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HUMAN CXCR4 PEPTIDE SPECIFIC ANTIBODIES TO STUDY DIFFERENTIAL GLYCOSYLATION IN NEUROBLASTOMA CELL LINES

By

TORI OWENS

A THESIS

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DEDICATED TO

My parents,

Dale Roberts-Owens and Keith Owens,

to whom, without their guidance

and support I would not be the successful

woman I am today.

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ABSTRACT

Neuroblastoma is the second most common pediatric malignancy within the United States and occurs mostly in children age 2 or younger with approximately 650 new cases per year. The chemokine G-protein-coupled receptor, CXCR4, can be overexpressed in human neuroblastomas. Overexpression of CXCR4 is thought to be an initiator of metastasis of neuroblastoma to bone tissue due to a chemotactic response induced by CXCR4's ligand, SDF-1 (stromal cell-derived factor-1). CXCR4 contains two putative N-linked glycosylation sites, one near the extracellular N-terminus, and another on the second extracellular loop. We believe these two CXCR4 N-glycans are differentially glycosylated across developmental stages in growing neuroblastoma cells and that we can use these unique glycan structures to create electrophoretic gel patterns to use to identify cell staging in childhood neuroblastomas. To accomplish this goal and to better understand CXCR4's role in metastasis, we have raised polyclonal antibodies (pAbs) to its 2 complement amino acid regions containing N-glycans using 2 contracted companies: ABR (Affinity Bioreagents, Inc. and Fisher Thermoscience. For this study, two different metastatic neuroblastoma cell lines were used: IMR-32 and SK-N-SH (ATCC). We used our human CXCR4 pAbs to examine gel and blot patterning of CXCR4 or its proteolytic products. Moreover, we developed a novel antibody capture method to help characterize CXCR4's complement of N-glycans using affinity chromatography with attached CXCR4 pAbs. We successfully examined the external, native expression of CXCR4 in our IMR-32 cells using immunocytochemistry, thus confirming that our synthetic peptide generated pAbs bind the *in situ* functional membrane form of human CXCR4. In addition, our antibodies also recognized solubilized forms of human CXCR4 in IMR-32 and SK-N-SH cell membrane preparations. Initial attempts to identify trypsin-released glycopeptides were unsuccessful. In summary, we have produced targeted pAbs to use to identify *in situ* expressed and *in vitro* denatured human CXCR4, binding to the glycosylated regions of this important cancer-related protein. We believe these important antibodies can be used to possibly inhibit metastatic events in neuroblastoma cells and can be used in the future to identify differential glycosylation in childhood neuroblastomas.

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ABBREVIATIONS AND SYMBOLS

Ab: Antibody

AP: Alkaline Phosphatase

~: Approximately

Asn: Asparagine

C: Celsius

C-terminus: carboxyl terminus

ddH_2O: distilled, deionized water

DNA: deoxyribose nucleic acid

EDTA: $C_{10}H_{14}O_8Na_2N_2$-$2H_2O$, ethylendiamine tetraacetic acid disodium salt

et al: and others

GAR-AP: Goat anti-rabbit antibodyconjugated with alkaline phosphatase

Glycine: amino acetic acid, $C_2H_5NO_2$

GPCR: G-protein coupled receptor

h: hour

IMR-32: human neuroblastoma cell line

IU: International unit

K: Kilo, 1000

KCL: Potassium Chloride

kDa: Kilodalton

LDS: Lithium Dodecyl Sulfate, C12H25LiO4S

mA: MiliAmperes

mAb: Monoclonal antibody

μg: microgram

μl: microliter

mg: milligram

ml: milliliter

mm: millimeter

mM: millimolar

M_r: Apparent molecular weight

MW: Molecular weight

N-linked: Asparagine linked

N-terminus: Amino terminus

NVS-g1: Peptide antibody specific for the first N-linked glycosylation site of CXCR4

NYT-g2: Peptide antibody specific for the second N-linked glycosylation site of CXCR4

NaCl: Sodium chloride

$NaHCO_3$: Sodium Bicarbonate

NaN_3: Sodium azide

ng: nanogram

nm: nanometer

pAb: Polyclonal antibody

PBS: Phosphate buffered saline

PNGase *F*: peptide-*N*-glycosidase *F*

RT: Room temperature

SDS: sodium dodecyl sulfate, $CH_3(CH_2)_{11}OSO_3Na$

SDS-PAGE: SDS Polyacrylamide gel electrophoresis

TBS: Tris buffered saline

TM: Transmembrane

Tris-Base: hydroxymethyl aminomethane crystallized free base, $C_4H_{11}NO_3$

Tris-HCl: Tris hydroxymethyl aminomethane hydrochloride, $NH_2C(CH_2OH)_3$-HCl

U: Unit

v/v: volume per volume

w/v: weight per volume

INTRODUCTION

Human Cancer

Generally defined, cancer is a class of diseases encompassing hundreds of different malignant neoplasms (Fox, 2010; NCI: Cancer, 2010). Three major properties of the disease include 1) uncontrolled growth of cells within the body, 2) invasion of the cell growth into nearby tissues, and 3) the possible spreading of malignant cells through circulation to distant locations in the body, also known as *metastasis*. Unlike many other diseases, cancer is not a foreign invader, it is the inter-workings of the body's own cells gone awry.

Cancer cannot be defined as a single disease, because it is, in fact, many diseases that consist of more than one hundred different types (Goldman and Schafer, 2011). A large majority of malignancies are categorized according to the cell type, tissue type, or organ of origin. Approximately 13% of all deaths within the United States are from cancer and moreover cancer is a prevalent cause of death throughout the world (WHO: Cancer, 2011). While an assortment of clinical tests and biological markers are currently in place and used for diagnosis and treatment of cancers, the incidence and mortality rates associated with cancer remain unacceptably high, and the need remains great for new and innovative ways to assess risk, detect cancer in its earliest stages, and treat cancers (Rothenberg *et al.*, 2003; Mendelsohn *et al.*, 2011).

All malignant neoplasms and cancer types begin in the body's cells (NCI: Cancer, 2010). Our bodies are made up of a multitude of different types of cells, which grow, divide, and die in a controlled way. Many different processes within the cell, such as damage to the cells' genetic material (deoxyribonucleic acid - DNA), can cause mutations in corresponding proteins that, in turn, cause the cell growth and division processes to become aberrant (Weinberg, 2006; Alberts *et al.*, 2008). Cells are equipped to deal with DNA damage and mutations using the cells' own DNA repair machinery; monitored by DNA polymerases. But, if a cell is damaged beyond repair, apoptosis, or programmed cell death, is initiated. Moreover, if a particular mutation that either enhances cell proliferation or decreases cell death is able to slip by either of the aforementioned mechanisms, it may allow the cell a selective advantage. This can lead to uncontrolled cell growth and division of those 'advantaged' cells, resulting in a growing mutant "clone" or a mass of tissue, otherwise known as a cancerous *tumor*.

Cancers are caused by either hereditary (genetic) influence or environmental factors: chemical agents, physical agents, infectious agents, radiation, poor diet/obesity, lack of physical activity, and even hormones are all environmental factors that can damage the genetic material (Anand *et al.*, 2008). Complex, tightly-regulated genetic processes occur when cells divide by mitosis or meiosis. Included in the many different classes of genes that regulate mitosis, are oncogenes and tumor suppressor genes (Weinberg, 2006), that when damaged, either by hereditary-inherited damage or environmental damage, can lead to the development and progression of cancer.

Malignant cancer types are classified into five broad groups: Carcinomas are the most common form of human cancers, and are of skin or epithelial cell origin (Weinberg, 2006; Alberts *et al*., 2008). Sarcomas are cancers that arise from cells of mesodermal origin. Leukemias are cancers of blood or bone marrow origin. Lymphomas are cancers of the immune system cell origin. Central nervous system cancers of the brain and spinal cord cell origin are sometimes known as neuroblastomas. There are also non-malignant tumors that form, known as benign tumors. Benign tumors contain neoplastic cells that have not become malignant or invasive to adjacent tissue types (Figure 1).

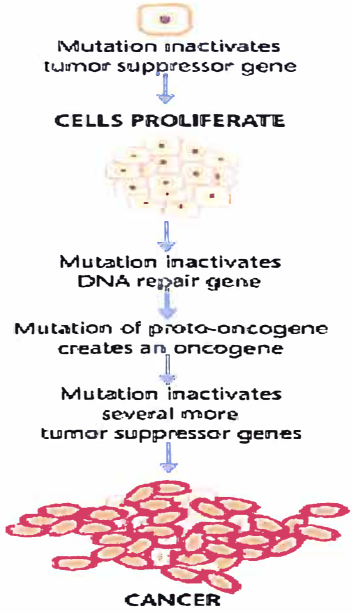


Figure 1. The progression of a cell from normal phenotype to uncontrolled growth. In normal cellular growth, if the cell is damaged beyond repair, apoptosis can occur. In uncontrolled cellular growth, multiple mutations in the DNA occur, either a mutation of a tumor suppressor gene causing inactivation of the gene or a mutation in an oncogene, causing it to become more active. Once multiple mutations have occurred, and cannot be fixed by the cells proof-reading machinery, uncontrolled cell growth results and a tumorous mass of cells are produced (NCI: http://www.cancer.gov/).

Metastasis

The original tissue, where a cancer has been detected, is known as the *primary site* or *primary tumor* (Kumar *et al.*, 2005; Weinberg, 2006; Chiang and Massague, 2008; and Klein, 2008). Most malignancies, once advanced in size and stage, can acquire the ability *to invade* surrounding tissue or penetrate through basement membranes and lymphatic blood vessels, enter the blood stream, migrate through circulation to a distant area in the body, and begin to proliferate throughout the body. These newly-formed tumors are known as local or distant metastases (Figure 2) and are referred to as *secondary tumors* (Alberts *et al.*, 2008). The cells of the secondary or metastatic tumor are very similar to the original primary tumor. For example, if an adrenal gland neuroblastoma metastasizes to bone, the secondary tumor is made up of abnormal adrenal gland cells, not of abnormal bone cells (Ramaswamy, *et al.*, 2002). Due to chemotactic cues, many malignancies preferentially metastasize to particular sites within the body (Geminder *et al.*, 2001).

Being able to assess the metastatic ability of a malignancy is of huge importance, and finding biomarkers for distinct malignant tumors is of great need. Discovering the cause and bio-mechanism of metastatic events is a high priority in cancer research.

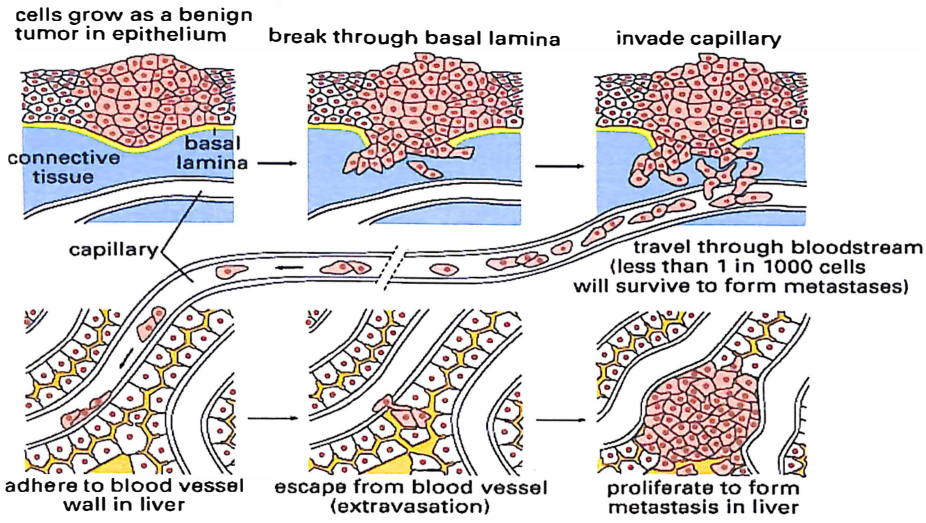


Figure 2. Schematic diagram showing how tumors can break through blood vessels and travel to a distant, novel secondary site and begin to proliferate metastatically. (http://thefutureofthings.com/articles/1012/smart-bombing-cancer.html)

Neuroblastomas

Neuroblastoma is a neuroendocrine tumor that can originate in any division of the sympathetic nervous system (SNS), including the adrenal glands or the nerve tissues of the neck, chest, pelvis, or abdomen; however, it most commonly arises in the adrenal gland (O'neill, 2003; Vincent, 2011). About half of all neuroblastoma instances occur in children approximately two years of age or younger and neuroblastomas are the most common extra-cranial, solid malignancy in childhood. Neuroblastomas detected in children have an annual incidence of about 650 new cases per year in the United States—

approximately 10% of all juvenile cancer occurrences per year—and accounts for about 15% of cancer-related deaths in children (van Golen *et al.*, 2006; Carlisle *et al.*, 2009).

Fortunately, pediatric neuroblastomas, unlike many human cancers, have been known to exhibit spontaneous regression from a malignant, undifferentiated state to a completely benign state (Bernard *et al.*, 2008; Mao *et al.*, 2011). The disease stages of pediatric neuroblastomas range from low to high risk. Most infants diagnosed with the disease fall into the low-risk category with a good prognosis—meaning, tumors usually subside with simple observation, chemotherapeutic treatment, or surgery. If the disease is caught before the age of one it is often curable, as long as the disease is localized to one central area. This is contrary to high-risk pediatric neuroblastomas, caught after the age of one, which are virtually resistant to the most intensive chemotherapeutic treatments, and is difficult to treat effectively (Geminder et *al.*, 2001; Cohn *et al.*, 2007). Several genes have been identified to help determine the metastatic ability of all neuroblastomas in general.

Recent studies have shown that amplification of the oncogene, *MYCN,* is associated with rapid progression of all neuroblatomas and poor prognosis, thus making the MYCN overexpressed protein product a key biomarker during assessment of neuroblastoma metastatic risk (Abel *et al.*, 2011). Recent studies of neuroblastomas, in general, have identified four molecular subgroups of neuroblastomas using discriminatory gene-expression profiling. These subgroups are identified as having a 6-gene signature and can be differentiated between each other into subgroups. In pediatric neuroblastomas, if the disease is diagnosed in an older child, and it has already

metastasized to a secondary site, a poor prognosis is given and mortality is the most likely result (Geminder *et al.*, 2001; van Golen *et al.*, 2006).

Neuroblastomas preferentially metastasize to bone marrow, bone, liver, and skin (Ma *et al.*, 2011). Bone invasion is the second most common site of metastasis in neuroblastomas and accounts for 56% of metastatic neuroblastoma cases (Sohara *et al.*, 2005). Preferential bone and bone marrow metastasis has been attributed to the CXCR4/SDF-1 receptor-ligand relationship (Ma*et al.*, 2011). It is thought that neuroblastoma cells employ a very similar mechanism, as used by hematopoetic stem cells (HSCs), when migrating to bone and/or bone marrow. The released metastatic cells are attracted to the bone and/or bone marrow by chemical signals celled chemokines.

Chemokines are small, pro-inflammatory 8-10 kDa chemotactic cytokines that bind to specific G-protein coupled receptors (GPCR) and have the ability to induce directed chemotaxis in near and distant responsive cells that contain their respective receptor. One such receptor is the CX receptor. There are four subgroups of chemokines related to CX receptors (CXC, CC, C, CX3C), which are named according to the positioning of the first two cysteines in the primary structure of these chemokines (Kucia *et al.*, 2003; Busillo and Benovic, 2007). Their corresponding chemokine receptors are named *complementary* to their respective ligand. Chemokines, in general, are promiscuous in binding with chemokine receptors; e.g., the CXCR4 receptor.

The homing process of HSCs, which express the CXCR4 receptor, is mediated by chemo-attraction to the CXCR4 receptor by one of its chemokine ligands, Stromal cell-

derived factor 1 (SDF-1), which is secreted from bone marrow stromal cells (Geminder *et al.*, 2001). In a similar fashion, neuroblastoma cells have been shown to overexpress the chemokine receptor CXCR4, which during dissemination from the primary site, are similarly chemo-tactically attracted to SDF-1, which is present in the secondary site location (destination).

In addition to its role in neuroblastoma cell migration, SDF-1 is also thought to be involved in neuroblastoma cell adhesion to bone marrow stromal cells, proliferation of neuroblastoma cells, and consecutive down-regulation of CXCR4, once successful secondary site neuroblastoma metastasis has occurred (Geminder *et al.*, 2001; Sohara *et al.*, 2005; and Struckhoff *et al.*, 2010). Yet, more recent analysis of this pathway has shown that it is possible that down-regulation of CXCR4 is directly correlated to neuroblastoma cell density, and may not be dependent on levels of SDF-1 in the surrounding extracellular environment (Carlisle *et al.*, 2009).

The CXCR4 Receptor

CXCR4, also known as fusin, is a 7-transmembrane chemokine receptor, glycoprotein that belongs to the rhodopsin-like, superfamily of G protein-coupled receptors (Wegner *et al.*, 1998; Laphem *et al.*, 2002; Huang *et al.*, 2003; and Busillo and Benovic, 2007). The membrane-transversing regions are hypothesized alpha helices by Wegner *et al.*, 1998. CXCR4 has an M_r of 39,700 using its complement of amino acids, but on blots has been shown to have molecular weights between 47 and 48 kDa, due to

the addition of 2 specific N-linked glycans (Chabot *et al.*, 2000; Figure 3). The highly conserved (as shown by BLAST P sequences search) predicted primary amino acid structure of human CXCR4, based on the known cDNA sequence of the human *CXCR4* gene, is 352 amino acids long (Figure 3).

CXCR4 in Normal Tissues

CXCR4 is responsible for a plethora of biological functions. Binding of CXCR4 by SDF-1 results in activation of many downstream signal transduction pathways, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and calcium mobilization (Liang *et al.*, 2004). SDF-1 mediates the homing of hematopoietic stem cells to bone marrow, migration of immune cells to inflammatory sites, and directing peripheral blood cells to the lymph nodes and spleen (Liang *et al.*, 2004; Carlisle *et al.*, 2009).

CXCR4 is up-regulated during implantation in the endometrium of a human blastocyst, suggesting that it plays a major role in *the adhesion phase* of human implantation. Furthermore, CXCR4 impairment in murine mice models has been proven lethal. In humans, a truncating CXCR4 mutation results in WHIM (Warts, hypogammaglobulinemia, infections, and Myelokathexis) syndrome due to loss of SDF-1-induced leukocyte signaling (Busillo and Benovic, 2007; Carlisle *et al.*, 2009;Bachelerie, 2010). WHIM syndrome, in turn, causes susceptibility to a number of other health complications such as human *Papilloma virus,* infection-induced warts and

carcinomas, hypogammaglobulinemia, recurrent bacterial infections, B and T-cell lymphopenia, and neutropenia coupled with preservation of senescent neutrophils in the bone marrow (Bachelerie, 2010).

The CXCR4/SDF-1 relationship is also an important factor in hematopoietic stem cell transplantation. During hematopoietic stem cell transplantation, it is important that peripheral blood stem cells are mobilized to the blood stream (Fiorina *et al.*, 2011). Since the CXCR4/SDF-1 relationship is responsible for the homing of those blood cells to the bone marrow, inhibition of the CXCR4/SDF-1 relationship is crucial for survival during transplantation in humans. Mobilization of peripheral blood stem cells during hematopoietic stem cell transplantation is achieved by Granulocyte-colony stimulating factor (G-CSF), which acts to block CXCR4/SDF-1 function by degradation of CXCR4 (Christopher *et al.*, 2011).

CXCR4's Role in Cancer

CXCR4 is a co-receptor involved in T-tropic HIV-1 and CD4-independent HIV-2 encapsulation, or acceptance of the virus/viral genetic information into a previously healthy host cell, and subsequent infection (Laphem *et al.*, 2002; Huang *et al.*, 2003; and Wegner *et al.*, 2005). Gaining insight into the above studies of CXCR4's involvement in HIV patients, CXCR4 has also become of extreme interest in cancer-related research.

CXCR4 is overexpressed in multiple forms of human cancer and stimulates many of the processes essential for cancer progression, such as tumor cell proliferation,

angiogenesis, and metastasis of multiple types of primary tumors to disseminate to secondary locations (Carlisle *et al.*, 2009; Struckhoff *et al.*, 2010).

CXCR4's Role in Neuroblastoma

Surface expression of CXCR4 has been shown in varying levels of multiple, human-derived neuroblastoma cell lines: differential levels of CXCR4 expression can, in turn, indicate different stages of neuroblastoma (Table 1). Moreover, transcriptome-wide gene screenings of a large neuroblastoma cell set has shown that CXCR4 is differentially overexpressed in metastatic CXCR4, in comparison to primary site neuroblastomas (Carlisle *et al.*, 2009).

Table 1. Neuroblastoma cell lines grouped into categories ranging from low to high expression values, based on varying levels of CXCR4 cell surface expression (Carlisle *et al.*, 2009).

CELL LINE	MEAN FLUORESCENT INTENSITY	CXCR4 SURFACE EXPRESSION CLASS
SH-SY5Y	0.9086	Low
SK-N-AS	1.290	Low
KCNR	1.796	Low
SK-N-FI	3.569	Low
CHP-134	4.066	Low
NGP	8.772	Moderate
NB-69	8.99	Moderate
KCN	11.899	Moderate
SK-N-SH	40.57	High
LAN-5	63.33	High

The expression of CXCR4 is further regulated in the promoter of the CXCR4 gene by a Nuclear Respiratory Factor 1 (NRF-1) binding site as a means of transcriptional control (Wegner *et al.*, 1998). NRF-1 is a transcription factor that homodimerizes and controls expression of key metabolic genes that are pivotal in contributing to normal cellular growth and possibly neoplastic growth (Biswass and Chan, 2010). It is also responsible for some nuclear genes necessary for mitochondrial respiration, DNA transcription, and replication. This suggests another possible role for CXCR4; to help promote expression of genes near the CXCR4 gene locus.

CXCR4 can be detected at the gene, transcriptional, translational, and surface expression levels using an armamentaria of different methods: e.g., Southern blotting and PCR for CXCR4 gene analysis and, Northern blotting for CXCR4 mRNA detection. For detection of the expressed protein, Western blotting, Fluorescence-Activated Cell Sorting (FACS), flow cytometry, and immunocytochemistry can be used (Lapham *et al.*, 2002; Carlisle *et al.*, 2009).

Overexpression of CXCR4

Previous PCR and microarray analysis techniques have both been employed in order to identify the CXCR4 gene. The CXCR4 gene is differentially overexpressed in metastatic neuroblastomas, in comparison to primary site neuroblastomas. This supports a role for CXCR4 in neuroblastoma metastasis (Wang *et al.*, 2006; Carlisle *et al.*, 2009). FACS has been used to classify different neuroblastoma cell lines into CXCR4 cell-surface expression level classification categories. The prior two techniques can also be used to compare CXCR4 cell surface expression levels in cancerous versus normal cell lines to demonstrate that high levels of CXCR4 are indicative of certain malignancies (Lee *et al.*, 2004).

Western blotting is a common, widely-used technique to detect CXCR4 protein expression. Due to CXCR4's glycan heterogeneity, Western blot analysis can lead to differential CXCR4 expression patterns, with specific isoform recognition by the same

CXCR4-specific antibodies for different cell types and each representative neuroblastoma cell line (Lapham *et al.*, 2002; Carlisle *et al.*, 2009).

It has been shown that there are various expressed isoforms of human CXCR4 from multiple cell types ranging from 38-101 kDa (Laphem *et al.*, 2002;Carlisle *et al.*, 2009). CXCR4 exhibits heterogeneic expression patterns, seen on blots. In addition to the heteroexpression of CXCR4 isotypes, CXCR4 has been shown to spontaneously form heterodimers with other chemokine receptors as well as homo-dimerizing with itself. The dimers form without stimulation from CXCR4's ligand, SDF-1, but binding of SDF-1 to CXCR4-involved dimers has shown to induce a dimer-stabilizing conformational change (Percherancier *et al.*, 2005; Busillo and Benovic, 2007). CXCR4's heterogeneity can also arise from different post-translational modifications (PTMs) such as sulfation, ubiquitination, and glycosylation (Carlisle *et al.*, 2009).

Protein Glycosylation

Carbohydrates densely layer the outer surface of all mammalian cells and can exist as part of glycolipids or glycoproteins (Chen *et al.*, 2007). Protein glycosylation is the connection of single, simple sugars or complex oligosaccharide chains to proteins and is the most abundant and complex form of post-translation modification; a more prolific cellular event than phosphorylation (Geyer and Geyer, 2006; Patwa, *et al.*, 2009).

There are two general types of possible glycol-linkages: The first is known as asparagine-linked glycosylation (N-linked or N-glycans). There are two subclasses of N-glycans: complex oligosaccharide attachment or high-mannose oligosaccharide attachment (Greer and Ivey, 2007). Differential glycosylation can occur in cancer with the addition and modification of these basic subtypes (Greer and Ivey, 2007).

N-linked oligosaccharide chains are added to proteins as they are simultaneously being translocated within the folds of the endoplasmic reticulum (ER) by one-pass transmembrane translocator proteins (Alberts *et al.*, 2008). Specific start and stop translocater amino acid sequence sites within the newly-formed protein allow for proteins to be properly translocated to the ER membrane as a final destination or to the ER lumen and then to the Golgi Complex for further protein post-translational modification. Dolichol, a lipid molecule, holds the oligosaccharide in the ER membrane and an enzyme called oligosaccharyl transferase catalyzes the transfer of the oligosaccharide chain from dolichol to the newly-formed protein as it is being translocated through the ER. N-glycans are attached to asparagine regions in proteins (N-linkage) having the consensus sequence (NH_2- Asn-XXX-Ser/Thr – XXX cannot be proline). If the protein *to be* glycosylated contains multiple N-linked consensus regions they are termed 'putative' regions until confirmed for covalent attachment of N-glycans.

The other type of protein glycosylation occurs when a sugar is linked to the hydroxyl group of a serine or threonine side chain, and is referred to as O-linked glycosylation (Alberts *et al.*, 2008). Less is known about the exact mechanism of original oligosaccharide attachment in the case of O-linked glycosylation.

Newly-attached oligosaccharides are sorted and processed by the membrane-embedded enzymes located in the lumen of the Golgi apparatus. Glycosyl transferases and glycosidases are enzymes that are organized within the membrane of the Golgi apparatus as enzyme complexes and are single-pass transmembrane proteins (Alberts *et al.*, 2008). Glycosidases remove or "trim" monosaccharide sugars from the glycoprotein oligosaccharide tree, and glycosyl transferases link new sugars onto the oligosaccharide tree.

Complex carbohydrates require different enzymes at each step within their synthesis and can exist as many different isomers, producing a complex and diverse amount of branched structures. The compound assemblies that are the outcome of constructing complex oligosaccharides from simple sugars amount to a large number and variety of biological glycomolecules such as glycosylated lipids, proteins, and thus cell surfaces (Glycomes) that exhibit a wide array of biological selectivity (Alberts *et al.*, 2008; Patwa *et al.*, 2009).

Over 50% of all membrane proteins exhibit some form of glycosylation (Geyer and Geyer, 2006). Protein glycosylation plays a very major role in a large multitude of biological processes including cell-cell recognition, inter- and intra- cellular signaling, fertilization, embryonic development, cell adhesion, injury, immune response, inflammation, cell division, cell growth, cell differentiation, cell death, and pathogens homing in on host cells (Geyer and Geyer, 2006; Chen *et al.*, 2007; Patwa *et al.*, 2009). Site-specific glycosylation plays an important role in key protein functions and improper

glycan attachments extending from a protein surface can cause a protein to fold improperly affecting its structure and function.

Protein glycosylation plays two major roles in the proper folding of proteins: 1) it prevents protein aggregation within a cell by making folding intermediates more soluble and 2) modifications of N-linked sugars in the Golgi apparatus establishes a sort of "glycol-code" that guides and marks the progression of protein folding, as well as mediating binding of protein chaperones and sugar-binding lectins for cell transport and targeting (Alberts *et al.*, 2008). Various cell types are dictated by where saccharides are located, and the amount of saccharide substitution on cell-surface proteins (Patwa *et al.*, 2009).

Although glycosylation is an abundant post translational modification (PTM) cellular event, analysis of the structural variation and complexity of carbohydrate structures and their derivatives makes their study a real challenge in terms of cell biochemistry (Patwa *et al.*, 2009). These aberrant glycosylation events give rise to cellular '**differential glycosylation**.' Therefore, the need for innovative and new ways to isolate, separate, and characterize glycoproteins for glycol analysis is a matter of increasing scientific interest.

Aberrant glycosylation is associated with parasitic infections, viral replication, cancer cell camouflaging, and tumor metastasis; and especially in cancers; e.g., breast cancer, prostate cancer, lung cancer, and colon cancer(Geyer and Geyer, 2006;Chen *et al.*, 2007). Specific changes in glycosylation have been shown to play key role in tracking disease progression and can hold an indicative value for an abundance of disease types,

including inflammation and cancer (Patwa *et al.*, 2009). Cell surface glycosylation alterations that are associated with tumors play a substantial role in the dissemination of cancerous cells because tumor cell adhesion or motility has been altered in such a way to either stimulate or prevent invasion and metastasis.

Aberrant/differential N-glycosylation events have been used as biomarkers for certain cancers. For example, in key serum protein that is upregulated in colon cancer, β-haptoglobin, is over-glycosylated in the serum of colon cancer patients, exhibiting a significant increase in the amount of fucose (α-1,6) linked to N-acetylglucosamine and N-acetyllactoseamine as identified by the lectin, Aleuria aurantia (AAL): These results were in comparison to healthy study participants, who exhibited normal levels of β-haptoglobin N-linked glycosylation (Park *et. al.*, 2009). Furthermore, lysosomal glycoproteins were shown to be differentially glycosylated across different colon cancer cell lines. More specifically, lysosomal glycoproteins of colon cancer cell lines synthesize more poly-N-acetyllactosamines, which is in concert with the more recent results of Park *et. al.*, 2009 (Saitoh *et al.*, 1992).

Aberrant glycosylation has not only been used as a biomarker of several different malignancies and of tumorigenesis. It has also been identified that removal/replacement of aberrant N-gylcans with chemotherapeutic treatment can slow tumor growth as well as increase the antitumor effects of chemotherapeutics. Emmett *et al.*, 2008 treated glioblastoma cells with a popular glycolysis inhibitor, 2-deoxy-D-glucose, and found that in addition to the inhibited growth of the glioblastoma cells, that 2-deoxy-D-glucose was also affecting N-glycan formation by replacing aberrant N-glycans, specifically D-

mannose. This replacement increased the antitumorigenic affects of 2-deoxy-D-glucose in glioblastoma cells. Furthermore, previous differential glycosylation profiling studies of glycoproteins in glioblastoma cancer stem cells was able to detect distinct changes in N-glycosylation patterning upon drug treatment with γ-secretase inhibitor, suggesting a shift of the glioblastoma cancer stem cells to a less tumorgenic phenotype (Dai *et al.*, 2011).

In terms of the different techniques applied to determine aberrant, differential N-glycosylation in different malignancies, there are only a few. Lectin arrays and removal (using PNGase)/Mass Spectrometric analysis of the N-glycans have previously been used to both recognize, as well as identify, the different N-glycan attachments. A more recent study has demonstrated the use of lectins to distinguish between patients with prostate cancer versus those patients with benign prostatic hyperplasia. The patients with prostate cancer showed a significant increase in fucosylation of prostate specific antigen (a key biomarker of prostate cancer) from serum when compared with the control. This study was of particular interest because such minute sample sizes were needed (50 μl) in order to achieve binding specificity of the lectin in high amounts (Dwek *et al.*, 2010). The need for more innovative and sensitive techniques to identify differential aberrant glycoylation in malignancies remains great, as time and multiple studies have revealed that aberrant glycosylation is a key biomarker of tumorigenesis.

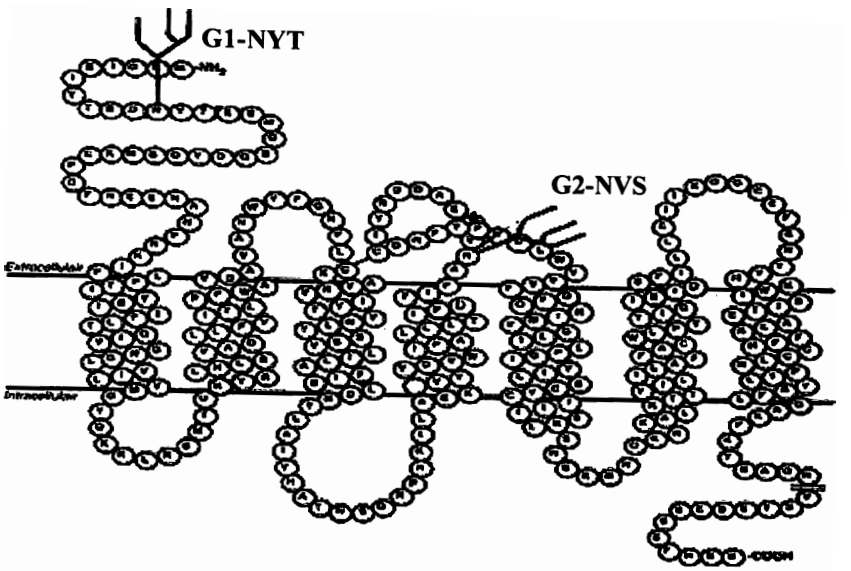


Figure 3. Proposed CXCR4 membrane model with its two N-glycan (Huskens *et al.*, 2007). The G1-NYT & G2-NVS are nomenclatures given to the 2 N-glycan sites, corresponding to our polyclonal antibody target regions, and are used throughout this thesis.

CXCR4 Glycosylation in Neuroblastomas

Differential glycosylation can occur by varying the sequence and composition of glycosylation building blocks, thus changing the glycan structure of the sugar on the protein in cell surfaces and in body fluids. It has been shown to correlate with both the development and progression of cancer and other disease states (Meany and Chan, 2011).

With particular concern to neuroblastomas, researchers found that membrane glycoproteins of human neuroblastomas experienced a high prevalence of fucosyl residues, by way of glycopeptide analysis and characterization (Santer and Glick, 1983).

This finding corresponds with reports that malignant cells experience aberrant glycosylation and different protein glycosylation patterns in comparison to non-malignant cells. Malignant cells also utilize abnormal glycosylation to evade detection by the immune system. Consequently, many clinical biomarkers and therapeutic targets in cancer are glycoproteins (Patwa *et al.*, 2009).

Differential, N-linked glycosylation is cell-type dependent, thus making it possible for multiple forms of CXCR4 to be the result of changes in asparagine-linked-glycosylation (N-linked glycosylation or N-glycosylation) or 'differential glycosylation' events (Huskens *et al.*, 2007). CXCR4 contains two N-glycans at Asn 11 and Asn 176; for clarity G1-NYT represents the first glycosylation site and G2-NVS the second N-glycan site (Chabot *et al.*, 2000). Interestingly, a mutation of CXCR4's Asn 11 to Ala caused a change in the molecular weight of CXCR4 from 47 kDa to 40 kDa, a drop of 7,000 daltons. Conversely, when Asn 176, the second N-glycan site, was mutated to Ala, no molecular weight change in CXCR4 was seen on blots, suggesting that the Asn 176 N-glycan site probably contains a small glycan (Chabot *et al.*, 2000; Busillo and Benovic 2007). However, both sites seem to be glycosylated as a more recent study agrees with previous studies that human CXCR4 contains 2 N-glycans, as stated above (Edo-Matas *et al.*, 2011). Mutation of Asn 11 of CXCR4 to glutamine or leucine inhibits SDF-1 binding, thus diminishing the corresponding signal transduction cascade (Busillo and Benovic, 2007).

The ability to characterize cell surface carbohydrate expression and distinguish between substantial differences in cell surface saccharide expression (the Cell Glycome)

is being shown to be a conceivable indicator of cancer development and metastasis, and could prove useful as a diagnostic tool in terms of therapeutic development (Chen *et al.*, 2007). Therefore, it is vital that the identification of different isoforms of glycoproteins for the diagnosis and management of human diseases, including cancer, become available because several diseases arise as the result of glycan structural alterations (Patwa *et al.*, 2009).

Since varying cell surface expression levels of CXCR4 have been shown in multiple, human-derived neuroblastoma cell lines, differential levels of CXCR4 expression are considered to be indicative of different stages of neuroblastoma (Carlisle *et. al.*, 2009). CXCR4 has also been shown to, with the help of its compliment of N-glycans, to stimulate tumor-masking processes, tumor cell proliferation, angiogenesis, and aid in metastasis of multiple types of primary tumors to disseminate to secondary locations (Carlisle *et al.*, 2009; Struckhoff *et al.*, 2010). Because removal of CXCR4's oligosaccharide on Asn 11 or mutation of Asn 11 to another amino acid inhibits SDF-1 binding, more concerns are raised in terms of targeting CXCR4 glycosylation site as a means to prevent metastasis of certain malignancies (Busillo and Benovic, 2007). Therefore, treatments for cancers, including neuroblastomas, that are suspected to employ CXCR4 during cancer progression, must be very specific and sensitive. Investigating CXCR4 *differential glycosylation* as a possible biomarker for aberrant CXCR4 expression could present itself as a novel way to specifically detect and subsequently treat certain malignancies and metastases. Antibodies raised against this cancer-related

differential glycosylation *trend* could be used to track and identify these possible biomarkers in cancer cells.

My Thesis Proposal

The purpose of this thesis is to present a *novel* approach, using polyclonal antibodies, to help characterize the possible 'differential glycosylation' of human CXCR4.

The overall goal of this thesis project was to determine if we could detect and characterize differential glycosylation in human CXCR4. To accomplish this goal, we characterized our panel of CXCR4-specific polyclonal antibodies to bind to and thus 1) detect the natively cell surface expressed CXCR4 in metastatic neuroblastoma cell lines (using immunocytochemistry - ICC), 2) to detect the denatured form of CXCR4 on Western blots using solubilized cell membrane preparations from the metastatic neuroblastoma cell lines, and 3) to attempt to identify specific CXCR4 glycopeptide patterns in the different metastatic neuroblastoma cell lines. The binding profiles of the CXCR4 glycopeptide antibodies to the cell surface (ICC) and in solubilized cell membrane preparations (Western blots) of metastatic neuroblastoma cell lines can possibly provide insight into the power and future use of these antibodies to prevent CXCR4 involved metastasis.

Differential glycosylation pattern recognition would imply that aberrant glycosylation in CXCR4 is a potential biomarker for staging events in metastatic neuroblastomas and that this differential glycosylation could aid in neuroblastoma metastases detection. If CXCR4 glycosylation is differential (heterogeneic) it could thus also serve as a possible new target for the detection, diagnosis, and treatment of metastatic neuroblastomas.

Thesis Hypothesis

We have raised polyclonal antibodies (pAbs) to the two segments of amino acids in human CXCR4 that contain N-glycans (see Figure 4). We hypothesize that 1) these specific polyclonal antibodies raised to glycan-attached amino acid stretches of human CXCR4 can be used to identify the native, cellular human CXCR4 membrane protein and 2) the denatured form of CXCR4.

G1-NYT: NH_2 – TSDNYTEEMGSGDYDSMC

G2-NVS: NH_2 – DFIFANVSEADDRYIC

Figure 4. The 2 antigenic *synthetic* amino acid sequences of CXCR4 target glycopeptides. The 2 N-glycans are proposed to be bound on the asparagine residue (the RED N) surrounded by the antigenic target amino acid sequences of CXCR4 shown.

Specific Aims

My specific aims were to:

1) Determine if the pAb panels raised to human CXCR4 bind to CXCR4 residing in the plasma membrane of the expressed, native confirmation of CXCR4 in IMR-32 neuroblastoma cell line and,
2) To determine if the same pAbs bind the denatured CXCR4 on Western blots, and,
3) To use the CXCR4-specific pAbs to determine if proteinase released glycopeptides of CXCR4, separated on Western blots, could be used to produce unique patterns to confirm the presence of differential glycosylation in over-expressed human CXCR4.

METHODS AND BACKGROUND

Antibodies Raised to CXCR4

To better study the molecular structure and function of CXCR4, two panels of CXCR4 polyclonal antibodies (pAbs) were raised to the 16 and 17-mer glycosylated amino acid segments given in figure 4 above of CXCR4 using synthetic, non-glycosylated peptide or both co-peptides injected into rabbits. Thus, resultant antibodies denoted as Gl-NYT and G2-NVS were raised against the two CXCR4 peptide regions containing N-linked oligosaccharides. The two putative glycosylated asparagines (11 and 176) are located on the extracellular epitopes of the CXCR4 receptor (Figure 3). The first oligosaccharide and the first 'N' are located near the amino terminus of the receptor. This is the first glycosylation site (denoted G1), hence the generated antibody name is **G1-NYT**, since the 'NYT' sequence is the first glycosylation sequence from the N-terminus. The second oligosaccharide is located on the second extracellular loop of CXCR4. Correspondingly, the second N-glycan segment pAb panel was denoted **G2-NVS (Figure 4 CXCR4 insert)**.

The conserved human CXCR4 16- and 17-mer synthetic peptide segments were made and injected into rabbits housed at ABR (Affinity BioReagents, Inc.) and Fisher Thermoscience. The bleed outs were collected on specific days, and the polyclonal antibodies were isolated from serum proteins at both companies using affinity columns whose beads were covalently attached to the synthetic peptide target antigenic sequences G1-NYT or G2-NVS, 17-mer or 16-mer segments, respectively. pAbs generated at Fisher Thermoscience were generated by co-injecting both amino acid segments in to two different rabbits.

The first panel (2009) was produced by Affinity BioReagents, Inc. (ABR), and a terminal cysteine (C) was added to the first synthetic peptide, which corresponds to the first putative glycosylation site (G1-NYT), making it an 18-mer peptide sequence (NH_2 – TSDNYTEEMGSGDYDSMC); The C was added to facilitate linking the C-reactive amino acid terminal to future columns. The second synthetic peptide, which corresponds to the second putative glycosylation site (G2-NVS), did not need to be supplemented with a terminal cysteine because the sequence used to create the antibodies already contained a terminal C (NH_2 – DFIFANVSEADDRYIC).

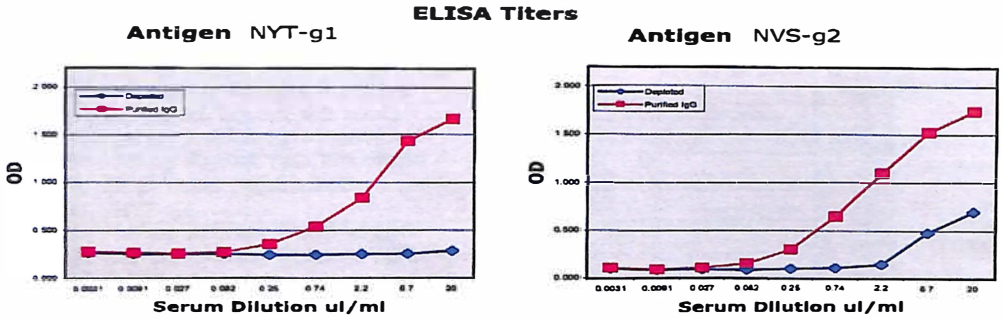
There were two sets of the first panel of antibodies. Bleedouts and the aforementioned peptide affinity columns used at ABR were sent to our lab for further collection of pAbs. Thus, a new ABR CXCR4 polyclonal glycopeptide antibody set of both G1-NYT and G2-NVS—referenced as G1-NYT-ABR (new) and G2-NVS-ABR (new), were isolated by Dr. Stan Ivey, my mentor for this project, December 2010. The originally isolated ABR CXCR4 polyclonal glycopeptide antibody set of both G1-NYT

and G2-NVS—referenced as G1-NYT-ABR (old) and G2-NVS-ABR (old); isolated by Affinity BioReagents, Inc., in 2008.

The second pAb panel was produced by ThermoFisher Scientific, with the co-peptide injection of synthetic unglycosylated peptides (NH_2 – TSDNYTEEMGSGDYDSM (G1) and NH_2 – DFIFANVSEADDRYIC (G2)); polyclonal antibodies were isolated in 2009. This last set is referenced in this thesis as G1-NYT-TF/ G2-NVS-TF. All purified pAbs were stored at 20° C until used.

Antibody ELISA titers for both rabbits (G1 and G2) were, produced for this study by ABR, Inc., and isolation profiles and titer values are given in Tables 2 and 3.

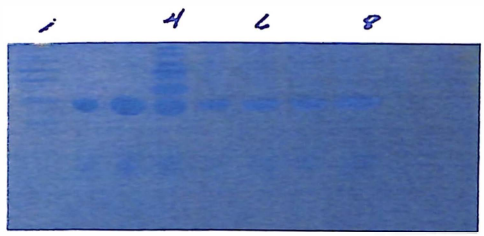
ELISA Titers



	Endpoint	Inflection Point	Endpoint	Inflection Point
Depleted Serum	<50	<50	<50	<50
Purified IgG	N/A	365	10780	634

SDS-PAGE

Lane 1: Molecular Weight Markers (220/97/66/45/30/20.1/14.3 kDa)
Lane 2: 10 µg Rabbit IgG Standard
Lane 3: 20 µg Rabbit IgG Standard
Lane 4: 1 µl Total Rabbit IgG
Antigen NYT-g1
Lane 5: 10 µg Affinity Purified IgG
Lane 6: 20 µg Affinity Purified IgG
Antigen NVS-g2
Lane 7: 10 µg Affinity Purified IgG
Lane 8: 20 µg Affinity Purified IgG

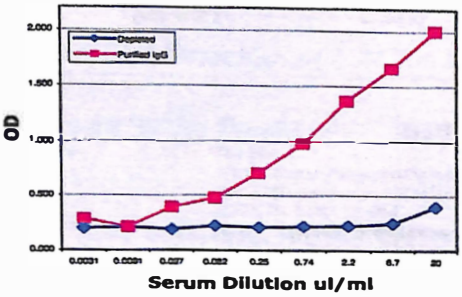


Purified IgG visualized by SDS-PAGE

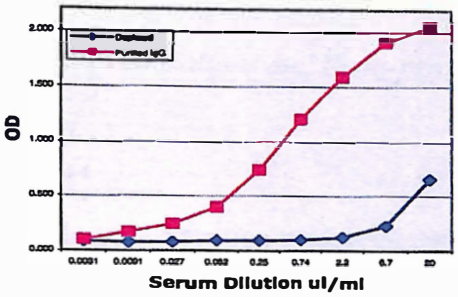
Table 2. ELISA titers of both peptide-affinity isolated pAbs G1-NYT-ABR and G2-NVS-ABR. Graphed is the amount of purified IgG followed by a complementary SDS-PAGE of the IgG's collected comparing the micrograms of isolated antibody (Data provided by ABR, Inc.). The purity of each antibody set (NYT-g1 and NVS-g2) can be seen in lanes 5-8 in the SDS-PAGE profile).

ELISA Titers

Antigen NYT-g1



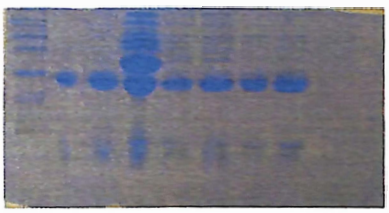
Antigen NVS-g2



	Endpoint	Inflection Point	Endpoint	Inflection Point
Depleted Serum	<50	<50	<50	<50
Purified IgG	891275	773	60027	1628

SDS-PAGE

Lane 1: Molecular Weight Markers (220/97/66/45/30/20.1/14.3 kDa)
Lane 2: 10 µg Rabbit IgG Standard
Lane 3: 20 µg Rabbit IgG Standard
Lane 4: 1 µl Total Rabbit IgG
Antigen NYT-g1
Lane 5: 10 µg Affinity Purified IgG
Lane 6: 20 µg Affinity Purified IgG
Antigen NVS-g2
Lane 7: 10 µg Affinity Purified IgG
Lane 8: 20 µg Affinity Purified IgG



Purified IgG visualized by SDS-PAGE

Table 3. ELISA Titer of both G1-NYT-ABR and G2-NVS-ABR for the final injection and bleed out/purification. Graphed is the amount of purified IgG with a corresponding SDS-PAGE analysis of pAb yeild and purity. As compared to Table 2, the avidity of the two pAb panels is significantly higher with multiple injections over time.

These graphs show us the amount of purified antibodies and avidity levels (graphed OD values) recovered after bleed out and synthetic peptide-affinity chromatography purification for both G1-NYT-ABR and G2-NVS-ABR, evaluated for yield and purity with SDS-PAGE (Tables 2 & 3).

Western Blot Detection of CXCR4 Membrane Preparations and Endo-proteinase Released CXCR4 Peptides

Cell culturing, harvesting, total protein concentration measuring, and endo-proteinase treatment were all done as described below. Briefly, 10-50 μgs of total, solubilized membrane proteins or peptides for each cell line were loaded onto a 12.0% SDS-PAGE followed by electrophoresis at 150 V (constant) for ~1 h using a Mini PROTEAN Tetra Cell (BIO-RAD) following manufacturer's instructions. Precision Plus Kaleidoscope Protein Standards (BIO-RAD) were run simultaneously with samples in separate lanes to monitor resolution for Western blot transfers. Precision Plus Unstained Protein Standards (BIO-RAD) were also run simultaneously with samples in a separate lane for Coomassie brilliant blue or silver staining for use in protein molecular weight calculations. Following electrophoresis, resolved proteins were transferred to either PVDF (polyvinyl diflouride) or Pure Nitrocellulose Membranes (BIO-RAD) at either 100 mA constant for 1h at RT or 30 mA constant overnight at 4°C using a Mini-PROTEAN II (BIO-RAD), again following manufacturer's instructions. Transfer efficiency was assessed either by visual confirmation of the transfer of the Precision Plus Kaleidoscope Protein Standard (BIO-RAD) on the blotting membrane or by briefly staining membranes

with a 0.1% Ponceau S solution in 5.0% acetic acid (Sigma) followed by rinsing in either TBS or ddH_20 to remove the temporary red staining; we also stained the transferred gel using Commassie BB to detect un-transferred proteins. After removal of Ponceau S, Western blot immune-staining detection was performed.

Membranes were blocked in 5.0% (w/v) Milk-TBS blocking solution, rinsed in TBS pH 7.5 (BIO-RAD), and incubated in 1.0% (w/v) Milk-TBS with a 1/100 dilution solution of either the control rabbit anti-human CXCR4 antibody Fusin (eBioscience) or the glycopeptide-directed rabbit anti-human CXCR4 antibodies G1-NYT and G2-NVS (Affinity BioReagents, Inc.) for 4 h at RT or over night at 4° C on a shaker or rocker (Adams Nurator or Reliable Scientific). Following binding of the primary antibody, membranes were rinsed in TBS pH 7.5 3x, 5 min each wash, and then incubated in 1.0% (w/v) Milk-TBS with a 1/2000 dilution of either Alkaline Phosphatase (AP) or Horseradish Peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (BIO-RAD) for 2 h at RT or over-night at 4°C with shaking. Following incubation with secondary antibody, membranes were rinsed as above and blots were developed as per the manufacturer's instructions. Membranes were incubated in Alkaline Phosphatase conjugated secondary antibodies developing solution consisting of Nitroblue tetrazolium chloride, BCIP, and Alkaline Phosphatase Development Buffer blots were monitored until bands appeared. Immunostaining with horseradish peroxidase, a developing solution consisting of hydrogen peroxide, methanol, HRP color development reagent (BIO-RAD), and ddH_20, was also used to develop blots; blots were allowed to develop over a 30-45 min period.

Determination of molecular mass was performed by a least squares linear interpolation from a curve of Precision Plus Unstained Protein Standard (BIO-RAD) run alongside samples during electrophoresis. Blots were photographed using a CCD camera (charge-coupled device; Stratagene Eagle Eye documenting system) or a Nikon 5 MP camera, dried and stored in plastic bags.

Metastatic Neuroblastoma Cell Lines

Our laboratory grew the metastatic neuroblastoma cell lines—IMR-32 (ATCC® CCL-127™) and SK-N-SH (ATCC® HTB-11™) in Table 4 below. We chose these 2 cell lines since both human cells overexpress CXCR4 and are metastatic. Moreover, our CXCR4 specific antibody panels (ABR-New/Old or ThermoFisher) bound the two different glycosylated epitopes (G1-NYT and G2-NVS) of CXCR4 with a high affinity. Of interest to future research is that our pAbs could possibly be used as agents to prevent neuroblastoma cell migration and subsequent neuroblastoma metastatic events.

Cell Type	Metastatic Ability	CXCR4 Expression
IMR-32	+	High
SK-N-SH	+	High

Table 4. Chart listing the 2 metastatic neuroblastoma cell lines used in this research, each representative of varying stages of neuroblastomas, with differential CXCR4 expression levels.

Proteases, also known as a proteinases or proteolytic enzymes, are a large group of enzymes that are used throughout the body for various metabolic processes (Barrett *et al.*, 2003). Proteases occur naturally in all organisms and are involved in multiple physiological reactions, ranging from simple food digestion to more complex processes such as the apoptotic pathway. They are grouped into the class of enzymes known as hydrolases. Proteases work by controlling protein catabolism by catalyzing the reaction of hydrolysis of various linking peptide bonds between the primary amino acid sequences, which include the participation of a water molecule. Proteases are involved in digesting/degrading long protein chains down into short peptide fragments.

Proteases are grouped into four separate major categories—serine proteinases, cysteine proteinases, aspartic proteinases, and metallo-proteinases—according to where they cleave and their catalytic active site characteristics (Barrett *et al.*, 2003). The structure of the catalytic site of the protease and the necessary amino acid(s) presents in the protein is inherent to protease attachment to a certain group in a protein.

Proteases can be specific or limited to breaking certain peptide bonds or they can be promiscuous/unlimited in which types of peptide bonds that they cleave. There are proteases that detach the terminal amino acids from the rest of the protein chain, known as exo-proteinases or those that attack the internal peptide bonds of a protein, known as endo-proteinases. Protease cleavage activity can be destructive to the functioning of a protein, meaning that principal domains or components of the protein are digested, abolishing the proteins ability to activate function or confer a signal in a signal

transduction pathway. For our experimental purpose, specific proteinases were used to release glycopeptides for specific studies.

Glycopeptides

There are many different strategies for analysis and characterization of glycoprotein glycosylation at different levels; such as intact proteins, released glycopeptides, or free oligosaccharide analysis. Analysis of glycoprotein glycosylation at the three different levels allows for better understanding of protein glycosylation function.

More specifically, glycopeptide analysis allows an identification of site-specific glycosylation properties. Several different techniques, methodologies, and experimental protocols have been developed for the analysis of glycopeptides. Amongst the different protocols, most involve glycoprotein digestion with specific endo-proteinases. The most commonly used endo-proteinases to achieve peptide fragments that include the protein glycosylation site are trypsin, chymotrypsin, Asp-N, Glu-C, and Lys-C and are specific to certain amino acid sites (Geyer and Geyer, 2006). The alternative to site-specific proteinases are non-specific proteases, which would be better for achieving very short glycopeptide fragments or glycans. Using the non-specific method can significantly reduce the molecular weight of the desired glycopeptide, but can also produce several peptide fragments that correspond to the same glycosylation site (Geyer and Geyer, 2006).

For my research, site-specific endo-proteinases—trypsin, chymotrypsin, and Glu-C, as well as the chemical agent, cyanogen bromide (CnBr)—were considered for use in

order to yield the desired glycopeptide fragments (see Table 5 below). Ideally, the endo-proteinase of choice should be one that cuts the protein of interest with predictable fragmentation, while leaving the desired glycopeptide sequences intact.

I specifically chose trypsin, a serine protease that cleaves off peptide chains on the carboxyl side of the amino acids lysine (K) and arginine (R), except when either is followed by proline (Barret *et al.*, 2003). Trypsin should yield the most ideal peptide fragment(s) of CXCR4 that contains both glycosylation sites (G1-NYT and G2-NVS), without cleaving within the antibody-directed target sequences. Chymotrypsin, Glu-C, and CNBr are predictably the least efficient in terms of my research because they all cut heavily *within* both glycosylation sites, yielding broken glycol antibody-directed peptide fragments, and thus yield undesireable glycopeptides (please see Table 5).

Following protease treatment, the proteinase-cut CXCR4 glycopeptides were to be subjected to capture by affinity chromatography, run on SDS-PAGE/Western blots to visualize migration differences amongst the predicted differential CXCR4 glycosylation between the different neuroblastoma cell line amino acid target segments seen in Table 6. The affinity chromatography capture and its complications are discussed below.

Table 5. Predicted protease fragments releasing glycopeptide segments from human CXCR4 expressed in IMR-32 and SK-N-SH cell lines used in my thesis. The asparagines containing the N-glycans are in **RED**; the remaining amino acids in the synthetic 17-mer (G1-NYT) and 16-mer (G2-NVS) segments are shown in **BLUE**. The cleavage sites are depicted by the asterisks.

Trypsin:

1. G1-NYT: NH_2-
   MEGISIY**TSDNYTEEMGSGDYDSM**KEPCFR*EE…
2. G2-
   NVS: …K*VVYVBVWIPALLLTIP**DFIFANVSEADDR*YIC**D
   R*F…

Trypsin is an endo-proteinase that cleaves peptide chains on the carboxyl side of the amino acids lysine (K) and arginine (R) amino acid residues, except when either is followed by proline (P).

Cell Culture and Preparation of the Solubilized Membrane Proteins

A typical growth and harvesting protocol of the SK-N-SH and IMR-32 human neuroblastoma cell lines was followed. Cells were grown in monolayers in 150 cm^2 plastic tissue culture flasks at 37°C under 5.0 % CO_2 in either DMEM or EMEM media, supplemented with 10.0 % (v/v) fetal bovine serum and 6.0 % (v/v) penicillin-streptomycin (Fisher Scientific, Inc.). Cells were grown to confluencyat 37°C with

changes of media every other day. The cells were harvested with 0.8 mM EDTA collection solution that contained 137 mM NaCl, 5.6 mM Dextrose, 5.4 mM KCl, and 6.7 mM $NaHCO_3$, pH 7.3.

Cells were pelleted at low speed centrifugation (Sorvall-centrifuge; 3000 xg). The supernatant was then decanted and the cells were resuspended in lysing buffer, containing 5.0 mM EDTA, 5.0 mM HEPES, pH 7.5, and 0.02 % (w/v) sodium azide. The cell suspension was centrifuged at 20,000 xg (Sorvall Superspeed RC2-B centrifuge, [Sorvall, Newton, CT]) for 20 min at 4° C. The supernatant was decanted and the lysed, pelleted cell membranes were resuspended in solubilization buffer: 5.0 % LDS (w/v), 2.0 % (v/v) β-mercaptoethanol, 60 mM Tris-HCl, pH 6.8 (~1.0 ml solubilization buffer per 6 x 10^6 harvested cells). The supernatant, containing the solubilized cell membranes, was heated at 80° C for 3 min, then allowed to cool to room temperature (RT). The solubilized membranes were then filtered through sterile Corning disposable 25 mm, 0.8 micron cellulose acetate membrane syringe filters attached to a 10 cc syringe (Becton Dickinson & Company) to remove most of the free DNA and RNA. The samples were stored at 4°C until time for electrophoretic separation.

Table 6. Potential yielded glycopeptides after proteinase treatment. Asterisks confer to cleavage sites. The Red and Blue amino acids are as depicted in Table 5 above.

Trypsin:

1. G1-NYT: MEGISIY**TSDNYTEEMGSGDYDSM**KEPCFR*
2. G2-NVS:
   - *VVYVBVWIPALLLTIP**DFIFANVSEADDR***
   - ***YICDR***

Protein Assays

Aliquots of total, solubilized membrane protein were taken for the Lowry D_c protein assay. Each of the control blanks, the series of protein standards (bovine serum albumin concentrations of: 14.3, 28.6, 57.2, 71.5, and 85.8 µg/ml), and the total protein aliquots were volumized to 1.0 ml final volume with Reagent A, Reagent B and Reagent S (BIO-RAD). The protein assay followed the direct procedure of Hsin-Lin Cheng's modification of the Lowry method (Protein Assay Products, BIO-RAD).

Reagent A, Reagent B, and Reagent S (BIO-RAD) were mixed with each blank, standard, and sample tube. A purple-blue color reaction was allowed to develop in the tubes for a time period of 10 min at RT. Using plastic cuvettes the optic density / absorbancy of the blanks, standards, and samples were measured at 750 nm wavelength

using a spectrophotometer (SmartSpec™ 3000, BIO-RAD). A calibration curve was created from the optic density/absorbance values of the protein standards and their corresponding protein concentrations: The protein concentrations of the sample tubes were determined from this curve. An equation of mean concentration readings/25µl (final amount of protein and ddH_2O) = 50 µg (amount of protein run on a gel to properly visualize bands on gels upon staining)/Unknown Volume(X) was used to determine the amount in µls that was needed to run in one lane of a gel.

Endo-proteinase Cleavage

Cell culturing, harvesting, and total protein concentration measuring were all performed as previously described. Briefly, all neuroblastoma cell lines (IMR-32, SK-Y-5Y, and SK-N-SH (the SK-Y-5Y cells were used initially)) were subjected to osmotic shock to create cell lysates and the subsequent lysates solubilized using the above-described SDS/β-Mercaptoethanol. The endo-proteinase trypsin was used to completely digest CXCR4 in all three cell lines, yielding N-glycan peptides by cutting the protein on the carboxyl side of lysines and arginines for future characterization on gels and blots. 20 µg of Mass Spectrometry Grade lyophilized trypsin (Fisher Scientific, Inc.) was reconstituted using 20 µl of 50 mM acetic acid, yielding a final concentration of 1.0 µg trypsin/ml. Reconstituted trypsin was aliquoted and stored at -20°C until needed. The non-ionic detergent, NP-40, was added to the harsh, SDS-digestion buffer to neutralize the SDS contained within the neuroblastoma membrane preparation samples, which did not denature the added trypsin. 5x trypsin digestion buffer consisted of 500 mM Sodium

Bicarbonate, 2.5% (v/v) NP-40, and ddH_2O. Two 1.0 ml sterile tubes (one containing trypsin and one tube, with no added trypsin used as the control) contained corresponding calculated amounts of sample, ddH_2O, 5x NP-40 trypsin digestion buffer, and trypsin (to one tube) were mixed together and incubated in a 37°C water bath overnight for 12 h.

Affinity Chromatography

Amino Link® Plus Immobilization Kit (ThermoFisher Scientific) Protocol was used to link G1-NYT and G2-NVS CXCR4 glycopeptide directed antibodies to beaded agarose support (resin in resin PBS buffer) in order to capture released endo-proteinase treated CXCR4 from proteinase-treated neuroblastoma cell lines and to isolate more pAbs from the bleedout serums from each set of rabbits from ABR, Inc. All centrifugations were performed at 3000 xg for 1.0 min using 15 ml collection tubes (Falcon, Fisher Scientific). All equilibrations/incubations were performed by placing columns on a rocker/rotator (Adams Nutator). For column preparation or attachment of the antibodies to the agarose beads, columns were equilibrated to RT to reconstitute the beads into the resin buffer. The column was spun down to remove resin buffer and 2.0 ml of BupHTM Citrate-Carbonate "coupling" buffer, pH 10 (0.1M Sodium Citrate, 0.05M Sodium Carbonate, 0.05% Sodium Azide) was added. Once the agarose beads were reconstituted, the resin was transferred to a 15 ml sterile tube (Falcon) and 4.0 ml of coupling buffer was added. Finally, the ABR pAbs were added (ABR, Inc./Amount-2 ml; Tube 7, 5.6 mg/ml antibody – 11.2 mg total pAb added) and incubated 4 h at RT or over night at 4° C (100 µl of antibody was saved at this step to later evaluate coupling

efficiency of the antibody to the agarose beads using the protein assay). After overnight incubation, the resin solution was transferred from the 15 ml tube (Falcon) and added back into the column 2.0 ml at a time and centrifuged to remove the coupling buffer (additionally, all supernatant was saved at this step to compare to the 100 µl of saved original antibody for comparison to determine the coupling efficiency using the protein assay—normalizing the2 volume sets). The resin beads were then washed with BupHTM PBS, pH 7.2 (0.1M Sodium Phosphate, 0.15 Sodium Chloride, 0.05% Sodium Azide) 3x. Then 2.0 ml of the pH 7.2 BupHTM PBS solution was added; spiked with 40 µl of 2.0% (w/v) Sodium Azide to the column for storage purposes.

For column use or capture of released endo-proteinase treated CXCR4 from neuroblastoma cell line solubilized proteins, the column was first reconstituted at RT and then centrifuged to remove the storage buffer. The column was then reconstituted with 6.0 ml of binding/wash buffer (BupHTM PBS, pH 7.2) and centrifuged. The peptide sample was heated at 80°C for 5 min to inactivate the trypsin, and 3.0 ml of the peptide-containing sample was added along with 200 µl of the binding/wash buffer (BupHTM PBS, pH 7.2) and the column was incubated overnight.

Immunocytochemistry

Immunocytochemistry was used to show the *in vivo* expression level of human CXCR4, and to determine the ability of our ABR, Inc. and Fisher Thermoscience pAb panels to recognize cell surface expression; native CXCR4 in IMR-32 neuroblastoma

cells. The experiment was done in duplicates. The cover slips (Fisher Brand) were cleaned using 90 % ethanol in the laminar flow hood and then UV-treated overnight to sterilize the environment. The cover slips were then transferred to 6-well cell culture plates (Corning, Inc.). IMR-32 cells were grown to confluency in 75 cm^2 sterile flasks (Corning Inc.) and were used to seed all cover slips and placed in our CO_2 incubator to grow overnight. Cells were fixed to the cover slips using PBS (phosphate-buffered saline, pH 7.2) with 4.0% (v/v) paraformaldehyde for 20 min at RT and subsequently rinsed 3x with PBS. Then cells were incubated in PBS containing 0.1% Triton X-100 for 15 min at RT in order to permeabilize the fixed cells. Cells were again rinsed 3x with PBS. Fixed cells were incubated in PBS with 10.0% goat serum for 1 h at RT for blocking.

Antibodies Fusin-control antibody, G1-NYT and G2-NVS (from Affinity BioReagents, Inc. and ThermoFisher Scientific) were diluted to the necessary concentration in 10.0% goat serum and cells were incubated with primary antibody at 4°C overnight or 2 h RT and subsequently rinsed 3x in PBS containing 1.0% (v/v) goat serum. All steps after primary antibody incubation were performed in the absence of light. Flourophore-conjugated secondary antibody (Invitrogen, Inc.) was diluted in 10% goat serum and cells were incubated for 2 h at RT and subsequently rinsed 3x in PBS containing 1.0% (v/v) goat serum.

To specifically stain the cell nuclei, DAPI nuclear stain was diluted in 1.0% (v/v) goat serum at a concentration of 300 ng/ml: Cells were incubated in DAPI for 30 min. Cover slips were then applied to appropriately labeled glass slides containing one drop of mounting medium (Fisher Brand) using forceps and sealed with clear nail polish. A

Leica SP5 confocal microscope was used to observe cells with Laser 405 for DAPI and Argon 488 for Alexa Flour, with the assistance of Dr. Chandran Sabanayagam.

RESULTS

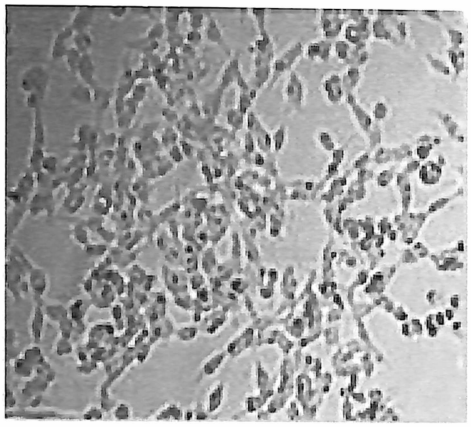
Human CXCR4 Detection in IMR-32 Cells using Polyclonal Antibodies

We tested the binding efficacy of our ABR pAb set to determine the surface level expression of CXCR4 in the metastatic neuroblastoma cell line IMR-32. If our pAbs bound the surface expressed human CXCR4 then we knew that our pAbs would bind to the natively-expressed, cancer-related protein, CXCR4, we could visualize the distribution of cell surface glycosylated human CXCR4, and in future studies, determine if the pAbs could inhibit metastasis of these migrating neuroblastoma cells. Therefore, IMR-32 neuroblastoma cells, overexpressing CXCR4, were grown and used in immunocytochemistry (ICC) studies using all our 3 pAb panels.

The cell culture morphology of our ATCC obtained IMR-32 cells is seen in figure 6 compares my cells (IMR-32) with previously published micrographs of IMR-32 cell lines. As can be seen in the figure, the two cell line morphologies are comparable.

Using ICC, I tested my hypothesis that our pAb panels bind the expressed, native CXCR4 in IMR-32 cells, a metastatic neuroblastoma cell line. For our control antibody we used Fusin, a commercial CXCR4 pAb purchased from Santa Cruz Biotechnology, that bind the second extracellular loop of human CXCR4.

Our IMR-32 Cultured Cells



IMR-32 Cells from ATCC

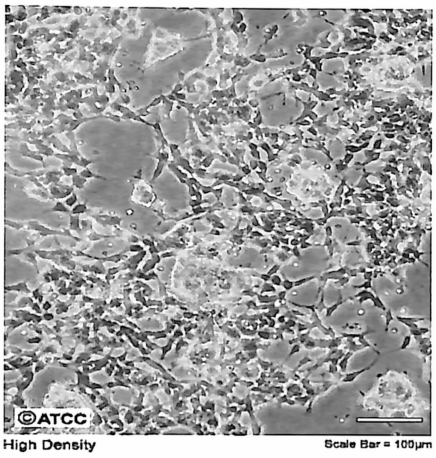


Figure 5. IMR-32 neuroblastoma cell lines growing in culture; a comparison of our cell line morphology vs. ATCC micrograph of IMR-32 cells in culture (ATCC, product description, 2011). Our cells were grown in 75cm^2 or 225 cm^2 cell culture flasks, fed every other day with DMEM, and harvested/reseeded once a week. Micrograph of our IMR-32 cell line actively growing in culture was taken using an Olympus IX71 Inverted Basic Research Microscope with Hamamatsu Digital Camera Attachment (C4742-95 line cell micrograph (ATTC, HTB-11).

The results of our first set of ICC control experiments are presented in figure 6. The first micrograph was stained with DAPI blue nuclear stain (Figure 6a) to identify the IMR-32 nuclei. The second micrograph shows cells stained with DAPI blue nuclear stain followed by incubation with goat anti-rabbit (GAR) secondary fluorescently- conjugated

antibodies (Figure 6b; Alexa Fluor 488 –Invitrogen, Inc.) to show non-specific binding of the secondary GAR-fluorescent pAbs, used later to locate our bound primary pAbs. Minimal to no background is seen in Figure 6b by GAR pAbs when used without our primary pAbs. Both of the control immunocytochemistry experiments—no antibody treatment with DAPI nuclear stain and only secondary antibody treatment with DAPI nuclear stain—appear similar.

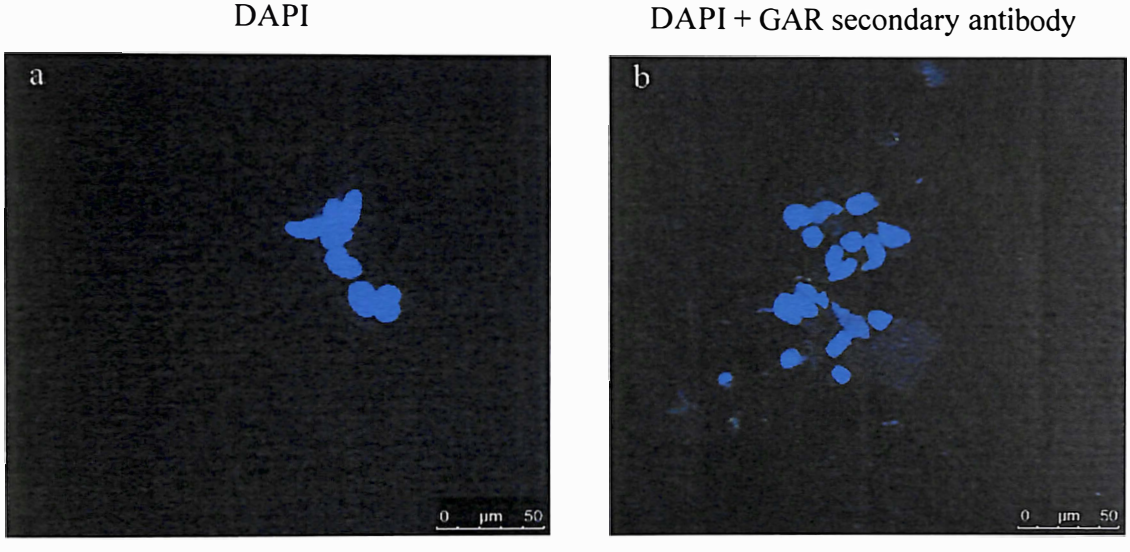


Figure 6. Control staining of our IMR-32 cells. Panel (a) was stained using DAPI nuclear stain, with no antibodies present and panel (b) DAPI nuclear stain + secondary GAR antibody treatment to assess the background, non-specific binding. Cells were located using a Leica SP5 microscope (NA 63x, water lens 1.3), and images were taken using Leica LAS AF software. The cellular nuclei are detected as blue fluorescence, stained with DAPI dye in each panel. Any residual secondary binding would be green fluorescence, stained with Alexa Fluor, covalently attached to the secondary goat anti-rabbit (GAR) antibody. These images represent a wide field view of multiple cell images.

DAPI + Fusin Control Polyclonal Antibody

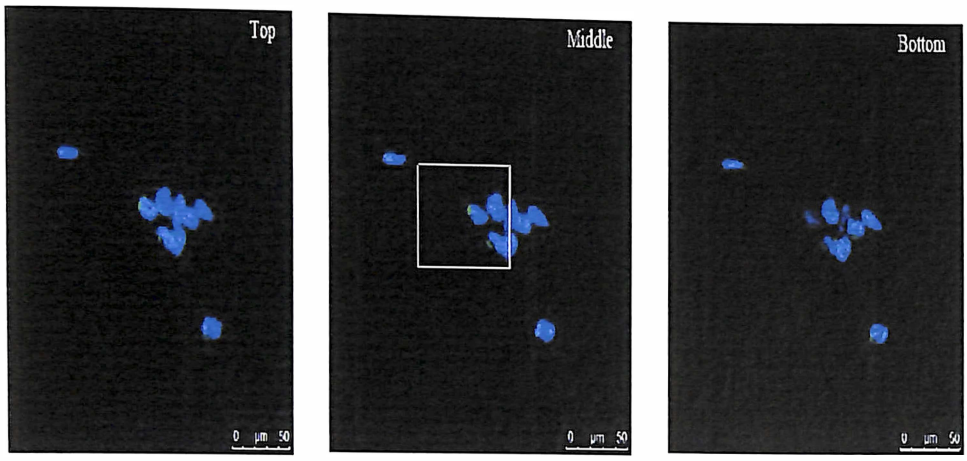


Figure 7. Fluorescent antibody immunocytochemistry imaging of IMR-32 cells. These micrographs include DAPI nuclear stain and control polyclonal antibody, Fusin. Again, cells were located using Leica SP5 microscope (NA 63x, water lens 1.3), and images were taken using Leica LAS AF software. These images represent multiple cell image sections; from the top of the cells, the middle view, and the bottom view. The white, boxed-in cells are a zoomed-in image seen in Figure 9, panel 9b below.

Previous studies have shown Fusin to bind CXCR4 in normal mouse intestine membranes using ICC imaging (Figure 8a). In our hands, the Fusin control antibody bound weakly on our IMR-32 cell surfaces. One can clearly visualize the binding differences of the control Fusin antibody to CXCR4 in normal mouse intestine membranes versus our IMR-32 cell membranes (Figure 8; panels 8a & 8b).

In stark contrast to the control antibody, Fusin, binding to CXCR4 on the cell surface of IMR-32 human neuroblastoma cell line, our CXCR4 polyclonal glycopeptide antibodies G1-NYT and G2-NVS (Affinity BioReagents - New and Old) bound surface

DAPI + Fusin Control Polyclonal Antibody: A comparison with previous studies

Previous Our Results

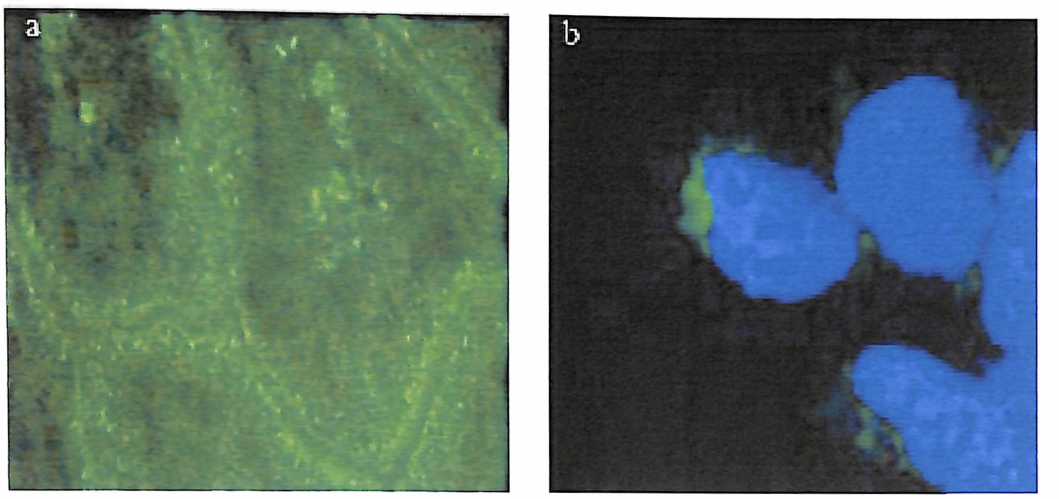


Figure 8. Fusin fluorescent antibody immunocytochemistry imaging of IMR-32 cells. (a) Normal mouse intestine frozen section, showing membrane staining (Santa Cruz Biotechnology Website, Fusin H-118). (b) DAPI nuclear stain and Fusin control polyclonal antibody (our results). Minimal Fusin binding is observed. *Image (b) represents a zoomed-in image seen boxed off in Figure 7 above.*

membrane CXCR4 in IMR-32 neuroblastoma cells quite well (Figures 9 & 10). Our polyclonal glycopeptide antibody that recognizes the first glycosylation site (Asn 11) located on the first extracellular loop of CXCR4 (G1-NYT: Affinity BioReagents-New and Old) bound with a much higher affinity and presence (Figures 9 & 10) than our polyclonal glycopeptide antibody that recognizes the second glycosylation site (Asn 176) located on the second extra-cellular loop of CXCR4 (G2-NVS: Affinity BioReagents -

New and Old; Figures 9 & 10). This is in agreement with the low binding affinity seen with Fusin, which also binds the second glycan loop of CXCR4.

Figure 9. G1 **pAb (first glycosylated loop specific) Affinity BioReagents, Inc.**

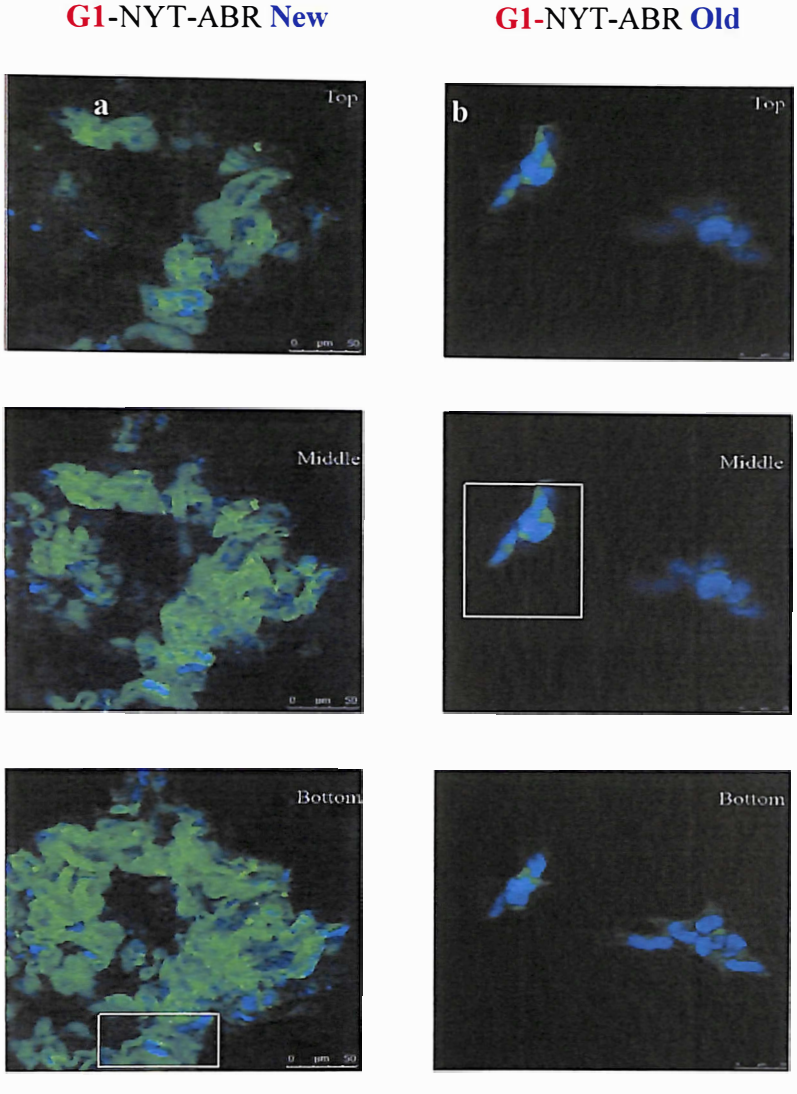


Figure 9. G1-NYT New and Old pAb ICC imaging of our IMR-32 cells. (a) DAPI nuclear stain + G1-NYT-ABR **New** polyclonal glycopeptide antibody treatment (Top, middle and bottom views for clarity – 0-50 µm magnification). (b) DAPI nuclear stain + G1-NYT-ABR **Old** pAb challenge (Top, middle and bottom views for clarity - 0-25 µm magnification. Again, these images represent a wide-field view of multiple cell images. The white, boxed-in cells are zoomed-in in figure 10.

G1-NYT-ABR **New**

G1-NYT-ABR **Old**

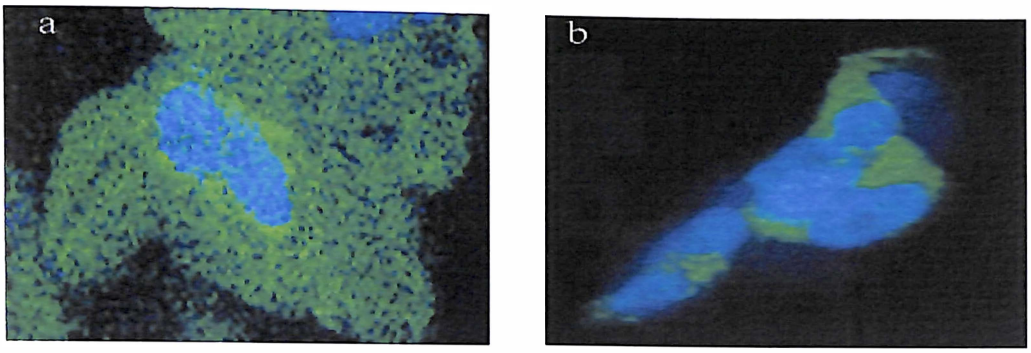


Figure 10. Close-ups of figure 9 above of our IMR-32 cells immunostained with G1 pAbs. (a) DAPI nuclear stain + G1-NYT-ABR New polyclonal glycopeptide antibody treatment. (b) DAPI nuclear stain + G1-NYT-ABR Old polyclonal antibody stained: middle sections for both.

Comparing the binding of control Fusin pAbs (figures 7 & 8) to the binding of our ABR CXCR4 pAbs (**New**-2010 and **Old**-2008; Figures 9-12), our pAbs recognized more membrane surface-level expressed CXCR4 that the control, Fusin pAbs. Yet, when comparing the binding of control CXCR4 polyclonal antibody Fusin (Figures 7 & 8) to the binding profile of our ThermoFisher Scientific (2009) CXCR4 plyclonal glycopeptides antibodies (Figure 13), the Fusin control pAbs recognized more surface-level expressed CXCR4 than the Thermofisher Scientific CXCR4 pAbs. Both are noteworthy findings that will be further elaborated upon in the discussion section.

Figure 11. G2 **pAb (second glycosylated loop specific) Affinity BioReagents,**

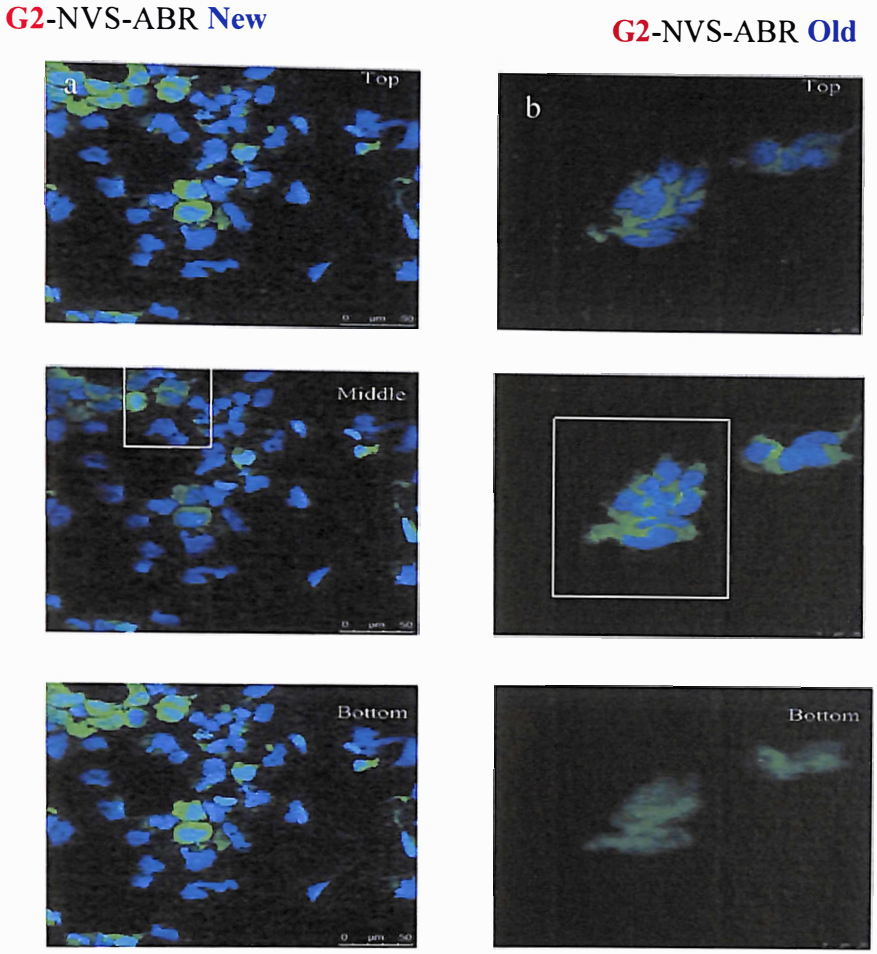


Figure 11. ICC imaging of our IMR-32 cells using our G2 pAbs. (a) DAPI nuclear stain + G2-NVS-ABR **New** polyclonal glycopeptide antibody treatment (Top, middle, and bottom views for clarity - 0-50 µm magnification). (b) DAPI nuclear stain + G2-NVS-ABR **Old** polyclonal glycopeptide antibody treatment (Top, middle, and bottom views for clarity - 0-25 µm magnification). The white, boxed-in cells represent the zoomed-in images seen in Figure 12 below.

G2-NVS-ABR New

G2-NVS-ABR Old

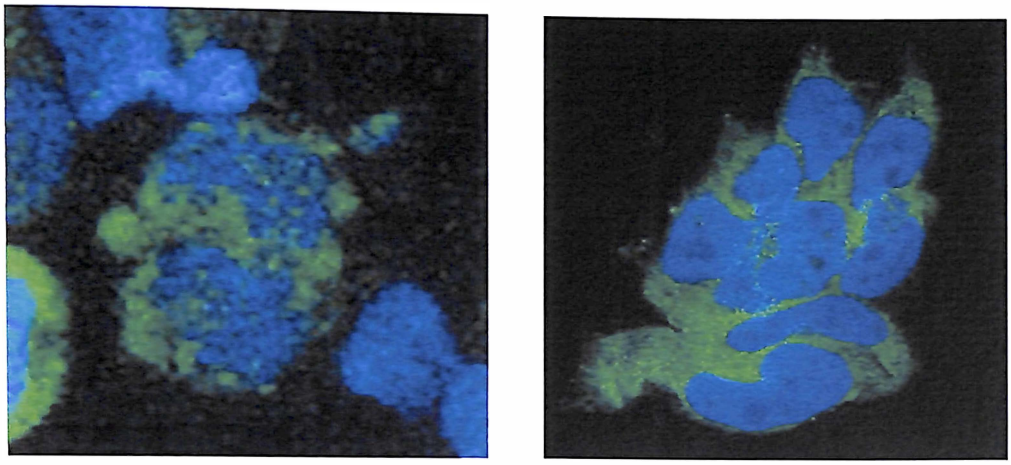


Figure 12. Close-up of ICC imaging of IMR-32 cells boxed in figure 11. (a) DAPI nuclear stain + G2-NVS-ABR New polyclonal glycopeptide antibody treatment. (b) DAPI nuclear stain + G2-NVS-ABR Old polyclonal glycopeptide antibody treatment: middle sections for both.

In stark contrast to the ABR generated pAbs to CXCR4, the ThermoFisher-raised pAbs did not bind well to the IMR-32 cell surface in our ICC experiments (Figure 13), nor did they exhibit high binding affinity to expressed CXCR4 in IMR-32 or SK-N-SH solubilized cell membrane preparations on our Western blots. The ThermoFisher CXCR4 polyclonal glycopeptide antibodies show similar profiles to that of the first two control immunocytochemistry experiments that exhibit no antibody treatment, and only secondary antibody treatment, respectively (Figure 6).

ThermoFisher Generated Polyclonal Antibodies

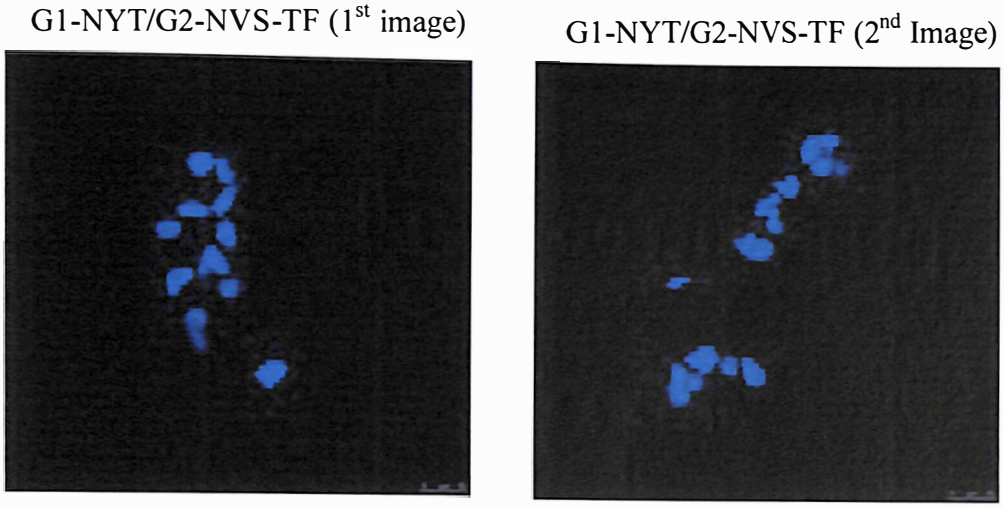


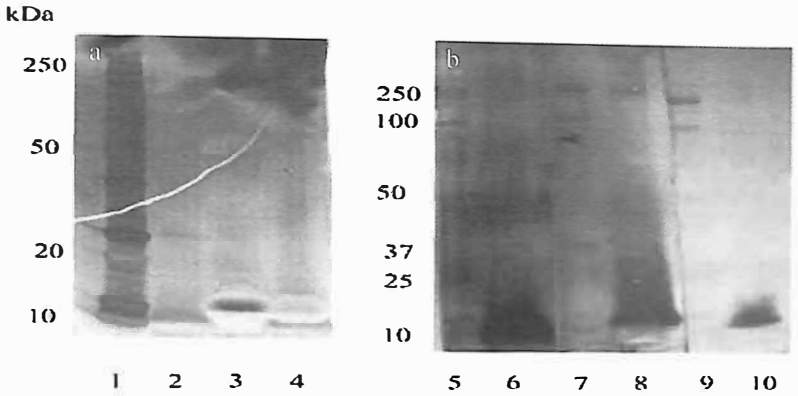
Figure 13. ThermoFisher Scientific IC of our IMR-32 cells. The 2 panels are different cell populations of IMR-32 cells, both challenged with combination G1-NYT/G2-NVS ThermoFisher CXCR4 pAbs (2009); 0-25 µm magnification. Cells were located using Leica SP5 microscope (NA 63x, water lens 1.3), and images were taken using Leica LAS AF software. Very little green is seen in these micrographs, indicating poor CXCR4 surface-expressed recognition by the ThermoFisher pAbs.

Western Blot Detection of Synthetic, Non-glycosylated CXCR4 Peptides (ABR)

After a successful ICC recognition of cell-surface overexpression of human CXCR4 in our IMR-32 neuroblastoma cells using our ABR pAb panels, we turned our focus to determining if the antibodies recognized both the naked synthetic peptide and its accompanying target CXCR4 protein sequence in mature CXCR4 on blots (Figure 14).

First, the ABR synthetic, non-glycosylated peptides were separated using SDS-PAGE and transferred to blots. The blots were challenged with G1-NYT-ABR Old, G2-

NVS-ABR Old, and a mixture of both, respectively, as seen in Figure 14. Using the amino acid content of each peptide, we calculated 1,900 daltons (G1-NYT-ABR) and 1,878 daltons (G2-NVS-ABR) for the 2 synthetic peptides. On our SDS-PAGE their apparent molecular weights (M_r) were much different: 7,000 (G1) and 13,000 (G2).



ABR Synthetic Peptides on gel and blots, challeneged with ABR raised polyclonal antibodies G1 & G2

Figure 14. 12% SDS-PAGE and sister blot of antigenic synthetic peptides. (a) Silver-stained gel with control peptides. Lane 1 contained MW stds, lane 2 contained G1-NYT control peptide, lane 3 contained G2-NVS control peptide, and lane 4 contained a combination of both peptides. (b) Western blot with control peptides. Lanes 5, 7, and 9 are Kaleidoscope MW Stds, lane 6 contained G1-NYT control peptide challenged with G1-NYT peptide antibody (7 kDa), lane 8 contained G2-NVS control peptide challenged with G2-NVS pAbs (13 kDa), and lane 10 contained both G1-NYT and G2-NVS control peptides challenged with both G1-NYT and G2-NVS pAbs. All peptides were loaded on the gel at 1.0 μg/lane.

These large discrepancies in calculated and gel apparent molecular weights could be due to the fact that the peptide's amino acid compositions did not bind as much SDS as normal proteins and thus ran much slower on our gels. Each peptide was also difficult to stain on gels and blots using non-specific staining.

Nonetheless, our ABR pAbs raised to synthetic amino acid sequences in human CXCR4 recognize the SDS-PAGE separated peptides transferred to blots. This supports the fact these pAbs are specific for their targeted glycosylated sequences in human CXCR4 expressed in living cancer cells, as seen in our ICC studies above.

Profiling Human CXCR4 in Gels and Western Blots using our pAbs

For the profiling of our pAbs on gels and Westerns we raised cells and used both our neuroblastoma cell lines, IMR-32 and SK-N-SH, to make membrane preparations (Figure 20). Western blots were run against total membrane protein preparations isolated from IMR-32 and SK-N-SH solubilized cells to confirm that CXCR4 was detectable by our control anti-CXCR4 antibody, Fusin, as well as by the isolated peptide antibody panels: G1-NYT-ABR and G2-NVS-ABR (New and Old), and G1-NYT/G2-NVS mixture (ThermoFisher, Sci.). The blots were also used to confirm the ICC binding data to insure our pAbs were indeed binding CXCR4 proteins.

Once the solubilized IMR-32 and SK-N-SH membrane proteins were separated using 12% SDS-PAGE (Figure 15), they were transferred to either nitro-cellulose or PVDF membrane using standard Western blotting protocols as described in the Methods/Background section. The membranes were immunostained using control FusinpAb, peptide antibodies G1-NYT-ABR/G2-NVS-ABR (New), G1-NYT-ABR (Old),G2-NVS-ABR (New), or the combination G1-NYT/G2-NVS-TF pAb (Figures 15-17). Gels were routinely Coomassie brilliant blue or silver stained. Silver staining is ten

Fusin Challenged (control) IMR-32 and SK-N-SH membrane proteins for Human CXCR4 Detection in our Western blots

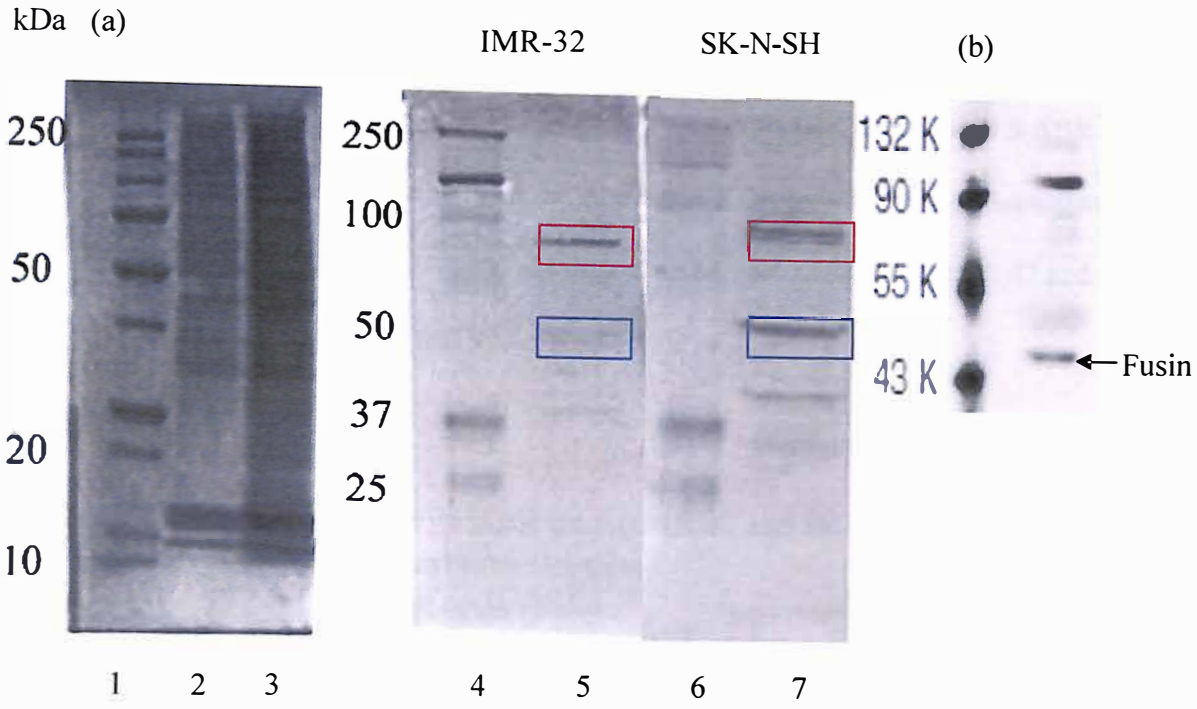


Figure 15. 12.0% SDS-PAGE (Coomassie-stained gel) and Western blots of solubilized IMR-32 and SK-N-SH neuroblastoma cell membranes. (a) Lane 1 contained MW Stds, lanes 4 & 6 contained Kaleidoscope MW Stds, lanes 2 & 5 contained solubilized membrane proteins from IMR-32 cells (50 μg/lane), and lanes 3 & 7 contained solubilized membrane proteins from SK-N-SH cells (50 μg/lane).

Western blots were challenged with the control pAb Fusin. (b) Insert comparison of Western blot of Jurkat (Santa Cruz Biotechnology) whole cell lysates showing similar recognition of CXCR4 using Fusin commercial CXCR4 pAb (Santa Cruz Biotechnology Website, Fusin H-118). The Native CXCR4 (47 kDa) is boxed in blue and the possible dimer of CXCR4, 101 kDa, is boxed in RED.

times more sensitive than Coomassie brilliant blue staining (BIO-RAD, 1997). After comparing our silver-stained gels to our Coomassie-stained gels, the CBB turned out to be sufficient for charactizing the detection and patterning of human CXCR4 when compared to the more sensitive silver staining.

Western blots immunostained using Fusin, G1-NYT-ABR and G2-NVS-ABR (New) all exhibited binding to denatured, solubilized CXCR4 with specific expression patterns comparable to previous studies seeing recurring, detectable bands at ~47 and ~101 kDa; as seen in Figures15 and 16.

Western Blots Probed With ABR pAb (IMR-32 and SK-N-SH Membrane Proteins)

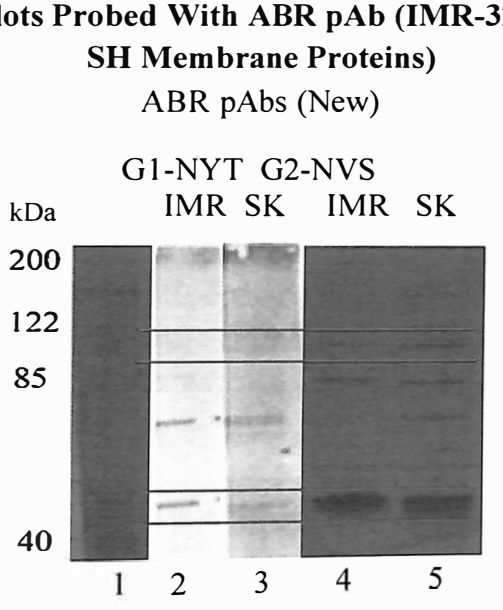
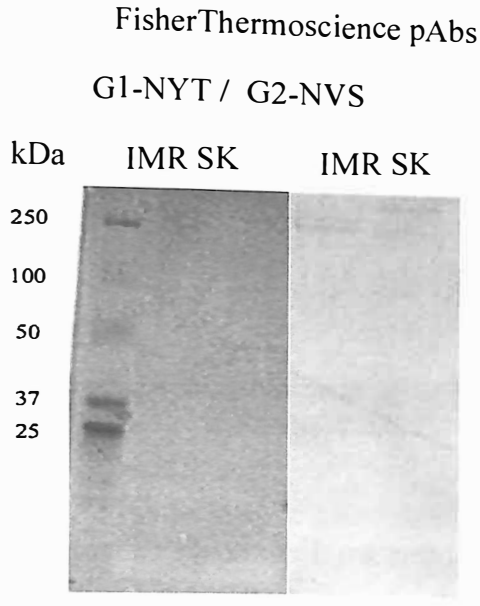


Figure 16. Western blots of IMR-32 and SK-N-SH neuroblastoma cell membrane proteins immunostained using G1-NYT-ABR (new) and G2-NVS-ABR (new) pAbs. Lane 1 contained Kaliedoscope MW Stds, lanes 2&3 contained solubilized membrane proteins from IMR-32 cells (50 µg/lane), lanes 3&5 contained proteins from SK-N-SH cells (50 µg/lane. Boxed in BLUE is CXCR4 47 kDa and boxed in RED is the probable CXCR4 dimer at 101 kDa; similar protein profiles are seen in our Fusin-stained blots in figure 15 above.



Duplicate Experiment showing little to no binding of CXCR4 proteins using the pAbs derived from Fisher Thermoscience

Figure 17. Duplicate Experiment showing little to no binding of CXCR4 proteins using the pAbs derived from Fisher Thermoscience. Western blot results of IMR-32 and SK-N-SH neuroblastoma cell line preparations challenged with CXCR4 pAb mixed G1-NYT/G2-NVS-TF. Lanes 1 & 4 contain Kaliedoscope MW Stds, lanes 2 & 5 contained solubilized membrane proteins from IMR-32 cells (50 μg/lane), lanes 3 & 6 contained solubilized membrane proteins from SK-N-SH cells (50 μg/lane). Both blots were challenged with CXCR4 glycopeptide polyclonal mixedG1-NYT/G2-NVS-TF antibody (two blots are the same experiment run twice. Minimal band detection is seen with the Fisher Thermoscience CXCR4 pAbs, G1-NYT/G2-NVS-TF.

The blot profiles of Fusin compared favorably with our ABR pAbs (G1 & G2; Jurkat-Santa Cruz Biotechnology). This is in good agreement with our ICC studies using these important human CXCR4 polyclonal antibodies. These specific Human CXCR4 polyclonal antibodies bind both the native AND the denatured CXCR4 glycoprotein. Unfortunately, no detection of discernable banding on our blots were seen using the Fisher Thermoscience pAbs. This, too, is in agreement with minimal cell surface binding

of these CXCR4 pAbs raised by Fisher Thermoscience. This suggests that the batch of pAbs is important for binding specificity, even though both companies, ABR and Fisher Thermoscience, used similar polyclonal antibody generating protocols.

Trypsin Endoproteinase Treatment of IMR-32 Solubilized Membrane Preparations and Subsequent Peptide pAb Recognition

To visualize glycan shift patterns of CXCR4 in neuroblastoma cell line IMR-32, a novel technique was employed. Instead of removing the sugar from the native protein and analyzing its structure via spectroscopic analysis, we took a more indirect approach to yield similar information.

We used trypsin to digest the entire array of IMR-32 membrane proteins to ultimately include fragmented CXCR4 protein: CXCR4 glycopeptides and non- CXCR4 peptides. CXCR4 protein was cut using trypsin, an endo-proteinase and separated using SDS-PAGE (Figure 18). As seen in figure 18, all the membrane proteins seen in lane 2 are completely cleaved: a total digest.

Western blotting was used to determine if our pAbs to CXCR4 recognized any of the glycopeptide fragments after trypsin treatment. The difference in molecular weights of the endo-proteinase cut CXCR4 peptides in comparison to the control synthetic peptide would come from two important factors: size of the composition of glycan monosaccharides and mass of the attached glycans. Peptide size differences are

generated due to the location trypsin cleaves (on the carboxyl side of R and K) in native CXCR4.

In addition to the trypsin treatment, we treated our membrane proteins with PNGase (Figure 19), which removes N-glycans, to produce a smaller proteainase fragment that would shift lower in our gels and blots to molecular weights, similar to our 2 target synthetic peptides.Unfortunately, the CXCR4 polyclonal glycopeptide antibodies G1-NYT and G2-NVS were unable to locate the trypsin cut CXCR4 and trypsin cut + PNGase treated CXCR4.

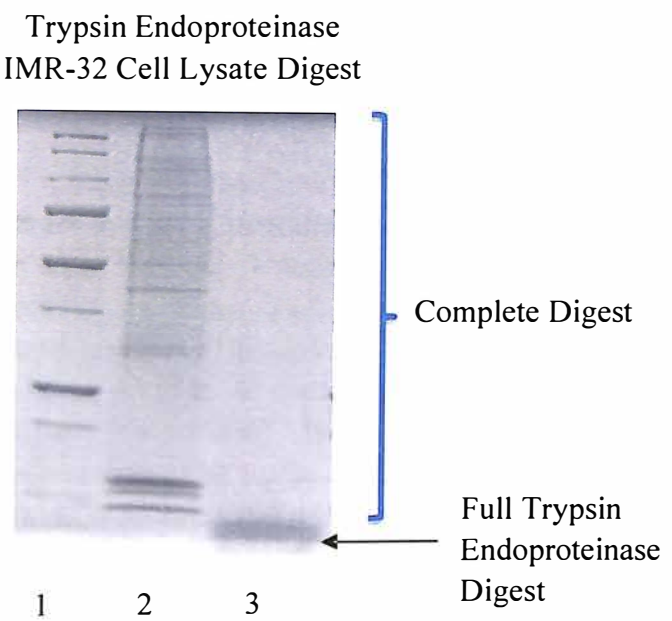


Figure 18. Trypsin endoproteinase IMR-32 cell lysate digest. Samples were separated using a 12% SDS-PAGE. Lane 1 contained Molecular Weight Standard (BIO-RAD), lane 2 contained uncut IMR-32 cell lysate membrane preparation, and lane 3 contains trypsin endoproteinase cut IMR-32 cell lysate membrane preparation giving a complete digestion.

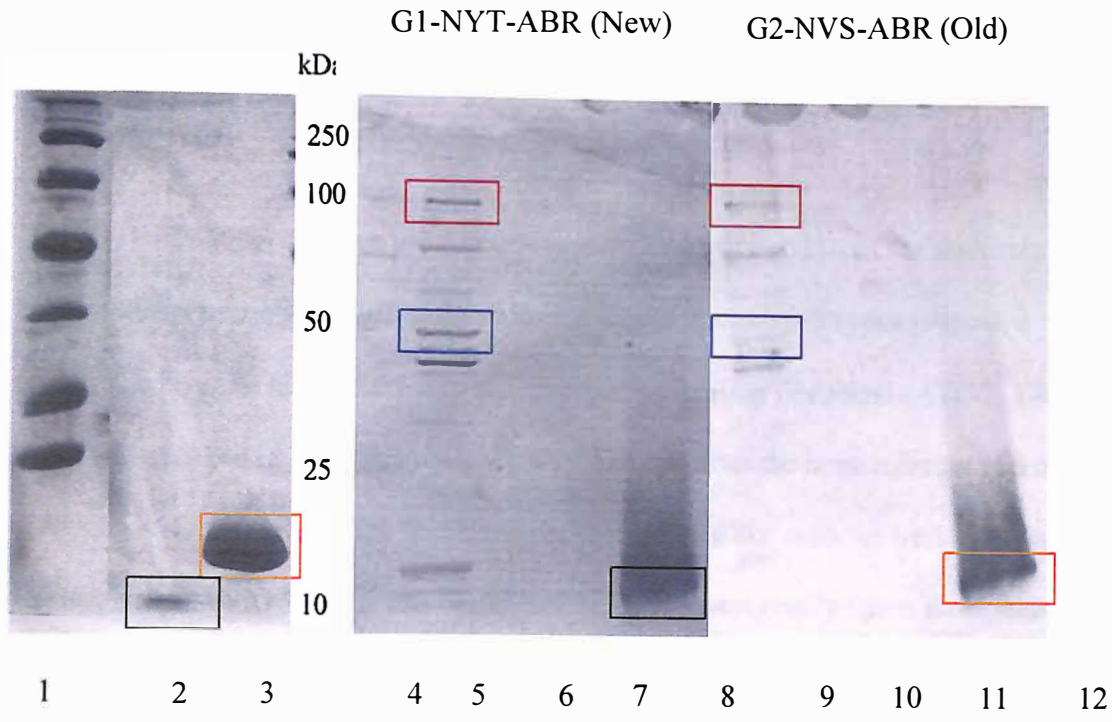


Figure 19. Coomassie stained 12% SDS-PAGE and accompanying sister blots of our 2 target synthetic peptides and accompanying Western blots of trypsinand PNGase cut IMR-32 solubilized cell membrane preparations. Lane 1 containedMWstds, lanes 2 & 3 contained G1-NYT-ABR and G2-NVS-ABR synthesized peptides (1.0 μg/lane), respectively. Lane 4 contained Kaliedoscope MW stds, lanes 5 & 9 contained uncut IMR-32 solubilized cell membrane preparations (50 μg/lane), lanes 6 & 10 contained trypsin-treated IMR-32 membrane proteins (50 μg/lane), lanes 7 & 11 contained trypsin and PNGase-treated IMR-32 proteins (50 μg/lane), and lanes 8 & 12 contain G1-NVT-ABR and G2-NVS-ABR synthetic peptides, respectively (1.0 μg/lane). Boxed in BLUE is CXCR4 47 kDa and boxed in RED is a CXCR4 dimer at 101 kDa. Boxed in black are synthetic peptides G1-NYT-ABR at 7,000 d and boxed in orange is the G2-NVS-ABR peptide (13,000 d).

DISCUSSION

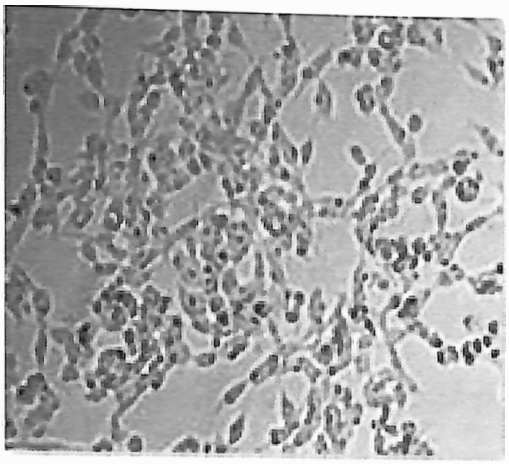
The IMR-32 cell line used in this research was derived from the abdomen of a human, thirteen-month old, Caucasian male. There are two cell types present, a predominant, small neuroblast-like cell and a large hyaline fibroblast (ATCC, 1967). The SK-N-SH cell line used in this research was derived from the bone marrow of a human, four-year old female (1970). There are also neuroblast-like cells, as well as fibroblasts present in the SK-N-SH cell line. Both cell lines were originally taken from metastatic neuroblastomas. Each of these cell lines has previously been shown to overexpress the chemokine receptor CXCR4 at different levels: IMR-32 at high-level expression and SK-N-SH at intermediate level expression; as characterized by Carlisle *et al.*, 2009. Pictures of our cultured IMR-32 cell lines,—self-taken cell micrograph pictures (CMG), and ATCC product taken CMG pictures,—featured in Figure 20, were used to demonstrate my ability to successfully grow cells in culture (IMR-32) and also to show the morphology of our IMR-32 cells in culture.

The self-taken CMG IMR-32 cell picture in terms of cell quality and morphology is comparable to the ATCC product taken CMG IMR-32 pictures. Therefore, my work is comparable to cell culture results published in previous literature. Even though, SK-N-SH is a separate cell line, distinguishable from the IMR-32 cell line, the morphology and CMG picture quality of my self-taken picture (IMR-32) is also comparable with that of the CMG pictures of the SK-N-SH CMG pictures taken from the ATCC published product description (ATCC, 2011).

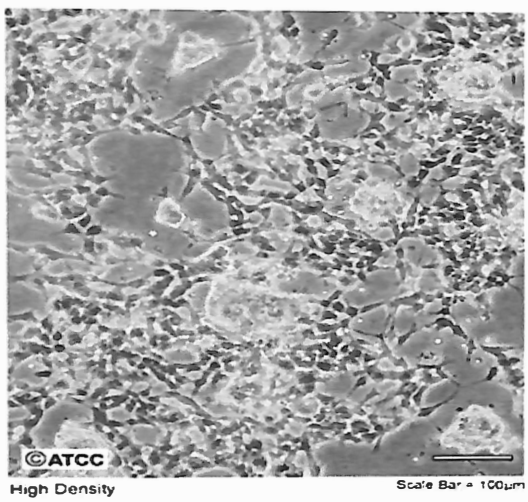
IMR-32 cell lines have previously been shown to be the neuroblastoma cell line representative of having the highest level of CXCR4 over expression (Carlisle, *et al.*, 2009). Therefore, in terms of our immunocytochemical results, only the IMR-32 neuroblastoma cell line was used to visualize surface level CXCR4 expression and use in cell membrane preparations to further characterize our polyclonal CXCR4 glycopeptide antibodies.

Our experimental results were rather surprising and promising. The polyclonal antibodies G1-NYT and G2-NVS from Affinity BioReagents (New-2010) bound to the plasma membranes of IMR-32 neuroblastoma cells extremely well, with full coverage showing plentiful CXCR4 membrane/cell surface level expression. Contrary to these strong results, our ThermoFisher CXCR4 polyclonal glycopeptide antibodies (2009) as well as the control CXCR4 antibody Fusin, did not bind IMR-32 cells well.

Our IMR-32 Cultured Cells



IMR-32 Cells from ATCC



SK-N-SH Cells from ATCC

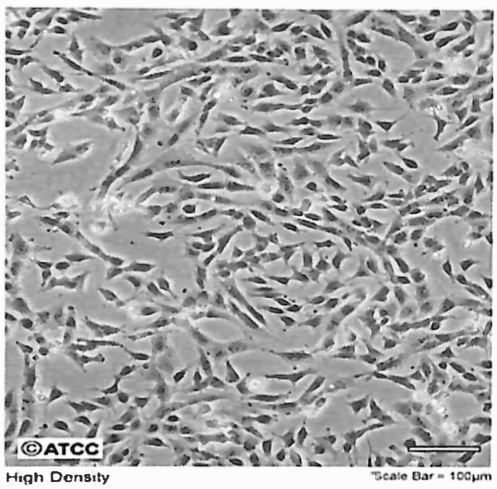


Figure 20. Morphology of CMG pictures of our IMR-32 cell line growing in culture in comparison to the CMG pictures taken by ATCC of the IMR-32 and SK-N-SH cell lines growing in culture.

Of the two sets of Affinity BioReagent (ABR) CXCR4 polyclonal glycopeptide antibodies (New-2010 and Old-2008), the New ABR pAbs bind to the cell-surface expressed, native CXCR4, and also recognize the denatured CXCR4 in our Western blots. In contrast, our Old ABR CXCR4 polyclonal glycopeptide antibodies showed high affinity in terms of binding to CXCR4 in the IMR-32 cell line. Yet, Old ABR pAbs exhibited extreme cross reactive binding in terms of CXCR4 detection in IMR-32 and SK-N-SH cells on Western blots.

In the case of the ThermoFisher (TF) CXCR4 polyclonal glycopeptide antibody (2009)—which contained both G1-NYT and G2-NVS antibodies in the rabbit sera, they did not bind well to the cell surface native CXCR4 in IMR-32 cells in immunocytochemistry studies (Figure 13), which agreed with Western blots, as this antibody also did not bind denatured solubilized CXCR4 in IMR-32 cells and also did not bind denatured solubilized CXCR4 in SK-N-SH cells (Figure 18). Also peculiar was the low binding affinity to CXCR4 in IMR-32 cells exhibited by the control antibody, Fusin (Santa Cruz BioTechnology) in immunocytochemistry results (Figures 7 & 8), as compared to high binding affinity of Fusin to CXCR4 in normal mouse intestine (Figure 9-Santa Cruz BioTechnology). The Fusin ICC results (Figures 7 & 8) were in contrast to Fusin binding to denatured CXCR4 on Western blots of both the IMR-32 and SK-N-SH neuroblastoma cell lines (Figure 15). Our results are consistent with previous Western blot results using the same Fusin control CXCR4 antibody (Pérez-Martínez, M., *et al.* 2010). Also, the data sheet characterizing the Fusin control antibody from Santa Cruz Biotechnology states:

Fusin (H-118) is recommended for detection of fusin of mouse, rat and human origin by **Western Blotting** (starting dilution 1:200, dilution range 1:100-1:1000), immunoprecipitation [1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)], **immunofluorescence** (starting dilution 1:50, dilution range 1:50-1:500), immunohistochemistry (including paraffin-embedded sections) (start-ing dilution 1:50, dilution range 1:50-1:500) and solid phase ELISA (starting dilution 1:30, dilution range 1:30-1:3000) (Santa Cruz Biotechnology, Inc.).

This Fusin antibody binds the second extracellular loop and also an intramembrane portion of CXCR4 (see figure 21), the epitope corresponding to amino acids 176-293 (Santa Cruz Biotechnology, Inc.). In standard ICC procedure, cells are permeabilized prior to primary antibody incubation using the mild detergent Triton X-100. Therefore, low affinity of Fusin binding to membrane CXCR4 in IMR-32 cells, seen in figures 7 & 8, was quite confusing; considering the cells were permeabilized, the antibody should have been able to bind both the extracellular and intramembrane portions of CXCR4 with ease. Permeabilization technique may have been the pitfall of this antibody not being able to bind with a high affinity to membrane bound CXCR4. The specific permeabilization solution used in my ICC experiments was Triton X-100, a mild hydrophilic detergent. Detergents such as Triton X-100 are generally used to permeabilize the cells when a researcher wants to characterize intracellular proteins, and not directly embedded within the cellular membrane (Burry, 2010). Triton X-100, a water soluble polar (hydrophilic) lipid, dissolves lipid bilayers. It does so by disrupting the cellular lipid bilayer; its hydrophilic tail binds and penetrates the cellular membrane lipid bilayer's hydrophobic tails located within the center of the cellular membrane. This dissolving action allows the membrane to be penetrated, but also allows the membrane

lipids to surround membrane embedded proteins similar to a micelle formation. Therefore, the intramembrane portion of CXCR4 may still not be accessible for binding by the Fusin antibody upon Triton X-100 permeabilization. This could affect Fusin binding because the epitope of CXCR4 where Fusin is within the intramembrane space, and could potentially be blocked by lipids surrounding the protein, therefore blocking binding of the Fusin antibody. Also, the shortcoming of using Triton X-100 as a lipid dissolving detergent is that it is non-selective in nature and may in fact remove lipid bound proteins along with lipids (Jamur and Oliver, 2010).

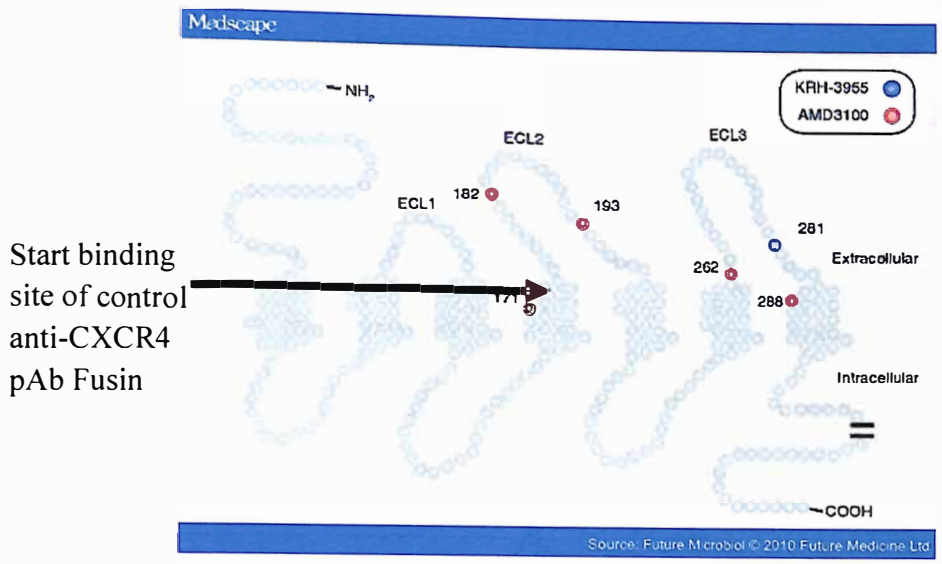


Figure 21. Proposed CXCR4 membrane model with an arrow indicating the beginning intramembrane amino acid sequence where the control CXCR4 antibody, Fusin, binds (Murakami and Yamamoto, 2001).

Minimal background in the control secondary pAb only ICC was seen. Both of the control ICC experiments—no antibody treatment with DAPI nuclear stain and only secondary antibody treatment with DAPI nuclear stain—showed minimal background staining (figure 6). Previous immunoflourescent staining (see figure 22) with CXCR4 directed antibodies have proven to show potent binding when characterizing the expression levels in various neuroblastoma cell lines (Carlisle *et al.*, 2009). Immunohistochemical (IHC) staining (see figure 23) of neuroblastoma primary tumor tissue samples has also been implemented to gauge the clinical diagnostic ability of CXCR4 expression patterns as an indicator of metastatic neuroblastoma (Russell *et. al.*,

2004). Interesting to note is that the anti-CXCR4 antibodies used to stain the cells of the neuroblastoma tumors in Russell et al. 2004 reacted primarily with cytoplasmic CXCR4. As stated in the introduction section, in normal pathology, CXCR4 is involved in the homing of hematopoietic stem cells to the bone marrow. For this reason, in the Russell et al. 2004 study, the researchers were able to classify and compare the differentiating levels of CXCR4 in primary neuroblastoma tumor cells v.s. metastatic neuroblastoma tumor cells, and subsequently assign a clinically significant diagnostic value to each tumor. Meaning that the intensity of the CXCR4 immunostaining was used to differentiate between high and low-grade neuroblastoma tumor pathology.

Yet, what is so fascinating and special about our laboratories polyclonal antibodies is that they were raised to the glycosylated regions of CXCR4, and glycosylation has been previously been shown to play a major role in the mediation of cancer cell progression, adhesion, camouflaging, and most important to the point of this thesis, metastasis. If these antibodies can mask or bind the N-linked glycans, which aid in neuroblastoma secondary dissemination, then they could possess the potential to inhibit neuroblastoma cell metastasis.

To test the binding abilities of our peptide-generated polyclonal antibodies, we used gel electrophoresis coupled to Western blotting transfers. Using 12% SDS-PAGE, 50 micrograms of IMR-32 solubilized membrane preparations were separated on the gels and transferred to Western blot, and immunostained using our set of CXCR4 specific pAbs.

The New ABR (G1-NYT and G2-NVS-2010) antibodies were very successful at detecting CXCR4 on Westerns as compared to the control Fusin pAb (Santa Cruz BioTechnology). I was able to reproduce Fusin polyclonal control antibody results in terms of CXCR4 Western blot detection in the IMR-32 and SK-N-SH cell lines, comparable to previously documented Fusin CXCR4 detection in Jurkat whole cell

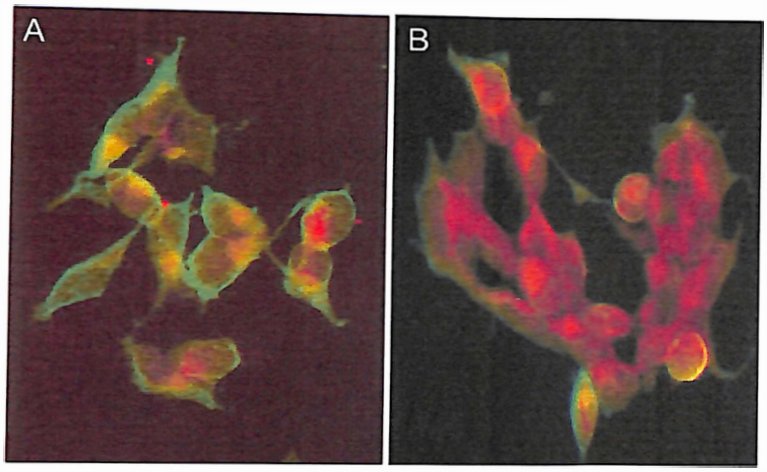


Figure 22. Previous results of metastatic neuroblastoma cell lines (A) SK-N-SH and (B) SH-SY5Y immunoflourescently stained using FITC conjugated antibodies. MAB172 monoclonal antibody (R & D Systems, Inc.) was used to immunostain CXCR4 (green) and MAB310 (R & D Systems, Inc.) was used to immunostain its ligand, SDF-1 (red) (Carlisle *et al.*, 2009).

lysates (Figure 16b-Santa Cruz BioTechnology). The Old ABR (2008) antibodies, detected CXCR4 in both the IMR-32 and SK-N-SH cell lines on Western blots (not shown). I chose to use the New ABR polyclonal antibodies (G1-NYT and G2-NVS-2010)

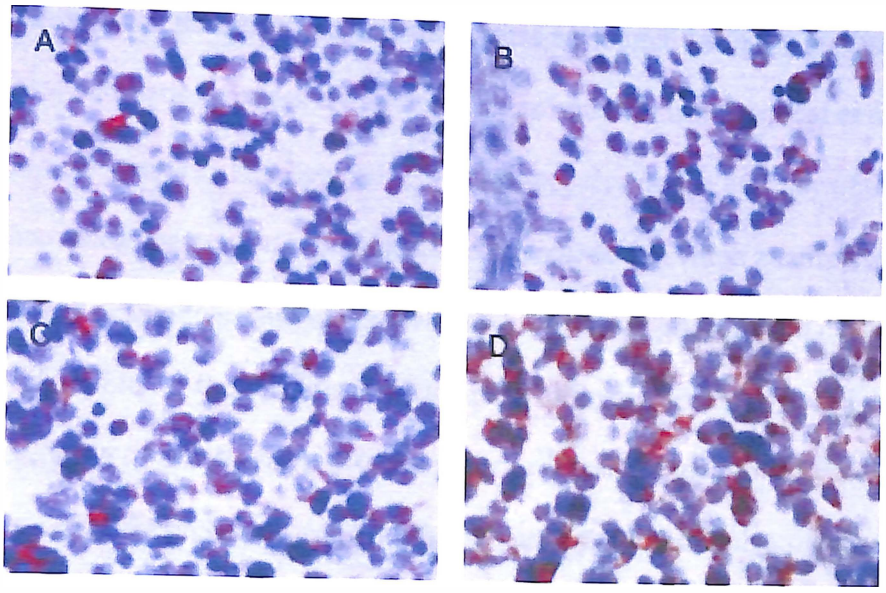


Figure 23. Previous results of CXCR4 immunohistochemistry in primary neuroblastoma tumor cells. Tumors were graded according to the proportion of reactive cells to anti-CXCR4 antibody (A) Grade 1, greater than 1% to 25% reactive; (B) grade 2, 26% to 50% reactive; (C) grade 3, 51% to 75% reactive; (D) grade 4, ≥76% reactive. (Russell *et al.*, 2004).

because when optimizing the experimental condition during Western blotting, I found that the New ABR antibody set exhibited stronger binding and reaction at both glycosylation sites. Due to this fact, Western blot experiments done with the Old ABR polyclonal antibodies are not featured in this thesis.

The ThermoFisher (2009) antibody set did not bind denatured CXCR4 on our Westerns blots (Figure 17). This is in agreement with these peptide antibodies also not recognizing the native, cell surface expressed CXCR4 in IMR-32 cells. These results were very interesting and indicate that producing glycopeptides polyclonal antibodies may depend on the company-used rabbits, techniques specific to each company for the

generation of the peptide antigens, and the subsequent polyclonal antibody production. Careful design of synthetic peptides for animal immunization and subsequent antibody production and collection is a key determining factor concerning the efficacy of the antibody. When using the synthetic peptide experimental design when attempting to produce polyclonal antibodies, capping the terminal side of the peptide with cystiene residues was preferable for these studies, because the sulfhydryl group of cysteine aids in the covalent coupling of the peptides to the Sepharose™ resin beads. The ABR (Old and New) antibody sets were produced using synthetic peptides that not only contained the two glycosylated amino acid areas (G1-NYT and G2-NVS), but also were modified to include cystiene residues at the terminal end of the peptide (NH_2 – TSDNYTEEMGSGDYDSMC and NH_2 – DFIFANVSEADDRYIC), while the Thermofisher synthetic peptides were not (NH_2 – TSDNYTEEMGSGDYDSM and NH_2 – DFIFANVSEADDRYIC). Yet, to note, is that the ending amino acid of the G2-NVS ThermoFisher synthetic peptide happened to end in a cysteine residue due to the natural primary amino acid sequence of CXCR4 chosen to produce the synthetic peptide for rabbit immunization. Recent technological advances suggest that peptide library animal inmunization, antibody collection, and subsequent purification can produce 'motif-specific' antibodies that will specifically bind one protein motif and that have the potential to be one amino acid (fixed) specific in the environment on surrounding amino acid sequences (Cell Signaling Technologies, Inc.). Also, with concern to diagnostic potential, the peptide immunization method for producing site-specific antibodies has been used to produce site-specific antibodies specific for use in the detection of levels of circulating parathyroid hormone (PTH) in terms of calcium release tracking (John, et al.,

1999, Immunotopics, Inc.). Both of these techniques were developed within different antibody producing companies. This enunciates the fact that the company, as well as the methodology of antibody production utilized can both have a large indicative factor on how site-specific said produced antibodies are, and can also have an effect on their potential diagnostic and therapeutic value. This point is also a central theme within my thesis, as the antibodies produced from Affinity BioReagents showed a clear higher binding affinity to both native and denatured CXCR4 in terms of experimentation, when compared to the antibodies produced by ThermoFisher Scientific.

There are advantages and disadvantages to companies choosing to produce monoclonal v.s. polyclonal antibodies. Monoclonal antibodies are produced through a hybridoma approach. The animals are first immunized with a specific antigen continuously, which in turn causes a high proliferation rate of potent antibody-presenting B lymphocytes. Subsequently, the B lymphocytes are removed from the mouse and fused with non-antibody producing malignant Myeloma cells. These newly formed cells are known as hybridomas, and they are now immortal due to being fused with a mutant. myeloma cell. The resultant hybridoma fusion cell population can then be used to collect purse, homogeneous antibody population continuously. Advantages to this technology are that this technology allows this is a constantly renewable source in which all antibody populations will be identical and the antibodies originally identify only one epitope of the antigen; allowing for less background and more consistent, reliable, reproducible results with concern to experimentation. Disadvantages include possibly being too epitope specific, meaning the produced antibodies may not recognize the protein of interest in a

wide range of species. Polyclonal antibodies are produced by antigen immunization of the animal of choice, subsequently, activated B lymphocytes present multiple antibodies to different portions of the antigen, creating a non-homogeneous population of antibodies specific to the same antigen. These antibodies are then secreted from the B lymphocytes into the blood stream and collected by bleed-out and subsequent antibody purification using affinity chromatography. The advantages of antibody production technique is that these antibodies recognize many epitopes of the protein of interest, yielding a higher signal/higher recognition. These antibodies are also more tolerant of possible mutations in the protein of interest due to the fact that they have been derived from a heterogeneous population of B lymphocytes. Disadvantages of this technique are that each batch of antibody produced per immunization may contain differing amounts of antibodies that recognize different epitopes of the antigen, leading to a lack of consistency. In addition to this, a large amount of non-specific antibodies are produced such that high background can result in terms of recognition/detection of the antigen. Also, because these antibodies recognize multiple epitopes, this may result in cross-reactivity with other proteins during detection.

Our laboratory chose to utilize the polyclonal antibody production method because, as stated earlier, they bind many more epitopes of the desired protein/antigen target. In terms of our research, they bind many more epitopes surrounding the glycan amino acid target, thus yielding higher potency recognition as observed on our ICC and Western blot experiment results. Yet, the disadvantage of our laboratory choosing to use

the polyclonal antibody production method was that there was high cross reactivity, with other similar protein amino acid segments.

Membrane, protein samples were prepared, CXCR4 glycopeptides were obtained using trypsin endoproteinase treatment, and the efficacy of endoproteinase treatment was assessed by SDS-PAGE (Figure 18). The trypsin cut protein profile consisted of only small molecular weight fragments near the dye front in the gel lane. This tight band near the dye front meant two things: 1) that we had a complete protein digestion using Trypsin, and that 2) the collection of digests were concentrated near the dyefront, ranging in molecular weights from ~7-17 kDa. Since detection of the CXCR4 predicted amino acid glycan segment(s) were not clearly detected using Western blotting, we tried to specifically isolate the CXCR4 glycopeptides using affinity chromatography.

By covalently linking the polyclonal glycopeptide antibodies to Sepharose™ beads, using trypsin-cut CXCR4; using the glycan specific antibodies, we attempted to isolate only the freed glycosylated regions of CXCR4. Yet yield of the glycopeptides after isolation was too miniscule to view by separation using SDS-PAGE electrophoretic separation (not shown).

Previous to this study, glycosylation changes had not been studied using this exact technique. Meaning, producing glycopeptides via. endoproteinase treatment, isolation of those glycopeptides using affinity chromatography, and visualization of molecular weight shifts by electrophoretic separation is a novel technique to study differences in the glycobiology of neuroblastoma cells. Although similar techniques of removing the sugar from native proteins using PNGase and then visualizing a major shift in molecular weight

of the protein thereafter due to the absence of glycosylation using SDS-PAGE has been successfully completed (Miley *et al.*, 2003; Gelperin, *et al.*, 2005; Baaij *et al.*, 2012).

I believe that the aforementioned endoproteinase treatment technique was not very successful for several reasons. After successful endoproteinase cleavage (Figure 19) the next step of the technique was to isolate CXCR4 glycopeptides using affinity chromatography. This was a tough task considering that our laboratory had to manually complete the task of covalently linking our polyclonal glycopeptide antibodies to the Sepharose^{TM} beads ourselves and then assess the binding of the antibodies to the beads. The binding affinity of G1-NYT was ~60% as assesed by protein assay prior and subsequent to the covalent binding of the antibody to the Sepharose^{TM} beads. This already puts us at a disadvantage, because the amount of CXCR4 glycopeptide that can be isolated has been decreased before the technique has even been implemented. Also, even though the amount of cells collected from 225 cm^2 flasks upon confluent growth is great, the amount of protein that can be collected from solubilized cell membrane preparations is far less. This is another aspect to factor in upon considering why our lab was not able to visualize differential CXCR4 glycosylation banding on SDS-PAGE or identify the glycopeptides using our polyclonal glycopeptide antibodies on Western blots. Essentially dilution of sample through the CXCR4 glycopeptide isolation technique may have presented major problems once we attempted to both visualize and detect the CXCR4 glycopeptide differential patterning. Meaning, that the amounts of not only endoproteinase cut protein, but also isolated CXCR4 glycopeptide amounts may have

been too small to vizualize once separated even on high acyrlimide percentage SDS-PAGE.

Polyclonal CXCR4 glycopeptide antibodies (Affinity BioReagents and Thermo Scientific) were raised in rabbits to the two glycosylated segments of CXCR4, but not to a complete molecule of human CXCR4, for use in these studies. Therefore, the lack of a definitive antibody response to the trypsin endoproteinase digested, and affinity chromatography purified CXCR4 glycopeptides on Western blots was highly unexpected. Predictably, the antibody should recognize the endoproteinase treated CXCR4 glycopeptides as they were able to recognize the synthetic CXCR4 antigenic peptide sequences. The CXCR4 glycopeptide pAbs (Affinity BioReagents and Thermo Scientific) did not bind trypsin endoproteinase cut isolated CXCR4 glycopeptides after affinity chromatography due to small sample size recovery. Lack of response of the CXCR4 glycopeptide directed pAbs to CXCR4 unpurified glycopeptides on blots was confusing because the antibodies were made to the glyco-containing epitopes of CXCR4. Increasing the ratio of antibody during primary antibody incubation as well as increasing the primary antibody incubation time did not increase CXCR4 trypsin endoproteinase cut glycopeptide antibody recognition.

Improvements upon this technique would include but not be limited to: troubleshooting about how to go about more efficient covalent linking of the antibodies to the affinity columns, obtaining more highly concentrated cell membrane preparation sampls, optimizing the affinity chromatography elution step in order to increase the concentration of isolated CXCR4 peptides, as well as optimizing what percentage of

acrylamide in SDS-PAGE is necessary to separate the peptides for band differentiation and visualization.

Trouble experienced in terms of pAb (G1-NYT and G2-NVS) non-recognition of the trypsin digested CXCR4 glycopeptides on Western blots due to the lack of antibody response may have to do with the harsh membrane preparation techniques used in our procedures/methods. Collected cell samples were subjected to SDS containing lysis buffer. SDS denatures all proteins and coats them in with negatively charged SDS molecules (Dimitriadis, 1979). This negative charge coating could cause the epitopes that the CXCR4 polyclonal glycopeptide raised antibodies recognize to become unavailable for binding on Western blots. Another possibility is the chance of "mixed micelle" formation. Mixed micelles are any micelle compromised of at least two different molecular species (Alberts *et al.*, 2008). Lipids can stick to the denatured proteins and form a mixed micelle compromised of the glycosylated protein, SDS molecules (negatively charged surfactant), and lipids from the lysed cell. If the protein is hypothetically contained within a mixed micelle, or covered in SDS molecules and lipids, proteases may not be blocked from their cleavage sites and thus not able to achieve the predicted peptide. In the same respect, the glycopeptides formed by the protease cleavage may be too crowded with sugars, SDS, and lipids to become recognized or bound by the polyclonal antibodies.

The glycopeptides themselves should also be considered in the inability of the antibodies to recognize the CXCR4 glycopeptides. Sugar chains have limited flexibility, even small N-linked oligosaccharides that exist and protrude out from the surface of

proteins can limit the scope of macromolecules to the protein surface (Alberts *et al.*, 2008). This can imply two things: one, we may not acheive the desired or preferred size CXCR4 glycopeptides due to oligosaccharide presence blocking, making CXCR4 more resistant to digestion by proteolytic enzymes and/or two, the antibodies directed towards the glycosylated regions, as macromolecules, may have trouble binding or experience lower affinity binding due to the existence of the protruding oligosaccharide chains.

The CXCR4 antibodies were raised to non-oligosaccharide containing synthetic peptides that mimicked the 17-mer and 16-mer glyco-containing regions of CXCR4. Therefore, when the antibodies were used to challenge actual human CXCR4 glycopeptides that indeed are glycosylated, they may have a significantly harder time binding given the new glycosylated environment in which the peptides contain N-linked oligosaccharides. However, both CXCR4 peptide raised antibodies recognize native CXCR4 in total protein solubilized membrane preparations. For this reason it may have proven useful to have increases the amino acid size of the synthetic peptide antigen to avoid the possibility that the antibodies would not be able to bind/recognize the glycosylated form due to the possible large size and complexity of the N-linked glycan structures. Even though trypsin endoproteinase primarily cleaves on the carboxy side of lysines and argeninies, the physical conditions of its cutting environment, such as pH and temperature (which for trypsin, are pH 8 and temperature 37 degrees Celsius, respectively) must also be optimized or the most advantageous cleavage points, determined from the primary amino acid sequence of CXCR4, may not be achieved. It may also be a possibility that the total CXCR4 sequence is necessary for the antibodies to bind,

meaning that regardless of which peptides are yielded, because the antibody is specific to total CXCR4 protein, that it will not bind.

Future study of CXCR4's complement o N-linked glycans and their implications on neuroblastoma metastasis would entail further development of the endoproteinase technique previously detailed in this thesis. Improvement upon the endoproteinase cut CXCR4 peptides and subsequent affinity column peptide capture method would be one important aspect to the discovery of possible 'differential glycosylation' CXCR4 patterning. Improvements of this method would mean improving the efficacy of the endoproteinase CXCR4 digestion by either increasing the amount of endoproteinase added, or by increasing the digestion incubation period. As noted, for successful peptide capture to occur (or a high yield of peptides during the capture), a minimum of 80% covalent antibody attachment to the resin beads is preferred (Thermo Scientific, Inc.: AminoLink Plus Immobilization Kit).

Another avenue for improvement of this peptide capture technique would be on the actual 'capture' portion of the method. First, the content of protein in the original sample must be high. Second, the endoproteinase digest must be successful. Third, the peptides must efficiently the Sepharose™ arose bound antibodies within the affinity column (which, again, requires that the antibodies have successfully covalently bound the Sepharose™ beads). And fourth, the peptides must be carefully eluted off of the column with the lowest possible volume, but also with the highest possible yield. This is a difficult task within itself that requires much practice in terms of the affinity chromatography technique, as there are many avenues for error within the process.

Even if one completes all of the aforementioned tasks with virtually no error and yields a high amount of captured CXCR4 peptides, they will still need to be separated, using SDS-PAGE technique. One of details that we found while completing this research was that the peptides do not seem to bind SDS well, thus they do not migrate and separate along a gel as a full protein would. Also, once separated using SDS-PAGE, it is difficult not only stain the gel and visualize the peptides, but also to gain antibody recognition of the peptide on Western blots. The first of those problems, staining and visualizing peptides on gels, can possibly be alleviated by using Silver Stain, instead of Coomassie Brilliant Blue Staining. Silver Stain is much more sensitive to proteins than Coomassie, and thus more ideal for research of this nature. The latter of these issues can possibly be alleviated by either increasing the amount of antibodies used, or by capturing more peptides, which brings us back to the original pitfall to this technique.

Yet even with the specific challenges of the N-glycan capturing using this technique in my thesis study, I believe the knowledge of differential glycosylation events within key protein biomarkers of different malignancies still holds great power as an indication of the biology, morphology, malignant potential, and stage of many different malignancies (Couldrey and Green, 2000). Aberrant glycosylation occurs in many different types of cancers (Park *et al.*, 2011), and exploring more efficient techniques of how to unmask what these differential glycosylation events are, and how they are working to aid the progression of different malignancies is a key factor in discovering

novel properties concerning cancer biology; but also new and innovative ways to detect and treat cancer as well as possibly prevent neoplasms from ever occurring.

Our polyclonal glycopeptides antibodies G1-NYT and G2-NVS do bind to denatured CXCR4 on Western blots, and thus present the potential to be useful in the detection of cancer progression, not only in neuroblastomas, but also in other cancers or diseases in which CXCR4 is a known biomarker or is hallmark of the diseased state. Thus, these antibodies could prove to be very powerful as they recognize both the cell surface expressed and denatured forms of CXCR4 in the IMR-32 neuroblastoma cell line. If they also bind CXCR4 just as potently in other neuroblastoma cell lines, or cancer cells in which CXCR4 is an over-expressed protein and thus a target for treatment, then our antibodies could prove to be a powerful treatment of cancers, particularly by preventing metastasis, by directly targeting N-glycan structures of CXCR4. Previous studies have shown that polyclonal antibodies directed at over-expressed protein targets can in fact prevent metastasis of cancer cells (Hanaki *et al.*, 2011).

This technique could prove very useful as a novel tool for the exploration of differential glycosylation, and its impact on tumor biology as well as act as an implicative measure of metastatic ability within different malignancies, targeting CXCR4. Saeland et al., Tian et al., Pan et al., Wang et al., and Mak et al. have shown that differential glycosylation can be used as a biomarker cancer cells through lectin binding, PNGase glycosylation removal/mass spectrometry, and site-specific mutagenesis experimental techniques. Sealand et al. used the lectin binding technique to demonstrate that glycosylation of key tumor biomarker proteins within colorectal cancer differs

significantly from the same proteins in normal colon mucosa; citing the fact that cell surface protein glycosylation changes/aberrant glycosylation may affect tumor progression by aiding tumor cell migration. Moreover, the changes in glycosylation may affect cancer cell interactions with the tumor microenvironment.

One strong aspect of my research approach is that our technique has an advantage over the lectin binding technique because it does not rely on the specificity of the glycan make-up but instead focuses on the size change to differentiate between normal and aberrant glycosylation by fishing out any changed CXCR4 glycan by binding to its stably attached amino acid segment. Our technique goes a step further to attempt to use the different possible visualized patterns to classify disease states within the cancerous phenotype.

Indifferent to key advantages seen using site-directed mutagenesis, used to study proteins with many different glycosylation sites, one at a time, and their implications on malignancies (Pan *et al.*, 2011; Wang *et al.*, 2011). An advantage of our CXCR4 specific N-glycan capture is the endoproteinase step, along with the affinity column glycospecific antibody isolation, allowing one to separate and isolate the two specific glycosylation sites at the same time; which would save time as these techniques are much more rapid than that of site-directed mutagenesis and instead of inhibiting the glycosylation actually allows analysis of the released peptidoglycan. In addition, this latter reasoning also gives insight as to why this novel technique may prove to be more effective than previous studies (Pan *et al.*, 2011; Wang *et al.*, 2011; Tian *et al.*, 2012) using PNGase treatment to remove the N-glycan from total protein and subsequent mass spectrometry analysis.

While removing the glycosylation site using PNGase to study glycan function within tumor biology may prove useful for proteins that have one single putative glycosylation site, our novel technique could be more effective for proteins with multiple glycosylation sites, which is typically the case of glycoproteins. Implementing the use of PNGase to the full glycoprotein sequence would cause removal of all N-linked glycan structures on the protein, which would still be the case with our novel technique, but the added use of endoproteinase treatment prior to PNGase N-glycan removal will allow for the study of multiple differential glycosylation site comparisons, once the affinity isolation of peptides is carried out. We have produced separate polyclonal antibody panels to separate the two in situ CXCR4 N-glycans. Because such is the case that CXCR4 has two N-linked glycosylation sites and also because of the afore mentioned reasons, I believe that further optimization and troubleshooting to improve upon this novel technique to explore differential glycosylation event in progressing stages of cancer should be pursued.

Our novel production of these important CXCR4 pAbs allows the use of endoproteinase treatment, affinity capture isolative techniques, and SDS-PAGE/Western blotting to study glycan changes of proteins in progressive disease states. This is a method that employs some new technical approaches in which the primary focus is not on the specific type of sugar tree that is attached to the protein to define glycosylation changes, but rather uses size difference to denote differential glycosylation amongst progressive cancer states to possibly be used a biomarker. Our methodology is simpler to use than previous techniques studying glycosylation and can be further utilized when

coupled to the powerful sequencing capabilities of mass spectrometry (Pan *et al.*, 2011; Wang *et al.*, 2011; Tian *et al.*, 2012).

When total membrane protein preparations were immunostained using G1-NYT and G2-NVS pAbs on blots, more CXCR4 banding was observed in comparison to the control Ab, fusin used to immunostain sister blots for comparison. This can possibly be contributed to hetero- and homodimerization, post-translational modifications (glycosylation, phosphorylation, ubiquitination, etc.), as well as the existence and multiple isoforms of CXCR4 ranging from 38 kDa to 87 kDa. The homodimer band on Western blots of solubilized cell membrane preparations of CXCR4 is at ~101 kDa. This is a suspected homodimer of the calculated ~47 kDa CXCR4 as previously documented in Carlisle et al., 2009. Other reasons for the extraneous banding may be because our CXCR4 glycopeptide antibodies are polyclonal and thus will recognize multiple epitopes of the peptide that it was raised to. The protein Basic Local Alignment Search Tool (BLASTp) uses an algorithm to search and compare primary amino acid sequences to check if a sequence is highly conserved or if sequences share homologous domains/regions with other proteins (Altschul *et al.*, 1997; Altschul *et al.*, 2005). When the CXCR4 primary amino acid sequence was subjected to BLASTp analysis, it was found that the sequence was extremely conserved and had no significant homologous regions shared with other proteins. In addition to this, when the two synthetic peptide sequences (G1-NYT: TSD**NYT**EEMGSGDYDSM; G2-NVS: DFIFA**NVS**EADDRYI) were subjected to BLASTp analysis, the results were that these two sequences were specific to the CXCR4 GPCR. Thus, given the highly conserved nature of this protein,

and the antigenic peptide sequences used to produce the antibodies in this study, there should have been little to no cross-reactivity with non-CXCR4 proteins.

Our CXCR4 polyclonal glycopeptide antibody binding profiles were characterized using native human CXCR4 solubilized membrane preparations from neuroblastoma cell lines IMR-32 and SK-N-SH. Using the CXCR4 polyclonal glycopeptide antibodies, two major forms of CXCR4 were identified on Western blots. One ~47 kDa which is suspected to be the glycosylated form of CXCR4 as the calculated molecular weight from amino acid primary structure of CXCR4 is ~39 kDa (Bachelerie, 2010). Another reason for the calculated molecular weight on the Western blots differing from that of the primary amino acid sequence of CXCR4 could be due to other PTM's such as ubiquitination and phosphorylation. Both bands correlated with Western blots containing human CXCR4 from the IMR-32 and SK-N-SH neuroblastoma cell lines that were challenged with the control antibody Fusin (figure 16).

In summary, we successfully raised rabbit polyclonal antibodies to the 2 human N-glycan CXCR4 amino acid sequences. These antibodies bound both N-glycan sites *in vivo* (ICC studies) and *in vitro* on our Western blots. We believe this method of targeting key N-glycan amino acid segments to use for pAb production is novel and that these antibodies should prove exceedingly important in the determinations of differential glycosylation in both neuroblastoma cells (used in this study) as well as any cancer cells over producing human CXCR4.

We also found, through our extensive characterization of these unique antibodies, that the companies producing the pAbs for laboratory use differ in their resultant product:

in this case our two different libraries of human CXCR4 polyclonal antibodies. We believe that if we used ABR, Inc. again to synthesize new peptide antigens we would have another successful library of human CXCR4 pAbs to use in cancer research.

For future studies, we believe this novel approach to using synthetically produced antibodies to bind N-glycan amino acid segments of key cancer proteins ccan be used to study in vivo, in vitro, and ex vivo cellular events to identify important differential changes in any cell line over producing human CXCR4.

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