exactly how the activation of PI-3 kinase leads to the activation of FAK was unclear [110, 111]. The study also showed that Src activity is required for the activation of FAK downstream of CXCR4 [111]. We do not know how FAK is activated by the feed-forward loop of KLF8/CXCR4 in response to CXCL12 stimulation in the breast cancer cells used in this study. It is possible that the breast cancer cells use the same mechanisms involving direct activation of PI-3 kinase and/or Src by $G\alpha_q$ as described above. Alternatively, the G proteins could transactivate other receptor proteins such as integrins or EGFR [113] resulting in subsequent downstream activation of FAK [108]. We have demonstrated that both PI-3 kinase and Src play a role in FAK-induced upregulation of KLF8 in fibroblasts and ovarian cancer cells and in the latter activation of SP1 downstream of Akt plays a likely role [43, 54]. Whether FAK upregulates KLF8 in the breast cancer cells by the same mechanism remains to be determined. In addition, it would also be interesting to investigate whether this feed-forward signaling loop can self-stand. Even though KLF8 is required for initially triggering the feed-forward loop, it is possible that, once activated, the loop can self-stand even if the ectopic KLF8 expression level is subsequently diminished. Activation of the feed-forward loop causes the downstream expression of endogenous KLF8 which may possibly sustain the loop independently even in the absence of ectopic KLF8. In that case, the feed-forward loop can be blocked only when the CXCL12 ligand is removed from the system. In view of this, it is possible that on a physiological level, the cell attenuates the KLF8-CXCR4-FAK loop primarily in response to diminished extracellular CXCL12 ligand expression or through rapid receptor internalization. Nevertheless, it will be important to determine the detailed signaling pathways and mechanisms that operate the critical feed-forward wheel of KLF8/CXCR4/FAK in breast cancer cells.

This is the first demonstration of the role for KLF8 in promoting TEM requiring CXCR4 engagement by CXCL12. For such TEM to occur, the cancer cell must be able to make a local trip along a CXCL12 gradient to approach the vascular vessels. This trip requires the cancer cell to be able to leave the primary tumor, migrate and degrade or re-model the extracellular matrix while invading through the stroma and the availability of the local gradient of CXCL12 and vascular vessels. KLF8 is well known to promote cell dissociation and migration by inducing EMT [40, 49, 77, 78] and invasion by upregulating MMP expression and activity [50, 51]. FAK is a potent migration-promoting protein upstream of various signaling effector proteins such as p130Cas and Crk [108, 112], which was also shown in CXCL12-stimulated cells [110, 111]. Given the novel feed-forward signaling wheel identified (Figures 5 and 8), it is likely that CXCR4 engagement by CXCL12 has an important impact on the regulation of the effector proteins downstream of both KLF8 and FAK, investigation of which is in progress. Cell movement associated with migration, invasion and TEM requires cytoskeletal rearrangement such as the formation and dynamic change of filopodia and lamellipodia. It will be interesting to determine whether the feed-forward signaling loop serves as an important molecular/signaling mechanism underlying this critical cellular change in metastatic cancer cells. Tissue damaging conditions such as hypoxia, toxins and ionized irradiation induce a CXCL12-rich environment for recruiting CXCR4⁺ stem cells and leukocytes to the site that requires tissue repair or regeneration [63, 89, 100]. Indeed, vascular endothelial cells under these stress conditions produce increased level of CXCL12 [114]. In addition, the high level of CXCL12 secreted by tumor-associated stromal cells upon hypoxia is critical for tumor angiogenesis [83]. It is thus important to investigate if KLF8 plays a role in tumor angiogenesis and resistance to anti-cancer therapies such as radiotherapy and if CXCR4 is involved.

We demonstrate that KLF8 plays a critical role in both invasive growth of the local primary tumor (Figure 6) and the lung metastasis (Figure 7) in a CXCR4-dependent manner. Cancer-associated fibroblasts can release CXCL12 at the primary tumor site to promote the primary tumor growth and invasion [83]. In view of this, the feed-forward signaling loop may also be a key in promoting primary tumor outgrowth. In addition to EMT, invasion and potential tumor angiogenesis discussed above, the remarkable impact of the feed-forward signaling on TEM indicates that KLF8 may be critical in promoting intravasation and/or extravasation via CXCR4-dependent TEM during metastasis. On reaching the foreign tissue, successful metastasis requires the tumor cells to respond to chemotactic signals in order to survive and ultimately colonize. Interestingly, FAK has recently been implicated in promoting the breast cancer colonization in the lungs [115]. It will be interesting to test if KLF8 also participates in regulating this final rate-limiting metastatic step and whether CXCR4 and CXCL12 are needed. The expression of CXCL12 has been found to be highest in the tissues of the lungs, liver, bone marrow and brain [64, 65]. Indeed, these organs tend to be the metastatic sites of CXCR4-high cancer cells [64, 72, 106, 116-118]. It will be worth of determining if KLF8-high cancer cells tend to colonize in the other CXCL12-high organs as well in addition to the lungs.

This study opens a new opportunity for understanding molecular mechanisms critical for targeted therapeutic control of breast cancer. Given the key role for both FAK and CXCR4 in breast cancer metastasis, FAK- and CXCR4-targeted therapies have been explored in pre-clinical tests and clinical trials [64, 102, 119-122]. However, major obstacles remain due mainly to unbearable side effects on normal tissues. The primary cause is the wide, if not ubiquitous, expression and physiological function of these two proteins. Indeed, knockout of either FAK or

CXCR4 is embryonically lethal [123, 124]. Thus, there is an urgent need to identify upstream targets and processes which can induce the expression and/or activation of FAK and CXCR4 specifically in tumor cells. Importantly, the expression of KLF8 in normal tissues is much lower than that in cancer tissues [40] and knockout of KLF8 is not embryonically lethal [125]. Thus, the identification of KLF8 as a critical feed-forward inducer of CXCR4 expression and activation of FAK in breast cancer cells presents an excellent therapeutic target to correct aberrant signaling of both FAK and CXCR4 specifically in cancer cells. Together with our previous reports demonstrating a critical implication of KLF8 in malignant progression of breast cancer [40, 43, 49, 50, 52, 53, 56, 79], this study further underscores KLF8 as an important regulator of breast cancer pathogenesis and malignancy and a potential therapeutic target with less side effects.

CHAPTER 3: KRÜPPEL-LIKE FACTOR 8 PROMOTES AGGRESSIVE COLONIZATION OF TUMOR CELLS POST EXTRAVASATION THROUGH A CXCL12-CXCR4 DEPENDENT PATHWAY

Abstract

Metastatic disease remains the most challenging impediment towards formulating effective therapy for breast cancer. The re-initiation of post-extravasation proliferation of dormant micrometastatic tumor cells lodged at the secondary tissue parenchyma marks the final rate-limiting step of the metastatic cascade. The process represents the maximum attrition rate of tumor cells, whereby majority of the extravasated cells are unable to proliferate and successfully generate macroscopic metastasis. The molecular mechanisms that allow a subset of dormant micrometastatic cells to re-initiate proliferation in the secondary tissue remains poorly understood. Here we report that the presence of CXCL12 in the foreign microenvironment, together with overexpression of KLF8 in micrometastatic cells, drives metastatic colonization. KLF8 triggers the formation of filopodium-like protrusions (FLP) in recently extravasated dormant tumor cells in response to extracellular CXCL12. *In vitro* Matrigel-on-Top assays showed that FLP formation leads to loss of polarity in cell colonies and the failure to arrest growth, thereby enabling the proliferation of KLF8-high tumor cells in response to extracellular CXCL12. CXCR4 mediates KLF8's role in post extravasation proliferation by engaging CXCL12 from the surrounding stroma to initiate proliferative pathways downstream. Furthermore, we identified that FAK is recruited to the newly formed FLPs and mediates KLF8's role in post-extravasation proliferation downstream of CXCL12-CXCR4 engagement. These results shed new light on the role of KLF8 and CXCR4-CXCL12 signaling in regulating the critical final step of macroscopic colonization.

Introduction

In the past few decades, adjuvant therapies and surgical resection have been proved to be effective for well-confined primary breast tumors. However metastatic tumor progression remains largely incurable and is the most life-threatening aspect of the disease. Indeed metastasis is responsible for over 90% of cancer-associated mortality [126] [30]. This proves that eventual success in developing effective therapeutic solutions for combating breast cancer is largely dependent on the ability to prohibit the metastatic process.

Metastasis represents a complex, multi-step process involving successive inter-related cell-biological events whereby invasive tumor cells i) dissociate from the primary tumor, ii) locally invade into the surrounding stroma, iii) intravasate into the lamina of blood vessels to travel with the blood as circulating tumor cells (CTCs), iv) extravasate out into the foreign tissue parenchyma, v) initially survive and sustain in the foreign microenvironment to form dormant microenvironment, and finally, vi) successfully reinitiate proliferation to form clinically detectable macrometastatic lesions at the secondary sites (metastatic colonization) [31, 75]. The later steps of the metastatic cascade, specifically micrometastatic formation and metastatic colonization represent the major rate-limiting steps of the metastatic process. A substantial proportion of successfully extravasated cells undergo apoptosis, whereas only three percent of the cells are able to persist in the foreign tissue parenchyma in a viable but growth-arrested state. The subsequent process of macroscopic metastasis formation is the most inefficient, whereby less than one percent of the cells that initially survive in the foreign microenvironment are able to reinitiate their proliferative programs to establish macroscopic metastasis [35]. Since, the highest attrition of cancer cells occurs in the steps following extravasation, it is clear that adapting to an unfamiliar foreign microenvironment is one of the major obstacles towards

establishing successful metastasis [36, 127]. It has been postulated that the ability of extravasated cancer cells to survive and reinitiate proliferation in the metastatic sites is largely dependent on i) establishing productive connections with the surrounding extracellular matrix and, ii) adapting to the unfamiliar cytokine environment to acquire mitogenic signals that promote reinitiation of cell proliferation [38].

Krüppel-like Factor 8 (KLF8) is a dual transcription factor that is aberrantly overexpressed in a large number of human cancers, including breast cancer. In recent years, KLF8 has emerged as a critical regulator of cancer pathogenesis with several studies confirming that KLF8 plays a key role in promoting cell cycle progression [43], oncogenic transformation [56], EMT [49], cancer stem cell induction [55] and DNA damage response [79]. Importantly, KLF8 expression has been found to be highly correlated with metastatic potential, as evidenced by patient tissue array analysis [50, 79], Indeed, xenograft studies have demonstrated that KLF8 promotes distant metastatic dissemination[50]. The positive role of KLF8 in the initial steps of the metastatic cascade, i.e., local tumor invasion and transendothelial migration, has been studied extensively[128]. However, the contribution of KLF8 towards the later rate-limiting steps of metastasis, specifically, the reinitiation of proliferative growth of extravasated cells to eventually establish successful macroscopic colonization, remains hitherto unknown.

CXCR4 is a seven-transmembrane G-protein coupled receptor that specifically binds to CXCL12 and has been strongly implicated in breast cancer metastasis. Elevated levels of CXCR4 expression has been found in breast cancer cells, as well as in patient tumors. The CXCR4 receptor directs the trafficking of CXCR4-high leucocytes towards CXCL12-rich inflammatory sites via CXCL12 gradient-dependent chemotaxis [63, 84]. It is also critical to the

homing of hematopoietic stem cells in the bone marrow[82]. The connection of the CXCR4-CXCL12 pathway has generated massive interest with the discovery that CXCR4-high breast cancers preferentially metastasize to organs which exhibit highest levels of CXCL12 expression, namely the lung, liver, bone marrow and lymph nodes. Several reports have indicated that inhibition of the CXCR4-CXCL12 interaction dramatically reduced metastatic incidence [71] . Indeed, several studies have demonstrated that CXCR4-high cancer cells can “hijack” cellular highways to migrate along the CXCL12 gradient to establish distant metastasis in CXCL12-rich tissues [65]. However, the precise role of the CXCR4-CXCL12 interaction in specifically establishing macroscopic metastatic lesions at the secondary site is largely unknown.

Our previous reports identified CXCR4 as a novel target of direct transcriptional activation by KLF8. By testing KLF8-overexpression in inducible breast epithelial cell lines and KLF8-knockdown inducible invasive breast cancer cell lines, we found that CXCR4 is a key mediator in promoting KLF8’s role in CXCL12-dependent breast cancer cell invasion and migration in vitro and invasive primary tumor growth in vivo. We also uncovered the critical role of KLF8 in promoting trans-endothelial migration along a CXCL12 gradient, indicating that KLF8 is crucial for both the intravasation and extravasation steps leading up to metastatic dissemination. Importantly, on testing the role of secondary metastatic dissemination in the lung using mouse xenograft studies, we found that there was a dramatic inhibition of metastatic rate by knockdown of KLF8 in tumor cells which was well prevented by the overexpression of CXCR4 in the tumor cells.[128] In this study, we investigated the role of KLF8 in promoting colonization of extravasated cancer cells in the secondary tissue. We utilized the *in vitro* three-dimensional (3D) “Matrigel-on-Top culture model to provide a physiological context to model

the post-extravasation behavior of cancer cells disseminated in the secondary tissue sites [129]. Single-cell suspensions of the cancer cells were plated at low densities (500-2000 cell/cm²) over a layer of undiluted matrigel and then covered with matrigel diluted 1:5 with the culture medium. Therefore, each individual cell was surrounded completely by ECM components, thereby emulating the conditions encountered by cancer cells following extravasation into the foreign microenvironment. MoT assay conditions have been shown to accurately reproduce the post-extravasation behavior of carcinoma cell lines having similar extravasation efficiencies but markedly differing rates of subsequent proliferation and colonization potential [37, 130].

In the present study, we identified that KLF8 promotes the formation of actin-rich protrusions or FLPs in micrometastatic cancer cells in response to the presence of CXCL12 in the surrounding microenvironment. Such protrusions are known to be critical in establishing productive connections with the ECM at the foreign site. CXCL12-treated KLF8-high cells adopted a non-polar, proliferative and disordered colony architecture that closely resembles the malignant tumor phenotype. Furthermore, we demonstrated that KLF8 promotes FLP formation and subsequent proliferation of cancer cells in CXCL12-enriched MoT conditions by upregulating intracellular CXCR4 expression. Finally, we demonstrated that KLF8's role in enhancing post-extravasation proliferation through CXCR4-engagement is mediated by the recruitment of FAK to nascent FLPs. These novel findings suggest that CXCL12 may be a crucial environmental cue in the foreign tissue environment which engages the CXCR4 receptor expressed on KLF8-high micrometastatic cancer cells to initiate proliferation and establish metastatic colonization.

Materials & Methods

Antibodies and reagents

Antibodies include mouse monoclonal for HA-probe (F-7) (sc-7392), β -actin (C4) (sc-47778), and rabbit polyclonal for FAK (sc-557) (Santa Cruz Biotechnology Inc, Dallas, TX, USA), CXCR4 rabbit polyclonal Ab (Ab-2074) (Abcam, Cambridge, MA, USA), and pY397-FAK rabbit monoclonal Ab (Invitrogen, 44625G, Carlsbad, CA, USA). For actin staining Alexa Fluor® 568 Phalloidin (Thermo Fisher Scientific, A12380) was used. The CXCR4 inhibitor AMD3100 (or octahydrochloride hydrate) (A5602) and the FAK inhibitor PF573228 (PZ0117) were purchased from Sigma (St. Louis, MO, USA). Recombinant human CXCL12 (300-28A) was purchased from Peprotech (Rocky Hill, CT, USA).

Plasmid construction, cell line generation, cell culture and transfection

The MCF-10A cell-line that expresses doxycycline-inducible KLF8 (10A-iK8) and the MDA-MB-231 that expresses doxycycline-inducible shRNA against KLF8 (231-K8ikd) were described previously [50]. The 231-K8ikd cell line expressing ectopic CXCR4 or its dN20 mutant was generated as discussed in the previous report [128]. Briefly, the CXCR4 cDNA was PCR-synthesized from mRNA isolated from MDA-MB-231 cells and cloned into the pKH3 vector between the SmaI and EcoRI sites to form the pKH3-CXCR4 plasmid. The amino terminal 20 amino acids of CXCR4 in the plasmid were removed by deletion PCR to obtain the dominant-negative mutant plasmid pKH3-CXCR4-dN20. To construct lentiviral vectors pLVZP-CXCR4 and pLVZP-CXCR4-dN20, we sub-cloned HA-CXCR4 and HA-CXCR4-dN20 from the pKH3-CXCR4 and pKH3-CXCR4-dN20 plasmids respectively, into the lentiviral vector pLVPZ between the PstI and NotI sites by PCR. Finally, the 231-K8ikd/CXCR4 and 231K8-

ikd/dN20CXCR4 cell lines were generated by infecting the parental 231-K8ikd cells with the lentivirus vectors pLVZP-CXCR4 or pLVZP-CXCR4-dN20 followed by puromycin selection. These cells were maintained in Dulbecco's modified Eagle's medium/F-12 with 5% horse serum (for cell lines derived from MCF-10A cell-lines) or Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The inducible cell lines were maintained under U or I conditions as explained before. Doxycycline hydrochloride was purchased from Sigma (D3072) (St. Louis, MO, USA). Plasmid DNA transfections were performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA).

Western blotting

Cell lines and antibodies used have been described above. Western Blotting was performed as previously described [128].

In vitro Matrigel-on-Top assay

The Matrigel-on-Top Assay was performed as described [131]. Matrigel Basement Membrane Matrix (Product #356234, Corning) was stored at 4°C overnight before the day of the experiment. Precooled 8 well chamber slides (#C7057, Nunc® Lab-Tek® II Chamber Slide™ system) were coated with 40ul of matrigel per well and incubated at 37°C for at least 30-40 minutes so as to allow the gel to solidify. Subsequently a single suspension of the cells was generated, whereby 400 cells were suspended in 200 ml of culture media containing 2% Matrigel. This matrigel-containing single cell suspension was seeded into the well of the chamber slide. 10 ng/ml CXCL12 was added directly into the matrigel-containing culture media. The slides were then maintained at 37°C for the times indicated with the media being changed

every fourth day. For inhibitor treatment, the cells were pretreated with AMD3100 (35 ng/ml) or PF573228 (1 μ M) for three hours prior to culturing cells under MoT conditions. For the monolayer culture, cells were plated on uncoated plates and seeded with medium that did not contain Matrigel.

In situ immunofluorescence staining of three-dimensional cell colonies

For detection of actin polymerization, the cells cultured in 8-well chamber slides under Matrigel on-top conditions were stained with Alexa Fluor 568 phalloidin. At the end of the incubation period, the culture media was removed and after fixation with 2% paraformaldehyde in PBS for 20 min the cells were permeabilized with 0.1% Triton X-100 in PBS for 3 to 5 minutes. PBS containing 1% BSA was used to block the cell colonies for 30 minutes before staining the colonies directly with Alexa Fluor 568 phalloidin for 20-30 mins. For in situ immunofluorescence staining, special care was taken during the washing and staining steps so that the matrigel does not get dislodged from the well. Post staining, the cells were washed with PBS and the divider of the chamber slide was removed. Cells were cultured for 5 days unless otherwise indicated. Media was changed every fourth day

Confocal imaging and analysis

Stained chamber slides were mounted with ProLong® Gold Antifade Mountant with DAPI (#P36931, Invitrogen) and imaged in the Ultraview Confocal Microscope (Perkin Elmer). Three dimensional images of cell colonies cultured under MoT conditions were taken by capturing sequential images along the Z-plane at .25 μ m interval (Z-stacks). For cells cultured in

monolayer, the threshold value to be considered “filopodia-positive cells” was taken to be 3 and for MoT cultured cell colonies, the threshold value was considered to be 1 FLP per colony. Images and videos were captured using the Volocity software. Quantification was done using Volocity as per the manufacturer’s protocols.

Metastatic lung nodule analysis

The collection and preparation of the metastatic tumor tissues, and the histological analysis procedures were previously described [128]. The vimentin antibody has been described above. The Volocity software was used to calculate the area of each nodule on two non-sequential lung tissue sections from 4 mice per group.

Statistical analysis

Mean +/- standard deviation is presented with a minimum of three observations per group. Student's t-test, unpaired, paired or single sample, with the Bonferroni correction for the multiple comparisons was applied as appropriate. The two by two tables were analyzed by Fisher's exact test. The alpha level of 0.05 was used to determine difference with statistical significance.

Results

KLF8 expression leads to filopodia formation in cells in monolayer culture in response to

CXCL12

It has been postulated that one of the most important impediment that obstructs the path towards successful metastatic colonization is the ability of extravasated cancer cells to survive in

the foreign tissue by adapting to the cytokine environment to acquire mitogenic and trophic signal, as well as to initiate proliferation by establishing productive connections with the foreign tissue parenchymal ECM. Breast cancer cells have been shown to form successful metastatic colonies specifically in CXCL12-rich secondary tissue environments like lung, liver, bone marrow, lymph nodes. We wanted to investigate the reason why breast cancer cells show high metastatic rate in CXCL12-rich organs. Is it possible that the CXCL12-rich tissue environment in these secondary organs presents a certain survival advantage to the tumor cells? We investigated whether a CXCL12 rich cytokine environment in the foreign tissue presents certain advantages to the newly extravasated tumor cells, which can lead to higher colonization potential of these cells. In view of this, we examined our KLF8-overexpression inducible breast epithelial cell line 10A-iK8 with or without treatment with the CXCL12 cytokine. Interestingly, on direct exposure to CXCL12, we observed that KLF8-overexpression in 10A-iK8 cells lead to the formation of actin-rich cytoplasmic protrusions, also known as filopodia. In the absence of CXCL12, KLF8-high 10A-iK8 cells did not show any filopodia formation. Furthermore, the CXCL12-dependent filopodia formation in the KLF8-high10A-iK8 cells was blocked on treatment with the CXCR4-specific antagonist AMD3100 (Fig 1A, B, C). Conversely, filopodia formation of 231K8-iKd cells exposed to CXCL12 treatment, was markedly reduced upon KLF8 knockdown or treatment with AMD3100 (Fig 1D, E, F). Importantly, in the absence of CXCL12 treatment, the 231K8-iKd cells showed significantly less filopodia formation.

These results indicate that firstly, CXCL12 treatment leads to morphological changes in the cell, namely filopodia formation in monolayer culture and secondly, this filopodia formation is dependent on KLF8 expression.

KLF8 promotes FLP formation in breast cancer cells in three dimensional cultures in response to CXCL12

Filopodia are thin actin-rich cytoplasmic protrusions that have been described to be like “antenna” or “tentacles” for cells to probe their environment. They have been implicated in several physiological processes like cell adhesion and migration. Recent reports suggest that the filopodia extended by monolayer-cultured cells are morphologically very similar to filopodia-like-protrusions or FLPs extended by extravasated cells that have been found to play a critical role in enabling cell to ECM connection in the foreign tissue [131]. It is known that metastatic cancer cells proliferate only if they can interact productively with the extracellular matrix. Since we already observed filopodia formation in these CXCL12-treated 231K8-iKd cells in monolayer culture, we investigated under similar conditions whether; these cells can extend FLPs or other morphological structures when cultured in the 3D MOT culture conditions. Our result showed that on exposure to CXCL12, the 231K8-iKd cells exhibited actin-rich projections or FLPs within 48 hours of MOT culture. The number of FLPs per cell colony as well as the overall FLP-positive colonies were significantly decreased on KLF8 knockdown, as well as on blocking the CXCR4 receptor with the CXCR4 specific antagonist, AMD3100. Our results show that, KLF8 promotes early extension of FLPs in invasive breast cancer cells under MOT culture conditions in response to presence of the CXCL12 ligand in the external environment. Our observation seems to indicate that CXCL12, when present in the foreign tissue parenchyma, can promote FLP formation in recently extravasated cancer cells in KLF8-dependent mechanism.

KLF8 expression leads to FLP formation and polarized, invasive tissue architecture in breast cancer cells after sustained treatment with CXCL12

Previous studies have reported that when cultured in 3D matrigel conditions, mammary epithelial cells organize into polarized acini-like structures, whereas carcinoma-derived mammary epithelial cells are incapable of forming organized structures, instead, forming disordered colonies that continue to proliferate. Indeed on culturing normal breast epithelial and breast cancer cells under 3D matrigel conditions, complete growth arrest was observed in the case of normal cells for the same time period. On the other hand, carcinoma derived cells formed disorganized structures and continued to proliferate. Indeed, non-malignant epithelial cells undergo a few rounds of cell division before arranging into polarized, growth-arrested colonies, while, malignant cells adopt a variety of colony morphologies, but share certain common features, namely, loss of polarity, a disordered structure and a failure to arrest growth. By utilizing our three-dimensional Matrigel-on-Top culture model, which mimics the conditions encountered by invasive cells post extravasation into the foreign tissue, we explored the morphological phenotypes exhibited by 231K8-ikd cells with or without KLF8 knockdown under sustained CXCL12 treatment. On culturing the cells in CXCL12-enriched Matrigel-on-Top conditions for five days, we found that the highly invasive 231K8-ikd cells adopted disorganized colony morphology with a complete loss of polarity. Interestingly, even though we observed a loss in polarity in 231K8-ikd cell colonies cultured in the absence of CXCL12, the disordered colony architecture was much more pronounced when the cells were exposed to sustained CXCL12 treatment. In contrast, upon KLF8 knockdown, the cells appeared to go through a few rounds of division, before arranging into organized, polarized colonies exhibiting

acinar morphology. (Fig 3A, B). In addition, our results also demonstrated that sustained exposure to CXCL12 promoted an increase in the number of FLP-positive cell colonies. In spite of sustained exposure to CXCL12, the overall FLP-positive colonies were significantly reduced on knocking down KLF8 in the 231K8-ikd cells, further implicating the critical role of KLF8 in promoting FLP formation in extravasated cancer cells in the presence of CXCL12 in the foreign tissue environment. (Fig 3C).

Overall, our results indicate that CXCL12 may be a crucial environmental cue in the foreign tissue environment to which KLF8-high cells respond *in vivo* to adopt malignant colony morphologies exhibiting a loss of polarity and disorganized structure. This may be one of the hallmarks of the initiation of macrometastasis formation and colonization in the secondary tissue.

KLF8 triggers proliferation of breast cancer cells in 3D culture in response to CXCL12

Loss of polarity of cell colonies in three-dimensional culture has been associated with a concurrent failure to arrest proliferation [132],[133]. To analyze whether KLF8 expressed by the extravasated cancer cells as well as the presence of CXCL12 in the microenvironment are both critical for the success of the colonization process at the secondary site, we cultured the 231K8-ikd cells under MoT conditions for a 10 day period and evaluated the percentage of proliferating cells. The use of the MoT assay provides a physiologically relevant context in which to emulate cells lodged in the secondary tissue site *in vivo* post extravasation. Our results showed that 231K8-ikd cells proliferated rapidly and formed significantly larger cell colonies in the presence of CXCL12, which was severely diminished on KLF8 knockdown. Importantly, regardless of the presence of CXCL12 treatment, KLF8 knockdown in 231K8-ikd cells resulted in polarized, growth-arrested acinus-like colonies resembling the phenotype exhibited by non-malignant

epithelial cells (Fig 4.A, B). On comparing the growth and proliferation of the 231K8-ikd cells at successive timepoints, we observed that intracellular KLF8 expression as well as the presence of extracellular CXCL12, contributed together to enhance the rate of proliferation of the 231K8-ikd cells under MoT conditions (Fig 4C).

Overall, our results indicate that the presence of CXCL12 in the external environment together with endogenous KLF8 expression are both critical for the cancer cells to initiate post-extravasation proliferation and subsequent colonization in the foreign tissue.

KLF8 triggers FLP formation and subsequent proliferation through CXCR4-CXCL12 engagement

We have previously identified that the CXCR4 receptor is a direct transcriptional target of KLF8 [128]. In view of this, we tested whether KLF8 promotes CXCL12-dependant proliferation of 231K8-ikd cells in MoT culture via CXCR4 upregulation and engagement. Consistent with our previous results, we observed that after a 48 hour MoT culture, the FLP formation was significantly reduced upon knockdown of KLF8. However, this knockdown-induced decrease in FLP formation was fully recovered on overexpressing CXCR4, but not its ligand-binding domain-deficient mutant dN20 [128] (Fig B new graph). Subsequently, a similar trend was observed in the rate of proliferation of the 231K8-ikd cells on prolonged culture under CXCL12-enriched MoT conditions. In contrast to the non-polar, proliferative, tumor-like colony architecture adopted by the 231K8-ikd cells, KLF8 knockdown induced a dramatic decrease in the proliferation rate, whereby the cells underwent a few rounds of division before forming growth-arrested, polar, acini-like colonies. Overexpression of CXCR4, but not its dN20 mutant, completely rescued the knockdown-induced decrease in proliferation and protected the ability of

the 231K8-ikd cell colonies to adopt a non-polar, malignant phenotype. As a complimentary approach to further confirm that KLF8 promotes CXCL12-dependant post-extravasation proliferation through CXCR4 engagement, we determined the relative Ki67 positivity as a measure of the proliferation rate by direct immunofluorescence in situ against the Ki67 antigen. The proportion of nuclei staining positive for Ki67 was dramatically reduced upon knockdown of KLF8. This decrease in Ki67 positivity was fully recovered by stable overexpression of CXCR4 in the MoT cultured 231K8-ikd cells, but not by the overexpressing the dN20 mutant of CXCR4.

These results strongly suggest that KLF8 promotes proliferation and subsequent secondary colonization of extravasated cells lodged in CXCL12-rich foreign tissue parenchyma by upregulating the expression of CXCR4 in the cell. This further suggests that CXCL12 may be a vital environmental cue which engages with its receptor CXCR4 expressed on newly extravasated cancer cells, thereby leading to the activation of downstream processes including filopodia formation and cellular proliferation which provides a survival advantage and a higher colonization potential to the KLF8-high cells lodged at the secondary tissue site.

KLF8 promotes aggressive macrometastases in the lung via CXCR4 expression

To determine the extent to which CXCR4 contributes in establishing macrometastases downstream of KLF8, we injected the 231K8-ikd cells, the 231K8-ikd cells with stable overexpression of CXCr4 or its dN20 mutant into the mammary fat pad, induced the knockdown of KLF8 in vivo and examined the extent of macrometastases formation in the lung. Importantly, the lung represents one of the organs that have the highest expression of CXCL12 and is also one of the most common metastatic sites for breast cancer. Knockdown of KLF8 markedly reduced

the size of metastatic nodules. However, on stably overexpressing CXCR4, this decrease in nodule size was fully restored. In contrast, stable overexpression of the ligand binding domain-deficient dN20 mutant could not bring about a recovery of the decrease in the nodule size. The size of the metastatic nodules is largely proportional to the ability of metastatic tumor cells to proliferate in the foreign tissue microenvironment. Our observations lend further credence to the notion that KLF8 plays a key role in establishing macrometastatic growth and colonization in CXCL12-rich secondary metastatic sites by upregulating the expression of CXCR4 in disseminated breast cancer cells.

KLF8's role in promoting CXCL12-dependent post-extravasation proliferation is mediated by the recruitment of FAK to nascent FLPs and subsequent FAK signaling

Recent reports have suggested that FAK signaling is a critical trigger for post-extravasation proliferation. Furthermore, FAK activation in extravasated cells appear to depend on the components of the ECM in the lung parenchyma, which in turn, is mediated by the prior assembly of actin-rich FLPs[131]. In addition, several reports have indicated that engagement of CXCR4 by CXCL12 leads to the downstream activation of FAK. In order to probe the molecular mechanism by which KLF8 promotes post-extravasation proliferation in the presence of CXCL12, we initially tested the level of FAK activation in response to CXCL12 treatment in 231K8-ikd cells cultured in monolayer. We observed that knockdown of KLF8 greatly decreased CXCL12-dependant FAK activation in 231K8-ikd cells. However, CXCL12 exposure lead to increased FAK activation in CXCR4 overexpressed cells in spite of KLF8 knockdown, an effect which was significantly diminished on overexpressing the dN20 mutant of CXCR4 (Fig 8A). From monolayer culture, we proceeded to maintain the cells in CXCL12-enriched MoT

conditions. Direct immunofluorescence staining in situ revealed that FAK was recruited to the nascent FLPs extended by 231K8-ikd cells as well as the KLF8 knockdown induced 231K8-ikd cells that stably express CXCR4 (Fig 8B). These results point to the critical role of FAK in mediating post-extravasation proliferation downstream of KLF8 and CXCR4 engagement. To further confirm the key role of FAK in regulating proliferation, we pretreated the 231K8-ikd cells and the KLF8 knockdown-induced 231K8-ikd/WTCXCR4 cells with the specific FAK inhibitor, PF-228 before culturing the cells under MoT conditions in the presence of CXCL12. In situ immunofluorescent staining against the Ki67 antigen revealed that inhibition of FAK activation severely hampers the ability of 231K8-ikd cell colonies to maintain a proliferative state in the presence of extracellular CXCL12. In case of the KLF8-knockdown induced 231K8-ikd/WTCXCR4 cells, FAK inhibition lead to a dramatic decrease in the proportion of nuclei staining positive for Ki67, regardless of the stable overexpression of CXCR4 (Fig 8.C,D).

Taken together, these results indicate that KLF8's role in promoting post-extravasation proliferation through CXCR4 engagement by extracellular CXCL12 is mediated by the recruitment of FAK to nascent FLPs and subsequent FAK activation and downstream signaling.

Figures

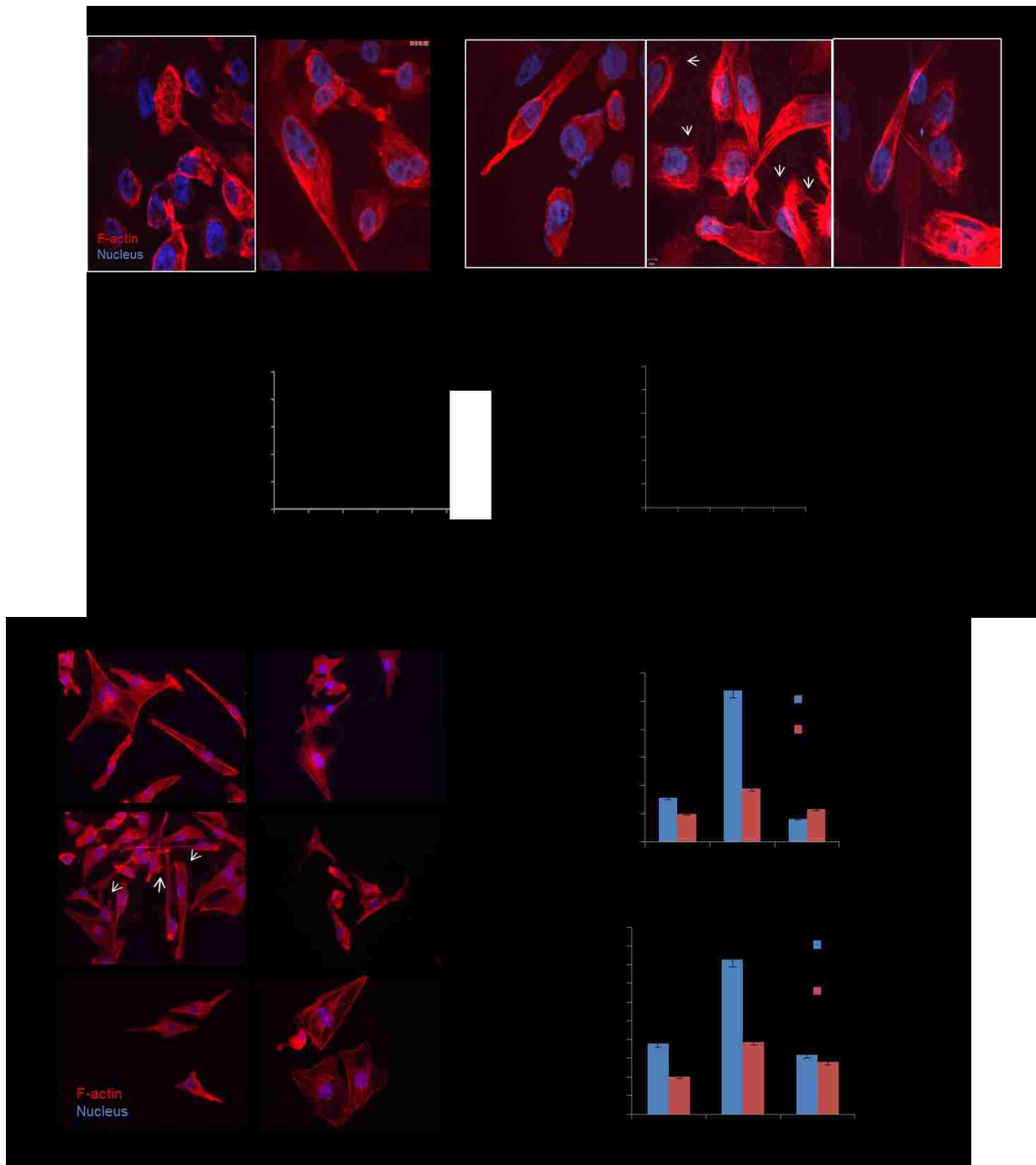


Figure 9. KLF8 expression leads to filopodia formation in cells in monolayer culture in response to CXCL12.

(a), Overexpression of KLF8 enhances filopodia formations in 10A-ik8 cells in response to CXCL12 exposure, which is diminished on treatment with AMD3100 . 10A-ik8 cells grown under induced (I) or uninduced (U) conditions were either exposed to PBS or CXCL12 or CXCL12 supplemented with AMD3100 for 24 hours. The cells were then stained for actin in-situ with Alexa Fluor 568-labelled phalloidin. Representative confocal images and quantification for each condition have been shown. (b,c), Quantification of filopodia-positive cells and number of filopodia showing that both determinants are significantly increased on CXCL12 treatment in the presence of KLF8 overexpression. Quantification was done on Volocity software. The results represent quantifications from three fields for each condition in three separate experiments. (d,e) KLF8 downregulation in 231K8-ikd cells resulted in significantly less filopodia formation despite CXCL12 treatment. 231K8-ikd cells grown under induced (I) or uninduced (U) conditions were either exposed to PBS or CXCL12 for 24 hours before phalloidin staining to visualize cytoplasmic actin protrusions (filopodia). Quantifications were done using Volocity imager. Results represent quantifications from three fields for each condition in three separate experiments. *P<0.01

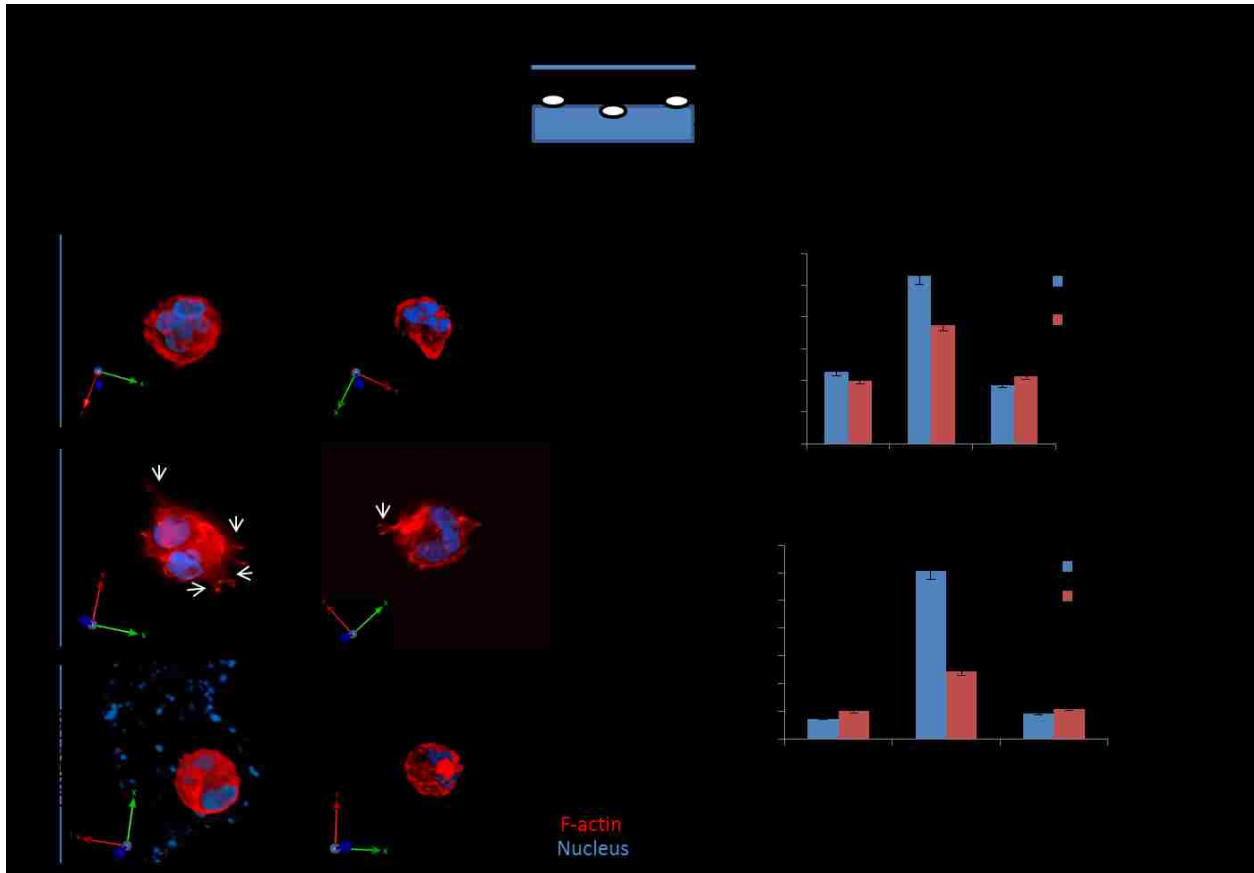


Figure 10. KLF8 expression leads to FLP formation in breast cancer cells in three dimensional culture in response to CXCL12.

(a), Schematic representation of the construction of the Matrigel-on-Top assay (b), 231K8-ikd cells show FLP formation in the presence of CXCL12, which is significantly diminished on KLF8 downregulation. 231K8-ikd cells grown under induced (I) or uninduced (U) conditions were either exposed to PBS or CXCL12 for 24 hours before MoT culture for 48 hours. The 3D matrigel inserts were stained with phalloidin to visualize 3D images of the cell clusters as well as FLP formation. (c,d) Quantification of

FLP-positive cells and number of FLP-positive cell colonies showing that both determinants are significantly increased on CXCL12 treatment in the presence of KLF8 overexpression. Downregulation of KLF8 significantly diminishes FLP formation in spite of presence of CXCL12. Quantification was done on Volocity software. Quantifications were done using Volocity imager. *P<0.01

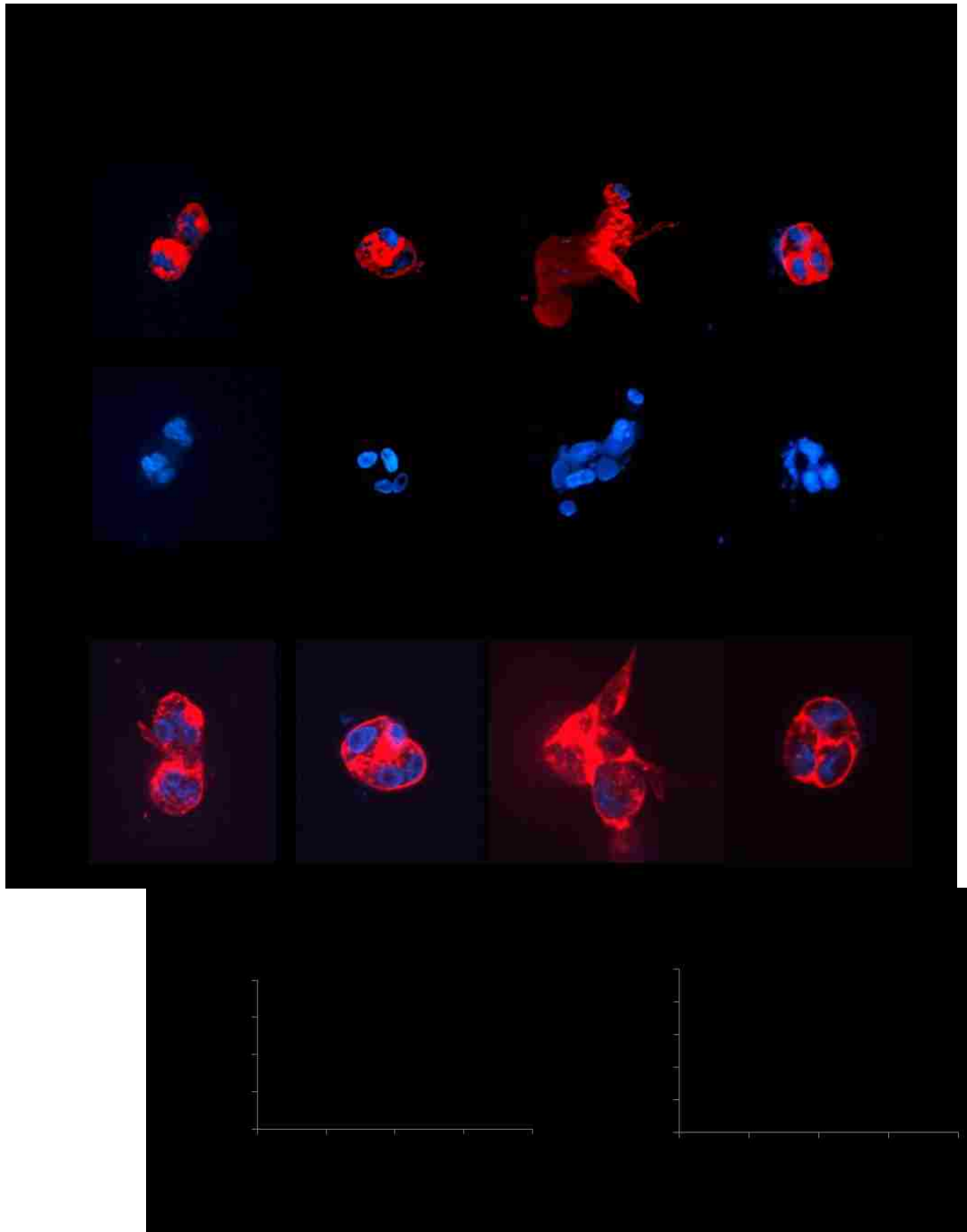


Figure 11. KLF8 expression leads to FLP formation and polarized, invasive tissue architecture in breast cancer cells after sustained treatment with CXCL12 in MoT culture.

(a), 231K8-ikd cells show FLP formation and non-polarized architecture in the presence of CXCL12, which is significantly diminished on KLF8 downregulation. 231K8-ikd cells grown under induced (I) or uninduced (U) conditions were either exposed to PBS or CXCL12 for 24 hours before MoT culture for 5 days. The 3D matrigel inserts were stained with phalloidin to visualize 3D images of the cell clusters as

well as FLP formation, (back) Quantification of colony polarity and number of FLP-positive cell colonies showing that both determinants are significantly increased on CXCL12 treatment in the presence of KLF8 overexpression. Downregulation of KLF8 significantly diminishes the non-polar architecture despite presence of CXCL12. Quantification was done on Volocity software. Quantifications were done using Volocity imager. *P<0.01

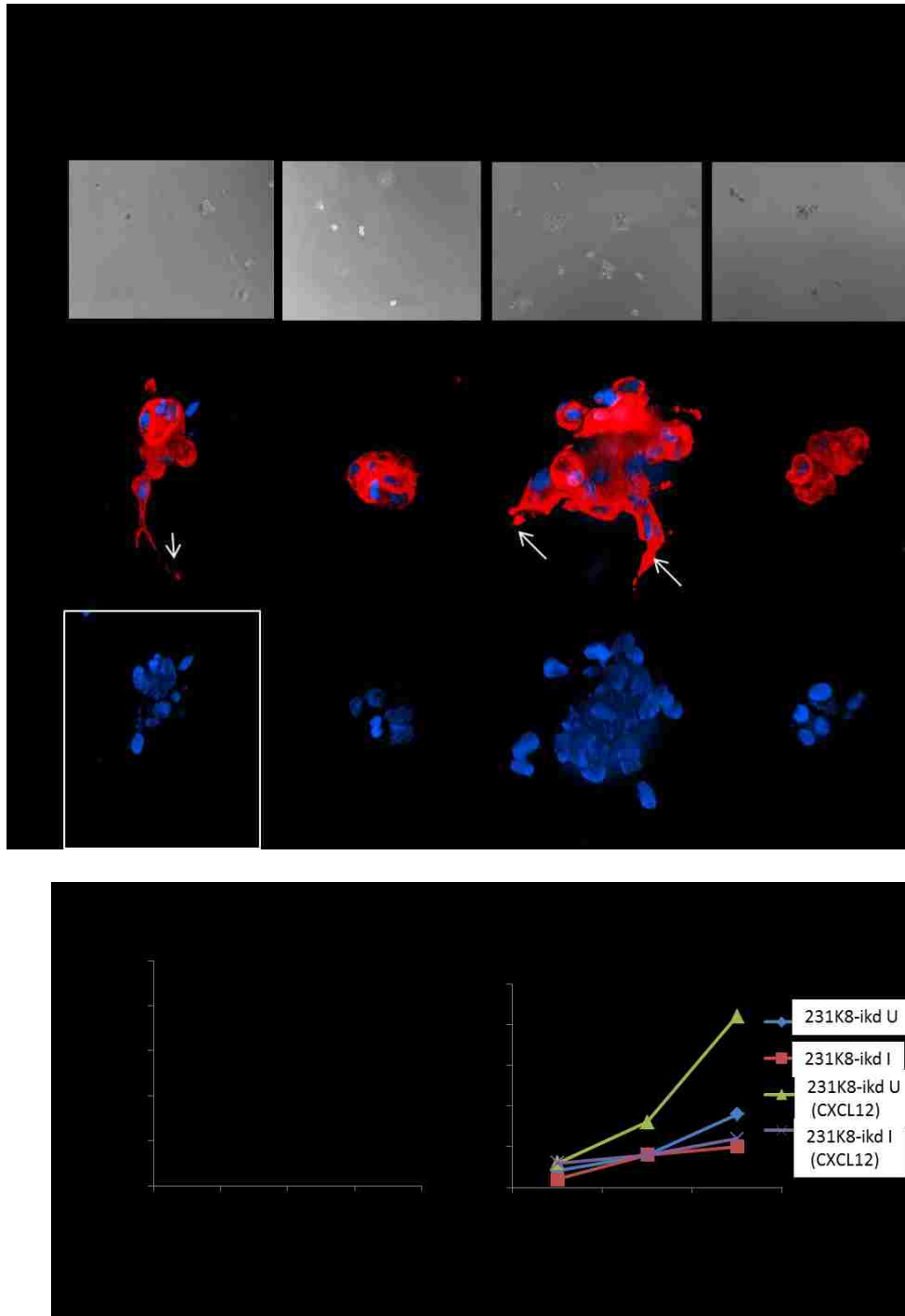
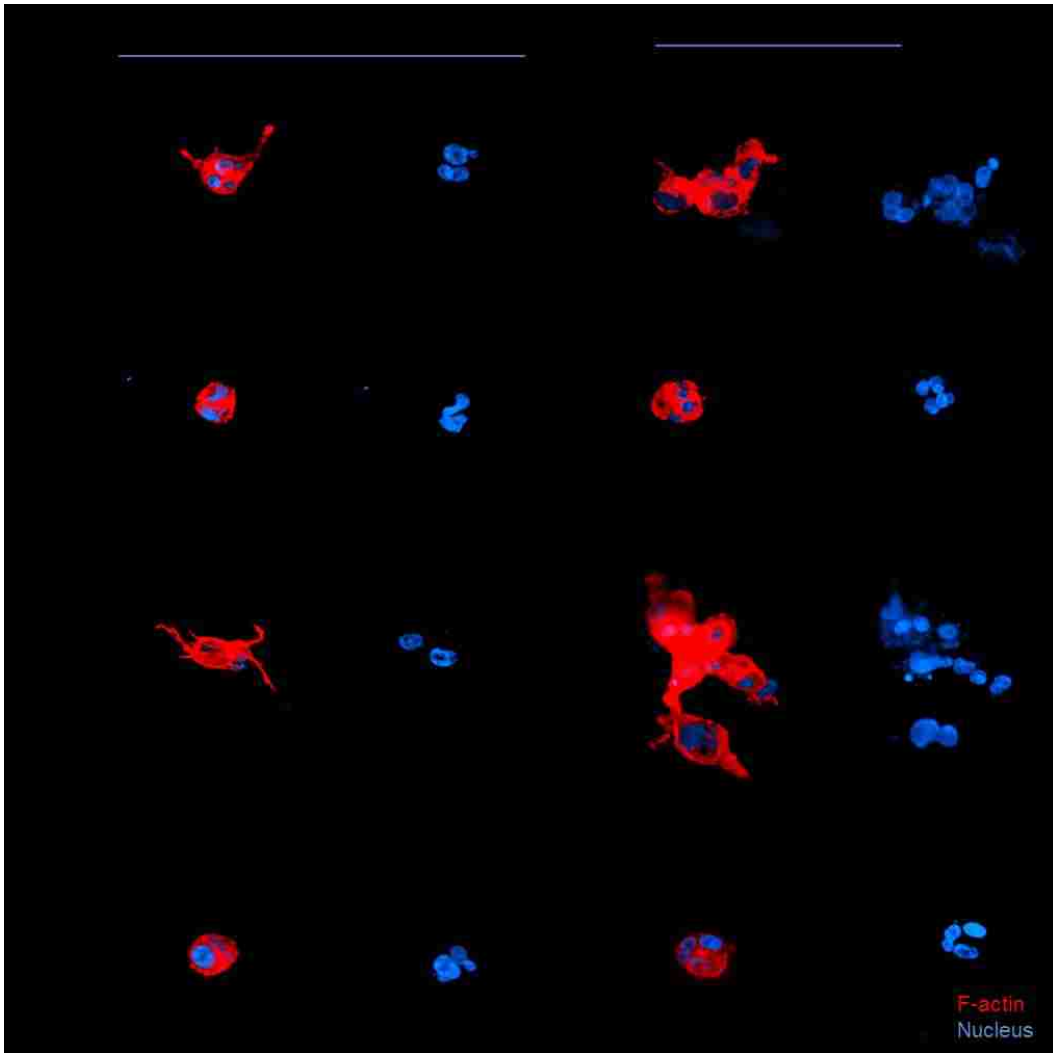


Figure 12. KLF8 expression leads to significantly enhanced proliferation rate of breast cancer cells in 3D under prolonged treatment with CXCL12,

(a), 231K8-ikd cells show FLP formation and non polarised architecture as well as significantly larger cell colonies in the presence of CXCL12, which is significantly diminished on KLF8 downregulation. 231K8-ikd cells grown under induced (I) or uninduced (U) conditions were either exposed to PBS or CXCL12 for 24 hours before MoT culture for 5 days. The 3D matrigel inserts were stained with

phalloidin to visualise 3D images of the cell clusters as well as FLP formation. **(b.)** Quantification cells per colony in 3D showing that it is significantly increased on CXCL12 treatment in the presence of KLF8 overexpression. Downregulation of KLF8 significantly diminishes cell number/ individual colony (as a marker for proliferation rate) Quantification was done on Volocity software. **(c)**, : KLF8 expression in leads to significantly enhanced proliferation rate of breast cancer cells in 3D progressively *P<0.01

A



B

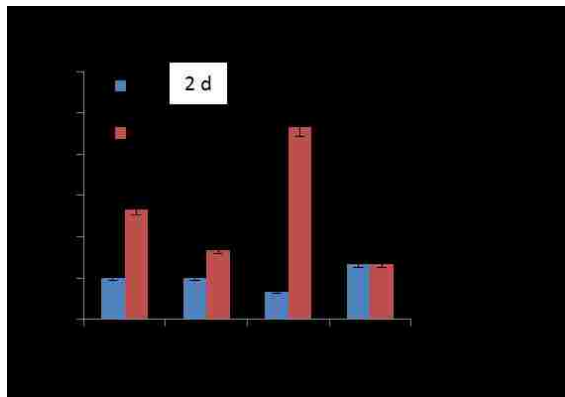


Figure 13. KLF8 triggers FLP formation and subsequent proliferation through CXCR4-CXCL12 engagement.

(a,b) Downregulation of KLF8 in 231 cells leads to diminished proliferation and FLP formation in 3D progressively, which is rescued on overexpression of WT-CXCR4 in KLF8-knockdown cells. Quantification was done on Velocity software. *P<0.01

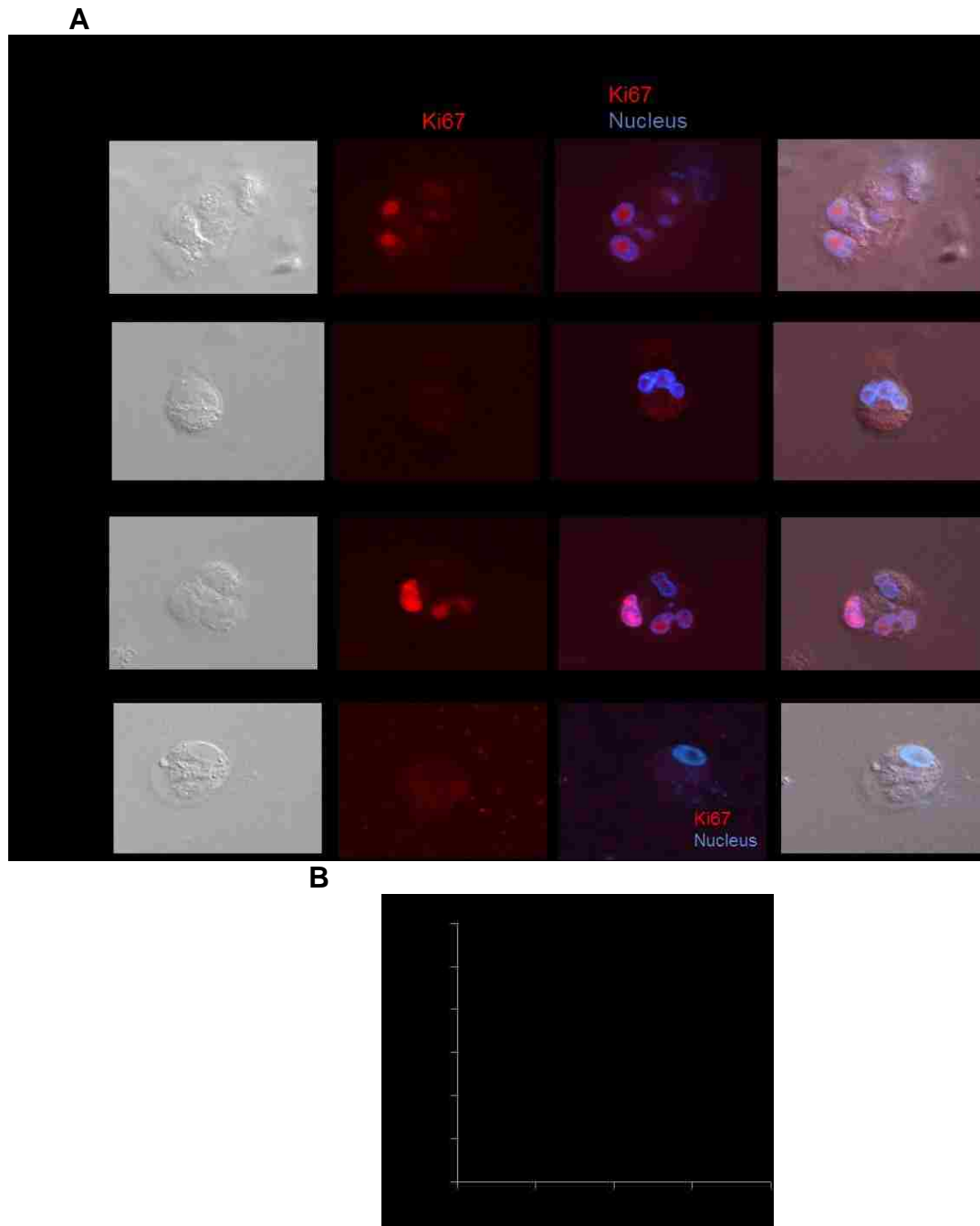


Figure 14. Exposure to CXCL12 promotes proliferation of extravasated breast cancer cells through KLF8 expression

(a,b) CXCL12 treated 231K8-ikd U cells are at a proliferative state, an effect which is diminished on KLF8 knockdown. The diminished proliferation rate is rescued on overexpressing WT-CXCR4 in the

KLF8-knockdown cells. The cells were cultured in MoT culture conditions and stained in situ with phalloidin and Ki67 antibody. Quantification was done on Velocity software *P<0.01

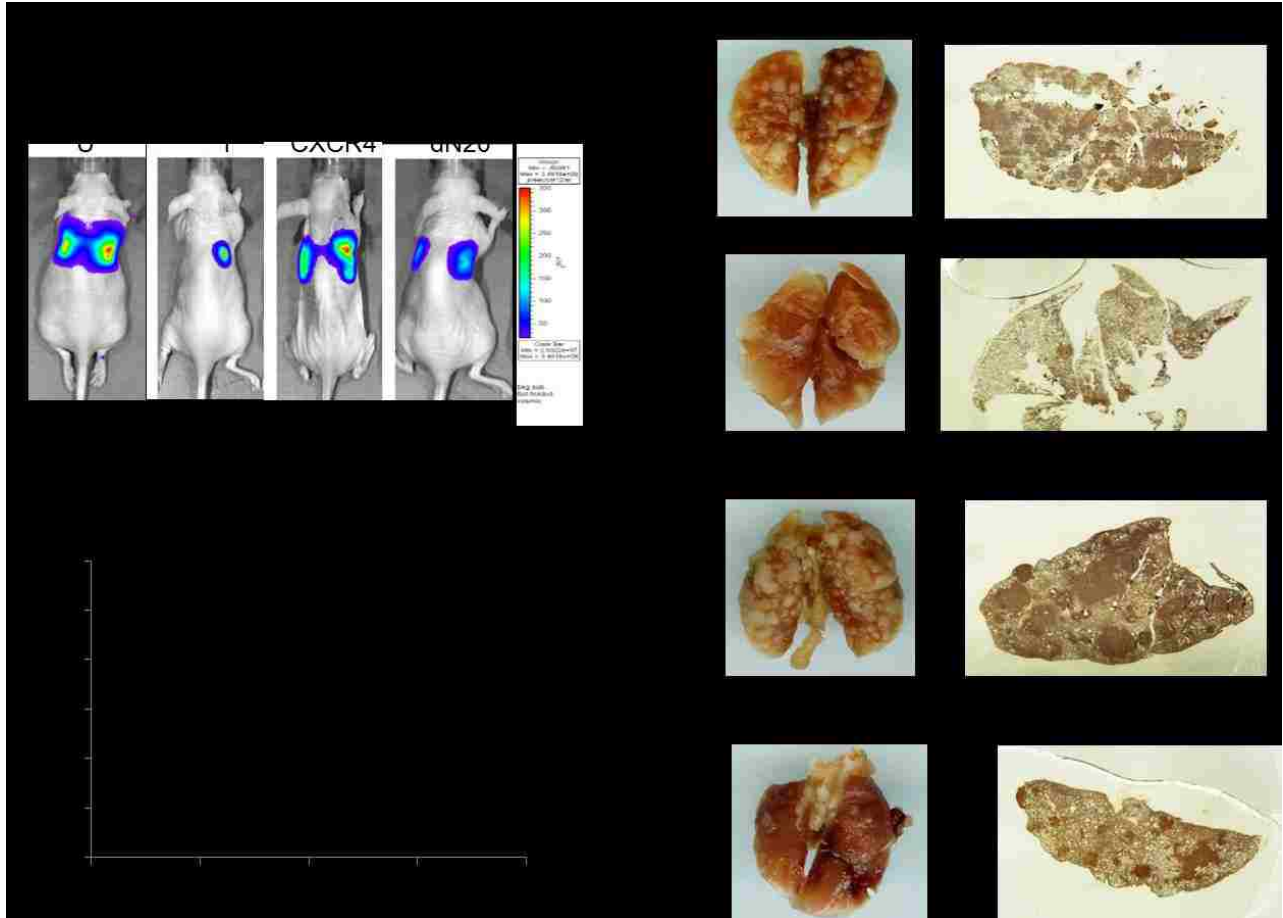


Figure 15. Higher proliferation rate of metastatic colonies, ie, larger macrometastases was seen in mice injected with 231K8-ikd U and 231K8-ikd/WTCXCR4 I cells

(a) KLF8 activation of CXCR4/CXCL12 signaling is required for lung metastasis. 231K8-ikd, 231K8-ikd/WTCXCR4 and 231K8-ikd/d20CXCR4 cells were injected into the mammary fat pad of four individual groups of mice as indicated. The second, third and fourth groups were fed with Dox diet (I, KLF8 knockdown induction). Representative images are shown for BLI imaging done on mice 6 weeks post injection. (b, c) Larger macrometastases was seen in mice injected with 231K8-ikd U and 231K8-ikd/WTCXCR4 I cells indicating that KLF8 promotes higher colonization rate of breast cancer cells via CXCR4 receptor. The area of macrometastasis was enumerated and quantified using the Velocity software. *P<0.01

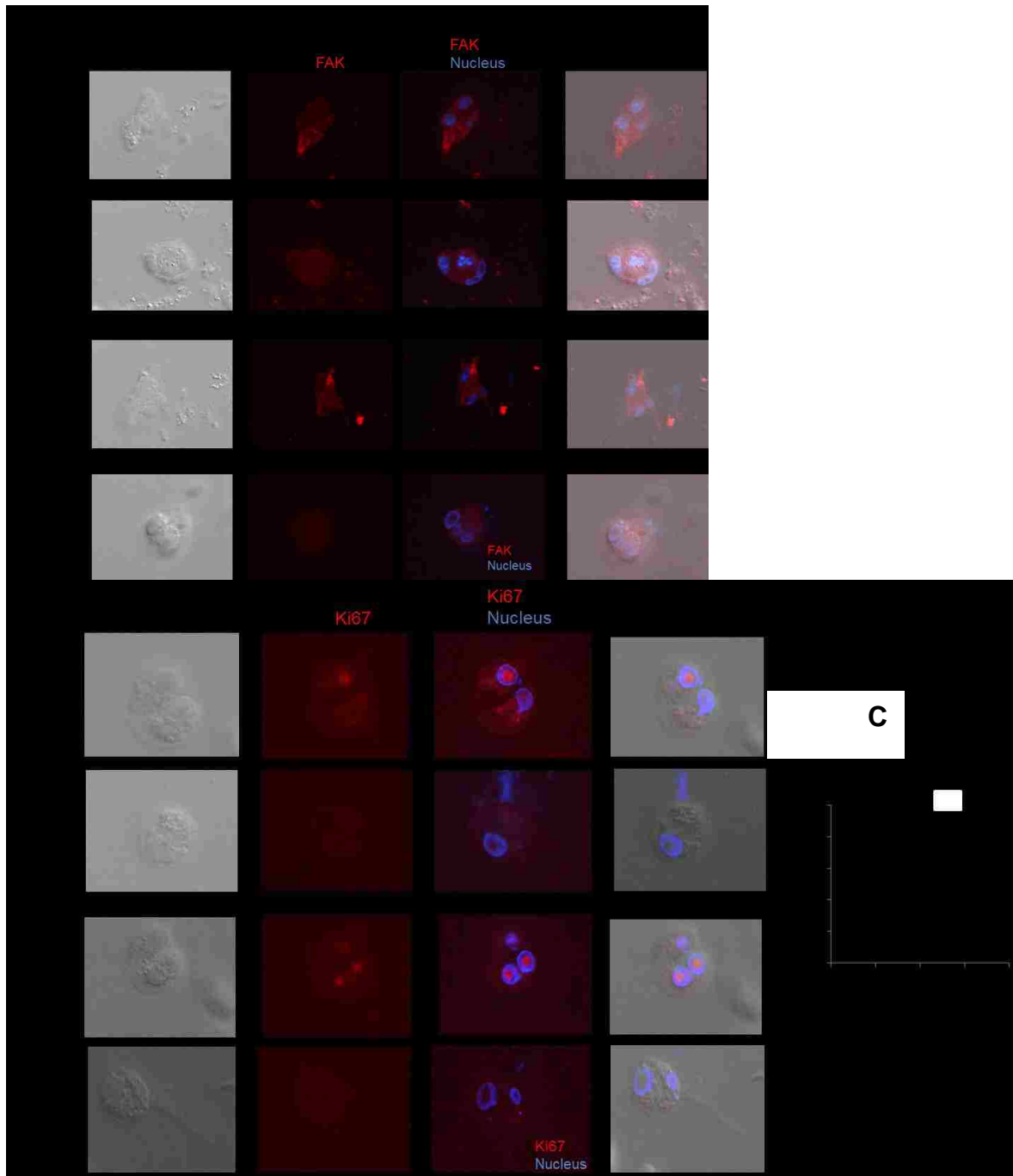


Figure 16. FAK is recruited to the newly formed FLP in 231K8-ikd U cells treated with CXCL12, pointing to the downstream role of FAK in regulating proliferation of cancer cells post colonization

(a) FAK is recruited to the nascent FLP in 231K8-ikd U as well as in 231K8-ikd/WT CXCR4 I cells treated with CXCL12, pointing to the downstream role of FAK in regulating proliferation of cancer cells post colonization. (b,c) FAK activation is critical for 231K8-ikd U and 231K8-ikd/ WTCXCR4 I to

maintain a proliferative state. The cells were pretreated with PF-228 for 4 hours before culturing in MoT conditions for 48 hours and stained in situ with phalloidin and Ki67 antibody. Quantification was done on Velocity software *P<0.01

Discussion

This study identified that KLF8 triggers FLP formation and the initiation of proliferation of recently extravasated dormant cancer cells disseminated in the foreign tissue parenchyma. CXCR4 acts as a key mediator of KLF8's role in enhancing post-extravasation proliferation by engaging its cognate ligand CXCL12 which is present at elevated levels in the foreign tissue microenvironment. Furthermore, we demonstrated that FAK activation is critical in mediating KLF8's role in post-extravasation proliferation downstream of CXCL12-CXCR4 engagement, whereby, recruitment of FAK to the newly-formed FLPs and subsequent activation triggers proliferative growth downstream.

This is the first study demonstrating that the CXCL12 present in the novel microenvironment, as well as endogenous KLF8 expressed in the growth-arrested micrometastatic cancer cells, together drive the establishment of successful macroscopic metastases. As reported previously, CXCL12 acts as an attractant and survival factor for CXCR4-expressing cells [64]. Recent studies have indicated that CXCL12 supports breast cancer cell survival in the bone marrow microenvironment, either in dormant state or as indolent micrometastasis through CXCR4 engagement [134]. Importantly, CXCL12-CXCR4 interaction has been shown to promote colonization of the bone marrow by hematopoietic stem cells and gonads by primordial germ cells (PGCs) during development. In addition, CXCL12 stimulates PI3K-AKT signaling which is

known to support the survival of cancer cells in harsh and unfamiliar microenvironments [135]. Our results indicate that, in addition to initially supporting dormant micrometastasis formation, the CXCR4-CXCL12 engagement is equally critical in stimulating subsequent overt macrometastasis in recently extravasated cancer cells having elevated levels of KLF8. The underlying reason behind breast cancer cells metastasizing preferentially to CXCL12 tissues like lung, liver, bone marrow and lymph nodes has been largely attributed to the role of the CXCR4 receptor in guiding the trafficking of breast cancer cells towards CXCL12-rich tissues along an increasing CXCL12-mediated chemotactic gradient. Our observations demonstrate that, in addition to directing breast cancer cell migration to the secondary host tissue, the CXCR4-CXCL12 axis also participates in triggering the final rate-limiting step of the metastatic-invasion cascade, whereby recently extravasated KLF8-high tumor cells respond to chemotactic signals from extracellular CXCL12 in the foreign microenvironment to reinitiate proliferative programs to ultimately colonize the host tissue.

The ability to extend actin-rich protrusions or FLPs has been found to be critical to the initiation of post extravasation proliferation of growth-arrested breast cancer cells lodged at the foreign tissue parenchyma [115, 131]. Interestingly, FLPs have been found to harbor integrin $\beta 1$ receptors that enable productive interactions between the cytoskeleton and the surrounding extracellular matrix components. Establishing meaningful connections with the foreign

microenvironment is one of the key steps that contribute towards subsequent proliferation of previously dormant cells disseminated at the metastatic site. Therefore, the ability of FLP formation is a prerequisite for eventual metastatic colonization and the inability of majority of cancer cells to initiate proliferation on reaching the foreign tissue site is closely linked to the inability to generate FLPs. Our report is the first to demonstrate that KLF8 plays a key role in triggering FLP formation in recently extravasated breast cancer cells through CXCR4 engagement by CXCL12 which is abundantly expressed in the host microenvironment.

Consistent with our results, FAK has recently been implicated in promoting proliferation and colonization of breast cancer cells in the lung. Multiple studies have reported that CXCL12 stimulation causes FAK activation as a downstream effect. In hematopoietic stem cells, FAK activation downstream of CXCR4-CXCL12 engagement is mediated by the PI3K-AKT signaling – a pathway well-known to enhance cancer cell survival in challenging environments. This study also shows that Src activity is critical in mediating FAK activation downstream of CXCR4 engagement. Importantly, cancer cells with elevated Src signaling and high CXCR4 expression levels are especially primed to utilize the physiological survival signals in the bone marrow, thereby increasing the probability of successfully establishing macroscopic metastases. In view of this, it will be interesting to study whether CXCR4-CXCL12 interaction stimulates FAK activation in extravasated cells through the PI3K-AKT pathway or Src signaling, especially since

both these pathways have been implicated in promoting metastatic colonization. Importantly, in our previous report we identified a critical feed-forward signaling wheel of KLF8, CXCR4 and FAK that drives metastatic dissemination of breast cancer cells in the lung[128]. It will be interesting to investigate whether this feed-forward loop also serves as an important molecular/signaling mechanism underlying the rapid proliferative growth seen in recently extravasated KLF8-high cells in response to extracellular CXCL12 in the foreign microenvironment.

This study opens up a new chapter in our understanding of the role of KLF8 and CXCR4 in the latter steps of the metastatic-invasion cascade. Elevated levels of CXCL12 in the metastatic site act as a crucial environmental cue that assists the newly extravasated cells in adapting to the unfamiliar microenvironment. KLF8-high cells express the CXCR4 receptor which engages its cognate ligand CXCL12 from the surrounding ECM to trigger the formation of actin-rich FLPs, which are critical for generating productive connections with the parenchymal ECM ultimately leading to the establishment of macroscopic metastases. These novel findings have identified KLF8 as a critical regulator influencing the outcome of the crucial rate-limiting final step of the metastatic process, whereby elevated levels of KLF8 expression is a prerequisite for the initiation of proliferation of extravasated breast cancer cells. Together with our previous report demonstrating the role of KLF8 in enhancing primary tumor invasion and transendothelial

migration [136], this study further underscores the importance of KLF8 as a critical regulator of the metastatic process in its entirety, thereby presenting KLF8 as an excellent target of therapeutic intervention against breast cancer metastasis.

CHAPTER 4: CONCLUSION

Metastasis represents a complex, multi-step process involving successive inter-related cell-biological events whereby invasive tumor cells i) dissociate from the primary tumor, ii) locally invade into the surrounding stroma, iii) intravasate into the lamina of blood vessels to travel with the blood as circulating tumor cells (CTCs), iv) extravasate out into the foreign tissue parenchyma, v) initially survive and sustain in the foreign microenvironment to form dormant microenvironment, and finally, vi) successfully reinitiate proliferation to form clinically detectable macrometastatic lesions at the secondary sites (metastatic colonization)[136]. The later steps of the metastatic cascade, specifically micrometastatic formation and metastatic colonization represent the major rate-limiting steps of the metastatic process. A substantial proportion of successfully extravasated cells undergo apoptosis, whereas only three percent of the cells are able to persist in the foreign tissue parenchyma in a viable but growth-arrested state. In the case of subsequent macroscopic colonization, less than one percent of the micrometastatic cells are able to reinitiate their proliferative programs to establish macroscopic metastasis.

Krüppel-like Factor 8 is a member of the Kruppel-like family of transcription factors. It has been found to be upregulated in different types of cancers, including breast cancer. Our study identified CXCR4 as a novel direct target of transcriptional activation by KLF8 and a key mediator of KLF8's role in promoting CXCL12-dependant breast cancer cell migration and invasion required for the invasive growth of the primary tumor as well as TEM essential for the lung metastasis involving a feed-forward activation of FAK. KLF8 directly interacts with the GT-box 1 site of the CXCR4 promoter to trigger CXCR4 transcription. Membrane-bound CXCR4 can then interact with CXCL12 in the immediate local environment to activate

directional cell migration along an increasing chemotactic gradient of CXCL12. Furthermore, CXCL12 engagement of the CXCR4 receptor leads to FAK activation downstream, resulting in cytoskeletal rearrangement and filopodia formation in the cell, ultimately triggering cell migration towards the CXCL12 gradient. In addition, FAK activation can induce heightened KLF8 expression downstream, pointing to the existence of a feed forward loop which can further enhance the cell-invasiveness and CXCR4-mediated migrational ability of breast cancer cells in response to CXCL12.

This study is the first to report that CXCR4 is a target of direct transcriptional activation by KLF8 which positively regulates the metastatic process through the CXCR4/CXCL12 axis. This is also the first report of a hitherto unknown role of KLF8 as a critical mediator of transendothelial migration. Indeed, our study provides substantial evidence to suggest that KLF8 plays a crucial role in the key steps of primary tumor dissemination, including tumor cell invasion of local stroma, chemotactic gradient-dependent directional transendothelial migration to cross the endothelial barrier and enter into the bloodstream (intravasation) and to exit from the blood vessel lumina into the tissue parenchyma of secondary organs (extravasation).

CXCR4 receptor was initially discovered as a co-receptor for HIV entry. The chemokine CXCL12 was established as the specific ligand for CXCR4. The expression of CXCL12 has been found to be highest in tissues like lung, liver, bone marrow and brain where it retains or attracts CXCR4-expressing cells. The CXCR4/CXCL12 axis is indispensable as an effective lymphocyte chemoattractant and hematopoiesis regulator. Tissue damaging conditions like hypoxia, toxins or irradiation induce an increase in local expression of CXCL12, thereby recruiting CXCR4-positive stem cells and lymphocytes to the site that requires tissue repair or regeneration. This study is consistent with other reports that indicate that cancer cells can “hijack” the

CXCR4/CXCL12 axis to establish distant organ-specific metastasis. Indeed, our study concurs with previous reports showing that inhibition of the CXCR4-CXCL12 axis can block cancer metastasis to secondary organs. This stresses the importance of being able to efficiently control the expression of a single chemokine receptor CXCR4, which can define the metastatic program of a tumor cell. In this study, we identified a novel role of KLF8 as the direct positive regulator of CXCR4 expression. Furthermore, we demonstrated the crucial role played by KLF8 in promoting organ-specific metastasis through the CXCR4-CXCL12 axis. Together with our previous reports, this study further highlights the importance of KLF8 as a master regulator of cancer pathogenesis and metastatic progression.

This is the first study demonstrating that the CXCL12 present in the novel microenvironment, as well as endogenous KLF8 expressed in the growth-arrested micrometastatic cancer cells, together drive the establishment of successful macroscopic metastases. KLF8 triggers FLP formation and the initiation of proliferation of recently extravasated dormant cancer cells disseminated in the foreign tissue parenchyma. CXCR4 acts as a key mediator of KLF8's role in enhancing post-extravasation proliferation by engaging its cognate ligand CXCL12 which is present at elevated levels in the foreign tissue microenvironment. Furthermore, we demonstrated that FAK activation is critical in mediating KLF8's role in post-extravasation proliferation downstream of CXCL12-CXCR4 engagement, whereby, recruitment of FAK to the newly-formed FLPs and subsequent activation triggers proliferative growth downstream. This study opens up a new chapter in our understanding of the role of KLF8 and CXCR4 in the latter steps of the metastatic-invasion cascade. Elevated levels of CXCL12 in the metastatic site act as a crucial environmental cue that assists the newly extravasated cells in adapting to the unfamiliar microenvironment. KLF8-high cells express the

CXCR4 receptor which engages its cognate ligand CXCL12 from the surrounding ECM to trigger the formation of actin-rich FLPs, which are critical for generating productive connections with the parenchymal ECM ultimately leading to the establishment of macroscopic metastases. These novel findings have identified KLF8 as a critical regulator influencing the outcome of the crucial rate-limiting final step of the metastatic process

More than 100 years back, Stephen Paget explained the metastatic process through a “seed and soil” hypothesis, whereby he stated that circulating tumor cells seek specific conditions that are favorable to their growth and survival and create metastatic lesions in these specific secondary sites. Consistent with others reports, our results support this hypothesis, indicating that tumor cells travel throughout the body under the influence of chemotactic gradients which make up the “cellular highways” for cell trafficking. However, on reaching secondary sites, successful metastasis requires that the tumor cells must respond to chemotactic signals in the foreign tissue in order to survive and ultimately proliferate to form macrometastatic lesions. It is possible that CXCL12 may participate in this process. Further investigation is required to probe whether KLF8, in association with the CXCR4-CXCL12 axis, can contribute to the process of macrometastatic development post extravasation of tumor cells. In addition, it has been reported that CXCL12 can be released at the primary tumor site by cancer-associated fibroblasts which play a key role in recruiting endothelial progenitor cells into the tumor mass, thereby promoting angiogenesis and tumor progression. In view of this, KLF8 may also play a key role in promoting angiogenesis, thereby enhancing tumor growth and further promoting transendothelial migration at the primary tumor site.

Given that the CXCR4-CXCL12 signaling axis has been identified to play a key role in breast cancer metastasis, disrupting the CXCL12-CXCR4 interaction by treating with antagonists

of the CXCR4 receptor may seem to be the ideal choice for curbing breast cancer metastasis. However, several practical difficulties exist in using this form of therapy. Firstly, long-term treatment of CXCR4-receptor antagonists has proven to be severely detrimental to patient health due to the severe toxicity to the immune system. The excessive toxicity rendered due to global CXCR4 inhibition has emerged as one of the major obstacles to the application of CXCR4-antagonist based therapy. Secondly, CXCR4 antagonists, like AMD3100, block essential processes like hematopoietic stem cell homing in the bone marrow. Therefore, CXCR4 inhibitors and antagonists are not well suited for use in targeted therapy. Thus, there is an urgent need to identify upstream targets and processes which can induce the expression CXCR4 and CXCR4 signaling specifically in tumor cells. The identification of KLF8 as a critical upstream inducer of CXCR4 expression in breast cancer cells presents an excellent therapeutic target to abolish aberrant upregulation and downstream signaling of CXCR4 receptor specifically in tumor cells, without global CXCR4 inhibition, thereby presenting KLF8 as a promising target of therapeutic intervention against breast cancer metastasis.

APPENDIX A : PUBLICATIONS

Debarati Mukherjee, Heng Lu, Lin Yu, Satadru K. Lahiri, Tianshu Li, and Jihe Zhao. Krüppel-like factor 8 activates the transcription of C-X-C cytokine receptor type 4 to promote breast cancer cell invasion, transendothelial migration and metastasis. *Oncotarget*; 2016 Mar 14. doi: 10.18632/oncotarget.8083. [Epub ahead of print]

Satadru K. Lahiri, Heng Lu, **Debarati Mukherjee**, Lin Yu and Jihe Zhao. Phosphorylation of KLF8 at serine 48 protects the protein stability. *Am J Cancer Res*. In press, (2016)

Tianshu Li, Heng Lu, **Debarati Mukherjee**, Satadru K. Lahiri, Chao Shen, Lin Yu and Jihe Zhao. Identification of epidermal growth factor receptor and its inhibitory microRNA141 as novel targets of Krüppel-like factor 8 in breast cancer. *Oncotarget*. Aug 28;6(25):21428-42, (2015)

Li T, Lu H, Shen C, Lahiri SK, Wason MS, **Mukherjee D**, Yu L, Zhao J. Identification of epithelial stromal interaction 1 as a novel effector downstream of Krüppel-like factor 8 in breast cancer invasion and metastasis. *Oncogene*. Oct 7 doi:10.1038/onc.2013.415, (2013)

Debarati Mukherjee and Jihe Zhao. The Role of chemokine receptor CXCR4 in breast cancer metastasis. *Am J Cancer Res*; 3(1):46-57, (2013)

Lu H, Hu L, Yu L, Wang X, Urvalek AM, Li T, Shen C, **Mukherjee D**, Lahiri SK, Wason MS, Zhao J. KLF8 and FAK cooperatively enrich the active MMP14 on the cell surface required for the metastatic progression of breast cancer. *Oncogene*. Jul 1 doi: 10.1038/onc.2013.247, (2013)

Lu H, Hu L, Li T, Lahiri S, Shen C, Wason MS, **Mukherjee D**, Xie H, Yu L, Zhao J. A novel role of Krüppel-like factor 8 in DNA repair in breast cancer cells. *J Biol Chem.* Dec 21;287(52):43720-9, (2012)

APPENDIX B : IACUC PERMISSIONS



8/10/2015

Dr. Jihe Zhao
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd, BSS 313
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr. Jihe Zhao:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #: 13-26
Title: Role of KLF8 in the regulation of EMT and Tumor Metastasis

First Approval Date: 9/20/2013

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

A handwritten signature in black ink that reads "Cristina Caamaño".

Cristina Caamaño
Associate Director, Research Program
Services

Copies: Facility Manager (when applicable.)



THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Re-Approval to Use Animals

Dear Dr. Jihe Zhao,

Your application for IACUC Re-Approval has been reviewed and approved by the UCF IACUC Reviewers.

Approval Date: 8/10/2015

Title: Role of KLF8 in the regulation of EMT and Tumor Metastasis

Department: Burnett School of Biomedical Sciences

Animal Project #: 13-26

Expiration: 9/19/2016

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expiration date regarding your need to file another renewal.

Christopher Parkinson, Ph.D.
IACUC Chair



8/20/2012

Dr. Jihe Zhao
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd. BSS 313
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr. Jihe Zhao:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #: 10-44
Title: Role of KLF8 in the regulation of EMT and Tumor Metastasis

First Approval Date: 10/26/2010

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call me at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

A handwritten signature in cursive script, appearing to read 'Cristina Caamaño'.

Cristina Caamaño
Assistant Director

Copies: Facility Manager (when applicable.)



THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Re-Approval to Use Animals

Dear Dr. Jihe Zhao,

Your application for IACUC Re-Approval has been reviewed and approved by the UCF IACUC Committee Reviewers.

Approval Date: 8/19/2012

Title: Role of KLF8 in the regulation of EMT and Tumor Metastasis

Department: Burnett School of Biomedical Sciences

Animal Project #: 10-44

Expiration: 10/25/2013

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expiration date regarding your need to file another renewal.

Christopher Parkinson, Ph.D.
IACUC Chair

Approved Renewed

APPENDIX C: COPYRIGHT PERMISSIONS

Re: Copyright policy to reproduce figures in published manuscript

 Business Office <business@e-century.org>
To: debarati; 

  Reply all | 

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Thanks for reaching out to us. Since you are going to use your own data, it is free of charge to use it.

Best regards,

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From: debarati <debarati@knights.ucf.edu>
To: "business@e-century.org" <business@e-century.org>
Sent: Thursday, March 10, 2016 6:55 AM
Subject: Copyright policy to reproduce figures in published manuscript

Hi,

I published a paper in the American Journal of Cancer Research in 2013 entitled,
"The Role of chemokine receptor CXCR4 in breast cancer metastasis"
Am J Cancer Res. 2013; 3(1): 46-57.

I am currently preparing my dissertation for graduating with a doctoral degree. I would like to request your permission to use the figures and tables and some text portions from this manuscript. Do I need to formally ask for the copyright?

Best,
Debarati



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On Tue, Mar 22, 2016 at 2:53 PM, debarati <debarati@knights.ucf.edu> wrote:

Dear Sir/Ma'am,

I have recently published a first author research paper in your journal entitled,

"Krüppel-like factor 8 activates the transcription of C-X-C cytokine receptor type 4 to promote breast cancer cell invasion, transendothelial migration and metastasis" **Oncotarget**. 2016 Mar 14. doi: 10.18632/**oncotarget**.8083. [Epub ahead of print]

I would like to use material from the above article in my PhD dissertation. As such, I would be immensely grateful if you could grant me the permission to use figures and text from this paper.

Thank you,

Debarati

APPENDIX D: DEFENSE ANNOUNCEMENT

**Announcing the Final Examination of Ms. Debarati Mukherjee for the degree of
Doctor of Philosophy in Biomedical Sciences**

Dissertation Title: Role Of KLF8-CXCR4 Signaling In Breast Cancer Metastasis

Date: April 4th, 2016 Time: 2:30 PM Location: BBS 103 (Live)/BMS 136 Simulcast

ABSTRACT

Krüppel-like factor 8 (KLF8) has been strongly implicated in breast cancer metastasis. However, the underlying mechanisms remain largely unknown. Here we report a novel signaling from KLF8 to C-X-C cytokine receptor type 4 (CXCR4) in breast cancer. Overexpression of KLF8 in MCF-10A cells induced CXCR4 expression at both mRNA and protein levels. This induction was well correlated with increased Boyden chamber migration, matrigel invasion and transendothelial migration (TEM) of the cells towards the ligand CXCL12. In contrast, knockdown of KLF8 in MDA-MB-231 cells reduced CXCR4 expression associated with decreased cell migration, invasion and TEM towards CXCL12. Histological and database mining analyses of independent cohorts of patient tissue microarrays revealed a correlation of aberrant co-elevation of KLF8 and CXCR4 with metastatic potential. Promoter analysis indicated that KLF8 directly binds and activates the human CXCR4 gene promoter. Furthermore, CXCR4-CXCL12 engagement downstream of KLF8 leads to the feed-forward activation of FAK. Interestingly, KLF8 expression, through CXCR4 engagement, triggered the formation of filopodium-like protrusions (FLP) and thereby enhanced the proliferation rate of breast cancer cells in 3D Matrigel-on-Top culture, under prolonged treatment with CXCL12. This indicates that KLF8 is critical in promoting aggressive colonization of tumor cells in a CXCL12-enriched foreign tissue microenvironment, thereby aiding in macrometastasis formation. Xenograft studies showed that overexpression of CXCR4, but not a dominant-negative mutant of it, in the MDA-MB-231 cells prevented the invasive growth of primary tumor and lung metastasis from inhibition by knockdown of KLF8. Apart from the lungs, KLF8-CXCR4 signaling is also required for spontaneous metastasis to other CXCL12-rich organs. These results collectively suggest a critical role for the KLF8-CXCR4 pathway in promoting breast cancer metastasis and shed new light on potentially more effective anti-cancer strategies.

Dissertation Committee Members:

Dr. Jihe Zhao (Chair)
Dr. Deborah Altomare
Dr. Annette Khaled
Dr. Shadab Siddiqi

Peer-Reviewed Publications

Published:

Debarati Mukherjee, Heng Lu, Lin Yu, Satadru K. Lahiri, Tianshu Li, and Jihe Zhao. Krüppel-like factor 8 activates the transcription of C-X-C cytokine receptor type 4 to promote breast cancer cell invasion, transendothelial migration and metastasis. *Oncotarget*; 2016 Mar 14. doi: 10.18632/oncotarget.8083. [Epub ahead of print]

Satadru K. Lahiri, Heng Lu, **Debarati Mukherjee**, Lin Yu and Jihe Zhao. Phosphorylation of KLF8 at serine 48 protects the protein stability. *Am J Cancer Res*. In press, (2016)

Tianshu Li, Heng Lu, **Debarati Mukherjee**, Satadru K. Lahiri, Chao Shen, Lin Yu and Jihe Zhao. Identification of epidermal growth factor receptor and its inhibitory microRNA141 as novel targets of Krüppel-like factor 8 in breast cancer. *Oncotarget*. Aug 28;6(25):21428-42, (2015)

Li T, Lu H, Shen C, Lahiri SK, Wason MS, **Mukherjee D**, Yu L, Zhao J. Identification of epithelial stromal interaction 1 as a novel effector downstream of Krüppel-like factor 8 in breast cancer invasion and metastasis. *Oncogene*. Oct 7 doi:10.1038/onc.2013.415, (2013)

Debarati Mukherjee and Jihe Zhao. The Role of chemokine receptor CXCR4 in breast cancer metastasis. *Am J Cancer Res*; 3(1):46-57, (2013)

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Lu H, Hu L, Li T, Lahiri S, Shen C, Wason MS, **Mukherjee D**, Xie H, Yu L, Zhao J. A novel role of Krüppel-like factor 8 in DNA repair in breast cancer cells. *J Biol Chem*. Dec 21;287(52):43720-9, (2012)

Pending:

Debarati Mukherjee, Heng Lu, Lin Yu, Satadru K. Lahiri, and Jihe Zhao. KLF8-CXCR4 signaling promotes breast cancer metastasis through FLP-dependent colonization. *In preparation*.

Approved for distribution by Dr. Jihe Zhao, Committee Chair

The public is welcome to attend

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