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Contribution Of Cx3cr1/fractalkine (cx3cl1) Axis To The Progression Of Non-Small Cell Lung Cancer (nsclc)

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CONTRIBUTION OF CX3CR1/FRACTALKINE (CX3CL1) AXIS TO THE PROGRESSION OF NON-SMALL CELL LUNG CANCER (NSCLC)

by

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THESIS

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Approved by:

______________________________________
Advisor

Date
DEDICATION

I would like to dedicate this to my parents, Gene and Viola Prince, who were always supportive of whatever endeavor I choose to undertake. Their encouragement enabled me to persevere through whatever challenges I faced.
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INTRODUCTION
Cancer

Cancer is the second leading cause of death in the United States. A total of 1,685,210 new cancer cases and 595,690 cancer deaths are projected to occur in 2016 (Siegel et al., 2016). Usurped only by heart disease, cancer poses a significant adversary to clinicians as well as researchers. Cancer incidence is driven by four types: lung and bronchus, prostate, breast and colorectal. Over the past 3 decades, the 5 year relative survival rate for all cancers combined has increased 23 percentage points (Siegel et al., 2016). In contrast to the steady increase in survival for most cancers, advances have been slow for lung and pancreatic cancers, for which the rates are 18% and 8% respectively (Siegel et al., 2016). This is primarily the result of initial diagnoses occurring when the cancer has progressed to an advanced stage.

Lung cancer is the leading cause of cancer death among both men and women in the United States (Torre et al., 2016). Lung cancer is divided into two types based on the morphology of the cells: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Of the two, NSCLC accounts for 85% of lung cancer incidence, with SCLC accounting for the additional 15%. The incidence has decreased for SCLC (Saintigny and Burger, 2012).

NSCLC is further subdivided into pulmonary adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The incidence rates are 40, 25 and 10 percent respectively (Carper and Claudio, 2015). Recently there has been a change in dominant cell type from squamous cell carcinoma that is generally central in the lung, to adenocarcinoma, primarily in the periphery of the lung (Saintigny and Burger, 2012). This change has been a result of deeper inhalation of cigarette smoke due to a decrease in tar content, as well as an increase in nitrosamines.
Despite advances in surgery, chemotherapy and radiotherapy over the last decades, the death rate from lung cancer has remained largely unchanged, primarily a consequence of metastatic disease (Saintigny and Burger, 2012). According to the National Cancer Institute (NCI), “metastatic cancer is cancer that has spread from the place it originated to another place in the body.”

**Metastatic Cancer**

The lung is a common site for metastasis, yet primary lung cancer cells also metastasize. It has been observed that lung cancer cells metastasize to the brain, bone, liver, adrenal gland as well to the other lung (Hillers et al., 1994). Cancer cells spread through three common routes: via transcoelomic, which is spreading and formation of metastatic tumors in the body cavities (mostly peritoneal and pleural cavities) (Jiang et al., 2015) which can lead to carcinomatosis, the lymphatics and the blood circulation (or the vasculature) also termed as hematogenous dissemination.

Hematogenous dissemination of cancer cells consists of distinct steps that enable the cells to enter the tissue and form a macro-metastases or colonies. The following steps constitute the metastatic cascade: (1) Intravasation or entry into the vasculature (Figure 1 Intravasation of cancer cells into the vasculature (or lymphatics), (2) travel through the blood circulation, (3) capture and firm adhesion under physiological flow, (4) extravasation or diapedesis out of the vasculature and into the connective tissue proper (Nguyen et al., 2009). Research has found that the rate limiting step in metastatic cancer is the ability of the metastatic cells to colonize and form macro-metastases that are clinically detectable (Chambers et al., 2002).
FIGURE 1 INTRAVASATION OF CANCER CELLS INTO THE VASCULATURE (OR LYMPHTICS)

Interpretation of the intravasation process modeled after (Seyfried and Huysentruyt, 2013)

In order for hematogenous dissemination to occur the cells must be able to migrate to the vasculature. Researchers have termed this process of migration as the “migratory cycle” (Xue and Hemmings, 2013). The following steps have been proposed to contribute to the cycle: initiation and polarization, formation of protrusions at the leading edge and retractions at the trailing edge, contraction of the cell body and the re-establishment of adhesions between the cell and the substratum (Xue and Hemmings, 2013).

As with anything a moving target is hard to pin down. Consequently, metastasis has proven to be a formidable foe in the battle against cancer. Much research has been devoted to identifying the mechanisms and/or molecules that can confer a migratory potential to cancer cells. Theoretically much has been accomplished in understanding the mechanisms of metastatic disease, yet clinically few therapeutic targets have been identified with successful therapeutic options.

Metastatic diseases account for almost 90% of cancer related deaths. Yet research into the field of metastasis, in comparison to other key events such as proliferation, etc., is lagging. This is partly due to the complexity of the metastatic process, but also due to a lack of sufficient funding and efforts into this area of research (Jiang et al., 2015).
For metastasis to occur cells have to possess the ability to have directional migration. This type of migration is composed of a number of cellular events, such as detection of extracellular signals by the cells, synthesis of cell surface proteins and the co-ordination of intracellular signaling and cytoskeletal proteins (Jiang et al., 2015).

**Current Therapeutic Options**

Currently clinical therapeutic options for advanced or metastatic NSCLC consists of systemic therapy and/or radical radiotherapy (Mery et al., 2015). These options have had limited success in increasing survivability, and have mostly been palliative in nature. Of late there has been an investigation into immunotherapeutic options, some of which have been FDA approved for clinical utilization.

The poor prognosis associated with NSCLC is primarily a consequence of the disease remaining asymptomatic for long periods, resulting in initial diagnosis when the disease has advanced to a point where surgery is not an option (Saintigny and Burger, 2012). This has led to an attempt at immunotherapy utilizing vaccination with tumor cells and dendritic cell activation (Pennell, 2015). Even though these options have shown evidence of inducing antitumor responses, the techniques have not been successful in NSCLC patients. Lack of immunogenicity has been postulated as a reason for the disappointing results.

Researchers then began to investigate immunotherapy utilizing inhibitors of immune checkpoint proteins (Pennell, 2015). Two specific molecules have been targeted. One, cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) out competes CD28 for binding the B7 ligands, thereby attenuating the secondary signal required for T-cell activation, and inhibiting priming and proliferation of naïve T-cells and thereby maintaining peripheral tolerance. By blocking CTLA-4, T-cell activation is able to proceed and activity is increased. The other molecule is the programmed cell death protein 1 (PD-1) pathway. The PD-1 pathway acts by inhibiting the CD8+
T-cells effector functions when PD-1 binds to one of its ligands, PD-L1 or PD-L2. The ligands are normally expressed by immune cells, yet it has been found that tumor cells upregulate the ligand PD-L1 on their surface and evade detection by the cytotoxic T-cells. Nivolumab, also known as Opdivo®, has been approved by the Food and Drug Administration (FDA) for use as a therapeutic option to target the PD-1 pathway. Nivolumab was approved for treatment of metastatic squamous NSCLC (Villadolid and Amin, 2015).

The mechanism of checkpoint inhibitors is in the context of countering immunoediting of the tumor (Pennell, 2015). Immunoediting is described by the three “E”s: elimination, equilibrium and escape. Elimination amounts to the immune system’s ability to identify and eliminate the cancer cells. If the immune system is unable to eliminate all of the cells then an equilibrium phase occurs, whereby the cancer cells are able to survive, albeit in a dormant state, despite the immune system. During this phase, cancer cells of low immunogenicity are able to colonize and then the escape phase occurs. Now cancer cells of low immunogenicity are able to escape the immune system and grow to a clinically detectable size. Immune checkpoint inhibition has shown great promise in tumor types not typically believed to be immunogenic, including NSCLC (Pennell, 2015).

In spite of the successes associated with checkpoint inhibitors, targeted immunotherapy has come with a price. Altered immunoregulation provoking immune dysfunction has opened the door to opportunistic autoimmune disorders (Kong and Flynn, 2014). Hence the term immune related adverse events (irAE’s) has been associated or designated as descriptive of the toxicities associated with checkpoint inhibition, as well as other methods of immunotherapy. There appears to be different pathogenic pathways to autoimmune manifestations, with PD-1 deficiency favoring a pathogenic autoantibody profile and CTLA-4 blockade favoring T-cell mediated organ damage.
Early recognition of irAE’s and initiation of treatment are critical to reduce the risk of complications since virtually all irAE’s are reversible with the use of steroids and other immune suppressants (Weber, 2012).

Use of tyrosine kinase inhibitors (TKI’s) against activation mutations of tyrosine kinase domains of the epidermal growth factor receptor (EGFR) is another therapeutic option used for NSCLC. First generation TKI’s that target the activation mutations in EGFR are initially successful, yet the patients were found to develop refractory tumors resistant to the drugs one to two years later (Kuwano et al., 2016). The fusion gene echinoderm microtubule associated protein like 4 (EML4) – anaplastic lymphoma kinase (ALK), another target for receptor TKI therapy, also leaves patients resistant to the drugs (Kuwano et al., 2016). What causes this resistance to the drugs? According to the literature it appears the dynamic genomic profile of cancer cells, which results in secondary mutations, has been postulated as the culprit.

The list of mutations in cancer cells is ever growing. Lately the conventional view of tumorigenesis being a direct result of a cascade of somatic mutations has been re-evaluated (Schmall et al., 2015). The presence of mutation alone was found to be insufficient for primary tumor growth and metastasis (Schmall et al., 2015).

Combination therapy has been a driving force to try to contain as many variables as possible for the benefit of the patient. One variable, which has been under investigation for years, has resurfaced as potentially contributing to the progression of cancer. Chemokine receptors have been linked to the organ specific metastasis of numerous carcinoma cell lines.

**Chemokines**

Chemokines are cytokines that have chemoattractant functionality. In other words, they are growth factors that provide a chemotactic gradient for cells to migrate towards, a process known as chemotaxis. The cells that normally follow this gradient are hematopoietic cells, i.e.
myeloid and lymphoid cells. However, it has been observed that carcinoma cells also migrate towards a chemotactic gradient (Zlotnik, 2006).

The chemokine superfamily consists of about 50 members that are subdivided into groups according to a conserved cysteine motif structure in the amino terminal region of the molecule. The groups are CC, CXC, CX_3C, and XC, where the “X” denotes amino acids between the cysteine residues. The CC and CXC groups are large and the chemokines, as well as the associated receptors, exhibit binding promiscuity. Hence, a single chemokine can bind to multiple receptors, as well as a single receptor being able to bind to multiple chemokines (Zlotnik and Yoshie, 2012).

Chemokines can be defined by their functional characteristics into two categories: homeostatic and inflammatory. Inflammatory chemokines are inducible and expressed under conditions of inflammation. These are the chemokines responsible for recruiting effector leukocytes to infection, inflammation, tissue injury and tumors. (Moser et al., 2004) Homeostatic chemokines are constitutively expressed and navigate leukocytes during hematopoiesis in the bone marrow and thymus, during initiation of adaptive immune responses in the spleen, lymph nodes (LN’s) and Peyers Patches (PP’s) and immune surveillance of healthy peripheral tissues (Moser et al., 2004). Despite this proposed delineation, there are some chemokines that overlap both categories and functionality. Dual function chemokines participate in immune defense functions and also target non-effector leukocytes at sites of leukocyte development and immune surveillance (Moser et al., 2004). Fractalkine, also known as CX_3CL1, is classified as a dual function chemokine (Zlotnik and Yoshie, 2012).

Most of the chemokines bind to glycosaminoglycans or GAG’s, specifically heparin sulfate, at the cell surface or in the extracellular matrix (ECM). This interaction has been proposed to control the diffusion potential of chemokines through the ECM and to determine local
concentrations, thus providing positional information within tissues in such forms as chemotactic gradients along which cells can migrate directionally (Monneau et al., 2015). Essentially, heparin sulfate (HS) presents the chemokine ligands to the cells expressing the associated chemokine receptors.

Fractalkine, or CX3CL1, differs from all other chemokines because it is expressed as a transmembrane protein (Shulby et al., 2004). Binding to plasma membrane anchored molecules is not necessary for fractalkine to resist sheer forces to the blood flow and to interact with circulating cells expressing the associated receptor CX3CR1 (Shulby et al., 2004).

The unique structure of fractalkine provides the potential for a transmembrane and soluble form of the chemokine. The transmembrane form can be cleaved by specific proteinases (Figure 2) producing the soluble form and providing for a chemotactic gradient for cells expressing the associated receptor to migrate towards. The transmembrane molecule consists of an extracellular N-terminal domain, a mucin stalk, a transmembrane α-helix, and a short cytoplasmic tail (Jones et al., 2010). Constitutive shedding of fractalkine occurs primarily via A desintegrin and metalloproteinase domain containing protein (ADAM)-10, while increased shedding under inflammatory and excitotoxicity conditions is mediated primarily by ADAM-17 (D'Haese et al., 2010). The soluble form consists of the chemokine domain, i.e. the CX3C motif, and the extracellular mucin like stalk (Jones et al., 2010).
The soluble form acts as a chemoattractant for natural killer (NK) cells, monocytes, macrophages and T-cells. Membrane bound fractalkine can be induced by inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1 (Jones et al., 2010). The transmembrane form acts as an adhesion molecule, which along with the integrin ligands intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, provide for a firm adhesion enabling cells to extravasate through the vasculature into the tissue proper. Since the binding of fractalkine to its receptor is extremely tight due to a low dissociation from the receptor, cleavage of fractalkine might be the only way to disrupt cell-cell contact and allow for migration into the tissue proper (Schwarz et al., 2010).

**Chemokine Receptors**

Chemokines signal through receptors that are attached to the membrane through seven transmembrane helical segments. These receptors are coupled to G proteins, the subfamily of which normally associated with chemokine receptors is the $G_{\alpha i}$ group. The aspartate-arginine-
tyrosine sequence, or DRY motif, in the second intracellular loop is required for activation of inhibitory $G_\alpha$ ($G_{\alpha i}$) proteins, whereas the asparagine-proline-X(2-3)-tyrosine (where X = leucine), or NPX(2-3)Y motif, located in the seventh transmembrane of most G protein coupled receptors (GPCR’s) contributes to ligand binding, activation, and internalization of the receptor (Schwarz et al., 2010). Both of these motifs and also several C-terminal Ser residues are found within CX$_3$CR1, the receptor associated with fractalkine, and are involved in its signaling and function (Schwarz et al., 2010).

The Ser residues can become phosphorylated by G protein coupled receptor kinases (GRK’s) and mediate interaction with β-arrestin resulting in desensitization towards the ligand (Schwarz et al., 2010). β-arrestin interacting with activated GPCRs promotes uncoupling of GPCRs from G proteins, thereby terminating the G protein dependent signaling initiated at the cell surface. Arrestins can only bind to phosphorylated GPCRs and are instrumental in GPCR desensitization (Roux and Cottrell, 2014).

Upon ligand binding, all GPCRs transiently induce their own phosphorylation, also known as homologous desensitization. When ligand is removed, the resensitization of the receptor is accomplished by fast recycling of the receptors and digestion of the ligand (Kamp et al., 2016).

Chemokine binding to the receptor stimulates multiple signaling events, such as increased intracellular cyclic adenosine monophosphate (cAMP) level, phospholipase activation, increased tyrosine phosphorylation particularly coinciding with activation of focal adhesion kinase (FAK) and zeta-chain associated protein kinase 70 (ZAP70), stimulation of two phosphoinositide 3 kinase (PI3K) isoforms (P85/p110 and C2α), activation of JAK2/STAT3 pathway, and activation of ERK/MAPK cascade (Cambien et al., 2001).
Chemokine receptors have been observed to transduce signals that lead to cytoskeletal reorganizations, integrin activation and other functions that increase adhesion and migration of the cells that express them. The association of chemokine receptor to different G proteins depends on the cell line studied (Cambien et al., 2001).

G Proteins

Chemokine receptors function as allosteric molecular relays where chemokine binding to the extracellular portions modifies the tertiary structure of the receptor allowing the intracellular part to bind and activate heterotrimeric G proteins. In response the activated G proteins exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and dissociate into α- and βγ-subunits, each having different effector targets (Rot and von Andrian, 2004).

The heterotrimeric G protein is a complex consisting of three subunits; an α- subunit that binds and hydrolyzes GTP and a β- and γ- subunit. The dissociation of this complex enables the GTP-α and βγ- subunits to target a diverse set of effectors and activate a variety of transmembrane signaling pathways. The spontaneous hydrolysis of GTP to GDP can be accelerated by various effectors as well as by regulators of G protein signaling (RGS) proteins (Wettschureck and Offermanns, 2005).

The basic properties of the G protein are defined by the α- subunit. This subunit has members that are classified into one of four subfamilies: $G_{αs}$, $G_{αi}/G_{αo}$, $G_{αq}/G_{α11}$, and $G_{α12}/G_{α13}$. Members of one family are structurally similar and often share some of their functional properties (Wettschureck and Offermanns, 2005). Across families, however, there are very specific expression patterns. The $G_{αi}/G_{αo}$ family have high expression patterns, mediating high amounts of βγ complexes. Therefore it is believed that activation of this receptor family results in the activation of βγ mediated signaling processes (Wettschureck and Offermanns, 2005). The
The $G_{\alpha q}/G_{\alpha 11}$ subfamily is almost ubiquitously expressed. Activation of this subfamily couples the receptor to $\beta$-isoforms of phospholipase C (PLC). The $G_{\alpha 5}$ subfamily is ubiquitously expressed, the activation of which couples the receptor to adenylyl cyclase and increases the levels of intracellular cAMP activation. The $G_{\alpha 12}/G_{\alpha 13}$ subfamily is also ubiquitously expressed. Receptors that couple to $G_{\alpha 12}/G_{\alpha 13}$ usually also couple to $G_{\alpha q}/G_{\alpha 11}$. In addition, $G_{\alpha 12}/G_{\alpha 13}$ activates other G proteins and can activate multiple signaling pathways leading to the activation of downstream effectors. Some examples include phospholipase $A_2$, $Na^+/H^+$ exchanger, or C-jun NH$_2$-terminal kinase.

One important cellular function of the $G_{\alpha 12}/G_{\alpha 13}$ subfamily is their ability to regulate the formation of actomyosin based structures and to modulate their contractility by increasing the activity of the small GTPase RhoA. Activation of RhoA by $G_{\alpha 12}/G_{\alpha 13}$ is mediated by a subgroup of guanine nucleotide exchange factors (GEFs) for Rho which include p115-RhoGEF, PDZ-RhoGEF and LARG (Wettschureck and Offermanns, 2005).

The assembly of the $\beta\gamma$ complex is established through the pairing of one of five $\beta$-subunits to one of twelve $\gamma$-subunits. This complex induces signaling that is involved in the regulation of ion channels, inducing particular isoforms of adenylyl cyclase and phospholipase C (PLC), and phosphoinositide 3 kinase (PI3K). With few exceptions, the ability of $\beta\gamma$ combinations to regulate effector functions does not dramatically differ.

**Rho GTPases**

Rho family GTPases are a subgroup of the Ras superfamily of GTPases and consist of 23 members that can be subdivided into six groups: Rho proteins (RhoA, RhoB, RhoC), Rac proteins (Rac1, Rac2, Rac3 and RhoG), Cdc42 proteins (Cdc42, TC10, TCL, Wrch1, Chp), Rnd proteins...
(Rnd1, Rnd2, Rnd3/RhoE), Rho BTB proteins (Rho BTB1, Rho BTB2, Rho BTB3) and Miro proteins (Miro1 and Miro2) (Rathinam et al., 2011). Of the 23 members the ones that have generated the most research are Ras related C3 botulinum toxin substrate 1 (Rac1), Ras homology gene family member A (RhoA), and cell division control protein 42 (Cdc42). Abnormal expression of these proteins has been observed to induce reorganization of the actin cytoskeleton, an increase in cell migration, invasion and metastasis which are key contributions to the progression of cancer (Rathinam et al., 2011).

Relative to G proteins, the Rho GTPases are monomeric as opposed to heterotrimeric like the G proteins, i.e. they are not a complex of α, β, and γ subunits. Like G proteins they are metabolic switches, so they also switch between an inactive state that is bound to GDP and an active state that is bound to GTP. The GTP binding is induced by guanine exchange factors (GEFs) and GTP hydrolysis resulting in GDP binding is controlled by GTPase activating proteins (GAPs) (Rathinam et al., 2011). The Rho GTPases are ubiquitously expressed.

Although Rac1, RhoA and Cdc42 share significant amino acid sequence homology, their effects on the actin cytoskeleton are quite different. Activation of Rac1 mediates the actin polymerization at the pseudopod, or the leading edge. This provides a gradient sensing mechanism for the cell. Activation of Cdc42 establishes the orientation machinery at the pseudopod, this is important for directional migration, a hallmark of chemotaxis. Activation of RhoA mediates the formation of the uropod at the trailing edge, or rear, of the cell. Retraction of the uropod pushes the cell forward (Rot and von Andrian, 2004). Rho GTPases also regulate several biological processes relevant to cancer including cell cycle control, epithelial cell polarity, cell survival and angiogenesis (Rathinam et al., 2011).
The Rho GTPases also have diverse effector protein targets. The different signaling pathways mediate the diversity in functionality of the Rho GTPases. RhoA interacts with RhoA associated coiled coil containing protein kinase (ROCK) which in turn activates myosin light chain kinase (MLCK) leading to activation of myosin, increased contractility and formation of stress fibers. Cdc42 binds to neuronal Wiskott-Aldrich syndrome protein (N-WASP) and signals to the actin related protein 2/3 (Arp2/3) complex leading to formation of filopodia and actin polymerization. Rac1 activates p21 activated kinase (PAK) and WASP-family verprolin homologous protein (WAVE) which results in altered actin nucleation activity of the Arp2/3 complex (Rathinam et al., 2011).

Directed cell movement in response to an increased concentration of chemoattractant, or chemotaxis, is key to the metastatic potential of carcinoma cells. The G proteins associated, or coupled, to the chemokine receptors are intricately involved in the activation of the Rho GTPases to mediate this process. The interaction of G proteins with the inner side of the plasma membrane is facilitated by lipid modifications of both the α-subunit as well as of the γ-subunit of the βγ complex. From this vantage point G proteins are able to activate GEFs of the Rho GTPases facilitating exchange of GDP for GTP (Wettschureck and Offermanns, 2005).

Chemokine receptors are generally associated with the \( G_{al} \) subfamily of G proteins. As mentioned above this subfamily mediates high expression of Gβγ complexes. In the presence of phosphatidylinositol-3,4,5-triphosphate (PIP3) a signaling lipid that is a product of PI3K (Salamon and Backer, 2013), the Gβγ complex can directly bind and activate phosphatidylinositol 3,4,5 triphosphate-dependent Rac exchanger 1 (P-Rex1) and Pak interactive exchange factor (PIXα), which are GEFs for Rac1 and Cdc42 respectively (Figure 3) (Kamp et al., 2016). \( \frac{G_{a12}}{G_{a13}} \) is able
to activate Rho and its effector ROCK via the Rho Specific GEF p115RhoGEF, leading to myosin II assembly at the rear of chemotaxing cells (Kamp et al., 2016).

Extracellular signal regulated kinase (ERK) also plays a role in the regulation of the Rho GTPases. ERK phosphorylates $T^{108}$ of Rac1 in response to EGF stimulation. This phosphorylation alters Rac1 activity, its subcellular localization and its role in mediating cell migration (Tong et al., 2016). RhoA contains an ERK docking site in its C-terminus of the amino acid sequence $K^{185}KKSGCLL^{193}$. RhoA interaction with ERK is mediated by this docking site, and also affected by ERK phosphorylation sites $S^{88}$ and $T^{100}$. RhoA phosphorylation by ERK enhances its function in regulating the formation of stress fibers (Tong et al., 2016).

Tumor cell motility in a 3D matrix tends to frequently exhibit two modes of migration: mesenchymal and amoeboid (Rathinam et al., 2011). RhoA-ROCK signaling mediates a rounded morphology with bleb-like structures constituting amoeboid movement. Rac1 mediates cells moving in an elongated mesenchymal manner (Sahai and Marshall, 2003). These two modes are
interchangeable, as inhibition of one promotes switching to the other. Common to both modes is the activation of Cdc42, which provides directional migration necessary for chemotaxis.

The mechanotransducing mechanisms that are responsible for executing cell migration are plastic and allow the rapid adaptation to environmental changes and challenges. Such adaptations often result in cells switching between different modes of migration. This plasticity can occur in response to tissue microregions and/or responses to therapeutic challenge (Friedl and Alexander, 2011).

**CX\textsubscript{3}CR1/Fractalkine (CX\textsubscript{3}CL1) Axis in Cancer**

It has been established that carcinoma cells gain the aberrant expression of chemokine receptors, unlike their normal counterparts (Balkwill, 2012). The gain of expression of chemokine receptors has been postulated to confer a migratory potential to the cells, similar to that of leukocytes (Zlotnik et al., 2011). This section will provide some examples where the CX\textsubscript{3}CR1/fractalkine signaling axis has contributed to the progression of numerous carcinoma cell lines.

Using PC3-ML cells, a metastatic prostate cancer cell line, Shulby et al. found that the cells expressed CX\textsubscript{3}CR1 and migrated to a chemotactic gradient formed by fractalkine to the bone marrow, adhering firmly to the bone marrow endothelial cells. The authors noted that endothelial cells of the bone marrow and osteoblasts in the stroma express fractalkine. Of note, when CX\textsubscript{3}CR1 binds to the ligand the PI3K/Akt survival pathway was activated (Shulby et al., 2004). In a subsequent article the authors found an androgen dependent mechanism mediating fractalkine cleavage from bone resident cells that could possibly explain why antiandrogen therapy has been found to delay skeletal metastases from primary prostate cancer (Jamieson et al., 2008). Essentially lack of soluble fractalkine ablates the gradient formation and the cells do not migrate to the bone.
Pancreatic ductal adenocarcinoma (PDAC) has been observed to invade the nerve sheaths of peripheral nerves. Marchesi et al. found that PDAC cells express CX3CR1 and the neurons express fractalkine. They observed that CX3CR1 expressing PDAC cells migrated to the chemotactic gradient supplied by fractalkine, and adhered to membrane fractalkine expressing cells of neural origin via mechanisms involving activation of G proteins, beta 1 integrins, and focal adhesion kinase (FAK). Hence the authors speculate that a potential mechanism for perineural invasion has been elucidated (Marchesi et al., 2008).

Nevo et al. found that the CX3CR1/fractalkine axis mediates the transendothelial migration (TEM) of neuroblastoma cells. They verified CX3CR1 expression in several neuroblastoma cell lines and found fractalkine constitutively expressed by endothelial cells of the bone marrow. Fractalkine binding to CX3CR1 resulted in a dose dependent activation of ERK1/2 and Akt. Therefore the authors postulate that the CX3CR1/fractalkine axis takes part in the bone marrow metastasis of neuroblastoma (Nevo et al., 2009).

Glioma tumors are the most frequently occurring malignant tumors in the brain. Because of the known involvement of the CX3 CR1/fractalkine axis in central nervous system pathophysiology as well as cross talk between neurons and microglia (neurons express fractalkine, microglia express CX3CR1), Locatelli et al. investigated the expression of CX3CR1 in gliomas. They assessed the mRNA and protein levels of CX3CR1 in the central nervous glial tumors of 70 patients. They found CX3CR1 highly expressed in the tumor cells, and the corresponding normal cells did not express the receptor. They did not, however, determine the molecular basis and biological significance of the upregulation of the receptor (Locatelli et al., 2010).

Breast cancer also frequently metastasizes to the bone. Contemplating the adhesive interactions between cancer cells and bone marrow endothelial cells, as well as the extravasation
toward chemoattractant molecules produced by the stroma surrounding the bone, Jamieson-Gladney et al. investigated the role of the CX3CR1/fractalkine axis in this process. The authors found that the functional interactions between fractalkine, which is produced by both the endothelial and stromal cells of the bone marrow, and the CX3CR1 receptor on breast cancer cells are determining factors in the arrest and initial lodging needed for skeletal dissemination (Jamieson-Gladney et al., 2011).

Epithelial ovarian carcinoma (EOC) is notorious for local dissemination, or transcoelomic, spread into the peritoneum. Hence Barbolina et al. investigated the role of the CX3CR1/fractalkine axis in the progression of EOC. They found that EOC cells express CX3CR1 and robustly migrate to the chemotactic gradient formed by fractalkine, which is expressed by the peritoneal mesothelial cells. Importantly, adhesion of ovarian carcinoma cells to human peritoneal mesothelial cells was dependent on CX3CR1/fractalkine signaling (Kim et al., 2012).

Treatment of clear cell renal cell carcinoma (CCRCC) metastases is clinically challenging, with a mortality rate of 30%. Yao et al. investigated the role of the CX3CR1/fractalkine axis in this deadly disease. CX3CR1 is strongly expressed on CCRCC cells, and upon binding to the ligand the ERK1/2 and PI3k/Akt pathways are activated. The authors concluded that since fractalkine is expressed by neurons as well as endothelial cells of the lung and bone marrow, common metastatic sites of CCRCC, this axis could play a significant role in the metastatic progression of CCRCC (Yao et al., 2014).

Gastric cancer is an important public health burden, with a five year survival rate of less than 30%. Therefore, Wei et al. investigated the contribution of the CX3CR1/fractalkine axis to this devastating disease. They found that CX3CR1 was expressed in the gastric carcinoma cells, and, unexpectedly in the non-neoplastic gastric epithelium. The function of the receptor in the
non-neoplastic gastric tissue was unknown. The upregulation of the receptor expression in the cancer cells was associated with metastasis, proliferation and survival in the cancer cells. They also noted a parallel increase in phosphorylated Akt levels (Wei et al., 2015).

Since lung cancer is the primary focus of this research, it is fitting to close out this section with information pertaining to some of the work that has been done with regard to the CX3CR1/fractalkine axis involvement in lung cancer. A couple of years ago, Mauri et al. did a preliminary study to investigate the expression of the CX3CR1 receptor in primary lung cancer and the associated metastatic tissue samples. Using 98 post mortem specimens taken from patients with untreated lung cancer they found that non-small lung cancer (NCSLC) had a higher immunopositivity for CX3CR1 than small cell lung cancer (SCLC). They also found that greater than 75% of the metastatic sites in NSCLC stained positive for CX3CR1 (Mauri et al., 2012).

Earlier last year Schmall et al. found that when they co-cultured lung cancer cell lines with macrophages the expression of the chemokine receptor CCR2 was induced and the expression of CX3CR1 was enhanced. According to the authors, crosstalk between the macrophages and the cancer cells via the receptors enabled the cancer cells to migrate. Depletion of macrophages (via clodronate, or macrophage Fas-induced apoptosis mice) and genetic ablation of CCR2 and CX3CR1 all inhibited lewis lung cancer 1 (LLC1) cell line growth and metastasis. The authors concluded that macrophages have a central role in lung cancer growth and metastasis via crosstalk with cancer cells mediated by CX3CR1 and CCR2 (Schmall et al., 2015). From the authors’ view point macrophage conditioned medium enabled the gain of function of expression of CCR2 and enhanced the expression of CX3CR1. The receptors mediate the gain of migratory potential, albeit at the coaxing of the macrophages.
Hypothesis, Aims and Objectives

Lung cancer is the fourth leading cause of cancer deaths. Metastasis is a major contributing factor to that mortality. Identification of a pathway unique to cancer cells and not observed in their normal counterparts provides a valuable target for therapy to combat this disease. Therefore my hypothesis is that the $CX_3$CR1/fractalkine ($CX_3$CL1) chemokine axis is involved in the progression of lung cancer.

My objective is to investigate the phenotype that the gain of function of the expression of chemokine receptors confers on the carcinoma cells that express them. Hence, my specific aims are as follows: (1) To study the biological role of the CX3CR1/CX3CL1 axis in lung cancer progression (proliferation, invasion). (2) To explore the mechanism of CX3CR1/CX3CL1 axis in lung cancer progression.

To elucidate these aims my research methodology is as follows: (1) Verify expression of CX3CR1 in the non-small cell lung cancer cell line A549, a metastatic cell line, via Western blot. (2) Expose cells to ligand, fractalkine (CX3CL1), and identify response. (3) Perform a migration assay to see if the cells will migrate to the chemotactic gradient.

In the previous section the research by Schmall et al. provided impetus to pursue this research. The authors stated that the expression of $CX_3$CR1 was enhanced by the macrophage conditioned medium. This leads to the question, what does the constitutive expression of the receptor confer to the functionality of the cells? Therefore it is the purpose of this proposal to try to identify the underlying signal transduction pathways that are activated when the receptor binds to the ligand.

MATERIALS and METHODS

Antibodies and Reagents

Anti-rabbit p-ERK, pan ERK, p-Akt and pan AKT antibodies were purchased form Cell Signaling Technology (Danvers, MA). Rho-GTPase antibody sampler kit containing anti-rabbit
Cdc42, Rac1/2/3 and RhoA antibodies was also purchased from Cell Signaling Technology (Danvers, MA). Anti-human CX3CR1 antibody was purchased from eBioscience (San Diego, CA). Anti-mouse GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat IgG horseradish peroxidase (HRP) conjugated antibody was purchased from R&D Systems (NE Minneapolis, MN). Chemokines fractalkine (CX3CL1) and SDF-1α (CXCL12) were obtained from ProSpec (East Brunswick, NJ). Cell culture media RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen.

**Cell Culture**

A549 cells and the human embryonic kidney cell line HEK-293, epithelial cells, were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS, and penicillin/streptomycin at 37°C with 5% CO₂. THP-1 cell lysates, a monocytic cell line, were kindly provided by Xiaoqing Guan.

**Western Blot**

Cells were washed in PBS, after which cells were lysed with lysis buffer (TBS, 0.2% Tween) supplemented with protease inhibitors (10 μl phenylmethylsulfonyl fluoride [PMSF], 1 μl aprotinin, 1 μl leupeptin and 1 μl pepstatin). Cells were centrifuged at 14,000 g for 15 minutes and the clear supernatant was assayed for protein concentration using Quick Start Bradford Dye Reagent (BioRAD). Samples were mixed with NuPage LDS sample buffer 4x (Invitrogen) containing 10% 2-mercaptoethanol (BioRAD) and heated at 70°C for 10 minutes. Protein bands were separated by electrophoresis on either a 10% (for CX3CR1, Akt and ERK) or 12% (for RhoA, Rac1 and Cdc42) SDS-PAGE gel. Separated bands were blotted onto a polyvinylidene difluoride (PVDF) membrane. Membrane blots were probed with specific antibody and then the associated secondary antibody conjugated to HRP. Developed images were visualized using the BioSpectrum 500 imaging system (UVP).
Signaling Assay
Cells were seeded into a six well plate at a seeding density of $5 \times 10^5$ cells per well. Cells were incubated in RPMI 1640 10% FBS for 24 hours at 37°C. Cells were then incubated in culture medium deprived of serum for 4 hours; afterwards cells were exposed to 50 ng/ml or 100 ng/ml fractalkine or 20 ng/ml SDF-1α for different time periods at the temperature of 37°C. Cell lysates were obtained as described above.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Proliferation Assay
A549 cells were seeded into 96 well plates at a seeding density of $6 \times 10^3$ cells per well in medium deprived of serum. This seeding density was determined by the generation of a standard curve using MTT to optimize the seeding density for the wells. Lane 6 was seeded with medium supplemented with 10% FBS as a positive control. Cells were stimulated with ligand in the following concentrations: lane three 10 ng/ml, lane four 25 ng/ml and lane five 50 ng/ml. Lane one was starvation medium only, no cell suspension, to serve as a blank. Lane 2 was cells in starvation medium, no ligand stimulation to serve as a negative control. Cells were treated for 24 hours, after which 20 µl MTT (5 mg/ml) was added to each well and plates were placed in an incubator at 37°C for 3 hours. The medium was removed, after which 120 µl of MTT solvent was added to dissolve the crystals formed by the mitochondria uptake of MTT. Absorbance was measured at 490nm using a multiwell spectrophotometer.

Scratch Wound Assay
A549 Cells were seeded onto 12 well plates at a seeding density of $1 \times 10^5$ cells per well in medium supplemented with 10% FBS. Once the cells had reached 90% confluence (a monolayer) cells were starved for 24 hours in medium deprived of serum. Using a 200 µl pipette tip, two scratch wounds were created in the cell monolayer, perpendicular to each other. This
assay uses the concept of wound healing to measure the migration potential of the cells. Essentially the scratch generated in the monolayer will simulate a wound and the cells will react to close the wound by migrating to fill the gap. Cells were washed with medium to remove the debris, and then supplemented with medium deprived of serum, containing ligand in the following concentrations: lane one starvation medium only, lane two 50 ng/ml fractalkine, lane three 100 ng/ml and lane four 20 ng/ml SDF-1α.

**Statistical Analysis**

One way ANOVA was used to set the P-value, and Student t-Test was used to determine statistical significance between the groups. All t-Tests were two sided. Experimental results are expressed as mean ± SE. A P-value of 0.05 was used throughout the whole analysis.

**RESULTS**

**CX₃CR1 Expression in A549 Cells**

The chemokine receptor CX₃CR1 has been observed to be expressed in numerous carcinoma cell lines; breast, prostate, pancreatic and ovarian are some examples. Therefore, the hypothesis is that the CX₃CR1/fractalkine axis contributes to the progression of the non-small cell lung cancer cell line A549. The initial step is to verify the expression of the receptor on the cell line.

The first set of experiments were performed to establish whether the receptor for fractalkine, CX₃CR1 is expressed in vitro on the A549 cell line. A549 and HEK-293 cells were cultured, harvested and lysed with lysis buffer. Along with cell lysates for THP-1 cells the protein bands were separated via SDS-PAGE and blotted onto a membrane. HEK-293 cells (human embryonic kidney cells), which express CX₃CR1 at a low level, were used as a negative control. The THP-1 cells, a monocytic cell line known to express CX₃CR1, were used as a positive control.
The results of the Western blot are shown in Figure 4. The figure displays the western blot results after probing for CX₃CR1 and then stripping the membrane and re-probing for GAPDH as a loading control. Also displayed in the figure is a graph of the relative expression level across the cell lines after normalization with the loading control. The error bars represent the standard error of the mean. Numbers represent the optical density measurement obtained after normalizing the relative density (i.e., intensity) of the sample bands to the loading control bands.

**FIGURE 4 CX₃CR1 EXPRESSION FOR A549, THP1 AND HEK-293 CELLS.**

Western blot of cell lysates from A549, THP-1, and HEK-293 probed for CX₃CR1. 40 µg of protein were loaded into each well. GAPDH used for normalization. Results are from two independent experiments.

Normalization of the samples with the loading control was done via Image J. Using gel analysis in the Image J program the user is able to compare the density (i.e., the intensity) of the bands. One of the wells is used as a standard, for both the samples and the loading control. It would be the same well on both gels. Once that relative density is established, then the sample is normalized to the loading control by dividing the relative density of the sample by the relative density for the loading control. The result is the relative expression of the sample after normalization with the loading control.
For normalization the user decides which well will be the standard to use for generating the relative densities. Here the well for the THP-1 cells was used as the standard. The reason for this is because THP-1 cells are known to express CX₃CR1, therefore using that well as a standard provides for a relative expression level comparative to a cell line known to express the receptor.

A549 cells express CX₃CR1 at a level comparable to THP-1 cells. The expression level of CX₃CR1 in HEK-293 cells is so low as to be almost negligible. The results confirm that CX₃CR1 is expressed by the non-small cell lung cancer cell line A549.

**CX₃CR1/fractalkine axis contribution to growth and proliferation**

Once the expression of CX₃CR1 was verified in the NSCLC cell line A549 the question was resurfaced regarding what contribution this gain of function could confer on the cell line? Epithelial cells do not normally express chemokine receptors, yet carcinoma cells ultimately gain the ability to express the receptors and therefore respond to the signal transduction mediated by ligand binding to receptor (Balkwill, 2012). Chemokines are effective inducers of proliferation (Kim et al., 2012). Proliferation has a prominent role in the pathophysiology of NSCLC. The difficulty with NSCLC is that patients are not diagnosed until the cancer has reached an advanced stage, i.e. metastasized. Metastatic progression in NSCLC is characterized by a widespread metastasis that far exceeds the size of the primary tumor.

Therefore, the question arises - is it possible that fractalkine, via the receptor CX₃CR1, could mediate the growth and proliferation of the NSCLC cell line A549? Hence, a MTT assay was performed. Cells were stimulated with the following range of concentrations; 10 ng/ml, 25 ng/ml (Kim et al., 2012) and 50 ng/ml (Shulby et al., 2004) of fractalkine. Cells suspended in RPMI with 0% fetal bovine serum (FBS) were used as a negative control. For a positive control, cells were suspended in RPMI with 10% FBS. Cells were seeded at a density of 6 x 10³ per well,
which was determined by a standard curve (data not included). The results of three repetitions of the assay conditions are shown in Figure 5. There was not a statistically significant difference in the absorbance, which is assumed to be indicative of the proliferation of cells.

The MTT assay is founded on the principle that mitochondria activity is directly related to proliferation. The assay relies on the uptake of the MTT working solution by the cells; it’s conversion into crystals which, once they are dissolved by the MTT solvent, absorb light at 490 nm. It has been postulated that MTT is specific for metabolic activity, not proliferation. Accumulating evidence suggests that cancer is a metabolic disease involving respiratory insufficiency with compensatory fermentation (Seyfried and Huysentruyt, 2013). The discrepancy in the results between the serum supplemented and the stimulated cells deprived of serum could be indicative of more cells in the stimulated samples with less metabolic activity. In an attempt to
assess the effect of the CX3CR1/fractalkine axis on proliferation a second assay was performed and this time the cells were counted using a hemocytometer.

To count the cells 24 well cell culture plates were used with a seeding density of $5 \times 10^4$ cells per well. Cells were allowed to attach in medium deprived of serum and supplemented with the following concentrations of fractalkine; 10 ng/ml, 25 ng/ml and 50 ng/ml in lanes 2, 3 and 4 respectively. For a negative control, lane 1 contained cells suspended in culture medium deprived of serum. As a positive control, lane 5 contained cells suspended in medium supplemented with 10% fetal bovine serum (FBS). After 24 hours cells were trypsinized and counted with the hemocytometer.

The results are shown in Figure 6. Cells stimulated with 10 ng/ml and 25 ng/ml showed significant growth and proliferation, even above the positive control. Between the two, the 25 ng/ml stimulation had the most significant growth. In the case of the 50 ng/ml stimulated cells, although the cells showed more growth than the negative control, there was less growth than the positive control, and significantly less growth than the cells stimulated with 10 ng/ml or 25 ng/ml.
The difference in the results between the MTT assay and the cell count using the hemocytometer could be the result of growth without metabolic activity. The MTT assay is specific for metabolic activity, not proliferation (Terry L Riss, 2013).

The reason for the lower proliferation of the cells stimulated with the 50 ng/ml concentration could be the fact that the chemokine receptors become desensitized to the ligand. This known fact is a natural checkpoint provided by nature to assure that the chemokine response is attenuated. Receptors are phosphorylated by GPCR kinases (GRKs), inducing interaction between the receptor and the β-arrestin protein. The β-arrestin protein interacts with the chemokine receptor and effectively uncouples it from the G-protein; terminating G-protein initiated signaling, which could account for the decrease in growth and proliferation (Roux and Cottrell, 2014).

**CX₃CR1/fractalkine Axis Involvement in Metastatic Progression**
Since the 50 ng/ml concentration of fractalkine basically stunted the growth of the cells it brings to mind the perspective that at this concentration the response of cells to the receptor binding
to the ligand could be to migrate (Shulby et al., 2004). In order to investigate this potential, a scratch wound assay was performed. The scratch wound assay is a simple method of assessing the migratory potential of cells (Cory, 2011). The concept behind the assay is if a wound is created, by scratching the cell monolayer with a pipette tip, the cells will migrate to close the wound which is typical of wound healing. Cells are grown to 90% confluency, after which the medium is removed and a scratch wound is made using a 200 µl pipette tip and the cells are then stimulated with 50 ng/ml or 100 ng/ml fractalkine or 20 ng/ml stromal derived factor 1α (SDF-1α/CXCL12). Since a growth stimulation was observed with the 25 ng/ml concentration in the cell count assay, an earlier scratch wound assay was performed using this concentration as well as the 10 ng/ml concentration. No significant results were observed, i.e. no significant migration was observed over the negative control, that data is included in Figure 8.

Cells were plated in 12 well cell culture plates at a seeding density of 1 × 10^5 cells per well. Cells were suspended in RPMI 1640 supplemented with 10% FBS and allowed to grow to 90% confluency. The medium was then removed, and the cells were supplemented with RPMI 1640 + 0% FBS with the following concentrations of ligand added; 50 ng/ml or 100 ng/ml fractalkine, or 20 ng/ml stromal derived factor 1α (SDF-1α/CXCL12). SDF-1α was used as a relative positive comparison as it has been stated in the literature that it induces migration of numerous cancer cell lines (Reymond et al., 2013) and (Teicher and Fricker, 2010).

The results are shown in Figure 7, which is photographs of the cells at 0 and 24 hours. Figure 8 shows the average migration per concentration group. Migration distances were measured using Image J. This measurement is used for a relative comparison of the migration between the groups.
The measurements were obtained via Image J using the Region of Interest (ROI) manager. This enables the user to stack the images into the manager and generate measurements. Because the manager allows the user to show all measurements in the stack it provides the user with the ability to measure the same region on each image in the stack. This measurement is a means of
comparing distances in image J, however it is a unit less, i.e. not identified as mm or cm, the measurement is comparable only in image J.

The 50 ng/ml stimulated cells have a migration measurement that is comparative to the SDF-1α stimulated cells. The significance is in the measurement between the 0 hour and the 24 hour migration measurements in each concentration group. It can also be observed that relative to the negative control the 100 ng/ml stimulated cells had an increased migration measurement in Image J. Therefore, it is possible that the CX₃CR1/fractalkine axis does confer a migratory potential to the cells. This leads to the question of what signal transduction pathways are activated to mediate this migration potential?

**CX₃CR1/fractalkine Axis Potential Activation of PI3K/Akt and MAPK/ERK Signaling**

It has been observed that different concentrations of the ligand can affect cells differently. The results of hemocytometer assay were indicative that the 50 ng/ml concentration of the ligand shifted the cells from growth and proliferation. The results of the scratch wound assay showed that the functional shift of the cells may be towards migration. Hence, the next set of experiments were focused on identifying the pathways that are activated when the cells are stimulated with higher concentrations of the ligand. The objective is to verify if the higher concentrations set off signal transduction pathways that mediate a chemotactic response.

To that end a signaling assay was performed. Cells suspended in RPMI 1640 supplemented with 10% FBS were seeded in a six well cell culture plate at a density of 5 x 10⁵ cells per well. Cells were allowed to grow to 90% confluence, at which time the medium was removed and the cells were supplemented with RPMI 1640 deprived of serum and incubated for 24 hours at 37°C. The medium was again removed and fresh RPMI 1640 deprived of serum was added but this time it was supplemented with the following concentrations of ligand; 50 ng/ml, or 100 ng/ml
fractalkine, or 20 ng/ml SDF-1α. Here SDF-1α is used as a relative comparison of the strength of activation of the signal transduction pathways. Cells were stimulated for 5 and 15 minutes for the fractalkine concentrations and 5 minutes for the SDF-1α concentration.

When chemokine receptors bind to ligand it has been observed that the PI3k/Akt and MAPK/ERK pathways are activated (Lee et al., 2006). Therefore, cells were lysed and clear lysates were electrophoresed in a 10% SDS-PAGE gel to separate the protein bands. The bands were subsequently blotted onto a PVDF membrane. Membranes were probed for phosphorylated Akt (p-AKT) and p-ERK, after which membranes were stripped and probed for pan-Akt (total Akt) and pan-ERK as a loading control. Densitometry analysis was performed by normalizing the samples to the loading control using the method mentioned above. Here for the positive control the band for the CXCL12 expression was used. This band was chosen so that the other expression levels would be comparative to a known inducer of the signal being analyzed.

The results are shown in Figure 9 and Figure 10. Figure 9 displays the blots probed for phosphorylated ERK (p-ERK) and total or pan-ERK, the graph also shows the relative expression of the concentrations normalized to the loading control. The error bars reflect a confidence level that is significantly low enough to hinder the ability to say that stimulation with the ligand induces the upregulation of the p-ERK pathway. Here it appears that stimulation with the 50 ng/ml concentration for 15 minutes and the 100 ng/ml concentration for 5 minutes exceeded the expression intensity of the positive control. Yet the confidence level is low enough to place doubt on the results. Contributions to this discrepancy could be experimental error, as well as a small number of experiments. The mean represents data from two independent experiments.
Figure 9 Phosphorylated ERK (p-ERK) and Total ERK (pan ERK)

Results of western blot probed for phosphorylated ERK. Total (pan) ERK was used for normalization. Gels were loaded with 20 µg of cell lysate per well.

Figure 10 displays the blots probed for p-Akt and pan (or total) Akt proteins. The graph is a plot of the optical density for the normalized p-Akt to pan-Akt and shows the relative expression level for each concentration comparative to the positive control band, i.e. the CXCL12 well. The error bars represent the standard error for the mean optical density.

Figure 10 Phosphorylated Akt (p-Akt) and Total Akt (pan-Akt)

Results of western blot probed for phosphorylated Akt. Total (pan) Akt was used for normalization. Gels were loaded with 20 µg of cell lysate per well.

The error bars reflect the confidence level that the mean represents the actual relative expression of the other concentrations comparative to the standard. It appears that the 100 ng/ml concentration signal intensity is higher than the positive control, and the standard error concurs...
with the possibility that stimulation with the 100 ng/ml concentration of the ligand induces the upregulation of the p-Akt signal transduction pathway.

Although p-ERK does not appear to be upregulated, p-Akt does based on the standard error and statistical analysis (P < 0.05). The significance of activated Akt is twofold. First, the signaling pathway that mediates survival. Second, it is involved in processes that induce growth and proliferation as well as prevent apoptosis. Yet the higher concentrations of the ligand, i.e. 50 ng/ml and potentially 100 ng/ml, did not show a significant stimulation of growth and proliferation in the MTT assay or the cell count. Hence is it possible that this level of concentration activates the Akt pathway for its involvement in migration?

With that comes the second significance of the activation of these pathways. Both have effects on the Rho GTPases, which are intricately involved in the cytoskeletal dynamics that lead to migration. Both PI3k/Akt and the MAPK/ERK are activated by the βγ-subunit of the G-protein that is coupled to the chemokine receptor. The immediate step involved in βγ mediated activation of ERK involves phospholipase C (PLC) β. The PI3k/Akt pathway is directly activated by the βγ-complex (Rot and von Andrian, 2004).

With regard to the effects the pathways have on the Rho GTPases, PI3k through its product phosphatidylinositol 3,4,5 triphosphate (PIP3), provides a docking site that mediates the interaction between the guanine exchange factors (GEF’s) P-Rex1 and PIXα, GEF’s for Rac1 and Cdc42 respectively, with the βγ-subunit (Kamp et al., 2016). ERK has a regulatory effect on the Rho GTPases (Vial et al., 2003) (Figure 11). It was recently found that ERK can enhance the activation of RhoA by phosphorylating it, and by phosphorylating Rac1 it can change its subcellular location and role in mediating cell migration (Tong et al., 2016). Phosphorylation of Rac1 on Threonine 108 (T^{108}), mediated by ERK, targets Rac1 to the nucleus, which isolates Rac1
from other guanine nucleotide exchange factors (GEFs) and hinders Rac1's role in cell migration (Tong et al., 2013). Therefore, the effect that PI3k/Akt and MAPK/ERK signal transduction pathways have on the Rho GTPases could significantly contribute to a migration potential.

![Diagram](image)

**Figure 11 Effect of ERK on Rac1 and RhoA, Modeled After (Vial et al., 2003)**

**CX₃CR1/Fractalkine Axis and Rho GTPases**

Metastatic disease accounts for 90% of cancer related deaths. Therefore an understanding of the signal transduction pathways that induce migration in cells is invaluable. As noted above, both the PI3k/Akt and MAPK/ERK pathways could be activated when higher concentrations of fractalkine bind to CX₃CR1. Considering the numerous observations in the literature that these pathways affect the activity of the Rho GTPases, as well as the fact that the Rho GTPases mediate cytoskeletal remodeling that translates into migration, it is imperative to investigate how the Rho GTPases are affected by the CX₃CR1/fractalkine axis.

Hence another signaling assay was performed. Cells were seeded into six well cell culture plates at a seeding density of 5 x 10⁵ cells per well. The cells were suspended in RPMI 1640 supplemented with 10% FBS and allowed to grow to 90% confluency at 37°C. After which the
medium was removed and RPMI 1640 0% FBS supplemented with 50 ng/mL, or 100 ng/mL fractalkine or 20 ng/mL SDF-1α was added to stimulate the cells. Cells were stimulated for 1 and 5 minutes. As a negative control, cells suspended in RPMI 1640 0% FBS with no supplemental stimulation were used. Again, stimulation with SDF-1α was used as a relative comparison to a ligand known to induce cells to migrate.

The cells were then lysed, and the protein bands were separated via SDS-PAGE. Resulting bands were blotted onto membranes. The membranes were probed with primary antibodies to Rac1, RhoA and Cdc42 and the associated secondary antibody. Results were visualized using chemoluminescence. Normalization of the signal to the loading control, which for these experiments is GAPDH, was performed and the associated plot of the relative expressions are also displayed. The results are shown in Figure 12, Figure 14, and Figure 14.

![Western blot image of membrane probed with RhoA anti-body. 20 µg of cell lysate was loaded into each well. Bands were normalized to GAPDH, the loading control. Error bars represent mean ± standard error (S.E.). Numbers represent the optical density for the relative expression obtained after normalization.](image)

**Figure 12 RhoA and GAPDH**

Figure 12 for RhoA reflects a standard error that defies any conclusive argument that the ligand upregulates the pathway. In contrast to Rac1 the blot appears to show that 100 ng/ml concentration for the 5 minute stimulation interval is significantly stronger than the other concentration/intervals. The graph of the relative expression shows significant error bars,
indicating a low confidence level in the mean reflecting the actual differences in the relative expression.

Figure 13 shows the data for Rac1 which also reflects a low level of confidence that the mean represents the actual comparative expression. Ironically the gel for Rac1 indicates that there is no difference in the expression level between the concentrations. Yet when the signals were normalized to the loading control it appears as if there is a difference, particularly with the 100 ng/ml concentration for the 5 minute stimulation interval. However, the standard error diminishes any conclusive argument for an effect of the ligand on the Rac1 pathway.
Figure 14 displays the data for Cdc42. The error bars represent the standard error of the mean, and reflect a low confidence level that the mean represents the actual comparative expressions. Here it appears that all the stimulated bands have an expression higher than the positive control, yet the error bars diminish the ability to conclude that stimulation of the cells with fractalkine induces the activation of Cdc42. As always experimental error could be a contributing factor as well as a low number of observations.

These results highlight the issues encountered over the course of this research project. As mentioned above, a contributing factor could be experimental error. What constitutes experimental error? Some of the issues could be a result of saturation of protein samples, over exposure of blot images, improper concentration of gel used for the desired protein, and the known difficulty of isolating membrane proteins. In addition, a strong contributing factor is the number of observations. The standard error reflects the confidence interval, and it also reflects the distribution between the original data points. Additional experimental data could have significantly offset the standard error, possibly with a positive effect.

Cancer is the second leading cause of death in the US, and 90% of cancer related deaths are a direct result of metastatic disease. Most of the patients diagnosed with lung cancer are asymptomatic until an advanced stage of the disease is reached. Hence, metastasis is a common occurrence at the initial diagnosis of the lung cancer patient.
Chemokine receptors have been observed to be a strong contributor to organ specific metastasis. Primary lung cancer metastasizes to the bone, brain, liver, adrenal gland and the other lung (Hillers et al., 1994). Two of the sites, the endothelial cells of the bone marrow and neuronal cells and astrocytes of the brain, constitutively express fractalkine. Hence, my hypothesis is that the CX₃CR1/fractalkine axis contributes to the progression of lung cancer.

The first step in the investigation of this perspective was to verify if lung cancer cells express the chemokine receptor CX₃CR1. Using the NSCLC cell line A549, an adenocarcinoma subset of NSCLC, it was observed via western blot that the cells do express the receptor. Using THP-1 cells, a monocytic cell line, as a relative positive comparison it was found that A549 cells expression of the protein was comparable to the THP-1 cells.

The next step was to investigate what potential functionality could the cells gain by expressing the receptor. To investigate the potential for CX₃CR1/fractalkine axis contributing to the growth and proliferation of the cells, two proliferation assays were performed. One, the MTT assay, resulted in no significant increase in growth and proliferation of cells stimulated with fractalkine relative to the negative control, which were cells suspended in medium deprived of serum. This could be a consequence of the Warburg effect, which has a phenotype of inadequate respiration and increased fermentation (Seyfried and Shelton, 2010). The core concept of the MTT assay is the assumption that active mitochondria translates to proliferation, when it actually translates to metabolism.

Therefore a second proliferation assay was done. This time the cells were counted using the hemocytometer as it was thought that a cell count could be more indicative of growth. This time a significant growth spurt was observed when the cells were stimulated with lower concentrations of fractalkine, i.e. 10 ng/ml and 25 ng/ml. This growth was even more significant
in comparison to the growth induced by the positive control, which was cells suspended in medium supplemented with 10% FBS. This result is indicative of the CX3CR1/fractalkine axis potentially contributing to the growth and proliferation of the A549 cells, which happens to be a metastatic cell line.

One curious observation was the fact that the higher concentration, i.e. 50 ng/ml, seemed to have an adverse effect on the growth of the cells. This was not intuitive, as an increase in growth was seen when comparing 10 ng/ml stimulated cells to 25 ng/ml stimulated cells. Although both concentrations resulted in significant growth over the negative control, the 25 ng/ml stimulated cells displayed a stronger growth. How could 50 ng/ml mediate a drop in proliferation? Of course the known concept of desensitization could apply to this situation, nature provides this mechanism to attenuate the cells response to the chemokine. Could it be that a higher concentration of the ligand mediates some other function, migration for example?

With that perspective in mind a scratch wound assay was performed to assess the migratory potential of the A549 cells. As stated before, A549 is a metastatic cell line. In this assay stimulation with SDF-1α/CXCL12 was used as a relative positive comparison for the migratory potential induced by stimulation with fractalkine. SDF-1α has been cited in the literature to induce migration in numerous carcinoma cell lines. It was observed that both the 50 ng/ml and 100 ng/ml concentrations mediated a migratory potential comparable to SDF-1α. Therefore the CX3CR1/fractalkine axis could also contribute to the metastatic cascade.

Now the question is what signal transduction pathways are activated when the ligand binds to the receptor? Numerous signaling assays were performed and the resulting blots were probed for specific proteins. The literature cites numerous examples of the CX3CR1/fractalkine axis activating the PI3k/Akt and MAPK/ERK signal transduction pathways in other carcinoma cell
lines. To investigate if that is also the case with lung cancer cells, some of the blots were probed for Akt and ERK. The focus on these pathways in the literature is the growth and proliferation advantage conferred by them. The perspective here is what other phenotype a higher concentration of the ligand could confer to these pathways, as these concentrations do not appear to contribute to growth.

Results of the western blots showed the PI3k/Akt pathway was activated when cells were stimulated with the 100 ng/ml concentration of fractalkine. Yet after performing a densitometry analysis it was found that a high level of confidence was reflective in the standard error. This lead to a lesson learned that densitometry analysis should be performed concurrently as blots are being generated to better evaluate the result of the blots. The ERK pathway did not appear to be significantly upregulated by stimulation with fractalkine.

The significance of this is not so much from a survival aspect. The proliferation assays revealed a negligible contribution in that context, as higher concentrations did not stimulate growth. Instead the focus is on the ability of the pathways to activate the Rho GTPases. PI3k mediates activation of P-Rex1 and PIXα, GEF’s for Rac1 and Cdc42 respectively, via its product PIP3. (Kamp et al., 2016) Cortical remodeling of the actin cytoskeleton is strongly dependent on Akt proteins. (Xue and Hemmings, 2013) ERK can potentially regulate Rac1 and RhoA. ERK phosphorylation of Rac1 alters its activity, subcellular localization and its role in mediating migration. ERK phosphorylation of RhoA enhances its activity (Tong et al., 2016).

When probing for the Rho GTPases it was found that upon normalizing the signals of the samples against the loading control the level of confidence reflective in the calculated standard error diminished any conclusive determination that fractalkine mediates the activation of the Rho GTPases. Hence with these errors it has been proven that densitometry analysis is a necessary step
to evaluate the optimization of the experiments. The low resulting confidence level in the results renders it impossible to assess the effect that stimulation with the ligand has on the activation of the RHO GTpases.

**CONCLUSION**

It cannot be emphasized enough how important it is to elucidate a means of targeting metastatic disease. Although this project focused on lung cancer, any carcinoma cells can metastasize and thus provide a formidable foe for the clinician. Chemokines and chemokine receptors have been observed to mediate the metastatic cascade, yet limited therapeutic strategies have been utilized in the clinic to target this process. Chemokine attraction for carcinoma cells, enabling dissemination and metastatic disease, is a lethal attraction for the host.


CX3CR1 is normally expressed in hematopoietic cells. Carcinoma cell expression is not shared by the normal counterparts. This provides a lucrative target specific to carcinoma cells and not toxic to normal cells. Currently there is an investigation of a small molecule antagonist to CX3CR1 in an effort to curb the metastatic cascade (Shen et al., 2016). Although the authors primary focus is on breast cancer it could potentially alleviate metastasis of numerous CX3CR1+ carcinoma cells.

Highlights of the findings include the following:
1. Using the NSCLC cell line A549, an adenocarcinoma subset of NSCLC, it was observed via western blot that the cells do express CX$_3$CR1 ($P < 0.05$). Using THP-1 cells, a monocytic cell line, as a relative positive comparison it was found that A549 cells expression of the protein was comparable to the THP-1 cells.

2. After performing a proliferation assay and cell count a significant growth spurt was observed when the cells were stimulated with lower concentrations of fractalkine, i.e. 10 ng/ml and 25 ng/ml ($P < 0.05$), indicative of the CX$_3$CR1/fractalkine axis potentially contributing to the growth and proliferation of the A549 cells, which happens to be a metastatic cell line.

3. After performing a scratch wound assay, it was observed that both the 50 ng/ml and 100 ng/ml concentrations mediated a migratory potential comparable to SDF-1$\alpha$ ($P < 0.05$). Therefore the CX$_3$CR1/fractalkine axis could also contribute to the metastatic cascade.

4. Results of western blots showed pathway was activated when cells were stimulated with the 100 ng/ml concentrations of fractalkine, no significant upregulation of ERK was observed ($P < 0.05$ for Akt, and $P = 0.9$ for ERK). After performing a densitometry analysis it was found that a high level of confidence was reflective in the standard error for the p-Akt blot. For ERK, the data indicates that the pathway is not upregulated when stimulated as the error bars did not reflect a high error rate and the $P$ value concurs with these results.
5. When probing for the Rho GTPases it was found that upon normalizing the signals of the samples against the loading control the level of confidence reflective in the calculated standard error diminished any conclusive determination that fractalkine mediates the activation of the Rho GTPases (P = 0.3 for Cdc42, P = 0.8 for RhoA and P = 0.7 for Rac1). These results were impacted by the sample size, as the data was not normally distributed. Additionally the loading control did appear to show signs of saturation which would also impact the results.

Although the results were not as expected, the lessons learned provide a wealth of understanding with regard to the optimization of experiments. These include realization that densitometry analysis is an important step in evaluating the results, and it is important to perform this step along the way. Also, the number of observations can significantly impact the statistical significance of the results, rendering an appearance of useless data. Careful optimization helps the researcher to generate results that can be better evaluated, whether the results favor or negate the hypothesis. Experimental error could always apply to lab measurements, again making densitometry analysis an important step in the progression of a research project.
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ABSTRACT

CONTRIBUTION OF CX3CR1/FRACTALKINE (CX3CL1) AXIS TO THE PROGRESSION OF NON-SMALL CELL LUNG CANCER (NSCLC)

by

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A thesis is presented on the characterization of the contribution of the chemokine axis, CX3CR1/Fractalkine, to the progression of Non-Small Cell Lung Cancer (NSCLC). Cancer is the second leading cause of death in the United States, and Lung cancer is one of the four leading causes of the disease. Carcinoma cells, unlike their normal counterparts, gain the ability to express chemokine receptors. Understanding if/how this gain of expression contributes to the progression of cancer could provide a lucrative therapeutic target. Goals of the study include investigating if the NSCLC cell line A549, an adenocarcinoma cell line, express the chemokine receptor CX3CR1, and if so what functionality the gain of expression confers on the cells. Expression of the receptor was determined by western blot, investigation of growth potential was established by performing a proliferation assay and migration potential was investigated via a scratch wound assay. CX3CR1 is normally expressed in hematopoietic cells. Carcinoma cell expression is not shared by the normal counterparts. This provides a lucrative target specific to carcinoma cells and not toxic to normal cells.
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