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CXCR4 AND IGF-IR INVOLVEMENT IN NEUROBLASTOMA MIGRATION

By

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A THESIS

Submitted in partial fulfillment of the requirements for
the degree of Masters in Sciences in Molecular and cellular Neuroscience
Graduate Program of Delaware State University

DOVER, DELAWARE
2012

Dedication

To my mother, *Seema Amiri,* my father, *Abdul Satar Amiri*, my husband *Sayed Niamat* my daughter *Sadaf Niamat*. Without your love and support, I would not probably be in this position today. I am forever grateful for the sacrifices you've made providing me the opportunity to pursue my own dreams.

I dedicate this thesis to the rest of my family (*Saghar Anvari, Sohrab Amiri, Sunita Amiri, Ahmad Saeed Amiri, Shabana Amiri, and Sameera Amiri,*), who all have been a great source of motivation and inspiration. I am very proud of my accomplishments thus far and I hope you view this chapter of my life with the same level of pride that I do. I dedicate this work to you.

To *my mentor and advisor,* Cynthia vanGolen: Without your support and devotion to this project, I do not think, any of this would have been possible.

Acknowledgement

This project could not have been finished without the supervision/guidance and assistance of an amazing group of people. For the mentorship and guidance I have received along the way, I am grateful. Starting with my mentors, Dr. Cynthia vanGolen, I would like to personally thank you for your encouragement and support. I would like to thank my committee members, Dr. Leonard Davis, Dr. Stan Ivey,Dr. Kenneth L. vanGolen, and Dr. Robert MacBride.I would like to thank you for accepting to evaluate my Master's thesis work. Your critiques on the thesis project have been invaluable and made it much easier to piece this work together into a final manuscript.

The assistance from my colleagues, friends, and our support staff was priceless to the completion of this project. I would also like to express thanks to all of the faculty and staff in the Department of Biology at Delaware State University for their guidance during my studies. I am forever grateful and deeply indebted to everyone who has supported me and I hope to have the opportunity to return the favor in the future.

Abstract

Neuroblastoma is an embryonic tumor of the autonomic nervous system, and the primary location of occurrence may be along the migration path of neural-crest derived cells. Neuroblastoma tumors generally occur in very young children; the median age at diagnosis is between 17- 22 months and approximately 90% of cases are diagnosed before age of 5. Children die of metastatic disease with metastases occurring primarily in bone, and the presence of bone metastases in children with neuroblastoma indicates a stage IV tumor with poor prognosis. In fact, the patients with the secondary tumors have a survival rate of less than 7%. Therefore, understanding how bone metastases form and identifying potential targets for treatment of metastatic disease are critical for improving stage IV patient survival. Two proteins potentially involved in signaling in NBL bone metastasis are the type I Insulin like growth factor receptor (IGF-IR) and the cytokine receptor CXCR4. In this study, we investigated the effect of IGF-IR and CXCR4 inhibition on NBL cell migration by using three different types of substrates: Collagen I, Collagen IV, and Matrigel. We show that neuroblastoma cell migration toward calf serum (CS), IGF-I, and SDF-1 is substrate specific. Migration through collagen I and collagen IV is significantly inhibited by neutralization of either CXCR-4 or IGF-IR, while invasion through Matrigel appears to be driven primarily through the IGF-IR.

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List of abbreviations

CXCR4	C-X-C chemokine receptor type 4
CS	Calf serum
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal growth factor
ERK	Extracellular related kinase
ECM	Extracellular matrix
HBSS	Hanks balanced salt solution
IGF	Insulin-like Growth factor
IGFBP	IGF binding protin
IGFBPR	IGFBP realated protein
IGF-IR	Type I IGF receptors
IGF-I	Insulin-like grwth factor 1 ligand
MMPs	Matrix metalloproteinases
MMP 9	Matrix metallopeptidase 9
MAPK	Mitogen activated protein kinase
NB-NBL	Neuroblastoma cancer
PI-3K	Phosphatidylinostiol 3-kinase
SDS-PAGE	Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SDF-I	Stromal cell-derived factor-1

General Introduction

The disease of cancer was found in Egypt around 1600 B.C where the only form of treatment was cauterization, a method to obliterate tissue with a hot device called "the fire drill". It was noted that the disease had no cure and the only option were sedative treatments. The Greek physician Hippocrates first created the word cancer in the year of 460- 370 B.C. The term of "carciono" and carcinoma" was used by the Hippocrates to designate ulcer forming a non-ulcer forming tumors (Kardinal, 1979). The word "carcinos" refers to a crab in the Greek language, for the reason tumors have finger like projections (Shimkin, 1974). Around 28-50 B.C a roman physician by the name of Celsus translated the term "crab" in to cancer to introduce it to the medical field. (Gallucci,1985).

During the 20th century advances in the field of scientific researches expanded the understanding of etiology cancer better. This particular scientific revolution started after James Watson and Francis Crick solved the structure of DNA in the 1960s and was awarded Nobel Prize in 1962 for their work. DNA is the basis to the genetic code, it is transcribed to DNA and RNA is translated to protein, in which controls the functions of the cell. After scientists learned how to translate the genetic code, they were able to categorize certain genes as oncogenes or tumor suppressors. An oncogene is a gene that contributes to the invention of a cancer. Oncogenes are mutated forms of genes that cause

cells to grow abnormally and proliferate rapidly, thereby becoming cancerous (Roidl et al., 2010). Oncogenic mutations frequently arise in genes that control cell division and differentiation, and these genes are identified as proto-oncogenes (Sun et al., 2012). Another important category of genes that play a major role in tumor formation is the tumor suppressors. This set of genes is also involved in the control of cell division, repair of DNA damage and control of cell death mechanisms such as apoptosis [(Stephen et al., 2012), (Gao et al., 2012). Mutated tumor suppressor alleles tend to be recessive whereas mutated oncogenic alleles tend to be dominant (Yoshida et al., 2000). Some cancers have been found to be secondary to particular genetic mutations and in order for the tumor cells to thrive; they need a source of nutrients.

Cancer is characterized by uncontrolled cell growth. However their growth is very different in contrast to normal cell growth. For instance, cancer cells have the tendency to continue to grow and form new abnormal cells instead of dying. Moreover cancer cells can grow into other tissues whereas the normal cells are not capable of doing it. Another characteristic of cancer cells are that they keep growing out of control and invading into the surrounding tissues and metastasizing to distant sites (Gallucci, 1985).

Today, it is widely accepted that the insulin-like growth factor 1 (IGF1) has characteristics of a circulating hormone and a tissue growth factor. The insulin-like growth factor-I receptor (IGF-IR), is known to be a transmembrane tyrosine kinase. IGF-IR is selectively activated by its ligands IGF-I and IGF-II. IGF-IR has a important role in

the growth, survival, and metastasis of a variety of human tumors, as well as tumors in children and adolescents [(Kurmasheva & Houghton, 2007),(Pollak, 2008),(Samani et al., 2007)].

Introduction

Neuroblastoma (NB) is an embryonic tumor of the autonomic nervous system, and the primary location is along the migration path of neural-crest derived cells (Bowen and Chung, 2009). Neuroblastoma is one of the second most ordinary solid tumors in childhood in the United States today. In 1864 an abdominal tumor was described by a German physician named Rudolf Virchow and he called it ("glioma'). A German pathologist Felix Marchand noted the characteristics of tumors in 1981, which is forming in the sympathetic nervous system and the adrenal medulla. In 1910 James Homer Wright implicit the tumor to be originating from the primitive neural cells, and he named it neuroblastoma. Furthermore he noted the circular clumps of cells in bone marrow samples which are now termed as the "Homer-Wright pseudorosettes (Brodeur et al., 1994).

Neuroblastoma tumors normally occur in very young children; the median age at diagnosis is between 17- 22 months and approximately 90% of cases are diagnosed before age of 5 (Maris et al., 2007). When the neuroblasts become abnormal, they tend to grow nonstop and form a tumor, this is the stage where neuroblastoma occurs. The tumors arise in the tissues of the sympathetic nervous system, usually in the adrenal medulla or paraspinal ganglia, and it can present as a mass lesions in the neck, chest,

abdomen, or pelvis (Maris, 2010). The clinical presentation of neuroblastoma is highly variable, which is usually based on the tumor size and stages of the growth the other organs. The incidence rate of neuroblastoma is 10.2 cases per million children under 15 years of age, therefore it is the most common cancer diagnosed during the first year of life (Maris, 2010).

The most frequent genetic abnormalities that are associated with neuroblasoma are the deletion of chromosomes 1p, 11q, and 14q; allelic gain of 11p and 17q; and the amplification of the MYCN proto-oncogene. The deletion of distal 1p is highly linked with both MYCN amplification as well as the patient outcome (Attiyeh et al., 2005). Moreover about 25 percent of people with neuroblastoma have a deletion of 1p36.1-1p36.3, which is related with a more harsh form of neuroblastoma, along with it is believed that the deleted region might consist of a gene that maintain cells from growing and dividing too rapidly otherwise in an abandoned way (White et al., 2005).

Neuroblastoma cells have three different subtypes, S-type, N-type, and I-type, which each of them have different morphologies along with different behaviors. S-type is the non-neuronal cells representing the glial and melanoblastic precursor cells, as well as these cells are highly substrate adherent, and are non-invasive. The N-type cells are neuroblastic , and these cells have a neuronal morphology, which are less substrate adherent, and also they are highly invasive [(Corey et al., 2010),(Wilson et al., 1992)]. I-type cells have an intermediate morphology and mixed properties of N- and S-type cells

(Walton et al., 2004). They frequently grow as mixtures of both of these cell types in culture. However some I-type cell lines behave as stem cells in which they can develop into either S-type or N-type cells [(Acosta et al., 2009), (Walton et al., 2004)].

The etiology of neuroblastoma (NB) is not clearly understood; on the other hand, both family genetic tendency and environmental exposures have been predicted as a possible cause. In addition various environmental factors including drugs, chemicals, and viruses have been examined in epidemiologic studies and shown as a possible contributing factors as will, however the results of these studies have not defined any true etiologic factor [(al-Shammri et al., 1992),(Olshan et al., 1999),(De Roos et al., 2001)].

Children die of metastatic disease with metastases occurring primarily in bone, and the presence of bone metastases in children with NB indicates a stage IV tumor with poor prognosis. In fact, NB patients with secondary tumors in bone have a survival rate of less than 7%. Therefore understanding how bone metastases form and identifying potential targets for treatment of metastatic disease are critical for improving stage IV patient survival (van Golen et al., 2006). Two proteins potentially involved in signaling in neuroblastoma bone metastasis which are the type I insulin-like growth factor receptor (IGF-IR) and the chemokine receptor CXCR4.

Tumor Cell Migration

Cell migration is very essential to the morphogenesis of embryos. The movement of the migratory cells causes gastrulation, formation of the layers in the embryo and the development of organs and tissues. Furthermore, cell migration is a primary factor of the homeostasis of the individual adult. There are two common types of cell migration, the movements of cell sheets and the birth of homogeneous cells in epithelial layers, as well as their movement to distant targets (Mierke et al., 2008).

The capability of a cancer cell to go under migration and invasion permit it to change its position in the tissues. For instance, these procedures permit the cells to enter the blood vessels for distribution into the circulation, and then undergo metastatic growth in distant organs (Chambers et al., 2002). In order for the cells to spread within the tissues, the tumor cells use a migration mechanisms which is similar to the one that arise in normal cells throughout physiological processes such as embryonic morphogenesis, wound healing and immune-cell trafficking (Friedl & Brocker, 2000). However it is said that the metastatic tumor cells normally travel at a faster speed in contrast to the normal cells. Therefore this elucidate the theory of which an increased cell migration is required for cells to become metastatic [(Friedl & Brocker, 2000), (Aman & Piotrowski, 2011)]. First in order for a tumor cell to invade into local tissue at the primary site, first it must move away from the primary site of the tumor cells. Furthermore in order for the tumor

cell to go through the basement membrane, it must move independently across the membrane in a persistent process in which requires degradation of connective tissue along with movement through the tissues [(Chiang et al., 2012), (Rolli et al., 2010)]. Afterward the cells degradate through the blood stream and existing the blood stream at the secondary site which is normally the lung and liver (Birgersdotter et al., 2005). After arriving in the new site the tumor cells frequently migrate away from poorly oxygenated sites to the highly oxygenated sites. Furthermore the earliest metastases appear as single cells or small colonies of cells that wrap around arterioles in the secondary site [(Friedl & Brocker, 2000),(Baker et al., 2011)].

The extracellular matrix (ECM) provides the substrate, as well as a barrier towards the advancing cell body. However the cell migration throughout the tissues results from a permanent cycle of interdependent steps [(Derycke & Bracke, 2004) (Friedl & Brocker, 2000), (Friedl & Wolf, 2003)]. Fundamentally, metastasis proceeds via the migration and invasion of cancer cells through variable extracellular matrix (ECM) environments, and studies have shown that cell migration is definitely sensitive to matrix mechanical properties [(Lo et al., 2000), (Ulrich et al., 2009)].

The Chemokine Receptor

Chemokines are small, and they are cytokine-like proteins structuring a large super family. Chemokines were first found to be a main mediators of leukocyte

chemoattraction in inflammation and immune cell homing and recirculation [(Meier et al., 2007) (Carlisle et al., 2009)]. They are also found to induce integrin activation and promoting cell adhesion and directional migration, when they are in the lead of binding to their cognate seven-transmembrane spanning G-protein-coupled receptors [(Baggiolini et al., 1997), (Campbell & Butcher, 2000), (Zlotnik & Yoshie, 2000)]. In addition they are shown to have a role in tumor biology [(Muller et al., 2001),(Balkwill, 2004a)].

A chemokine receptor called CXCR4 is shown to be generally found on tumor cells, it express in about 23 different types of cancers (Balkwill, 2004b). CXCR4 is expressed on the cell surface of most human endothelium, epithelium and leukocyte populations[(Murdoch et al., 1999), (Eddleston et al., 2002) (Yun and Jo, 2003)]. Along with various other types of tumor cells, such as nonsmall cell lung cancer (Phillips et al., 2003), prostate cancer (Taichman et al., 2002), colon carcinoma (Ottaiano et al., 2005), neuroblastoma (Geminder et al., 2001), kidney cancer (Schrader et al., 2002), pancreatic cancer (Koshiba et al., 2000), breast cancer (Muller et al., 2001), rhabdomyosarcoma (Libura et al., 2002), and hepatocellular carcinoma (Xiang et al., 2009). The biding of the CXCR4 ligand CXCL12 (known as SDF-1) results to mediate directed migration of cancer cells to the sites of metastasis (Balkwill, 2004b),(Balkwill, 2003). However the primary role of chemokines and there receptor are found to be involved in regulating the recruitment and trafficking of the leukocyte subsets to the inflammatory sites. Whereas

activating the leukocyte integrins results in binding to their adhesion receptors on endothelial cells [(Kulbe et al., 2004) (Bajetto et al., 2001)]. Moreover some latest reports indicate the expression of CXCR4 on neuroblastoma metastasis in bone marrow, and reported to be contributing to neuroblastoma tumor cells homing to the bone marrow [(Wysoczynski et al., 2005), (Zhang et al., 2007)]. In addition CXCR4 and CXCL12 are believed to be involved in tumor growth and metastasis to the bone marrow (Zhang et al., 2007)

Role of CXCR4 in Neuroblastoma

The expression of CXCR4 is found to be regulated in neuroblastoma and can be regulated by different types of tissue stroma, chemokines are involved in neuronal cell migration and guiding [(Laudanna & Alon, 2006), (Zhang et al., 2007) (Laudanna & Alon, 2006)]. Neuroblastoma along with many other type of cancers tend to express a broad network of chemokines and chemokine receptors (Vicari & Caux, 2002). Moreover other investigatigator in in-vitro studies using neuroblastoma cell lines found that CXCR4 have a role in neuroblastoma pathophysiology (Balkwill, 2004b). However in some other studies it is purposed that neuroblastoma cell lines express CXCR4 and bind to CXCR4 ligand, stromal-derived factor-1 (SDF-1), which this is resulting in induces tumor cell migration (Geminder et al., 2001).

Recent information indicate that CXCR4 is generally expressed on neuroblastoma metastasis in the bone marrow, and it could be actively contributing to neuroblastoma tumor cell homing to the bone marrow (Zhang et al., 2007). Furthermore it is demonstrated that the CXCR4 over expression is results in promoting neuroblastoma primary and secondary tumor growth but on the other hand it does not promotes neuroblastoma invasion [(Joseph et al., 2005), (Meier et al., 2007)].

Role of CXCR4 in migration

The primary role of chemokines and its receptors is to regulate the recruitment and trafficking of leukocyte subsets to inflammatory sites in the course of chemoattraction. In which these happen as a result of leukocyte integrins activation that has bind to their adhesion receptors on endothelial cells (Laudanna & Alon, 2006). The expression of extensive network of chomokines and chemokine receptors have been found to be present in several other type of cancers (Vicari & Caux, 2002). Chemokines are able to couple to distinct signaling pathways (Wysoczynski et al., 2005).

The chemokine signaling system consists of over 43 extracellular protein ligands, along with18 G protein-coupled receptors which coordinate leukocyte trafficking and control immunity, inflammation, HIV infection and development, therefore it is concluded that chemokines are the central regulators of directed cell migration [(McGrath et al., 1999), (Simmons et al., 1998) (Murdoch & Finn, 2000)]. SDF-1

(Stromal Derived Factor 1, also known as CXCL12) is found to be a secreted protein and is a chemoattractant for T lymphocytes and monocytes in vitro and in vivo studies. SDF-1 shows to have an effects on proliferation and differentiation of a variety of stem cell populations, and CXCR4 is the receptor for SDF-1 and is believed to be G_i-coupled since signaling is inhibited by pertussis toxin [(Aiuti et al., 1997), (Lieberam et al., 2005)]. CXCR4 is reported to mediate directed migration of cancer cells to the sites of metastasis through the binding of its cognate ligand CXCL12 (SDF-1) [(Balkwill, 2003) (Marchesi et al., 2004)]. Chemokines are also involved in neuronal cell migration and patterning (Bajetto et al., 2001).

CXCR4 Inhibitors

The binding of the CXCL12, which is the ligand for the CXCR4, leads to the activation and migration of immune cells. In addition, it also yields other functions, such as growth, and embryonic development, mainly in the formation of blood vessels (Mancuso et al., 2010). Several type of CXCR4 inhibitor is being used in either research institution or clinical trials. T140 is a CXCR4 inhibitors which also acts as a carrier for AZT, and it is targeting the CXCR4 (Tamamura et al., 2001). T134 (EC50, 53 nM) is another potential inhibitor which work similar to the T140, and it interferes with HIV-1 infection by blocking CXCR4 (De Clercq et al., 1994). CGP64222 is developed with arginine and lysine, and it inhibits the Tat/TAR RNA (transactivation response element)

interaction to suppress the HIV-1 replication, competing with Tat for binding to the transactivation response element (TAR) it also blocks the co-receptor CXCR4 to inhibit viral infection (Litovchick et al., 2000). AMD3100 is also a highly potent and selective anti-HIV-1 and HIV-2 inhibitor, which blocks HIV-1 entry by binding to the co-receptor CXCR4 [(Li et al., 2008; Schols et al., 1997)]. Researchers reported that the CXCR4 inhibitor AMD3100 encourages disruption of the interaction of multiple myeloma MM cells (Azab et al., 2009). AMD070, or AMD3100 derivative, is a potent orally available CXCR4 antagonist which strongly inhibits virus infectivity with EC50 of 1–10 nM and currently being used in Phase I clinical trials (Murakami & Yamamoto, 2010). Neutralizing antibody CXCR4 is also shown to be used as an inhibitor for the CXCR4 receptor (Alsayed et al., 2007)

The Insulin-like growth factors

The insulin-like growth factors (IGFs) are important for normal fetal development, development and postnatal growth. Iinsulin-like growth factors (IGFs) are well known as key regulators of energy metabolism and growth (Pollak, 2008). The IGF family consists of two ligands, IGF-I and IGF-II, two receptors, IGF-IR and IGF-IIR, six binding proteins (IGFBPs), and several IGFBP related proteins (IGFBPRPs) (Jones and Clemmons, 1995); (Pollak et al., 2004). The IGFBP and IGFBP-rP are known to be a part of the cell surface receptor proteins, along with the IGFBP and IGFBP-rP proteases. In

which also these IGFBP proteases are shown to be a different integral factor of the IGF system (Vorwerk et al., 2002).

IGF Ligands

One family of factors theoretically involved in neuroblastoma metastatic progression is the insulin-like growth factor (IGF) family (van Golen et al., 2006). This IGF family comprises of two ligands, IGF-I and IGF-II, in which their known actions occur through type I IGF receptor (IGF-IR) activation and signaling [(van Golen et al., 2006), (Foulstone et al., 2005)]. Insulin-like growth factors (IGF-1 and IGF-2) are greatly homologous, low molecular weight, single chain polypeptides that are structurally similar to pro-insulin. IGF-1 and IGF-2 both bind to the IGF-1R, and IGF1 has individuality of both a circulating hormone and a tissue growth factor. The majority of IGF-1 which is found in the circulation is produced by the liver. Therefore the regulation of hepatic IGF-1 production is very complex (Wang et al., 2010). Up-regulating of IGF-I gene expression has been linked to a dominant role in the Growth hormone (GH), while its stimulatory influence is clearly reduced by starvation (Thissen et al., 1994). The insulin and the IGFs family's growth factors are well accepted as key regulators of energy metabolism and growth (Pollak, 2008). Insulin-like growth factor II (IGF-II)' and IGF-I are chemically similar peptides in they also share the same sequence homology with insulin [(Ullrich et al., 1986), (Morgan et al., 1987),(Conover et al., 1987). IGF-I

mediates the growth promoting effects of growth hormone during postnatal growth (Furlanetto et al., 1977), (Kurtz et al., 1988).

The IGF Receptors

The insulin-like growth factor-I receptor (IGF-IR), a transmembrane is a tyrosine kinase that is activated by its ligands IGF-I and IGF-II. The IGF-IR is also consists of an alpha- along with a Beta-subunits, which are disulfide-linked in a Beta-alpha-alpha-Beta arrangement in the mature receptor. The two ligands have been strongly associated in the, survival, growth, and metastasis of a wide variety of human tumors (Foulstone et al., 2005). IGF-IR overexpression promotes tumor growth, progression, invasion, and metastasis (Foulstone et al., 2005), (van Golen et al., 2006). On the other hand disruption of IGF-IR expression shows to reverses the transformed phenotype (Adams et al., 2000). Increased IGF-I, IGF-II, and IGF-IR expression is present in neuroblastoma, and their ligand IGF-I or IGF-II coupled to IGF-IR promotes neuroblastoma cell survival and growth [(Kiess et al., 1997), (Liu et al., 1998)]. Research have been consistently showing that down regulation of the IGF-1R in which causes growth inhibition and apoptosis of cancer cells (Mauro et al., 2003). Several other type of tumors or tumor cells have been shown to be affected by IGF-1R targeting, such as glioblastoma, (Resnicoff et al., 1994) melanoma,(Macaulay et al., 2001), neuroblastoma (Liu et al., 1998) prostate cancer (Reiss et al., 1998), rhabdomyosarcoma (Kalebic et al., 1994) lung cancer (Lee et al.,

1996) Ewing's sarcoma (Scotlandi et al., 2002), medulloblastoma (Wang et al., 2001) and others.

Role of IGF (system) In Neuroblastoma

The IGF family consists of two ligands, IGF-I and IGF-II, whose known actions happen through type I IGF receptor (IGF-IR) activation and signaling. Moreover the IGF family is potentially involved in neuroblastoma metastatic and progression. The overexpression of IGF-IR promotes invasion, progression, tumor growth, and metastasis (Foulstone et al., 2005) whereas the disruption of IGF-IR expression reverses the transformed phenotype, and a high level of expression of these two ligand has been to be present in neuroblastoma (Adams et al., 2000).

The insulin-like growth factors, IGF-I and IGF-II, are intricately involved in the proliferation and differentiation of a large number of cell types, and mainly in neuroblastoma cells. Moreover their mitogenic effects are transmitted via a type 1 membrane receptor that has tyrosine kinase activity [(El-Badry et al. 1991, (Stewart & Rotwein 1996)]. Furthermore IGF-I or IGF-II coupled to IGF-IR promotes neuroblastoma cell survival and growth , whereas the IGF-IR inhibition shown to induce tumor regression in mice (Piriz et al., 2011).

The IGFs and IGF receptors are expressed in both neuroblastoma cell lines and primary tumors, and IGF expression is present in all stages of neuroblastoma, signifying

that the IGF system is involved in many aspects of neuroblastoma tumorigenesis (Grellier et al., 2002),(Kim et al., 2004; Wang et al., 2010)].

Role of IGF (system) on migration

Insulin-like growth factor-1 (IGF-1) is a neurotrophic factor and plays an important role in promoting axonal growth from neurons (Ding et al., 2006). IGF-1 has been shown to promote the survival of motor neurons, neurite outgrowth and neuronal migration [(Leinninger et al., 2004), (Xiang et al., 2011)]. Investigators have found that these mechanisms might be due to an increase level of IGF-I in cyclase response element binding mediated transcription (CREB). The CREB in the DRG neuronal nuclei is stimulating the L-type calcium channels which improve the survival of the cells and also regulates the expression of myelin-specific proteins of Schwann cells (Sin et al., 2009)

IGF-IR Inhibitors

Ssynthesizing selective inhibitors for IGF-1R shown to be very difficult, due to the similarity of the structures of IGF-1R and the insulin receptor (IR), particularly in the regions of the ATP binding site and tyrosine kinase regions (Weroha and Haluska, 2008). Prominent in current research are three main classes of inhibitor. The first classes are Tyrphostins such as AG538 and AG1024 and these are in early pre-clinical testing (Blum et al., 2000). Both AG 538 and I-OMe AG 538 inhibit IGR-1R autophosphorylation in intact cells and in a dose-dependent manner but I-OMe-AG 538 is shown to be better

than the other inhibitors (Li et al., 2009). However the I-OMe AG 538 is found to be more hydrophobic and less sensitive to oxidation than AG 538 (Riedemann & Macaulay, 2006). The second class is Pyrrolo (2, 3-d)-pyrimidine derivatives such as NVP-AEW41, which show far greater (100 fold) selectivity towards IGF-1R (Garcia-Echeverria et al., 2004). The third class is monoclonal antibodies which are probably the most specific and promising therapeutic compounds.

Rationale

The tumor microenvironment is made up of a number of cell types including fibroblasts, pericytes, endothelial cells, macrophages, and neoplastic cells (Joyce, 2005). The interaction between cellular components of the microenvironment is critical for processes such as tumor growth, angiogenesis, and metastasis (Solinas et al., 2009). We hypothesize that IGF-IR and CXCR4 receptor signaling pathways interact in neuroblastoma to promote metastasis and it is also hypothesized that two distinct pathways affect one another to increase neuroblastoma migration, invasion, and metastasis. Therefore the purpose of the current study was to determine the effect of IGF-IR and CXCR4 inhibition on NBL cell migration by using three different type of substrate Collagen I, Collagen IV, and Matrigel.

We tested this hypothesis by performing the following aims;

a. Determine the effect of IGF-IR inhibition on CXCR4 expression

b. Determine the effect CXCR4 activation on IGF-IR expression

c. Determine the effect of IGF-IR and CXCR4 inhibition on NBL cell migration.

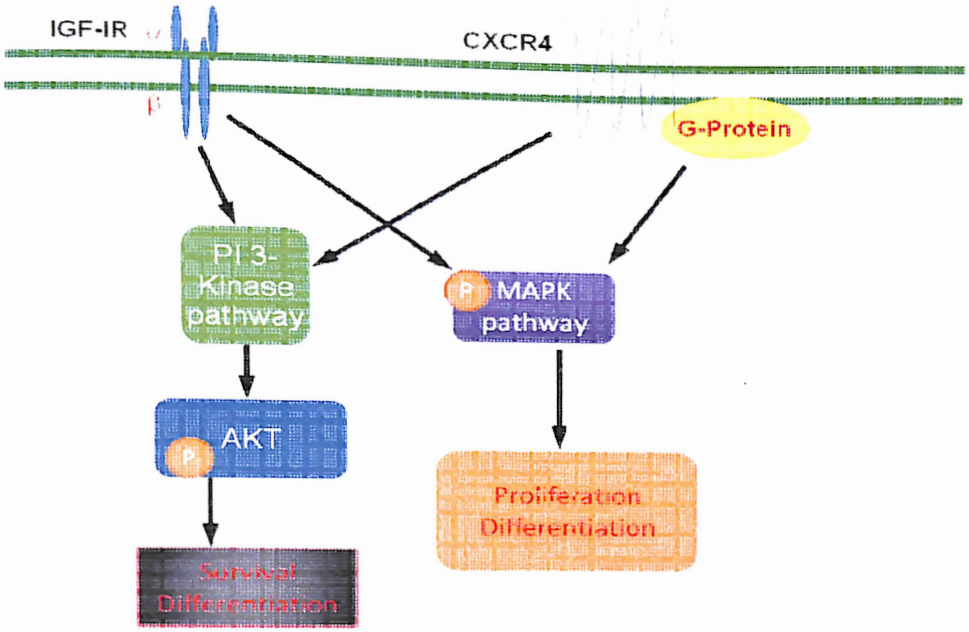

Figure 1: This figure indicate the location of the IGF-IR and CXCR4 protein at the receptor level and the downstream pathways which is involved for each of these proteins.

Materials and Methods

Table 1

The list of antibodies

Type	Target	Species Reactivity	Application
Antibody	SDF-1∞	Human	Migration assay
Antibody	SDF-1β	Human	Migration assay
Antibody	CXCR4	Anti-human	Western Blot & migration assay
Antibody	IGF-I	Human	Western Blot & migration assay
Antibody	IGF-IR	Human	Western Blot & migration assay
Antibody	Anti-rabbit IgG	HRP conjugated	Western Blot

IMR-32

In April, 1967 IMR-32 cell line was established by W.W. Nichols, J. Lee and S. D from a mass occurring in a 13-month-old Caucasian male abdominal area (Tumilowicz et al., 1970). The IMR-32 cell line was proposed to the American Type Culture Collection (ATCC) when it was in the 36th passage, it has been indicated that the IMR-32 cell line can proliferate up to or behind 80th serial subculture.

In our experiments the cells were cultured in EMEM (ATCC, Manassas, VA) containing 10% FBS, purchased from ATCC (Manassas, VA) and 1% Penicillin-Streptomycin purchased from MP Biomedical, llC (MP Solon Ohio). The cells were grown in DMEM and EMEM, 10% FBS + 1% Penicillin. All cell lines were incubated at 37C in a humidified atmosphere with 5% CO2. Cells were routinely subculture using trypsin-EDTA (ATCC Manassas, VA). The medium was replaced every 2-3 days and the cultured cells were split once their confluence level reached 70-90%.

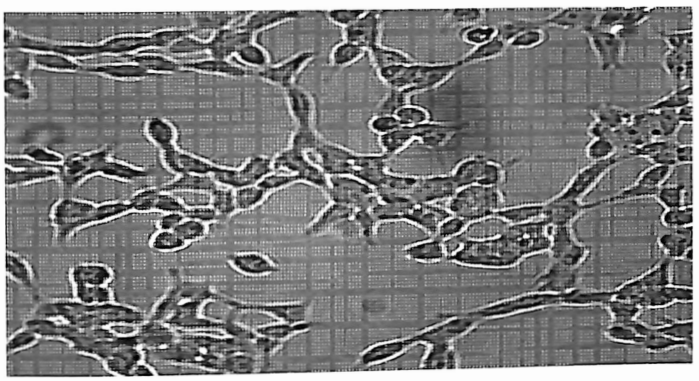

Figure 2: IMR-32 cultured in DMEM media with serum.

Western- Immunoblotting

The cells were typsinized to dislodge adherent cells from the culture. The cell pellets were washed with HBSS purchased form Sigma-Aldrich (St.Louis, MO). Six plates were labeled with different concentration and were serum deprived for 12 hours. One plate was feed from the media containing serum, and one with no serum and no treatment (as a control), and then the plates were set in the incubator for 12 hours. After the 12 hours or the next day the plates were treated with the antibody of the interest (CXCR4, IGF-IR, or SDF-1) at different concentrations and placed in the incubator for 30min. After 30min the plates were washed with cold PBS and resuspended with RIPA buffer solution (RIPA buffer solution was made from 2000μL RIPA buffer 1X + 20μL Phospatease cocktail inhibitor set 1 and 20μL protease cocktail inhibitor set II) and set on ice for 5 minutes. After the 5 minutes the cells were script off from each plate and put in separate conical tubes. The cell lysates were placed on a labeled box and stored at -20 °C. The protein concentration was determined using a SmartSpec Plus Spectrophotometer (Bio-RAD, Hercules CA). The cell lysates were fractionated on a 12% SDS polyacrylamide gel (Bio-RAD, Hercules CA). The proteins were transferred to a nitrocellulose membrane either overnight (35 volts) at 4°C. The nitrocellulose membrane was washed with 1x Tris Buffered Saline (TBS) for 5 minutes and incubated in blocking solution containing 5% milk (nonfat dry) dissolved in TBS containing 0.1% Tween 20 for 2 hours at room temperature. The membrane was washed 3 times for 5

minutes each in TBS/T (1x TBS + 0.1% Tween-20) buffer. The membrane was either incubated in primary antibody (appropriate dilution per the instructions of the manufacturer) over night at 4°C with gentle agitation. The membrane was washed with TBST and followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:2000) for 1 hour at room temperature with gentle shaking. Furthermore blots were developed using the eagle eye device, the blots which are shown in this thesis are one of at least three independent experiments done, a schematic drawing of western blotting is shown in figure 3 below.

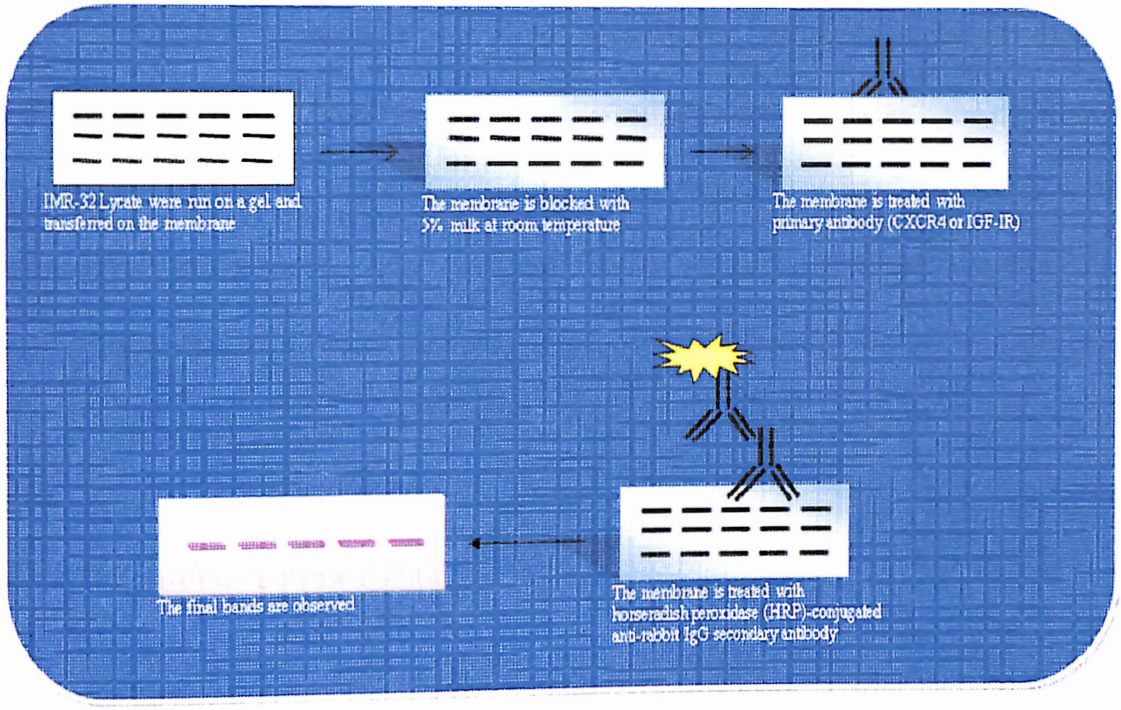

Figure 3: An outline of the procedure of the western immunoblating used to study the expression of CXCR4 and IGF-IR using IMR-32 cell line.

Migration assays

The migration assay was performed as described previously (van Golen et al., 2006). In this study three type of substrate were used for migration assay and then the results were compared, the substrates were Collagen I, Collagen IV, and Matrigel coated inserts purchased from BD, biosciences (Bedford, MA). IMR32 cells were trypsinized, counted using a Hemocytometer, and grown on the upper surface of transwell filters coated with collagen I, IV or Matrigel transwell filters. The transwell filters were incubated at 37°C in 5% CO_2 in serum medium for 48 hours for collagen I and IV and 22Hr for Matrigel. After 48 hours, IMR-32 cells that had not migrated through the membrane were washed off and removed from the upper surface of the membrane using a wet Q-tip. The IMR-32 cells that had migrated were then stained with 0.5% crystal violet prepared with methanol. After washing, the inserts were left to dry overnight at room temperature, the subsequent day they were quantitated under the microscope. 10% calf serum in DMEM was used as a positive control; a schematic of transwell migration assay is shown in figure 4 next page.

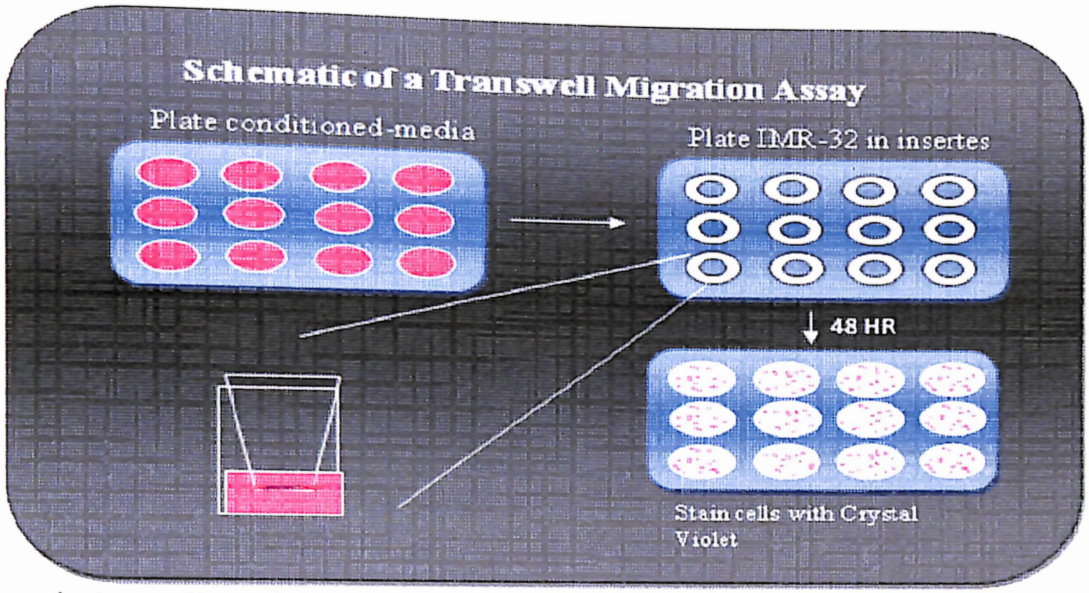

Figure 4: An outline of the procedure of the Boyden Chamber Chemotaxis assay used to study migration of IMR-32 cells.

Statistical Analysis

Data is presented as an average +/- standard deviation (S.D.). The student T- test analysis was used for comparisons using online software called "quick calcs" (GraphPad's). The results were considered statistically significant at a p-value < 0.04, and at a p-value < 0.001. T-test compares the means of two groups and is one of the most common tests which are being used by scientists today. The t-test can be used when two groups of data are used such as treated and untreated, which can be done by using one of the online software. The way the software work is that when the data points are placed in the t-test table the software calculate a t ratio, where the t ratio is the difference between sample means which are divided by the standard error of the difference, in which is

calculated by pooling the standard error means of the two groups. If the difference is large compared to the standard error of the difference, then the t ratio will be larger, and the P value is smaller. Therefore from this p-value the data can be concluded to be statistically significant or not significant.

Results

Effect of CXCR4 expression on IGF-IR inhibition

Several of the G-protein linked receptors are known to play an essential role in cancer metastasis, survival and proliferation. Therefore, some of these receptors are becoming the attractive targets for pharmacological approaches. Recently a potential targets have been identified for anti-metastatic therapies which is the $G_{\alpha i}$-protein linked receptor CXCR4 that binds to the α-chemokine stromal derived factor-1 (SDF-1)(Schier, 2003). This receptor family includes other members, such as or named as the seven-transmembrane-spanning proteins that are residing primarily in plasma membrane that transduce signals by coupling to guanine nucleotide-binding proteins (G-proteins)(Schier, 2003). Some of these chemokines are shown to modulate cell survival and growth (Horuk, 2001). It has been shown that cells from almost all cancer types were found to express CXCR4 and be responsive to SDF-1 gradient (Yasuoka et al., 2008). Neuroblasoma has been shown to be expressing both of the SDF-1 receptors, CXCR4 and CXCR7.Also the expression of CXCR4 has been shown by other studies on a few more neuroblastoma cell lines not just IMR-32: on SK-NSH and its noradrenergic subclone SH-SY5Y[(Sanders et al., 2000),(Catani et al., 2000)]. In the present study, we show the expression of CXCR4 using IMR-32 neuroblastoma cell line, which we are able to show the expression of CXCR4 on IGF-IR inhibition.

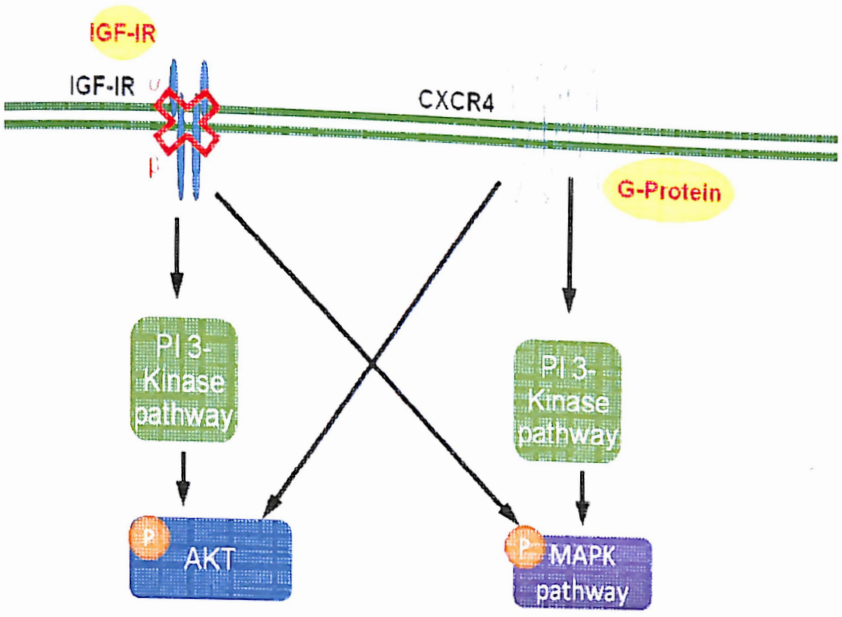

Figure 5: Effect of CXCR4 expression on IGF-IR inhibition.

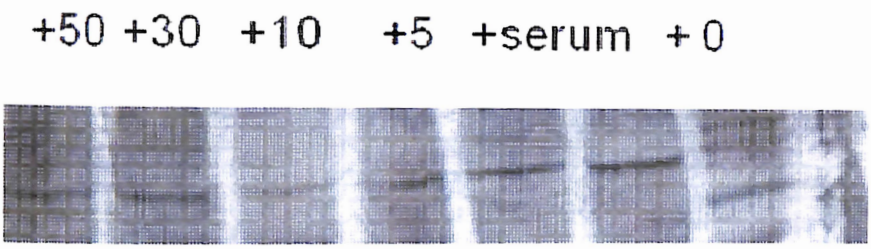

Figure 6: Whole cell lysates were collected from IMR32 cells and were treated with IGF-IR neutralizing antibody and the CXCR4 primary antibody was used to look at CXCR4 expression. Lysates were run on a SDS-PAGE gel and Western immunoblotted for the CXCR4.

The IMR-32 cell lysates were collected and treated with IGF-IR, meaning the IGF-IR receptor is blocked as shown in figure 5. Six plates of cells were treated with: zero concentration with serum, zero without serum (these two are used as control group), and the next 4 plates were treated with concentrations of 5μg, 10 μg, 30 μg, and 50 μg. Then the lysates were run on a SDS-PAGE gel and western immunoblotted to look at CXCR4 expression. Our result in figure 6 shows the expression of CXCR4 on IGF-IR inhibition. In addition our results in figure 6 also demonstrate that the IGF-IR inhibition does not affect CXCR4 levels.

Effect of IGF-IR expression CXCR4 activation

The pathogenesis of neuroblastoma is related to expression of the type 1 insulin-like growth factor receptor (IGF1R) and to transcription factor MYC-N amplification (Grandinetti et al., 2006). Previous studies have shown that MYC-N expression is disrupted by blockade of the IGF-1R with a specific monoclonal antibody, αIR3 (Hartford & Ratain, 2007).

In the present study, we are trying to show the expression of IGF-IR using IMR-32 neuroblastoma cell line. We are showing the expression of IGF-IR on CXCR4 activation.

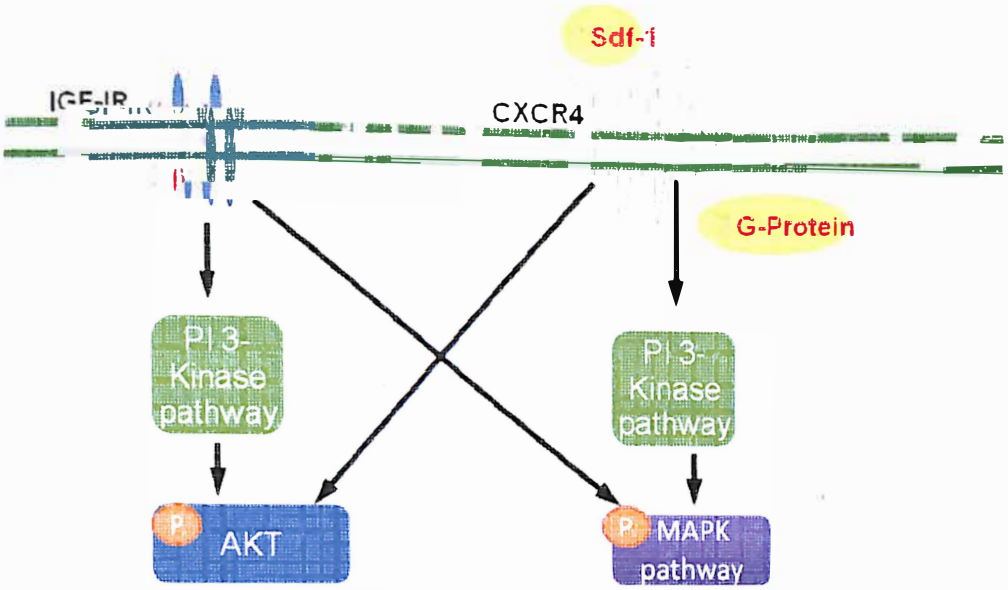

Figure 7: Effect of IGF-IR expression on CXCR4 activation.

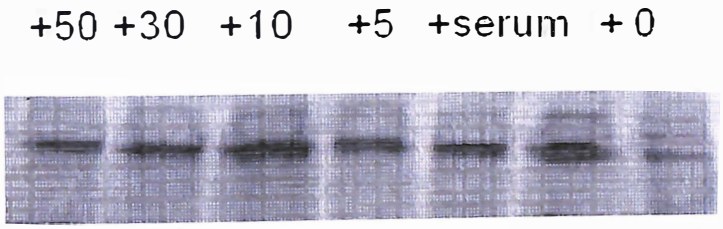

Figure 8: Whole cell lysates were collected from IMR32 cells and were treated with SDF-Iα, and used IGF-IR primary antibody to look at the expression of IGF-IR receptor.

The IMR-32 cell lysates were collected and treated with SDF-1α, which this will result in activation of the CXCR4, and we looked at the expression of the IGF-IR receptor when the CXCR4 is activated. Therefore six plate of cells were treated each at different concentration from zero concentration of antibody with serum and zero amount of antibody without serum which zero concentration are used as control and then another 4 plates containing 5μg, 10 μg, 30 μg, and 50 μg. Then, after the lysates were collected they were analyzed by using a spectrophotometer doing a Lowery protein assay to find the exact concentration of each of proteins. Then the lysates were run on a SDS-PAGE gel and western immunoblotted to look at the expression of the IGF-IR. In figure 8, our results show the expression of the IGF-IR at 100kd, which it has been also found by other members of the lab. In addition our results in figures 8 also demonstrate that CXCR4 activation does not alter the expression of IGF-IR.

Effect of IGF-IR and CXCR4 inhibition on NBL cell migration

CXCR4 receptor plays a fundamental role in bone marrow homing of the hemopoietic cells (Peled et al., 2000). Moreover one of the conditions for using of a similar mechanism by the neuroblastoma cells is the expression of CXCR4. Therefore in our study we identified CXCR4 expression as a possible general characteristic of neuroblastoma cells. We have further analyzed the effect of IGF-IR and CXCR4

inhibition on NBL cell migration through three different types of ECM substrate: Collagen I, Collagen IV, and Matrigel.

Collagen is a extracellular matrix (ECM) protein which is being used for a wide variety of research experiment, such as cell culture, cell attachment, growth, migration, and tissue morphogenesis [(Okuyama et al., 1981),(Buehler, 2008)]. Collagen I is suggested to be one of the most frequently used substrate by research investigators. It consist of up to 90% of the skeletons of the mammals along with it is widespread in all over the body, in addition to bones, it is also found in skin, tendons, ligaments, cornea, intervertebral disks, dentine, arteries and granulation tissues as the main locations [(Barnard et al., 1993; Park et al., 2003), (Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition New York.: W.H. Freman; 2000).

Type IV collagen is another type of collagen, in which type IV is a unique member of the large superfamily which it comprise at least 28 different types in vertebrates, distinct most of the other collagens, type IV collagen is an limited member of the basement membranes, it forms molecular networks that influence cell adhesion, migration, and differentiation, through a complex intermolecular and intramolecular interactions[(Veit et al., 2006), (Khoshnoodi et al., 2008)].

Matrigel matrix is a solubilized basement membrane. It is extracted from the Engelbreth-holm-Swarm mouse sarcoma. It contains laminin, collagen IV, entactin and

growth factors, including TGF beta, basic FGF and more (Kleinman & Martin, 2005). The involvement of ECM receptors as well as matrix degrading enzymes has been distinguished by the invasion of tumor cells into Matrigel (Joshi et al., 2010).

Metastasis is a nonrandom procedure; therefore certain types of cancer favorably metastasize to particular sites, although other types support other distant sites for metastasis formation. The bone marrow is a main preferential, and common metastatic site for several types of cancer, including breast and prostate carcinoma as well as neuroblastoma [(Seeger et al., 2000),(Fukuda et al., 2001)].

Other studies have shown that recombinant human SDF-1α induces the migration of CXCR4-expressing SH-SY5Y neuroblastoma cells in CXCR4- and heterotrimeric G -protein-dependent manners (Catani et al., 2000). Furthermore SH-SY5Y which is a type of neuroblastoma cell line shown to interact at multiple levels with bone marrow constituents, which this prove that bone marrow promote SH-SY5Y cell migration, adhesion to bone marrow stromal cells, and proliferation (Seeger et al., 2000).

In this study our goal was to determine the effect of IGF-IR and CXCR4 inhibition on NBL cell migration by using three different type of substrate Collagen I, Collagen IV, and Matrigel. To compare and see if the neuroblastoma cell migration toward calf serum (CS), IGF-I and SDF-1 is substrate specific. A transwell migration assay was performed by plating IMR32 treated with or without IGF-IR or CXCR4

neutralizing antibodies in the upper chamber and serum, IGF-I, or SDF-1 in the lower chamber. The migration assay was carried out for 48 hours and then the cells were fixed afterwards and stained and washed with deionized water, than the inserts were left to get dry overnight, the next day the cells were counted before plating using a hemocytometer and after migration using a microscope.

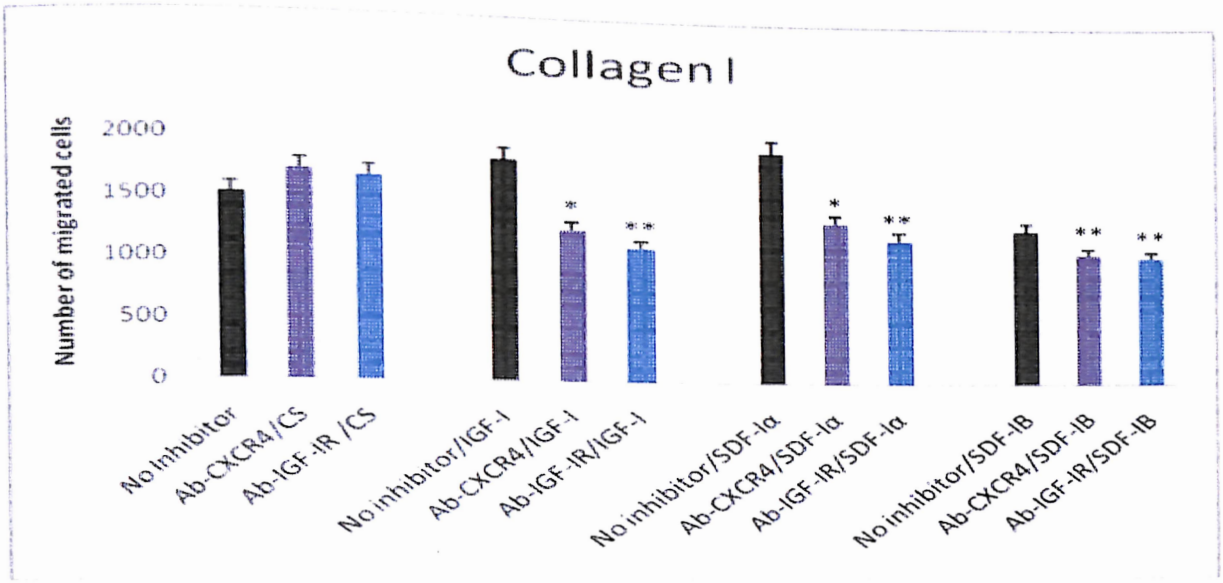

Figure 9: Effect of IGF-IR and CXCR4 inhibition on NBL cell migration using collagen I substrate. IMR-32 cells were treated with or without IGF-IR or CXCR4 neutralizing antibodies in the upper chamber and serum, IGF-I, or SDF-1 in the lower chamber. The cells were counted before plating using a hemocytometer and after migration using a microscope. The statistical analysis was performed using student T-test analysis by using an online database (quick calcs), values are represented as mean ± SEM for each condition. An asterisk (*) represents statistical significance ($P < 0.04$) compared to the control (no ligand), and the (**) represents statistical significance ($P < 0.001$) compared to control.

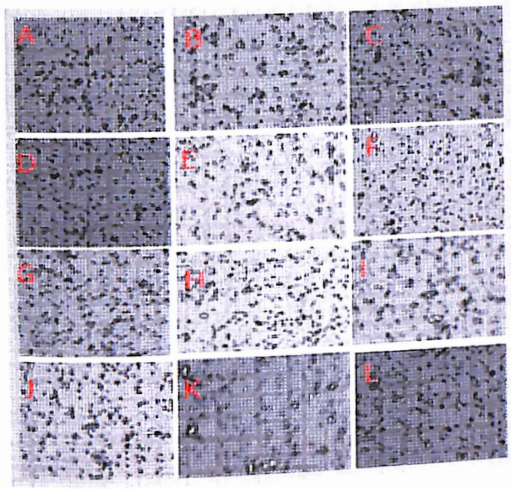

Figure 10: Images of migrated cells using collagen I inserts. (A) No Inhibitor. (B) Ab-CXCR4/CS. (C) Ab-IGF-IR /CS. (D) No inhibitor/IGF-I. (E) Ab-CXCR4/IGF-I. (F) Ab-IGF-IR/IGF-I. (G) No inhibitor/SDF-Iα. (H) Ab-CXCR4/SDF-Iα. (I) Ab-IGF-IR/SDF-Iα. (J) No inhibitor/SDF-IB. (K) Ab-CXCR4/SDF-IB. (L) Ab-IGF-IR/SDF-IB.

When the cells are plated on collagen I, we see more migration toward IGF-I and SDF-Iα than SDF-Iβ. Interestingly, both the CXCR4 neutralizing antibody and the IGF-IR neutralizing antibody have a noticeable effect on preventing migration toward SDF-I and IGF-I, as demonstrated by a one-third reduction in the number of cells that migrated through the collagen I-coated filters.

We next studied the role of collagen IV in migration. Collagen IV is a main element of the basement membranes throughout the body along with lamini, and it is shown to have the ability to bind to cell surface proteins and the extracellular matrix proteins (Rosca et al., 2012).

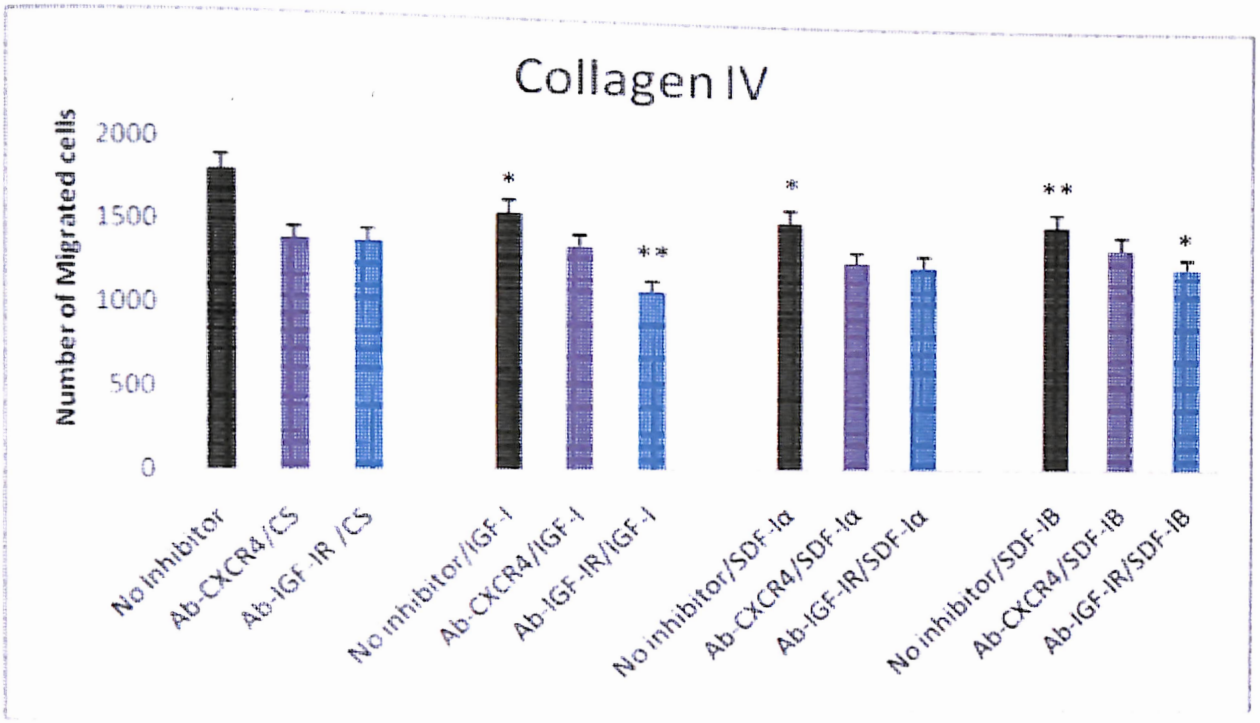

Figure 11: Effect of IGF-IR and CXCR4 inhibition on NBL cell migration using collagen IV substrate. IMR-32 cells were treated with or without IGF-IR or CXCR4 neutralizing antibodies in the upper chamber and serum, IGF-I, or SDF-1 in the lower chamber. The cells were counted before plating using a hemocytometer and after migration using a microscope. The statistical analysis was performed using student T-test analysis by using an online database (quick calcs), values are represented as mean ± SEM for each condition. An asterisk (*) represents statistical significance ($P < 0.04$) compared to the control (no ligand), and the (**) represents statistical significance ($P < 0.001$) compared to control.

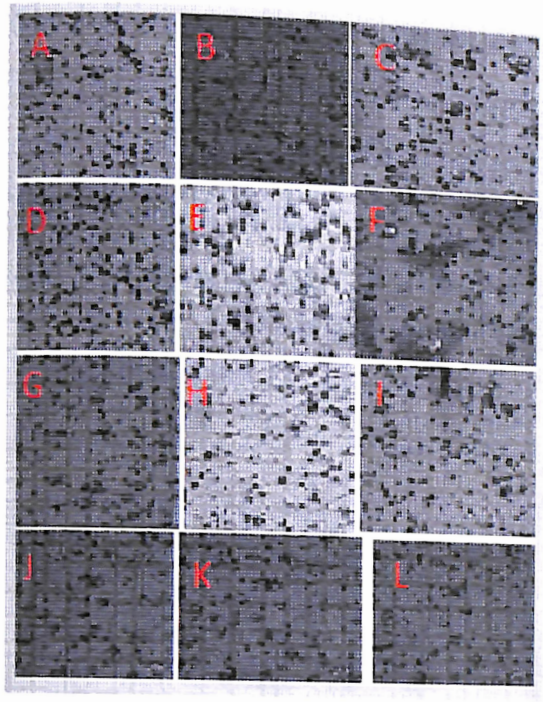

Figure 12: Images of migrated cells using collagen IV inserts. (A) No Inhibitor. (B) Ab-CXCR4/CS. (C) Ab-IGF-IR /CS. (D) No inhibitor/IGF-I. (E) Ab-CXCR4/IGF-I. (F) Ab-IGF-IR/IGF-I. (G) No inhibitor/SDF-Iα. (H) Ab-CXCR4/SDF-Iα. (I) Ab-IGF-IR/SDF-Iα. (J) No inhibitor/SDF-IB. (K) Ab-CXCR4/SDF-IB. (L) Ab-IGF-IR/SDF-IB.

When cells are plated on collagen IV, we see that the cells migrate to a greater extent toward calf serum (CS) than toward SDF-1 or IGF-I. Once again, both neutralizing antibodies decrease the number of cells migrating toward CS, IGF-I, or SDF-1.

The next substrate which we studied its role in migration is matrigel. There are many differences between matrigel and authentic basement membrane in relation of their comparative abundance and their collaborations among the factors. This might make it difficult to imitate the main purpose of basement membranes [(Khoo et al., 2011), (Sodek et al., 2008)].

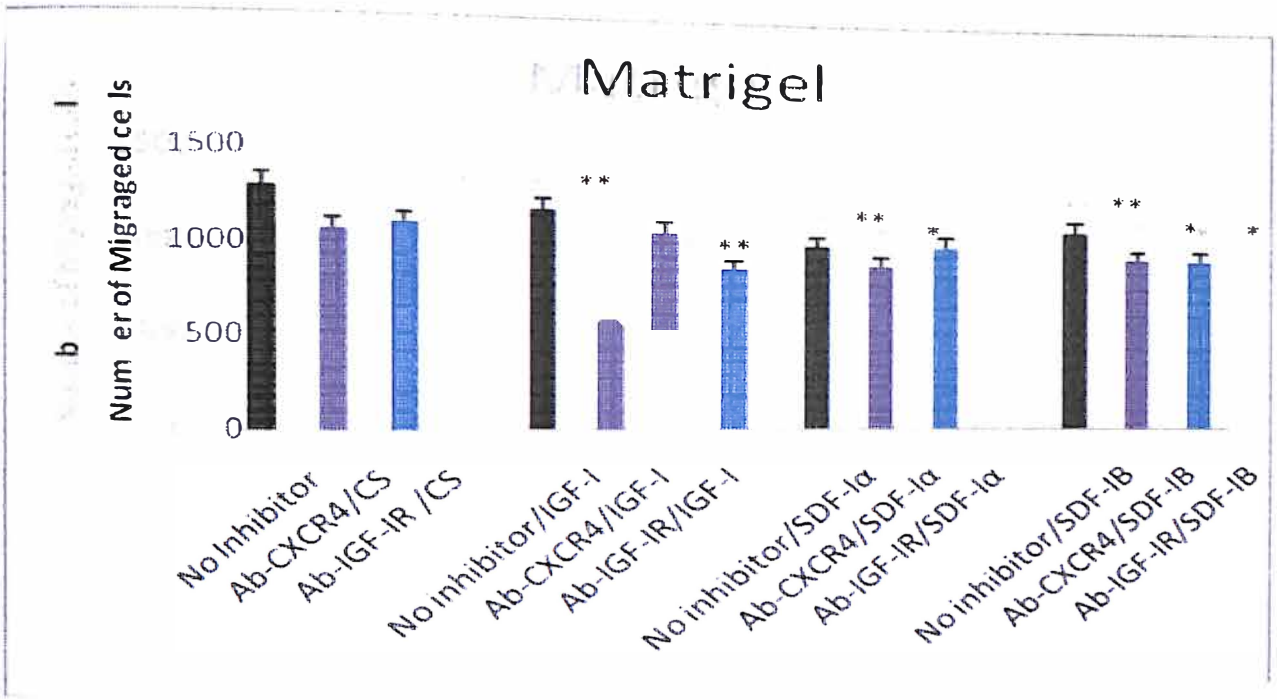

Figure 13: Effect of IGF-IR and CXCR4 inhibition on NBL cell migration using Matrigel substrate. IMR-32 cells were treated with or without IGF-IR or CXCR4 neutralizing antibodies in the upper chamber and serum, IGF-I, or SDF-1 in the lower chamber. The cells were counted before plating using a hemocytometer and after migration using a microscope. The statistical analysis was performed using student T-test analysis by using an online database (quick calcs), values are represented as mean ± SEM for each condition. An asterisk (*) represents statistical significance ($P < 0.04$) compared to the control (no ligand), and the (**) represents statistical significance ($P < 0.001$) compared to control.

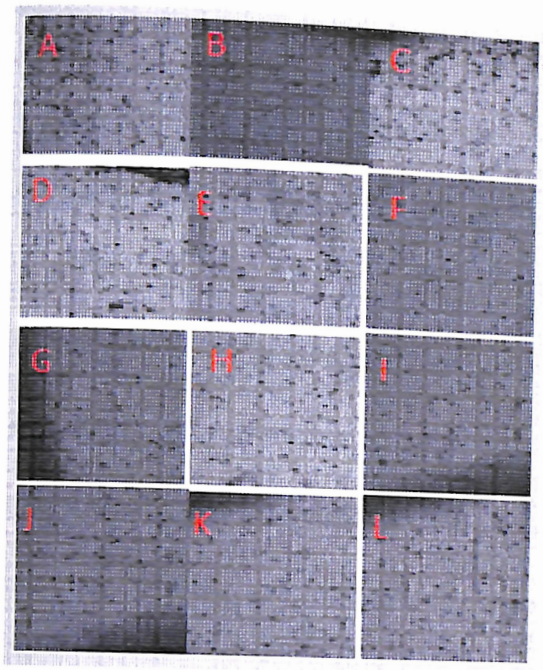

Figure 14: Images of migrated cells using Matrigel inserts. (A) No Inhibitor. (B) Ab-CXCR4/CS. (C) Ab-IGF-IR /CS. (D) No inhibitor/IGF-I. (E) Ab-CXCR4/IGF-I. (F) Ab-IGF-IR/IGF-I. (G) No inhibitor/SDF-Iα. (H) Ab-CXCR4/SDF-Iα. (I) Ab-IGF-IR/SDF-Iα. (J) No inhibitor/SDF-IB. (K) Ab-CXCR4/SDF-IB. (L) Ab-IGF-IR/SDF-IB.

When cells were plated on Matrigel, a higher number of cells migrated toward calf serum (CS) and IGF-I than SDF-1. Both neutralizing antibodies slightly diminished cell migration toward calf serum. However, the most dramatic result was the reduction in the number of cells migrating toward IGF-I in the presence of the IGF-IR neutralizing antibody, confirming results previously published by Dr. van Golen.

Discussion and Future Directions

Our goal was to understand the molecular mechanisms underlying the pathogenesis of neuroblastoma, which will provide the framework for the development of targeted therapies. The presence of metastatic disease in children with neuroblastoma is linked with a low probability of survival especially when it is metastasizing to bone. Furthermore the metastatic pattern is expected, with bone marrow and the cortical bone being the most common distant sites (Lane et al., 2000). However understanding the mechanism of bone and bone metastasis form and recognizing potential targets for treatment of metastatic disease are critical for improving patient survival specially stage IV neuroblastoma which has only 7% survival rate.

Recent studies show an array of chemokines playing a role in cancer biology [((Righi et al., 2011; Rossi & Zlotnik, 2000)], and they have been shown to induce metastasis of various tumor cells to the bone or other sites. These tumors cells are including; neuroblastoma, breast carcinoma, and prostate (Teicher and Fricker, 2010),(Meier et al., 2007)]. Some of the prominent chemokines that are reported to play a significant role in metastasis are CXCL12 and CXCR4 (Chen et al., 2012). The CXCL12 and CXCR4 are also known to be involved in development, tumor survival, metastasis, and angiogenesis [(Banisadr et al., 2000), (Teicher and Fricker, 2010)]. The bone marrow microenvironment also shows to have a correlation in facilitating the survival,

differentiation, and proliferation of normal hematopoietic cells, and tumor cell (Meads et al., 2008). CXCL12 and interleukin 6 (IL-6) are some of the factors that have been found to produce in the bone marrow and these factors are shown to mediate homing, survival, and proliferation of tumor cells [(Van Overstraeten-Schlogel et al., 2006), (Meads et al., 2008)].

We have previously shown the involvement of other molecules or family of protein receptor in neuroblastoma bone metastasis. These proteins were the chemokine receptor CXCR4 and type I insulin-like growth factor receptor (IGF-IR) protein family (van Golen et al., 2006).

In the current study, we provide evidence showing the expression of both of these proteins using a neuroblastoma primary cell line IMR-32. IMR-32 is one of the most aggressive of all the neuroblastoma cell lines (Wilson et al., 2011). The IGF-IR and CXCR4 receptor signaling pathways are known to interact in neuroblastoma to promote bone metastasis [(Theriault, 2012), (Yoneda and Hiraga, 2005), (van Golen et al., 2006)].

The expression of CXCR4 receptor on malignant epithelial cells influences the biology of cancer and may also play a central role in directing the metastasis of CXCR4 tumor cells to the organs that express CXCL12, the ligand for the CXCR4 (e.g., lymph nodes, lungs, liver, and bones). They used small molecular inhibitors of CXCR4, such as T140, BKT140, or AMD3100, and blocking antibodies toward CXCR4 [(Shim et al.,

2006), (Arscott et al., 2009)]. In the present study we were able to show the expression of CXCR4 using a neutralizing antibody to block the IGF-IR pathway. Our results indicate that IGF-IR inhibition does not have an effect on CXCR4 expression at the receptor levels (Fig. 6).

We further studied the expression of IGF-IR when activating the CXCR4 pathway using its ligand SDF-Iα at the receptor level (Fig. 8). In addition, we have also found the expression of IGF-IR on another type of neuroblastoma cell line SY5Y along with IMR-32 (data not shown). Over expression of IGF IR has been found to give resistance to apoptotic cell death in human neuroblastoma cell line (Kim & Feldman, 2009). IGF-I/II signaling through IGF-IR is shown to control neuroblastoma survival, differentiation, proliferation and motility [(van Golen et al., 2000), (Taddesse-Heath et al., 2010)]. CXCR4 plays an important role in neuroblastoma metastasis, and CXCR4 is a receptor linked with endothelial adhesion and migration. High levels of expression of CXCR4 are found to be highly associated with bone and bone marrow metastases and poor outcome prognosis, including in neuroblastoma [(Juarez et al., 2004),((Balkwill, 2004), (Taddesse-Heath et al., 2010)]. Our finding is of significance entailing that expression of IGF-IR is not cell specific, as our results show the presence of the IGF-IR in SY5Y neuroblastoma cell line as well (data not shown).

We next investigated the migration of neuroblastoma cells using transwell chamber assay in *vitro*, looking at the role of both IGF-IR and CXCR4 inhibition in

neuroblastoma cell migration toward chemoattractants using three different substrates (Collagen I, IV, and Matrigel). Collagens and Matrigel are the most profuse proteins present in the body of mammals providing structural support [(Qi et al., 2010),(Yoh et al., 2002)]. These proteins make up about 25 percent of proteins in the body. The fundamental of collagen with tumor cells interaction in peritoneal metastasis is mediated by the collagen receptors, α2β1 integrin and α3β1 integrin (Xie et al., 2011).

In our study we found different results when using collagen I and IV (Fig. 9, Fig. 11). In the presence of collagen I, when the cells were plated with no inhibitor but having the IGF-I ligand as a chemoattractant in the lower chamber, we found the difference in migration not being statistically significant compared to the control group, migrating towards 10% calf serum. However, when we used the IGF-IR inhibitor in the upper chamber, the difference is considered to be extremely statistically significant, resulting in a far lesser number of cells migrating. This finding is in line with our hypothesis when blocking the IGF-IR pathway; the number of migrating cells should be decreased. Interestingly when we used the CXCR4 inhibitor in the upper chamber and the IGF-I ligand in the lower chamber we still recorded a significant decrease in the number of cells migrating. This finding is surprising, that even when we block the CXR4, the cells are migrating toward IGF-I ligand (Fig. 9). Therefore, we hypothesized this to be due to the involvement of other proteins playing a role in migration; therefore, CXCR4 may not be

the only protein mediating migration behavior along with other proteins like IGF-IR. This finding has been previously reported by VanGolen *et al,* that when the IGF-IR was inhibited, only about 50% reduction in migration of cells was seen towards IGF-I (van Golen et al., 2006).

Based on our previous findings and published data we decided to explore the CXCR4 pathway and its role in migration. CXCR4 interacts with its ligand SDF-I, and once it is binding to the ligand the pathway gets activated and produce further responses in the downstream of the signaling pathway (Chen et al., 2012). The interaction of CXCR4 with its ligand SDF-I produces downstream intracellular enzyme activities. Therefore, CXCR4 is a good therapeutic target for blocking of these activities, which also have an important role in cancer migration and metastasis (Sun et al., 2010). When the CXCR4 pathway is blocked with an inhibitor, the expected results should be a decrease in migration of the cells toward its ligand, SDF-1. Therefore when we blocked the CXCR4 receptor we saw a big decrease in migration of the cells toward SDF-1 ligand (Fig. 9). These results confirmed that CXCR4 neutralizing antibody decrease the migration of IMR-32 cells towards the SDF-1 ligands (SDF-Iα and SDF-Iβ). Moreover our results were considered statistically significant compared to the control group, which is the cells migrating towards 10% calf serum. We further explored the role of SDF-1 ligands (SDF-Iα and SDF-Iβ) and found out that in the presence of collagen I, a higher

number of cells migrated towards IGF-I and SDF-Iα than SDF-Iβ. We think this could be because SDF-Iα is the primary ligand for CXCR4, which is also known to have a significant role in tumor metastasis and also recruitment of endothelial cells from the bone marrow to the tumor microenvironment (Muller et al., 2001). Our results coincides with the literature confirming that activation of CXCR4 by its ligand, SDF-1, may play a major role in migration and metastasis. We further verified that migration of IMR-32 cells is not only IGF-IR dependent as the IGF-IR was blocked; the IMR-32 cells still migrated towards SDF-1α via activation of CXCR4 (Fig. 9, Fig. 6). In summary our findings show that both the CXCR4 neutralizing antibody and the IGF-IR neutralizing antibody have a noticeable effect on preventing migration toward SDF-I and IGF-I, as demonstrated by a one-third reduction in the number of cells that migrated through the collagen I-coated filters (Fig. 9). Additionally these findings also supply a strong rationale for the use of collagen I matrices in investigations pertaining to invasive behavior by cancers that undergo peritoneal metastasis.

We next studied the role of collagen IV in migration. Collagen IV is a main element of the basement membranes throughout the body along with lamini, and it is shown to have the ability to bind to cell surface proteins and the extracellular matrix proteins (Rosca et al., 2012). When the cells were plated with no inhibitor in collagen IV transwell inserts and the cells migration was studied towards IGF-I, an increase in migration was observed, however in the prescience of IGF-IR inhibitor, a decrease in

migration of the cells toward IGF-I was seen (Fig. 11). These results indicate that in the presence of collagen IV and IGF-IR inhibitor, the migrations of tumor cells are partly inhibited by the IGF-IR inhibitor.

We further explored the role of collagen IV in the presence of SDF-1 and CXCR4. Our results show a great number of cells migrating towards the calf serum (CS) than the SDF-1 or IGF-I. Once again, both neutralizing antibodies to IGF-IR and CXCR4 decrease the number of cells migrating toward CS, IGF-I, or SDF-1. These findings are thought to be due to calf serum containing a broad spectrum of hormones, growth factors, nutrients, and attachment factors. Thus, a combination of these macromolecules in the CS triggers a variety of pathways in the cells, resulting in an induction of migration (Singh & Armstrong, 1997). On the other hand when the cells migrate toward SDF-I and IGF-I they bind specifically to that ligand and activate that specific pathway, therefore a lesser number of cells are expected to migrate.

The next substrate which we studied its role in migration is matrigel. There are many differences between matrigel and authentic basement membrane in relation of their comparative abundance and their collaborations among the factors. This might make it difficult to imitate the main purpose of basement membranes [(Khoo et al., 2011), (Sodek et al., 2008)]. To create cross linkages with laminin network, the firmness and flexibility of Collagen IV is very important for basement membrane. Although matrigel matrices are

less cross-linked than the basement membranes, and the methods of matrigel and basement membrane are shown to be very similar (Sasaki & Passaniti, 1998). Similar to our results from Collagen I and IV, our matrigel results presented a very similar pattern in migration. Our findings show that in the presence of matrigel, a higher number of cells migrated towards the calf serum and IGF-1 when compared to the cells migrating toward SDF-1. When an inhibitor of IGF-IR was used, the number of migrating cells decreased (Fig. 13). Neutralizing antibodies against IGF-IR and CXCR4 slightly diminished cell migration toward calf serum. However, the most dramatic result was the reduction in the number of cells migrating toward IGF-I in the presence of the IGF-IR neutralizing antibody, confirming results previously published in the literature (Kim et al., 2004). This could be due to modulation of the MMP family of proteins. Tumor cells require the capability to overcome ECM barriers by raging of proteases directly groups of the matrix metalloprotease family (Struckmann et al., 2008). MMPs have been shown to have a significant role in angiogenesis, tissue repair, and organogenesis, and the majorities of MMPs are found to be soluble and are secreted in the extracellular matrix (Kazes et al., 1998). In addition to having matrix remolding, MMPs has been found to have one of the most central functions of mealloproteases, in which the matrix also consists of many other different types of proteins (Zarrabi et al., 2011). In addition another function of MMPs is the deprivation of the cell surface proteins in which these cells surface proteins can be also a part of cell-matrix communication and cell-cell interactions (Werb, 1997).

Consequently disruption of proteins of the cell-matrix interactions by MMPs results in transforming of the cell migration because the cells are less attached to the matrix and therefore they are in a more motile state (Sengupta & MacDonald, 2007). However as previously mentioned, MMPs are critical for the deprivation of basement membrane and stromal matrices, however the gelatinases MMP-2 and MMP-9, and transmembrane MMPs are found to be significant mediators of basement membrane remodeling (Shankavaram et al., 2001). While the cleavage of stromal fibrillar collagen I networks found to be very limited to MMPs and the transmembrane MMPs (Geminder et al., 2001).

However there are other proteins beside MMPs that are involved in tumor cell attachment, which these proteins are called integrins. Research investigators found that tumor attachment and migration are controlled by various expression of integrin β subunits and the expression of β1 integrin is associated with the attached adherent cells (Meyer et al., 2004). While the lack of or the absence of this integrin causes the detachment of tumor cells and change in cellular morphology (rounding of cells) (Liu et al., 2009). In addition the expression of a very low level of β1 integrin is present in the neuroblastic N cells (Lyle & McCormick, 2010). High levels of h1 integrin are found in less tumorigenic adherent neuroblastoma cell lines, whereas the expression of h1 integrin is deficient in the less adherent neuroblastoma cell lines which are also more tumorigenic

(Meyer et al., 2004). Other investigators show that the insertion of recombinant h1 integrin into less adherent neuroblastoma cell lines, results in drastically increasing their attachment to fibronectin and collagen IV (Alford et al., 1998).

In this research we used IMR-32 cell lines, which are the neuroblastic N cells and are more tumorigenic. Thus from the previous research findings we can say that the cells we used have a very low level of β1 integrin, which is also causing these cells to have a lower tendency toward attachment. It has been found that the more tumorigenic cell lines also express lower level of h1 integrin, which is also causing the cells to have a lower tendency in attachment (Ara & DeClerck, 2006). Our research findings fall under both of these two categories, the cells that we used express a very low amount of β1 and h1 integrins, which are both, important in tumor attachment. Therefore this supports our results for the collagen IV substrate where we see a decrease in migration of cells toward their ligand. This is also due to the binding of these intgrins to collagen IV and the cells are loosely attached to the membrane.

In conclusion, our results demonstrate that neuroblastoma cell migration toward calf serum (CS), IGF-I, and SDF-1 is substrate specific. Migration through collagen I and IV are significantly inhibited by neutralization of either CXCR-4 or IGF-IR, while invasion through matrigel appears to be driven primarily through the IGF-IR. We think the main reason for the substrate specificity of the neuroblastoma cell migration is because of the involvement of the different types of proteins expression in each of the

substrates. Some of these proteins can be such as MMPs and integrins, which play an important role in tumor attachment and migration.

To further expand our knowledge of the current research findings, it would be interesting to determine the effect of IGF-IR inhibition on CXCR4 downstream signaling pathway, and also determine the effect of CXCR4 inhibition on IGF-IR activation. Additionally a comparison study utilizing other type of inhibitors against CXCR4 and IGF-IR can provide invaluable results further validating our findings. It will be also worthwhile to test out the affect of molecules that are being used in clinical trials with inhibitory target affects against CXCR4, IGF-IR and SDF-1.

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http://www.csse.monash.edu.au.

Curriculum Vita

EDUCATION

MS – Molecular and Cellular Neuroscience
Delaware State University, Dover, DE **Date of Graduation** **2012**

B.S. –Chemistry (Biology Minor) **Date of Graduation** **2008**
Delaware State University - Dover, DE

RESEARCH EXPERIENCE

Master's thesis Research **09/2008-11/2011**

Research Mentor: Dr. Cynthia VanGolen

Title: CXCR4 and IGF-IR Involvement in Neuroblastoma Migration (NBL)

- It is shown that IGF-IR and CXCR4 receptor signaling pathways interact in NBL to promote metastasis.
- Conducted research on determining the effect of IGF-IR and CXCR4 inhibition on NBL cell migration by using three different types of substrate Collagen I, Collagen IV, and Matrigel.
- Migration pattern of NBL was studied using different type of chemoattractant,.
- A higher number of NBL cells migration resulted toward IGF-I and sdf-Iα when collagen I was used.
- More migration was seen toward calf serum (CS) than toward SDF-1 or IGF-I when collagen IV was used.
- The research results were presented in a poster form at the NIH, NCRR Third Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE) at the Bethesda, Maryland on June 16 - 18, 2010 (Won a National research award).

Delaware State University – Dover, Delaware

Research Assistant (Biochemistry Lab) **09/2007– 05/2008**

On the Different Lipolytic Capability of Diverse Organs from Young Adult Guinea Pigs: A Chromatographic Study

Research Mentor: Dr. Fatma Helmy

- This research project involved chromatographic and densitometric analyses on the phospholipids profile of young adult guinea pigs heart, kidneys, spleen, and testis and their *in- vitro* deacylation by endogenous phospholipases.
- The *in-vitro* incubation of whole-tissue homogenate of young adult guinea pig heart, kidney, testis, and spleen at pH 7.4 and 38°C for 60 min was conducted followed by thin-layer chromatography (TLC) analysis.
- Research results were presented in a poster form at the Experimental Biology Conference at San Diego, Sand Diego, in April 05-09, 2008 (Published on JPC Journal 2010).

North Carolina State University – Raleigh, North Carolina

Research Assistant (Biochemistry lab) **06/2007 - 08/2007**

Developing Effective Mass Spectrometry Sample Preparation Methods for Enhanced Sequence Coverage of Proteins: Evaluating the Use of Alternative Chemical Denaturants

Summer Research Mentor: Dr. Michael B. Goshe

- In this study two proteins were used with four different chemical denaturants to determine which one of these chemical denaturants works the best to facilitate tryptic digestion.
- The proteins were a ribosomal protein L7Ae from *Methanocaldococcus jannaschii*, a thermophilic bacterium, and human serum albumin (HSA).
- Each protein was reduced, alkylated, and proteolytically digested with trypsin in the present of each denaturant then analyzed by LC/MS/MS using an ion trap mass spectrometer.
- Presented in poster form at The Annual Biomedical Research Conference for Minority Students at Austin, Texas on November 09, 2007

Delaware State University – Dover, DE

Research Assistant (Biology Lab) **09/2006 -05/2007**

Impact of a hole drilled through the air cell of fertile domestic fowl eggs on hatchability and development

Research Mentor: Dr. Sabrina Brougher

- The purpose of this research was to investigate if drilling a hole in a fertile egg could disrupt embryonic development, potentially increasing embryonic mortality and reducing hatchability.
- A 2mm diameter hole was drilled through the eggshell on day 1 of incubation using a Dremel tool and Control (no hole) eggs were used as our positive control.
- Samples (n = 4 eggs/treatment/day) were collected from embryonic day 14 through hatch (day 21), and sampled embryos were weighed and culled by cervical dislocation.
- During the daily necropsy, lungs, and brains were also weighed and frozen at -80°C for future analysis. In addition, the percent of hatchability of treatment and control eggs were compared.
- The results were presented in a PowerPoint presentation on April 21, 2007DSU Honor's day, hosted by Delaware State University at Dover, DE.

Delaware State University – Dover, DE

Research Assistant (Physical Chemistry lab) **06/2005 -08/2006**

The Determinations of Equivalent Weights and Fluoride ion analysis of polymers

Research Mentor: Dr. Preston Hayward

- The purpose of this research was to determine the equivalent weights of Nafion polymers and to measure fluoride concentrations from fluoropolymer systems such as Nafion.
- The equivalent weight of Nafion polymers was measured by using an acid- base titration method and colorimetery using of Micro lab colorimeter was used to measure fluoride concentrations.
- Presented in poster form at the HBCU-UP National Research Conference, hosted by Bowie State University held on February 9-12, 2006 at Baltimore, MD.

AWARDS AND SCHOLARSHIPS

• Bridge to Doctorate Scholar	**2008 - 2010**
• National research award Winner	**June 2010**
• Member of Society for Neuroscience	**2009-2011**
• Member of ASBMB	**2008 - 2010**
• Minority Access to Research (MARC) Scholar	**2007- 2008**
• Member of Alpha Chi Honor Society	**2007- 2008**
• Dean's List	**2005 & 2007**

PUBLICATION

F.M. Helmy, S. White, S. Amiri, **Rohina Amiri**, A. Saliu. On the different lipolytic capability of diverse organs from young adult guinea pigs. A chromatographic study. Journal of Planar Chromatography 23 (2010) 4, 277–28