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The chemotropic effect of SEMA7A, SEMA4D, and SEMA3A on human neutrophils

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The Chemotropic Effect of SEMA7A, SEMA4D, and SEMA3A on Human Neutrophils

A Thesis

Presented to

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Andrea Lee French

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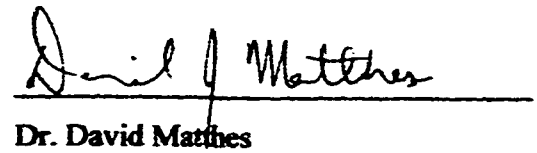
APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES



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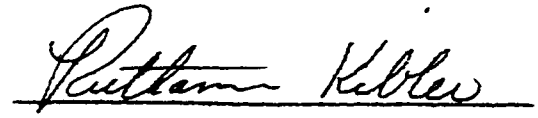

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ABSTRACT

THE CHEMOTROPIC EFFECT OF SEMA7A, SEMA4D, AND SEMA3A ON HUMAN NEUTROPHILS

by Andrea Lee French

The semaphorin gene family encodes proteins known to function as axon guidance molecules in the developing nervous system and to mediate diverse immunological processes including B cell aggregation, and upregulation of CD54 (ICAM-1) and induction of Il-6 and Il-8 production by monocytes. As semaphorin genes have been identified in two families of viruses, poxviridae, and herpesviridae, we have further explored the role of semaphorins in modulating the immune response. Semaphorins act primarily as chemorepellent factors in the developing nervous system. Therefore, we examined the migration-inhibitory and chemorepellent roles of three human semaphorins, SEMA4D, SEMA7A, and SEMA3A, on neutrophil migration in Boyden chamber-based *in vitro* migration assays and analyzed the results by checkerboard analysis. We have demonstrated that while SEMA4D and SEMA7A show no ability to significantly alter neutrophil migration in these assays, SEMA3A can function as a neutrophil chemorepellent. (Supported by a grant from Exelixis Pharmaceuticals, Inc. and NIH MBRS grant 08192-19)

ACKNOWLEDGEMENTS

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BACKGROUND AND SIGNIFICANCE

In the early 1960's, Roger Sperry hypothesized that neurite guidance was regulated by chemoattractant molecules (Sperry, 1963). However, not until recently have researchers begun to identify and isolate these guidance proteins. Although many of the first guidance molecules discovered had adhesive or chemoattractant activities, two research groups, in the early 1990's, independently discovered a family of chemorepellent guidance proteins now called semaphorins. This new family of proteins was first found to help guide specific subtypes of neurons in grasshopper embryos. Since this finding, researchers have identified multiple semaphorin genes in mammalian and viral genomes. In addition, semaphorins have been found to serve a broad range of functions including roles in the immune system, neural guidance and viral infections.

The first group of researchers, Kolodkin et al. (1992), discovered fasciclin IV, now called semaphorin I (Sema-1a), by creating monoclonal antibodies to homogenized grasshopper nerve cords. In a screen of monoclonal antibody-stained grasshoppers, Kolodkin et al. found that one specific antibody revealed both a subset of central nervous system axon pathways and circumferential stripes of epithelial cells in the grasshopper limb bud. This antibody was then used to clone the new axonal glycoprotein, fasciclin IV and to study its function during growth cone guidance. When researchers blocked fasciclin IV with an anti-fasciclin IV antibody during grasshopper embryogenesis, they found that the Til axon pathway in the limb bud was altered at the location where the growth cone contacted the fasciclin IV expressing cells. Instead of the tight fasciculation and turning of the two Til axons which occurs with the normal expression of fasciclin

IV, they observed multiple axon branches that formed within the fasciclin IV-expressing stripe in the trochanter of the limb bud. This altered morphology of the Ti1 axons showed these researchers that the new protein, fasciclin IV, had a role in axon guidance.

As research continued on fasciclin IV, a second group of researchers worked to isolate neural guidance molecules acting in the chick brain. Through a process of biochemical purification and molecular cloning, Luo, Raible, and Raper (1993) discovered a new protein that was found to have a collapsing effect on sensory and retinal ganglion cell growth cones. This protein, identified as chick collapsin, was later discovered to be 60% identical to Sema-1a at the amino acid level. Sema-1a and chick collapsin became the founding members of the semaphorin protein family.

Since their first discovery, semaphorins have been isolated from many species including humans, Drosophila, nematodes and viruses (Hall et al., 1996; Kolodkin, Matthes, & Goodman, 1993; Ensser & Fleckenstein, 1995). In vertebrates alone, scientists have identified over twenty semaphorins with a broad range of expression patterns (Luo et al., 1993; Behar, Golden, Mashimo, Shoen & Fishmen., 1996).

Semaphorin Structure in Metazoan Organisms

Recently the Semaphorin Nomenclature Committee (1999) unified the nomenclature for the semaphorin and collapsin proteins. Through this process all known semaphorin proteins have been grouped and re-named. Both the old names and the new names will be given in the "Background and Significance" section of this paper. In the new system, the rodent and invertebrate semaphorins are designated by the "Sema" format. The other vertebrate semaphorins are designated in the "SEMA" format. For the

purposes of this masters thesis, the old names for the semaphorins will be written in long form and the new names will be emboldened and in parentheses. For example, in this text "semaphorin Z7" will be written "semaphorin Z7 (**SEMA4E**)."

The semaphorins are a highly conserved family of proteins that share a common core structure called the sema domain. The sema domain is an extracellular region that consists of 500 amino acids containing 16 cysteine residues with characteristic spacing (Kolodkin et al., 1993). Beyond this domain, the structure of the different semaphorins varies. Metazoan semaphorins have thus far been separated into seven distinct classes (Adams, Betz, & Puschel, 1996; Semaphorin Nomenclature Committee, 1999). See figure 1.

Class I semaphorins consist of the sema core and a transmembrane region followed by a short cytoplasmic domain (Kolodkin et al., 1993). This class of invertebrate semaphorins include grasshopper semaphorin I (**Sema-1a**), Tribolium semaphorin I (**Sema-1a**), Drosophila semaphorin I (**Sema-1a**) and Sema-1b.

The Class II semaphorins contain the sema domain and a C2 type immunoglobulin domain followed by a unique amino acid sequence. Thus far, the three semaphorins that have been classified in this group are Drosophila semaphorin II (**Sema-2a**), Caenorhabditis elegans semaphorin II (**Sema-2a**), and grasshopper semaphorin II (**Sema-2a**) (Kolodkin et al., 1993; Semaphorin Nomenclature Committee, 1999).

The Class III semaphorins differ from the others in that they have a carboxy-terminus tail of positively charged, basic amino acids in addition to the sema and immunoglobulin domains (Kolodkin et al., 1993). Because no transmembrane, or

hydrophilic region appears to exist in these Class III semaphorins, they are thought to be secreted, perhaps, as membrane associated or soluble proteins (Adams et al., 1996). Examples of semaphorins in this class include murine semaphorin A (**Sema3B**), murine semaphorin D (**Sema3A**), murine semaphorin E (**Sema3C**), chick collapsin I (**SEMA3A**), chick collapsin II (**SEMA3D**) and human semaphorin III (**SEMA3A**).

Class IV semaphorins have both a transmembrane and an immunoglobulin domain. Some of the semaphorins included in this class are CD 100 (**SEMA4D**), murine semaphorin B (**Sema4A**), murine semaphorin C (**Sema4B**), and murine semaphorin W (**Sema4F**) (Hall et al., 1996; Puschel, Adams, & Betz, 1995; Herold, Elhabazi, Bismuth, Bensussan, & Boumsell, 1996).

Class V semaphorins have a sema domain, a transmembrane domain, a cytoplasmic tail and seven thrombospondin type 1 repeats (Adams et. al., 1996). In thrombospondin, the type 1 repeats have been found to be responsible for both the promotion of the outgrowth of neurites and the attachment of neural and non-neural cells (Neugebauer, Emmett, Venstrom & Reichardt, 1991; O'Shea, Liu & Dixit, 1991). Because the Class V semaphorins share this homology to thrombospondin, Adams et al. (1996) hypothesized that the Class V semaphorins serve as positive axonal guidance cues. The two semaphorins currently in this class are murine semaphorin F (**Sema5A**) and murine semaphorin G (**Sema5B**).

Class VI semaphorins have a sema domain, a transmembrane domain and a cytoplasmic domain much like Class 1. However, the semaphorins in this class include those found only in vertebrates. Murine semaphorin 6A (**Sema6A**), murine semaphorin

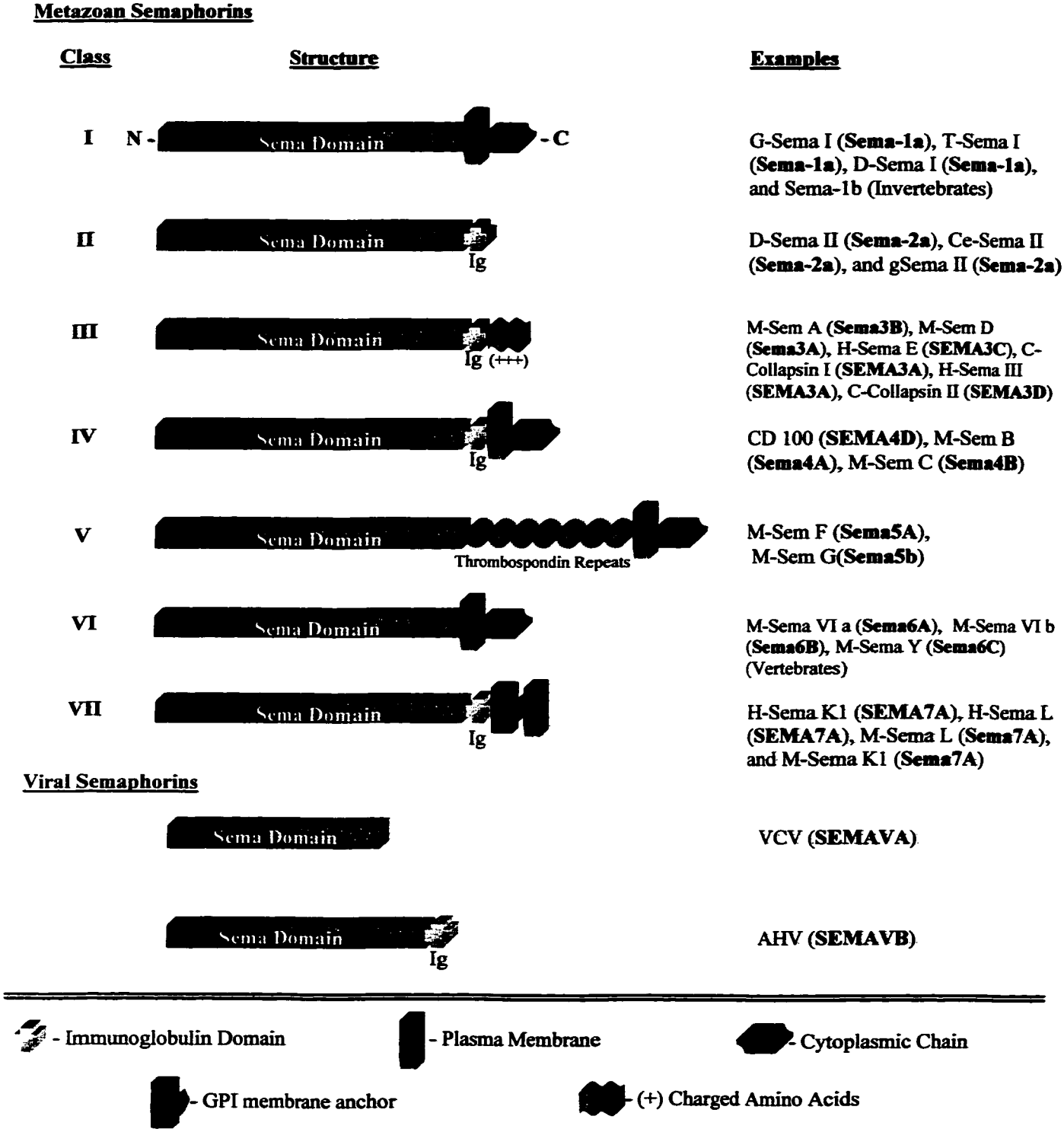


Figure 1: The structure of both metazoan and viral semaphorins

6B (**Sema6B**), rodent semaphorin Z (**Sema6B**), murine semaphorin Y (**Sema6C**) and rodent semaphorin Y (**Sema6C**) are the semaphorins that share this structure.

Class VII semaphorins have a sema domain, an immunoglobulin domain, and a glycosylphosphatidylinositol(GPI) anchor to the plasma membrane. This linkage is hypothesized to give this class of semaphorins the ability to modulate the immune system in localized regions (Xu et al., 1998) . The semaphorins included in this class are human semaphorin K1 (**SEMA7A**), human semaphorin L (**SEMA7A**), murine semaphorin K1 (**Sema7A**), and murine semaphorin L (**Sema7A**).

Structure of Semaphorins in Viruses

In addition to the seven classes of semaphorins isolated from the metazoan organisms, semaphorins have been isolated from both the herpes and pox family of viruses. The alcelaphine herpesvirus type 1 genome contains a semaphorin homologue (**SEMAVB**) which codes for a protein that is 653 amino acids long. This protein has a sema domain that contains 13 of the 16 conserved cysteine residues found in metazoan semaphorins. Given its amino-terminal sequence and absence of a transmembrane domain, it is thought to be a secreted protein that may act in a diffusible fashion to subvert the host immune response (Ensser & Fleckenstein, 1995; Xu et al.,1998).

In the pox family of viruses, the genomes of both the vaccinia and variola viruses contain genes that code for semaphorin-like proteins. The vaccinia viruses contain a sequence that codes for a semaphorin protein (**SEMAVA**) with a sema domain that is 441 amino acids long. The variola virus contains a gene sequence that codes for a sema domain that is 375 amino acids long. These two semaphorins each contain 6 of the 16

conserved cysteine residues found in the metazoan semaphorins (Kolodkin et al., 1993). As with the AHV Sema, these semaphorins appear to be secreted.

Neural Development Guidance Molecules

The most studied and well-known function of the semaphorin proteins is their role as a neural development guidance molecule. During early embryogenesis one of the mechanisms by which neurons grow involves a process called neural extension. In this process, the leading edge of the axon, the growth cone, sends out finger-like projections called filopodia and veil-like projections called lamellipodia in order to sense the surrounding environment. Through direct contact with the surrounding cells and by sensing chemical gradients in the extracellular fluid, these lamellipodia and filopodia determine the direction of the neurite extension (Bray & Hollenbeck, 1992; Tessier-Lavigne, 1992; Goodman & Shatz, 1993). Depending on whether they come in contact with a positive or negative cue, the filopodia will selectively polymerize or depolymerize their actin matrix. Neural extension follows the direction of actin polymerization and positive cellular cues.

Sema-1a

A broad range of semaphorins have been implicated in repulsive axon guidance (Giger, Pasterkamp, Holtmaat, & Verhaagen, 1998; Matthes, Sink, Kolodkin, & Goodman, 1995; Messersmith et al., 1995; Puschel et al., 1995). The first semaphorin identified as a chemorepellent axon guidance molecule was fasciclin IV (**Sema-1a**). A study completed by Kolodkin et al. (1992) documented the effects of fasciclin IV (**Sema-1a**) on Ti1 pioneer neurons growing in cultured grasshopper limb buds. They showed

that when Ti1 pioneer neurons encounter a stripe of semaphorin-expressing epithelial cells, they radically changed direction traveling along but not across the stripe. However, when anti-semaphorin antibodies were added, Ti1 pioneer neurons exhibited an altered morphology with the extension of multiple, defasciculated axons which often extended through the region of the semaphorin stripe (Kolokin et al., 1992). This experiment shows that Sema-1a plays an integral role in the guidance of developing Ti1 pioneer neurons.

Sema-2a

Another semaphorin identified as a negative axon guidance cue was semaphorin II (**Sema-2a**). In the study completed by Matthes et al. (1995), researchers created transgenic Drosophila embryos to show that semaphorin II (**Sema-2a**) caused selective inhibition of specific synaptic arborizations. By using the P element-mediated transformation, they generated ectopic expression of Sema-2a in ventral embryonic Drosophila muscles that under normal conditions did not express the gene. They found that the effects of Sema-2a in these transgenic muscles was limited to the inhibition of SNb and SNd motor neurons. Instead of forming a normal synaptic terminal arborization in the cleft between muscles 6 and 7, the RP3 growth cones exposed to Sema-2a remained external to the muscles. Matthes et al. (1995), also showed that the other neurons in these muscles (SNa, SNc, and the intersegmental nerves) were unaffected by the expression of Sema-2a. This study confirmed Sema-2a acts as a selective inhibitor of synapse formation during target recognition.

SEMA7A

A recent study completed by Xu et al. (1998) identified a new class of semaphorins. The semaphorin K1 (**SEMA7A**) protein shares a high homology to the AHV viral semaphorin and contains a GPI linkage. In addition to identification of this semaphorin, Xu et al.(1998) used both Northern blot analyses and *in situ* hybridization to define its biological role. A cDNA fragment corresponding to the mouse homologue of SEMA7A was used as a probe for Northern blot analyses. In this study, they found that SEMA7A is highly expressed in brain, spinal cord, lung, and testis tissue and moderately expressed in heart, muscle, adrenal gland, lymph nodes, thymus and intestine tissue of adult mice. To examine the distribution of SEMA7A mRNA during development, Xu et al. (1998) performed *in situ* hybridization analyses on tissue sections from embryonic day 11 and day 15 embryos, and on the brain and spinal cord sections of postnatal day 3- and 5- week old mice. They found that although SEMA7A mRNA was not significantly expressed in developing embryos, the SEMA7A mRNA signal became stronger at P3 and was highest in the 5-week old mice. The widespread and abundant expression of SEMA7A mRNA in adult brain suggests that it could play a role in the maintenance and plasticity of connections in the adult nervous system.

SEMA3A

Semaphorin III (**Sema3a/SEMA3A**) is the best characterized axonal chemorepellent semaphorin. Sema3a/SEMA3A has been shown to induce growth cone collapse and axon repulsion of a variety of neurons. These include sensory and motor components of several cranial nerves, spinal nerves, sympathetic chain and ciliary

ganglion (Giger et al., 1998). In addition to neuron repulsion, Sema3A/SEMA3A is thought to be involved with the histogenesis of heart, bone and cartilage tissue (Behar et al., 1996).

A study completed by Messersmith et al. (1995) found that murine semaphorin III (**Sema3A**) controls the development of dorsal root ganglion by selectively repelling the axons that normally terminate dorsally in the spinal cord. By first isolating Sema3A transcripts from the ventral spinal cord and then creating Sema3A-secreting COS cells, researchers showed that Sema3A, secreted by the ventral spinal cord explants of embryonic day 14 mice, inhibited the outgrowth of axons of neural growth factor (NGF)-responsive sensory neurons.

A study completed by Puschel et al. (1995), showed the chemorepellent nature of semaphorins in murine embryogenesis when they found that murine semaphorin D (**Sema3A**) prevented axonal extension in dorsal root ganglion explants. The neurites from explants of dorsal root ganglia were grown on a mixture of collagen and Matrigel, a mouse tumor-cell produced extracellular matrix cocktail (Becton Dickenson Biosciences, CA). When these neurite cells were placed next to Sema3A-secreting HEK 293 cell aggregates, the researchers showed that after 48 hours of culture, a nearly complete inhibition of outgrowth was found on the proximal side of the explant facing the transfected cells (Puschel et al., 1995). In their study, the inhibition effect was not recreated when mock transfected cells were used.

Finally, a study completed by Tanelian, Barry, Johnston, Le & Smith (1997) showed that SEMA3A could repulse and inhibit adult sensory afferents *in vivo*. In this

study, both the normal and regenerating A-delta and C fiber sensory nerve rabbit cornea models were used to show the ability of SEMA3A to repulse or inhibit sensory afferents. Using gene gun transfection and production of SEMA3A in corneal epithelial cells in adult rabbits, the researchers showed that SEMA3A can cause repulsion of established A-delta and C fiber trigeminal sensory afferents. In addition, they showed that following epithelial wounding and denervation SEMA3A was able to inhibit collateral nerve sprouts from innervating the re-epithelialized tissues proving that the small-diameter adult sensory neurons retained the ability to respond to SEMA3A.

Semaphorins in the Immune System

In addition to the extensive studies that have shown semaphorins' role in nerve development, semaphorin proteins have, also, been identified in the immune system. To date, the major semaphorin expressed in cells of the mammalian immune system is human CD 100 (**SEMA4D**) and its murine homologue, murine semaphorin G (**sema4D**).

SEMA4D

SEMA4D has been shown to be expressed in lymphoid tissue, the interfollicular T cell zones and in B cells in the germinal centers of secondary lymphoid follicles (Furuyama et al., 1996; Dorfman, Shahsafaei, Nadler & Freeman, 1998). A study completed by Elhabazi et al. (1997) showed the role of SEMA4D in lymphocyte activation and aggregation. A research study completed by Hall et al. (1996) showed that when combined with the T-cell surface protein CD45, Sema4D created an enhanced B cell aggregation effect and increased B cell life.

Herold et al. (1996) showed that Sema4D linked to a phosphatase, CD45, on the T cell surface when antigenically stimulated. This phosphatase is a key molecule in the T cell receptor activation pathway, which has led these researchers to hypothesize that Sema4D may be integral in the T cell activation. A more recent study completed by Elhabazi et al. (1997) shows that the Sema4D molecule stimulates a cytosolic cascade activating a pathway within the T cell.

Finally, murine semG (**sema4D**) has been located primarily in thymocytes in the thymic cortex (Furuyama et al., 1996). When thymocytes or immature T cells develop, they travel from the thymic cortex to the medulla via the deep cortex. The actual pathway through which this migration occurs has yet to be determined, but the results of this study led scientists to hypothesize that sema4D may be important as a guidance molecule for developing thymocytes.

Neutrophils and Semaphorins

Neutrophils are essential phagocytic cells involved with a host's non-specific immune defense system. These leukocytes that follow chemotropic factors like chemokines to the sight of infection or damage, are responsible for removing opsonized foreign organisms and substances from tissue. Although many proteins that act as neutrophil chemoattractant have been identified, current research has begun to focus on proteins that repel neutrophils (Lipton & Catania, 1998). In pursuing the identification of these chemorepellent molecules, researchers have been searching for the endogenous signals that induce neutrophil dispersion after the resolution of an infection.

Because semaphorins have been found to be strong chemorepellent molecules to other cell types and because they have been recently identified in the immune system, this study focuses on the effect semaphorins have on neutrophil chemotaxis. SEMA3A and SEMA4D were chosen for this study because of their roles in the immune system. SEMA7A was chosen because of its systemic expression pattern in tissues throughout the body. Ultimately, the chemotropic effect of semaphorins studied in this masters thesis project will help to reveal semaphorins' roles in the human immune system.

SPECIFIC AIMS

The primary question addressed was:

Do SEMA7A, SEMA3A and SEMA4D have a chemotropic effect on human neutrophils?

The goal of this research was to pursue the hypothesis that mammalian semaphorins can act as chemotropic agents in the immune system. Specifically, the human semaphorins: **SEMA4D**, **SEMA7A**, and **SEMA3A** were studied using Boyden chamber assays to examine the chemotaxis of the neutrophils presented with each of these semaphorins.

The project can be divided into three separated components.

Part 1: Verify the identity, DNA, and protein coded for each semaphorin/pEX.mh expression construct.

Part 2: Create transiently transfected COS-1 cells that synthesize SEMA4D, SEMA3A, and SEMA7A protein.

Part 3: Identify the chemotropic effect semaphorins have on human neutrophils using Boyden chamber assays.

EXPERIMENTAL OVERVIEW

The aim of this project was to elucidate the role that SEMA3A, SEMA7A, and SEMA4D have in chemotaxis of neutrophils (Appendix 1a and Appendix 1b) Each of these semaphorins were previously cloned into the pcDNA3.1 (Invitrogen, CA) based mammalian expression vector pEX.mh by workers at Exelixis Pharmaceuticals. This project began with the transformation of *E.coli* and the production of semaphorin plasmid DNA. The identity of each plasmid was then verified via restriction digests and DNA sequencing. Transient transfections of COS-1 cells with the pEX.mh-sema constructs were performed to provide an *ex vivo* source of semaphorin protein. Verification of the transfection success and protein production was determined by β-galactosidase activity assays performed on the transfected COS-1 cells.

Human neutrophils were isolated from four healthy human volunteers three hours before each chemotaxis assay. Human neutrophils were, then, isolated by differential centrifugation from the blood.

The Boyden chamber assay was used to determine the chemotropic effect the different semaphorins have on isolated neutrophils. Briefly, a polycarbonate filter placed in between the upper and lower compartment of the Boyden chamber served as the barrier through which the motile neutrophils traveled. To test for chemoattraction, three combinations of substances were placed in the lower well of different Boyden chambers: (1) the extract of semaphorin secreting COS cells; (2) fMLP; (3) the extract of mock transfected COS-1 cells. Human neutrophils suspended in a dilution of the extract of the mock transfected COS-1 cells were placed in the upper well of the Boyden chamber. To

test the effect of varying chemoattraction semaphorin gradients, defined dilutions of semaphorin secreting COS-1 cell extracts were placed in both the upper and lower wells of the Boyden chamber.

To test for chemorepulsion, two combinations of substances were placed in the upper chamber along with the human neutrophils: (1) the extract of the semaphorin secreting COS cells and (2) the extract of mock transfected COS-1 cells. The lower chamber contained dilutions of the extract of mock-transfected COS-1 cells. The effect of varying chemorepulsion semaphorin gradients was tested by placing defined dilutions of the semaphorin secreting COS-1 cell extracts in both the upper and lower well of the Boyden chamber.

After incubation of the apparatus, the cells that crossed the filter were stained and counted. The chemotactic response to the semaphorins was then quantified by counting a proportion of migrating neutrophils.

MATERIALS AND METHODS

Constructs

In order to test the chemotropic effects of SEMA7A, SEMA3A and SEMA4D on human neutrophils, native proteins were required for use in the Boyden chamber. Unfortunately, purified forms of these proteins were not commercially available. As a result, mammalian expression constructs were synthesized by inserting the SEMA7A, SEMA3A and SEMA4D genes into the pcDNA 3.1 (Invitrogen, CA) based expression plasmid, pEX.mh (Exelixis, San Francisco). This semaphorin expression construct was

then used to induce COS-1 cells to synthesize the semaphorin proteins required for this project.

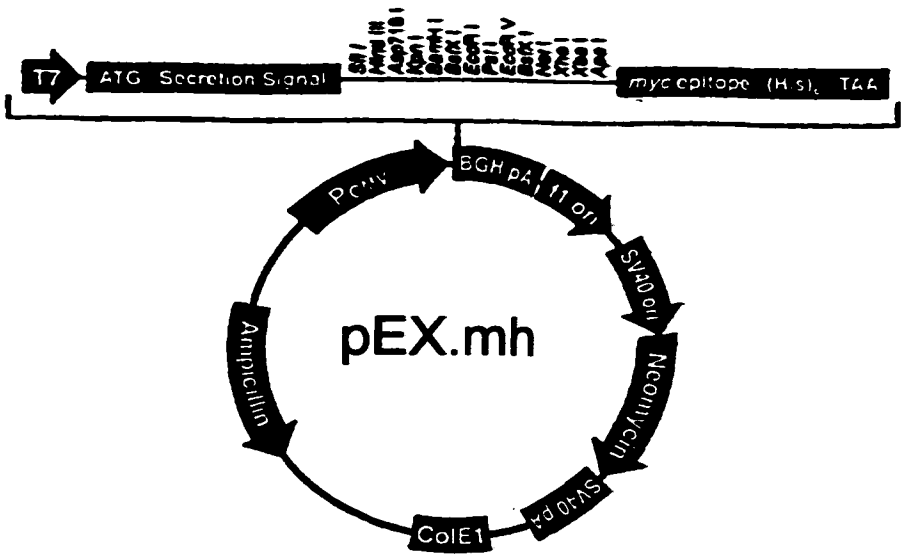


Figure 2: The pEX.mh plasmid vector (Exelixis Pharmaceuticals, San Francisco, CA)

The pEX.mh is a 5.4 kb Epstein-Barr virus based subcloning vector that contains many components that were advantageous in this project (See figure 2). Researchers have found that Epstein-Barr virus based vectors transform cells with a high efficiency (Margolskee, Kavathas, & Berg, 1988). Margolskee et al. were able to isolate 10^6 to 10^7 independent transformants in human lymphoblastoid cells when they created cDNA library with Eptein-Barr based pcD vector. In the pEX.mh vector, a human cytomegalovirus promoter has been added to the Epstein-Barr construct to increase the protein synthesis in primate cell lines and those from other species (Boshart et al., 1995).

In addition, the pEX.mh vector carries both ampicillin and neomycin resistance genes. This allows one to use ampicillin screening to identify bacterial transformants and neomycin screening to pick successful transfectants.

Finally, the pEX.mh mammalian expression vector incorporates both a *myc* and *poly-his* tag into the synthesized proteins. When the desired protein is synthesized in high enough quantity, these tags can be used to identify the synthesis of the coded semaphorin protein. By using antibodies against these tags in immunoblotting, the presence of semaphorin in the extracts of the COS cells could be determined.

Exelixis Pharmaceuticals (San Francisco, CA) provided three 10ug aliquots of SEMA7A, SEMA3A, and SEMA4D genes cloned into the pEX.mh.

Plasmid Verification and Preparation

Preparation of the plasmids began with the production of competent Top10F *E.coli* cells (Stratagene, CA) and generation transformants containing the semaphorin-pEX.mh constructs. (Chung & Miller,1988).

Transformation

The bacterial cells were grown to the early log phase (OD_{600} = 0.3-0.6) in Luria broth (LB). The cells were then spun for 10 minutes at 1,000 x g at 4°C in a IEC HN-SII clinical centrifuge (International Equipment Company, NY), resuspended in 1/10th the volume of transformation and storage buffer (TSB): LB broth (pH 6.1) containing 10% polyethylene glycol, 5% dimethylsulfoxide, and 20 mM Mg^{++} (10mM $MgCl_2$ + 10 mM $MgSO_4$) at 4°C, and incubated on ice for 10 minutes. The cells were then stored at –70° C until used.

On the day of the transformation, 0.1mL aliquots of the cells were removed from –70°C storage, thawed on ice for 10 minutes and transferred to pre-chilled polypropylene tubes. Then, 1 ul (approx. 100 pg) of plasmid DNA was added to the cells and the mixture was placed on ice for 30 minutes. Nine hundred microliters of room temperature TSB medium was placed in each of the polypropylene tubes containing the DNA/cell mixture. The transformed cells were then incubated for 1 hour in a 37°C shaker bath rotating at 225rpm. The cell mixture (50µl) were then plated on LB/ampicillin agar plates and allowed to grow overnight in a 37 °C incubator.

Plasmid Isolation

Three colonies from each of the LB + ampicillin plates were grown in 5 mL of LB + ampicillin broth. After sixteen hours, glycerol stocks were made from each of the broth cultures using 750µl culture and 750 µl glycerol. These cultures were then stored at -70°C. Aliquots (1.5 ml) from the remaining culture were used to isolate the semaphorin constructs using Qiagen™ mini prep kits (Qiagen, CA). Preliminary Xba I/Hind III digests were completed on the plasmid isolated from each of the mini preps to verify similarity between each of the three colonies picked.

One of the three glycerol stocks made of each of the semaphorin transformants was then used to inoculate cultures overnight which then seeded the 300 mL cultures used to produce plasmid. The plasmids (SEMA4D, SEMA7A, and SEMA3A) were then isolated using the 500-tip column Qiagen™ Maxi prep kit. Each column ideally yields 500ug of plasmid per column per 300ml culture. The plasmid DNA was resuspended in 100ul of deionized water. Five microliters of the plasmid DNA sample were diluted with

995µl of water and then quantified using a Shimadzu™ UV 160U UV-Visible Recording Spectrophotometer. The purity of the sample was determined by calculating the 260/280 ratio.

Restriction Mapping and Sequencing of Clones

Both partial sequencing and restriction digests were used to verify the identity of each of the gene inserts in the Exelixis semaphorin constructs. Plasmid DNA for each of the semaphorin clones was sent to the sequencing facility at California State University, Northridge for end sequencing. The first primer had the sequence: 5'TTGACGCAAATGGGCGGTAGGCGT 3'and bound the cytomegalovirus promoter on the 3' end of the multiple cloning site of the vector. The sequence that was verified extended from the 5' to 3' end including approximately 100 base pairs of the vector and 300 base pairs of the insert. The second primer had the sequence 5'ACAACAGATGGCTGGCAACTAGAA 3' and bound to the BGH polyadenlyation site located on the 5' end of the multiple cloning site of the vector. This primer extended from the 3' to 5' end and, also, included approximately 100 base pairs of the vector and 300 base pairs into the insert.

The BLAST analysis program (National Center of Biotechnology Information, www.ncbi.com) was used to verify the identity of each of the Exelixis clones. The program compared the sequence of the Exelixis clones (SEMA7A, SEMA4D, SEMA3A) obtained from the C.S.U. Northridge sequencing facility to the GenBank published sequence for each of the respective semaphorins used. This program determined the inserts, mutations,

gaps and percent identities of the semaphorin genes in the Exelixis clones as compared to the GenBank sequences.

Once the end sequences were identified, they were combined with the Genbank published sequence to form a composite full sequence of the semaphorin insert in the Exelixis clones. These composite sequences were then analyzed with the Webcutter 2.0 program (www.firstmarket.com/cutter/cut2.html) to determine (1) which enzymes cut each sequence, (2) where those enzymes cut in the sequence and (3) the expected sizes for each semaphorin fragments of the clones.

Semaphorin	Enzyme I	Enzyme II	Restriction Buffer	Size of Expected Semaphorin Fragment
SEMA4D	Hind III	XhoI	B	1290
	Xba I	XhoI	H	836
	Xba I	Hind III	M	2126
	Hind III	Asp 718	B	1013
	Xba I	Sca I	B	No Band from Insert
	Hind III	Sca I	B	No Band from Insert
	Xba I	Asp 718	B	1113
SEMA7A	Hind III	Xba I	M	1827
	Hind III	Bgl II	M	620
	Xba I	Sac I	A	482
	Hind III	Sac I	A	1345
	Xba I	Bgl II	H	1207
	Nco I	—	H	522
	Pst I	—	H	No Band from Insert
SEMA3A	DraI	AvaI	A	641
	Hind III	Ava I	B	1643

Table 1- Restriction digests performed on Exelixis constructs

To verify the sequence of the semaphorin genes, restriction digests were run on each of the semaphorin clones using the restriction enzymes and buffers found in Table 1. Ten microgram aliquots of each of the plasmids were used per restriction digest. As a result, the digestion mix for double digests included approximately 5 ul plasmid, 3 ul enzyme I (30 Units), 3 ul enzyme II (30 Units), 5 ul buffer, and 34 ul sterile Millipore water. After the two hour incubation in a 37 °C water bath, a 6 ul aliquot was mixed with

6 ul of 5x loading buffer [0.25% Bromophenol Blue, 60% (v/v) Glycerol/water] and loaded in a 1.2% 11 cm x 16 cm Tris-Borate-EDTA gel. The loaded gels were then run in an electric field of 35V for 16 hours. Once removed from the power supply, the gels were stained with 1 μg/ml ethidium bromide stain for 1 hour and then visualized on a Macintosh™ computer using the Gel Doc Imaging System (BIO-RAD, CA) and the Molecular Analyst image analysis software version 2.1.1 (BIO-RAD, CA). Video prints were made to document each gel run.

Protein Synthesis

COS-1 cell lines

For synthesis of protein in this project, transiently transfected COS-1 cells were used. COS cells are African Green Monkey kidney cells which have been infected with the DNA tumor virus SV40 (ACTG,1997; Glaxo Wellcome, 1998). The SV 40 virus infecting the COS-1 cells facilitates the cellular mechanics of replication without stimulating the production of virions. As a result, when COS cells are transfected with mammalian expression constructs or plasmids containing the SV 40 virus promoter and origin of replication such as the pEX.mh, they replicate the construct at a high rate As a result of the high concentration of replicated plasmid, transfected COS cells theoretically gain the ability to synthesize large quantities of the protein encoded by the construct.

Since COS cells are derived from mammalian cells, proteins synthesized by COS cells do not contain the bacterial N-terminal residue, formyl-Methionine (fM). This peptide label has been found to be a potent neutrophil chemoattractant. If found on the semaphorins used in this study, the fM tag would destroy the ability to distinguish

between the effect semaphorins have on neutrophils and the fM affect. By using COS cells, this problem was avoided.

Culturing of COS-1 Cell lines

COS-1 cells were purchased from American Type Culture Collection (Manassas, VA). The 1 ml ampule of frozen cells was thawed and the cells cultured in 12 ml of RPMI 1640 medium combined with 10% heat inactivated fetal calf serum, 100 units/ml Penicillin, 100 units/ml Streptomycin, and 2 mM L-glutamine. The cells were grown in Falcon 25cm^2 tissue culture flasks and kept in a 37°C/CO_2 incubator where they were monitored daily to assess the pH of the medium and cell health. Because COS-1 cells are an adherent cell line, replacement of the medium required only removing the old medium and adding new medium to the flask. Medium was replaced daily.

The cells were allowed to expand from $1.2x10^5$ cells/ cm^2 to $3.6x10^5$ cells/ cm^2. As a result, the cells were passaged every 48 hours. First the old medium was removed and each of the flasks was rinsed twice with 10 ml of autoclave-sterilized PBS. Then 2 ml of 2x trypsin were placed into the flask and the cells incubated in a 37°C/ 5% CO_2 incubator for 10 minutes. The trypsin released the cells from the bottom of the flask so that the cell suspension could be transferred to a new flask. Seven milliliters of complete RPMI 1640 medium with serum was added immediately to the trypsinized cell suspension to inactivate the trypsin. To continue the cell line 3ml aliquots of the cell suspension (1×10^6 COS-1 cells/mL) were placed into three flasks such that a total of $3x10^6$ COS-1 cells were added to each flask or $1.2x10^5$ cells/ cm^2. Ten milliliters of complete RPMI medium with serum were then added to each flask and the cells were

allowed to grow until confluency 48 hours later. To prepare cells for transfection, $1x10^6$ cells per plate (approximately 1ml) were added to each of 6-60mm x 15mm Falcon tissue culture dishes. Five milliliters of complete RPMI with serum were then added to each plate. These cells were allowed to grow for 24 hours to an approximate density of 2.5 x 10^5 cells/cm^2 or approximately 70% confluency after which they were transfected.

Transfection of COS-1 Cells

LipofectAMINE™ (Gibco/BRL, MD) is a liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethy-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE). Although the exact mechanism is not known, it is believed that the negative charge on the DNA groups bind to the positively charged surface of the liposome. The residual negative charge on the liposome is then believed to bind to the negatively charged sialic acid residues on the cell surface (Ausubel et al., 1991).

Liposome transfections have been found to have high transfection efficiencies. Depending on the cell line, liposome transfections have transfection frequencies in mammalian cells from 5 to 100 times greater than other methods (Felgner et al., 1987; Rose, Buonocore, & Whitt 1991). As many as 60% to 80% of transiently transfected cells have been shown to express the desired protein. For this project, LipofectAMINE™ was chosen because it was less toxic to the cells than the calcium phosphate precipitation method. As a result, LipofectAMINE™ allowed for greater expression of the semaphorin protein.

The day before each transfection, COS-1 cells were passaged and 1.00 x10^6 cells were added to each dish. On the day of the transfection, 15 microliters of LipofectAMINE™ was mixed with 100 µl Opti-MEM™ medium (Gibco/BRL, MD) in a 1.5 ml microcentrifuge tube and allowed to equilibrate to room temperature. In a separate 1.5 ml microcentrifuge tube, 2 µg of semaphorin/pEX.mh pDNA was mixed with 100ml Opti-MEM™ medium. The 102µl pDNA/Opti-MEM™ mix was then put into the 1.5ml microcentrifuge with the LipofectAMINE™ and Opti-MEM™ medium. This mixture was then incubated at room temperature for 30 minutes to allow for the formation of the micelles.

After the incubation, 800 µl of the Opti-MEM™ medium was added to each microcentrifuge tube containing the DNA/LipofectAMINE™ mix. Then, the medium on the incubating cells was removed and the plates were rinsed with 2 ml of Opti-MEM™ medium. One milliliter of the pDNA/LipofectAMINE™ mix was layered over each rinsed dish and incubated for five hours in a 37°C/5% CO_2 incubator. After the incubation, 1 ml of RPMI 1640 medium containing 2x serum, L-glutamine, and no antibiotics was added to each plate which was then returned to the incubator for another 24 hour incubation. Finally, the DNA/ LipofectAMINE™ mix was removed from each plate and replaced with 5ml of complete RPMI 1640 medium and the cells were allowed to incubate for another 24 hours.

The cells were harvested using a rubber policeman 48 hours after the addition of the DNA/LipofectAMINE™ mix and tested. As a result of the small quantity of DNA added per plate, the LipofectAMINE™ protocol by Gibco/BRL, MD discouraged co-

transfections of the test and reporter plasmids. Consequently, the β-galactosidase reporter plasmid was transfected in a separate dish along side the dishes with cells transfected with the semaphorin DNA.

Cell Extracts

Cell extracts were prepared by resuspending each cell pellet in 60μl of hypotonic solution containing 3 parts complete RPMI medium and 1 part sterile, deionized water. Then the cells were put through a sequence of three freezing/thawing sequences alternating between 3 minutes in the –80°C freezer and 2 minutes in the 37°C water bath. The cells were then spun in a microcentrifuge for five minutes and the supernatant fluid was then removed and used immediately to prevent excessive protein degradation by proteases. The extract of the β-galactosidase transfected cells was tested before each Boyden chamber assay.

Immunoblotting

Dot Blot Analysis

COS-1 cell extracts and culture supernatants were first tested by dot blot analysis to verify expression of a myc tagged protein in each transfection. A nitrocellulose membrane was cut to 2.5 inches x 4 inches and eight circles were drawn on the membrane with a Sharpie™ fine tip permanent marker. The membrane was then pre-wetted with milipore water and hung dry for 10 minutes. Two aliquots (5μl/aliquot) of extract were then applied in each circle (1 extract/circle) and allowed briefly to dry. The *myc* labeled protein was then detected via BIORAD Alkaline Phosphatase Development described below.

Nitrocellulose Alkaline Phosphatase Development

The nitrocellulose membranes from both the Dot Blot and Western Blot preparations were exposed to a 1:5000 dilution 1° antibody solution [4 ul c-myc mouse antibody, 20 ml 1X PBS, .1% Tween 20]. One nitrocellulose membrane with protein was placed in a 300 ml polystyrene bottle. The 1° antibody solution was added, and the bottle was loaded horizontally onto a FLEX COAT Finishing Motor (Shoff Tackle Co., www.shofftackle.com) that rotated at 18 rpm. The motor/bottle set-up was put in a 4°C cold room and the nitrocellulose membrane was exposed to the 1° antibody for 16 hours. The 1° antibody solution was then poured out of the bottle. Four 20 ml-15 minute washes of PBS and .1% Tween were used to remove any excess 1° antibody. Eight microliters of Goat anti-mouse alkaline phosphate 2° antibody (BioRad) was then suspended in 20 ml of PBS and .1% Tween and applied to the membrane for 1 hour. The membrane was then washed in TBS and .1% Tween twice for five minutes. The final 5 minute wash was with 20 ml of 1xTBS alone. After the final wash, the membrane was transferred to a glass petri dish and exposed to 100 ml of BIORAD AP Color Development Solution (1 ml reagent A, 1 ml reagent B, 100 ml development buffer). After 30 minutes, the membrane was removed and placed in milipore water to stop the reaction. Finally the membrane was allowed to dry and then stored in a dark place until photographed.

Western Blots Analysis

Following a positive dot blot result, western blots were run to verify the size of the myc tag expressing protein. Fifteen milliliters of 4.5% SDS-PAGE stacking gels were poured over 30 ml of a 7% to 20% SDS-PAGE gradient resolving gel. Thirty microliters

of each extract were mixed with 15 ul protein loading buffer [2% SDS, 7% glycerol, 5% β mercaptoethanol, 62 mM Tris, Bromophenol Blue (0.1g/100ml), pH 6.8]and 15ul of deionized water. The samples were placed in a 65°C water bath for 15 minutes to allow for denaturation. Once cooled, the samples were loaded on to the SDS-PAGE gradient gel, run at 80V for 1hr, and then run at 160V for 8 hrs.

After the samples had been run, the SDS-PAGE gel was then equilibrated in Towbin transfer buffer for 1hr to remove excess salt. The protein transfer from the SDS-PAGE gel to the nitrocellulose membrane was achieved using a Hoeffer™ Trans-Blot cell. One piece of nitrocelluose membrane and five pieces of filter paper were soaked with transfer buffer and were stacked as follows: 2 pieces of pre-wetted filter paper, nitrocellulose, SDS-PAGE gradient gel with protein, 3 pieces of pre-wetted filter paper. The stack then had a piece of thin sponge placed on top and on the bottom and the completed stack was locked into the gel holding cage of the Hoeffer™ system. The electrodes on the lid of the Hoeffer™ system were oriented such that the proteins ran from the positive electrode to the negative electrode. The protein transfer system was run for 16 hours at 100mA in a 4°C cold room.

To verify the transfer, the nitrocellulose was exposed to Ponceau stain for 1 minute and then immediately placed in deionized water. Red stained protein bands were visualized upon exposure to the deionized water. The nitrocellulose membrane was rinsed until all of the Ponceau stain was removed. In addition, the post-transfer SDS-PAGE gel was placed in Coomassie blue stain for four hours and then de-stained using a

5%methanol/ 7% acetic acid solution de-staining solution. De-staining of the SDS-PAGE gel took 24 hours.

The nitrocellulose membrane was then placed in a polystyrene bottle with 50mL of blocking solution made by dissolving 500 mg of instant milk in 50 ml PBS and .1% Tween 20. The bottle was loaded horizontally onto a FLEX COAT Finishing Motor and allowed to rotate at 18 rpm for 1 hour at room temperature. The blocking solution was then replaced with 20 ml of 1° antibody solution and the nitrocellulose was developed with the BIORAD alkaline phosphatase protocol described above.

β-Galactosidase Assay

For each day transfections were performed, both a positive and a negative control plate were included. The negative control was a plate of cells that underwent the transfection procedure without any DNA added. These cells were considered to be "mock" transfected cells. The positive control was a plate of cells that were transfected with the control DNA, pcDNA3.1 containing the lacZ gene. This gene encodes the β-galactosidase protein that cleaves β-galactose. The transfection was judged successful if the extract of the positive control plate showed β-galactosidase activity when assayed.

The β-galactosidase assays were carried out on 96-well plates. 100x magnesium solution [0.1M $MgCl_2$; 4.5M βMercaptoethanol], 1x o-nitrophenyl-β-o-galactopyranoside (ONPG) [4 mg/ml ONPG in 0.1M sodium phosphate (pH = 7.5)] and 0.1 M sodium phosphate (pH = 7.5) were mixed in a 15ml conical tube and allocated to the wells. For each sample tested, 3ul 100x magnesium solution; 66ul 1x ONPG; 201ul 0.1M sodium phosphate were added to a single well. Thirty microliters of extract were then added to

the combined reagents and mixed. Positive results were determined primarily by observing the well contents turning yellow due to the accumulation of o-nitrophenol. However, a few of the extracts were read using a BIO-TEK Instruments Microplate Autoreader EC 311at an optical density of 460nm. The number of wells used depended on the number of extracts being tested.

Isolation of Neutrophils

Human neutrophils were only used in the final stages of this project during the months of May, June, and July 1999. The day the first blood sample from each volunteer was drawn, the volunteers signed a Human Subject Authorization form verifying their agreement to have their blood drawn and their approval of the use of their blood sample for this project (See Appendix 8+9). Blood was drawn from four healthy, human volunteers two hours before each Boyden chamber assay. The blood was drawn by venipuncture using a Vacutainer Brand 21 gauge butterfly needle with Luer adapter (Becton Dickinson, Rutherford, NJ) and one – 10 ml sodium/EDTA Vacutainer Brand tube. The blood was immediately layered over 3 ml of Mono-Poly Resolving Medium (ICN Biomedicals, OH) (d = 1.14 g/ml) mixed with 300 µl of sterile deionized water. The layered cells were spun for 45 minutes at 1,200 x g without the use of the break in the centrifuge and the neutrophil band was removed. The neutrophils were then placed in a separate tube and washed with PBS. After spinning for 10 minutes at 1200xg in a clinical centrifuge, the supernatant fluid was removed. Cells were resuspended in 6ml of deionized water for 20 seconds causing any remaining red blood cells to be lysed. The neutrophils were then returned to isotonicity with 2 ml of 3.4% saline solution and

washed with PBS. Finally the cells were suspended in 1 ml of RPMI 1640 medium with 10% heat inactivated fetal calf serum.

The isolated neutrophils were counted using 0.4% trypan blue stain (Sigma Chemical Company, MO) and a hemocytometer. The trypan blue stains only the dead cells allowing one to determine the number of viable neutrophils. Eighty thousand neutrophils were loaded into the upper well of each Boyden chamber.

The primary goal of this step was to maintain neutrophil motility. One of the main factors keeping the neutrophils motile and responsive to chemotropic agents is speed. Therefore, the neutrophils were isolated and the Boyden chamber assays completed within three hours of the blood being drawn to ensure maximum motility.

Boyden Chamber Assay

General

Chemotaxis of neutrophils has been measured by a number of techniques ranging from viewing changes in morphology of cells fixed with gluteraldehyde to observations of a single cell's movement and behavior in a gradient of chemoattractant with video photography (Haston & Shields, 1985; Zicha, Dunn & Segal, 1997). One of the most common methods for observing neutrophil motility, however, is the Boyden chamber assay (Boyden 1962; Lackie, Chaabane & Crocket, 1987).

The generic Boyden chamber is set up in two vertical compartments separated by a filter with a pore size that ranges between 3 and 8μm depending on the size of the motile cell type being tested (Figure 3). Typically, the upper chamber holds the chemoresponsive cells and the lower chamber holds the chemoattractant. Incubation

times in different research projects range from 30 minutes to two days depending primarily on the hardiness of the cells and strength of the chemoattractant.

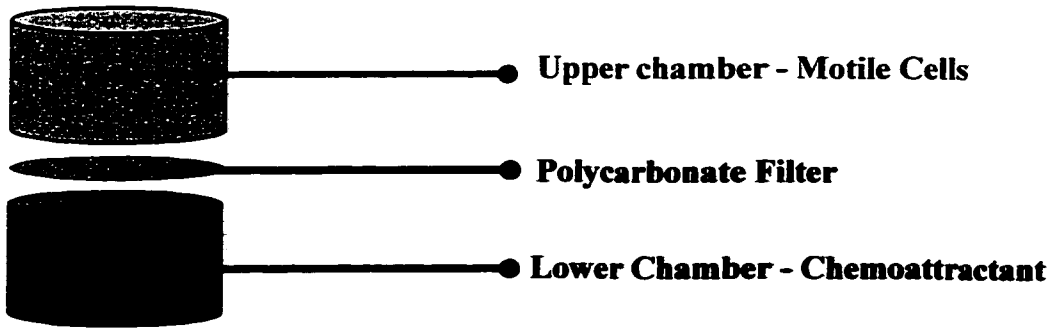


Figure 3: The Blind-well Boyden chamber

Filters

Although other filter options such as nitrocellulose and cellulose ester were available for the Boyden chamber, the polyvinylpyrrolidone-free polycarbonate filter was used in this project. This filter has been shown to be best at assessing chemotaxis because it is only 10 μm thick, as compared to the 110 μm thick nitrocellulose filters and it allows the neutrophils to pass through the filter relatively quickly. In addition, the neutrophils easily adhere to the polycarbonate.

Experimental Design

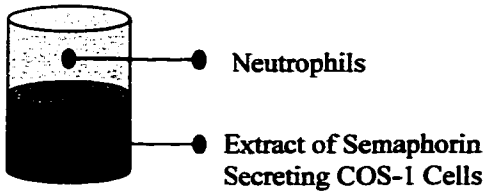
The general experimental design of this research project is shown in figure 4. The chemoattraction assays were performed by placing the motile cells in the upper compartment and the chemoattractant in the lower compartment. In these experiments, if the number of neutrophils that crossed the filter were greater than those in the control the semaphorins would have be shown to have a chemoattractive effect on neutrophils.

Thus far, however, semaphorins have been shown to primarily act as chemorepellent molecules. As a result, the second group of experiments was run to test the chemorepulsive effect of semaphorins on neutrophils. In these assays, the neutrophils and semaphorin were both placed in the upper chamber. If the number of neutrophils that passed through the filter were greater than the number that passed through the filter in the control, then SEMA4D, SEMA3A, and/or SEMA7A would have been shown to have a chemorepulsive effect.

EXPERIMENTAL DESIGN

CHEMOATTRACTION ASSAYS

Experiment 1.1



CHEMOREPULSION ASSAYS

Experiment 1.2

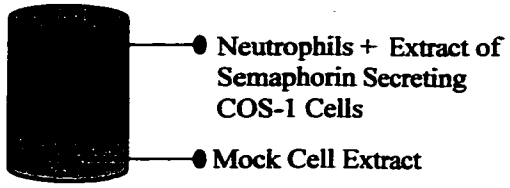


Figure 4 - Experimental Design

Finally if the number of neutrophils that passed through the filter did not alter in the presence of semaphorin in either the chemoattraction or chemorepulsion assay, it would suggest that semaphorins have no effect on the directed motility of neutrophils *in vitro* as assessed by the Boyden chamber assay.

Checkerboard Analysis

A checkerboard analysis is primarily used to see the effect of a chemotropic agent on chemotaxic cells over varying concentrations and gradients. It, also, allows one to distinguish between guided cellular movement, chemotaxis, and a change in the random

movement of cells, chemokinesis. In this project the checkerboard analysis was performed for each experiment to determine both the concentration of semaphorin which stimulated the greatest neutrophil response and the chemokinetic effect semaphorins have on isolated neutrophils (Wilkinson, 1974; Andresen & Ehlers, 1998).

The generic design of the checkerboard analysis is four by four grid (total chambers = 16). In this design, the Boyden chambers in the vertical columns have the same respective concentration of chemotropic agent in their upper wells while the chambers in the horizontal rows have the same respective concentration of chemotropic agent in their lower wells (See Table 2). This design allows for the study of both (1) the discrete effect of a chemotropic agent by putting it in only one of the two chambers and (2) the gradient effects of a chemotropic agent by placing it in varying concentrations in both the upper and lower wells of the chamber. For this research, the checkerboard analysis was used to show the effects of several semaphorin environments on neutrophils.

Upper Well of Boyden Chamber

Lower Well: Chemotropic Agent Concentration	0	10^{-7} mM	10^{-6} mM	10^{-5} mM
0	1	2	3	4
10^{-7} mM	5	6	7	8
10^{-6} mM	9	10	11	12
10^{-5} mM	13	14	15	16

Table 2: Checkerboard analysis design
a) One Square represents one set of conditions tested in triplicate
b)The number within each square indicates a portrait set of 3 chambers in a 48 well chemotaxis plate

As seen in Table 2, column 1 contains four Boyden chambers with no semaphorin in the upper wells and an increasing concentration of semaphorin in each of the four lower wells. Therefore, the data from this column of Boyden chambers revealed the lower well concentration of semaphorin that stimulated the greatest amount of chemotaxis in the human neutrophils in the chemoattraction experiments. On the other hand, row 1 had four Boyden chambers with no semaphorin in their lower wells and an increasing concentration of semaphorin in the upper wells. This data showed the upper well concentration of semaphorin that stimulated the greatest chemorepulsive effect in human neutrophils.

In addition to the straight chemoattraction and chemorepulsion effects of semaphorins, the checkerboard analysis also determined the consequence of both less steep positive and less steep negative concentration gradients of semaphorin on neutrophils (Table 2). In the Boyden chambers where there is a lower concentration of semaphorin in the upper wells versus the lower wells, the effects of a positive gradient was seen. This occurred in chambers 7,8, and 12. In the wells where there was a higher concentration gradient in the upper wells versus the lower wells, the effects of a negative gradient were determined. The varying negative concentration gradients occur in chambers 10,14, and 15.

The chemokinetic effect semaphorins have on neutrophils was determined by the number of neutrophils which cross the filter for each well of the chamber that falls on the diagonal of the checkerboard analysis plan (1, 6, 11, 16). Because no gradient is present in these chambers, any increase or decrease in the number of neutrophils which cross the

filter when compared to the mock transfected COS cell control will be due to the chemokinetic effect of the semaphorin. Once the number of cells that crossed the filter in the chemokinetic chambers was quantified, statistical analyses were performed of the movement in the chemokinetic chambers versus both the negative control and the chemotaxis chambers, as described in the results.

Purified semaphorin protein was not used in this project because it was not commercially available. As a result, the research relied strictly on COS cell synthesis of semaphorin protein. Therefore, the amount of semaphorin in each well was varied by diluting the semaphorin-transfected cell extract from the COS cells with complete RPMI 1640 medium with serum (Table 3).

Upper Chamber of Boyden Chamber

Lower Chamber Semaphorin Supernatant Dilution	**0**	**1:20**	**1:200**	**1:2000**
0	$0_{1:20}$ / $0_{1:20}$	1:20 / $0_{1:20}$	1:200 / $0_{1:200}$	1:2000 / $0_{1:2000}$
1:20	$0_{1:20}$ / 1:20	1:20 / 1:20	1:200 / 1:20	1:2000 / 1:20
1:200	$0_{1:200}$ / 1:200	1:20 / 1:200	1:200 / 1:200	1:2000 / 1:200
1:2000	$0_{1:2000}$ / 1:2000	1:20 / 1:2000	1:200 / 1:2000	1:2000 / 1:2000

Table 3: Experimental set-up of semaphorin Boyden chamber analysis using checkerboard design. Each experimental box represents 3 wells on the 48-well Boyden chamber plate.

The dilution scheme of the Boyden chamber analysis used in this research project is shown in Table 3. The mock-transfected COS-1 cell extract was diluted to match the concentration of COS-1 cell cellular protein found in the transfected cells. This was done to prevent false data caused by the effect of a cellular protein gradient on neutrophil chemotaxis. In addition, the positive control of 10^{-5} mM formyl-Methionine-Leucine-Proline (fMLP) in the lower chamber and 80,000 neutrophils suspended in RPMI 1640 medium with 10% fetal calf serum without any extract was placed in the wells in the lower right corner of the plate. fMLP is a bacterial peptide label that has been shown to be a powerful chemoattractant to neutrophils. This positive control was used to verify the success of the Boyden chamber assay plate.

Setting up the Boyden Chamber Assay

a) b)

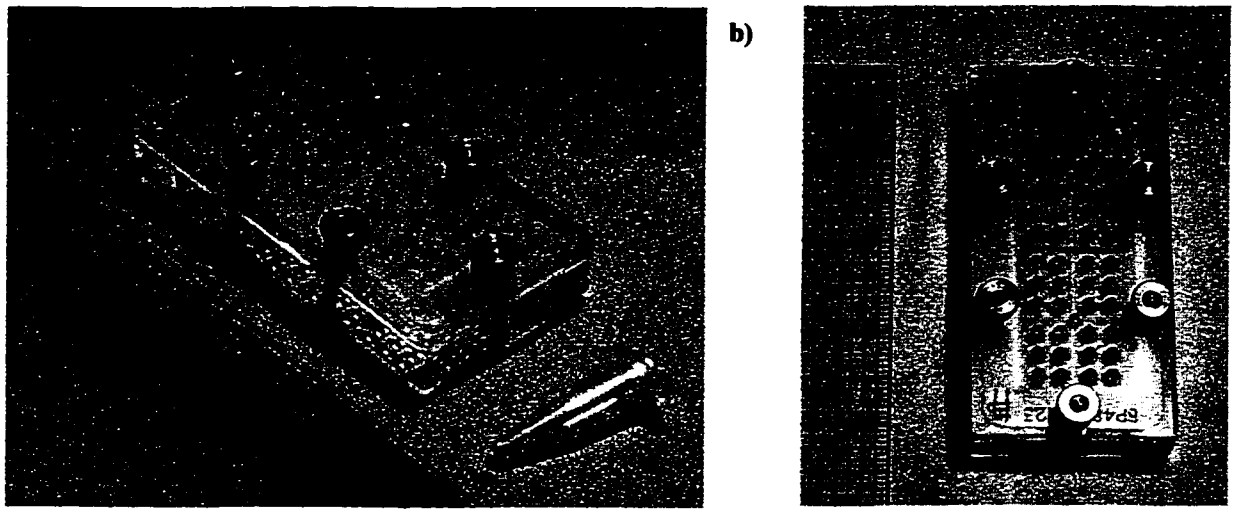


Figure 5 - Picture of 48-well Boyden Chamber (Neuroprobe, NH). (a) 45° view of Boyden chamber (b) Top view of Boyden chamber

In this project, the 48 blind well Boyden chamber apparatus was used to assess neutrophil chemotaxis (Figure 5). While the neutrophils were being isolated, the lower chambers of the plates were filled with 28ul of the prescribed dilutions of the cell extracts indicated in Table 3. Once filled, the polyvinylpyrrolidone-free polycarbonate filter (5 μm pores) was gently placed over the lower plate with the matte side down. Each of the lower wells was then checked for trapped air bubbles. If bubble were found then they were noted on the template

Once the filter was placed, the rubber gasket was put carefully over the filter and, the upper chamber was firmly affixed over the gasket and filter. Still maintaining pressure on the top plate, the six bolts were tightened securely to prevent any leakage or mixture of either lower or upper chamber content. The Boyden chamber was then rechecked for bubbles. If bubbles were present, then they were forced to one side of the chamber by tipping the chamber on its side. The location of the bubbles was then re-noted on the template.

Eighty thousand neutrophils were mixed with the appropriate dilution of either semaphorin or mock transfected cell extract as described in Table 3 and added to the upper well. Although the upper well of the Boyden chamber has a maximum capacity of 50ul, only a total 40ul of extract and neutrophils were added.

The filled apparatus was then incubated in a 37°C/ 5%CO_2 incubator for 45 minutes. After the incubation the Boyden chamber was disassembled and the filter removed. The cells were stained with Diff-Quick™ (Baxter Healthcare Company, IL) and counted as described below.

To prevent any cross contamination of semaphorin proteins between assays, only one semaphorin was tested per 48-well Boyden chamber assay plate. The chamber was cleaned with TERG-A-ZYME™ (Alconox Incorporated, NY) and rinsed with deionized water and then re-rinsed with filtered deionized water between each experiment. The chamber was dried using a sterile Pasteur pipette connected to a vacuum to remove the water from each well. The external portions of the chamber were dried with Kimwipes™. All Boyden chambers assays performed were set up to allow the Checkerboard analysis method.

Cell Quantification

To quantify the number of neutrophils that passed through the filter during the one hour incubation period the filter was carefully removed and the cells on the top of the filter wiped off by dragging the filter over an edge of a microscope slide. A small clamp was placed on the lower edge of the filter for weight. A larger clamp was affixed to the top of the filter to allow for dipping into the stain. The filter was then dipped ten times into the Diff-Quick™ fix solution. This was followed by fifteen dips into the Diff-Quick™ Solution 1 and 30 dips into the Diff-Quick Solution 2. The modifications to the original Diff-Quick™ protocol for both the Solution 1 (15 dips instead of 10) and Solution 2 (30 dips instead of 10) were made due to the difficulty in staining the cytosol of the neutrophils on the membrane. After staining, the filter was affixed to a microscope slide using mineral oil. The upper side of the membrane faced the slide. Five high powered fields (40x) were then counted to determine the number of neutrophils that crossed the membrane in response to the different stimuli.

Data Analysis

A repeated measures variance (ANOVA) with a block design was used to test the statistical significance of the effect of SEMA7A, SEMA4D, and SEMA3A on human neutrophils. This analysis estimates the variability for each subject allowing for more variance in the raw data to be explained. As a result, this statistical test allows the researcher to use a smaller sample size to obtain a statistically significant finding.

The repeated measures ANOVA best fit the structure of this research data because there were 36 repeated measures of the same 16 semaphorin environments for each semaphorin tested in the Boyden chamber assays. Also, the same four volunteers were used to test all three semaphorins.

RESULTS

Plasmid purification

Yield

As seen on Table 4, the mean yield of plasmid per 300 ul culture was 446 μg. The maximum yield of 692 μg was obtained for the SEMA7A plasmid construct on 10/14/98. The lowest yield of 159 μg was obtained for the SEMA3A plasmid on 10/7/98. The variation in the yields found for each of the plasmids could be due to one or more of three major factors. The first factor is a variation in the time the cultures were allowed to grow. The protocol called for 300 ml cultures to be grown overnight, anywhere from 12 to 18 hours. The amount of bacterial growth in the six hour difference is considerable and could affect the number of plasmid isolated.

The second factor affecting the yield is the growth phase the cultures were in when they were harvested. If the cells were in the later stages of log growth the cultures would have been healthy and the death rate minimal. The yield of cultures harvested in this phase would have been expected to be high. If the cells were harvested during either the stationary or death phase, then the cultures would not be as healthy and rapidly growing due to exhaustion of the medium. If harvested during these phases of bacterial growth, the desired plasmid DNA in these cultures could have already been degrading due to the released DNAse of the dying cells. As a result, the plasmid yield in these cultures would be reduced.

Construct Preparation	Date of Plasmid Isolation	Optical Density Reading (260 nm)	Yield	Purity (260/280)
SEMA3A	10/7/98	0.159 A	159 µg	2.0217
	10/14/98 – Prep 1	0.240 A	360 µg	2.0588
	10/14/98 – Prep 2	0.149 A	210 µg	1.957
SEMA4D	10/6/98	0.405A	405 µg	1.7756
	10/7/98	0.489 A	489 µg	1.8225
	11/29/98 – Prep 1	0.346 A	692 µg	1.8221
	11/29/98 – Prep 2	0.326 A	652 µg	1.8078
SEMA7A	10/6/98	0.497 A	497 µg	1.7115
	10/14/98 – Prep 1	0.663 A	633 µg	1.8876
	10/14/98 – Prep 2	0.402 A	402 µg	1.8348
	11/29/98 – Prep 1	0.449 A	449 µg	1.6471
	11/29/98 – Prep 2	0.405 A	405 µg	1.8693

Table 4 – Optical density reading, yield, and purity of each of the SEMA3A, SEMA4D and SEMA7A maxi plasmid preparations used for COS-1 cell transfections

The third major factor affecting the yield would be the efficiency of replication each plasmid had in the TOP10F cells. Although the plasmid backbone of each of the constructs was the same, factors such as the size of the gene could affect the replication

of the plasmid. A decrease or increase in the replication rate would mean a respective decrease or increase the yield of the plasmid being isolated.

Purity

The purity of the samples was measured by the 260/280 ratio. The ideal value for this ratio was considered 1.8 because of the 1:200 dilution of the original sample. If the value was greater than 2, then RNA contamination was considered a problem. If the value was less than 1.5, the sample was not used for transfections. The mean calculated purity for the diluted plasmids was 1.85. The high 260/280 ratio in the SEMA3A plasmid samples from 10/7/98 and 10/14/98 were due to the decreased activity of the RNAse enzyme used for these samples (Table 4). Once new RNAse was prepared the purity of the samples returned to around 1.8. This can be see in the SEMA7A sample from 10/14/98 were the new RNAse was first used.

Plasmid verification

Restriction mapping and sequencing of each of the semaphorin inserts were used to verify the identities of the clones donated by Exelixis. Sequencing data obtained from California State University, Northridge showed the size of each of the semaphorin inserts, whether or not the gene was fused with a secretory signal, and the molecular weight of the coded protein.

SEMA4D

The published sequence for SEMA4D (Genbank accession # HSU060800) is 4157 base pairs (bp) with the open reading frame between base pair 88 and base pair 2676. The SEMA4D insert for the clone was 2126 base pairs starting from base pair 157

and ending at base pair 2283 (Appendix 2a). An exogenous secretory signal of 98 base pairs was placed at the N-terminus of the gene. The unglycosylated protein product of this gene is determined to be 85.3 kilodaltons (kD). The Exelixis SEMA4D clone coded for a protein which had 21 amino acids deleted from the N-terminus (Appendix 2b). This deletion generates a secreted form of the normally membrane bound SEMA4D.

All the digests of SEMA4D yielded a band approximately equal to the predicted fragment (Figure 6). The Hind III/Xho1 double digest of the clone yielded the 1290bp fragment (Column 10, Figure 6). The Xba1/Xho1 digest yielded a 836bp fragment (Column 9, Figure 6). The HindIII/Xba1fragment yielded a 2126 bp fragment (Column 11, Figure 6). The Hind III-Asp718 digest yielded a 1013 bp fragment (Column 13, Figure 6). And the Xba1/Asp718 digest yielded a 1113 bp digest (Column 12, Figure 6). Two digests on the SEMA4D part of Figure 6 were run in error. The Sca I enzyme does not have a restriction site in the SEMA4D insert. As a result the Xba I/Sca I digest and the Hind III/ Sca I digest do not yield a band from the insert. However, due to the agreement of the predicted fragment size and the actual fragment size of the correctly planned enzymes, the SEMA4D insert in the pEX.mh vector was determined to be a viable molecular construct.

SEMA7A

The published sequence of SEMA7A (Genbank accession # AF017542) is 2498 bp with the open reading frame between base pair 1 and base pair 2001. The SEMA7A insert in the Exelixis clone was 1688 bp beginning at 139bp of the gene and ending at bp 1827(See Appendix 4a). An exogenous secretory signal was also placed at the N-

terminus of the protein. The unglycosylated protein product of this gene is estimated to be 72.7 kD. The Exelixis clone protein product deletes 45 amino acids of the N terminus of the protein and 25 amino acids from the C terminus when compared to the published protein sequence (See Appendix 4b). These cleaved regions are believed not to be active regions of the protein.

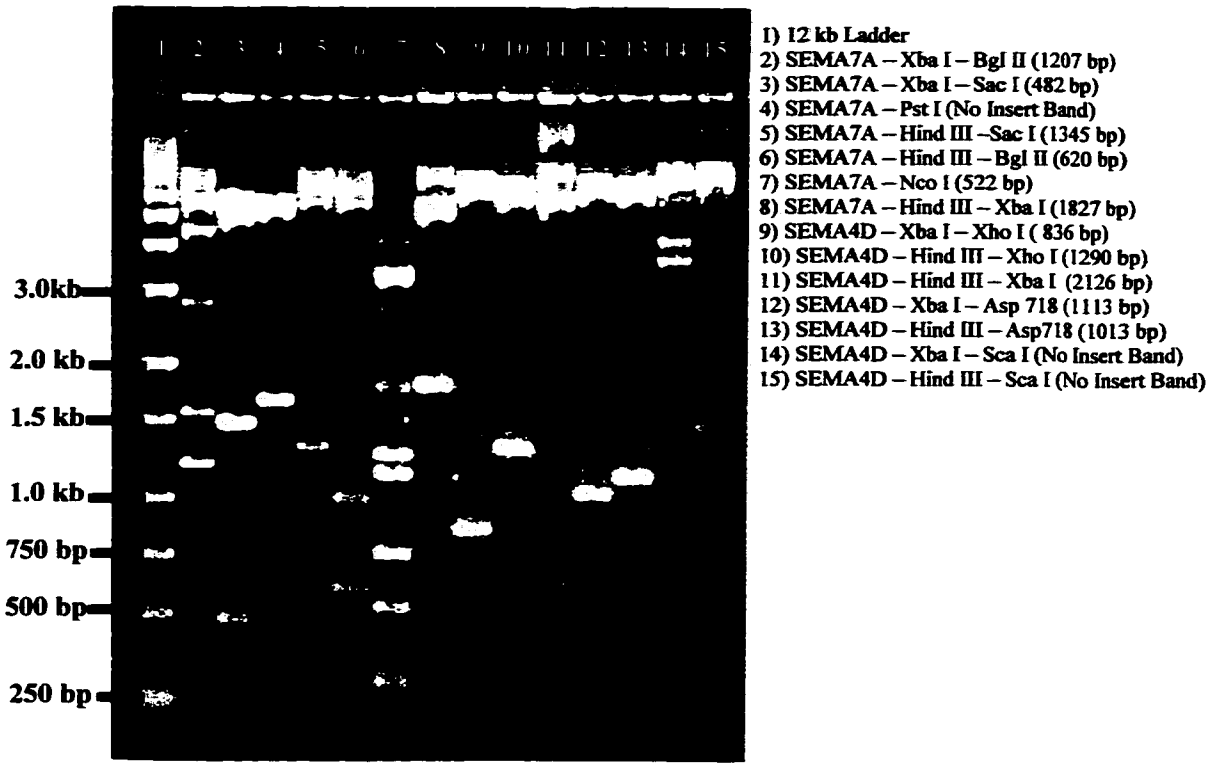


Figure 6 – Restriction digests of SEMA7A and SEMA4D pEX.mh expression constructs. (#) = the size of the expected digested semaphorin fragment. The other bands on the gel are from the pEX.mh vector.

All of the digests run on the SEMA7A clone yielded bands approximately equal to the predicted fragments (Figure 6). The HindIII/Xba1 double digest yielded the 1827 bp fragment (Column 8, Figure 6). The Hind III/Bgl II digest yielded a 620bp fragment

(Column 6, Figure 6). The XbaI/SacI digest yielded a 482 bp fragment (Column 3, Figure 6). The Hind III/SacI digest yielded a 1345bp fragment (Column 5, Figure 6). The Xba 1/Bgl II digest yielded a 1207 bp fragment (Column 2, Figure 6).

Finally, the Nco I digest yielded a 522 bp fragment (Column 7, Figure 6). As with the SEMA4D digests, one of the digests for SEMA7A was run in error. There is only one Pst I site in the SEMA7A insert. As a result, a single digest with Pst I would only linearize the construct and not produce a band from the insert alone. However, because of the correlation of the predicted and actual fragment sizes in the properly planned digests, the Sema7A insert in pEX.mh vector was determined to be a viable molecular construct.

SEMA3A

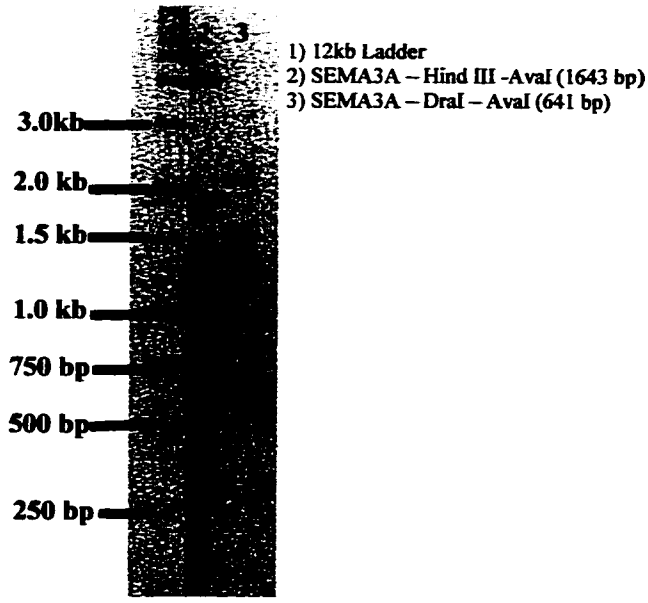


Figure 7 - Restriction digest of SEMA3A.
(#) – the size of the expected digested semaphorin fragment. The other bands on the gel are from the pEX.mh vector.

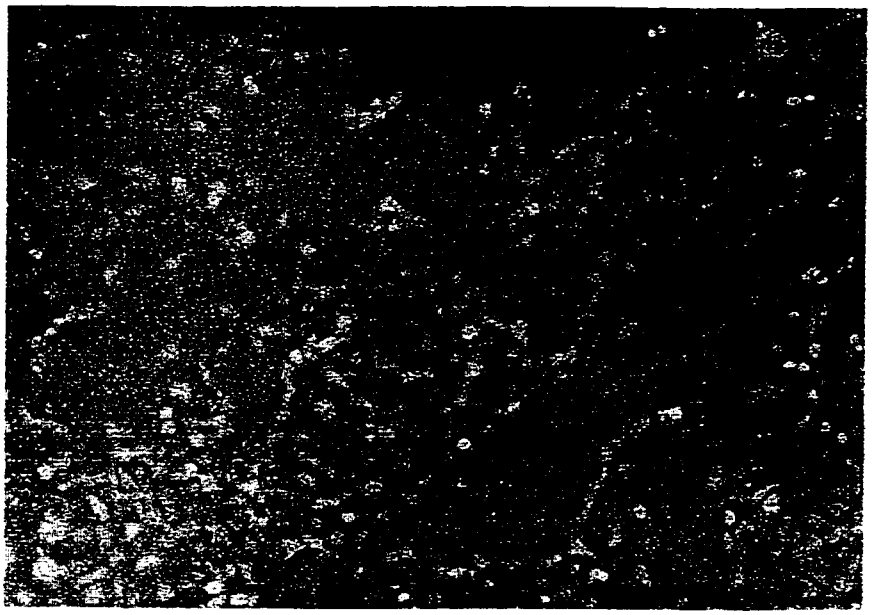
The published sequence of the SEMA3A (Genbank accession # L26081) is 2530bp with the open reading frame between base pair 16 and base pair 2331. The SEMA3A insert in the Exelixis clone was 2252bp beginning at base pair 76 and ending at base pair 2328 (See appendix 3a). As with the others, semaphorin 3 is a fusion protein with a 99 base pair exogenous secretory signal at the N-terminus of the protein. The predicted protein product of this gene is estimated to be 89.9 kD. The Exelixis clone protein product had 20 amino acids deleted from the N-terminus of the protein (Appendix 3b).

All of the digests run on the SEMA3A clone yielded bands approximately equal to the predicted fragment size (Figure 7). The HindIII/AvaI digest yielded a fragment 1643bp. The DraI/AvaI digest yielded a fragment 641bp in size. Because of the correlation of the predicted and actual fragment sizes, SEMA3A insert in the pEX.mh vector was determined to be a viable molecular construct.

COS-1 Cell Culture

COS-1 cell cultures were grown and sustained for transfections through the ninth passage. Initially, the cells were passaged and then grown for 3-5 days before the next passage. The medium was replaced only once during this period. However, cells raised in this manner were not healthy. The overcrowding of the cells and the acidic pH of the medium (<6.1). As a result of not changing the medium daily caused the live cells to decrease their division rate and severely reduce their viability. Often times during this period, these cells were seeded at 2.0×10^6 cells per 60 mm x 15 mm transfection plate and they would only achieve 30% confluency overnight.

a)



b)

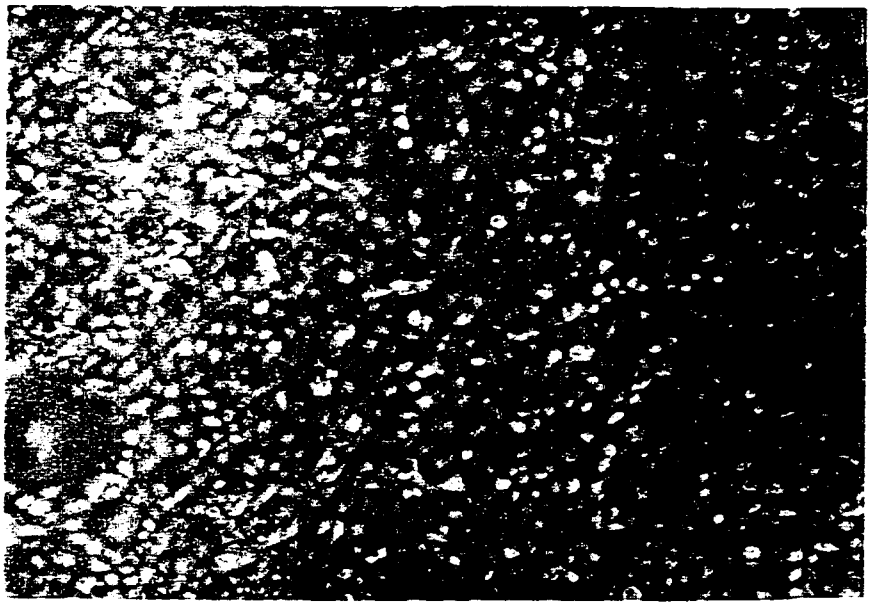


Figure 8 – Cultured COS-1 cells
a) 24 hours post passage (30% Confluent)
b) 48 hours post passage (Confluent culture)

As the realization that cell health was directly correlated with successful transfections, the procedure for growth and maintenance of the COS-1 cell cultures was

changed. Hardy, healthy cell cultures were obtained when the medium was replaced daily. Each passage, new flasks would be seeded with 3 x 10^6 cells and allowed to grow for 48 hours before another passage. These COS-1 flasks would be 30% confluent 24 hours post-passage (Figure 8a), and fully confluent 48 hours post-passage (Figure 8b). For the LipofectAMINE™ transfections between 750,000 and 1.25x10^6 cells were placed in each 60mm x15mm tissue culture plates and allowed to grow overnight. These plates would be between 60% confluent and 80% confluent by the time of the transfection.

Transfections

Transfections were completed for SEMA7A, SEMA3A, and SEMA4D using both the calcium phosphate precipitation method described in Current Protocols and the LipofectAMINE™ transfection method (Gibco/BRL). After many months of transfections first using the calcium phosphate transfection method (Current Protocols, 1990) and then comparing it with the LipofectAMINE™ transfection, the LipofectAMINE™ transfections were ultimately used to synthesize the semaphorin protein used in the Boyden chamber.

Due to the increased protein yield, decreased requirement of DNA, and the ease of the LipofectAMINE™ protocol when compared to the calcium phosphate precipitation, LipofectAMINE™ was the primary means of transfection.

Immunoblotting

Dot Blot

The dot blot analyses showed darkly stained AP developed purple dots on the membrane where the extracts had been loaded. However, the negative control or the

extract of the mock transfected COS-1 cell showed a high background signal. The mock transfected cell were only exposed to the LipofectAMINE™ treatment without the addition of pEX.mh/insert pDNA. This meant that although there were strong alkaline phosphatase developed dots where the extracts were place, there was an indiscernible difference to the naked eye between the color of the dots of the experimental samples and the negative control. Because the dots developed so darkly, testing proceeded with the Western analysis in hopes of visualizing the synthesized semaphorin proteins in the crude extract.

Western Analysis

The Western analysis of the crude protein extract was completed to identify the sized of the *myc* tag expressing proteins. When the SDS-PAGE gradient gel was stained with Coomassie blue stain post-transfer, a high concentration of COS-1 cellular protein could be identified in each well. In fact the protein content was so high in each well that the lanes were darkly stained smears from the 210 kD marker to the 15 kD marker. If the extracts had been frozen or not used immediately, then the molecular weight of the heaviest protein was reduced to approximately 120 kD and the concentration of the lower weight proteins was increased as a result of protease activity. The expected sizes of the desired proteins were approximately 72 kD for SEMA7A, 90 kD for SEMA3A, and 85 kD for SEMA4D.

With the verification of the Ponceau stain, protein was confirmed on the nitrocellulose membrane after the transfer. However, none of the nitrocellulose membranes developed in this masters thesis project showed alkaline phosphatase positive

bands. This includes the lack of development of the positive control, which was a concentrated c-myc tagged SEMAIII protein extract donated by Exelixis. The reasons for the failure of this assay in this project are hypothesized in the Discussion section. Despite the failure of the Western analysis, the Boyden chamber assays were completed because the success of the transfections was clearly indicated by the β-galactosidase positive transfection control.

β-galactosidase Assay

β-gal Extract	Undiluted	1:10	1:100	1:1,000	1:10,000
6/18/99	12.6 *	1.142	0.126	0.013	.006
6/17/99	13.3 *	1.344	0.133	0.014	.003
6/16/99	12.22 *	1.222	0.122	0.013	.009

Table 5 – β-galactosidase activity measured at O.D. 460 on 3 LipofectAMINE™ extracts after 15 minute incubation at room temperature (* Calculated Values exceeded Spectrophotometer measuring capabilities)

β-gal Extract	Undiluted	1:10	1:100	1:1,000	1:10,000
6/18/99	67.4 *	6.74 *	0.674	0.020	-0.045
6/17/99	74.9 *	7.49 *	0.749	0.031	-0.047
6/16/99	68.8 *	6.88 *	0.688	0.022	-0.023

Table 6 – β-galactosidase activity measured at O.D. 460 on 3 LipofectAMINE™ extracts after 2 hour incubation in a 37 °C / 5% CO_2 incubator. (* Calculated Values exceeded Spectrophotometer measuring capabilities)

For each day of a transfection, a β-galactosidase positive control was run. Before the semaphorin extracts were used, the extract of this positive control was tested in the β-galactosidase assay and read at 460nm in a BIO-TEK Instruments Microplate Autoreader EC 31. Even in successful calcium phosphate precipitation transfections, the β-galactosidase activity of COS-1 cells transfected with 30 μg of DNA could not be detected unless the β-galactosidase assay was incubated for 15 - 30 minutes in a 37°C

incubator. However, the β-galactosidase activity of COS-1 cells transfected with 1/15th the amount of DNA (2 μg) using the LipofectAMINE ™ transfection protocol was detected within 5 minute of the extract being added to the assay reagent mix at room temperature. As a result, the LipofectAMINE ™ transfections were used instead of the calcium phosphate precipitation method.

For every culture plate transfected with the β-galactosidase pDNA using the LipofectAMINE™ assay in this project, the extracts were uniformly positive giving the same β-galactosidase expressions at the same rate. Serial dilutions of three β-galactosidase transfected COS-1 cell extracts were run to determine the concentration of extract needed to detect β-galactosidase activity.

Table 5 and 6 show the consistency of the extracts of the 3 separate LipofectAMINE™ transfections performed with 2 μg β-galactosidase pDNA. Table 5 shows the 450 nm reading of differing dilutions of the β-galactosidase activity of three extracts after 15 mintues incubation at room temperature. Table 6 shows the 450 nm reading of differing dilutions of β-galactosidase activity of three extracts after 2 hours.

Boyden Chamber Analysis

After the Boyden chamber filters were stained and affixed to a microscope slide, they were viewed on a Leitz –Wetzlar SM-LX compound microscope (Germany). Under the 40x objective, a neutrophil nucleus is dark purple and the cytoplasm ranges from pale pink to pale purple. As seen in figure 9, these cells' darkly stained lobed nuclei make them easy to identified and count.

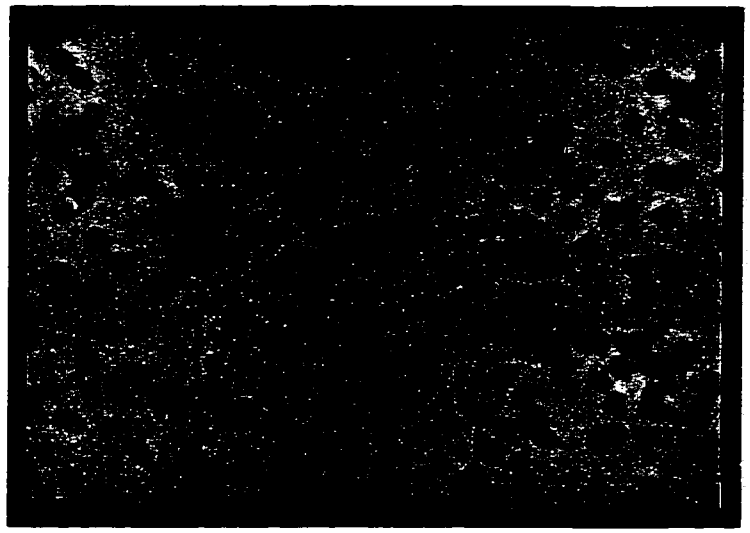


Figure 9a – Boyden Chamber Negative Control - Lower side of filter after staining. The Boyden chamber negative control consists of neutrophils placed in Boyden chamber with only medium. Dark Purple Dots = Stained Neutrophil Nuclei. Clear Core Spots = 5 μm pores in the polyvinylpyrrolidone-free polycarbonate Boyden chamber filter.

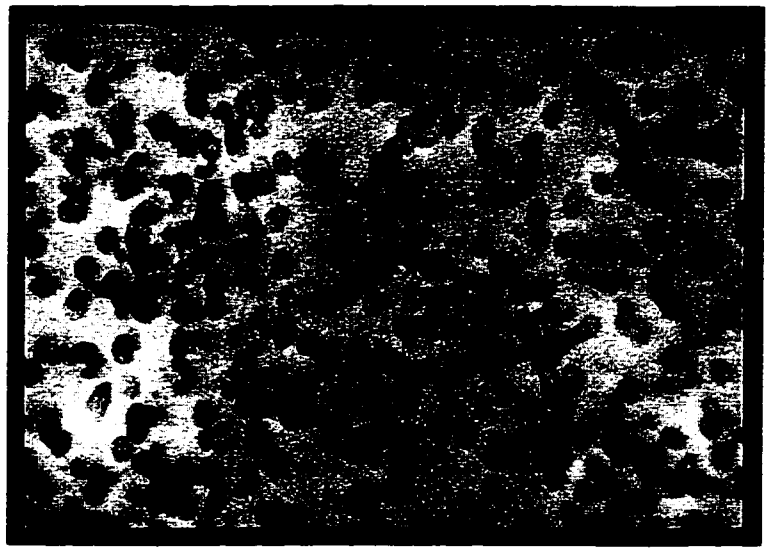


Figure 9b – Boyden chamber positive control - Lower side of Boyden chamber filter after staining – Neutrophils place in Boyden chamber with 10^{-7} mM fMLP in lower chamber. Dark Purple Dots = Stained Neutrophil Nuclei. Clear Core Spots = 5 μm pores in the polyvinylpyrrolidone-free polycarbonate Boyden chamber filter.

When the neutrophils are placed in a Boyden chamber with only medium in the upper and lower chambers, they move randomly via chemokinesis such that a few cells cross the filter (Figure 9a). However, when the neutrophils are placed in a Boyden

chamber with a strong positive chemoattractant like fMLP in the lower well, the cells move toward the chemoattractant in a directed motion which allows a significant number to cross the filter (Figure 9b).

Because of the strong response neutrophils have to fMLP, fMLP was used as a positive control to verify the motility of the tested neutrophils and the successful use of the Boyden chamber chemotaxis plate. When the filter of the positive wells had less than 60 neutrophils /field crossing the filter, the entire run of 48 chambers on the chemotaxis plate was rejected and redone. Ultimately, the data from four runs or 192 Boyden chamber assays were discarded due to a failure of the positive control.

The failure of the four plates was later found to be a result of TERG-A-ZYME™ (Alconox, Inc. NY) detergent residue remaining on the plates after washing. Due to the extremely small volume (25 ul) of the lower well, fluid was not easily rinsed from these wells. As a result, the detergent residue remained and the enzymatic action of the detergent affected the ability of neutrophils to adhere to the lower side of the filter.

To prevent this difficulty, the washing procedures for the Boyden Chamber plate became more stringent. The plate was first rinsed with deionized water in a beaker. Then, it was rinsed thoroughly with millipore water from a squirt bottle. This water was then removed with the vacuum system, and the chamber was re-rinsed five times with the millipore water from the squirt bottle. This washing procedure removed the excess detergent residue and prevented the failure of subsequent Boyden chamber assay runs.

Experimental Boyden chamber assay runs

Nineteen hundred and twenty Boyden chamber assays were completed in order to test the effect of SEMA4D, SEMA7A, and SEMA3A on human neutrophils. Using the checkerboard assay template shown in Table 2, each of the 16 different semaphorin environments were tested in 36 separate wells. Five random 40x fields of neutrophils were counted per well of each of these filters. This analysis yielded a total of 180 separate fields counted for each different semaphorin concentration gradient shown in the checkerboard assay template (Table 2).

To minimize the variation expected among different individuals, the same four volunteers were used for each semaphorin tested. This decreased the statistical variance calculated for the raw data so the statistical significance of the neutrophils' response to SEMA4D, SEMA7A, and SEMA3A could be detected more accurately. Ultimately, the checkerboard analysis allowed for discrimination among possible chemoattraction, chemorepulsion, and chemokinetic effects of SEMA4D, SEMA7A, and SEMA3A.

Measurement of Chemoattraction

The checkerboard analysis system tested for both the direct chemoattraction effect of the semaphorins (Figure 10a) and the effect of a lesser chemoattraction semaphorin gradient on human neutrophils (Figure 10b). The direct chemoattraction effect was measured when the neutrophils mixed with dilute mock extract were loaded into the upper well and varying dilutions of semaphorin secreting COS-1 cell extract were loaded in the bottom well. In this test, the neutrophils were only exposed to the semaphorins when they enter and pass through the filter.

The effect of the chemoattraction gradient was measured when the neutrophils were mixed with a more dilute concentration of semaphorin secreting COS-1 cell extract than was loaded into the lower well. In this circumstance, the neutrophils were exposed to semaphorin protein in both wells. However, these Boyden chamber assays tested the effect of a less steep chemoattractive semaphorin gradient on neutrophils.

Direct Chemoattraction effect of SEMA4D, SEMA7A and SEMA3A

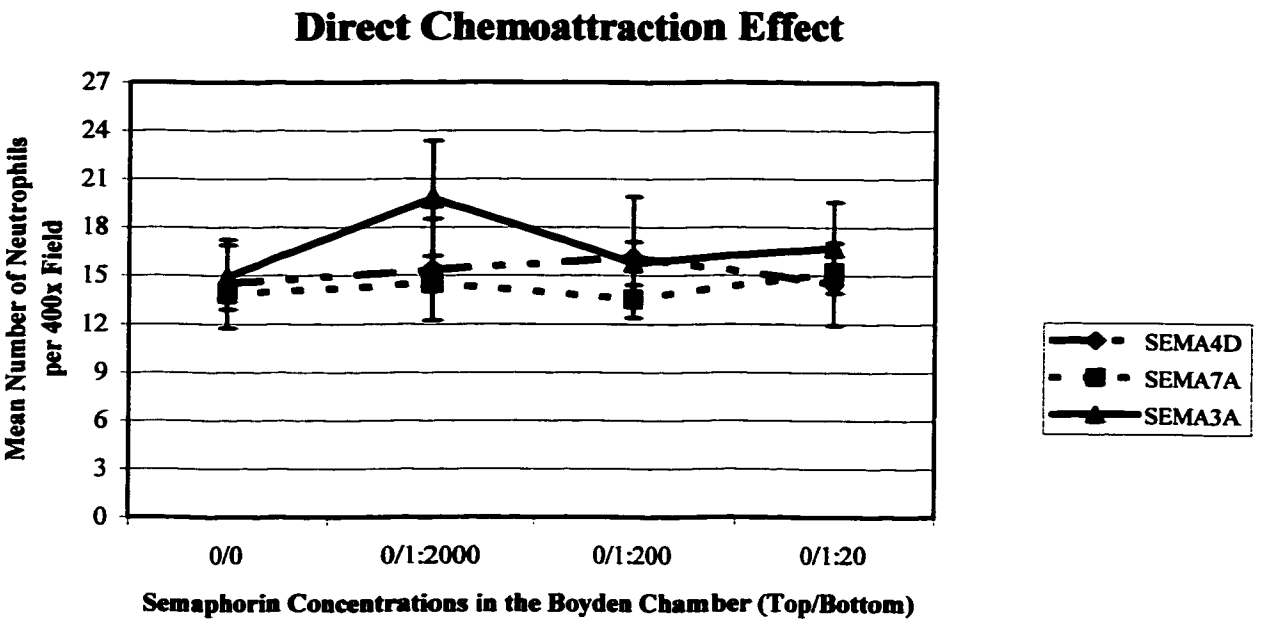


Figure 10a – Direct chemoattraction effect of SEMA4D, SEMA7A, and SEMA3A
Each colored line represents the combined mean value of all the experimental repetitions of the four subjects tested for the each different semaphorin. The vertical bars represent the standard error of the means.

The mean number of neutrophils that crossed the filter in a 400x field in wells 1,5,9 and 13 of Table 2 were statistically analyzed with a repeated measures ANOVA with blocks for each of the tested semaphorins. In this analysis there was no significance found in the different semaphorin concentrations investigated for any of the tested semaphorins when compared to the negative control. As a result, SEMA4D, SEMA7A,

and SEMA3A were shown to not act as a chemoattractant in the direct chemoattraction set-ups of the Boyden chambers.

Chemoattraction gradient effect of SEMA4D, SEMA7A and SEMA3A

The mean number of neutrophils that crossed the filter per 400x field in wells 1,7,8, and 12 of Table 2 were statistically analyzed with a repeated measures ANOVA with blocks for each of the tested semaphorins. In this analysis no significant difference was found in the positive gradient concentrations tested. The data does not, therefore, suggest that SEMA4D, SEMA7A, and SEMA3A act as a chemoattractant in those assays.

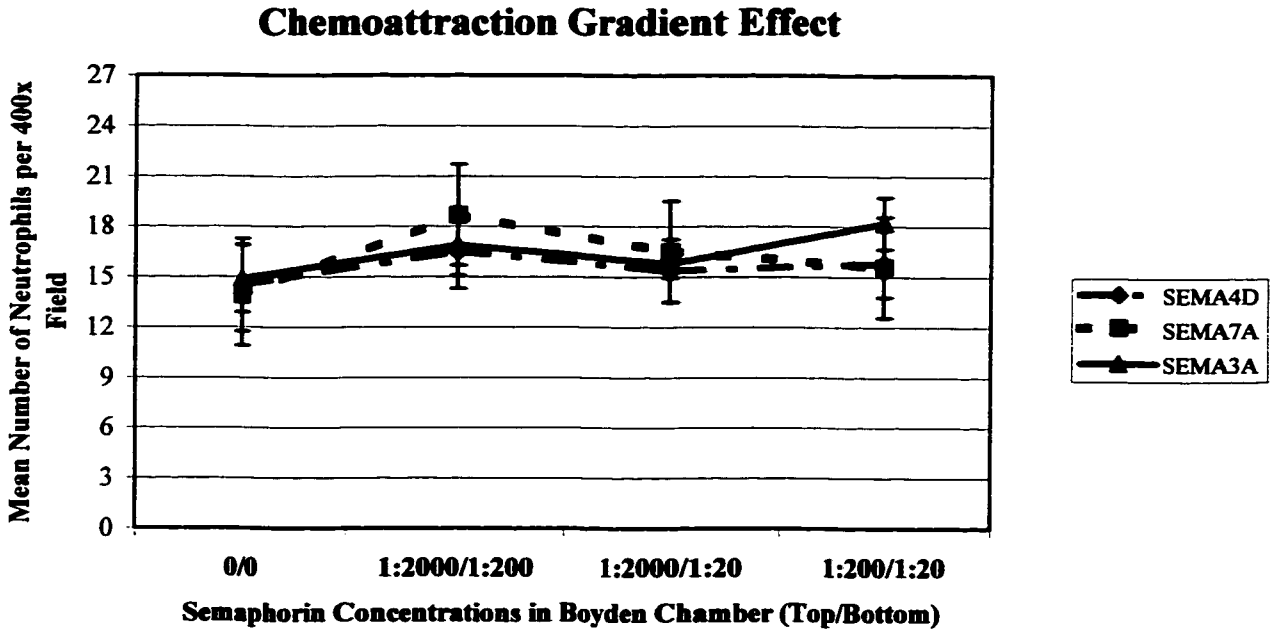


Figure 10b – Effect of a chemoattractive gradient of SEMA4D, SEMA7A and SEMA3A on human neutrophils Each colored line represents the combined mean value of all of the experimental repetitions of the four subjects for each of the respective semaphorins. The vertical bars represent the standard error of the means.

Measurement of Chemorepulsion

The checkerboard analysis system tested for both the direct chemorepulsion effect of the semaphorins (Figure 10a) and the effect of a less steep chemorepulsion semaphorin gradient on human neutrophils (Figure 10b). The direct chemorepulsion effect was measured when the neutrophils mixed with varying dilutions of semaphorin secreting COS-1 cell extracts were loaded into the upper well and varying dilutions of mock transfected COS-1 cell extracts were loaded in the bottom well. In this test, the neutrophils were only exposed to the semaphorins in the upper chamber and when they passed through the filter they entered a relatively semaphorin free environment

The effect of the chemorepulsion gradient was measured when the neutrophils were mixed with a more concentrated extract of semaphorin secreting COS-1 cells than was loaded into the lower well. In this circumstance, the neutrophils were exposed to semaphorin protein in both wells. However, these Boyden chamber assays tested the effect of a less steep chemorepulsive semaphorin gradient on neutrophils.

Direct Chemorepellent Effect

The mean number of neutrophils that crossed the filter per 400x field in wells 1,2,3, and 4 of Table 2 were analyzed with a repeated measures ANOVA with blocks for each of the tested semaphorins. In this analysis, no significant difference was found in the quantity of neutrophils that crossed the filter as a result of the direct negative semaphorin gradients for both SEMA4D and SEMA7A (See Figure 11a).

SEMA3A, however, did show a significant chemorepulsive effect in the Boyden chambers that had a 1:20 dilution of SEMA3A extract and a 1:200 dilution of SEMA3A

extract ($p = 0.02$) in the upper chamber. The data showed a mean increase of 50% in the number of neutrophils that crossed the filter.

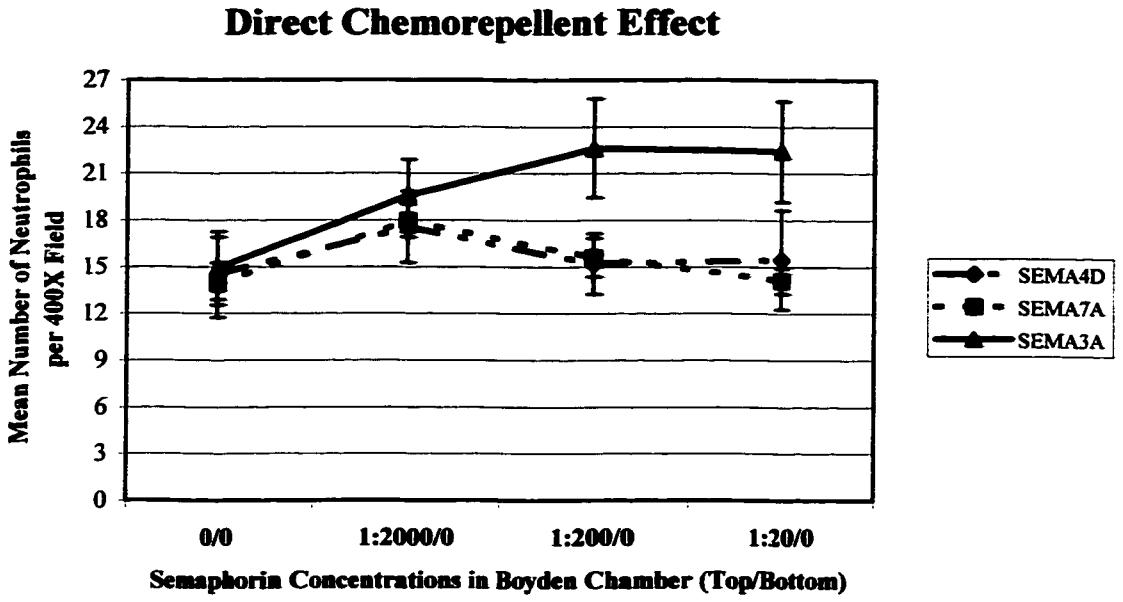


Figure 11a – The direct chemorepellent effect of SEMA4D, SEMA7A and SEMA3A on human neutrophils- Each colored line represents the combined mean value of all the experimental repetitions of the four subjects for each of the respective semaphorins. The vertical bars represent the standard error of the means.

The Boyden chamber with the 1:2000 dilution of the extract of SEMA3A, also, showed a significant change over the negative control ($p < 0.05$). In this chamber, there was a 30% increase of neutrophils crossing the filter.

Chemorepulsion gradient effect of SEMA4D, SEMA7A and SEMA3A

The mean number of neutrophils that crossed the filter in a 400x field in wells 1,10,14 and 15 of Table 2 were statistically analyzed with a repeated measures ANOVA with blocks for each of the tested semaphorins. In this analysis, there was no significant difference in the quantity of neutrophils that crossed the filter as a result of the less steep negative semaphorin gradients for both SEMA4D and SEMA7A.

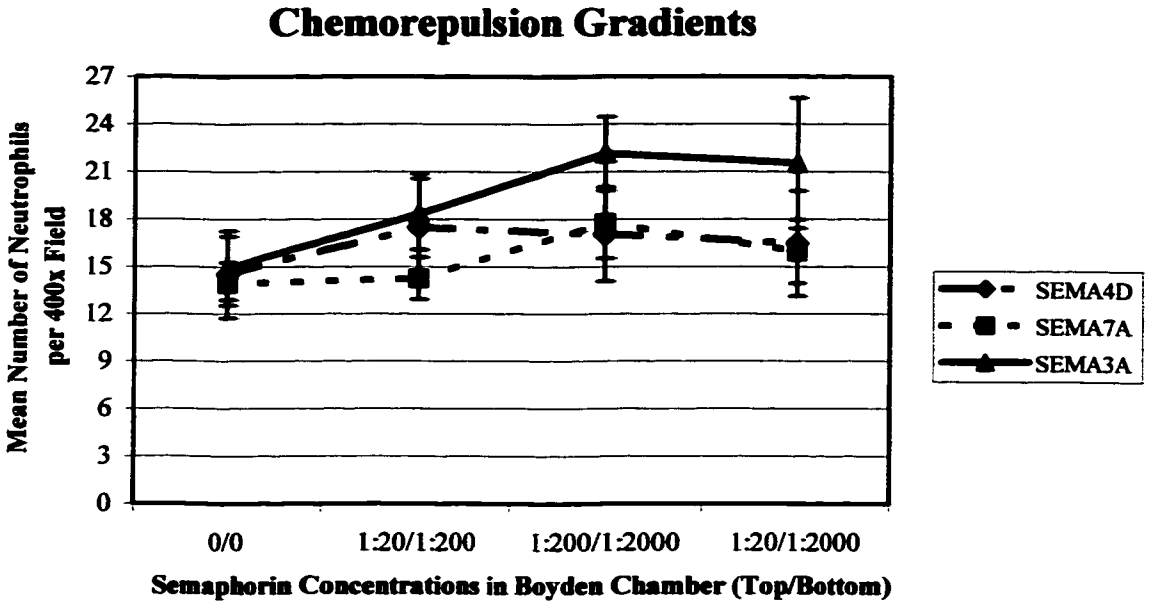


Figure 11b – The effect of a chemorepulsive gradient of SEMA4D, SEMA7A and SEMA3A on human neutrophils- Each colored line represents the combined mean value of all the experimental repetitions of the four subjects for each of the respective semaphorins. The vertical bars represent the standard error of the means.

SEMA3A, however, did show a significant chemorepulsive effect in the Boyden chamber with the following chemorepulsive gradients: (1) 1:20 dilution in the upper chamber and 1:2000 dilution in the lower chamber; (2) 1:200 dilution in the upper chamber and 1:2000 dilution in the lower chamber ($p < 0.05$). The data for the 1:20/1:2000 Boyden chamber showed a mean increase in the number of neutrophils that crossed the filter of 44% when compared to the negative control. The data for the 1:200/1:2000 Boyden chamber showed a mean increase of 49% when compared to the negative control.

The 1:20/1:200 Boyden chamber also showed an increase in the mean for the number of neutrophils that crossed the filter over the negative control. However the variance of the data causes the difference in means not to be statistically significant.

Chemokinetic Effect of SEMA4D, SEMA7A, and SEMA3A

The mean number of neutrophils that crossed the filter in a 40x field in wells 1,6, and 11 of Table 2 were statistically analyzed with a repeated measures ANOVA with blocks for each of the tested semaphorins. In this analysis, there was no significance in the time the assays were completed nor was there a significant difference in the quantity of neutrophils that crossed the filter as a result of the chemokinetic gradients for any of the tested semaphorins. As a result, SEMA4D, SEMA7A, and SEMA3A were not shown to alter the random movement of neutrophils in the Boyden chamber apparatus.

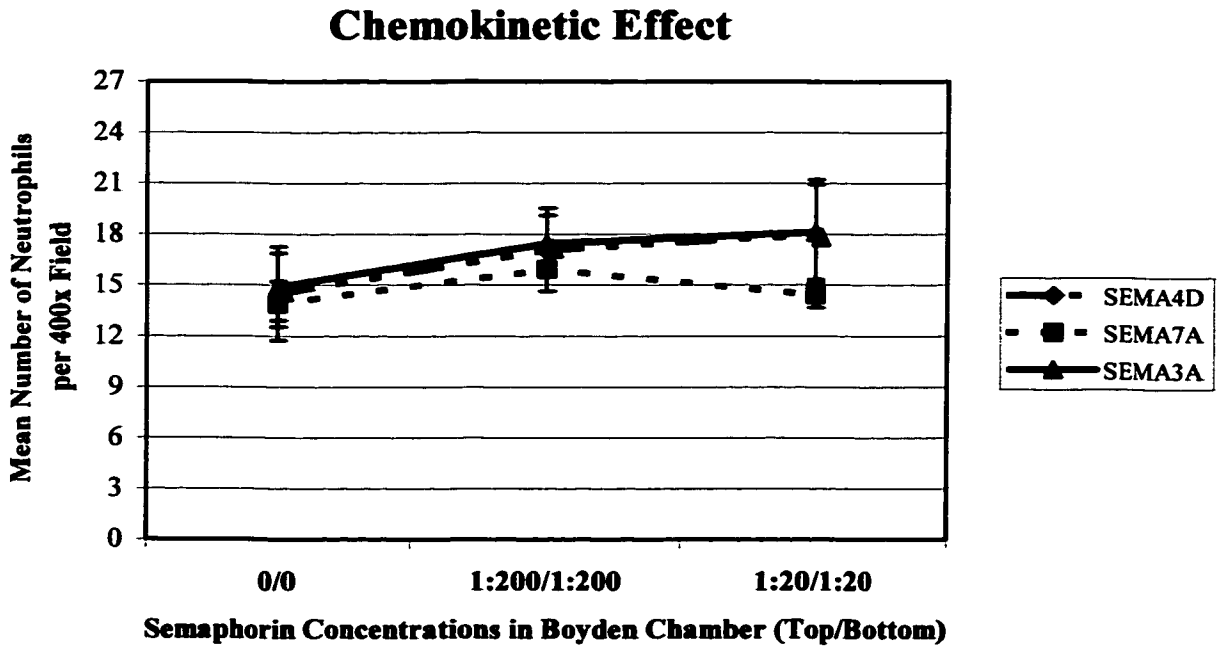


Figure 12 – The chemokinetic effect of SEMA4D, SEMA7A, and SEMA3A- Each colored line represents the combined mean value of all of the experimental repetitions of the four subjects for each of the respective semaphorins. The vertical bars represent the standard error

DISCUSSION

The focus of this masters thesis project was to show the effect SEMA7A, SEMA3A, and SEMA4D had on human neutrophil migration. The first phase of the project verified the sequences of the semaphorin/pEX.mh molecular constructs for SEMA7A, SEMA3A, and SEMA4D through sequencing and restriction digests. In the second phase, the respective semaphorin proteins were synthesized by transiently transfected COS-1 cells. Finally, the extracts of the transiently transfected COS-1 cells were tested for their chemotactic activity using the Boyden chamber assay and checkerboard analysis. This design tested for the chemoattraction, chemorepulsion, and chemokinetic effects of the SEMA7A, SEMA3A, and SEMA4D.

Effects of SEMA4D and SEMA7A on Neutrophils

The results of these assays clearly showed that SEMA7A and SEMA4D had no chemotactic effect on neutrophils. The repeated measures ANOVA analysis and graphs of the SEMA7A and SEMA4D data showed that there was no significant change over concentration or time in any of the Boyden chamber environments tested.

This lack of neutrophil responsiveness could have been the result of several factors. The first is that neutrophils may not have receptors for SEMA7A and SEMA4D. Researchers have so far identified two semaphorin receptors, neuropilin-1 and neuropilin-2, in axons (Fugisawa et al., 1997; Chen, Chedotal, He, Goodmaric & Tessier-Lavigne, 1997). However, researchers have neither identified the semaphorin receptors nor the cells in the immune system that express those receptors. As a result, future studies may

find that neutrophils do not have the receptors that make them sensitive to SEMA7A and SEMA4D.

The second factor may have been that this project did not use purified protein. Although the success of the transfections was determined by a successful β-galactosidase assay, the amount of secreted SEMA4D and SEMA 7A was not quantified. As a result, the amount of secreted SEMA4D and/or SEMA7A might not have been high enough to elicit a neutrophil response.

The third factor that might explain the lack of neutrophil response to SEMA7A and SEMA4D is high degree of variance found in the raw data obtained in the Boyden chamber assay. A detection method with less (within-individual) variance or one with a greater range of response might reveal neutrophil response to SEMA4D and SEMA7A.

A fourth possible explanation as to the failure of SEMA4D and SEMA7A to stimulate a chemotactic response in neutrophils could be missing co-factors. The Boyden chamber assay is an *in vitro* test. As with any *in vitro* study, it does not recreate the complexity of the *in vivo* environment. Co-factors such as chemokines or extracellular matrix proteins might be needed for the cellular response of neutrophils to SEMA4D and SEMA7A. If these necessary cofactors were present in the Boyden chamber assay, then neutrophils might show a significant response to these semaphorins.

Finally, the negative result for SEMA4D and SEMA7A could indicate that semaphorins have cell specificity. Researchers have described many populations of neurons that are non-responsive to the powerful neural chemorepellent SEMA3A (Luo et al., 1993, Messersmith et al., 1995; Puschel et al., 1995; Shepherd, Luo, Raper & Chang,

1996; Varela-Echavarria, Tucker, Puschel & Guthrie, 1997; Kobayashi et al., 1997). The same cellular specificity may, also, occur in the semaphorins of the immune system. SEMA4D has been identified as a membrane bound protein in lymphocytes (Hall et al., 1996). SEMA7A has been isolated from numerous tissues throughout the body including the lymph nodes (Xu et al., 1998). Even though SEMA7A and SEMA4D have been found to function in lymphocytes of the immune system, it is possible that the functions of these two semaphorins do not extend to chemotaxis of neutrophils.

SEMA3A's Role as Chemorepellent for Neutrophils

The significant finding of this project was the discovery that SEMA3A acts as a chemorepellent to human neutrophils. In five of the six SEMA3A chemorepellent environments, neutrophils show a statistically significant increase in their movement. The sixth Boyden chamber chemorepellent gradient environment, 1:20/1:200, combined the two concentrations of SEMA3A that seemed to elicit the maximum chemorepellent response in the other assays. Interestingly enough, the graphs of both the direct chemorepellent effect and the chemorepellent gradients seem to show a binding curve where the receptors on the neutrophils saturate and the neutrophils cannot be repelled to any greater extent at an extract dilution of 1:200.

This finding supports the published data describing SEMA3A as a strong chemorepellent. However, SEMA3A has been, primarily, shown to be a chemorepellent to specific subsets of neurites throughout the body. Only recently has a role of SEMA3A in the immune system been introduced. An abstract published in the proceedings of the 1999 FASEB national meeting reported that SEMA3A inhibited the migration of B cells

and monocytes (Boumsell, Delaire, Chedotal & Bensussan, 1999). This masters thesis is the first to report that SEMA3A acts as a chemorepellent to human neutrophils.

In vivo immune function of SEMA3A

Since the chemotactic role of SEMA3A was tested in an *in vitro* assay, the function of SEMA3A in the immune system can only be hypothesized to be similar. The structure of SEMA3A is that of the secreted protein with a sema domain, immunoglobulin domain and a chain of positively charged, basic amino acids. Researchers believe that the positively charged, basic amino acids might be loosely and reversibly bound to the surface of the cell membrane (Kolodkin et al., 1993). These loosely bound semaphorins might act as a dispersive signal to neutrophils at the end of an infection or inflammation. An interstitial gradient created by cells secreting SEMA3A could serve as a sign that those cells are no longer under attack by invading pathogens.

Another possible function of SEMA3A could be that the cellular secretion of this semaphorin may be an indicator of overall cell health. A research project looking into the expression of SEMA3A in distressed or dying cells might find that the SEMA3A secretion could be decreased or terminated before death. The cessation of the SEMA3A secretion would allow neutrophils to approach the dying cell and to remove it from the system.

Modifications in future SEMA3A research

The major need for future studies of SEMA3A in the immune system is purified protein. The main detraction of using the cellular extracts of the COS-1 cells secreting semaphorin was the high proportion of "junk" or cellular proteins in the extract.

Although the negative controls used the diluted mock cellular extract, a more precise analysis of the magnitude of the chemorepellent effect of SEMA3A could be identified with the purified protein. Another limitation of the data obtained with the COS-1 extracts was the variance between chambers, days, and people. If the semaphorin had been produced in high enough quantities to be purified, then some of this variance might have been reduced.

Another possibility for making a more precise determination of the chemorepellent effect would be to use another assay system. The Boyden chamber is a fairly crude piece of equipment relative to today's technology. Although factors are in place to minimize some of problems, the Boyden chamber yields precision that is limited by the person counting the cells. The manual counting of the cells is in many cases very subjective. By making a set of rules such as to count purple dots (full nuclei) only and not purple rings (partial nuclei), one may increase the precision of the assay. However, because of the inherent variation in the assay itself, the quantity of data that needs to be collected to obtain a significant measure is large and the gathering of this data can be very tedious. Other methods of assessing chemotaxis such as viewing the changes in morphology of cells fixed with gluteraldehyde or observing a single cell's movement and behavior in a gradient of chemorepellent with video photography allow for a more objective analysis without the tedium of counting tens of thousands of fields with hundreds of thousands of cells (Haston & Sheilds, 1985; Zicha et al. 1997).

Another limitation of this project was the failure to obtain a strongly positive western blot. Although the success of the transfections was indicated by the β-

galactosidase positive control, an indisputable demonstration of protein secretion would have been a positive western blot using either the *myc* or *poly-his* tag coded for in the pEX.mh molecular construct. A western would have not only detected the presence of a *myc* or *poly-his* tagged protein, but it would have, also, shown the size of the protein.

The primary reason for the failure of the western blots in this project probably has to do with human error or failure of the reagents. For every western blot run there was a positive and negative control. In the early stages of this research, the transfections were unsuccessful due to cell death. However, the westerns run during this time always yielded a band correctly identifying the positive control. However, just at the point where the transfections were succeeding the reagents had to be replaced. New nitrocellulose and new primary antibody had to be re-ordered. In addition, the positive control that had worked previously had to be replaced. After, these replacements were made, the positive control band was no longer found on the alkaline phosphatase developed nitrocellulose. Despite repeated attempts varying multiple factors, a positive western blot could not be obtained.

Another factor that might have caused the failure of the western blots could have been if the semaphorin in the COS-1 cell extracts were too dilute to transfer the minimum of 100 pg to the nitrocellulose membrane required for successful development. In the future, protein purification could eliminate this problem. Commercially produced kits that purify protein using the *poly-his* tag on the secreted protein are available through biotechnology companies such as Qiagen™ (Carlsbad, CA).

COS-1 cell extract chemokinetic inhibitor?

A curious observations made from this research was the apparent trend of an increase in the mean number of neutrophils which crossed the filter in those Boyden chambers which had extremely low total protein concentration in their upper and lower wells. The wells with the 0/1:2000 dilution in their top and bottom well respectively (Figure 10a) or which had 1:2000/0 dilution in their top and bottom well respectively (Figure 11b) showed a noticeable increase in the neutrophil chemotaxis. All three semaphorins in Figure 11a and the SEMA3A point in figure 10a show an increasing trend in the mean number of neutrophils that crossed the filter in these wells when compared to the negative control. Although this increase is not statistically significant, this increase could be the result of the extremely small amount of COS-1 cell protein in the wells.

If this project were to continue, or if the Boyden chamber were to be used again, a test of the chemokinetic effect of the mock transiently transfected COS-1 cell extracts should be performed. This could be done by comparing the number of neutrophils that cross the filter in serial dilutions of the mock cell extract with the number of neutrophils that cross the filter with simply RPMI 1640 medium containing 10% fetal calf serum. It is possible that the COS-1 extracts may serve to decrease the chemokinetic activity of human neutrophils.

Future directions for basic and clinical research

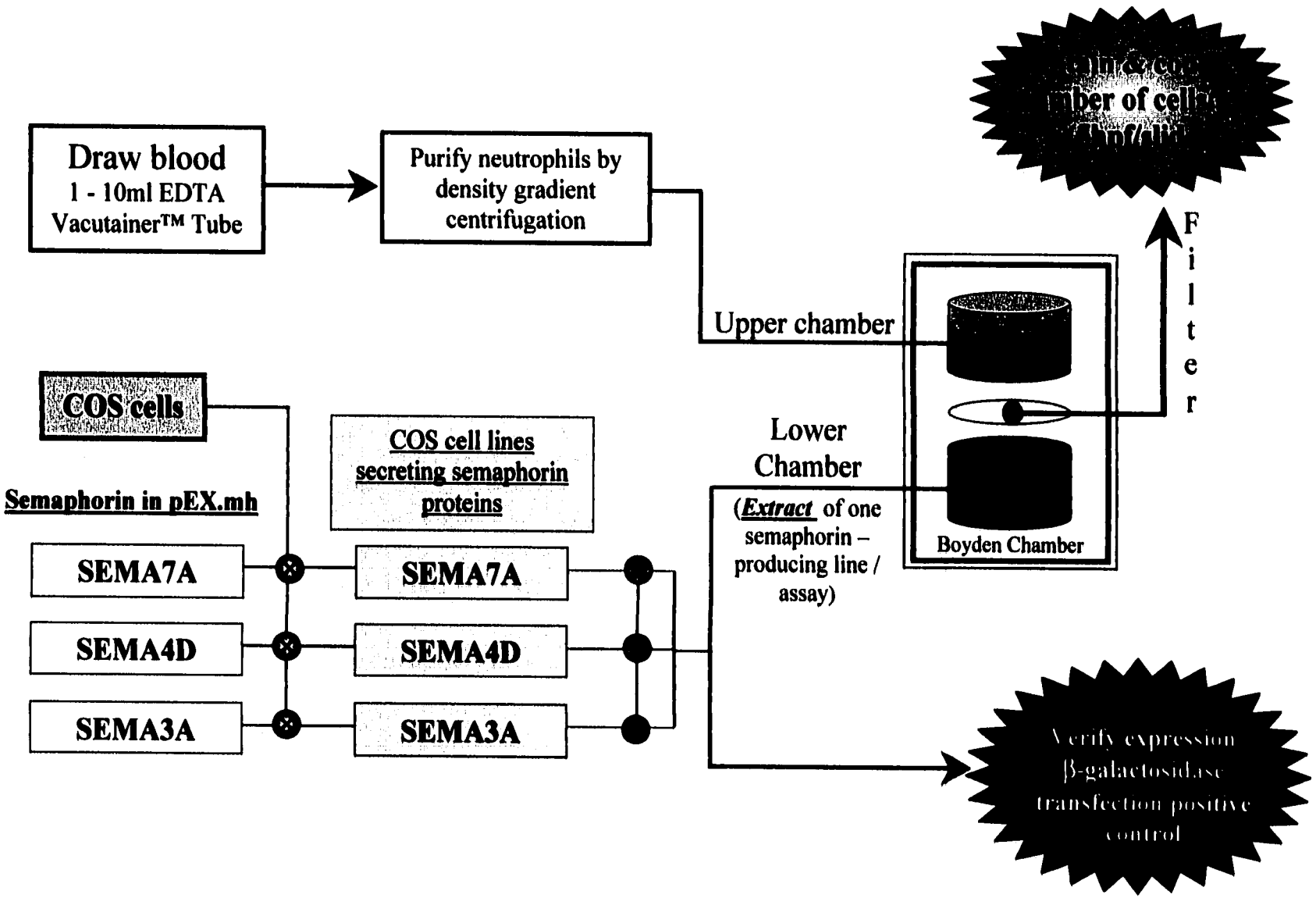
The discovery of a significant neutrophil chemorepellent function for SEMA3A has reaching implications for basic medical research. Studies could be undertaken to determine the signal transduction pathway of SEMA3A in neutrophils. Research might

also be done to investigate possible semaphorin inhibitors, in addition, to isolating and classifying unknown cell surface receptors on human neutrophils. Neuropilin I was recently identified as an axonal receptor for SEMA3A (He & Tessier-Lavigne, 1997). Another interesting question to investigate is whether neuropilin I is the SEMA3A receptor on human neutrophils or if another protein acts as a receptor on neutrophils.

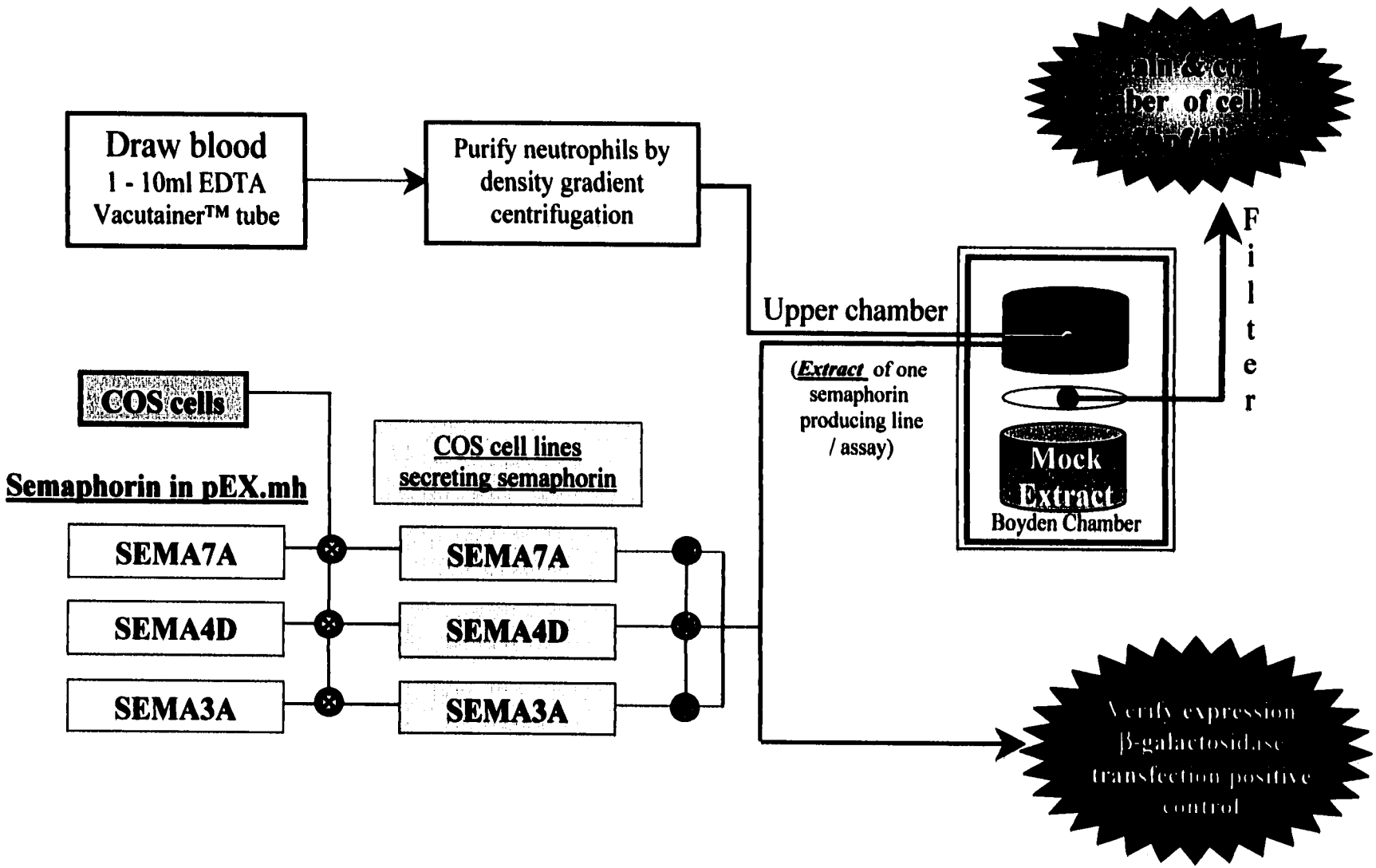
In addition to the future directions in basic science, clinical applications for semaphorins also present themselves. The newly discovered function of SEMA3A as a chemorepellent to human neutrophils could ultimately lead to its use as a possible anti-inflammatory agent. Other neutrophil chemorepellents such as α-melanocyte stimulating hormone (α-MSH) have been shown to decrease inflammation in multiple areas including the brain (Lipton & Cantina, 1998). In addition to its possible use as an anti-inflammatory, SEMA3A might serve to limit the effects of autoimmune disease. By repelling neutrophils from joints and organs, SEMA3A could limit the tissue damage caused by neutrophils in these areas. Further study of the function of SEMA3A in the immune system, and the further identification of the roles of SEMA7A, and SEMA4D in the body is required.

Appendix 1a and Appendix 1b

EXPERIMENT 1.A - MEASUREMENT OF NEUTROPHIL CHEMOTAXIS WITH EXTRACT OF SEMAPHORIN-SECRETING COS CELLS IN BOYDEN CHAMBERS - CHEMOATTRACTION ASSAY



EXPERIMENT 1.B - MEASUREMENT OF NEUTROPHIL CHEMOTAXIS WITH EXTRACT OF SEMAPHORIN-SECRETING COS CELLS IN BOYDEN CHAMBERS - CHEMOREPULSION ASSAY



Appendix 2a – Translated DNA Sequence of the SEMA4D insert in pEX.mh vector using an alignment of the sequencing data and the published Genbank Sequence (HSU60800).

```
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CCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCT - 120
 H  C  L  L  A  Y  R  N  *  Y  D  S  L  *  G  D  P  S  W  L
AGCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTC - 180
 A  T  M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S
CACTGGTGACGCGGCCCAGCCGGCCAGGCGCGCCGTACGAAGCTTGTTTGCACCCATACC - 240
 T  G  D  A  A  Q  P  A  R  R  A  V  R  S  L  F  A  P  I  P
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CAACTACTCAGCCTTGCTGCTGAGCGAGGACAAGGACACCTTGTACATAGGTGCCCGGGA - 360
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GGCGGTCTTCGCTGTGAACGCACTCAACATCTCCGAGAAGCAGCATGAGGTGTATTGGAA - 420
 A  V  F  A  V  N  A  L  N  I  S  E  K  Q  H  E  V  Y  W  K
GGTCTCAGAAGACAAAAAAGCAAAATGTGCAGAAAAGGGGAAATCAAAACAGACAGAGTG - 480
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CCTCAACTACATCCGGGTGCTGCAGCCACTCAGCGCCACTTCCCTTTACGTGTGTGGGAC - 540
 L  N  Y  I  R  V  L  Q  P  L  S  A  T  S  L  Y  V  C  G  T
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 N  E  D  G  K  G  R  C  P  F  D  P  A  H  S  Y  T  S  V  M
GGTTGATGGAGAACTTTATTCGGGGACGTCGTATAATTTTTTGGGAAGTGAACCCATCAT - 720
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CTCCCGAAATTCTTCCCACAGTCCTCTGAGGACAGAATATGCAATCCCTTGGCTGAACGA - 780
 S  R  N  S  S  H  S  P  L  R  T  E  Y  A  I  P  W  L  N  E
GCCTAGTTTCGTGTTTGCTGACGTGATCCGAAAAAGCCCAGACAGCCCCGACGGCGAGGA - 840
 P  S  F  V  F  A  D  V  I  R  K  S  P  D  S  P  D  G  E  D
TGACAGGGTCTACTTCTTCTTCACGGAGGTGTCTGTGGAGTATGAGTTTGTGTTCAGGGT - 900
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GCTGATCCCACGGATAGCAAGAGTGTGCAAGGGGGACCAGGGCGGCCTGAGGACCTTGCA - 960
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 K  K  W  T  S  F  L  K  A  R  L  I  C  S  R  P  D  S  G  L
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GGAGCAGTCCCACACCAAGTGGGTGCGCTATAATGGCCCGGTACCCAAGCCGCGGCCTGG - 1260
 E  Q  S  H  T  K  W  V  R  Y  N  G  P  V  P  K  P  R  P  G
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 A  C  I  D  S  E  A  R  A  A  N  Y  T  S  S  L  N  L  P  D
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CAACAGGCCCAGGTTAATCAAGAAAGATGTGAACTACACCCAGATCGTGGTGGACCGGAC - 1440
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```

```
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GCACAAAGCCATCAGCCTCGAGCACGCTGTTCACATCATCGAGGAGACCCAGCTCTTCCA - 1560
 H  K  A  I  S  L  E  H  A  V  H  I  I  E  E  T  Q  L  F  Q
GGACTTTGAGCCAGTCCAGACCCTGCTGCTGTCTTCAAAGAAGGGCAACAGGTTTGTCTA - 1620
 D  F  E  P  V  Q  T  L  L  L  S  S  K  K  G  N  R  F  V  Y
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CTGCGTGGCTCTGCACCAGACCGAGAGCCCCAGCAGGGGTTTGATTCAGGAGATGAGCGG - 1800
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 D  A  S  V  C  P  D  K  S  K  G  S  Y  R  Q  H  F  F  K  H
CGGTGGCACAGCGGAACTGAAATGCTCCCAAAAATCCAACCTGGCCCGGGTCTTTTGGAA - 1920
 G  G  T  A  E  L  K  C  S  Q  K  S  N  L  A  R  V  F  W  K
GTTCCAGAATGGCGTGTTGAAGGCCGAGAGCCCCAAGTACGGTCTTATGGGCAGAAAAAA - 1980
 F  Q  N  G  V  L  K  A  E  S  P  K  Y  G  L  M  G  R  K  N
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 K  S  S  D  S  R  G  P  E  Q  K  L  I  S  E  E  D  L  N  S
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```

Appendix 2b: Translated protein sequence of SEMA4D coded for in the pEX.mh construct (**construct protein**) compared to the Genbank SEMA4D (HSU60800).

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SEDKDTLYIGAREAVFAVNALNISEKQHEVYWKVSEDKKAKCAEKGKSKQT

ECLNYIRVLQPLSATSLYVCGTNAFQPACDHLNLTSFKFLGKNEDGKGRCPF

DPAHSYTSVMVDGELYSGTSYNFLGSEPIISRNSSHSPLRTEYAIPWLNEPSFV

FADVIRKSPDSPDGEDDRVYFFFTEVSVEYEFVFRVLIPRIARVCKGDQGGLR

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NNVGLSAVCAYNLSTAEEVFSHGKYMQSTTVEQSHTKWVRYNGPVPKPRP

GACIDSEARAANYTSSLNLPDKTLQFVKDHPLMDDSVTPIDNRPRLIKKDVN

YTQIVVDRTQALDGTVYDVMFVSTDRGALHKAISLEHAVHIIEETQLFQDFE

PVQTLLLSSKKGNRFVYAGSNSGVVQAPLAFCGKHGTCEDCVLARDPYCA

WSPPTATCVALHQTESPSRGLIQEMSGDASVCPDKSKGSYRQHFFKHGGTA

ELKCSQKSNLARVFWKFQNGVLKAESPKYGLMGRKNLLIFNLSEGDSGVYQ

CLSEERVKNKTVFQVVAKHVLEVKVVPKPVVAPTLSVVQTEGSRIATKVLV

ASTQGSSPPTPAVQATSSGAITLPPKPAPTGTSCEPKIVINTVPQLHSEKTMYL

KSSDNRLLMSLFLFFFVLFLCLFFYNCYKGYLPRQCLKFRSALLIGKKKPKS

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Appendix 3a- Translated DNA sequence of the SEMA3A insert in the pEX.mh vector using an alignment of the sequencing data and the published Genbank Sequence (L26081).

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GAAGAACAATGTGCCAAGGCTGAAATTATCCTACAAAGAAATGTTGGAATCCAACAATGT - 300
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 I  G  H  H  P  E  D  N  I  F  K  L  E  N  S  H  F  E  N  G
CCGTGGGAAGAGTCCATATGACCCTAAGCTGCTGACAGCATCCCTTTTAATAGATGGAGA - 720
 R  G  K  S  P  Y  D  P  K  L  L  T  A  S  L  L  I  D  G  E
ATTATACTCTGGAACTGCAGCTGATTTTATGGGGCGAGACTTTGCTATCTTCCGAACTCT - 780
 L  Y  S  G  T  A  A  D  F  M  G  R  D  F  A  I  F  R  T  L
TGGGCACCACCACCCAATCAGGACAGAGCAGCATGATTCCAGGTGGCTCAATGATCCAAA - 840
 G  H  H  H  P  I  R  T  E  Q  H  D  S  R  W  L  N  D  P  K
GTTCATTAGTGCCCACCTCATCTCAGAGAGTGACAATCCTGAAGATGACAAAGTATACTT - 900
 F  I  S  A  H  L  I  S  E  S  D  N  P  E  D  D  K  V  Y  F
TTTCTTCCGTGAAAATGCAATAGATGGAGAACACTCTGGAAAAGCTACTCACGCTAGAAT - 960
 F  F  R  E  N  A  I  D  G  E  H  S  G  K  A  T  H  A  R  I
AGGTCAGATATGCAAGAATGACTTTGGAGGGCACAGAAGTCTGGTGAATAAATGGACAAC - 1020
 G  Q  I  C  K  N  D  F  G  G  H  R  S  L  V  N  K  W  T  T
ATTCCTCAAAGCTCGTCTGATTTGCTCAGTGCCAGGTCCAAATGGCATTGACACTCATTT - 1080
 F  L  K  A  R  L  I  C  S  V  P  G  P  N  G  I  D  T  H  F
TGATGAACTGCAGGATGTATTCCTAATGAACTTTAAAGATCCTAAAAATCCAGTTGTATA - 1140
 D  E  L  Q  D  V  F  L  M  N  F  K  D  P  K  N  P  V  V  Y
TGGAGTGTTTACGACTTCCAGTAACATTTTCAAGGGATCAGCCGTGTGTATGTATAGCAT - 1200
 G  V  F  T  T  S  S  N  I  F  K  G  S  A  V  C  M  Y  S  M
GAGTGATGTGAGAAGGGTGTTCCTTGGTCCATATGCCCACAGGGATGGACCCAACTATCA - 1260
 S  D  V  R  R  V  F  L  G  P  Y  A  H  R  D  G  P  N  Y  Q
ATGGGTGCCTTATCAAGGAAGAGTCCCCTATCCACGGCCAGGAACTTGTCCCAGCAAAAC - 1320
 W  V  P  Y  Q  G  R  V  P  Y  P  R  P  G  T  C  P  S  K  T
```

```
ATTTGGTGGTTTTGACTCTACAAAGGACCTTCCTGATGATGTTATAACCTTTGCAAGAAG - 1380
 F  G  G  F  D  S  T  K  D  L  P  D  D  V  I  T  F  A  R  S
TCATCCAGCCATGTACAATCCAGTGTTTCCTATGAACAATCGCCCAATAGTGATCAAAAC - 1440
 H  P  A  M  Y  N  P  V  F  P  M  N  N  R  P  I  V  I  K  T
GGATGTAAATTATCAATTTACACAAATTGTCGTAGACCGAGTGGATGCAGAAGATGGACA - 1500
 D  V  N  Y  Q  F  T  Q  I  V  V  D  R  V  D  A  E  D  G  Q
GTATGATGTTATGTTTATCGGAACAGATGTTGGGACCGTTCTTAAAGTAGTTTCAATTCC - 1560
 Y  D  V  M  F  I  G  T  D  V  G  T  V  L  K  V  V  S  I  P
TAAGGAGACTTGGTATGATTTAGAAGAGGTTCTGCTGGAAGAAATGACAGTTTTTCGGGA - 1620
 K  E  T  W  Y  D  L  E  E  V  L  L  E  E  M  T  V  F  R  E
ACCGACTGCTATTTCAGCAATGGAGCTTTCCACTAAGCAGCAACAACTATATATTGGTTC - 1680
 P  T  A  I  S  A  M  E  L  S  T  K  Q  Q  Q  L  Y  I  G  S
AACGGCTGGGGTTGCCCAGCTCCCTTTACACCGGTGTGATATTTACGGGAAAGCGTGTGC - 1740
 T  A  G  V  A  Q  L  P  L  H  R  C  D  I  Y  G  K  A  C  A
TGAGTGTTGCCTCGCCCGAGACCCTTACTGTGCTTGGGATGGTTCTGCATGTTCTCGCTA - 1800
 E  C  C  L  A  R  D  P  Y  C  A  W  D  G  S  A  C  S  R  Y
TTTTCCCACTGCAAAGAGACGCACAAGACGACAAGATATAAGAAATGGAGACCCACTGAC - 1860
 F  P  T  A  K  R  R  T  R  R  Q  D  I  R  N  G  D  P  L  T
TCACTGTTCAGACTTACACCATGATAATCACCATGGCCACAGCCCTGAAGAGAGAATCAT - 1920
 H  C  S  D  L  H  H  D  N  H  H  G  H  S  P  E  E  R  I  I
CTATGGTGTAGAGAATAGTAGCACATTTTTGGAATGCAGTCCGAAGTCGCAGAGAGCGCT - 1980
 Y  G  V  E  N  S  S  T  F  L  E  C  S  P  K  S  Q  R  A  L
GGTCTATTGGCAATTCCAGAGGCGAAATGAAGAGCGAAAAGAAGAGATCAGAGTGGATGA - 2040
 V  Y  W  Q  F  Q  R  R  N  E  E  R  K  E  E  I  R  V  D  D
TCATATCATCAGGACAGATCAAGGCCTTCTGCTACGTAGTCTACAACAGAAGGATTCAGG - 2100
 H  I  I  R  T  D  Q  G  L  L  L  R  S  L  Q  Q  K  D  S  G
CAATTACCTCTGCCATGCGGTGGAACATGGGTTCATACAAACTCTTCTTAAGGTAACCCT - 2160
 N  Y  L  C  H  A  V  E  H  G  F  I  Q  T  L  L  K  V  T  L
GGAAGTCATTGACACAGAGCATTTGGAAGAACTTCTTCATAAAGATGATGATGGAGATGG - 2220
 E  V  I  D  T  E  H  L  E  E  L  L  H  K  D  D  D  G  D  G
CTCTAAGACCAAAGAAATGTCCAATAGCATGACACCTAGCCAGAAGGTCTGGTACAGAGA - 2280
 S  K  T  K  E  M  S  N  S  M  T  P  S  Q  K  V  W  Y  R  D
CTTCATGCAGCTCATCAACCACCCCAATCTCAACACGATGGATGAGTTCTGTGAACAAGT - 2340
 F  M  Q  L  I  N  H  P  N  L  N  T  M  D  E  F  C  E  Q  V
TTGGAAAAGGGACCGAAAACAACGTCGGCAAAGGCCAGGACATACCCCAGGGAACAGTAA - 2400
 W  K  R  D  R  K  Q  R  R  Q  R  P  G  H  T  P  G  N  S  N
CAAATGGAAGCACTTACAAGAAAATAAGAAAGGTAGAAACAGGAGGACCCACGAATTTGA - 2460
 K  W  K  H  L  Q  E  N  K  K  G  R  N  R  R  T  H  E  F  E
GAGGGCACCCAGGAGTGTCGGGCCCGAACAAAAACTCATCTCAGAAGAGGATCTGAATAG - 2520
 R  A  P  R  S  V  G  P  E  Q  K  L  I  S  E  E  D  L  N  S
CGCCGTCGACCATCATCATCATCATTGAGTTTAAACCGCTGATCAGCTCGAATGGGC - 2580
 A  V  D  H  H  H  H  H  H  *  V  *  T  A  D  Q  L  E  W  A
```

Appendix 3b- Translated protein sequence of SEMA3A coded for in the pEX.mh construct (**construct protein**) compared to the Genbank SEMEA3A sequence (L26081)

MGWLTRIVCLFWGVLLTARANYQNGKNNVPRLKLSYKEMLESNNVITFNGL

ANSSSYHTFLLDEERSRLYVGAKDHIFSFDLVNIKDFQKIVWPVSYTRRDECK

WAGKDILKECANFIKVLKAYNQTHLYACGTGAFHPICTYIEIGHHPEDNIFK

LENSHFENGRGKSPYDPKLLTASLLIDGELYSGTAADFMGRDFAIFRTLGHH

HPIRTEQHDSRWLNDPKFISAHLISESDNPEDDKVYFFFRENAIDGEHSGKAT

HARIGQICKNDFGGHRSLVNKWTTFLKARLICSVPGPNGIDTHFDELQDVFL

MNFKDPKNPVVYGVFTTSSNIFKGSAVCMYSMSDVRRVFLGPYAHRDGPNY

QWVPYQGRVPYPRPGTCPSKTFGGFDSTKDLPDDVITFARSHPAMYNPVFP

MNNRPIVIKTDVNYQFTQIVVDRVDAEDGQYDVMFIGTDVGTVLKVVSIPKE

TWYDLEEVLLEEMTVFRPTAISAMELSTKQQQLYIGSTAGVAQLPLHRCDIY

GKACAECCLARDPYCAWDGSACSRYFPTAKRRTRRQDIRNGDPLTHCSDLH

HDNHHGHSPEERIIYGVENSSTFLECSPKSQRALVYWQFQRRNEERKEEIRV

DDHIIRTDQGLLLRSLQQKDSGNYLCHAVEHGFIQTLLKVTLEVIDTEHLEE

LLHKDDDGDGSKTKEMSNSMTPSQKVWYRDFMQLINHPNLNTMDEFCEQV

WKRDRKQRRQRPGHTPGNSNKWKHLQENKKGRNRRTHEFERAPRSV

Appendix 4a- Translated DNA sequence of the SEMA7A insert in the pEX.mh vector using an alignment the sequencing data and the published Genbank sequence (AF017542).

```
AGAGTGTGCTTTGACGCGTGGGAGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAAC - 60
 E  C  A  L  T  R  G  S  L  Y  K  Q  S  S  L  A  N  *  R  T
CCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCT - 120
 H  C  L  L  A  Y  R  N  *  Y  D  S  L  *  G  D  P  S  W  L
AGCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTC - 180
 A  T  M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S
CACTGGTGACGCGGCCC AGCCGGCCAGGCGCGCCGTACGAAGCTTACACCTAAGGAGCGG - 240
 T  G  D  A  A  Q  P  A  R  R  A  V  R  S  L  H  L  R  S  G
ACCCCGCATCTTCGCCGTCTGGAAAGGCCATGTAGGGCAGGACCGGGTGGACTTTGGCCA - 300
 P  R  I  F  A  V  W  K  G  H  V  G  Q  D  R  V  D  F  G  Q
GACTGAGCCGCACACGGTGCTTTTCCACGAGCCAGGCAGCTCCTCTGTGTGGGTGGGAGG - 360
 T  E  P  H  T  V  L  F  H  E  P  G  S  S  S  V  W  V  G  G
ACGTGGCAAGGTCTACCTCTTTGACTTCCCCGAGGGCAAGAACGCATCTGTGCGCACGGT - 420
 R  G  K  V  Y  L  F  D  F  P  E  G  K  N  A  S  V  R  T  V
GAATATCGGCTCCACAAAGGGGTCCTGTCTGGATAAGCGGGACTGCGAGAACTACATCAC - 480
 N  I  G  S  T  K  G  S  C  L  D  K  R  D  C  E  N  Y  I  T
TCTCCTGGAGAGGCGGAGTGAGGGGCTGCTGGCCTGTGGCACCAACGCCCGGCACCCCAG - 540
 L  L  E  R  R  S  E  G  L  L  A  C  G  T  N  A  R  H  P  S
CTGCTGGAACCTGGTGAATGGCACTGTGGTGCCACTTGGCGAGATGAGAGGCTACGCCCC - 600
 C  W  N  L  V  N  G  T  V  V  P  L  G  E  M  R  G  Y  A  P
CTTCAGCCCGGACGAGAACTCCCTGGTTCTGTTTGAAGGGGACGAGGTGTATTCCACCAT - 660
 F  S  P  D  E  N  S  L  V  L  F  E  G  D  E  V  Y  S  T  I
CCGGAAGCAGGAATACAATGGGAAGATCCCTCGGTTCCGCCGCATCCGGGGCGAGAGTGA - 720
 R  K  Q  E  Y  N  G  K  I  P  R  F  R  R  I  R  G  E  S  E
GCTGTACACCAGTGATACTGTCATGCAGAACCCACAGTTCATCAAAGCCACCATCGTGCA - 780
 L  Y  T  S  D  T  V  M  Q  N  P  Q  F  I  K  A  T  I  V  H
CCAAGACCAGGCTTACGATGACAAGATCTACTACTTCTTCCGAGAGGACAATCCTGACAA - 840
 Q  D  Q  A  Y  D  D  K  I  Y  Y  F  F  R  E  D  N  P  D  K
GAATCCTGAGGCTCCTCTCAATGTGTCCCGTGTGGCCCAGTTGTGCAGGGGGGACCAGGG - 900
 N  P  E  A  P  L  N  V  S  R  V  A  Q  L  C  R  G  D  Q  G
TGGGGAAAGTTCACTGTCAGTCTCCAAGTGGAACACTTTTCTGAAAGCCATGCTGGTATG - 960
 G  E  S  S  L  S  V  S  K  W  N  T  F  L  K  A  M  L  V  C
CAGTGATGCTGCCACCAACAAGAACTTCAACAGGCTGCAAGACGTCTTCCTGCTCCCTGA - 1020
 S  D  A  A  T  N  K  N  F  N  R  L  Q  D  V  F  L  L  P  D
CCCCAGCGGCCAGTGGAGGGACACCAGGGTCTATGGTGTTTTCTCCAACCCCTGGAACTA - 1080
 P  S  G  Q  W  R  D  T  R  V  Y  G  V  F  S  N  P  W  N  Y
CTCAGCCGTCTGTGTGTATTCCCTCGGTGACATTGACAAGGTCTTCCGTACCTCCTCACT - 1140
 S  A  V  C  V  Y  S  L  G  D  I  D  K  V  F  R  T  S  S  L
CAAGGGCTACCACTCAAGCCTTCCCAACCCGCGGCCTGGCAAGTGCCTCCCAGACCAGCA - 1200
 K  G  Y  H  S  S  L  P  N  P  R  P  G  K  C  L  P  D  Q  Q
GCCGATACCCACAGAGACCTTCCAGGTGGCTGACCGTCACCCAGAGGTGGCGCAGAGGGT - 1260
 P  I  P  T  E  T  F  Q  V  A  D  R  H  P  E  V  A  Q  R  V
GGAGCCCATGGGGCCTCTGAAGACGCCATTGTTCCACTCTAAATACCACTACCAGAAAGT - 1320
 E  P  M  G  P  L  K  T  P  L  F  H  S  K  Y  H  Y  Q  K  V
GGCCGTCCACCGCATGCAAGCCAGCCACGGGGAGACCTTTCATGTGCTTTACCTAACTAC - 1380
 A  V  H  R  M  Q  A  S  H  G  E  T  F  H  V  L  Y  L  T  T
AGACAGGGGCACTATCCACAAGGTGGTGGAACCGGGGGAGCAGGAGCACAGCTTCGCCTT - 1440
 D  R  G  T  I  H  K  V  V  E  P  G  E  Q  E  H  S  F  A  F
```

```
CAACATCATGGAGATCCAGCCCTTCCGCCGCGCGGCTGCCATCCAGACCATGTCGCTGGA - 1500
 N  I  M  E  I  Q  P  F  R  R  A  A  A  I  Q  T  M  S  L  D
TGCTGAGCGGAGGAAGCTGTATGTGAGCTCCCAGTGGGAGGTGAGCCAGGTGCCCCTGGA - 1560
 A  E  R  R  K  L  Y  V  S  S  Q  W  E  V  S  Q  V  P  L  D
CCTGTGTGAGGTCTATGGCGGGGGCTGCCACGGTTGCCTCATGTCCCGAGACCCCTACTG - 1620
 L  C  E  V  Y  G  G  G  C  H  G  C  L  M  S  R  D  P  Y  C
CGGCTGGGACCAAGGCCGCTGCATCTCCATCTACAGCTCCGAACGGTCAGTGCTGCAATC - 1680
 G  W  D  Q  G  R  C  I  S  I  Y  S  S  E  R  S  V  L  Q  S
CATTAATCCAGCCGAGCCACACAAGGAGTGTCCCAACCCCAAACCAGACAAGGCCCCACT - 1740
 I  N  P  A  E  P  H  K  E  C  P  N  P  K  P  D  K  A  P  L
GCAGAAGGTTTCCCTGGCCCCAAACTCTCGCTACTACCTGAGCTGCCCCATGGAATCCCG - 1800
 Q  K  V  S  L  A  P  N  S  R  Y  Y  L  S  C  P  M  E  S  R
CCACGCCACCTACTCATGGCGCCACAAGGAGAACGTGGAGCAGAGCTGCGAACCTGGTCA - 1860
 H  A  T  Y  S  W  R  H  K  E  N  V  E  Q  S  C  E  P  G  H
CCAGAGCCCCAACTGCATCCTGTTCATCGAGAACCTCACGGCGCAGCAGTACGGCCACTA - 1920
 Q  S  P  N  C  I  L  F  I  E  N  L  T  A  Q  Q  Y  G  H  Y
CTTCTGCGAGGCCCAGGAGGGCTCCTACTTCCGCGAGGCTCAGCACTGGCAGCTGCTGCC - 1980
 F  C  E  A  Q  E  G  S  Y  F  R  E  A  Q  H  W  Q  L  L  P
CGAGGACGGCATCATGGCCGAGCACCTGCTGTCTAGAGGGCCCGAACAAAAACTCATCTC - 2040
 E  D  G  I  M  A  E  H  L  L  S  R  G  P  E  Q  K  L  I  S
AGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTAAACCGC - 2100
 E  E  D  L  N  S  A  V  D  H  H  H  H  H  H  *  V  *  T  A
TGATCAGCTCGAATGGGCCCCAA - 2123
 D  Q  L
```

Appendix 4b- Translated protein sequence of SEMA7A coded for in the pEX.mh construct (**construct protein**) compared to the Genbank SEMA7A sequence (AF17542)

MTPPPPGRAAPSAPRARVPGPPARLGLPLRLRLLLLLWAAAASAQG**HLRSGPRI**

FAVWKGHVGQDRVDFGQTEPHTVLFHEPGSSSVWVGGRGKVYLFDFPEGK

NASVRTVNIGSTKGSCLDKRDCENYITLLERRSEGLLACGTNARHPSCWNLV

NGTVVPLGEMRGYAPFSPDENSLVLFEGDEVYSTIRKQEYNGKIPRFRRIRG

ESELYTSDTVMQNPQFIKATIVHQDQAYDDKIYYFFREDNPDKNPEAPLNVS

RVAQLCRGDQGGESSLSVSKWNTFLKAMLVCSDAATNKNFNRLQDVFLLP

DPSGQWRDTRVYGVFSNPWNYSAVCVYSLGDIDKVFRTSSLKGYHSSLPNPR

PGKCLPDQQPIPTETFQVADRHPEVAQRVEPMGPLKTPLFHSKYHYQKVAV

HRMQASHGETFHVLYLTTDRGTIHKVVEPGEQEHSFAFNIMEIQPFRRAAAI

QTMSLDAERRKLYVSSQWEVSQVPLDLCEVYGGGCHGCLMSRDPYCGWD

QGRCISIYSSERSVLQSINPAEPHKECPNPKPDKAPLQKVSLAPNSRYYLSCP

MESRHATYSWRHKENVEQSCEPGHQSPNCILFIENLTAQQYGHYFCEAQ

Appendix 5

Agreement to Participate in Research

a) Darcy Levy

b) Victoria Wu

c) Kristen French

d) Andrea French

Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

6. The results of this study may be published but no information that could identify the subject will be included.

7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 9244872. Complaints about the research may be presented to the Biology Department Chair, Lee Dorosz (408) 924-2429. Questions or complaints about the research, subjects rights, or research-related injury may be presented to Serena Stanford, Ph.D, Associate Academic Vice President for Graduate Studies and research(408) 924-2480.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to the subjects relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

A signature of a subject on this docLiment indicates agreement to participate in the study.

The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

Darcy J Lewis — Subject's Signature — 5/31/99 — Date

Andrea L Benech — Investigator's Signature — 5/31/99 — Date

Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

6. The results of this study may be published but no information that could identify the subject will be included.

7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 9244872. Complaints about the research may be presented to the Biology Department Chair, Lee Dorosz (408) 924-2429. Questions or complaints about the research, subjects rights, or research-related injury may be presented to Serena Stanford, Ph.D, Associate Academic Vice President for Graduate Studies and research(408) 924-2480.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to the subjects relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

A signature of a subject on this document indicates agreement to participate in the study.

The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

Subject's Signature — Date 6/8/99

Investigator's Signature — Date 6/8/99

Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

6. The results of this study may be published but no information that could identify the subject will be included.

7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 9244872. Complaints about the research may be presented to the Biology Department Chair, Lee Dorosz (408) 924-2429. Questions or complaints about the research, subjects rights, or research-related injury may be presented to Serena Stanford, Ph.D, Associate Academic Vice President for Graduate Studies and research(408) 924-2480.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to the subjects relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

A signature of a subject on this document indicates agreement to participate in the study.

The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

Kristen French — Subject's Signature
July 9, 1999 — Date

Andrea L. French — Investigator's Signature
7/9/99 — Date

Agreement to Participate in Research

Responsible Investigator: Dr. David J. Matthes
Protocol: Blood draw for cell fractionation and testing of blood cell behavior in response to treatment with test compounds.

1. I have been asked to participate in a research study investigating the effect of recombinant Semaphorins on white blood cells.

2. I will provide 30 milliliters (or amount I specify) of blood to be drawn by a licensed phlebotomist using a syringe needle and Vacuutainer. Standard blood drawing and handling procedures will be followed.

3. There may be slight discomfort upon sticking with the needle. There is a possibility of hematoma formation (bleeding under skin) resulting from the blood draw.

4. There are no benefits to me consequent to participation in this study aside from the feeling of having contributed to a research program.

5. No alternative procedures will be done.

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7. There will be no compensation for participation in this study.

8. Questions about the research may be addressed to the principal investigator at San Jose State University, David Matthes (408) 9244872. Complaints about the research may be presented to the Biology Department Chair, Lee Dorosz (408) 924-2429. Questions or complaints about the research, subjects rights, or research-related injury may be presented to Serena Stanford, Ph.D. Associate Academic Vice President for Graduate Studi[illegible] and research(408) 924-2480.

9. No service of any kind, to which one is otherwise entitled, will be lost or jeopardized if I choose not to participate in the study.

10. Consent is given voluntarily. I may refuse to participate in the study or in any part of the study. If I decide to participate in the study, I am still free to withdraw at any time without prejudice to the subjects relation to San Jose University or any other participating institution.

11. I have received a signed and dated copy of the consent form.

A signature of a subject on this document indicates agreement to participate in the study.

The signature of a researcher on this document indicates agreements to include the above named subject in the research and attestation that the subject has been fully informed of his or her rights.

Andrea L. Stench — Subject's Signature — Date 6/8/99

Andrea L. Stench — Investigator's Signature — Date 6/8/99

Appendix 6

San Jose State University Subjects Institutional Review Board Permission Letter

San José State
UNIVERSITY

Office of the Academic Vice President
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FROM: Nabil Ibrahim,
AVP, Graduate Studies & Research

DATE: December 7, 1999

The Human Subjects-Institutional Review Board has approved your request to use human subjects in the study entitled:

"The Chemotropic Effect of SEMA7A, SEMA4D, and SEMA3A on Human Neutrophils"

This approval is contingent upon the subjects participating in your research project being appropriately protected from risk. This includes the protection of the anonymity of the subjects' identity when they participate in your research project, and with regard to any and all data that may be collected from the subjects. The Board's approval includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Nabil Ibrahim, Ph.D., immediately. Injury includes but is not limited to bodily harm, psychological trauma and release of potentially damaging personal information.

Please also be advised that all subjects need to be fully informed and aware that their participation in your research project is voluntary, and that he or she may withdraw from the project at any time. Further, a subject's participation, refusal to participate, or withdrawal will not affect any services the subject is receiving or will receive at the institution in which the research is being conducted.

If you have any questions, please contact me at (408) 924-2480.

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