The Function and Regulation of CXCR4 in Ewing Sarcoma

by

Melanie Anne Krook

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Doctoral Committee:

Associate Professor Elizabeth R. Lawlor, Chair
Professor David G. Beer
Professor Thomas E. Carey
Professor Maria Castro
Associate Professor Gary D. Luker

To Marcia Hetletvedt
always embracing life, 1960-2011

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
β2M	Beta-2 microglobulin
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL12	Chemokine (C-X-C motif) ligand 12
CTLC	Cutaneous T-cell lymphoma
CXCR4	Chemokine (C-X-C motif) receptor 4
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DTT	Dithiothreitol
ECM	Extracellular matrix
EFS	Event-free survival
EMT	Epithelial-mesenchymal transition
ES	Embryonic stem cell
EZH2	Enhancer of zeste homolog 2
FBS	Fetal bovine serum
FDA	Food and drug administration
G-CSF	Granulocyte-colony stimulation factor
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
H2AK119Ub1	Mono-ubiquitination of histone 2A on lysine 119
H3K27ac	Acetylation of histone H3 on lysine 27
H3K27me1/2/3	Mono-, di-, trimethylaton of histone H3 on lysine 27

H3K4me1	Monomethylation of histone H3 on lysine 4
H3K4me3	Trimethylation of histone H3 on lysine 4
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
hESC	Human embryonic stem cell
HIF1-α	Hypoxia-inducible factor 1-α
HIV-1	Human immunodeficiency virus-1
HMT	Histone methyltransferase
HSC	Hematopoietic stem cell
JNK	C-Jun N-terminal kinase
LUC	Luciferase
MAPK	Mitogen-activated protein kinase
MBD	Methyl CpG-binding domain
MDS	Myelodysplastic syndrome
MLL	Mixed lineage leukemia
MNase	Micrococcal nuclease
PcG	Polycomb group proteins
PI3K	Phosphoinositide 3-kinase
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
qPCR	Quantitative polymerase chain reaction
RNAi	RNA interference
SDF-1	Stromal cell-derived factor 1
TALENs	Transcription activator-like effector nucleases
TrxG	Trithorax group proteins
XCI	X-chromosome inactivation

ABSTRACT

Ewing sarcoma is an aggressive and deadly bone and soft tissue cancer predominantly effecting the pediatric population. While intensification of chemotherapy has improved the outlook for patients with localized disease, little progress has been made for patients with metastatic or relapsed disease. The prognosis for these patients remains dismal, with fewer than 20% surviving. However, little is known about the biological mechanisms driving Ewing sarcoma metastasis and in order to improve outcomes for Ewing sarcoma patients, it is imperative that we understand these metastatic processes. The chemokine receptor, CXCR4, is the most commonly expressed chemokine receptor in human cancer. There is evidence demonstrating that the CXCL12/CXCR4 signaling axis contributes to tumor metastasis in over twenty human malignancies but its function in Ewing sarcoma is not yet known. Through the work of this thesis, we sought to investigate the role of the CXCL12/CXCR4 signaling axis as a mediator of Ewing sarcoma metastasis.

Evaluation of CXCR4 transcript and surface protein in Ewing sarcoma cell lines and primary tumors revealed variability of CXCR4, ranging from undetectable expression to high-level expression. We have demonstrated the highly dynamic nature of CXCR4 expression in Ewing sarcoma cells and have shown that this molecular plasticity allows Ewing sarcoma to switch back and forth between CXCR4- and CXCR4+ states in response to changes in the

microenvironment. Significantly, Ewing sarcoma cells that express high levels of CXCR4 are highly migratory and invasive toward a CXCL12 gradient. The CXCR4 antagonist, AMD3100, impeded the migratory and invasive properties of the CXCR4+ cells.

Having demonstrated that CXCR4 is dynamic and that its expression is induced in response to microenvironmental cues we investigated the potential contribution of epigenetic regulation to these cell-state transitions. Chromatin immunoprecipitation studies revealed that the *CXCR4* promoter exists in a bivalent state, as characterized by simultaneous enrichment of the activating, H3K4me3, and the repressive, H3K27me3 marks at the *CXCR4* promoter. Of significance, exposure of Ewing sarcoma cells to microenvironmental cues resulted in loss of bivalency, as evidenced by loss of H3K27me3 and retention of the H3K4me3. Additionally, we demonstrated that an active enhancer exists upstream of CXCR4 and also contributes to *CXCR4* regulation. In support of this, treatment of Ewing sarcoma cells with the bromodomain inhibitor, JQ1, resulted in marked down-regulation of *CXCR4* expression in ambient conditions and blocked stress-dependent induction. These findings support the potential use of epigenetic modifiers as therapeutics that could be used to antagonize cell state transitions.

This thesis reveals the highly plastic and dynamic nature of CXCR4 expression in Ewing sarcoma and shows that cells transition between CXCR4- and CXCR4+ states in response to changes in the microenvironment. These data support a model in which reversible, epigenetically-driven, cell state

transitions contribute to metastasis. I have developed novel genetic and *in vivo* tools that will enable testing of this hypothesis in the future. By elucidating mechanisms of CXCR4 regulation and the contribution of CXCL12/CXCR4 signaling to Ewing sarcoma metastasis this thesis identifies new areas for biologic investigation and new opportunities for therapeutic intervention.

CHAPTER ONE

INTRODUCTION

Rationale

Cancer represents a major public health problem and is a leading cause of morbidity and mortality worldwide (1). Additionally, this is reflected in pediatric population, as cancer is the number one cause of death by disease in children between the ages of 0 and 19 years in the U.S. (2). Furthermore, metastasis accounts for 90% of cancer related deaths. This thesis focuses on Ewing sarcoma, an aggressive and deadly pediatric cancer. Ewing sarcoma is the second most common bone malignancy in children and young adults (3). Over the last several decades, the outcome for Ewing sarcoma patients with localized disease has improved; however for the quarter of patients who present with metastatic disease, the outcome remains dismal (4, 5). Unfortunately these statistics are attributed to the lack of new therapies being developed to combat metastatic pediatric cancer as we are still trying to understand the molecular and cellular biology driving Ewing sarcoma metastasis.

Chemokines and chemokine receptors play essential roles in many physiological processes with the most critical being the ability to induce chemotaxis. Given this role in chemotaxis, there is a rich literature implicating

chemokines and their receptors in tumor metastasis (6, 7). One of the best-studied chemokine and chemokine receptor pairs is CXCL12/CXCR4. The CXCL12/CXCR4 signaling axis has been shown to contribute to tumor cell proliferation and angiogenesis (8-10). Furthermore, numerous studies demonstrate that CXCR4 expression is upregulated in metastatic tumors when compared to localized tumors (11, 12). Despite this phenomenon being documented in many human malignancies, little is known about the contribution of CXCR4 to Ewing sarcoma metastasis. Therefore, in this thesis we begin to investigate the contribution of CXCR4 to Ewing sarcoma metastasis. Defining the contribution of CXCR4 to Ewing sarcoma metastasis will provide a new understanding of the molecular mechanisms driving Ewing sarcoma with the long term hope of developing new therapeutic strategies to tackle metastatic disease.

Ewing sarcoma

Pathologist James Ewing first described Ewing sarcoma in the 1920s as a bone tumor, which he initially termed diffuse endothelioma (13). Ewing sarcoma is an aggressive small round cell tumor of presumed stem cell origin affecting the bones and soft tissues. Although a rare tumor type with only an average of 200 new cases in the United States per year, Ewing sarcoma is the second most common bone malignancy in children and young adults (14). Ewing sarcoma has a predilection for males (1.6:1) and a peak incidence between the ages of 5 and 25 (15). Ewing sarcoma most commonly presents in the long bones (femur, humerus, and tibia), pelvis, and chest wall (16). The bone tumors preferentially

present in the adolescent patients whereas the soft tissue tumors preferentially present in the adult patients (17).

Ewing sarcoma genetics

Ewing sarcoma is genetically characterized by a somatic reciprocal translocation between the EWSR1 gene and an ETS family member, which is considered pathognomonic for the disease (18). The translocation encodes a protein, which acts as an aberrant transcription factor that deregulates key genes involved in Ewing sarcoma oncogenesis (19). About 85-90% of Ewing sarcoma cases have an EWS-FLI1 translocation between chromosome 11 and 22 t(11;22)(q24;q12) (19, 20). Variant EWS-ETS fusions have been reported including EWS-ERG, EWS-ETV1, EWS-ETV4, and EWS-FEV (21-25). In less than 1% of Ewing sarcoma patients, non-EWS fusions have been identified. These fusions occur between the TET (TLS/EWS/TAF15) family member, TLS and one of two ETS family members, ERG or FEV (26). Aside from the translocation, Ewing sarcoma tumors are typically genetically silent tumors with a low mutational burden; however, recurrent somatic mutations across Ewing sarcoma tumors and cell lines have been reported in *STAG2* (20%), *CDKN2A* (12%), and *TP53* (7%) (27-29).

Ewing sarcoma treatment

Ewing sarcoma is treated with aggressive multi-agent chemotherapy and local control. A combination of neoadjuvant and adjuvant multi-agent systemic

chemotherapy consisting of vincristine, doxorubicin, and cyclophosphamide, and alternating rounds of ifosfamide and etoposide is considered the standard of care for Ewing sarcoma patients (30-32). In addition to chemotherapy, aggressive local control measures including radiation and/or surgery are also part of standard care procedures. However, as Ewing sarcoma is common in the axial skeleton, surgical resection is often times unachievable. While this combination is often successful at controlling local, non-metastatic disease, unfortunately, there is a high level of toxicity associated with chemotherapeutic agents. Patients who do survive often suffer from debilitating or lethal health conditions including cardiovascular disease, growth abnormalities, infertility, and secondary malignancies (33-35).

Ewing sarcoma prognosis

Prior to the use of chemotherapy as treatment for Ewing sarcoma patients, survival was less than 10% (4). Intensification of therapy has led to five-year event-free survival (EFS) rates of 70-80% in patients with localized disease (4, 5). Unfortunately, this progress has not yet been achieved for patients with metastatic disease. For the quarter of patients who present with metastatic disease at diagnosis the outcome is poor with five-year EFS of less than 30% (4, 5). Additionally, up to a third of patients who present with localized disease will relapse at distant sites following an initial clinical remission. The outcomes for these patients are equally grim. Currently, aside from the presence of overt

metastasis there is no way to predict which Ewing sarcoma patients will relapse and which will survive.

Metastasis

Metastasis is a complicated, multi-step process in which tumor cells spread from a primary location to a non-adjacent secondary location. Metastasis is responsible for over 90% of cancer related deaths and, unfortunately, is the aspect of cancer pathogenesis that is least understood (36). In brief, the metastatic cascade consists of the following steps: tumor cells locally invade through the extracellular matrix (ECM), intravasate into the blood supply, survive and circulate through the vasculature, extravasate into the parenchyma of distant tissues, form micrometastatic colonies, and ultimately, generate macroscopic growths to complete the metastatic cascade (37). Understanding the critical components contributing to the metastatic cascade is imperative in order to improve patient outcome. Despite new discoveries, and an enhanced understanding of Ewing sarcoma biology, little remains known about the molecular mechanisms driving Ewing sarcoma metastasis.

Chemokines

Chemokines are *chemo*tactic chemo*kines* that are involved in many processes such as immune surveillance, inflammation, embryogenesis, angiogenesis, and every step of the metastatic cascade—the most critical being their ability to induce directed chemotaxis (6, 38-43). To date, over 50 human

chemokines have been characterized along with over 20 chemokine receptors (summarized in Figure 1.1) (44).

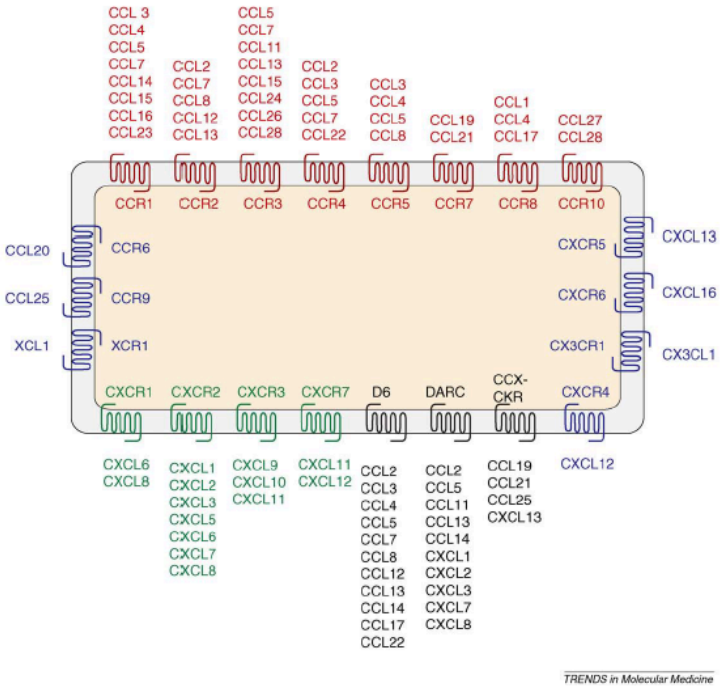

Figure 1.1. Chemokines and their receptors. A comprehensive list of the different classes of chemokines and the chemokine receptors, which they bind to. There is chemokine redundancy with one chemokine binding multiple chemokine receptors. This figure was adapted from (44).

Chemokines are a family of small proteins with molecular weights ranging between 8 and 10 kD that generally share a common structural characteristic of conserved cysteine residues. Chemokines are separated into four classes based on the arrangement of these cysteine residues, which help form their three-dimensional structure: CC (β), CXC (α), CX_3C (δ), and C (γ) (structures are summarized in Figure 1.2) (45). The CC family is the largest chemokine family (28 members), the CXC family is the second largest family (17 members) and the CX_3C and C families each have one member (44). The two cysteines in the N-terminus of the CC family are adjacent to each other, there is one amino acid

separating the cysteine residues in the CXC family, the CX_3C has three amino acids separating the cysteines, and the C family has a single cysteine in the N terminus (45).

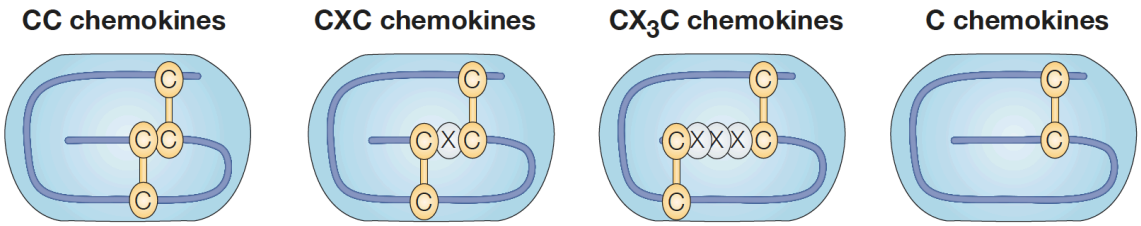

Figure 1.2. Chemokine classes based on structure. Chemokines are separated into four groups based on the first two cysteine residues. Typically (the C chemokines are the exception) four cysteine residues interact with the first and the third and the second and the fourth residues forming disulfide bonds. Chemokines bind to chemokine receptors that also contain the same conserved structure. Adapted from (45).

Functionally, chemokines are classified as either inflammatory or homeostatic. Inflammatory chemokines are activated and recruit cells to the site of injury or inflammation whereas homeostatic chemokines are constitutively expressed (46). Homeostatic chemokines are involved in more diverse functions than inflammatory chemokines such as secondary lymphoid tissue architecture as well as regulating hematopoietic cell trafficking (47). In addition to their critical role in the immune system, cytokines have been known to play a role in a number of pathologies including cancer, rheumatoid arthritis, multiple sclerosis, vascular disease, pulmonary disease, Crohn's disease, and human immunodeficiency virus-1 (HIV-1) infection (48-54).

Chemokine receptors

Chemokines function by binding to and subsequently activating surface bound G protein-coupled receptors (GPCRs). GPCRs share a common, seven-

transmembrane structure with seven helical regions connected by three extracellular and three intracellular loops (55). As with chemokines, the chemokine receptors are also broken into four classes—CCR, CXCR, CX_3CR, and XCR, each of which contains two conserved cysteine residues that form a disulfide bond critical for ligand binding (summarized in Figure 1.1). As defined in their name, GPCRs are coupled with G proteins located on the inner surface of the plasma membrane. The G proteins are heterotrimeric, containing Gα, Gβ, and Gγ subunits (56, 57). The Gα subunit has four family members: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, with each family member imparting the GPCR signal through different signal transduction pathways (58, 59). Under basal conditions, GDP (guanosine diphosphate) is bound to the Gα subunit, and the Gβ and Gγ subunits form a dimer. Upon chemokine binding, GPCRs undergo conformational changes, in which GTP (guanosine triphosphate) replaces GDP in the α subunit, displacing the Gβ/Gγ subunit from the GPCR (60). The Gα and Gβ/Gγ subunits remain tethered to the plasma membrane but are no longer bound to the GPCR (60, 61) (signaling of G proteins is summarized in Figure 1.3). Multiple intracellular signaling pathways are activated upon chemokine binding such as MAPK (mitogen-activated protein kinase), and cAMP (cyclic adenosine monophosphate), and JNK (c-Jun N-terminal kinase) (62). The signal remains active as long as GTP is bound to the Gα subunit, but can revert back to an inactive form when GTP is hydrolyzed back to GDP and the Gα and Gβ/Gγ subunits are re-associated with the GPCR. GPCRs can also signal independently of G proteins (63).

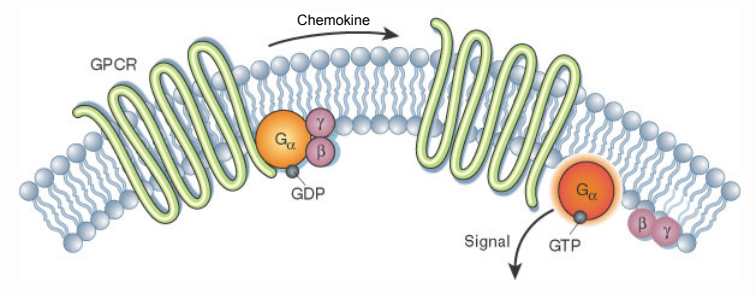

Figure 1.3. Signaling through GPCRs. Under basal conditions, the Gβ/Gγ subunit (purple) and the Gα subunit (with GDP bound, orange) are tethered to the GPCR (green). Upon chemokine binding, GDP is converted to GTP at the Gα subunit resulting in signal transmittance. Upon GDP conversion, the Gα and Gβ/Gγ subunits are no longer bound to the GPCR. The complex can reassemble back to the basal state (left side) upon hydrolysis of GTP back to GDP. Adapted from (60).

Like chemokines, chemokine receptors have been implicated in many diseases, including cancer (CXCR4, CCR4, CCR7, CCR10), HIV (CXCR4 and CCR5), multiple sclerosis (CCR2), and rheumatoid arthritis (CCR1) (64-68). GPCRs are considered to be highly druggable targets given their cellular location, role in many physiological processes, and their importance in disease. In fact, out of the currently available prescription and over-the-counter drugs, 30-50% of them either directly or indirectly target GPCRs (69, 70). CCR5 and CXCR4 antagonists have been successfully developed as drugs for HIV inhibition and stem cell mobilization, respectively (71, 72).

CXCL12 and CXCR4

One of the best-studied chemokine and chemokine receptor pair is CXCL12/CXCR4. CXCR4 is a G-protein coupled chemokine receptor encoded by chemokine (C-X-C motif) receptor 4 on chromosome 2. CXCR4 is widely expressed on cells in the immune and central nervous system, hematopoietic

cells, as well as cells in the brain, lung, colon, heart, liver and kidney (62). CXCL12, also referred to as stromal cell-derived factor 1 (SDF-1), is encoded by chemokine (C-X-C motif) ligand 12 on chromosome 10 and is the sole chemokine for CXCR4. CXCL12 is a homeostatic chemokine that is produced by endothelial cells of multiple organs including the bone marrow, lung, liver, brain, kidney, and heart (73).

The CXCL12/CXCR4 signaling axis plays a critical role in the retention and homing of hematopoietic stem cells (HSCs) in the bone marrow, as well as chemotaxis of numerous cell types (74). Additionally, the CXCL12/CXCR4 axis plays a role in other physiologic processes like immune surveillance, HIV-1 infection, tissue homeostasis, embryogenesis, tumor growth, and metastasis (8, 75-81). A number of signaling pathways are activated upon CXCL12 binding to CXCR4. These signaling pathways regulate intracellular calcium flux, transcription, proliferation, survival, and chemotaxis (summarized in Figure 1.4) (62). Given the role of this axis in many diverse cellular processes, it is unsurprising that CXCR4 and CXCL12 knockout mice are embryonic lethal (82, 83). These mice exhibit defects in hematopoiesis, heart development, and brain development.

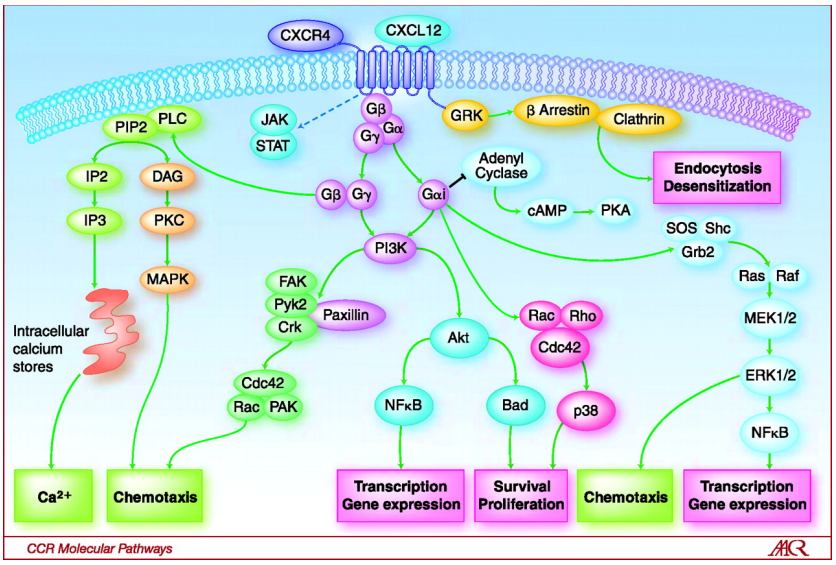

Figure 1.4. CXCL12/CXCR4 signaling pathways. Upon binding of CXCL12 to CXCR4, a variety of downstream signaling pathways are activated leading to several biological processes. This figure was adapted from (62).

The CXCL12/CXCR4 signaling axis in cancer

CXCR4 is the most commonly expressed chemokine receptor in human cancers, and growing research provides evidence that high CXCR4 expression is associated with metastatic disease and poor outcome in over 20 human cancers, including breast cancer (84), pancreatic cancer (85), prostate cancer (86), leukemia (87), melanoma (88), rhabdomyosarcoma (89-91), neuroblastoma (92), and osteosarcoma (93, 94). Specifically in breast cancer, the expression of CXCR4 is a key signature of metastatic cells, and early extravasation of liver and colon cancer cells is regulated by CXCR4 (84, 95). In addition to the role of CXCR4 in metastasis, there is evidence that CXCR4 contributes to tumor growth (96). Interestingly, the sites in which CXCL12 are highly expressed—the lungs, bone, bone marrow, liver, lymph nodes, and brain—are all common metastatic sites in human malignancies (97). Notably, the most common metastatic sites in

Ewing sarcoma patients are the lung, bone, and bone marrow (98). These findings alone support the hypothesis that CXCR4 may play a critical role in the metastasis of Ewing sarcoma. In further support of this, increased expression of *CXCR4* transcript was recently found to be associated with metastatic disease in both Ewing sarcoma derived cell lines and primary tumors (12). Taken together, these clinical and experimental observations implicate CXCR4 as a potential key mediator of Ewing sarcoma metastasis.

Targeting CXCR4

Disrupting the CXCL12/CXCR4 signaling axis has received a great deal of therapeutic interest. The molecule, 1,1'-[1,4-phenylenebis(methylene)]-bis-1,4, 8,11-tetraazacyclotetradecane octahydrochloride dihydrate, also referred to as Plerixafor or AMD3100, was first discovered as a potential HIV-1 inhibitor by means of inhibiting CXCR4 (99, 100). Plerixafor consists of two cyclam rings linked by a 1,4-phenylenebis (methylene) linker (Figure 1.5). AMD3100 is a potent and selective CXCR4 inhibitor and works by preventing the binding and function of CXCL12. Specifically, the protonated cyclam rings of AMD3100 interact with carboxylate groups in the ligand-binding crevice of CXCR4 (101). It has been demonstrated that AMD3100 does not bind any other relevant chemokine receptors, confirming the CXCR4 specificity (99).

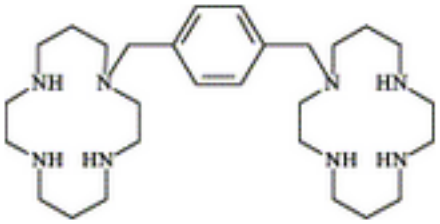

Figure 1.5. Structure of AMD3100. Structure of 1,1'-[1,4-phenylenebis(methylene)]-bis-1,4, 8,11-tetraazacyclotetradecane. Adapted from (99).

Due to cardiac toxicities, solubility issues, and limited bioavailability, Plerixafor was not further developed as an HIV-1 inhibitor (102, 103). Despite these limitations, Plerixafor is an FDA approved compound used for stem cell mobilization. Specifically, it is approved in combination with G-CSF (granulocyte-colony stimulation factor) for the mobilization of HSCs for autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma (104, 105). Given the abundant evidence for CXCR4 in the pathogenesis of many cancers, there has been a great deal of interest in continuing the development of novel CXCR4 antagonists as potential cancer therapeutics.

CXCR4 regulation

To further understand the role of CXCR4 in cancer it is necessary to understand the precise mechanisms that regulate CXCR4. Thus far, there is abundant work in the literature describing the regulation of CXCR4 protein expression as well as the regulation of CXCR4 signaling. CXCR4 protein expression is regulated by several co-translational modifications including glycosylation and tyrosine sulfation (106). Additionally, three processes that

regulate GPCR signaling—desensitization, internalization, and degradation—have been shown to regulate CXCR4 signaling (106).

Despite the biological significance of CXCR4, little is known about the transcriptional regulation of CXCR4. There is some evidence suggesting that CXCR4 transcription is dynamically regulated in response to stimuli. Cytokines (e.g. TGF-1β, IL-2, and IL-10) and growth factors (e.g. bFGF, VEGF, and EGF) have been shown to upregulate CXCR4 expression, while some inflammatory cytokines have been shown to downregulate CXCR4 (e.g. TNF-α and INF-γ) (106-111). This upregulation is accompanied by an increase in migratory and invasive potential. The precise mechanism by which these factors upregulate CXCR4 remains unknown; however, given that the *CXCR4* locus is rarely mutated in cancer, it suggests that overexpression is not genetically determined. Therefore, the potential role of epigenetic mechanisms of CXCR4 gene regulation was investigated in this thesis.

Epigenetics

Traditionally, it was thought that the sole underlying cause of cancer is the accumulation of genetic mutations (112). These genetic mutations occur in tumor suppressor genes or oncogenes resulting in loss of function or gain of function, respectively. Recently there has been a paradigm shift, with new evidence demonstrating that in addition to genetic mutations, epigenetics play a critical role in cancer. Epigenetics refers to heritable alterations in gene function and gene expression that occur in the absence of changes to the underlying DNA

sequence (113-115). Epigenetic regulation of gene expression occurs via three main processes: chromatin remodeling, DNA methylation, and histone modifications (summarized in Figure 1.6) (116).

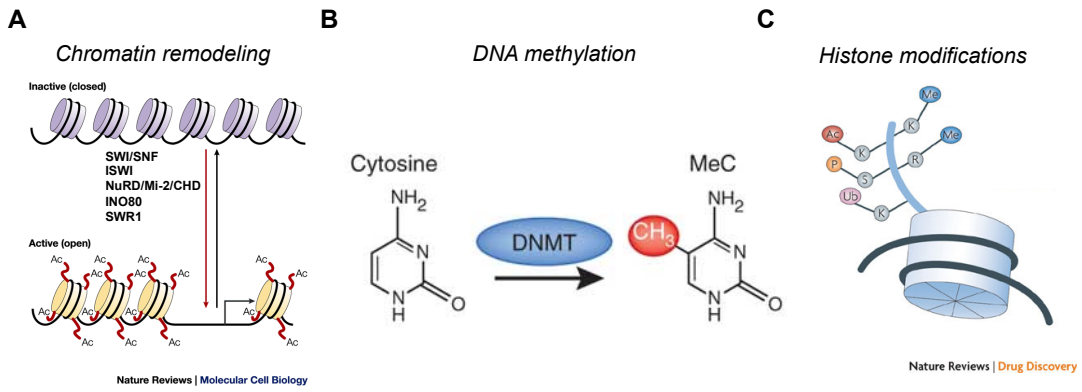

Figure 1.6. Three epigenetic processes that regulate gene expression. A. Chromatin remodeling alters nucleosome positioning to allow or disallow transcriptional machinery access to the DNA. This remodeling is mediated by SWI/SNF, ISWI, NuRD/Mi-2/CHD, INO80 and SWR1 family members. B. DNA methylation is the addition of a methyl group to the fifth position of cytosine. This process is carried out by DNMTs. C. Histone modifications are the post-translational modifications that occur on the N-terminal tails of histone. Modifications include acetylation, methylation, phosphorylation and ubiquitination. Images are adapted from (117) in A, (118) in B and (119) in C.

Chromatin remodeling

Alterations in chromatin structure influence the accessibility of transcriptional machinery to the condensed genomic DNA. These alterations are controlled by ATP-dependent remodeling complexes that are termed chromatin remodelers (120). Chromatin remodelers all contain a highly conserved ATPase subunit, and use energy generated from ATP hydrolysis to reposition nucleosomes (move, destabilize, eject, or restructure) (121). Through their effects on nucleosome positioning, these complexes ultimately serve to both activate and repress gene expression. There are five described families of chromatin remodeling complexes: SWI/SNF, ISWI, NuRD/Mi-2/CHD, INO80, and SWR1 (122) (Figure 1.6A). The SWI-SNF complex has recently emerged as a

tumor suppressor in some cancers with a high frequency of inactivating mutations, close to that of p53 (123). Ultimately, chromatin remodeling is tightly linked to both DNA methylation and histone modifications and the interactions between the three processes critically regulate gene expression (124).

DNA methylation

DNA methylation is an epigenetic modification in which cytosines are methylated at the fifth position and subsequently converted to 5-methylcytosines by DNA methyltransferases (DNMTs) (Figure 1.6B) (118). In mammalian systems, there are three major DNMTs: DNMT1, DNMT3a and DNMT3b that are further classified as either *de novo* or maintenance DNMTs (125, 126). *De novo* methyltransferases modify unmethylated DNA, while maintenance DNMTs bind to hemi-methylated DNA and add a methyl group to the cytosine of the newly synthesized strand. DNMT1 is the most abundant DNMT and is responsible for maintenance methylation throughout life (127). DNMT3a and DNMT3b are *de novo* DNMTs and play critical roles in development (128). A fourth DNMT, DNMT2, has been discovered but has no transmethylase activity (127, 129). GC rich genomic sequences are preferentially targeted for DNA methylation resulting in gene silencing. This silencing commonly occurs through the hypermethylation of promoter sequences. Methylation of promoter regions impedes the binding of transcriptional machinery to initiate transcription, and further compacts the chromatin through binding of methyl CpG-binding domain proteins (MBD) (130, 131). DNA methylation regulates many cellular processes including

development, chromosome instability, genomic imprinting, and X-chromosome inactivation (XCI) (132-135). Traditionally DNA methylation has been viewed as an irreversible mark; however, recent studies have discovered that TET family proteins have the ability to demethylate DNA by converting the 5'-methylcytosine to 5'-hydroxymethylcytosine (136).

DNA methylation has been heavily implicated in cancer, most commonly through silencing of critical tumor suppressor genes (137, 138). There is evidence, however, that DNA hypomethylation plays a role in the regulation of tumor-promoting genes (139, 140). It has been reported that DNA methylation regulates CXCR4 and CXCL12 in breast cancer and melanoma. In breast cancer, patients with tumors in which *CXCR4* was unmethylated had a poorer prognosis than patients with tumors in which *CXCR4* was methylated (141). In colon cancer, it has been shown that *CXCL12* is hypermethylated in the colon epithelium, which promotes tumor metastasis (142). The methylation of promoter CpG islands is a rare event in normal cells as compared to tumor cells; thus the development of tumor-specific DNA methylation inhibitors has been of great therapeutic interest (138). Azacitidine and its derivatives have been used to inhibit DNA methylation resulting in restored gene expression of aberrantly silenced genes (143). These inhibitors have shown anti-tumor effects both *in vitro* and *in vivo* and are approved in the clinic for the treatment of high-risk myelodysplastic syndrome (MDS)(144).

Histone modifications

DNA is wrapped around histone cores H2A, H2B, H3 and H4 in order to compact DNA. Two of each of the histone cores come together to form an octomeric nucleosome composed of a central H3 and H4 tetramer, and two flanking H2A-H2B dimers. This nucleosome core has approximately 147 base pairs of DNA wrapped in a 1.67 left-handed superhelical turn (145). In addition to the four main histone cores, there is an H1/H5 linker histone, which binds to the regions between the nucleosomes and helps maintain chromatin structure (146). The main histone cores (H2A, H2B, H3, and H4) have histone tails that come out of the nucleosome, which can be covalently modified (147). Post-translational modifications of the N-terminal tails such as acetylation, methylation, phosphorylation and ubiquitination as well as others, play a critical role in regulating chromatin state and altering DNA accessibility (148-150) (Figure 1.6C).

The most common and best-studied histone modifications are the acetylation and methylation of lysine residues on histone H3 and H4, which are associated with either transcriptional activation or repression. Lysine residues can be mono-, di- and tri-methylated, each of which confers different functions. There are three classes of epigenetic regulators that carry out the regulation of the post-translational histone modifications: *epigenetic readers* recognize the histone marks, *epigenetic writers* lay down the histone marks, and *epigenetic erasers* erase the histone marks (151). Histone acetyltransferases (HATs) (e.g. CBP/p300) are the writers for the acetylation mark and histone deacetylases (HDACs) (e.g HDAC1-3) erase the mark (152). The writers for methylation are

histone methyltransferases (HMTs) and the histone demethylases (e.g LSD1, JMJD1A, and JMJD2C) erase the methyl marks (153-155).

The trithorax group (TrxG) and the polycomb group (PcG) proteins are responsible for the opposing actions of gene activation and gene repression (156). Functioning within two multi-protein complexes (PRC1 and PRC2) the PcG proteins mediate transcriptional silencing (157). This silencing is specifically mediated through the PRC2 complex by the methyltransferase, enhancer of zeste homolog 2 (EZH2). EZH2 has the ability to mono-, di-, and tri-methylate H3K27 (H3K27me1/2/3). Silencing is also mediated through the PRC1 complex which mono-ubiquitinates histone H2A on lysine 119 (H2AK119Ub1) (158, 159). Transcriptional activation occurs through the methylation or acetylation of lysine residues in histone H3 and histone H4. The trimethylation of lysine residue 4 on histone H3 (H3K4me3) is specifically mediated by the TrxG proteins (160). The enzymes responsible for catalyzing this mark include SET1A, SET1B, and mixed lineage leukemia (MLL) proteins 1-4 (161).

This opposition plays an extremely critical role in development, with many promoters in human embryonic stem cells (hESCs) exhibiting both TrxG and PcG marks—a bivalent chromatin state. Bivalency is defined as the simultaneous presence of the repressive, H3K27me3 mark and the activating, H3K4me3 mark. Genes that are in this bivalent state are considered to be in a repressed “off” state, but are poised and ready for activation in response to development and microenvironment cues (162). In response to these cues, the bivalent domains can resolve into either active domains characterized by the H3K4me3 mark, or

repressed domains characterized by the H3K27me3 mark, resulting in gene activation or gene silencing, respectively. One example of this occurs in colorectal tumors in which the loss of the H3K27me3 mark from bivalent promoters results in the activation of cancer-promoting genes (163). Furthermore, it has been demonstrated that there is a link between the genes that are bivalent in hESCs and genes that are deregulated in cancer. In cancer, many of these bivalent promoters are targeted for DNA methylation, resulting in gene silencing and loss of dynamic plasticity (164, 165).

In addition to promoter regulation by histone modifications, enhancers are also regulated by histone modifications. It is estimated that there are hundreds to thousands of enhancers in the genome. Enhancers are short (50-1500 bp), cis-acting DNA sequences that function to increase gene transcription. Enhancers can reside upstream or downstream of the genes they act on. While there is no single mark that characterizes enhancers, genome-wide mapping of histone modifications has demonstrated that the monomethylation of histone H3 on lysine 4 (H3K4me1) and the acetylation of histone H3 of lysine 27 (H3K27ac) are marks of active enhancers (166-168). In addition to these histone marks, it has been shown that p300 binding sites reside at enhancers (169). Active enhancers that are bound by transcriptional coactivators, such as Mediator, are termed super-enhancers (170). In cancer, the acquisition of super-enhancers at key oncogenic drivers is a frequent tumorigenic mechanism (171). Given the critical role that enhancers and super-enhancers play in cancer progression, therapeutic

strategies have been developed to target enhancer activity; most notable BET bromodomain inhibitors such as JQ1 are showing promise (172).

Developmental programs are under tight epigenetic regulation and cancer can be considered a developmental disease. Taking these two together, it is no surprise that the hijacking of these epigenetically controlled developmental programs is a key feature of human malignancies. Many human malignancies have aberrant expression or mutations in critical components of both the PcG and TrxG complexes. In many cancers, including Ewing sarcoma, BMI-1 and EZH2, both components of PRC1 and PRC2, respectively, are aberrantly overexpressed (173). The MLL family of proteins are histone methyltransferases, responsible for regulating gene expression and are frequently aberrant in several human tumors. For example, MLL1 is commonly rearranged in leukemia and MLL2 and MML3 are mutations occur in leukemia (174). Given the deregulation of many epigenetic processes in cancer, it is no surprise that there is a great deal of interest in targeting these epigenetic mechanisms as means of therapy.

Thesis Summary

The overall aim of this thesis is to elucidate the contribution of CXCR4 to Ewing sarcoma pathogenesis, in particular tumor metastasis. In Chapter Two, I describe our investigations into the expression pattern of CXCR4 in Ewing sarcoma cell lines and primary tumor samples, which demonstrated heterogeneous expression. Interestingly, we determined that the expression of

CXCR4 was highly dynamic and responsive to microenvironmental cues such as serum deprivation, hypoxia, and growth constraints, all of which are factors seen in a growing tumor. Functionally, we demonstrated that CXCR4-positive cells are migratory and invasive in a CXCR4-dependent manner (to a CXCL12 gradient), and that the CXCR4 antagonist, AMD3100, impeded these phenotypes. In Chapter Three, I detail our investigations to determine the molecular mechanisms that underlie dynamic regulation of CXCR4 in response to the microenvironmental cues discussed in Chapter Two. We focused our efforts on evaluating epigenetic regulation of the *CXCR4* locus at both the promoter region and at an upstream enhancer element. We determined that the *CXCR4* promoter resides in a bivalent state in Ewing sarcoma cells and that in response to external cues (serum deprivation, hypoxia, and EZH2 inhibition) the H3K27me3 mark is lost, resulting in an increase in *CXCR4* expression. Furthermore, we determined that an enhancer upstream of *CXCR4* contributes to the regulation of CXCR4. Specifically, the enhancer antagonist, JQ1, inhibits CXCR4- to CXCR4+ cell state transitions in response to stress. Together the results of the studies detailed in these two chapters demonstrate that epigenetic regulation of the *CXCR4* locus in response to microenvironmental cues allows Ewing sarcoma cells to adopt a more migratory and invasive phenotype. In Chapter Four, I present tools and techniques that we have developed to further study the role of CXCR4 in Ewing sarcoma metastasis and disease progression in future studies. To assess the contribution of CXCR4 to the metastatic cascade, we optimized a mouse model of spontaneous metastasis that can be

used to address mechanistic questions regarding the contribution of CXCR4 to disease progression *in vivo*. Additionally, we are generating CXCR4 knockout cell lines to definitively study the contribution of CXCR4 to biological processes, including but not limited to proliferation, migration, invasion, and metastasis. Finally, we are developing a CXCR4-GFP knock-in system that will allow us to track the plastic and dynamic nature of CXCR4 on a single cell basis in both *in vitro* and *in vivo* systems.

The final chapter of this thesis, Chapter Five, discusses the future implications of this work. Specifically, it focuses on extending these findings to other tumor types as well as the rationale for the development of novel CXCR4 antagonists as potential metastasis prevention agents. Because CXCR4 has been implicated in the metastasis of many cancers and metastasis is the leading cause of death in cancer patients, targeting the CXCL12/CXCR4 signaling axis could be a promising avenue for cancer treatment.

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CHAPTER TWO

STRESS-INDUCED CXCR4 PROMOTES MIGRATION AND INVASION OF EWING SARCOMA[2]

ABSTRACT

Ewing sarcoma is the second most common bone cancer in pediatric patients. Although the primary cause of death in Ewing sarcoma is metastasis, the mechanism underlying tumor spread needs to be elucidated. To this end, the role of the CXCR4/SDF-1a chemokine axis as a mediator of Ewing sarcoma metastasis was investigated. CXCR4 expression status was measured in primary tumor specimens by immunohistochemical staining and in multiple cell lines by quantitative reverse transcriptase PCR and flow cytometry. Migration and invasion of CXCR4-positive Ewing sarcoma cells toward CXCL12/SDF-1a were also determined. Interestingly, while CXCR4 status was disparate among Ewing sarcoma cells, ranging from absent to high-level expression, its expression was found to be highly dynamic and responsive to changes in the microenvironment. In particular, upregulation of CXCR4 occurred in cells that were subjected to growth factor deprivation, hypoxia, and space constraints. This upregulation of

[2] Krook, M.A.; Nicholls, L.A.; Scannell, C.A.; Chugh, R.; Thomas, D.G.; Lawlor, E.R. (2014) "Stress-induced CXCR4 promotes migration and invasion of Ewing sarcoma." Molecular Cancer Research, 6: 953-64. doi: 10.1158/1541-7786.MCR-13-0668

CXCR4 was rapidly reversed upon removal of the offending cellular stress conditions. Functionally, CXCR4-positive cells migrated and invaded toward an SDF-1a gradient and these aggressive properties were impeded by both the CXCR4 small-molecule inhibitor AMD3100, and by knockdown of CXCR4. In addition, CXCR4-dependent migration and invasion were inhibited by small-molecule inhibitors of Cdc42 and Rac1, mechanistically implicating these Rho-GTPases as downstream mediators of the CXCR4-dependent phenotype.

INTRODUCTION

Ewing sarcoma is an aggressive bone and soft tissue malignancy that primarily affects children and young adults (1). Over the past several decades, overall survival has improved dramatically for patients who present with localized disease. Multiagent systemic chemotherapy and aggressive local control measures have led to 5-year event-free survival rates of 70% to 80% in these patients (1, 2). However, for the approximately 25% of patients who present with metastatic disease, the outcome is significantly worse. Event-free survival for these patients remains less than 25%, and intensification of chemotherapeutic regimens has failed to improve outcome (1). In addition, up to a third of patients who present with localized disease will relapse at distant sites following an initial clinical remission and outcomes for these patients are equally dismal. Innovative approaches to therapy and improved understanding of the metastatic process

are needed to improve outcomes for patients with primary and relapsed metastatic Ewing sarcoma.

Despite its clinical importance, the biologic mechanisms underlying Ewing sarcoma metastasis remain largely unknown. Chemokine receptors are seven-transmembrane, G-protein–coupled cell surface proteins that are defined by their ability to induce chemotaxis through the binding of small chemoattractant cytokines or chemokines (3). Chemokine (C-X-C motif) receptor 4 (CXCR4) is the most commonly expressed chemokine receptor in human cancer, and increased expression of the CXCR4-encoding transcript was recently found to be associated with metastatic disease in Ewing sarcoma–derived cell lines and tumors (4). Significantly, high CXCR4 expression has also been associated with metastatic disease and poor outcome in many other human cancers of both epithelial and nonepithelial origin (3, 5), including breast cancer (6), pancreatic cancer (7), leukemia (8), rhabdomyosarcoma (9-11), and osteosarcoma (12-14). Interestingly, the ligand for CXCR4, CXCL12 (SDF-1α), is highly expressed in common sites of Ewing sarcoma metastasis, including lung, bone, and bone marrow, further implicating the potential role of this axis in mediating the distant spread of primary tumor cells.

In this study, we evaluated the expression characteristics of CXCR4 in Ewing sarcoma primary tumors and cell lines, and specifically addressed whether the CXCR4/SDF-1α axis promotes tumor cell migration and invasion. Our findings demonstrate that expression of CXCR4 is both highly variable in Ewing sarcoma and highly dynamic, being reversibly induced in response to

microenvironmental stresses, including growth factor deprivation, hypoxia, and space constraints. Moreover, our studies confirm that Ewing sarcoma cells that express high levels of CXCR4 display increased chemotactic migration and invasion, which is mediated, at least in part by activation of the Rho-GTPases, Rac1, and Cdc42. Importantly, inhibition of the CXCR4/SDF-1α axis inhibits the aggressive cellular phenotype, thus revealing the potential contribution of CXCR4 signaling to Ewing sarcoma metastasis.

EXPERIMENTAL PROCEDURES

Cell culture

Ewing sarcoma cell lines were kindly provided by Dr. Timothy Triche Children's Hospital Los Angeles (CHLA, Los Angeles, CA) and the Children's Oncology Group (COG) cell bank (www.cogcell.org) and identities confirmed by short tandem repeat profiling (courtesy of Dr. Patrick Reynolds, Texas Tech University, Lubbock, TX). Cells were maintained in RPMI-1640 media (Gibco) supplemented with 10% FBS (Atlas Biologicals, Inc.) and 6 mmol/L l-glutamine (Life Technologies) in an incubator at 37°C in 5% CO_2. For CHLA-25 studies, plates were coated with 0.2% gelatin before cell seeding. For serum-starved conditions, cells were cultured in the same conditions without the addition of FBS. For hypoxia studies, cells were incubated in 1% O_2 in an xVivo system (Biospherix) at 37°C and 5% CO_2. For growth constraint conditions, cells were

cultured under standard culture conditions and CXCR4 analyzed when cells reached 100% confluence.

Quantitative real-time PCR and Western blotting

RNA was isolated from cell lines (RNeasy Mini; Qiagen) and cDNA was generated (iScript; Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using validated Taqman primers (*CXCR4, 18S*, and *B2M*; Life Technologies). Analysis was performed in triplicate using the LightCycler 480 System (Roche Applied Science) and average Cp values were normalized relative to reference genes (*18S* and *B2M*) within each sample using ΔΔ Cp method. Levels of phospho-ERK [Cell Signaling; Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb #4370], phospho-Akt [Cell Signaling; Phospho-Akt (Ser473) (D9E) XP Rabbit mAb #4060], Akt [Cell Signaling; Akt (pan) (C67E7 Rabbit mAb #4691), Erk (Cell Signaling; p44/42 MAPK (Erk 1/2) #9102], and ACTIN [Abcam; Anti-beta Actin antibody (HRP) (ab20272)] were determined in whole cell lysates using standard Western blot assays as previously described (15).

Cell sorting and assessment of Rac1 activation in sorted populations

Cells were dissociated with Accutase (EMD Millipore Corporation) and resuspended in staining media (L-15 media, 0.1% bovine serum albumin, 10 mmol/L HEPES; Life Technologies), then blocked for 15 minutes at 4°C with agitation (in 0.5% FBS; Atlas Biologicals, Inc.). After blocking, human CXCR4 Alexa Fluor 488 monoclonal antibody (R&D Systems; clone 44717) was added (5 μL per 1.0×10^6 cells) and incubated for 30 minutes at 4°C with agitation. After

two washes, cells were resuspended in staining media and passed through a 0.40-μm sterile nylon mesh strainer (Thermo Fisher Scientific). Flow cytometry analysis was performed using a BD Accuri C6 Flow Cytometer (BD Biosciences). Fluorescence-activated cell sorting (FACS) of cells into CXCR4-positive and CXCR4-negative fractions (top 10% and bottom 10%) was done using a Beckman Coulter MoFlo Astrios (Flow Core, University of Michigan, Ann Arbor, MI) with gating determined by analysis of unstained controls.

For evaluation of Rac1 activation, FACS-sorted TC-32 cells were serum-starved overnight in the presence or absence of SDF-1α (200 ng/mL; R&D Systems). Levels of Rac1 activation were determined using a G-LISA kit (Cytoskeleton) according to the manufacturer's instructions.

Immunohistochemistry

For tumor immunohistochemistry, formalin-fixed, paraffin-embedded tumor microarray slides were deparaffinized, hydrated, epitope retrieved, and stained with an antibody against CXCR4 (dilution 1:500; Abcam; AB-2074) as previously validated and described (16). Specificity of the antibody was confirmed in our hands by immunostaining of cell pellets collected from CXCR4-high, CXCR4-low as well as control and CXCR4 knockdown TC-32 cells. Adjacent tumor microarray slides were incubated with CD99 (Mouse monoclonal antibody; clone 12E7; DAKO; Cat # M3601; 1:100) and hematoxylin and eosin to identify tumor cells. Sections were scored for the presence of CXCR4 using the Allred schema (17). The proportion of tumor cells was assigned a score between 0 and 5, and the staining intensity was assigned a score between 0 and 3. These 2 values

were added to produce a staining score. Given recent studies describing nuclear localization of CXCR4 in some cancers (18, 19), both cytoplasmic and nuclear staining were assessed and equally weighted. Nuclear staining of CXCR4 was evident in >10% of nuclei in 35% of cases.

CXCR4 knockdown

For CXCR4 knockdown studies, cell lines were transduced with pLKO.1 puro vectors that contained one of two independent short hairpin RNAs targeted to CXCR4:shCXCR4–1: 5′-TGGAGGGGATCAGTATATACA-3′ and shCXCR4-2: 5′-GTTTTCACTCCAGCTAACACA-3′ (Addgene; plasmid 12271 and 12272; ref. (20)) or an inert nonsilencing sequence: shNS: 5′-CAACAAGATGAAGAGCACCAA-3′. Cells were selected in puromycin (2 μg/mL; Sigma) for 72 hours before subsequent experiments.

In vitro migration and invasion

Real-time cell analysis (RTCA) of cell migration and invasion was monitored using a CIM-plate 16 and xCELLigence DP System (Acea Bioscience, Inc.). Cells were serum-starved overnight in RPMI-1640 with 0.2% Media Grade (K) Probumin (Millipore). Before cell seeding, electrodes were coated with 0.2% gelatin and RPMI-1640 containing 0.2% Probumin was placed in the upper chamber, and media containing SDF-1α (100 ng/mL; R&D Systems) were added to lower chambers. The CIM-plate was allowed to equilibrate for 1 hour in an incubator at 37°C in 5% CO_2. For migration studies, 1×10^5 cells/well were placed in the upper chamber of a CIM-16 plate and then the plate was equilibrated for 30 minutes at room temperature. For migration assays done with

combination of stresses, cells were serum-starved and placed in either normoxic or hypoxic conditions overnight before evaluation of migration. For invasion studies, 1×10^5 cells/well were plated in the upper chamber of wells that had been previously coated with 5% (v/v) Growth Factor Reduced Matrigel Matrix (diluted 1:20 in basal RPMI media; BD BioSciences). Matrigel-coated plates were allowed to equilibrate for 4 hours in an incubator at 37°C in 5% CO_2 before addition of cells. For compound assays, cells were pretreated overnight with either 2.5 μg/mL AMD3100 (Sigma-Aldrich), 30 μmol/L Rac1 inhibitor [NSC 23766 (hydrochloride); Cayman Chemical), or 7 μmol/L Cdc42 inhibitor (ML 141; EMD Millipore) and then seeded in CIM-16 plates as above. Parallel migration assays were performed with 2×10^5 cells on 0.8 μm cell culture inserts (Thermo Fisher Scientific) for 24 hours. After incubation, noninvading cells were removed from the upper surface and inserts were stained (Crystal Violet Stain; 0.5% crystal violet, 20% methanol) and migratory cells were imaged by light microscopy.

Statistical analysis

Data are reported as mean ± SEM from three independent experiments, and *P* values were calculated using the Student *t* test.

RESULTS

CXCR4 expression is highly heterogeneous in Ewing sarcoma

Recent studies of gene expression showed that expression of the *CXCR4* transcript varies among Ewing sarcoma cell lines and tumors (4). To determine if expression of the CXCR4 protein is equally heterogeneous, we assessed a panel of four well-established Ewing sarcoma cell lines. qRT-PCR analyses corroborated earlier studies and demonstrated a wide range of *CXCR4* expression (Figure 2.1A). The variability in transcript expression was mirrored by flow cytometry studies of protein expression, with relatively low levels of CXCR4 detected in TC-71 and A673 cells and high-level expression evident in CHLA-25 and TC-32 cells (Figure 2.1B). Analysis at the level of individual cells showed that the variation in CXCR4 signal intensity between the different cell lines was a result of different frequencies of CXCR4-positive cells within each culture (Figure 2.1B). Specifically, in the two low-expressing cell lines, fewer than 5% of cells expressed CXCR4. Conversely, 20% to 40% of cells in CHLA-25 and TC-32 expressed detectable levels of the receptor at the cell surface. In addition, the level of expression in CXCR4-positive populations ranged from weak to robust, as demonstrated by the continuum of fluorescence intensities displayed by CXCR4-positive cells (Figure 2.1C). To evaluate whether this same heterogeneity in CXCR4 protein expression exists in primary tumors, we evaluated a tissue microarray comprised of 64 Ewing sarcoma samples. Sufficient viable tumor was present to score 43 tumor samples from 32 unique patients. Consistent with cell line data, CXCR4 staining showed marked intertumor variability, ranging from absent (N = 13) to strongly positive in the majority of tumor cells (N = 13). The remainder of the samples (N = 17) showed

an intermediate staining pattern in which both CXCR4-positive and CXCR4-negative tumor cells were identified in the same core specimen Figure 2.1D). No difference in staining pattern was identified between 28 samples that were obtained from primary tumor specimens and 15 that were isolated at the time of disease recurrence (Figure 2.1E). The average CXCR4 score was 5.0 in 4 diagnostic samples that were obtained from patients with metastatic disease and 3.8 in 17 localized tumor samples. Although this analysis showed a trend to increased expression in primary tumors of patients who present with metastatic disease, the sample size is inadequate to draw conclusions about associations between CXCR4 expression and clinical stage. Thus, like cell lines, CXCR4 protein expression is highly heterogeneous in Ewing sarcoma tumors, and individual cells within the same tumor also vary in CXCR4 expression.

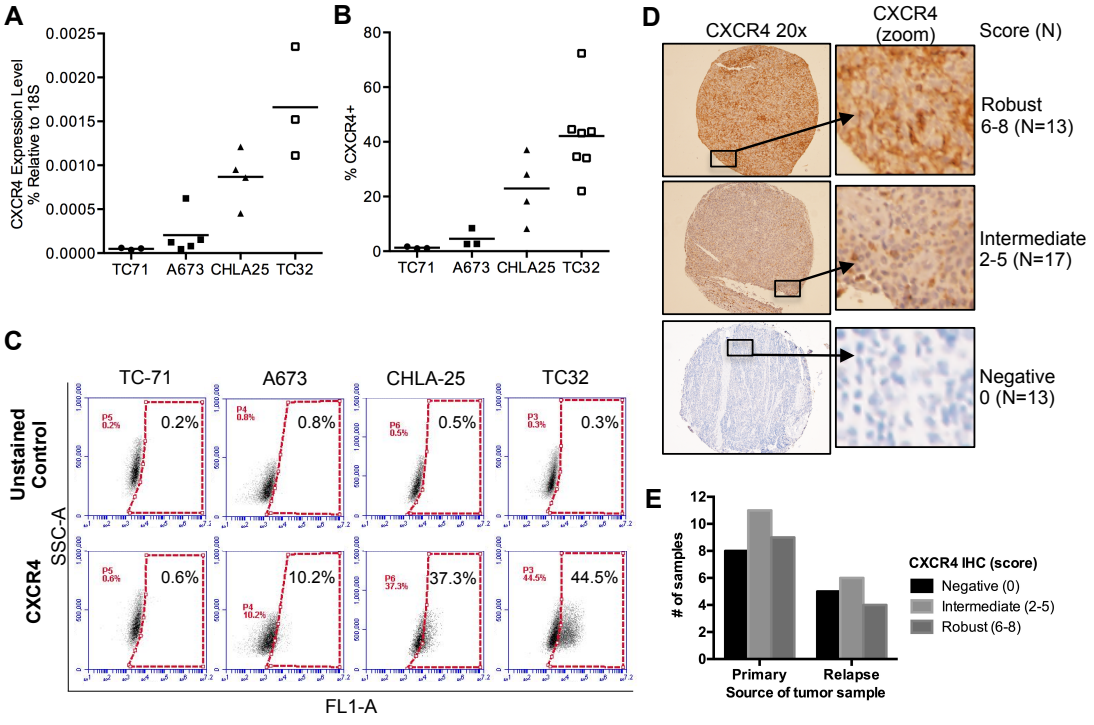

Figure 2.1. Heterogeneous expression of CXCR4 in Ewing sarcoma. A, qRT-PCR of *CXCR4*

expression in Ewing sarcoma cell lines. Expression was normalized to the housekeeping 18S rRNA in each sample. Individual replicates are shown and the horizontal line represents average expression of replicate experiments. B, flow cytometry of CXCR4 cell surface expression in Ewing sarcoma cell lines. Data are expressed as the percentage of positive cells. Gating was determined based on unstained control cells in the same experiment. Individual replicates are shown and the horizontal line represents average expression of replicate experiments. C, representative dot plots for each of the 4 cell lines are shown, demonstrating the heterogeneity of CXCR4 expression both between cell lines and also showing the range of positivity within the CXCR4-positive cell in each cell line. D, immunohistochemical (IHC) staining of 43 Ewing sarcoma tumor samples showed marked variability ranging from complete absence to robust staining in all tumor cells. Representative examples of robust (score 6–8), intermediate (score 2–5), and negative (score 0) tumors are shown along with the total number of tumors in each category. Both CXCR4-positive and -negative tumor cells were evident in intermediate tumors. E, summary of IHC scores for 28 primary tumor samples and 15 relapse samples shows no difference in CXCR4 expression between the two categories.

CXCR4 expression is dynamic and induced in response to growth factor deprivation

Tumor cell heterogeneity is a key factor that contributes to drug resistance and tumor progression. We observed significant interexperiment heterogeneity in CXCR4 expression in our *in vitro* studies of Ewing sarcoma cell lines (Figure 2.1A and B). In particular, we noted that the relative proportion of CXCR4-positive cells varied substantially between replicate experiments, particularly in the two high-expressing cell lines. This observation, together with the highly variable nature of expression in tumor samples, led us to hypothesize that expression of CXCR4 may be dynamic in Ewing sarcoma and subject to regulation in response to changes in the local microenvironment. To begin to address this possibility, we tested whether the variability in expression might be a consequence of the relative availability of growth factors. To achieve this, we measured CXCR4 expression in cells that had been deprived of serum. As shown, serum deprivation led to an increased frequency of CXCR4-positive cells in three of the four cell lines (Figure 2.2A). Only TC-71 cells remained unchanged with fewer than 2% of cells expressing CXCR4 in both serum-rich and serum-

deprived conditions. To determine if the upregulation of CXCR4 protein expression was a consequence of increased *CXCR4* transcription, we compared mRNA levels in the two conditions. Consistent with transcriptional upregulation, *CXCR4* mRNA levels increased in all four cell lines following serum deprivation (Figure 2.2B). In addition, the degree of transcriptional induction corresponded to that of increased protein expression. TC-71 showed the least and TC-32 cells showed the most robust upregulation of transcript Figure 2.2B). We next evaluated whether restoration of growth factor availability would reverse the induction of CXCR4. To achieve this, serum was added to the media of cells that had been starved for 24 hours. Following the addition of serum, rapid downregulation of CXCR4 expression was observed with levels being restored to baseline within 24 hours (Figure 2.2C).

Next, we addressed whether the reversible changes in CXCR4 expression seen in heterogeneous cell populations reflected dynamic regulation at the level of individual cells. TC-32 cells were FACS-sorted into pure populations of CXCR4-positive and CXCR4-negative cells, and then monitored over 3 weeks in ambient culture conditions to determine if positive cells would become negative and *vice versa.* Consistent with dynamic and bidirectional regulation of CXCR4, both populations of FACS-sorted TC-32 cells gradually reverted to their basal pattern of CXCR4 expression (Figure 2.2D). Specifically, the initial CXCR4-positive population generated CXCR4-negative cells and the initial CXCR4-negative population generated CXCR4-positive cells with both cultures

reestablishing the baseline equilibrium state of approximately 30% to 40% CXCR4-positive cells within 3 weeks.

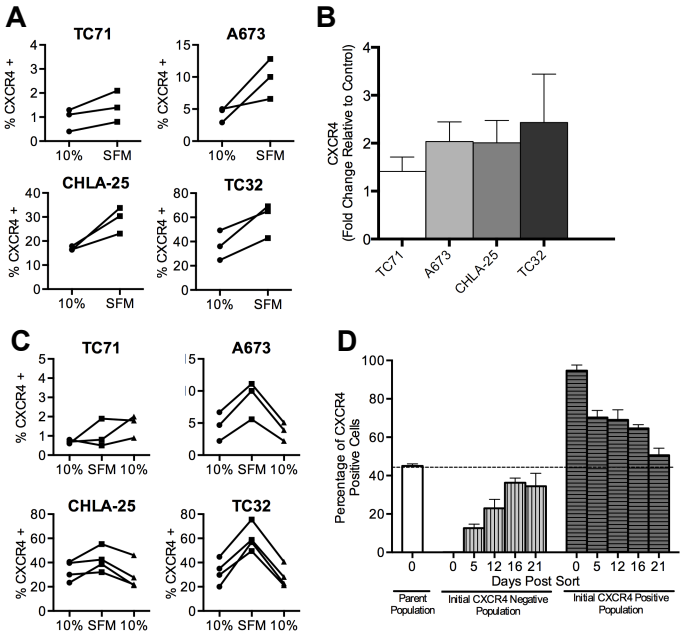

Figure 2.2. CXCR4 expression is reversibly induced in response to growth factor deprivation. A, surface expression of CXCR4 was determined by flow cytometry as in Figure 2.1 for Ewing sarcoma cells plated under standard (10% FBS) and serum-deprived (serum-free media, SFM) conditions. Exposure of cells to SFM for 24 hours resulted in upregulation of CXCR4. Each line and pair of data points represents the data for an independent experiment. B, qRT-PCR analysis of *CXCR4* expression in Ewing sarcoma cells grown in SFM conditions for 24 hours. Expression in each sample was normalized to the housekeeping β 2 microglobulin (*B2M*) and expressed as fold change relative to expression in standard 10% FBS conditions. Results are shown as mean ± SEM from three independent experiments. C, flow cytometry of CXCR4 expression in serum-starved Ewing sarcoma cells (SFM) after being returned to standard culture conditions (10%) shows reversion of expression to baseline state. Each line and pair of data points represents the data for an independent experiment. D, TC32 cells were FACS-sorted into CXCR4-high (top 10%) and CXCR4-low (bottom 10%) populations and then both populations were maintained in standard culture conditions for 3 weeks. CXCR4 expression was monitored by flow cytometry on days 5, 12, 16, and 21 after sorting, revealing reversion over time to baseline heterogeneity. Results are shown as mean ± SEM from three independent experiments.

Thus, CXCR4 expression in Ewing sarcoma cells is dynamic and is rapidly and reversibly induced in response to growth factor deprivation. Moreover, Ewing

sarcoma cells in standard tissue culture transition back and forth between CXCR4-negative and CXCR4-positive cell states in response to changes in the microenvironment, ultimately maintaining a basal equilibrium state that is specific for each cell line and condition.

CXCR4 is induced in Ewing sarcoma cells that are exposed to hypoxia and growth constraints

Having established that growth factor deprivation leads to induction of CXCR4, we next questioned whether other stresses that might be encountered by a growing Ewing sarcoma, such as hypoxia and space constraints, would also affect CXCR4 expression. CXCR4 is induced by hypoxia-inducible factor 1-α (HIF1-α) in mesenchymal stem cells and cancer cells that are exposed to hypoxic environments (21, 22). Consistent with these observations, we discovered that exposure of Ewing sarcoma cells to hypoxia resulted in an increase in *CXCR4* transcript (Figure 2.3A) and an increased frequency of $CXCR4^+$ cells (Figure 2.3B). Removal of the hypoxic insult resulted in a return to basal levels within 48 hours (Figure 2.3B). Interestingly, in direct contrast to growth factor deprivation, TC-71 cells were more susceptible to hypoxia-induced changes than were TC-32 cells, indicating that the inherent plasticity of CXCR4 expression in response to different stimuli varies among the different cell lines. Finally, subjecting cells to space constraints, by growing them to confluence, also resulted in reproducible upregulation of *CXCR4* transcript (Figure 2.3C) and protein expression (Figure 2.3D) that was reversed when cells were returned to subconfluent, log-phase growth conditions (Figure 2.3D).

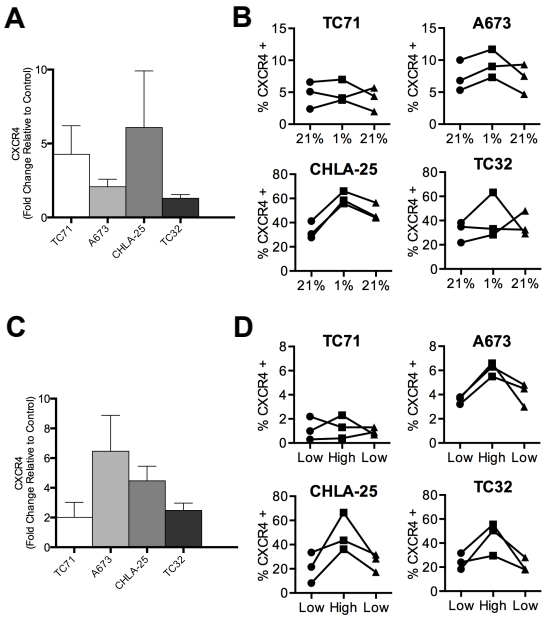

Figure 2.3. CXCR4 expression is reversibly induced in response to hypoxia and cell confluence. A, qRT-PCR analysis of *CXCR4* expression in Ewing sarcoma cells grown in hypoxic conditions for 24 hours shows upregulation of the transcript. Gene expression calculated in each sample was normalized to the housekeeping β 2 microglobulin (*B2M*) and expressed as fold change in hypoxia relative to expression in normoxia (control). Histograms represent mean fold change ± SEM for three independent experiments. B, flow cytometry of CXCR4 expression in Ewing sarcoma cells before (21%) and after (1%) exposure to hypoxia for 24 hours shows upregulation of CXCR4 expression in hypoxic conditions. The CXCR4-positive cell frequency reverted to baseline 48 hours after cells were returned to ambient (21%) conditions. C, qRT-PCR analysis of *CXCR4* expression in Ewing sarcoma cells grown in log phase, low density (low) compared with confluent, high-density (high) conditions for 48 hours. Gene expression calculated as in Figure 3A and expressed as mean fold change ± SEM in high-density cells relative to low-density (control) cells. D, flow cytometry of CXCR4 expression in log-phase (low) and confluent (high) conditions shows upregulation of CXCR4 expression that is then reversed when cells are returned to low-density growth conditions after 48 hours. For A and C, results are shown as mean ± SEM from three independent experiments. For B and D, each line and pair of data points represents the data for an independent experiment.

Thus, like growth factor deprivation, exposing Ewing sarcoma cells to hypoxia and space constraints also results in upregulation of *CXCR4* transcription and an increased frequency of CXCR4-positive cells. These changes are reversed when these microenvironment stresses are removed, demonstrating the highly plastic and dynamic nature of CXCR4 regulation in Ewing sarcoma cells.

CXCR4 promotes Ewing sarcoma cell migration and invasion

Given its well-established role as a mediator of metastasis in numerous other cancers, we next investigated whether CXCR4 might also contribute to an invasive phenotype in Ewing sarcoma. First, we assessed whether Ewing sarcoma cells demonstrate chemotactic migration toward SDF-1α. As expected, given the very low frequency of CXCR4-positive cells, neither TC-71 nor A673 cells migrated toward SDF-1α (data not shown). In contrast, the CXCR4-high cell lines, CHLA-25 and TC-32 both demonstrated substantial and rapid migration toward SDF-1α (Figure 2.4A and B). Moreover, exposure of the cells to AMD3100, a small-molecule inhibitor of CXCR4, significantly inhibited this chemotactic migration (Figure 2.4A and B). To further validate these findings, we induced stable knockdown of CXCR4 in both CHLA-25 and TC-32 cell lines using two different short hairpin RNA constructs (Figure 2.4C and D). Consistent with pharmacologic inhibitor studies, knockdown of CXCR4 significantly impaired the migration of both CHLA-25 and TC-32 cells toward SDF-1α (Figure 2.4E and F).

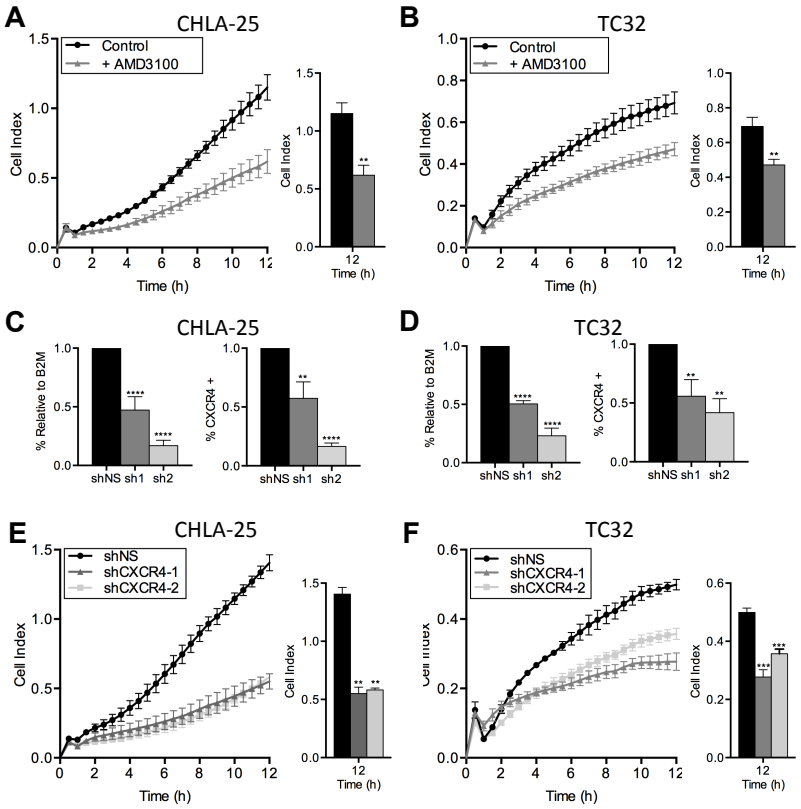

Figure 2.4. CXCR4 promotes chemotactic migration of Ewing sarcoma cells. A and B, migration of CHLA-25 (A) and TC32 (B) cells toward SDF-1α (100 ng/mL) was measured using real-time cell analysis (xCELLigence CIM-Plate 16) in the presence and absence of the CXCR4 inhibitor AMD3100. AMD3100 significantly inhibited chemotaxis. C and D, knockdown of CXCR4 was effectively achieved in CHLA-25 (C) and TC32 cells (D) using lentiviral transduction of 2 different shRNA sequences directed against CXCR4 (sh1 and sh2). Control cells were transduced with an inert nonsilencing shRNA vector (shNS). Successful knockdown was confirmed by qRT-PCR (left) and flow cytometry (right). E and F, migration of CHLA-25 (E) and TC32 (F) cells toward SDF-1α (100 ng/mL) was inhibited following knockdown of CXCR4. In all plots, graphs represent mean ± SEM of three independent experiments with four replicates per condition. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ as compared with controls.

Invasion of cancer cells through basement membranes comprised of extracellular matrix proteins is a critical step in the metastatic cascade (23). To model this process *in vitro*, we used Matrigel, a gelatinous protein mixture mimicking extracellular components found in tumors (24). Both CHLA-25 and TC-32 cells invaded through the Matrigel layer toward SDF-1α, and invasion was abrogated by both AMD3100 (Figure 2.5A and B) and by CXCR4 knockdown

(Figure 2.5C and D). In contrast, SDF-1α had no effect on the invasive potential of A673 cells (data not shown). Thus, CXCR4-positive Ewing sarcoma cells are stimulated to migrate and invade toward SDF-1α, but modulation of the CXCR4/SDF-1α axis by pharmacologic or genetic means can profoundly inhibit this response.

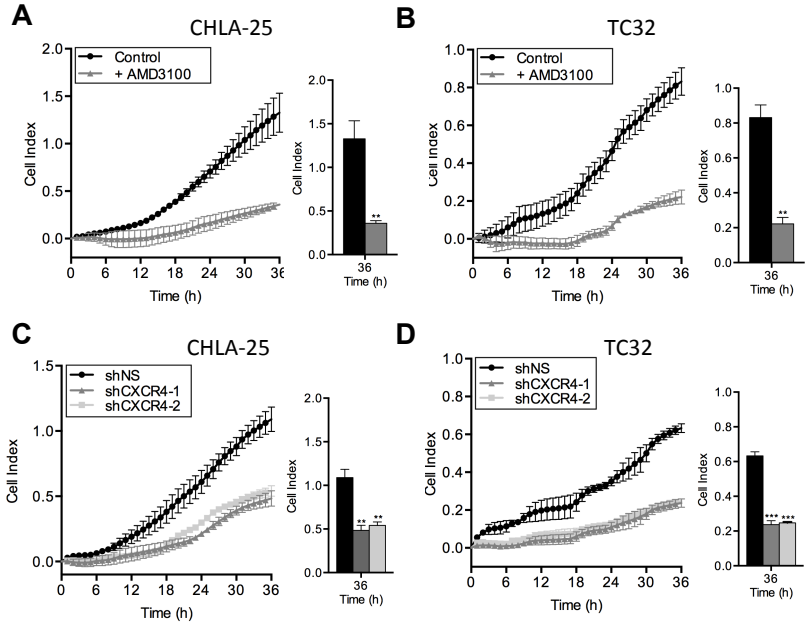

Figure 2.5. CXCR4 promotes invasion of Ewing sarcoma cells. A and B, invasion of CHLA-25 (A) and TC32 (B) cells toward SDF-1α (100 ng/mL) through a Matrigel layer was monitored by real-time cell assays as in Figure 4. AMD3100 inhibited migration of both cell lines. C and D, knockdown of CXCR4, as in Figure 4, resulted in significant inhibition of invasion of CHLA-25 (C) and TC32 (D) cells. Graphs represent mean ± SEM of three independent experiments with four replicates per condition. **, $P < 0.01$ and ***, $P < 0.001$ as compared with controls.

Rac1 and Cdc42 mediate CXCR4-dependent migration and invasion

The mechanisms by which the CXCR4/SDF-1α axis contributes to tumor growth and metastasis are pleiotropic, and cell type and context dependent (17). Activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) cascades are both observed downstream of CXCR4 activation (17). In addition, studies of breast and liver cancer have shown that the small

GTPases, Rho, Rac1, and Cdc42 are activated in these tumors following SDF-1α engagement of CXCR4, and that Rho-GTPase signaling is, at least in part, responsible for mediating the invasive/metastatic phenotype (25, 26). Interestingly, recent studies of Ewing sarcoma have also implicated Rac1 as a key mediator of tumor metastasis (27). To begin to address the mechanisms by which CXCR4 promotes the invasive cellular phenotype in Ewing sarcoma, we assessed the effects of SDF-1α treatment on the MAPK and PI3K pathways by evaluating phosphorylation of extracellular signal–regulated kinase (ERK) and AKT. As shown, SDF-1α treatment for 24 hours, which promoted cell migration and invasion, had no significant impact on activation of either kinase in CHLA-25 or TC32 cells (Figure 2.6A). Next we investigated whether SDF-1α–dependent chemotactic migration and invasion were dependent on Rac1 and/or Cdc42. Exposure of Ewing sarcoma cells to either NSC 23766 or ML 141, small-molecule inhibitors of Rac1 and Cdc42, respectively, resulted in significant inhibition of both migration (Figure 2.6B-D) and invasion (Figure 2.6E). In particular, inhibition of Rac1 nearly completely abrogated the chemotactic invasion of CXCR4-positive Ewing sarcoma cells. To determine if Rac1 activation is induced by SDF-1α, TC-32 cells were FACS-sorted on the basis of CXCR4 and Rac1 activity measured in the different populations in the presence or absence of SDF-1α. As shown, CXCR4-high cells displayed higher Rac1 activity than CXCR4-low cells, even in unstimulated conditions (Figure 2.6F). Exposure to SDF-1α potentiated Rac1 activity in both cell populations but activation of Rac1 was reproducibly most pronounced in SDF-1α–stimulated CXCR4-high

cells. Together, these studies demonstrate that the invasive cellular phenotype imparted to CXCR4-positive Ewing sarcoma cells following SDF-1α engagement is, at least in part, mediated by downstream activation of Rac1 and Cdc42 Rho-GTPases, in particular Rac1.

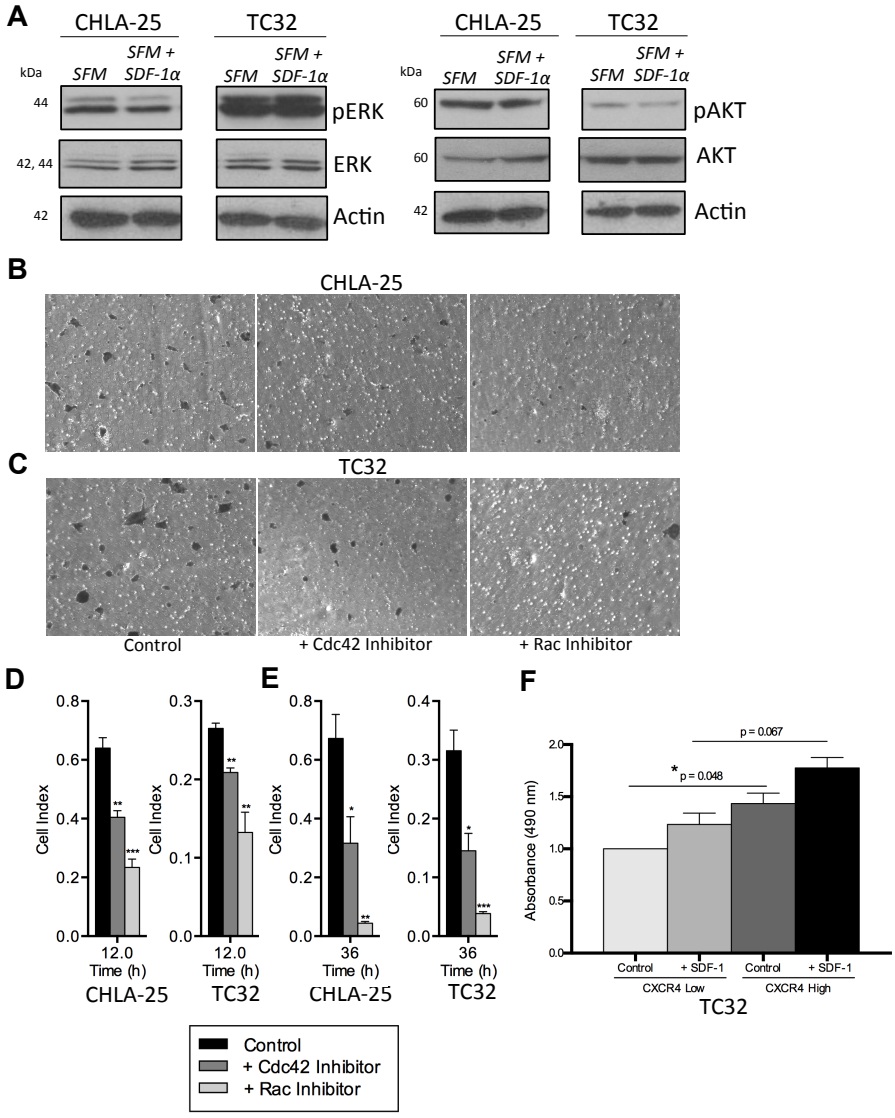

Figure 2.6. CXCR4-mediated chemotaxis is dependent on Rac1 and Cdc42. A, Western blot of CHLA-25 and TC32 cells shows no significant induction of either P-ERK (left) or P-AKT (right) following 24-hour exposure of serum-starved cells (SFM) to SDF-1α (100 ng/mL). B and C, Endpoint analysis of cell migration toward SDF-1α in the presence or absence of Rac1 (NSC 23766) or Cdc42 (ML141) inhibitors was performed as described in Materials and Methods using transwell assays and crystal violet staining. Inhibition of Rac1 and Cdc42 both impeded CXCR4-dependent cell migration. D and E, pharmacologic inhibition of Rac1 (NSC 23766) and Cdc42

(ML141) inhibits CXCR4-dependent migration (D) and invasion (E) of CHLA-25 and TC32 cells. Summary histograms show mean ± SEM of three independent experiments with four replicates per condition. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ as compared with controls. F, Rac1 activity was measured in TC32 cells sorted on the basis of CXCR4. Absorbance values are normalized to control condition (0% in CXCR4-low) and summary histograms show mean ± SEM of two independent sorts with three replicates per condition. *, $P < 0.05$.

CXCR4-dependednt migration is increased in Ewing sarcoma cells that are exposed to multiple stresses

Cells in the center of rapidly growing tumors are subjected to a diminished blood supply and must simultaneously endure conditions of both growth factor and oxygen deprivation. Given our findings that CXCR4 and CXCR4-dependent migration are induced by each of these stresses independently, we next investigated whether chemotactic migration of Ewing sarcoma cells would be further enhanced in cells that were simultaneously exposed to both serum starvation and hypoxia. As predicted, migration of serum-starved (and thus CXCR4-upregulated) CHLA-25 and TC32 cells toward SDF-1α was increased under hypoxic as compared with normoxic conditions (Figure 2.7A). Together, these studies suggest an additive role of microenvironmental stresses in promoting CXCR4-mediated Ewing sarcoma cell migration.

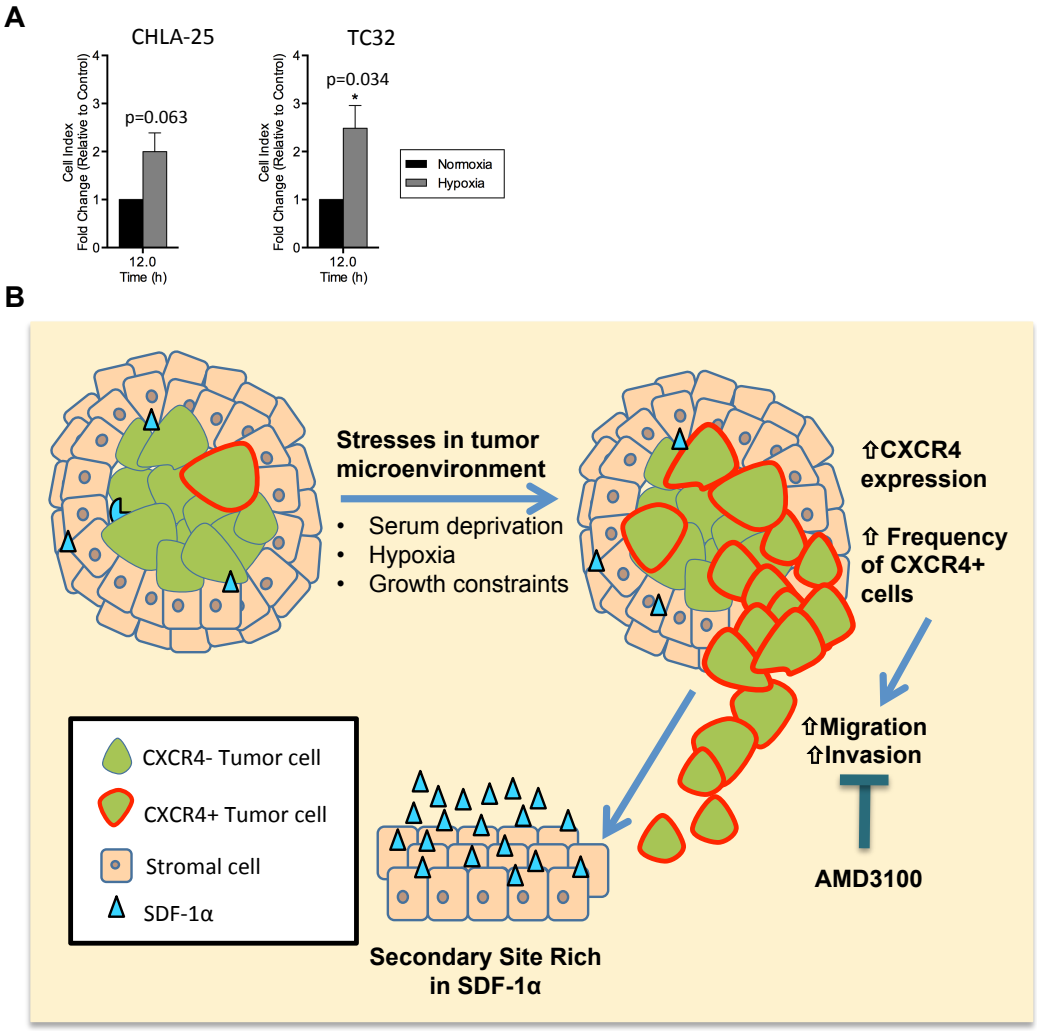

Figure 2.7. Hypothetical model of stress-induced, CXCR4-dependent invasion and metastasis. A, migration of CHLA-25 and TC32 cells toward SDF-1α (100 ng/mL) was measured using real-time cell analysis (xCELLigence CIM-Plate 16) in normoxic (21% O_2) or hypoxic conditions (1% O_2). Chemotactic migration of each cell line was further increased in hypoxia relative to normoxia. Graphs represent mean ± SEM of three independent experiments with four replicates per condition. Cell index was normalized to migration in normoxic conditions for each cell line. *, $P < 0.05$ as compared with controls. B, a growing tumor begins to deplete its resources, including growth factors and oxygen. Continued tumor growth leads to space constraint at the primary site. Upregulation of CXCR4 in response to these microenvironmental stresses promotes invasion of Ewing sarcoma cells through basement membranes and extracellular matrix and chemotaxis toward SDF-1α–rich secondary sites such as lung and bone marrow.

DISCUSSION

In these studies, we have shown that expression of CXCR4 is heterogeneous, both in Ewing sarcoma cell lines and primary tumors, and that expression is also highly dynamic. In particular, CXCR4 transcript and protein expression are reversibly increased when cells are exposed to serum deprivation, hypoxia, and confluent growth conditions. All of these stresses are encountered by a growing tumor *in vivo* as it outstrips its blood supply and expands to abut surrounding adjacent tissues, resulting in growth factor and oxygen deprivation and space constraints. Using both pharmacologic and genetic tools, we have also demonstrated that CXCR4-positive Ewing sarcoma cells display a highly migratory and invasive chemotactic phenotype when exposed to the CXCR4 ligand, SDF-1α/CXLC12. Our finding that Ewing sarcoma cells dynamically regulate CXCR4 leads us to propose a new model of Ewing sarcoma tumor cell invasion in which local microenvironment-induced cell stress results in upregulation of CXCR4, promoting chemotactic migration and invasion of CXCR4-positive Ewing sarcoma cells to distant sites of metastasis. In particular, this model proposes a mechanistic basis for the preferential metastasis of Ewing sarcoma cells to lungs and bone marrow, microenvironments rich in SDF-1α/CXLC12 (Figure 2.7B).

Studies of Ewing sarcoma tumors and cell lines have previously identified a potential role for the CXCR4/SDF-1α axis in Ewing sarcoma pathogenesis (4, 16, 28, 29). In particular, interrogation of gene expression databases identified an association between high levels of the *CXCR4* transcript and metastatic disease (4). In addition, concomitant clinical correlative studies in the same study

suggested that Ewing sarcoma tumors that express high levels of *CXCR4* and a related chemokine receptor, *CXCR7*, which also binds SDF-1α, are associated with worse overall survival (4). More recently, an immunohistochemical study of 30 Ewing sarcoma tumors revealed robust CXCR4 staining in approximately one third of cases, and these investigators also reported an association between CXCR4 expression and poor outcome, although no correlation with metastatic disease was identified (16). In our own study, we also detected robust expression of CXCR4 in approximately one third of cases and an absence of CXCR4-positive cells in another third. However, CXCR4-positive cells were also identified in the remaining third of cases, but tumor cells were found to be heterogeneously positive. Consistent with the study by Berghuis and colleagues (16), the pattern of CXCR4 expression in our tumor cohort did not correlate with the source of the tumor sample. Samples from both primary and recurrent lesions showed equally heterogeneous expression patterns. Together, these studies confirm the heterogeneous nature of CXCR4 protein expression in primary Ewing sarcoma tumors and support further investigation of the contribution of CXCR4 signaling to Ewing sarcoma progression. Whether or not high-level expression or an increased frequency of CXCR4-positive cells at the time of diagnosis portends a worse prognosis for patients still requires further investigation. Specifically, given the complexities of prognostic biomarker discovery, it is critical that this question next be addressed prospectively in a large cohort of equivalently treated patients (30). Moreover, given the highly heterogeneous nature of CXCR4 expression, a single core-needle biopsy sample may or may not be representative of CXCR4

expression in other areas of the tumor. Ideally, multiple cores should be assessed when a dynamically regulated and heterogeneous protein like CXCR4 is being evaluated as a potential prognostic biomarker.

Berghuis and colleagues identified a role for CXCR4/SDF-1α in promoting cell proliferation, rather than metastasis (16). Given the pleiotropic nature and cell context-specific response of CXCR4-dependent signaling, it is not surprising that different experimental designs have uncovered different results and elucidated different functions for the CXCR4/SDF-1α axis in Ewing sarcoma pathogenesis. We have shown that exposure of CXCR4-positive Ewing sarcoma cells to SDF-1α results in robust induction of chemotaxis, and that both migration and invasion are promoted by activation of CXCR4 signaling. In addition, studies with small-molecule inhibitors AMD3100, NSC 23766, and ML 141 showed that migration and invasion toward SDF-1α are dependent on CXCR4 and its downstream effectors, Rac1 and Cdc42, respectively. Interestingly, our studies also indicated that the basal activity of Rac1 is higher in CXCR4-positive Ewing sarcoma cells than CXCR4-negative cells, even in the absence of ligand and that Rac1 was maximally activated by SDF-1α in the CXCR4-positive population. Moreover, we have also found that inhibiting Rac1 blocks SDF-1α –independent invasion of serum-starved Ewing cells that do not express high levels of CXCR4 (data not shown). In addition, Rac1 was also recently implicated as a key mediator of Ewing sarcoma cell invasion and metastasis downstream of the tyrosine kinase receptor ERBB4 (27). Thus, activation of Rac1 is implicated in both nonchemotactic and SDF-1α–mediated Ewing sarcoma migration and

invasion, downstream of and in parallel to CXCR4-dependent signaling, suggesting that this Rho-GTPase may be a critical downstream hub, present at the convergence of multiple Ewing sarcoma metastatic pathways.

The origins of tumor heterogeneity are multifactorial, and contributing factors include genetic variation, stochastic processes, different microenvironments, and cell plasticity (31). Indeed, dynamic regulation of metastasis-inducing genes in response to exogenous cues is a hallmark of epithelial cancer cell plasticity, resulting in epithelial–mesenchymal transition (EMT), a critical initiating event in the onset of carcinoma metastasis (32). Unlike most adult solid tumors, pediatric solid tumors mainly arise from nonepithelial tissues, predominantly neural and mesenchymal lineages, thus obviating a role for EMT. We have discovered that, like EMT genes in epithelial cancers, CXCR4 expression in Ewing sarcoma is highly plastic and this phenotypic plasticity results in functional changes that can contribute to cell invasion and metastatic dissemination. In particular, CXCR4 expression is highly responsive to stresses in the local microenvironment, reverting to its basal state when the stressor is removed. Consistent with this observation, dynamic regulation of CXCR4 has also been observed in neuroblastoma, a neural crest–derived solid tumor (33, 34), demonstrating that plasticity of CXCR4 is not limited to Ewing sarcoma. Interestingly, high levels of CXCR4 have also been identified in tumor- and metastasis-initiating cancer stem cell populations (7, 35, 36), suggesting that dynamic regulation of CXCR4 may contribute to the dynamic regulation of stemness that has been described in highly plastic cancer cell populations (37).

We hypothesize that dynamic regulation of CXCR4 in Ewing sarcoma, as well as other pediatric solid tumors, contributes to cellular heterogeneity and supports the dynamic transition of cells between nonmetastatic and metastatic states. Studies are ongoing in our laboratory to determine the precise molecular mechanisms that underlie the dynamic regulation of CXCR4 expression and to define whether it is under the control of epigenetic, transcriptional, and/or posttranscriptional regulatory pathways.

Current systemic cytotoxic agents have reached the limit of tolerability, and novel approaches to treatment, in particular approaches that prevent metastatic relapse, are desperately needed for Ewing sarcoma and other invasive solid tumors (38). The CXCR4/SDF-1α axis is a well-established mediator of tumor metastasis, and it offers a potentially attractive therapeutic target for the treatment and prevention of metastatic disease (17). Our current work, along with recent studies of other sarcomas and neuroblastoma (9, 10, 14, 34, 39), suggests that this axis represents a potential target for metastasis prevention in Ewing sarcoma as well as other aggressive pediatric tumors and should be further investigated in relevant preclinical therapeutic models of these cancers. In particular, studies of spontaneous metastasis using orthotopic, patient-derived xenograft models will be most informative and should be pursued for preclinical studies of CXCR4-targeted therapies.

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CHAPTER THREE

EPIGENETIC PLASTICITY OF THE *CXCR4* LOCUS CONTRIBUTES TO CELL STATE TRANSITIONS IN EWING SARCOMA[3]

ABSTRACT

Tumor heterogeneity and cellular plasticity are means by which tumors can evade upfront therapy and progress to metastatic disease. This diversity is a major therapeutic dilemma. Tumor cell heterogeneity can be mediated by both genetic and epigenetic mechanisms, which can be modulated by the tumor microenvironment. We recently reported that expression of CXCR4 is highly heterogeneous as well as dynamic and responsive to microenvironmental cues in Ewing sarcoma. In particular, *CXCR4* expression is induced in response to serum deprivation, hypoxia, and growth constraints. This expression induction leads to the transition of cells from a CXCR4- non-motile to a CXCR4+ highly motile state. In this study, we investigated the potential contribution of epigenetic regulation of CXCR4 cell-state transitions in response to changes in the microenvironment. We have demonstrated that the *CXCR4* promoter exists in a

[3] A version of Chapter Three is in preparation to be submitted for publication. The list of authors is: Melanie A. Krook, Allegra G. Hawkins, Raj Patel, David Lucas, Rashmi Chugh, and Elizabeth R. Lawlor.

bivalent state with simultaneous enrichment of both the activating H3K4me3 and the repressive H3K27me3 histone marks. Significantly, exposure of Ewing sarcoma cells to serum deprivation or hypoxia resulted in loss of histone mark bivalency as evidenced by loss of H3K27me3 with retention of H3K4me3 modifications. Finally, we demonstrated that an upstream enhancer also contributes to *CXCR4* regulation. In support of this, treatment of Ewing sarcoma cells with the enhancer antagonist, JQ1, resulted in marked down regulation of *CXCR4* expression in ambient conditions. Furthermore, JQ1 treatment blocked up-regulation of *CXCR4* in response to both serum deprivation and hypoxia. Collectively, these data demonstrate a key role for the epigenetic regulation of *CXCR4*, thus highlighting a novel opportunity for the development of innovative therapeutic strategies that tackle the complex issue of cellular plasticity and tumor heterogeneity in Ewing sarcoma.

OBJECTIVE STATEMENT

In Chapter Two, we demonstrated that in response to stress (serum deprivation, hypoxia and growth constraints), Ewing sarcoma cells dynamically regulate CXCR4 by transitioning from CXCR4- to CXCR4+ states. Additionally, this transition is accompanied by an increase in the migratory and invasive potential of Ewing sarcoma cells. Furthermore, CXCR4 heterogeneity is evident even between cells within the same tumor or cell culture (1). We next sought to determine the molecular mechanisms that drive the dynamic regulation of

CXCR4. We specifically propose that this dynamic regulation is governed, at least in part, by epigenetic plasticity at the *CXCR4* gene locus. Our recent findings on the molecular mechanisms regulating CXCR4 plasticity are summarized in this chapter.

INTRODUCTION

Thus far, Ewing sarcoma biological studies and clinical trials have largely failed to address the inherent heterogeneity and phenotypic plasticity of tumor cells. Tumor heterogeneity contributes to tumor progression and remains a major challenge in the treatment and diagnosis of cancer as well as of the development of novel cancer therapeutics (2, 3). Furthermore, both tumor and stromal cells can contribute to tumor heterogeneity, which results in phenotypic heterogeneity among cells. This phenotypic heterogeneity can include variability in gene expression, motility, and metastatic potential across cells in a tumor (4). This heterogeneity can be driven by both intrinsic genetic and epigenetic mechanisms as well as by contributions of the tumor microenvironment (3, 5). Current evidence suggests that Ewing sarcoma tumors are driven by epigenetic mechanisms rather than genetic mechanisms (6). On average, 33 to 66 genes exhibit somatic mutations in any given tumor type (7). Melanoma exhibits far more mutations than average with ~200 mutations per tumor and the majority of pediatric tumors exhibit far fewer than the average number of mutations (7). In Ewing sarcoma, the EWS-ETS translocation is considered pathognomonic and

aside from the occasional *STAG2* (15%), *CDKN2A* (10%) and *TP53* (6%) mutations, the translocation is the predominant genetic driver (8-10). This suggests that tumor heterogeneity in Ewing sarcoma is driven by factors other than strictly genetic mechanisms.

Cellular plasticity is an adaptive strategy adopted by cancer cells in order to survive and undergo disease progression. This cancer cell plasticity can be mediated by microenvironmental signals that modulate reversible epigenetic modifications (11). Epigenetic regulation of chromatin plays a critical role in normal development and tissue maintenance (12). Two of the most prevalent protein complexes that modulate chromatin structure are the Polycomb group (PcG) and Trithorax group (TrxG) complexes, which regulate thousands of genomic loci (13-15). Dysregulation of these protein complexes has been implicated in cancer pathogenesis. PcG and Trx proteins are recruited to the loci of developmental genes and act antagonistically by depositing chromatin marks that repress (trimethylation of histone H3 on lysine 27: H3K27me3) or activate (trimethylation of histone H3 on lysine 4: H3K4me3) gene expression, respectively. In embryonic stem cells, the promoters of many developmental genes are maintained in a bivalent state characterized by the simultaneous presence of both the H3K4me3 and H3K27me3 marks (16). This bivalent state allows for rapid gene activation or repression in response to developmental and microenvironmental cues. In cancer, the regulation of bivalent chromatin domains contributes to tumor cell heterogeneity and phenotypic fates (17).

In the current study, we have demonstrated that *CXCR4* locus is epigenetically regulated and that a bivalent promoter allows CXCR4 to be rapidly activated in response to stress. Additionally, we demonstrate that an enhancer upstream of CXCR4 contributes to its regulation and that the enhancer antagonist, JQ1, can be used to block CXCR4 state transitions.

MATERIALS AND METHODS

Cell culture

Ewing sarcoma cell lines were cultured in RPMI-1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Atlas Biologicals, Inc., Fort Collins, CO, USA) and 6mM L-glutamine (Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO_2. For CHLA-25 cells, prior to cell seeding, plates were briefly coated (~5 minutes) with 0.2% Gelatin (Gelatin from bovine skin, Type B). For serum starved conditions, cells were cultured in the same conditions without the presence of FBS for 24 hours. For hypoxia studies, cells were incubated in an xVivo system (Biospherix, Lacona, NY, USA) at 1% O_2, 37°C and 5% CO_2 for 48 hours. For GSK-126 studies, cells were treated with either vehicle control (DMSO; D128-500, Fisher Scientific, Waltham, MA) or 10μM GSK-126 (A-1275, Active Biochem, Maplewood, NJ) daily for 72 hours prior to functional studies. For (+)-JQ1 studies, cells were treated with either vehicle control (DMSO; D128-500, Fisher Scientific, Waltham, MA), or 1 μM (+)-JQ1 (11187, Cayman Chemical, Ann Arbor, MI) for 24 hours.

Quantitative real-time PCR

RNA was isolated using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA) and cDNA was generated using iScript (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed using validated *CXCR4* and beta-2-microglobulin (*B2M*) Taqman assays (Life Technologies, Grand Island, NY). Analysis was performed in triplicate using the Lightcycler® 480 System. Using the ΔΔCt method, gene expression was normalized to the reference gene.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed according to the methods of Gilfillan et al. 2012 (18). In brief, Ewing sarcoma cells ($3.6x10^5$ per IP) were digested with Micrococcal nuclease (MNase) (70196Y, Affymetrix, Santa Clara, CA) for 5 minutes at 37°C, sonicated for 20 seconds (Qsonica cup horn sonicator (Qsonica Sonicators, Newtown, CT, USA)), blocked for 1 hour with Dynabeads A+G (10001D and 10003D; Life Technologies, Carlsbad, CA), incubated with 1 µg of desired antibody overnight, incubated with Dynabeads A+G for 3 hours, washed (5 minute wash; 5 x RIPA buffer, 1 x LiCl buffer, 1 x TE buffer), digested proteins with Proteinase K for 1 hour at 55°C and purified immunoprecipitated DNA according to manufacturer's instructions (Zymo Genomic DNA Clean & Concentrator, D4011). For ChIP-re-ChIP studies, after the 3 hour incubation with Dynabeads A+G, the beads were incubated with dithiothreitol (DTT) (10mM final concentration, 15508-013, Life Technologies, Carlsbad, CA) for 30 minutes at

37°C. Chromatin was then incubated with the desired second antibody overnight and the protocol continued as above. Primer pairs for the *CXCR4* promoter and enhancer region are listed in Table 3.1.

Promoter Region			
Primer	**Forward**	**Reverse**	**Size (bp)**
#3	5'- GGG TGG TCG GTA GTG AGT CC -3'	5'- CAG AGA GAC GCG TTC CTA GC -3'	120
#5	5'- ACG CCT TCT CTG CAG TTG TG -3'	5'- TTC CAG TGG CTG CAT GTG TC -3'	105
#6	5'- GGG CTG CGC TCT AAG TTC AA -3'	5'- TAG CAA AGT GAC GCC GAG G -3'	144
#3_1	5'- GGG ATG TCT TGG AGC GAG TT -3'	5'- AAC AGT CAC CAG GCG CTT AA -3'	122
#8	5'- TCA CTA GGG TCA GGT GCA GA -3'	5'- TCG CGA ATT GGT TAC CGC TA -3'	131
GAPDH	5'- TAC TAG CGG TTT TAC GGG CG -3'	5'- TCG AAC AGG AGG AGC AGA GAG CGA -3'	166
Enhancer Region			
#1	5'- GGC TAG GAG GTG TGG ATG AA-3'	5'- CCA GAG TTG CCA CAG GAG AT-3'	126
#2	5'- CCC TTT CTG GGC TTT TTC TT-3'	5'- TTT GTG AAG GTG CCC TAT CC-3'	122
#3	5'- AAG GCT TTG AAG GCA CTG AA -3'	5'- GAC ATG AGG CAA TGC AAG AC-3'	124
#4	5'- TGT GGC AGC TGT GAA GAA TC-3'	5'- CTG GGA AGT AGG GCA GTC AG-3'	125
#5	5'- AGA GGG TAG GAG GGC TGT GT -3'	5'- CCC ACT GCT AGG CTT TTC TG-3'	129
#6	5'- CAT ATC CCC TGG AGG ACC TT-3'	5'- TTT TCT GCT GTC CCA ACT CC-3'	125
#8	5'- CAG TAT GAC CAG GGG TTT GC-3'	5'- TCT GGC AAT GAT TCC TCC TC-3'	125

Table 3.1, Related to Figure 3.1 and Figure 3.6. Primers used for ChIP quantitative genomic PCR and ChIP-re-ChIP analysis.

Antibodies

Antibodies were used for chromatin immunoprecipitation per manufacturer's instructions; H3K4me3 Rabbit anti-Human Polyclonal Antibody (49-1005; Life Technologies, Carlsbad, CA), Anti-trimethyl-Histone H3 (Lys27) Antibody (07-449; Millipore, Billerica, MA), Anti-Histone H3 (acetyl K27) antibody (ab4729; Abcam, Cambridge, MA), Anti-Histone H3 (mono methyl K4) antibody (ab8895; Cambridge, MA), normal mouse IgG (sc-2025; Santa Cruz Biotechnology, Dallas, TX), Rabbit IgG (ab37415; Abcam, Cambridge, MA).

Cell sorting

Cell sorting was performed as previously described (1). In brief, cells were blocked for 15 minutes at 4˚C with agitation (0.5% FBS), incubated with human CXCR4 Alexa Fluor 488 monoclonal antibody (5 µL/ $1.0x10^6$ cells) for 30 minutes at 4˚C with agitation, passed through a 0.40 µm sterile nylon mesh strainer and sorted into $CXCR4^{negative}$ (bottom 10%) and $CXCR4^{positive}$ (top 10%) on a Beckman Coulter MoFlo Astrios.

In vitro migration and invasion

Migration and invasion assays were performed as previously described (1). In brief, $1.0x10^5$ pretreated CHLA-25 or $2.0x10^5$ TC32 cells in RPMI-1640 media containing 0.2 % Probumin (Millipore, Billerica, MA) were seeded in the upper chamber of a CIM-16 plate (Acea Bioscience, Inc., San Diego, CA) and media containing SDF-1α (100 ng/mL, R&D Systems) was added to the lower chamber. For the invasion assays, the upper chamber was coated with a layer of Matrigel™ (Growth Factor Reduced Matrigel Matrix was diluted 1:20 in media, BD Biosciences, San Jose, CA). Prior to the assay, the electrodes were coated with 0.2% gelatin for 30 seconds at room temperature. Migration and invasion assays were carried out in an xCELLigence DP system with measurements every hour over a period of 12 hours and 36 hours, respectively.

Statistical analysis

Data are reported as mean ± SEM from a minimum of three independent experiments unless otherwise indicated. *P*-values were calculated using ratio paired t-test unless otherwise indicated.

RESULTS

The *CXCR4* promoter is bivalent in Ewing sarcoma cells

Epigenetic histone modifications play a well-established role in gene expression. Given our findings that *CXCR4* mRNA is rapidly and reversibly up regulated in response to cellular stresses (1), we sought to assess the role of epigenetic mechanisms in regulating *CXCR4* plasticity. Having determined that the *CXCR4* promoter resides in a bivalent state in human embryonic stem cells (Figure 3.1A), we sought to determine if the *CXCR4* locus is bivalent in Ewing sarcoma cells. We first assessed whether the histone modifications H3K4me3 and H3K27me3 are present at the *CXCR4* locus in Ewing sarcoma cells. Five primer sets (#3, #5, #6, #8, and #3_1) were designed for the *CXCR4* promoter (Figure 3.1B). We performed chromatin immunoprecipitation (ChIP) studies followed by quantitative genomic PCR (qPCR) in a panel of Ewing sarcoma cells, with the cell lines ranging from low *CXCR4* expression to high *CXCR4* expression (Figure 3.1C). We determined that both histone marks (H3K4me3 and H3K27me3) were present at the *CXCR4* locus in cells grown under standard culture conditions (unstressed and unsorted) (primer set #8, Figure 3.1D). Additional primer sets demonstrated similar results (Figure 3.2). These results

may reflect populations of cells with either H3K4me3 or H3K27me3 marks. Therefore, to determine true bivalency, we performed sequential ChIP experiments on the same chromatin in TC71, A673, CHLA-25, and TC32 cells. These ChIP-re-ChIP studies confirmed that the *CXCR4* locus is bivalent in Ewing sarcoma (Figure 3.1E). In HeLa cells, the *CXCR4* promoter is characterized by a univalent state, with enrichment of only the H3K4me3 mark (ENCODE Database, Figure 3.3). Additionally, HeLa cells have very high expression of CXCR4 with nearly 100% of the cells expressing CXCR4 (Figure 3.1F and 3.1G). We confirmed the ENCODE findings with ChIP-qPCR and ChIP-re-ChIP experiments demonstrating that HeLa cells are not bivalent and are marked solely with the H3K4me3 mark (Figure 3.1H and 3.1I).

Thus, these data demonstrate that in Ewing sarcoma cells the *CXCR4* promoter exists in a bivalent state and, as such, may be poised ready for activation in response to microenvironmental cues.

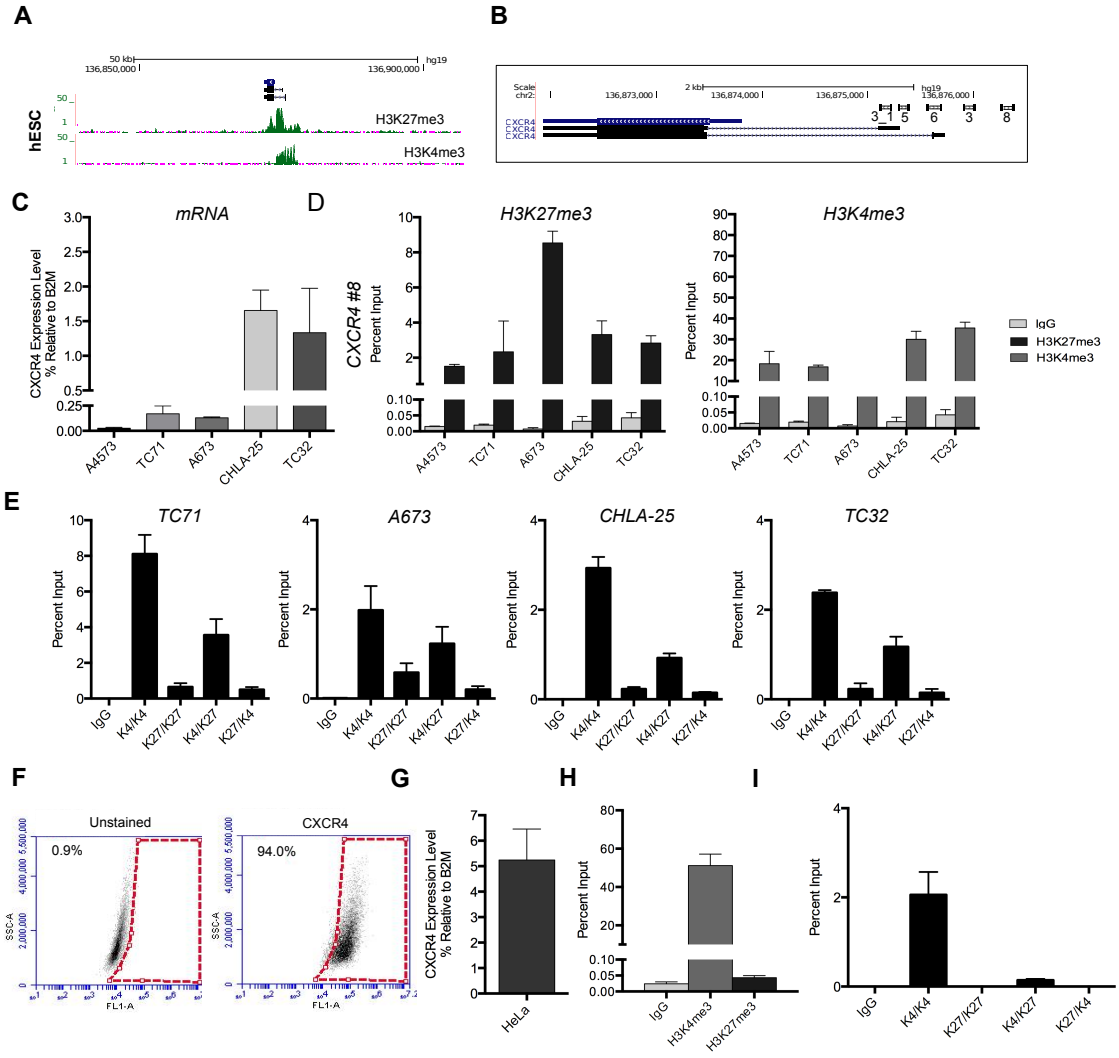

Figure 3.1. The *CXCR4* promoter resides in a bivalent state in Ewing sarcoma. Gene tracks for H3K4me3 and H3K27me3 at the *CXCR4* promoter in human embryonic stem cells (hESC) assembled from the ENCODE database demonstrate a bivalent state (A). Map of primer pairs designed for the *CXCR4* promoter region (B). *CXCR4* expression was determined in a panel of Ewing sarcoma cell lines using qRT-PCR (Low Expression: A4573, TC71, A673; High Expression: CHLA-25, TC32) (C). Chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) was used to assess the presence of the activating mark H3K4me3 and the repressive mark H3K27me3 in Ewing sarcoma cell lines (C). Under standard culture conditions, the H3K4me3 and H3K27me3 marks were enriched at the *CXCR4* promoter in a panel of Ewing sarcoma cells (D). Sequential Chromatin immunoprecipitation (ChIP-re-ChIP) for H3K4me3 followed by H3K27me3 (K4/K27) and vice versa, H3K27me3 followed by H3K4me3 (K27/K4) was performed on TC71, A673, CHLA-25, and TC32 cells confirming that the *CXCR4* promoter is bivalent as marked by simultaneous presence of both H3K4me3 (activating) and H3K27me3 (repressive) histone modifications (E). IgG, K4/K4, and K27/K27 served as controls. HeLa cells express high levels of CXCR4 (F and G). HeLa cells are not bivalent and are marked solely with the H3K4me3 mark (H and I). Data represented as mean ± SEM of three independent experiments.

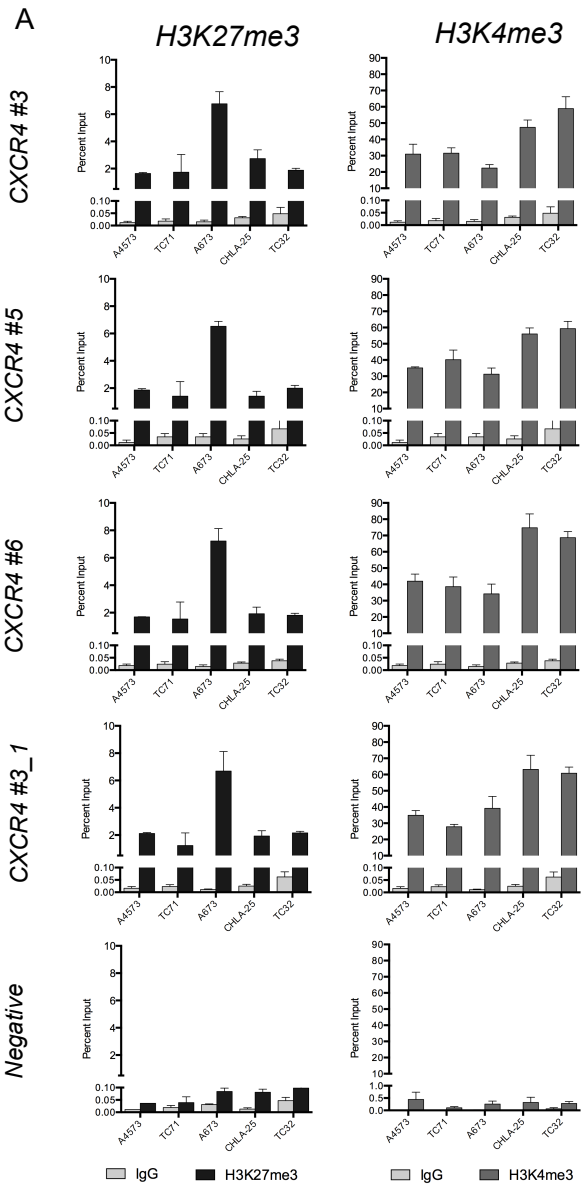

Figure 3.2, Related to Figure 3.1. Enrichment of H3K4me3 and H3K27me3 marks at the *CXCR4* promoter. ChIP-qPCR for the H3K4me3 and H3K27me3 histone modifications and IgG control at the *CXCR4* promoter in a panel of Ewing sarcoma cell lines under standard culture conditions for primer sets #5, #6, #8, #3_1, and negative controls. Data represented as mean ± SEM of three independent experiments.

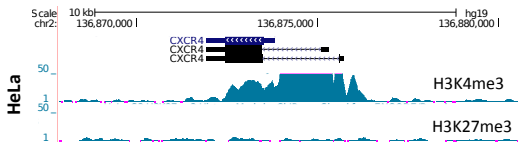

Figure 3.3, Related to Figure 3.1. H3K4me3 and H3K27me3 gene tracks at the *CXCR4*

promoter in HeLa Cells. Gene tracks assembled from the ENCODE database demonstrate that the *CXCR4* promoter in HeLa cells is marked by the enrichment of H3K4me3 and the absence of H3K27me3 histone marks.

Histone marks correlate with CXCR4 expression

Having demonstrated that CXCR4 expression is heterogeneous in Ewing sarcoma cells we wanted to assess whether expression correlated with epigenetic marks. To this end, we analyzed the histone marks at the *CXCR4* promoter in more homogenous populations of cells that had been sorted on the basis of CXCR4 expression. CHLA-25 and TC32 cells were FACS sorted into $CXCR4^{negative}$ and $CXCR4^{positive}$ fractions (bottom 10% and top 10%) with gating parameters determined by unstained controls (Figure 3.4A and B). As expected, *CXCR4* mRNA expression correlated with surface levels of CXCR4 in the two populations, with the $CXCR4^{negative}$ population expressing low levels of *CXCR4* and the $CXCR4^{positive}$ population expressing high levels of *CXCR4* (Figure 3.4C and D). Consistent with our hypothesis that epigenetic mechanisms contribute to the regulation of *CXCR4* expression, analyses of histone modifications at the *CXCR4* promoter demonstrated preferential enrichment of H3K27me3 in the $CXCR4^{negative}$ population (Figure 3.4E and F), while the $CXCR4^{positive}$ population demonstrated preferential enrichment of the H3K4me3 mark (Figure 3.4G and H). These data indicate that *CXCR4* expression levels are inversely correlated with the H3K27me3 mark and directly correlated with the H3K4me3 mark at the gene promoter.

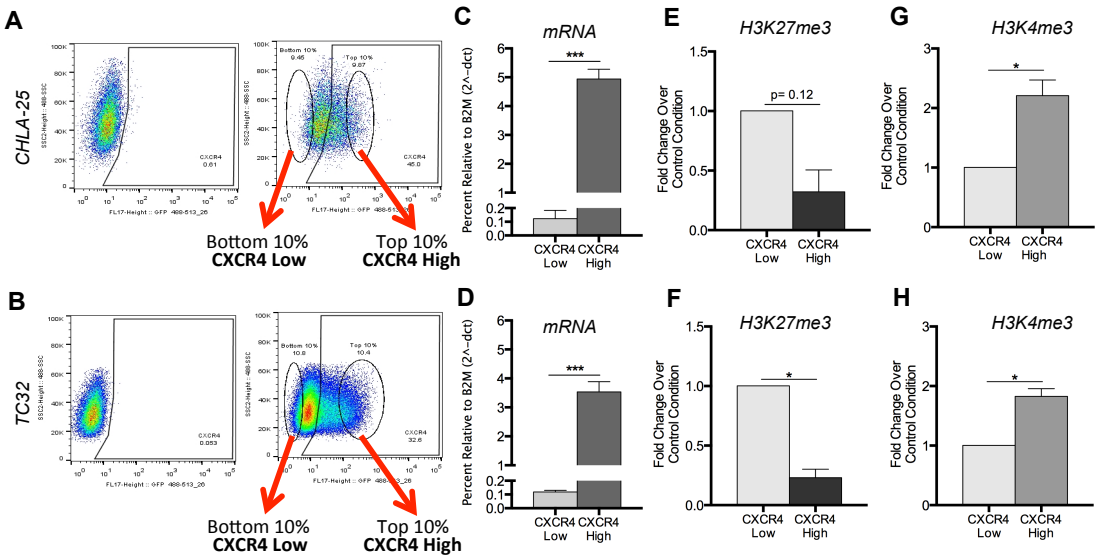

Figure 3.4. Sorting on the basis of CXCR4 correlates with histone marks. CHLA-25 (A) and TC32 (B) cells were FACS-sorted into CXCR4^{negative} (bottom 10%) and CXCR4^{positive} (top 10%) using the Beckman Coulter MoFlo Astrios. *CXCR4* expression by qRT-PCR correlated with CXCR4 surface protein expression as determined by flow cytometry (C and D). ChIP experiments were performed on both the CXCR4^{negative} and the CXCR4^{positive} populations revealing that in sorted CHLA-25 and TC32 cells, CXCR4 expression is inversely correlated with H3K27me3 (E and F) and directly correlated with H3K4me3 (G and H) marks (H3K4me3: positive and H3K27me3: negative). Data represented as mean ± SEM of three independent experiments.

Conditions that induce upregulation of *CXCR4* in Ewing sarcoma cells also induce loss of bivalency at the *CXCR4* promoter

Having demonstrated that the *CXCR4* locus is bivalent in Ewing sarcoma cells, we next explored if changes in histone modifications occurred in response to cellular stress, converting the *CXCR4* locus from a bivalent, repressed state to a univalent, active state. We previously demonstrated that in response to serum deprived and hypoxic conditions, *CXCR4* expression was upregulated, though to varying levels across cell lines (1). We subjected four Ewing sarcoma cell lines to serum deprived conditions for 24 hours and then performed ChIP-qPCR for the H3K27me3 and H3K4me3 histone marks. A673 cells robustly upregulated *CXCR4*, CHLA-25 and TC32 moderately upregulated *CXCR4*, and *CXCR4*

expression was unchanged in TC71 cells (Figure 3.5A). We found that in response to serum deprivation, there was a marked reduction in H3K27me3 enrichment in A673, CHLA-25 and TC32 cells relative to standard culture conditions (10%) (Figure 3.5B), however there was no change in the H3K4me3 enrichment in response to serum-deprived conditions (Figure 3.5C). TC71 cells, which did not upregulate *CXCR4*, had no change in H3K27me3 enrichment in serum-deprived conditions (Figure 3.5B). These data suggest that loss of the H3K27me3 mark at the *CXCR4* promoter contributes to upregulation of *CXCR4* in response to serum deprivation.

Next, Ewing sarcoma cells were subjected to hypoxia for 48 hours. In response to hypoxia, CHLA-25 cells upregulated *CXCR4* while *CXCR4* expression was unchanged in A673, TC32, and TC71 cells (Figure 3.5D). In CHLA-25 cells, the H3K27me3 mark was lost in response to hypoxic stress, but was unchanged in A673, TC32 and TC71 cells (Figure 3.5E). Additionally, H3K4me3 marks were unchanged in response to hypoxic stress (Figure 3.5F). These data suggest that Ewing sarcoma cell response to hypoxic stress was highly variable across cells lines and only CHLA-25 reproducibly upregulated *CXCR4* in these conditions. Collectively, these data demonstrate that the bivalent state of the *CXCR4* promoter allows Ewing sarcoma cells to rapidly switch states from a CXCR4-negative to a CXCR4-positive state. Further, they show that the stimuli that can induce *CXCR4* upregulation and loss of bivalency are highly cell-context dependent, even among Ewing sarcoma cell lines.

Given our findings that the H3K27me3 mark was lost in response to microenvironmental stresses, we sought to determine if this was mediated by EZH2, the methyltransferase responsible for the H3K27me3 mark. We took advantage of GSK-126, the EZH2 inhibitor that is 1000-fold more selective for EZH2 than other histone methyltransferases (19). Ewing sarcoma cells were treated with subcytotoxic doses of GSK-126 (10 μM) for 72 hours resulting in global loss of H3K27me3 (data not shown). Interestingly, treatment with GSK-126 resulted in the upregulation of *CXCR4* in A673, TC71, TC32 and CHLA-25 cells (Figure 3.5G). Further, this upregulation was accompanied by the corresponding loss of the H3K27me3 mark at the *CXCR4* promoter (Figure 3.5H). As anticipated, there was no effect of GSK-126 on the enrichment of the H3K4me3 mark (Figure 3.5I). These data demonstrate that EZH2 mediates the H3K27me3 mark at the *CXCR4* locus.

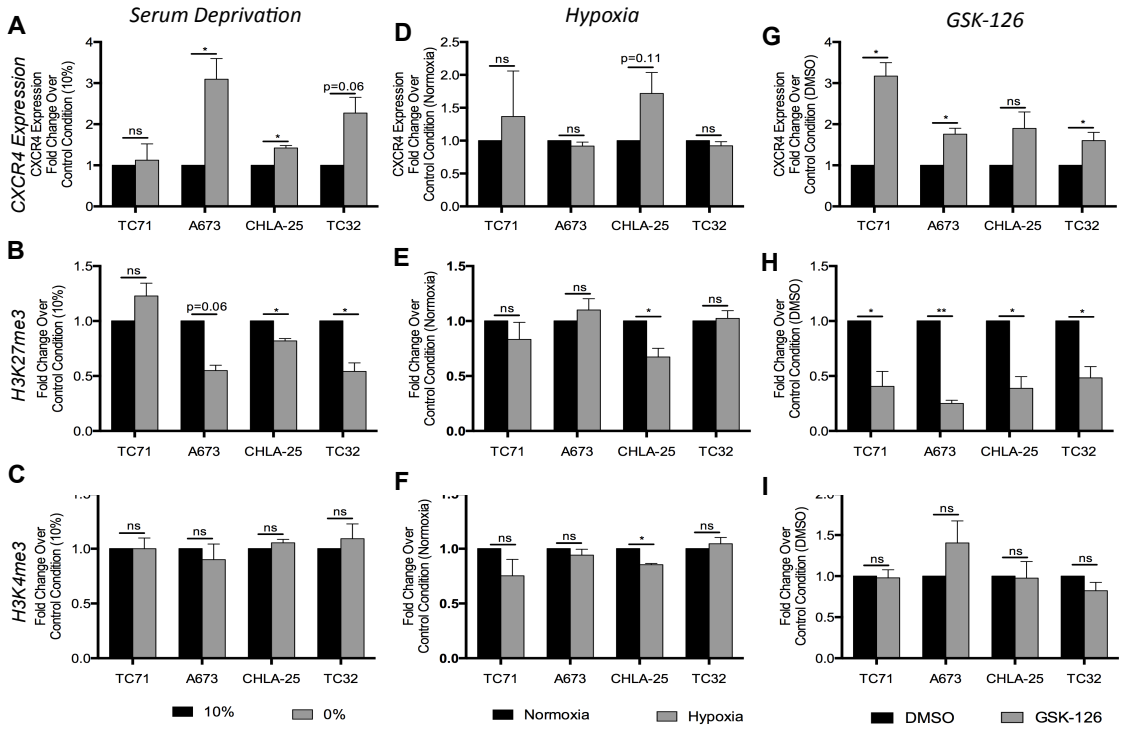

Figure 3.5. Ewing sarcoma cells lose the repressive H3K27me3 mark at the *CXCR4* promoter in response to stress. Ewing sarcoma cells were cultured in standard conditions for 24 hours. Cells were then exposed to different micro-environmental stresses: serum free media (SFM) for 24 hours, or hypoxia (1% O_2) for 48 hours, or GSK-126 (10 μM) for 72 hours prior to performing ChIP experiments. Expression of CXCR4 was determined using qRT-PCR under non-stressed and stressed conditions. In response to serum deprivation, A673, CHLA-25, and TC32 cells lost the H3K27me3 (B), which resulted in *CXCR4* upregulation (A). TC71 cells did not upregulate *CXCR4* (A) and there was no change in H3K27me3 enrichment (B). There was no change in H3K4me3 enrichment after serum deprivation (C). Additionally, under conditions of hypoxia CHLA-25 cells upregulated *CXCR4* (D) and the H3K27me3 mark was lost (E) whereas TC71, A673, and TC32 cells did not upregulate *CXCR4* (D) and there was no change in H3K27me3 enrichment (E). As expected, there was no change in H3K4me3 enrichment in response to hypoxic stress (F). Treatment of Ewing sarcoma cells with subcytotoxic doses of the EZH2 inhibitor, GSK-126, resulted in an increase in *CXCR4* expression (G). This increase in *CXCR4* expression was accompanied by a substantial loss in H3K27me3 enrichment (H). Additionally, there was no difference in H3K4me3 enrichment after treatment with GSK-126. Results shown as mean ± SEM (n=3).

Our current findings demonstrate that the *CXCR4* locus resides in a bivalent state and that exposure of Ewing sarcoma cells to serum deprivation or hypoxic stress increases *CXCR4* expression resulting in a population of cells that possess an increased tumorigenic potential. This increase in *CXCR4* is driven by the loss of H3K27me3, which is mediated by EZH2.

CXCR4 sorted cells upregulate *CXCR4* in response to serum deprivation

The above data show that *CXCR4* expression correlates directly with H3K4me3 and inversely with H3K27me3, and that in response to serum deprivation *CXCR4* is upregulated and the H3K27me3 mark is lost. We therefore sought to determine whether serum deprivation would convert $CXCR4^{negative}$ cells to $CXCR4^{positive}$ cells. TC32 cells were sorted into $CXCR4^{negative}$ and $CXCR4^{positive}$ populations and replated under standard culture conditions overnight. The cells were then exposed to either serum containing or serum deprived conditions for 24 hours (Figure 3.6A). We confirmed our previous findings that *CXCR4* expression correlated directly with H3K4me3 and inversely

with H3K27me3 marks in the serum containing culture conditions even 48 hours post sort (data not shown). We found that *CXCR4* expression was upregulated in response to serum deprived conditions in both the CXCR4^{negative} and CXCR4^{positive} populations, though the CXCR4^{negative} population upregulated *CXCR4* more robustly (Figure 3.6B). Despite the upregulation of *CXCR4* in both the CXCR4^{negative} and CXCR4^{positive} populations, the enrichment of H3K27me3 was decreased only in the CXCR4^{negative} population in response to serum deprivation (Figure 3.6C). As expected, the H3K4me3 mark was unaffected in either population (CXCR4^{negative} or CXCR4^{positive}) in response to serum deprived conditions (Figure 3.6D). These data suggest that the majority of the CXCR4^{negative} cells reside in a bivalent state which allows them to be more responsive to microenvironmental cues than the CXCR4^{positive} population. The observation that the CXCR4^{positive} population upregulated *CXCR4* suggests that there might be other factors, epigenetic or otherwise contributing to CXCR4 regulation.

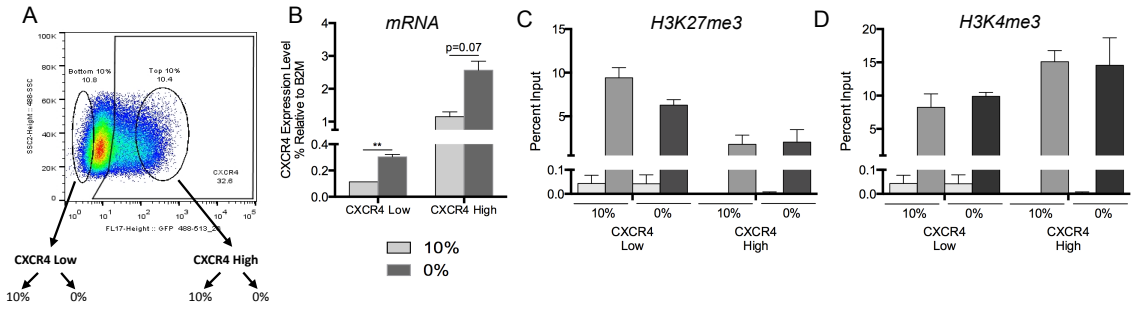

Figure 3.6. CXCR4 sorted cells upregulate *CXCR4* in response to serum deprivation. TC32 cells were sorted into CXCR4^{negative} and CXCR4^{positive} populations (A). The two sorted cell populations were then maintained under standard culture conditions for 24 hours post sort and were then placed into serum replete or serum deprived conditions for 24 hours (A). In response to serum deprived conditions both the CXCR4^{negative} and CXCR4^{positive} populations upregulated *CXCR4* (B). In response to upregulation of *CXCR4*, only the CXCR4^{negative} population lost the repressive, H3K27me3, mark at the *CXCR4* promoter (C). There was no change in H3K27me3 enrichment in the CXCR4^{positive} population (C). Additionally, there was no change in H3K4me3

enrichment in response to serum deprivation in either the CXCR4^{negative} or CXCR4^{positive} populations (D). Results shown as mean ± SEM (n=3 for *CXCR4* expression, n=2 for ChIP experiments).

An upstream enhancer contributes to CXCR4 regulation

In addition to promoter regulation of gene expression, enhancers have been shown to regulate transcription independently of promoter regions. We therefore hypothesized that alterations at an enhancer region may also contribute to CXCR4 regulation in response to stress. In HeLa cells there is a presumed enhancer upstream of *CXCR4* as characterized by the presence of H3K4me1 and H3K27ac histone marks (Figure 3.7A) (20-22). To determine if an enhancer exists upstream of *CXCR4* in Ewing sarcoma cells, four sets of primers were designed for this region (Figure 3.7A). In a panel of five Ewing sarcoma cell lines as well as HeLa cells, there was enrichment of the enhancer histone marks H3K4me1 and H3K27ac suggesting an enhancer element exists upstream of *CXCR4* and may play a role in *CXCR4* regulation (Figure 3.7B).

To address whether this enhancer is contributing to CXCR4 regulation, we took advantage of an enhancer antagonist, JQ1. JQ1 is a potent and selective inhibitor of the BET family of bromodomains functioning to displace bromodomains from chromatin by binding competitively to acetyl-lysine recognition marks (23). We treated Ewing sarcoma cells with sub-cytotoxic doses of JQ1 (1 μM) for 24 hours and examined *CXCR4* expression. JQ1 treatment resulted in down-regulation of *CXCR4* expression in HeLa, A673, CHLA-25, and TC32 cells but had no effect on *CXCR4* expression in TC71 cells (Figure 3.7C). We also demonstrated that JQ1 blocks the CXCR4- toCXCR4+

cell state transitions in response to hypoxic stress (Figure 3.7D) and serum deprivation (Figure 3.7E). Furthermore, exposure of the CHLA-25 and TC32 cells to JQ1 significantly inhibited their chemotactic migration (Figure 3.7F) and invasion (Figure 3.7G) to SDF-1. Collectively these data suggest that an enhancer also contributes to CXCR4 regulation. Furthermore, enhancer dependent regulation of *CXCR4* transcription opens the door to the potential of epigenetic modifiers as novel therapeutics that could be used to antagonize metastasis-promoting cell state transitions.

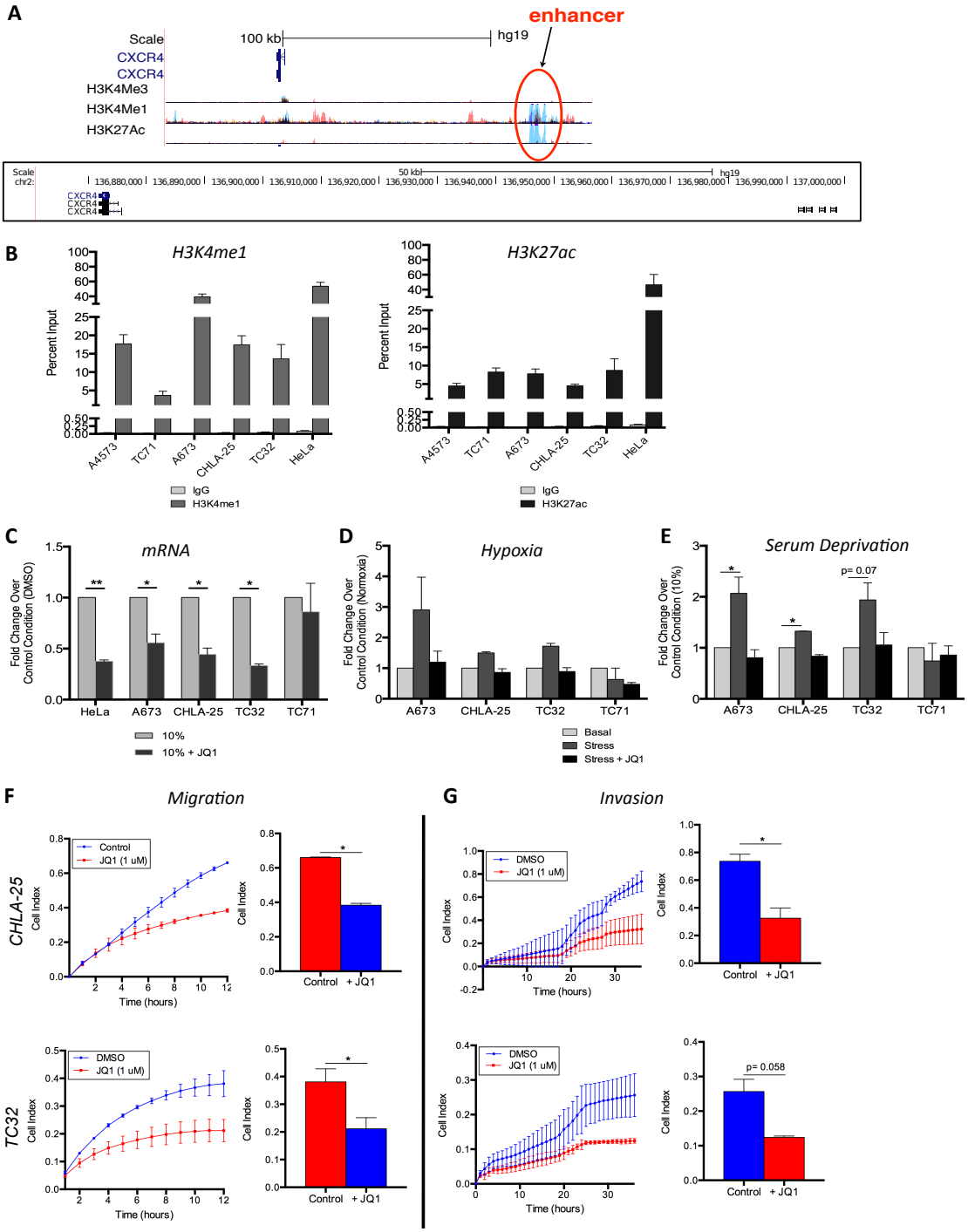

Figure 3.7. An enhancer exists upstream of *CXCR4* and contributes to CXCR4 regulation. An enhancer element exists upstream of the CXCR4 gene in HeLa cells (encode database) as characterized by the presence of the H3K4me1 and H3K27ac marks and may be an additional site of CXCR4 regulation (A). Primers were designed for the presumed *CXCR4* enhancer region (A). Chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) was used to assess the absence or presence of the H3K4me1 and H3K27ac marks at the presumed enhancer element in Ewing sarcoma cells and in HeLa cells (B). The enrichment of H3K4me1 and H3K27ac at this locus confirmed the presence of an enhancer element (B). In support of

enhancer function, exposure of Ewing sarcoma cells to the bromodomain inhibitor JQ1, which antagonizes enhancer activity, inhibited *CXCR4* transcription (C). Treatment of Ewing sarcoma cells with JQ1 prevented the upregulation of *CXCR4* in response to stress; hypoxia (D) and serum deprivation (E). Additionally, JQ1 inhibited *in vitro* chemotactic migration (F) and invasion (G) of CHLA-25 and TC32 cells to SDF-1α. Results shown as mean ± SEM (n=3).

DISCUSSION

In this chapter, we have demonstrated that stress-dependent induction of *CXCR4* in Ewing sarcoma is, in part, epigenetically regulated. In particular, the *CXCR4* promoter in at least some CXCR4^{negative} Ewing sarcoma cells resides in a bivalent state, which permits rapid and reversible transitions between CXCR4- and CXCR4+ cell states. Specifically, exposure of Ewing sarcoma cells to serum deprivation or hypoxia resulted in loss of bivalency in both bulk populations as well as FACS-sorted CXCR4- cells, as evidenced by loss of H3K27me3 with retention of H3K4me3 modifications. Loss of bivalency was accompanied by upregulation of *CXCR4*. In addition, we noted that an active enhancer exists upstream of the *CXCR4* locus and demonstrated that this enhancer also contributes to the epigenetic regulation of CXCR4 in Ewing sarcoma. In support of this, JQ1 treatment of Ewing sarcoma cells resulted in marked down-regulation of *CXCR4* expression in ambient conditions. In addition, JQ1 treatment blocked up-regulation of *CXCR4* in response to both serum deprivation and hypoxia. Our findings lead us to propose a new model of Ewing sarcoma cell heterogeneity in which microenvironmental cues lead to CXCR4- to CXCR4+ cell state transitions, transitions which are, in part, epigenetically regulated at the *CXCR4* locus (promoter and enhancer) (Figure 3.8).

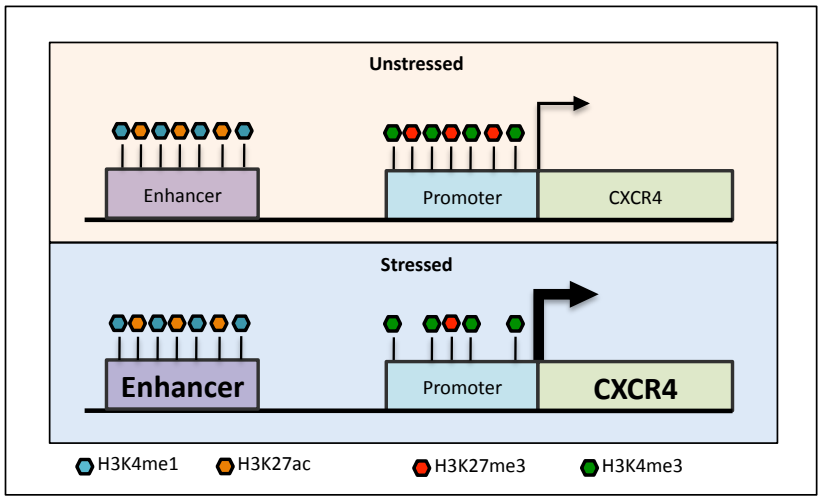

Figure 3.8. Model of CXCR4 regulation at the promoter and enhancer. Under standard culture conditions Ewing sarcoma cells maintain the *CXCR4* promoter in a bivalent state with the simultaneous presence of both the H3K27me3 and H3K4me3 histone modifications. Additionally, there is an enhancer upstream of CXCR4 that is characterized by the enrichment of the H3K4me1 and H3K27ac histone modifications. Under conditions of stress such as, serum deprivation and hypoxia, CXCR4 is converted from a poised, inactive state to a univalent, active state through the loss of the repressive, H3K27me3, mark. The loss of this mark results in the upregulation of *CXCR4* expression. The upregulation of CXCR4 creates a subpopulation of cells with increased metastatic potential. Additionally, the enhancer contributes to the regulation of CXCR4, though the precise mechanisms of this upregulation remain to be elucidated.

Thus far, studies of cancer biology and clinical trial development have failed to address the inherent heterogeneity and phenotypic plasticity of tumor cells (24). This is, in part, due to that fact that the underlying mechanisms mediating tumor heterogeneity and plasticity remain largely unknown (25). However, recent published findings, in addition to our current data, demonstrate that cancer cell plasticity is mediated epigenetically through the resolution of bivalent domains. In particular, Chaffer and colleagues identified that non-CSCs are plastic populations with the ability to convert to CSCs (26). They demonstrated that this plasticity was dependent on a master regulator of the epithelial-to-mesenchymal transition, Zeb1 (26). In basal $CD44^{lo}$ non-CSCs, Zeb1 is maintained in an inactive, poised (bivalent) state and in response to the

microenvironmental cue, TGFβ, the bivalent Zeb1 promoter resolves into an activate chromatin configuration resulting in basal $CD44^{hi}$ CSCs (26). Of importance, luminal $CD44^{lo}$ non-CSCs did not possess the ability to convert to $CD44^{hi}$ CSCs due to the findings that the Zeb1 promoter was maintained in a repressed, "off" state rather than a bivalent state (26). Another study recently assessed whether or not ovarian cancer cells were responsive to microenvironmental cues and if this response was epigenetically mediated (27). They demonstrated that culturing ovarian cancer cells in three-dimensional culture conditions as compared to two-dimensional culture conditions altered histone modifications and affected gene expression (27). These findings demonstrate that epigenetic mechanisms control tumor cell plasticity, whether in the context of Zeb1 transitions in breast cancer, multivalent marks in ovarian cancer cells, or CXCR4 transitions in Ewing sarcoma. Given these findings, it would be important to consider the use of adjuvant therapies targeted at preventing these conversions.

It has been well documented that in addition to the heterogeneous expression of CSC markers, the makeup of CSCs varies from patient to patient (28-30). In the context of Ewing sarcoma we have also demonstrated that CXCR4 expression is heterogeneous across cell lines and tumors (1). Furthermore, CXCR4 plasticity is not a universal property across Ewing sarcoma cell lines. Notably, our previous work demonstrated that not all Ewing sarcoma cells uniformly upregulate CXCR4 in response to serum deprivation (1). Some cells are more plastic than others: TC32 and CHLA-25 cells are highly plastic and

responsive while TC71 cells are relatively unresponsive. This was also evident in the pattern of histone modifications. TC32 cells rapidly converted from a bivalent inactive state to a univalent active state whereas TC71 cells were "stuck" in a repressed, inactive state even after exposure to serum-deprived conditions. These findings underscore to the complex nature of tumor heterogeneity as seen in the clinic.

Thus far, our preliminary studies have demonstrated that an enhancer exists upstream of *CXCR4* and contributes to the regulation of CXCR4. We have specifically demonstrated that the enhancer region upstream of *CXCR4* resides in an inactive/poised state under basal culture conditions characterized by the H3K4me1 and H3K27ac marks. We have some preliminary data to suggest that enrichment of activating marks is increased at the enhancer under conditions of stress; however, further studies are necessary to confirm these findings. Furthermore, to definitively demonstrate that JQ1 is functioning by disrupting the BET family of bromodomain containing proteins at the *CXCR4* enhancer mentioned above, ChIP-PCR experiments are needed to assess the binding of BET bromodomains at the *CXCR4* enhancer prior to and following JQ1 treatment. JQ1 has high specificity for the BET bromodomain family member, BRD4, and thus these experiments should be initially conducted to analyze BRD4 binding (31). These studies will help further define the precise contribution of the enhancer to CXCR4 regulation.

The mechanisms underlying gene expression are complex and vast. It is very possible that epigenetic regulation is not the only process mediating CXCR4

plasticity, and that the dynamic regulation of *CXCR4* is dependent on transcription factor binding in addition to changes in the chromatin state. To address this, designing a *CXCR4*-promoter luciferase reporter would allow us to assess the contribution of chromatin-independent transcriptional regulation of *CXCR4* in the context of stress. Site-directed mutagenesis of the exogenous *CXCR4* promoter construct would then be performed to systematically determine which regions in the promoter, and thereby which transcription factors, are responsible for the dynamic regulation of CXCR4.

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CHAPTER FOUR

DEVELOPING NOVEL MODELS TO STUDY CXCR4 IN EWING SARCOMA METASTASIS *IN VIVO*

OBJECTIVE STATEMENT

In this chapter I describe the development and testing of novel models designed to analyze the role of CXCR4 in Ewing sarcoma metastasis. In addition, these tools will allow further evaluation of the emergence of CXCR4 heterogeneity during Ewing sarcoma progression *in vivo*.

PART ONE

BACKGROUND AND RATIONALE

In Chapter Two, we demonstrated that CXCR4 promotes migration and invasion *in vitro* (1). While migration and invasion are both integral parts of the metastatic cascade, to definitively determine the contribution of CXCR4 to Ewing sarcoma metastasis, *in vivo* models are essential. Human-mouse xenograft models are considered the gold standard for studying metastasis. These models

consist of the transplantation of human cell lines or tumors into immunocompromised animals (mice or rats). There are two types of experimental approaches to study metastasis using these xenograft models: the experimental metastasis model and the spontaneous metastasis model (2).

The experimental metastasis model is the most widely used model to study metastasis and refers to the injection of cells directly into the circulatory system (2). These models test the ability of cells to arrest, extravasate and grow in various organs. Unfortunately, these models bypass the first few steps of the metastatic cascade as discussed in Chapter One. The most common experimental metastasis model is the tail vein injection model, in which cells are injected in the lateral tail vein of mice or rats. Other common injection sites include intrasplenic injections, portal vein injections, and intracardiac injections (3-5). In these models, tumor cell colonization is often limited to the lungs as it is the first capillary bed the tumor cells reach following injection. Thus far, the majority of Ewing sarcoma metastasis studies have used the tail vein injection model (6, 7).

The spontaneous metastasis model allows cells to disseminate from a local, primary tumor to a distant, secondary site. Traditionally, these models have consisted of tumor cell injections or tumor implantations into a subcutaneous (flank) site. These injection or implantation sites have been favored because it provides an environment for rapid primary tumor growth. These subcutaneous tumor implantations are often used for screening potential anti-neoplastic agents (8). While this model is good for studying primary tumor

growth, spontaneous metastases to distant sites are very rare (2), limiting its use as a model of metastasis. The preferred spontaneous metastasis model is the orthotopic spontaneous model, in which cancer cells are injected into an anatomical location that mimics the clinical presentation of a particular cancer. For example, the mammary fat pad is an orthotopic site for breast cancer and the adrenal gland is an orthotopic site for neuroblastoma (9, 10). These orthotopic models readily recapitulate human disease as demonstrated through histology, growth characteristics, angiogenesis, and metastatic behavior (2). While these orthotopic spontaneous models are the best at recapitulating the characteristics of clinical disease, they are technically challenging, time consuming, and can be costly to perform. To understand the contribution of CXCR4 to Ewing sarcoma metastasis, it is essential to use the appropriate model. This is critical as studying Ewing sarcoma metastasis has proven to be challenging due to the lack of a genetically engineered mouse model.

In Ewing sarcoma literature, all of the aforementioned models have been used to study different aspects of Ewing sarcoma biology as well as others. Recently, Mendoza-Naranjo et al. described the use of renal subcapsular implantation model to study Ewing sarcoma metastasis (11). They argued that the tissue underlying the renal capsule is highly vascularized, allowing for the rapid formation of macroscopic metastases as compared to subcutaneous or other orthotopic models. In their model, Ewing sarcoma cells were implanted into the exteriorized kidneys of anaesthetized live mice. While this model was effective for their study, it was an extremely invasive procedure. We were

fortunate to have the opportunity to collaborate with Dr. Erika Newman who has optimized an innovative and minimally invasive orthotopic xenograft model which utilizes ultrasound guidance to implant cells into the subrenal capsule. This model has been previously described wherein neuroblastoma cells are orthotopically injected using ultrasound guidance into the adrenal or para-adrenal space (12). We decided to modify the previously described model in order to be able to inject Ewing sarcoma cells into the subrenal capsule. Successful development of this model in our laboratory would allow us to efficiently study Ewing sarcoma metastasis. As this model was first reported using neuroblastoma cells, our initial optimization and protocol development was conducted using neuroblastoma cells.

MODEL DEVELOPMENT

The experimental procedures for the subrenal capsule injection model are detailed as follows: $1.0x10^6$ GFP/Luciferase (LUC) tagged human tumor cells (neuroblastoma/Ewing sarcoma) were resuspended in PBS and Matrigel™ (1:1) (BD Worldwide, 354234) at a concentration of $2.0x10^4$ cells per microliter and placed on ice. Nine week-old NOD SCID mice (Charles River Breeding Labs, 394) were anesthetized in an induction chamber using 2% isoflurane in O_2 delivered at 2 liters/minute. Once anesthetized, the dorsal hair of the mouse was removed with the commercial depiliating agent, Nair. The animal was then transferred to the imaging table ventral side down, where isoflurane was

delivered via a nose cone for inhalation at 1.5% in O_2 delivered at 2 liters/minute. The mouse was restrained and taped to insure taut skin and optical ointment was placed in the animal's eyes to prevent them from drying out. A chilled 22-gauge catheter (BD Worldwide, 381423) was gently inserted through the skin and back muscle into the subrenal capsule to provide a channel for the needle injection. After removal of the hub, a chilled Hamilton syringe fitted with a 27 gauge bore needle (BD Worldwide, 60476-220) loaded with 10µl of the cell suspension (2.0 x 10^5 total cells) was guided steretoactically through the catheter and positioned into the subrenal capsule visualized using ultrasound (Figure 4.1A). The cells were injected into the subcapsular space and the needle was left in for one minute allowing the Matrigel™ to set. The needle was slowly removed, followed by removal of the catheter. All ultrasound procedures were performed using the Vevo 2100 High-Resolution In Vivo Imaging System with the MS 550D transduced with a center frequency of 40 MHz and a bandwidth of 22-55 MHz (Cardiovascular Center Research Core Lab, University of Michigan). Bioluminescent imaging was performed on the Perkin Elmer *In Vivo* IVIS Spectrum Optical Imaging System (Center for Molecular Imaging Core, University of Michigan).

We confirmed that these injections could be successfully completed in our laboratory. Our initial studies were conducted using neuroblastoma cells, which were injected into the peri-adrenal space (Figure 4.1A). We also confirmed that we could successfully inject methlyene blue/Matrigel™ in the subrenal capsule, which allowed us to proceed with experimental studies (Figure 4.1B and C).

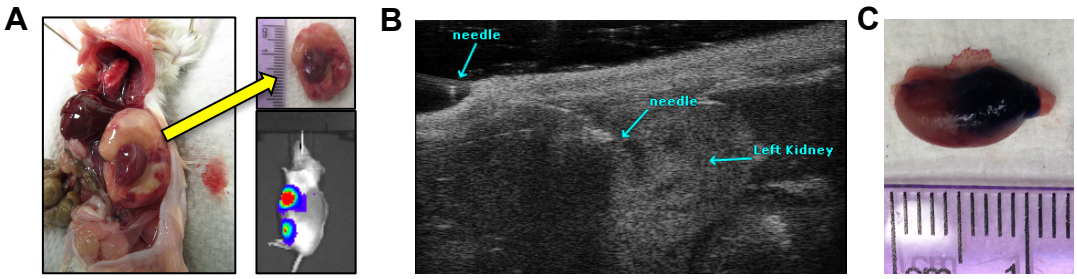


Figure 4.1. Subrenal capsule model of tumor progression *in vivo*. **A.** Guided by ultrasound, neuroblastoma cells were injected into the peri-adrenal space. A large peri-renal tumor is detected at necropsy and a metastatic lesion is detected by bioluminescent imaging. The subrenal capsule approach will be used for ES studies. **B.** Ultrasound image of percutaneous injection of the subrenal capsule. **C.** Image of excised kidney following injection of methylene blue/Matrigel™ into the subrenal capsule.

RESULTS

Ewing sarcoma *in* vivo metastasis models

Upon demonstrating that our laboratory can successfully inject "tumor cells" into the subrenal capsule, we wanted to determine if this innovative technique would provide a model to study Ewing sarcoma metastasis. Despite the aforementioned disadvantages of other xenograft models, we felt that it was necessary to compare the subrenal capsule model to other established Ewing sarcoma xenograft models. We decided to compare the following models: the subcutaneous injection model, the tail vein injection model, and the subrenal capsule injection model.

We tested a panel of Ewing sarcoma cell lines and compared their ability to metastasize in each of the models (detailed experimental procedures are outlined in the legend of Table 4.1). As expected with the subcutaneous injections, none of the cell lines metastasized (Table 4.1A). Interestingly, not all

of the cell lines developed tumors at the site of injection; CHLA-25 cells did not form any primary tumors (Table 4.1A). These findings suggest that tumorigenic heterogeneity exists across Ewing sarcoma cell lines. For the tail vein injection model, A673 and TC32 cells readily formed tumors while TC71 and CHLA-25 cells did not (Table 4.1B). This again highlights tumorigenic heterogeneity. For the subrenal capsule model, all of the cell lines we tested (A4573, A673, CHLA-25, and TC32) readily formed tumors at the site of injection (A4573 had two misinjections) (Table 4.1C). A4573 and TC32 were the only two cell lines to develop metastases. Metastases were evident in the lung with frequencies of 40% for TC32 cells and 33% for A4573 cells (Table 4.1).

A

Subcutaneous Injections		
	Primary Tumor	Metastases
Cell Line	*Number of mice/Total mice (%)*	
CHLA-9	10/10 (100%)	0/10 (0%)
A673	10/10 (100%)	0/10 (0%)
CHLA-25	0/10 (0%)	0/10 (0%)
TC32	10/10 (100%)	0/10 (0%)

B

Tail Vein Injections	
	Tumor Presence
Cell Line	*Number of mice/Total mice (%)*
TC71	0/3 (0%)
A673	3/3 (100%)
CHLA-25	0/3 (0%)
TC32	3/3 (100%)

C

Subrenal Capsule Injections		
	Primary Tumor	Lung Metastases
Cell Line	*Number of mice/Total mice (%)*	
A4573	3/5 (60%)	1/3 (33%)
A673	4/5 (80%)	0/5 (0%)
CHLA-25	5/5 (100%)	0/5 (0%)
TC32	5/5 (100%)	2/5 (40%)

Table 4.1. Summary of *in vivo* metastasis model pilot study. A. CHLA-9, A673 and TC32 cells readily formed tumors at the site of injection in the subcutaneous model. CHLA-25 cells did not form tumors. None of the cell lines exhibited metastases. **B.** A673 and TC32 formed tumors in the tail vein injection model whereas TC71 and CHLA-25 cells did not. **C.** A4573, A673, CHLA-25, and TC32 cells all formed primary tumors and only A4573 and TC32 cells metastasized from the subrenal capsule. The subcutaneous and tail vein injections were performed as previously described (13). In brief, for the subcutaneous model $1.0x10^6$ A673, CHLA-9, CHLA-25 or TC32 cells with injected with Matrigel™ (1:1 ratio) into the flank of 8 week-old NOD-SCID mice (10 mice per cell line). For the tail vein model, $1.0x10^6$ A673, CHLA-9, CHLA-25 or TC32 cells with injected into the lateral tail vein of 8 week-old NOD-SCID mice (3 mice per group).

From this pilot study, we have determined that the subrenal capsule model is the preferred model to study the contribution of CXCR4 to Ewing sarcoma

spontaneous metastasis. Furthermore, given that TC32 cells readily metastasize (40%) and have high expression levels of CXCR4, this cell line was chosen to study the contribution of CXCR4 to Ewing sarcoma metastasis.

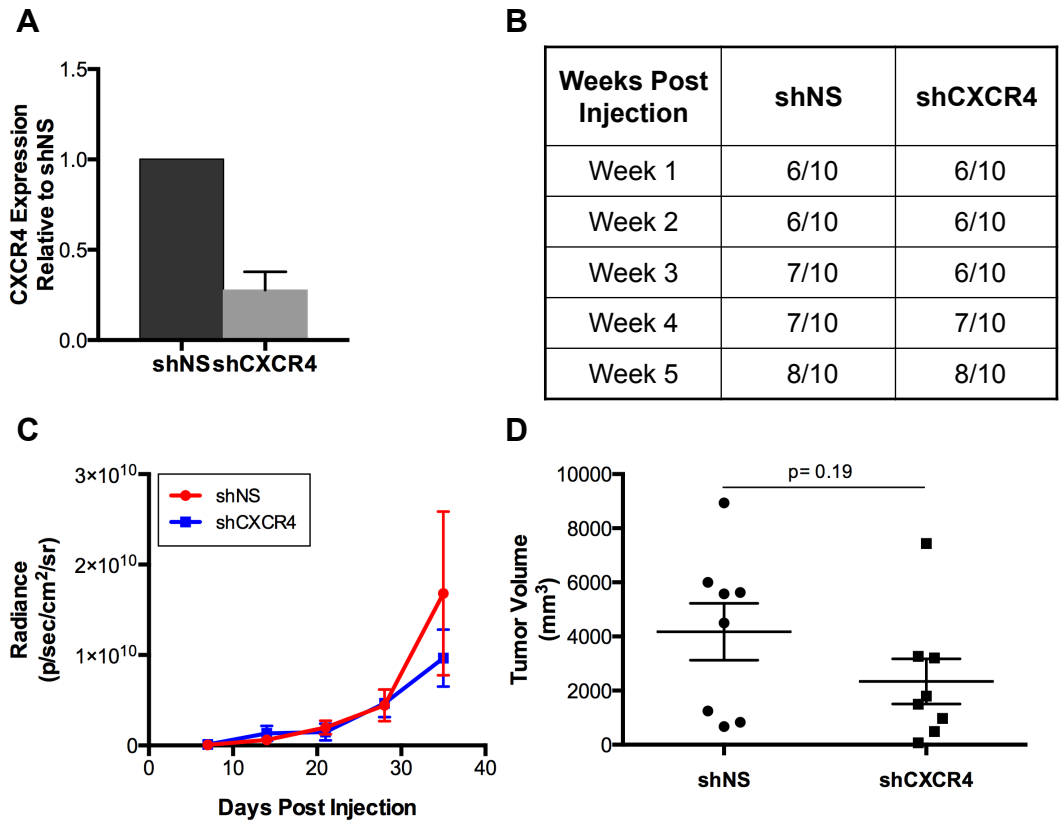
The innovative subrenal capsule model was used to determine the contribution of CXCR4 to Ewing sarcoma metastasis

To test the contribution of CXCR4 to the metastatic cascade *in vivo*, we used the aforementioned subrenal capsule xenograft model. We hypothesized that knockdown of CXCR4 in Ewing sarcoma cells would reduce the development of metastases. We used TC32 cells with stable knockdown of CXCR4 (shCXCR4) and TC32 cells with an inert non-silencing sequence (shNS) for the *in vivo* studies (both generated using previously described short hairpin RNA constructs) (1, 14). CXCR4 knockdown was confirmed in the cells prior to injection (Figure 4.2A). Ten mice were injected with $2.0x10^5$ GFP/luciferase (LUC) labeled TC32 shNS or TC32 shCXCR4 cells into the subrenal capsule of 8 week-old NOD-SCID mice. The TC32 shNS and TC32 shCXCR4 groups were compared with respect to time to engraftment, tumor size, and evidence of metastatic disease.

Weekly bioluminescence imaging demonstrated that both control and CXCR4 knockdown cells formed tumors with an 80% success rate (8 out of 10 mice per group developed tumors) (Figure 4.2B). Interestingly, there was little difference in total body bioluminescence signaling (p/sec/cm^2/sr) between the shNS and shCXCR4 tumors between weeks 1 and 4, post injection (Figure

4.2C). However, at 5 weeks post injection there appeared to be a trend towards the CXCR4 knockdown tumors decreasing size as determined by radiance (Figure 4.2C). These data suggest that CXCR4 may play a role in promoting Ewing sarcoma tumor growth at local sites.

At 6 weeks post injection, the primary tumors were excised, measured and *ex vivo* imaging was performed to determine the presence of any metastases. Upon mouse necropsy, it was confirmed that 2 mice per group had no evidence of primary tumor formation, which corroborated the bioluminescent findings (Figure 4.2B). Primary tumor volume (mm^3) was calculated using the formulation V = L x W x D where L is length, W is width and D is depth. There was marked variability in tumor volume in both groups (Figure 4.2D). Though not statistically significant ($p = 0.19$), there was a definite trend toward CXCR4 promoting tumor growth based on tumor volume (Figure 4.2D).



Weeks Post Injection	shNS	shCXCR4
Week 1	6/10	6/10
Week 2	6/10	6/10
Week 3	7/10	6/10
Week 4	7/10	7/10
Week 5	8/10	8/10

Figure 4.2. CXCR4 may contribute to primary tumor growth. **A.** CXCR4 knockdown was confirmed in GFP/LUC TC32 shCXCR4 cells as compared to the GFP/LUC TC32 shNS. **B.** An 80% injection success rate as observed in both shNS and shCXCR4 groups defined as $>10^6$ p/sec/cm^2/sr. **C.** Bioluminescent imaging (radiance) showed a trend towards CXCR4 knockdown inhibiting tumor growth. **D.** Tumor volume supports bioluminescent findings with tumor volumes trending towards smaller in the CXCR4 knockdown group.

For the *ex vivo* imaging, each mouse was injected with luciferin, and subsequently euthanized five minutes post injection. The primary tumor was then removed and the mouse carcass was imaged to determine if metastases were present. A single bioluminescent image was captured and whole body ROIs (region of interest) were determined based on the single capture per mouse. There was no difference in total body radiance between the shNS and shCXCR4 groups (Figure 4.3A). These findings were inconclusive due to the

residual blood that remained and generated a signal in the mouse cavity after primary tumor resection (Figure 4.3B).

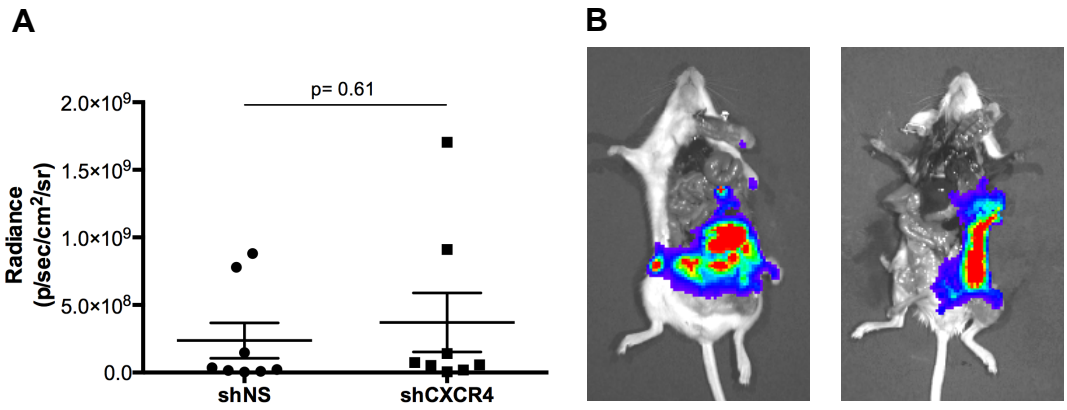
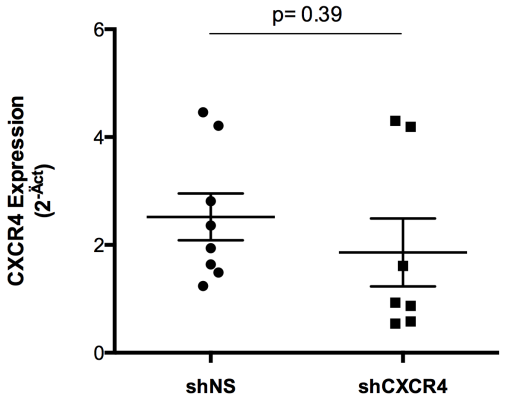


Figure 4.3. *Ex vivo* imaging demonstrated no difference in metastatic formation. **A.** There was no difference in *ex vivo* radiance after primary tumor resection. **B.** Residual blood spillage into the mouse cavity was evident on bioluminescent imaging following primary tumor resection.

qRT-PCR was performed to confirm that the tumors maintained CXCR4 knockdown in the TC32 shCXCR4 group. Surprisingly, there was no statistical difference (p=0.39) in *CXCR4* expression in the non-silencing tumors and the CXCR4 knockdown tumors. (Figure 4.4A). These findings suggest that there was a selective advantage *in vivo* in favor of CXCR4 expressing cells. Despite the outgrowth of CXCR4+ cells, the two largest tumors had the highest *CXCR4* expression (yellow), and the five smallest tumors had the lowest *CXCR4* expression (red) (Figure 4.4B). When plotting *CXCR4* expression against tumor volume (15 tumors; 8 shNS and 7 shCXCR4), there is a moderate correlation (r = 0.53) between tumor volume and *CXCR4* expression with the largest tumors having the highest *CXCR4* expression and the smallest tumors having the lowest

CXCR4 expression (Figure 4.4C-D). These data further support our radiance and volume findings that CXCR4 may be contributing to tumor growth *in vivo*.

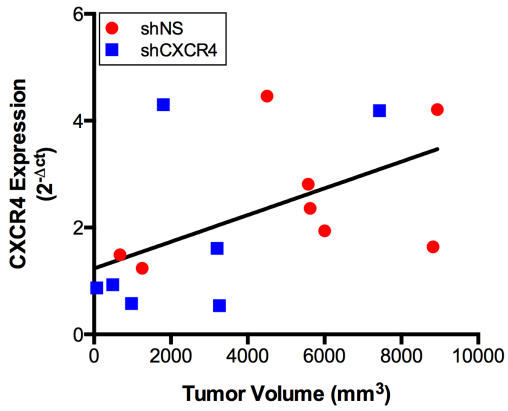
A



B

TC32 shNS		TC32 shCXCR4	
Tumor Volume (mm^3)	CXCR4 Expression ($2^{-\Delta ct}$)	Tumor Volume (mm^3)	CXCR4 Expression ($2^{-\Delta ct}$)
827	1.64	7433	4.19
1251	1.24	3263	0.54
5577	2.81	68	0.87
8935	4.21	3205	1.61
6000	1.94	975	0.58
5625	2.36	1800	4.30
4500	4.46	1500	N/A
675	1.49	488	0.93

C



D

Tumor Volume vs. CXCR4 Expression	
Pearson r	
r	0.5313
95% confidence interval	0.02612 to 0.8203
R squared	0.2823
P value	
P (two-tailed)	0.0416
P value summary	*
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	15

Figure 4.4. Tumor volume correlates with *CXCR4* expression. **A.** There was no difference in *CXCR4* expression between the shNS and shCXCR4 tumor groups. **B.** The tumors with the largest volume expressed high levels of *CXCR4* (yellow) and the tumors with the lowest volume expressed the lowest levels of *CXCR4* (red). **C.** A moderate positive correlation exists between tumor volume and *CXCR4* expression. **D.** The correlation coefficient values from 4.4C plot are reported.

DISCUSSION

Out of the three models tested, we determined that the subrenal capsule mouse xenograft model was the best *in vivo* platform to study metastasis. This

model allows us to study the metastatic cascade in its entirety. However, the kidney is not a usual site for Ewing sarcoma development. The most common primary sites for Ewing sarcoma are the long bones including the femur (20%), the humerus (6.0%) the tibia (10%) and the fibula (8.0%) (15). While there have been documented cases of Ewing sarcoma cases in the kidney, they are very rare (16). To further facilitate a clinically relevant site, intra-femoral or intra-tibial metastasis models have been widely used as the bone microenvironment plays a key in the Ewing sarcoma pathogenesis (17). While we believe that the subrenal capsule model is an appropriate model to study Ewing sarcoma metastasis, current studies are being implemented in the laboratory to optimize the intra-tibial model.

Using the subrenal capsule model to test the contribution of CXCR4 to Ewing sarcoma metastasis, our preliminary studies suggest that CXCR4 may contribute to Ewing sarcoma primary tumor growth *in vivo*. Our data is further supported by findings from D Berghuis, et al., which demonstrated that CXCL12 stimulation of $CXCR4^{hi}$ Ewing sarcoma cells promotes proliferation *in vitro* (18). Furthermore, they argued that CXCR4 does not promote metastasis in Ewing sarcoma. While not conclusive, due to blood spillage into the mouse cavity, we saw no difference in metastatic formation between shNS and shCXCR4 in our studies. However, our findings were complicated by the fact that there was a selective pressure against $CXCR4^{lo}$ cells *in* vivo. We demonstrated that the tumors lost CXCR4 knockdown as the shCXCR4 tumors had comparable *CXCR4* expression levels to the shNS tumors. To definitively determine the role

of CXCR4 in Ewing sarcoma tumorigenesis further studies are necessary to eliminate the outgrowth of CXCR4 expressing cells in the knockdown population.

PART TWO

BACKGROUND AND RATIONALE

Stable RNA interference (RNAi) is a powerful scientific tool to evaluate protein function. However, as demonstrated above, it is limited by incomplete loss-of-function of the targeted gene as well as off-target effects that can result from random integration of the shRNA sequence into the genome. Recently, new genome-editing technologies, including TALENS and CRISPR/Cas9 have been developed that allow for direct-targeted disruption and modification of endogenous genes. The primary advantages of using the CRISPR/Cas9 gene targeting approach as are as follows: (a) simple and cost-effective means to generate cell lines in comparison to conventional knockout approaches; (b) specifically targeted small mutations can be introduced; (c) no integration of vector DNA occurs; and (d) no selection is needed (19). We sought to generate a CXCR4 knockout cell line using the CRISPR/Cas9 system. These cell lines will be essential tools that can be used in future *in vivo* studies to eliminate the outgrowth of $CXCR4^{high}$ cells as demonstrated above. This will allow us to truly define the contribution of CXCR4 to Ewing sarcoma pathogenesis.

We have recently shown that expression of CXCR4 is dynamic and that Ewing sarcoma cells transition between a relatively non-motile CXCR4- state to a more migratory CXCR4+ state in response to microenvironmental cues, including hypoxia, growth factor deprivation, and growth constraints (1). These cell state transitions have been demonstrated *in vitro* in a pooled population of cells but have yet to be characterized *in vivo* and on a single cell level. To monitor these transitions on a single cell level we sought to generate a CXCR4-GFP knock-in reporter cell line (experimental details outlined below).

RESULTS

Generating CXCR4 knockout cells

In collaboration with Clontech, four different sgRNAS were developed to target CXCR4 (Table 4.2). The sgRNAs were designed to target both isoforms of CXCR4 using http://crispr.mit.edu/ and http://chopchop.rc.fas.harvard.edu/.

Control Oligos	
Sense	*Antisense*
CCGGTGACATCAATTATTATACAT	AAACATGTATAATAATTGATGTCA
Oligo #1	
Sense	*Antisense*
CCGGGTACAGGCTGCACCTGTCAG	AAACCTGACAGGTGCAGCCTGTAC
Oligo #2	
Sense	*Antisense*
CCGGGGGCAATGGATTGGTCATCC	AAACGGATGACCAATCCATTGCCC
Oligo #3	
Sense	*Antisense*
CCGGAGCATTTTCTTCACGGAAAC	AAACTCGTAAAAGAAGTGCCTTTG
Oligo #4	
Sense	*Antisense*
CCGGCACTTCAGATAACTACACCG	AAACCGGTGTAGTTATCTGTTGTG

Table 4.2. CXCR4 targeting sgRNA sequences. Table of oligo sequences designed to target CXCR4 for knockout studies. Red letters for each oligo are the overhang sequence. The antisense oligo is complementary to the sense oligo.

The sgRNA cleavage efficiency was tested using the Guide-it Mutation Detection Kit and determined that sgRNAs #1, #2, and #4 were effective in the cleavage of the target sequence where sgRNA #3 demonstrated ineffective cleavage (Clontech, 631438) (Figure 4.5A). The sgRNAs were then successfully cloned into the all in one Guide-it™ CRISPR/Cas9 System (Red) per the manufacturer's instructions (Clontech, 632602). Confirmation of cleavage was determined by using the Guide-it sgRNA *In Vitro* Transcription Kit. Plasmids were transfected using the Xfect™ Transfection Reagent (Clontech, 631318). Cells were then sorted on the basis of tdTomato as previously described (1).

To test the constructs, HeLa cells were used as a sample cell line due to the high expression of CXCR4 with nearly 100% of the cells expressing CXCR4

on their cell surface (20). We have successfully generated CXCR4 knockout HeLa cells (Figure 4.5B). Generation of CXCR4 knockout clones will be continued in two Ewing sarcoma cell lines, CHLA-25 and TC32. We chose these cell lines as they express high levels of CXCR4 at baseline. Upon successful generation of the knockout cell lines, functional studies will be used to assess for an inhibition of CXCR4-mediated *in vitro* migration and invasion. These cells will be used in *in vivo* future metastasis studies (See subrenal capsule injection model) to determine the contribution of CXCR4 to Ewing sarcoma metastasis by eliminating the potential of $CXCR4^{high}$ cell outgrowth.

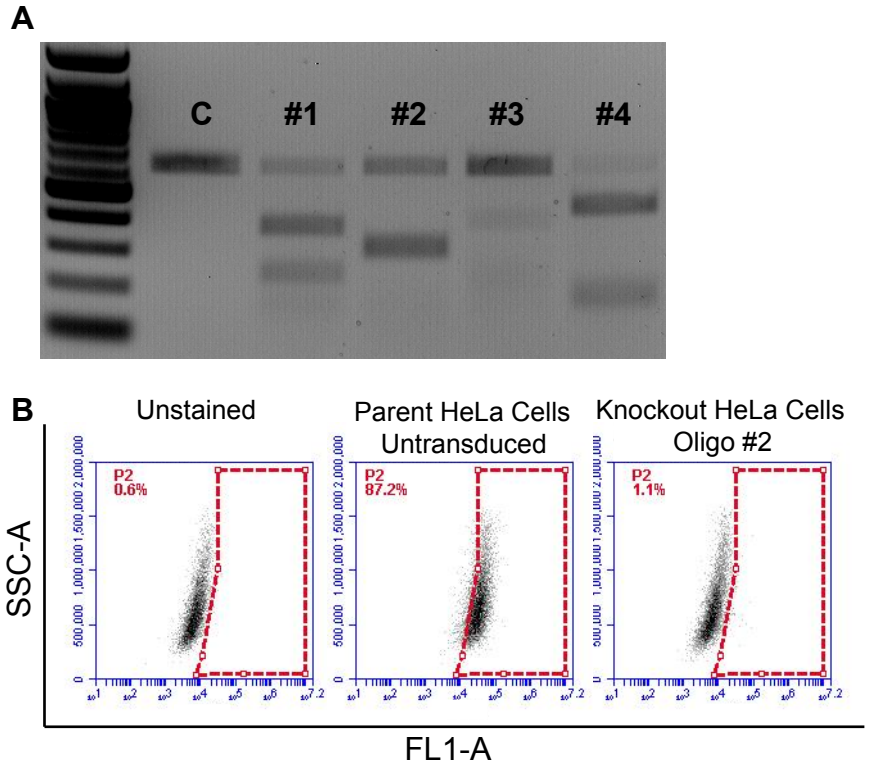


Figure 4.5. CXCR4 knockout cells. **A.** The cleavage efficiency of 4 sgRNAs was determined. Oligo #3 is the least effective at cleavage of the target sequence. **B.** HeLa knockout CXCR4 cells were successfully generated using oligos #2.

Generating CXCR4 knock-in reporter cells

In collaboration with Sigma, we are generating a CXCR4 knock-in cell line in which GFP is fused in-frame with CXCR4. Two sgRNAs were designed to target the CXCR4 stop codon (Figure 4.6A). Ongoing studies are being conducted to validate if the CRISPRs are cleaving at the appropriate site and how efficient they are at cutting using the Guide-it™ Mutation Detection Kit (Figure 4.6B). Upon validation of the sgRNAs, a donor plasmid with homologous arms will be designed to carry in GFP into the cut site (Figure 4.6C). Clones will be screened for GFP expression and then sequence validated (Figure 4.6D). These cells will be used for future *in vitro* and *in vivo* studies to visualize changes in CXCR4 expression on a single cell basis.

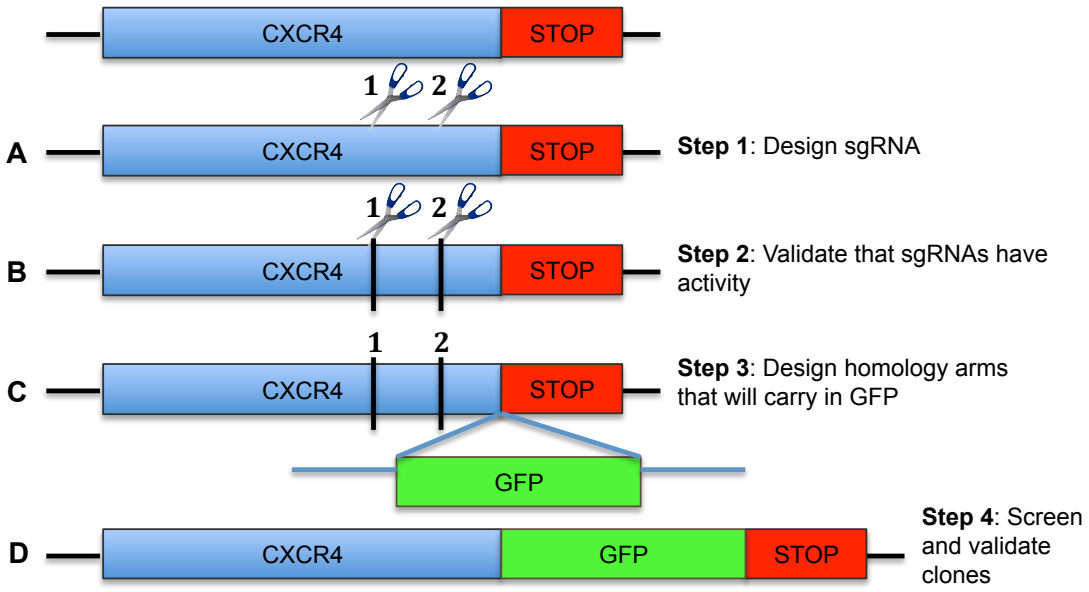


Figure 4.6. Schematic of CXCR4-GFP knock-in reporter cell line work flow. **A.** sgRNAs are designed to cut within 300 bp of the CXCR4 stop codon. **B.** The activity of the sgRNAs is validated using the Guide-it™ Mutation Detection Kit. **C.** Upon validation that the sgRNAs cut effectively, homology arms are designed that will carry in the GFP to the cut site. **D.** Clones will be screened for GFP expression and validated by sequencing to generate CXCR4-GFP knock-in Ewing sarcoma cell lines.

DISCUSSION

To overcome the challenges uncovered in the *in vivo* metastatic Ewing sarcoma study, we sought to generate CXCR4 knockout cells. These cells will allow us to establish the contribution of CXCR4 to the metastatic cascade in a more precise manner. In Chapters Two and Three, we demonstrated that Ewing sarcoma cells upregulated CXCR4 in response to stress. However, this upregulation of CXCR4 was not uniform across all Ewing sarcoma cell lines. Some cell lines robustly upregulated CXCR4 when exposed to serum deprived conditions, while others did not upregulate CXCR4 at all. How can we determine which cells will respond and which cells will not respond? We are generating a CXCR4-GFP knock-in construct using CRISPR/Cas9 in which GFP will be fused in frame to CXCR4. This construct will allow us to use GFP as a marker for the changes in CXCR4 expression to monitor the cell state transitions that occur with respect to CXCR4 on a single cell level. The development of these novel techniques will allow us to determine if CXCR4+ Ewing sarcoma cells are key contributors to Ewing sarcoma metastasis. We will also be able to examine the dynamic nature of CXCR4 regulation and its contribution to tumor heterogeneity.

SUMMARY STATEMENT

In summary, this chapter outlined the development and testing of a novel *in vivo* model to assess the contribution of CXCR4 in Ewing sarcoma metastasis. Furthermore, we are in the process of developing and implementing tools that will

allow us to further study CXCR4 heterogeneity during Ewing sarcoma progression *in vivo*.

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CHAPTER FIVE

CONCLUSIONS

INTRODUCTION

Ewing sarcoma is the second most common bone malignancy in children and adolescents (1). Although the overall survival for Ewing sarcoma patients has improved over the last several decades, metastasis remains the primary cause of death (2). However, as metastasis is a complex, multistep process influenced by hundreds of factors, many gaps still exist in understanding the molecular mechanisms driving Ewing sarcoma tumor metastasis. Through the work of this thesis, we sought to investigate the role of the CXCR4/CXCL12 chemokine axis as a mediator of Ewing sarcoma metastasis.

Specifically, we have discovered that CXCR4 promotes Ewing sarcoma migration and invasion *in vitro* (Chapter Two). Furthermore, we determined that CXCR4 is heterogeneously expressed in Ewing sarcoma cell lines and tumors and that its expression is highly dynamic (Chapter Two). We demonstrated that CXCR4 is reversibly induced in response to microenvironmental stresses, such as serum deprivation, hypoxia, and space constraints, promoting the dynamic

transition of cells between nonmigratory/noninvasive (CXCR4-) and migratory/invasive states (CXCR4+) (Chapter Two). We next sought to determine the precise molecular mechanisms underlying the observed dynamic regulation of CXCR4 expression.

We focused our efforts on defining the epigenetic contribution to CXCR4 plasticity. We identified that the CXCR4 promoter exists in a bivalent state, which permits rapid and reversible transitions between CXCR4- and CXCR4+ cell states in response to microenvironmental cues (Chapter Three). Furthermore, we discovered that an enhancer also contributes to CXCR4 regulation (Chapter Three). In support of this, the enhancer antagonist, JQ1, down-regulated *CXCR4* expression in ambient condition as well as blocked up-regulation of *CXCR4* in response to both serum-deprivation and hypoxia (Chapter Three). Additionally, JQ1 blocked CXCR4-mediated Ewing sarcoma migration and invasion (Chapter Three). In conclusion, we demonstrate an epigenetic mechanism for the regulation of CXCR4 plasticity. Taken together, these findings suggest a potential avenue for the development of new therapeutic strategies to block CXCR4 signaling in Ewing sarcoma.

Additionally, we have tested and developed *in vivo* models to study Ewing sarcoma metastasis. We used an innovative orthotopic xenograft model to test the contribution of CXCR4 to Ewing sarcoma metastasis. Furthermore, we are also in the process of developing new genetic tools and reporters that will be used for future experiments to further define the contribution of CXCR4 to Ewing sarcoma metastasis.

To our knowledge, this work is the first to provide evidence that CXCR4 contributes to Ewing sarcoma *in vitro* migration and invasion as well as preliminary evidence that CXCR4 contributes to Ewing sarcoma tumor growth *in vivo*. Additionally, we provide an epigenetic mechanism responsible for regulating the dynamic expression of the chemokine receptor, CXCR4 in Ewing sarcoma. Overall, this thesis is significant because it provides evidence that the CXCR4/CXCL12 signaling is important in Ewing sarcoma pathogenesis. Additionally, this thesis provides an epigenetic mechanism that can be therapeutically exploited to eliminate CXCR4- to CXCR4+ cell state transitions.

FUTURE DIRECTIONS

Is CXCR4 plasticity a characteristic of cancer stem cells (CSCs)?

Tumor heterogeneity remains a major therapeutic challenge, however, the mechanisms underlying how tumor heterogeneity arises are poorly understood (3). Cancer stem cells (CSCs) have been implicated as key contributors to tumor heterogeneity (4). Within a tumor, CSCs are a rare subpopulation of cells that are pluripotent, have the ability to self-renew, and possess tumor-initiating capabilities; thus generating heterogeneous tumors that consist of both CSCs and non-CSCs (cancer stem cell hypothesis) (5). Additionally, there are phenotypic differences between CSCs and non-CSCs including varied rates of proliferation, differential migratory and invasive behavior, and altered metastatic potential (4). Unsurprisingly, the percentage of CSCs within any given tumor can

drastically vary. CSCs have been identified in many tumor types including leukemia, breast, brain, liver, pancreatic, and colon cancer (6-10). Epigenetic plasticity is a hallmark of stem cells mediated through a specific epigenetic feature—a bivalent chromatin state (11). As mentioned in Chapter One, bivalent chromatin is defined as the simultaneous presence of the active, H3K4me3, and repressive, H3K27me3, histone modifications at gene promoters (12, 13). Bivalent domains generally silence genes while keeping them poised and ready for activation. In response to developmental or microenvironmental cues, these domains can then resolve into active states with a predominant H3K4me3 mark, or resolve into a repressed states with a predominant H3K27me3 mark (13).

In Chapter Three, we demonstrated that a bivalent CXCR4 promoter promotes transitions between CXCR4- and CXCR4+ cell states. These studies were conducted in Ewing sarcoma cells lines, which are thought to innately behave like stem cells. In addition to the involvement of the CXCR4/CXCL12 signaling axis in normal stem cell homing, there is evidence in the literature to suggest that CXCR4 may be associated with cancer stem cells (14). For example, CXCR4 surface protein levels have been shown to increase in CD133+ human glial-derived CSCs that have been exposed to hypoxic stress (15). In pancreatic cancer, populations of CD133+/CXCR4+ CSCs are found on the invasive edge of a tumor, which in turn promotes migration (15, 16). Additionally, a study conducted in our laboratory by Dr. Chris Scannell, demonstrated that *CXCR4* transcript was enriched in slowly proliferating (PKH-dye-retaining) Ewing sarcoma cells compared to more rapidly dividing counterparts. These findings

highlight the fact that CXCR4 may be enriched in a subpopulation of CSCs that possess increased metastatic potential. Therefore we hypothesize the epigenetic plasticity of CXCR4 is a characteristic of cancer stem cells. To address this hypothesis future studies could be done using breast cancer cell lines (e.g. MCF-7 and MDA-MB-231) and glioblastoma cell lines (e.g. U138MG) (17-19). These cell lines were chosen because CSCs have been identified in both breast cancer and glioblastoma (20, 21). Additionally, there are well-established protocols for the identification and viable isolation of the CSC population from these tumor types (22).

To test our hypothesis, we would initially subject these cell lines (bulk populations) to serum deprived and hypoxic conditions and assess any changes in *CXCR4* expression by qRT-PCR. Preliminary studies have demonstrated that some breast cancer cells upregulate *CXCR4* expression after serum deprivation for 24 hours (SUM159 and MCF7, data not shown). Based on our findings from Chapter Three, we hypothesize that the upregulation of *CXCR4* seen in breast cancer cells is epigenetically mediated by the loss of the H3K27me3 mark at the CXCR4 promoter. To test this hypothesis, Chromatin immunoprecipitation (ChIP) experiments would be used to examine changes in the enrichment of the H3K4me3 and H3K27me3 histone modifications at the CXCR4 promoter prior to and after exposure to serum deprived culture conditions. These experiments could be repeated in glioblastoma cells as well as extended to conditions of hypoxic stress. As we demonstrated in Chapter One and Chapter, not all cells within the population upregulate CXCR4. In order to determine if the CSCs are

the cells within a population that are plastic and responsive to stress, these experiments should also be conducted in sorted populations (non-CSCs vs. putative CSCs and CXCR4- vs. CXCR4+). Furthermore, in Chapter Two, we demonstrated that the upregulation of CXCR4 in response to microenvironmental stress increased the migratory and invasive potential of Ewing sarcoma cells in a CXCR4-dependent manner. It is important to test the functional implication of *CXCR4* upregulation in other tumor types such as proliferation, migration, and invasion. Should our hypotheses be correct, addressing the epigenetic plasticity of CXCR4 and its impact on CSC biology highlights the importance of developing therapeutic interventions targeting CXCR4 activation.

Is CXCR4 responsive to other stresses?

In Chapters Two and Three, we demonstrated that CXCR4 is upregulated in Ewing sarcoma in response to serum deprivation, hypoxia, and growth constraints. While these stresses are relevant, they are only a fraction of the conditions tumor cells face in their *in vivo* microenvironment. In addition to growth factor and nutrient deprivation, Ewing sarcoma tumors are bombarded with cytotoxic agents and radiation as part of standard treatment protocols (23-27). Despite great successes in treating cancer with chemotherapy and radiation, the challenges have been identifying the population of tumor cells that will survive, spread, and repopulate after initial therapy (28). CXCR4+ cells have been implicated as populations of cells that survive therapy and contribute to tumor relapse. In pediatric acute myeloid leukemia (AML) cell lines, CXCR4

expression was increased after treatment with conventional chemotherapy therapy (29). In addition, gemcitabine induced CXCR4 expression in pancreatic cancer, promoting tumor cell invasion (30). Lastly, non-small cell lung cancer cells that survived ionizing radiation treatment displayed an increase in CXCR4 expression (31). Collectively, these data suggest that the upregulation of CXCR4 after treatment with chemotherapy or radiation may be a mechanism of therapeutic resistance.

For Ewing sarcoma patients, up to a third of patients who present with localized disease will relapse at distant sites following an initial clinical remission. Are the cells that survive initial treatment and contribute to disease relapse CXCR4+ in Ewing sarcoma? We hypothesize that chemotherapy-induced upregulation of CXCR4 Ewing sarcoma cells is a mechanism of therapeutic resistance. To test this hypothesis we would subject Ewing sarcoma cell lines to chemotherapeutic agents that are commonly used in the treatment of Ewing sarcoma patients. The current protocol for Ewing sarcoma includes vincristine, doxorubicin, and cyclophosphamide (VDC), alternating with ifosfamide and etoposide (IE) (26, 32). We would test a variety of drug combinations and dosing regiments in a panel of Ewing sarcoma cell lines to assess any changes in that may occur in CXCR4 expression. Additionally, Ewing sarcoma cells could be subjected to varying doses of gamma irradiation to determine its effect on CXCR4 expression. Surface CXCR4 and mRNA expression would be determined by flow cytometry and qRT-PCR, respectively.

If our hypotheses stand correct, and CXCR4 is upregulated in response to chemotherapy, we would want to assess the mechanisms underlying this upregulation. Given our focus in Chapter Three on the epigenetic regulation of CXCR4 plasticity, we hypothesize that the upregulation of CXCR4 as a means of therapeutic resistance is epigenetically mediated. To test our hypothesis, we will perform ChIP experiments as described in Chapter Three, to compare changes in histone modifications at the CXCR4 locus in chemotherapy naïve cells and chemotherapy resistant cells. Additionally, since CXCR4 overexpression has been shown to promote migration, invasion, and metastatic potential of many tumor types, we would assess whether cells that have upregulated CXCR4 and survived chemotherapy have increased migration and invasion potential (33-38). These experiments would be conducted using the xCELLigence system as described in Chapters Two and Three. We predict that the cells treated with chemotherapy will upregulate CXCR4, which in turn will promote Ewing sarcoma cell migration and invasion. Furthermore, we predict that the upregulation of CXCR4 is, in part, epigenetically regulated through a bivalent promoter. We plan to extend these studies to other tumor types like breast cancer and glioblastoma.

Can novel approaches be used to target CXCR4 for cancer therapy?

Targeting the CXCR4/CXCL12 signaling axis is highly appealing and has generated a great deal of interest due to the vast roles it plays in cancer progression, in a multitude of tumor types (39, 40). The first CXCR4 inhibitor to enter clinical trials was AMD3100, which was originally developed for the

treatment of HIV. In 2008, AMD3100 was FDA approved in combination with G-CSF for autologous HSC mobilization for patients with non-Hodgkin lymphoma and multiple myeloma (41). CTCE-9908, a small peptide antagonist for CXCR4 was approved in 2005 for orphan drug status for the treatment of osteosarcoma (42). In addition to these two compounds, there are many clinical trials underway with CXCR4 pathway inhibitors (43-45). Despite these efforts to generate CXCR4 targeted therapies, very few drugs have been successfully implemented in the clinic.

Our findings in Chapter Three suggest a new, potentially promising approach to targeted CXCR4 inhibition. We have demonstrated a critical role for epigenetically determined cell plasticity in Ewing sarcoma. In Chapter Three, we demonstrated that the treatment of Ewing sarcoma with subcytotoxic doses of the enhancer antagonist, JQ1, blocked CXCR4- to CXCR4+ cell state transitions as well as inhibited CXCR4-mediated migration and invasion. These findings open the door to novel therapeutic approaches that exploit the potential of epigenetic modifiers as adjunct therapies that antagonize plasticity and thereby minimize tumor cell heterogeneity.

Given that cancer cells are exquisitely dependent on epigenetic pathways, the use of epigenetic modifiers, as pharmacologic agents is of great interest. Currently, the DNMT inhibitors, 5-azacitidine and decitabine, are approved for the treatment of myelodysplastic syndrome (MDS). Additionally, the HDAC inhibitors, vorinostat and romidepsin are approved for cutaneous T-cell lymphoma (CTCL) (46). Unfortunately, there are no epigenetic modifiers that are

currently approved for pediatric cancers, however, the approval of the above inhibitors provides hope that after the necessary preclinical data, the use of epigenetic modifiers may be clinical approved for the treatment of pediatric cancers.

A key challenge for future epigenetic therapies will be determining the appropriate time of administration in the clinic. Current studies suggest that epigenetic modifiers are most effective in combination with other anti-cancer strategies (47). Additionally, it will be of great importance to verify that these global epigenetic modifiers have no deleterious effects. In Chapter Three, we used the EZH2 inhibitor, GSK-126, to confirm that the loss of H3K27me3 enrichment we were seeing in response to serum deprivation and hypoxia was mediated directly through EZH2. Treatment with subcytotoxic doses of GSK-126 resulted in a marked upregulation of CXCR4 and was accompanied by a dramatic decrease in the enrichment of H3K27me3 at the CXCR4 promoter. Given the critical role of CXCR4 in promoting proliferation, migration, and invasion, the upregulation of *CXCR4* is not desired outcome. These data provide a cautionary tale to the use of epigenetic modifiers, which may activate or repress unintended targets. Despite our findings, pharmacological inhibition of EZH2 has been developed as a therapeutic strategy for the treatment of lymphomas with EZH2 mutations (48).

SUMMARY

In summary, the work conducted in this thesis demonstrates a role for CXCR4 in Ewing sarcoma pathogenesis. We show that CXCR4 is heterogeneously expressed in Ewing sarcoma cell lines and tumors ranging from absent to high-level expression. Interestingly, we noted that in Ewing sarcoma cells, CXCR4 expression is highly plastic and dynamic in response to changes in the microenvironment, which leads to the emergence of a population of cells that possess increased migratory and invasive potential. In addition, we demonstrated that the dynamic regulation of CXCR4 is governed, at least in part, by epigenetic plasticity at the CXCR4 locus. Specifically, the CXCR4 promoter resides in a bivalent state and in response to changes in the microenvironment is converted from a bivalent repressed state to a univalent, active state, which is mediated by the loss of the repressive histone modification, H3K27me3. Furthermore, an enhancer upstream of CXCR4 also contributes to its regulation. In support of this, we demonstrated that JQ1, an inhibitor of enhancer function, antagonizes CXCR4 state transitions as well as inhibits CXCR4-mediated Ewing sarcoma cell migration and invasion. Together, these studies reaffirm the importance of the CXCR4/CXCL12 signaling axis in cancer and the potential clinical implications of targeting this axis for the treatment and prevention of cancer progression.

This thesis has provided a foundation for understanding the role of CXCR4 in Ewing sarcoma metastasis. Of significance, this thesis contributes to the growing literature demonstrating the role of epigenetic mechanisms regulating tumor heterogeneity and cancer cell plasticity.

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