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In situ analysis of lymphoid expression of murine semaphorins 4A, 4D, 4F & 7A

Sreedevi Chalasani
San Jose State University

NOTE TO USERS

In Situ Analysis of Lymphoid Expression of Murine Semaphorins 4A, 4D, 4F, & 7A

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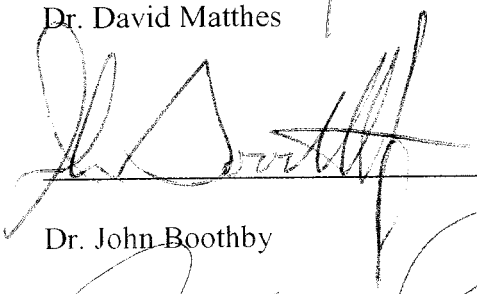
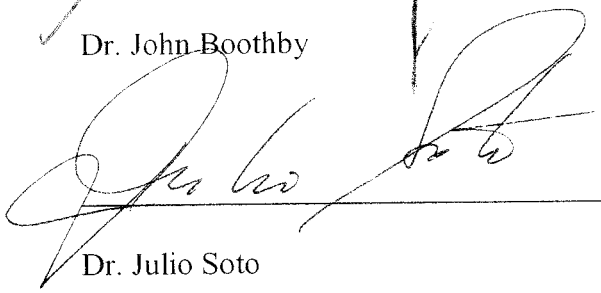
Dr. David Matthes



Dr. John Boothby



Dr. Julio Soto

APPROVED FOR THE UNIVERSITY

ABSTRACT

In Situ Analysis of Lymphoid Expression of Murine Semaphorins 4A, 4D, 4F, & 7A

by Sreedevi Chalasani

The semaphorin gene family encodes proteins that function as neural patterning and axon guidance molecules in the developing nervous system and that appear to have important functions outside of nervous system. The lymphoid expression and potential functions of Sema4A, Sema4D (CD100) and Sema7A (CDw108) have been partially characterized by others. Here we provide a detailed analysis of the lymphoid expression of Sema4F expressed in lymphoid tissues along with Sema4A, Sema4D, and Sema7A by *in situ* analysis. Our hypothesis is that Sema4A, 4D, 4F, and 7A might act as chemotropic agents and, along with other chemotactic and adhesion molecules, regulate the movement of T and B lymphocytes. The results of our analysis show the localization of Sema4A, 4D, 4F, and 7A to specific tissue microenvironments of thymus, spleen, and lymph nodes in mice. Our data combined with the published literature on the functions of semaphorins in immune system support the hypothesis.

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INTRODUCTION

Semaphorins were first identified in grasshoppers[1] with homologues subsequently being found in *Drosophila* and in vertebrates.[2] Semaphorins are characterized by highly conserved motifs within a 500 amino acid semaphorin domain. All the members of the family contain a signal sequence and are either transmembrane or secreted proteins. The secreted semaphorins contain a single Ig domain and a 70-120 amino acid carboxy-terminal region. Some transmembrane semaphorins contain an Ig domain and a stretch of 80 amino acids carboxy-terminal to the sema domain, a transmembrane domain and an 80-140 residue cytoplasmic tail. Transmembrane semaphorins generally act as short range or cell-to-cell signals, whereas secreted semaphorins are diffusible signals. The biological activity of some semaphorins is regulated by the proteolytic cleavage of the extracellular domain by furin-like convertases.[3, 4] Secreted and membrane-bound semaphorins exist as disulfide-linked homodimers and their oligomerization is required for functionality.[3, 5]

Based on the structural and evolutionary differences among the proteins of the family, semaphorins are grouped into eight general classes I-VIII (Semaphorin Nomenclature Committee, 1999). Classes I and II contain semaphorins identified in invertebrate species. Classes III-VII contain vertebrate semaphorins. Class VIII contains the viral semaphorins found in non-neurotrophic DNA viruses. Class III contains secreted semaphorins. Classes IV-VII contain transmembrane or membrane-anchored semaphorins.[6] By convention, abbreviations for human semaphorins have all capital letters and mouse semaphorins have only the first letter capitalized. This convention will be followed throughout this thesis.

Semaphorins are expressed in a wide range of adult and embryonic tissues, and are known to trigger signaling pathways through specific receptor combinations including the plexins, neuropilins or β1-integrins. The neuropilin family consists of two members, NP-1 and NP-2. The mammalian plexin family has nine members, divided into four subfamilies (plexinA1 to A4, plexinB1 to B3, plexinC1, and plexinD1).[6] Class III semaphorin responses are mediated by specific combinations of NP-1 and NP-2. Classes I, IV, VII, and viral semaphorins interact with plexins.[7-10] GPI-anchored (glycosylphosphotidyl-inositol-anchored) Sema7A interacts with receptor protein β1-integrin to induce olfactory axon growth.[11] Several proteins other than neuropilins, plexins, and β1-integrins have been identified as part of semaphorin receptor complexes. Sema4D promotes invasive growth in epithelial cells through a receptor complex consisting of plexinB1 and tyrosine kinase receptor Met.[12] In the immune system CD72 acts as a Sema4D receptor. Sema4A, expressed by B cells and dendritic cells, enhances the generation of antigen-specific T cells through Tim-2 receptor.[13]

Functions of Semaphorins

Many semaphorins act as repulsive guidance cues that arrest the advance of neuronal growth cones and thereby play an important role in progressively guiding the neuronal growth cones towards their appropriate targets. Semaphorins also play a role in the adult nervous system, inhibiting axon growth after injury and maintaining established neuronal pathways.[14] Semaphorins not only play an important role in axon guidance, but are also critical in the regulation of cell migration, angiogenesis, and in the modulation of the immune system.[37, 51-53] Semaphorins also regulate neuronal apoptosis and metastatic

growth of tumors.[47, 39, 54, 55] Several semaphorin family members continue to be expressed in the adult brain and spinal cord. Their expression is induced upon nervous system injury in rodents and in neuropathology in humans.

Some studies have suggested alternative functions for semaphorin family members. SEMA3D, for instance, functions in bone differentiation and nerve guidance[35] and SEMA3B functions as a tumor suppressor gene in lung tissue.[26] SEMA3B inhibits lung cancer cell growth and induces apoptosis. Sema3A, the semaphorin best known for its role in axon guidance, is also able to induce apoptosis of sensory and sympathetic neurons.[38, 39]

SEMA3C expression has been related to cell survival in several contexts.[36] For example, SEMA3C is overexpressed in metastatic cells. SEMA3C is also overexpressed in 33.3% of recurrent squamous cell carcinomas and is involved in non-multi drug resistance (MDR) of human cancers.[47] Transfection of cisplatin-sensitive human ovarian cancer cells with SEMA3C conferred upon them a drug-resistant phenotype. This suggests that SEMA3C participates in carcinogenesis and in the progression of certain human tumors and might be involved in tumor cell survival promoting tumor metastasis by helping the cells to avoid or subvert immune surveillance.[36]

Synovial cells also express SEMA3C. The cell death resistance promoted by this semaphorin and observed in cancer cells may also be involved in the irreversible destructive growth of synovial cells in patients with rheumatoid arthritis.[70]

Although it has been shown that tumor cells express high levels of SEMA3E, SEMA3E alone is not sufficient to produce a metastatic cell, but rather plays a facilitating

role in the metastatic process.[71] SEMA3B and 3F were found in the chromosome region 3p21.3, where small cell lung cancer cell lines almost uniformly exhibit homozygous deletions.[72] It is possible that a mutation or down regulation of SEMA3B may provide some growth or survival advantage to tumor cells.[72] Loss of contact inhibition, one of the most important features seen in neoplastic cells, could be due to an alteration in semaphorin expression or function.

These are just some examples of the diverse roles played by the semaphorin family members. Finding semaphorins in immune organs offers a whole new understanding of their functions outside the nervous system and has lead to an understanding of their possible role in cancer metastasis, immune suppression and lymphocyte migration.

Sema4A in the Immune System

Sema4A was identified originally in embryos with its expression levels increasing gradually throughout embryonic development.[13] Sema4A is also expressed in various adult tissues including brain, spleen, lung, kidney, and testis. Sema4A though not expressed by resting T cells, is expressed abundantly as a homodimer on the surface of dendritic cells and at low levels on resting B cells. Sema4A participates in T cell activation by enhancing interaction between T cells and dendritic cells. The Sema4A receptor is Tim-2, a member of the family of T cell, immunogloblulin domain and mucin domain (Tim) proteins and is expressed on activated T cells.[13]

Sema4D in the Immune System

Sema4D is a 150-kDa transmembrane protein, which can be proteolytically cleaved to generate a 120-kDa soluble protein. Sema4D is expressed at high levels in

lymphoid organs including the spleen, thymus, lymph nodes and in non-lymphoid organs, such as the brain, heart, and kidney. More specifically, Sema4D is abundantly expressed on resting T cells but weakly expressed on resting B cells and antigen-presenting cells like dendritic cells. Sema4D uses two receptors, plexinB1 and CD72 which are expressed in non-lymphoid and lymphoid tissues respectively. In contrast to Sema4A, Sema4D is involved in the activation of B cells and dendritic cells.[46] Antigen-specific antibody production and antigen-specific T cell generation is accelerated by administration of recombinant soluble Sema4D[56, 57] or recombinant soluble Sema4A in mice.[13] Sema4D, Sema4A or agonistic reagents that stimulate CD72 or Tim-2 in conjugation with conventional immunization may enhance immune responses generated against infectious agents by vaccines that are weakly immunogenic.[46] Sema4D inhibits spontaneous and monocyte chemotactic protein-3 (MCP-3) -induced migration of monocytes. Thus, Sema4D might be useful clinically as an anti-inflammatory agent.[15] Strong upregulation of Sema4D in oligodendrocytes following spinal cord transection and inhibition of embryonic DRG (dorsal root ganglion) neurite outgrowth *in vitro*, identifies transmembrane Sema4D as a myelin-associated outgrowth inhibitor.[6]

Sema4F in the Immune System

Sema4F is a transmembrane protein and its expression increases significantly after birth. The cytosolic domain of Sema4F is highly conserved between mouse, rat, and human suggesting an important role for this domain in Sema4F function. Sema4F contains a c-terminal PDZ domain-binding consensus sequence and interacts with the post-synaptic density protein SAP90/PSD-95. This interaction might lead to synaptic

localization and clustering of Sema4F. These events could influence signal transduction of Sema4F and suggest a possible role in the regulation of glutamatergic synapses.[62] There is very little known about the function of Sema4F in the immune system.

Sema7A in the Immune System

Sema7A is a membrane-associated GPI-linked glycoprotein and is the semaphorin most similar to the semaphorin xenolog encoded by alcelaphine herpesvirus-1 (SEMA VB).[50] Sema7A is an axon growth-promoting factor required for lateral olfactory tract development through β1-integrin.[11] There might be other molecules involved in mediating Sema7A functions. Sema7A similar to its viral Sema xenologs, binds to the receptor plexinC1 inducing activation of monocytes. Sema7A is preferentially expressed on activated lymphocytes and thymocytes, and is a potent immunomodulator.[46, 50, 66] Sema7A is highly expressed in monocytes and C13 cells (immortalized immature microglial cells) and is expressed at lower levels in peripheral blood mononuclear cells (PBMC), lung, and brain.[50] Sema7A affects immune cell function *in vitro*, including chemotaxis and cytokine production. Sema7A also defines the John-Milton-Hagen human blood group on erythrocytes, which has been implicated in a clinically benign autoimmune disorder named paroxysmal nocturnal hemoglobinuria.[20] Sema7A is suspected to be involved in HIV-1 infection as the host-lymphocyte Sema7A is incorporated in the HIV-1 envelope during the budding stage.[59] Sema7A is also present in microglia, the principal immune effector cells of the nervous system, which arise from blood monocytes. There is evidence that Sema7A can be released from cells by proteolysis, consistent with models that propose autocrine functions for the protein.[60] Indeed, Sema7A has been shown to be an autocrine

monocyte activator whose expression is upregulated following spinal cord injury, indicating the possible role played by Sema7A in post-injury neural immune responses.[11, 50] An antibody against Sema7A receptor plexinC1, inhibits viral semaphorin-induced induction of inflammatory cytokines by monocytes.[61]

All these findings suggest the functional importance of semaphorins in broad range of tissues beyond the nervous system. The clinical relevance of semaphorins and their ability to manipulate immune responses make semaphorins potential enhancers of host immunity against pathogenic organisms.

Histology of Thymus

The thymus is a bilobed organ situated above the heart. Each lobe is surrounded by a capsule and is divided into lobules, which are separated from each other by strands of connective tissue called trabeculae. Each lobule is organized into two compartments, the outer cortex which is densely packed with immature T cells called thymocytes and an inner medulla with many fewer thymocytes. The cortex and medulla of the thymus are crisscrossed by a three-dimensional stromal cell network composed of epithelial cells, interdigitating dendritic cells and macrophages, which make up the framework of the thymus and contribute to thymocyte maturation. Interdigitating dendritic cells are located at the junction of the cortex and medulla and have long processes that interact with developing thymocytes. There is decrease in both cortical and medullary size with aging. The thymus reaches its maximal size at puberty and then undergoes a significant reduction in size accompanied by an increase in total fat content.[41]

Histology of Spleen

The spleen is surrounded by a capsule of dense connective tissue that extends as trabeculae and divides the splenic pulp into incomplete compartments. Seventy five percent of the volume of the spleen is made up of red pulp.[40] Red pulp is loose reticular tissue rich in capillaries. It is penetrated by venous sinuses and populated with macrophages. The nodular white pulp is densely populated with lymphocytes and is composed of primary lymphoid follicles (B cell areas) and T cell areas. Upon antigenic challenge, primary lymphoid follicles develop into secondary lymphoid follicles. Each secondary lymphoid follicle is composed of a germinal center where B cells divide rapidly, surrounded by a mantle zone, which in turn is surrounded by a marginal zone.[41] The T cell areas lie around the central arterioles in the periarteriolar lymphocyte sheath (PALS).[45] The border between the red and white pulp is known as the perifollicular zone.

Splenic white pulp is involved in the primary immune response to blood-borne antigens and polysaccharide antigens. Splenic red pulp functions as a hematopoietic organ by providing specific environment for the destruction of cells or microorganisms coupled with antibody and for the destruction of erythrocytes that have decreased flexibility and lowered osmotic resistance. There are fewer secondary follicles in spleen of older animals.[41]

Histology of Lymph Node

Lymph nodes are encapsulated organs with connective tissue from the capsule extending interior to form trabeculae. Each lymph node contains an outer cortex, inner cortex, and a medulla. The outer cortex is formed by lymphoid tissue composed of

lymphoid nodules or follicles. The inner cortex is a continuation of the outer cortex and contains few follicles. The medulla is composed of medullary cords, cord-like branched extensions of the lymphoid tissue of the inner cortex, separated by medullary sinuses. Lymph enters the nodes through the afferent lymphatic vessel into the subcapsular sinuses. Lymph flows from cortex to medulla and leaves the lymph node by efferent lymphatic vessel. Lymphocytes enter lymph nodes from the blood stream by crossing through the walls of high endothelial venules (HEV). HEVs have an endothelial lining of tall cuboidal cells between which the lymphocytes can travel.[41, 67]

Lymphocyte Maturation

Lymphocyte precursors differentiate into B cells in the bone marrow. Some precursors are carried in the blood from the bone marrow to the thymus and differentiate there into T cells. Thus the bone marrow and thymus are the primary lymphoid organs where the major lymphopoiesis takes place. From there, mature lymphocytes enter the bloodstream and populate the secondary lymphoid organs such as spleen, lymph nodes, and organized lymphoid tissues associated with mucosal surfaces. The secondary lymphoid organs are the sites where naive lymphocytes encounter dendritic cells presenting antigenic peptides in conjunction with major histocompatibility (MHC) class II molecules.

Small lymphocytes stay in the secondary lymphoid organs briefly if not activated by antigen-presenting cells and migrate into lymphatic vessels and back to the blood stream within hours to start another cycle of circulation, a process called lymphocyte recirculation.[24] Upon activation by antigen-presenting cells, naive T cells start to

proliferate and eventually differentiate into effector/memory T cells, which are then seeded via the blood and lymph throughout the tissues of the body. The vast majority of recirculating lymphocytes are T cells, whereas B cells seem to recirculate at a much slower pace.[25] The migration of lymphocytes is not random but is closely related to their functional properties. Differential expression of adhesion molecules plays an important role in regulating the nonrandom migration of specific subsets of lymphocytes to specific tissue microenvironments. Many of the same adhesion molecules are also responsible for enhanced accumulation of lymphocyte subsets in inflamed tissues.[24, 42, 43] Chemokines are thought to play important roles in directing the migration of lymphocytes to specific tissue microenvironments as well.[25] Most chemokines are expressed constitutively in tissues such as primary and secondary lymphoid organs.[22, 23]

T Cell Maturation in Thymus

Chemokines direct the homing of early progenitors from the blood, their movement to the outer cortex and/or association with particular stromal elements. Some studies suggested that T cell progenitors enter the thymus through vessels at the cortico-medullary junction.[28] Early $CD4^-CD8^-$ double negative (DN) cells move towards the subcapsular region of the outer cortex after they enter the thymic parenchyma.[27] Positive selection and transition of $CD4^-CD8^-$ DN to $CD4^+CD8^+$ double positive (DP) thymocytes in the inner cortex is accompanied by marked changes in chemokine receptor expression and function. Following positive selection, thymocyte migration across the cortico-medullary boundary is facilitated by loss of responsiveness to cortical specific chemokine SDF-1[29-31] and acquired responsiveness to medullary specific chemokines like ELC, SLC, and MDC.[11]

$CD4^+CD8^+$ DP thymocytes interact with dendritic cells which are present in significant numbers at the cortico-medullary border and differentiate into $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) thymocytes. After entering the medulla, SP thymocytes continue to differentiate from the immature $CD69^+CD62L^{low}$ stage to a more mature $CD69^-CD62L^{high}$ stage.[32, 34] At this stage chemokines might be facilitating association with antigen-presenting cells (APCs) for negative selection or directing thymocytes to associate with medullary stromal cells that deliver signals for further maturation. The more mature SP cells are resistant to negative selection and they increase in number before they emigrate to the periphery.[32, 33] The mature T lymphocytes then leave the thymus to populate the periferal lymphoid organs.

B Cell Maturation in Spleen

In adult mouse, B cells are generated in the bone marrow and immigrate to the spleen to undergo maturation. The migration of naïve B cells to the spleen might be facilitated by chemokines such as SDF-1, SLC, AND CKβ-11. The type 1 (T1) transitional B cells ($IgM^{bright}IgD^-CD21^-CD23^-$) are the first bone marrow-derived B cell precursors arriving into the spleen. T1 B cells are in the outer periarteriolar lymphocyte sheath (PALS) close to the primary follicle.

T1 B cells develop into type 2 (T2) transitional B cells that are $IgM^{bright}IgD^+$ $CD21^{bright}CD23^+$. Type 2 transitional B cells are found exclusively in the primary follicles of the spleen and their migration into the follicles is facilitated by the chemokine BLC.[19, 44] Mature B cells that are IgM^+ IgD^{bright} $CD21^+$ $CD23^+$, can be generated from T1 or T2 B cells. B cell receptor mediated signaling functions are essential for the development of

T2, mature and marginal zone (MZ) B cells from T1 B cells. Mature B cells in the lymphoid follicles of the spleen are exposed to antigen, presented by follicular dendritic cells. This leads to the initiation of germinal center reaction and finally rapid production of antigen-specific, high-affinity antibodies. In the absence of antigen, mature B cells recirculate with lymph and blood. Mature B cells are also seen in the outer PALS and in red pulp. Mature B cells develop into MZ B cells that populate the marginal zone surrounding the lymphoid follicle, along with macrophages. MZ B cells are $IgM^{bright}IgD^{-}$ $CD21^{bright}CD23^{-}$ and are not present in spleens of newborn mice.[44]

Clinical Significance

Semaphorins found in non-neurotropic DNA viruses are grouped into class VIII semaphorins. Two distinct semaphorins encoded within the genomes of Vaccinia virus (A39R) and Alcelaphine herpes virus (AHV-sema) have been identified.[16] No biological activity has yet been described for the secreted semaphorin encoded within AHV, which is responsible for leukopenia and generalized lymphadenopathy in susceptible ruminant hosts.[16] A39R and AHV-sema share only 29% amino acid identity and they both bind to a receptor (VESPR / plexinC1) expressed on a variety of hematopoietic cells.[18] Most viral homologues to cellular genes have functions in the modification of apoptotic pathways or host immune response.[17] Apart from its probable role in the maturation and/or migration of T cells, SEMA7A which is homologous to AHV-sema could be playing a role in immunomodulation. Unlike SEMA7A, AHV-sema lacks transmembrane sequence. AHV-sema might be secreted by the virus-infected cells to bind to SEMA7A receptor, thereby exerting an analogous or antagonistic function,

compromising the immune response to the viral infection.[17] It was shown that an antibody against Sema7A receptor plexinC1 inhibits viral semaphorin-induced induction of inflammatory cytokines by monocytes.[61]

Vaccinia semaphorin A39R is biologically active on human monocytes, inducing expression of ICAM-I (an adhesion molecule) and cytokines IL-6, IL-8, and TNF-α[71] similar to Sema4D function. Antigen-specific antibody production and antigen-specific T cell generation is accelerated by administration of recombinant soluble Sema4D in mice.[56, 57] By mimicking the functions of Sema4D, A39R could be helping vaccinia in delaying the host immune response. A39R semaphorin binds to plexinC1 on primary mouse dendritic cells and acts as a potential regulator of the actin cytoskeleton in the immune system supporting our hypothesis that semaphorins could be involved in the regulation of leukocyte movement. A39R semaphorin is secreted at very high levels by poxvirus infected cells. Induced expression of A39R semaphorin by vaccinia strains which do not normally express, increases the severity and the persistence of skin lesions. One explanation could be that A39R semaphorin alters the movement of plexinC1-expressing cells (dendritic cells, monocytes and granulocytes) that respond to infection. This effect would benefit the vaccinia virus by inhibiting the recruitment of immune effector cells at the site of virus infection. Alternately, it is possible that semaphorin regulates the cytoskeleton and shape of neighboring cells, preparing them for infection by the virus.[63] This A39R semaphorin work is consistent with our hypothesis that semaphorins could be influencing or modifying lymphocyte response to signals during their maturation and also during an immune response.

Specific Aims

The goal for our research was to investigate, determine, and analyze the expression pattern of Sema4A, Sema4D, Sema4F, and Sema7A in lymphoid organs of six- and sixteen-week old mice and their specific localization within these tissues by tissue *in situ* hybridization.

Specifically, our goals were (1) to extract total RNA from mouse lymphoid tissue and to amplify fragments of semaphorins 4A, 4D, 4F, and 7A by RT-PCR; (2) to clone the Sema4D and 7A fragments into the pGEM vector, and the Sema4A and 4F fragments into the pCRII vector; (3) to generate digoxigenin (DIG)-labeled RNA probes and make 10 μm sections of mouse thymus, spleen, and lymph nodes; and finally (4) to hybridize sense and antisense probes to the tissue sections and determine, and document the expression patterns of the four semaphorins.

MATERIALS AND METHODS

EST Analysis

The tissue of origin of the expressed sequence tags (ESTs) corresponding to the 19 human and 19 mouse semaphorins were examined at Unigene (NCBI). Any EST with a lymphoid tissue source was taken as suggestive of the expression of that semaphorin in that tissue.

Probe Design and Synthesis

Semaphorin gene sequences were obtained from GenBank. The accession numbers for semaphorins studied in this research project (4A, 4D, 4F, and 7A) are NM_013658, NM_013660, NM_011350, and NM_011352 respectively. A multiple sequence alignment of all human semaphorins was performed to allow the identification of sequences within the semaphorin domain that were not well conserved among the semaphorins. This analysis allowed us to design probes that would be sema specific, but would be unique to the particular semaphorin. Such a region (about 450 base pairs) was chosen for each semaphorin to be amplified by RT-PCR. Primers were selected using the primer3input application. The important parameters for the design of primers and positioning included the following.

1. Product size range was specified to be between 300 and 500 base pairs.
2. The minimum primer size was set to be 20 nucleotides.
3. The maximum primer size was set to be 21 nucleotides.

Fifty nanomoles of each primer (Table 1) was ordered from Operon, CA, USA (Appendix 4).

Gene		Primer sequences 5' to 3'
Sema4A	Forward	GGGAATTCACAAAAAGGCCTCCGAGACT
	Reverse	GGAAGCTTATCAAGATGGGCAACAGGAG
Sema4D	Forward	AAGCAGACGGAATGCCTAAA
	Reverse	GATCACGTCAGCAAAGACGA
Sema4F	Forward	GGGAATTCGCTGACTCCTATCTCACCCG
	Reverse	GGAAGCTTGCTGGAAACTGGACACATCA
Sema7A	Forward	CGTGGCAAGGTCTACCACTT
	Reverse	TCCCGTTGTATTCCTGCTTC

Table 1. Primers for amplification of semaphorin gene fragments by RT-PCR.

Below, each of the probes designed is shown within the context of the total sema protein sequence. The Blue font represents sema domain, dark red font represents conserved amino acids within the sema domain and underlined sequence represents the *insitu* probe region (the nucleotide sequence of each probe is shown in Appendix 2).

A. Sema4A

MALPSLGQDSWSLLRVFFFQLFLLPSLPPASGTGGQGPMPRVKYHAGDGHRALSFFQQK
GLRDFDTLLLSDDGNTLYVGARETVLALNIQNPGIPRLKNMIPWPASERKKTECAFKKK
SNETQCFNFIRVLVSYNATHLYACGTFAFSPACTFIELQDSLLLPILIDKVMDGKGQSP
LTLFTSTQAVLVDGMLYSGTMNNFLGSEPILMRTLGSHPVLKTDIFLRWLHADASFVAA
IPSTQVVYFFFEETASEFDFFEELYISRVAQVCKNDVGGEKLLQKKWTTFLKAQLLCAQ
PGQLPFNIIRHAVLLPADSPSVSRIYAVFTSQWQVGGTRSSAVCAFSLTDIERVFKGKY
KELNKETSRWTTYRGSEVSPRPGSCSMGPSSDKALTFMKDHFLMDEHVVGTPLLVKSGV
EYTRLAVESARGLDGSSHVVMYLGTSTGPLHKAVVPQDSSAYLVEEIQLSPDSEPVRNL
QLAPAQGAVFAGFSGGIWRVPRANCSVYESCVDCVLARDPHCAWDPESRLCSLLSGSTK
PWKQDMERGNPEWVCTRGPMARSPRRQSPPQLIKEVLTVPNSILELRCPHLSALASYHW
SHGRAKISEASATVYNGSLLLLPQDGVGGLYQCVATENGYSYPVVSYWVDSQDQPLALD
PELAGVPRERVQVPLTRVGGGASMAAQRSYWPHFLIVTVLLAIVLLGVLTLLLASPLGA
LRARGKVQGCGMLPPREKAPLSRDQHLQPSKDHRTSASDVDADNNHLGAEVA

B. Sema4D

MRMCAPVRGLFLALVVVLRTAVAFAPVPRLTWEHGEVGLVQFHKPGIFNYSALLMSEDK
DTLYVGAREAVFAVNALNISEKQHEVYWKVSEDKKSKCAEKGKSKQTECLNYIRVLQPL
SSTSLYVCGTNAFQPTCDHLNLTSFKFLGKSEDGKGRCPFDPAHSYTSVMVGGELYSGT
SYNFLGSEPIISRNSSHSPLRTEYAIPWLNEPSFVFADVIQKSPDGPEGEDDKVYFFFT
EVSVEYEFVFKLMIPRVARVCKGDQGGLRTLQKKWTSFLKARLICSKPDSGLVFNILQD
VFVLRAPGLKEPVFYAVFTPQLNNVGLSAVCAYTLATVEAVFSRGKYMQSATVEQSHTK
WVRYNGPVPTPRPGACIDSEARAANYTSSLNLPDKTLQFVKDHPLMDDSVTPIDNRPKL
IKKDVNYTQIVVDRTQALDGTFYDVMFISTDRGALHKAVILTKEVHVIEETQLFRDFEP
VLTLLLSSKKGRKFVYAGSNSGVVQAPLAFCEKHGSCEDCVLARDPYCAWSPAIKACVT
LHQEEASSRGWIQDMSGDTSSCLDKSKESFNQHFFKHGGTAELKCFQKSNLARVVWKFQ
NGELKAASPKYGFVGRKHLLIFNLSDGDSGVYQCLSEERVRNKTVSQLLAKHVLEVKMV
PRTPPSPTSEDVQTEGSKITSKMPVGSTQGSSPPTPALWATSPRAATLPPKSSSGTSCE
PKMVINTVPQLHSEKTVYLKSSDNRLLMSLLLFIFVLFLCLFSYNCYKGYLPGQCLKFR
SALLLGKKTPKSDFSDLEQSVKETLVEPGSFSQQNGDHPKPALDTGYETEQDTITSKVP
TDREDSQRIDELSARDKPFDVKCELKFADSDADGD

C. Sema4F

MLARAERPRPGPRPPPVSLFPPPSSLLLLLLAMLSAPVCGRVPRSVPRTSLPISEADSY
LTRFAAPHTYNYSALLVDPASHTLYVGARDSIFALTLPFSGEKPRRIDWMVPETHRQNC
RKKGKKEDECHNFIQILAIANASHLLTCGTFAFDPKCGVIDVSSFQQVERLESGRGKCP
FEPAQRSAAVMAGGVLYTATVKNFLGTEPIISRAVGRAEDWIRTETLSSWLNAPAFVAA
MVLSPAEWGDEDGDDEIFFFFTETSRVLDSYERIKVPRVARVCAGDLGGRKTLQQRWTT
FLKADLLCPGPEHGRASGVLQDMTELRPQPGAGTPLFYGIFSSQWEGAAISAVCAFRPQ
DIRAVLNGPFRELKHDCNRGLPVMDNEVPQPRPGECITNNMKFQQFGSSLSLPDRVLTF
IRDHPLMDRPVFPADGRPLLVTTDTAYLRVVAHRVTSLSGKEYDVLYLGTEDGHLHRAV
RIGAQLSVLEDLALFPETQPVESMKLYHDWLLVGSHTEVTQVNTSNCGRLQSCSECILA
QDPVCAWSFRLDACVAHAGEHRGMVQDIESADVSSLCPKEPGEHPVVFEVPVATVGHVV
LPCSPSSAWASCVWHQPSGVTSLTPRRDGLEVVVTPGAMGAYACECQEGGAARVVAAYS
LVWGSQRGPANRAHTVVGAGLVGFFLGVLAASLTLLLIGRRQQRRRQRELLARDKVGLD
LGAPPSGTTSYSQDPPSPSPEDERLPLALGKRGSGFGGFPPPFLLDSCPSPAHIRLTGA
PLATCDETSI

D. Sema7A

MTPPPPGRAAPSAPRARVLSLPARFGLPLRLRLLLVFWVAAASAQGHSRSGPRISAVWKG
QDHVDFSQPEPHTVLFHEPGSFSVWVGGRGKVYHFNFPEGKNASVRTVNIGSTKGSCQDK
QDCGNYITLLERRGNGLLVCGTNARKPSCWNLVNDSVVMSLGEMKGYAPFSPDENSLVLF
EGDEVYSTIRKQEYNGKIPRFRRIRGESELYTSDTVMQNPQFIKATIVHQDQAYDDKIYY
FFREDNPDKNPEAPLNVSRVAQLCRGDQGGESSLSVSKWNTFLKAMLVCSDAATNRNFNR
LQDVFLLPDPSGQWRDTRVYGVFSNPWNYSAVCVYSLGDIDRVFRTSSLKGYHMGLPNPR
PGMCLPKKQPIPTETFQVADSHPEVAQRVEPMGPLKTPLFHSKYHYQKVVVHRMQASNGE
TFHVLYLTTDRGTIHKVVESGDQDHSFVFNIMEIQPFHRAAAIQAISLDADRRKLYVTSQ
WEVSQVPLDMCEVYSGGCHGCLMSRDPYCGWDQDRCVSIYSSQRSVLQSINPAEPHRECP
NPKPDEAPLQKVSLARNSRYYLTCPMESRHATYLWRHEENVEQSCEPGHQSPSCILFIEN
LTARQYGHYRCEAQEGSYLREAQHWELLPEDRALAEQLMGHARALAASFWLGVLPTLILG
LLVH

RNA Isolation

The RNAqueous-Midi kit (Ambion, Austin, TX) was used for purifying total RNA from mouse thymus and lymph nodes. Thymus and axillary, inguinal and mesenteric lymph nodes were isolated from 16-week old C57BL/6 mice (SJSU Institutional Animal Care and Use Committee, protocol #771, Appendix 3). Pooled lymphoid tissue (0.1 to 0.5 grams) was homogenized and suspended in PBS (phosphate buffered saline) and calf bovine serum. Cells were spun at 12,000 rpm at 4°C, for 5 minutes and cell counts were made using a hemocytometer. Cells were then resuspended in lysis/binding solution and centrifuged again at 12,000 rpm at 4°C, for 5 minutes. An equal volume of 64% ethanol was added to the clarified lysate and was mixed gently. The lysate was aspirated into a syringe through an 18 gauge needle. The needle was removed and a glass fiber filter unit was attached to the syringe. The lysate was slowly passed into a waste receptacle. The filter was then washed with an equal volume of wash solution #1. The filter was washed twice with a 70% volume of wash solution #2/3. Air was forced vigorously through the filter to remove residual wash solution and this was repeated until no additional droplets or fine spray was visible. The RNA was eluted from the filter by adding hot elution solution to the syringe. The eluted RNA was run on a 1% agarose/TAE gel to verify successful RNA purification (data not shown).

RT-PCR

PCR products of 350-400 base pairs were generated by RT-PCR using RetroScript kit (Ambion). One microgram total RNA, 4 µl dNTP mix, 2 µl R-primer,

and 16 µl nuclease-free water were mixed together for the reverse transcription (RT) reaction. The tubes were spun briefly and heated at 85°C for 3 minutes. Tubes were then transferred to ice, spun briefly and stored on ice. Two microlitres RT-PCR buffer 10x, 1 µl RNase inhibitor and 1 µl M-MLV Reverse Transcriptase were added to the tubes. The contents were mixed gently, pulsed and incubated at 42°C for one hour. The tubes were then incubated at 92°C for 10 minutes to inactivate the reverse transcriptase. Five microlitres RT reaction, 5 µl RT-PCR buffer 10x, 2.5 µl dNTP mix, 17.5 µl nuclease-free water, 2.5 µl PCR primers at 5 µM concentration of each primer and 1 unit Thermostable DNA polymerase were mixed for the amplification reaction. PCR was carried out at 95°C for 5 minutes, followed by 30 cycles of 94°C for 20 seconds, 55°C for 30 seconds, 72°C for 40 seconds, and a final extension of 72°C for 5 minutes. The cDNA was resolved on a 1% agarose/TAE gel to assess the success of the amplification, product size and the product yield (data not shown).

Cloning of Semaphorin Gene Fragments

Amplified gene fragments of semaphorins 4D and 7A were cloned into pCR2.1 vector and semaphorins 4A and 4F were cloned into pCRII vector using the TA cloning kit (Invitrogen, Carlsbad, CA). Taq polymerase amplified PCR products were ligated into pCR2.1 vector or pCRII vector according to the protocol given in the kit. Success of ligation and transformation reactions was assessed by digesting plasmids with restriction enzymes and resolving on agarose gels (data not shown).

The sema4D and 7A inserts were then subcloned into the pGEM vector (Promega, Madison, WI), so that the T_7 and SP_6 promoters would flank the insert. The pCR2.1

ligation products were digested using restriction enzymes Kpn I and Xho I. The pGEM vector was also digested using restriction enzymes Kpn I and Sal I. The RE digested products were precipitated using NaCl and were resolved on a 1% agarose/TAE gel (data not shown). Four microlitres of the sema insert released from the pCR2.1 and 0.5 µl of the linearized pGEM were combined with 1 µl of 10x ligation buffer, 3.5 µl of nuclease-free water and 1 µl of T_4 DNA ligase for the ligation reaction. The tubes were then incubated at 14°C overnight. Following transformation, all the plasmids were cut with diagnostic restriction enzymes and resolved on a 1% agarose/TAE gel to assess the success of ligation and transformation (data not shown).

Transformation

INVαF cells (provided with the TA cloning kit) were used for transformation. Transformations were performed according to the protocol provided by the manufacturer. White colonies (β-gal negative) that are ampicillin resistant were picked and a master plate was made simultaneously. Plasmid isolation was done by mini prep procedure. The isolated plasmid was digested with specific enzymes to check for the presence of the correct insert. The RE digested products were resolved on a 1% agarose/TAE gel (data not shown).

Maxi Prep and Sequencing

Cultures were grown in 100 ml luria broth containing 100 mg/ml ampicillin for two days before the maxi preps. Plasmids were isolated at large scale using Qiagen Maxi Prep kits with Qiagen-tip 500 (Qiagen Inc. Valencia, CA) according to the protocol given in the kit. The isolated plasmid was diluted several fold and quantified using Schimadzu

UV 160U spectrophotometer. All the prepared plasmids were seperated on a 1% agarose/TAE gel to check for genomic DNA contamination (data not shown). Each plasmid (1.6 µg) in 22 µl of nuclease-free water was sent to 'The Protein and Nucleic Acid (PAN) Biotechnology Facility' at Stanford University, CA where all four plasmids were sequenced in both directions using primers specific for T_7 and SP_6 promoters.

Probe Labeling

Digoxigenin-labeled sense and antisense riboprobes were generated for each semaphorin using the DIG-RNA labeling kit (SP_6/T_7), (Roche Applied Science, Indianapolis, IN). The DNA template was linearized using restriction enzymes BamHI and EcoRV. The linearized template DNA was resolved on a 1% agarose/TAE gel to assess the success of linearization (data not shown). DNA was purified by phenol/chloroform extraction. Tubes were filled with phenol/chloroform and spun at 13,000 rpm for 5 minutes. The upper aqueous layer containing DNA was removed into a fresh sterile tube containing 100% ethanol. The contents were gently mixed and spun at 13,000 rpm for 5 minutes. Ethanol was removed and 80% ethanol was added to wash the pellet. Tubes were shaken gently and spun again at 13,000 rpm for 5 minutes. Ethanol was then removed completely before the pellet was allowed to air dry. The pellet was resuspended in 15 µl 1x TE buffer. One microgram of purified template DNA at 0.05 µg/µl, 2 µl of 10x transcription buffer (400 mM Tris-HCl, pH 7.5/8, 60 mM $MgCl_2$, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1 unit/ml RNase inhibitor), 1 µl of RNase inhibitor, 2 µl of 10x DIG-labeling mix, 2 µl of RNA polymerase (SP_6, T_7 or T_3) at 2 units/µl final concentration and diethyl pyrocarbonate

(DEPC)-treated water (to get 20 µl total volume) were added to a sterile, RNase-free, labeled microfuge tube placed on ice. The tubes were mixed gently and spun briefly to collect the solution. The reaction mixture was incubated at 37°C for 2 hours in a water bath. Template DNA was removed by adding 2 µl of 10 units/µl RNase-free DNase I and incubating for 15 minutes at 37°C. The DIG-riboprobe was precipitated and the reaction was stopped by adding 2 µl of 0.2 M EDTA. The pellet was resuspended in 25 µl of DEPC-treated water. Fifty microlitres of carbonate buffer (0.06 M Na_2CO_3, 0.04 M $NaHCO_3$) pH 10.2 was added to each sample and the tubes were incubated at 60°C for the appropriate length of time. Hydrolysis times for each probe were calculated (using the following formula) (74).

$$\text{Hydrolysis time (min)} = \frac{\text{length of probe (kb)} - \text{length of desired end product (kb)}}{[0.11 \times \text{length of probe (kb)} \times \text{length of desired end product (kb)}]}$$

Riboprobes require hydrolysis to improve cellular penetration. An equal volume (75 µl) of hydrolysis-neutralization buffer (200 mM sodium acetate, 1% (v/v) acetic acid, pH 6.0) was added to stop the hydrolysis. A 3x volume (300 µl) of chilled 100% ethanol (chilled at -20°C) to precipitate RNA was then added. The contents were mixed and the tubes were incubated at -70°C for 30 minutes. The tubes were spun at 13,000 xg or 10,400 rpm for 15 minutes at 4°C in a microcentrifuge. Ethanol was removed and the pellet was washed with 100 µl of cold 70% ethanol. The tubes were spun again at 13,000 xg or 10,400 rpm for 5 minutes at +4°C in the microcentrifuge. The 70% ethanol was then removed. The pellets were dried and resuspended in 100 µl DEPC-treated water.

The probes were all stored at –70°C for up to 7 months.

Quantification of Probe Yield

Riboprobe synthesis yields were determined by fluorescence microplate assay using an RNA Quantitation kit (Molecular Probes Inc. Eugene, OR) and a CytoFluor fluorescence multi-well plate reader, Series 4000 (PE Biosystems).[75]

Nuclease-free pipettes, sterile disposable plastic bottles, or nuclease-free glassware were used to prepare all solutions in all experiments from RNA purification to riboprobe hybridization.

Reagent Preparation

An aliquot of the concentrated DMSO stock solution (RiboGreen RNA quantitation reagent) was diluted 200-fold using 1x TE buffer. The working solution of RiboGreen reagent was protected from light by covering with foil and working in dark when possible. The working solution was also made fresh each time.

RNA Standard Curve

The RNA solution used to prepare the standard curve was treated the same way as the experimental samples and contained similar levels of all compounds. The 100 µg/ml ribosomal RNA standard provided in the kit was diluted 50-fold using 1x TE to make 2 µg/ml working solution. The 2 µg/ml working solution was diluted into the appropriate volumes using 1x TE buffer in microplate wells, as shown in Table 2.

Volume (µl) of TE buffer	Volume(µl) of 2 µg/ml RNA stock	Volume (µl) of 200-fold diluted RiboGreen reagent	Final RNA Concentration In RiboGreen assay
0	100	100	1 µg/ml
50	50	100	500 ng/ml
90	10	100	100 ng/ml
98	2	100	20 ng/ml
100	0	100	blank

Table 2. Volumes of TE buffer, RNA stock, and RiboGreen reagent to be added to the microplate in the process of making a standard curve.

One hundred microlitres of the working solution of RiboGreen reagent was added to each microplate well containing RNA as shown in Table 2. The samples were incubated for 3 minutes at room temperature, protected from light. The sample fluorescence was measured using the fluorescence microplate reader and standard fluorescein wavelengths of excitation (480 nm) and emission (520 nm). To ensure that the sample readings remain in the range of detection for the fluorometer, the instrument's gain was set so that the sample containing the highest RNA concentration yields fluorescence intensity near the fluorometer's maximum. The fluorescence value of the reagent blank was subtracted from that of each of the samples. To create the standard curve, corrected fluorescence values were plotted on the Y-axis and RNA concentrations were plotted on the X-axis.

Sample Analysis

The experimental RNA solutions were diluted in 1x TE to a final volume of 100 µl. One hundred microlitres of the aqueous working solution of the RiboGreen

reagent was added to each sample. The samples were incubated for 3 minutes at room temperature, protected from light. Fluorescence of each sample was measured using instrument parameters identical to those used when generating the standard curve. The fluorescence value of the reagent blank was subtracted from that of each of the samples. The concentration of RNA in the samples was determined from the standard curve (data not shown).

Preparation of Samples for* In Situ *Hybridization

Making DEPC-treated Water

RNase-free water was made by adding 0.1% DEPC (Sigma, Ronkonkoma, NY) in Millipore water, shaking well and incubating overnight at room temperature before autoclaving. DEPC-treated water was used in all experiments from RNA purification to riboprobe hybridization. Work surfaces, gloves and items that cannot be DEPC treated were treated with RNase Zap (Sigma) and rinsed in DEPC-treated water to make them RNase free.

Coating Slides[74]

Glass slides were arranged in metal racks and soaked over night in 10% Terg-a-zyme (Alconox Inc. White Plains, NY). The slides were rinsed thoroughly in purified Millipore water three times and were allowed to dry. The slide racks were wrapped in aluminum foil and baked at 180°C, for 3 hours. The slides were then immersed in 2% solution of 3-amino propyl tri ethoxy silane (APES) (Sigma) in acetone for 20 seconds. Slides were rinsed once in acetone for 20 seconds, and then in DEPC-treated water, twice. The slides were dried in an oven at 37°C overnight and were stored

in an airtight container.

Making Embryo Powder

Five 12.5-week mouse embryos were homogenized in 5 ml PBS. Four volumes of ice cold acetone were added, mixed and incubated on ice for 30 minutes. The tubes were spun at 9,500 rpm in a Sorval centrifuge using S32 rotor, for 10 minutes at 4°C. The supernatant was removed and ice cold acetone was added to wash the pellet in the tube. The tubes were spun again at 9,500 rpm in the same conditions as before. The supernatant was removed and the pellet was spread on a stack of Whatman filter papers. Once the pellet was dry, it was ground to powder using a mortal and pestle. The embryo powder was then stored in aliquots in 0.5 ml centrifuge tubes at 4°C.

Animals

Two- to six-week old male C57BL mice were purchased from Simonsen laboratories (Gilroy, CA, USA). The animals were maintained in accordance with the IACUC guidelines as per the approved protocol # 771 (Appendix 3).

Six-week old mice were used, as we wanted to investigate the semaphorin expression in young adults. In addition we obtained tissue from 16-week mice which are representative of a more mature animal. Spleen was sectioned successfully only from 16-week mice. There is no data for splenic expression in 6-week mice. We were unsuccessful in extracting lymph nodes from these older mice, so there is no data for lymph node expression from 16-week mice.

Tissue Processing

Tissue processing was done using a protocol devised by Jennifer Robertson at

McMaster University, Canada (personal communication). Animals were euthanized and dissected under sterile conditions. Dissected mouse thymus, spleen and lymph nodes were fixed in freshly prepared 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.2 and incubated for 23 hours at 4°C. The tissue was dehydrated for 30 minutes in each of several ethanol dilutions – 20%, 50%, 70%, 100% and 100% at 4°C. The tissue was then washed twice in 100% xylene for 30 minutes each, at room temperature. The tissue was infiltrated in 50% xylene in paraffin twice, for 30 minutes each, at 60°C in a dry oven. Next the tissue was infiltrated with 100% paraffin for 30 minutes, at 60°C. Finally the tissue was embeded in paraffin.

Tissue Sectioning

All the instruments used during sectioning were treated with RNase-zap, rinsed with DEPC-treated water or wiped clean with Kimwipes from an unused box. The paraffin block was trimmed to expose the tissue and was soaked in cold water for 15 minutes to rehydrate the tissue. The soaking step was repeated after cutting a few sections and when the tissue started to shred. Ten micron sections were cut using "820" Spencer microtome from (American Optical Corporation, Southbridge, MA) and gently floated onto the DEPC-treated water on APES-coated slides. The sections were afixed by placing the slides on a heating table covered at 42°C, overnight.

Hybridization and Detection

Tissue sections were hybridized with sense and antisense DIG-labeled riboprobes as follows. All reagents and solutions until day 4 were RNase free. DEPC-treated water and new reagents were used to make the solutions. All the glass, metal, and plastic slide

racks, holders, and staining dishes were washed in Terg-a-zyme, rinsed in millipore water, treated with RNase Zap and were wiped dry with Kimwipes (assuming those from an unused box were RNase free). All the solutions required on days 1, 2, and 3 were made before the start of hybridization. The oven used for hybridizations was turned on and set to 65°C three days before the actual usage and was monitored for any instability in temperature.

Day 1 and Day 2

The water bath and the oven were set to 65°C.

DEPC treated water was made.

Day 3

Solutions that would be used on day 4 were prepared. These include the following:

0.2 M PBS	Na_2HPO_4 $7H_2O$ (Sigma), NaH_2PO_4 H_2O (Sigma), and NaCl (Sigma) in DEPC-treated water
10 N NaOH	NaOH (Sigma) in DEPC-treated water
4% PFA/PBS	Dissolved paraformaldehyde (Sigma) in 0.2 M PBS. A few drops of 10 N NaOH were added to clear the solution. The solution was subsequently filtered using 500 ml filters (Nalgene labware, Rochester, NY) and stored at 4°C.
Xylene	(Sigma)
Ethanol	100%, 95%, 85%, 70%, 50%, 25%
Proteinase K	2.5 µg/µl Proteinase K (Sigma) in DEPC-treated water

Prehybridization solution	50% Formamide (Sigma), 5x SSC, 5x Denhardt's (Sigma), 250 µg/ml Ecoli tRNA (Sigma), 500 µg/ml Herring testis DNA (Sigma)

Herring testis DNA and tRNA solutions were always made the day before they were used (day 3).

Day 4

The paraffin sections were deparaffinized in xylene for 5 minutes at room temperature. The sections were then rehydrated in ethanol 100%, 95%, 85%, 70%, 50%, 25% and in water, 3 minutes in each at room temperature. The sections were fixed with 4% PFA/PBS for 20 minutes at room temperature. The sections were then washed three times in 0.2 M PBS, 3 minutes each at room temperature. One millilitre of 2.5 µg/µl proteinase K was added to each slide and incubated at room temperature for 5 minutes. The sections were then washed twice in 0.2 M PBS for 5 minutes. The sections were refixed in 4% PFA/PBS for 20 minutes. They were then washed three times in 0.2 M PBS for 3 minutes. The sections were incubated in 0.75 ml prehybridization solution per slide overnight at room temperature. Probe was added to prehybridization solution to make a final concentration of 100 ng probe per 90 µl hybridization solution.

The prehybridization solution was removed from the slides by tilting them gently. Ninty microlitres of hybridization solution was added to each slide. Glass coverslips (24 x 50, Sigma) were placed carefully on the sections. Immediately the slides were transferred to the oven at 65°C and incubated overnight. SSC solutions (5x SSC

necessary for one wash and 0.2x SSC for three washes) were placed in the 65°C water bath and the glassware to be used next day was placed in an incubator at 65°C.

Day 5

The water bath was set to 68°C. Solutions that would be used on day 5 were prepared. These include the following:

SSC	5x SSC, 0.2x SSC (Sigma)
B1 buffer	0.1 M Tris (Sigma) pH 7.5 and 0.15 M NaCl in DEPC-treated water
Blocking solution	B1 + 10% sheep serum (Sigma)
B2 buffer	B1 + 0.1% sheep serum
Anti-DIG solution	B2 + 1/5000 of monoclonal anti-DIG AP-conjugate Ig fraction of mouse ascites fluid (Sigma)

The slides were washed once in 5x SSC at 65°C to let the coverslips off. The sections were then washed three times in 0.2x SSC, 30 minutes each at 65°C in an oven. Preabsorption of antibody was begun during the last 30 minute wash. In preparation for antibody preabsorption, embryo powder was added to blocking solution in a 15 ml tube and heating the mixture at 68°C for 30 minutes. The slides were washed once in 0.2x SSC for 30 minutes at room temperature. The embryo powder solution was votexed for 10 minutes and then cooled on ice for 15 minutes before anti-DIG antibody was added to make a 1/5000 dilution. Preabsorption was carried out for 1 hour at 4°C. The sections were equilibrated in B1 buffer for 5 minutes at room temperature. Slides were removed from the rack, dried (bottom and the side) with a Kimwipe and arranged on a stack of

Kimwipes in hood before adding blocking solution. The tube of preabsorbed antibody was spun for 1 minute in a swinging bucket centrifuge at 6,000 rpm. The supernatant was diluted with B2 buffer to make 35 ml of working anti-DIG antibody solution at 1/5000 final dilution. Each slide was tilted to let the blocking solution drip off. The slides were then arranged in clean glass staining trays. Six hundred microlitres of anti-DIG solution was added to each slide. The trays were covered with aluminum foil to retain moisture and left at 4°C overnight.

Day 6

Solutions that would be used on day 6 were prepared.

B1 buffer	0.1 M Tris pH 7.5 and 0.15 M NaCl
B3 buffer	0.1 M Tris pH 9.5, 0.1 M NaCl and 50m M $MgCl_2$ (Sigma)
NBT/BCIP	working dilution (Sigma)

The slides were rinsed three times in B1 buffer, 30 minutes each at room temperature. The sections were then equilibrated in B3 buffer for 5 minutes at room temperature. Slides were arranged in clean glass staining trays and 750 µl of NBT/BCIP working solution was added to each slide. A stack of moist Kimwipes were placed in each tray to maintain humidity level. The trays were then wrapped with aluminum foil and transferred to a dark room drawer. The staining reaction was allowed to develop in the dark for up to 2 days (60 hours). The color development was monitored every 2 hours for the rest of day 6.

Day 7 and Day 8

Color development was monitored for two days for a few sample slides, so that most

slides were left undisturbed until color development was stopped.

Day 9

The slides were examined without coverslips for staining pattern using a Zeiss microscope (Axioskop 2). Digital images of sections that had a good cell morphology and absence of contaminants and a pattern consistent among sections were captured in the next couple of days with a Zeiss MC80DX camera mounted on the microscope. Sense probed sections treated the same way as antisense probed sections served as controls for background and nonspecific staining.

Hybridizations were performed three times on sections from two different animals. The expression patterns of the semaphorins observed in this project were documented (Table 4; Figures 1-4).

RESULTS

EST Analysis

Expressed sequence tags reflect expression patterns of genes in particular tissues. EST analysis was done to get a preliminary idea of which semaphorins might be detected in lymphoid tissues by *in situ* hybridization (Table 3). Some semaphorins (indicated by red font in Table 3) were dropped from this project after unsuccessful attempts to amplify them from pooled adult spleen, thymus and lymph nodes by RT-PCR.

Expression Patterns Observed in this Research Project

The expression patterns of the semaphorins 4A, 4D, 4F, and 7A were observed in tissues extracted, processed, sectioned, and hybridized with sense and antisense DIG-labeled riboprobes (Table 4; Figures 1-4). Hybridization results were presented for two different animals. It is important to note that some of the figures (Figures 1-4) are a composite of two or more pictures from the same section.

While all the four semaphorins have identical staining patterns in animals 16-weeks of age in the thymus and spleen, at 6-weeks the patterns are different, with only some overlap. At 16-weeks of age Sema4A, 4D, 4F, 7A are expressed in the cortex of thymus and in follicles in the white pulp of spleen.

In the thymus at 6-weeks, Sema4A and Sema4D are expressed in the outer cortex, Sema7A in the inner cortex, and Sema4F in the inner cortex and medulla. In the lymph node at 6-weeks Sema4D is expressed in the outer cortex and around efferent lymphatic vessels on HEV, Sema4A and Sema7A in lymphoid follicles, and Sema4F in the medullary region with extensions going into the cortex.

Gene	Thymus	Spleen	Lymph node	Tonsil	Tumors
Sema3A		Mouse	Mouse		
Sema3B		Mouse			
Sema3C	Mouse	Human			
Sema3D	Mouse	Human	Mouse		
Sema3E	Mouse		Human		
Sema3F	Mouse	Mouse	Mouse		
Sema4A	Mouse	Mouse	Mouse	Mouse	
Sema4B	Mouse	Mouse, Human			
Sema4C		Human	Mouse		
Sema4D	Mouse, Human	Mouse	Mouse	Human	Prostate
Sema4F	Mouse	Mouse			
Sema4G		Human			
Sema5A		Mouse			Lung
Sema5B					
Sema6A		Human	Mouse		
Sema6B					
Sema6C	Mouse		Mouse		
Sema6D	Mouse	Mouse	Mouse		
Sema7A	Mouse	Mouse, Human	Human	Human	

Table 3. Results of EST analysis. Blue font indicates semaphorins that were successfully amplified in the subject of this research project. Red font indicates semaphorins that were dropped from the project. Black font indicates semaphorins we did not attempt to amplify.

Gene	Age	Tissue	Figures	Expression pattern
Sema4A	16 weeks	Thymus	1.1 (page 36) 1.2 (page 37)	cortex
		Spleen	1.3 (page 38)	follicles in white pulp
	6 weeks	Thymus	1.4 (page 39)	outer cortex
		Lymph node	1.5 (page 40)	lymphoid follicles
Sema4D	16 weeks	Thymus	2.1 (page 41)	cortex
		Spleen	2.2 (page 42)	follicles in white pulp
	6 weeks	Thymus	2.3 (page 43)	outer cortex
		Lymph node	2.4 (page 44) and (page 45)	cortex and around efferent lymphatic vessel on HEV
Sema4F	16 weeks	Thymus	3.1 (page 46) 3.2 (page 47)	cortex
		Spleen	3.3 (page 48)	follicles in white pulp
	6 weeks	Thymus	3.4 (page 49)	medulla and inner cortex
		Lymph node	3.5 (page 50)	medullary region with cortical extensions
Sema7A	16 weeks	Thymus	4.1 (page 51) 4.2 (page 52)	cortex
		Spleen	4.3 (page 53)	follicles in white pulp
	6 weeks	Thymus	4.4 (page 54)	inner cortex
		Lymph node	4.5 (page 55)	lymphoid follicles

Table 4. Expression patterns of semaphorins 4A, 4D, 4F, and 7A observed in mouse lymphoid tissue in this research project.

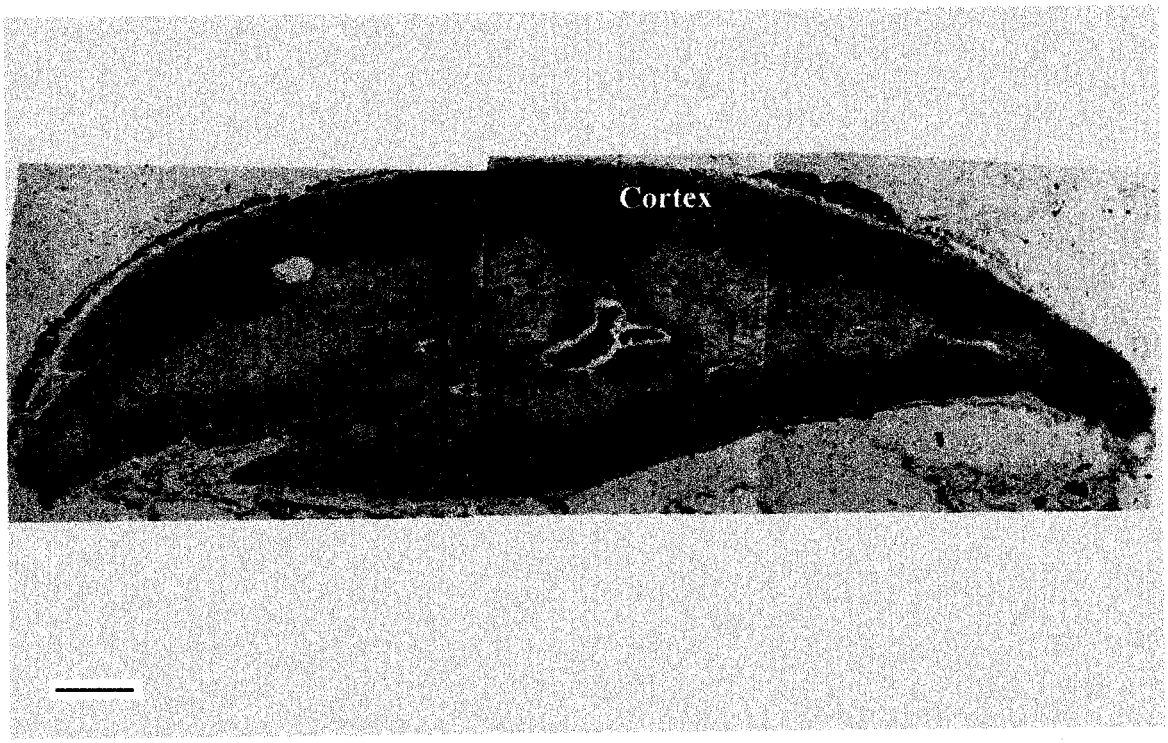

Figure 1.1a. Sema4A antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 µm.

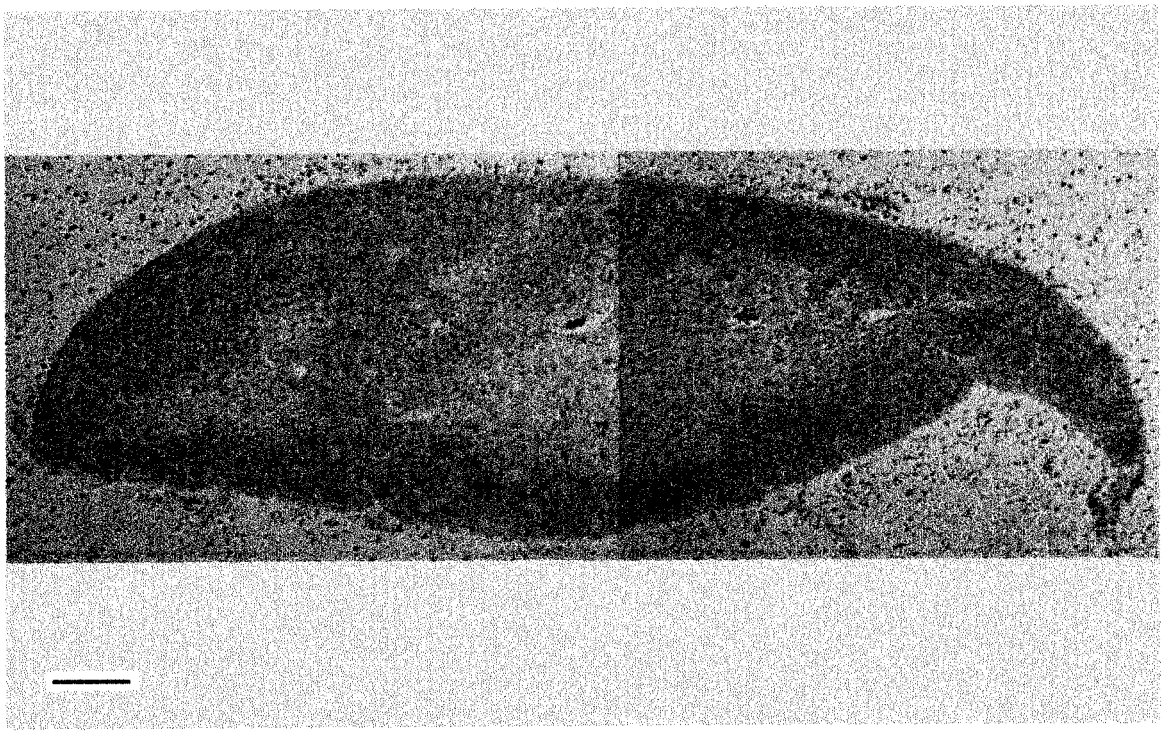

Figure 1.1b. Sema4A sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 µm.

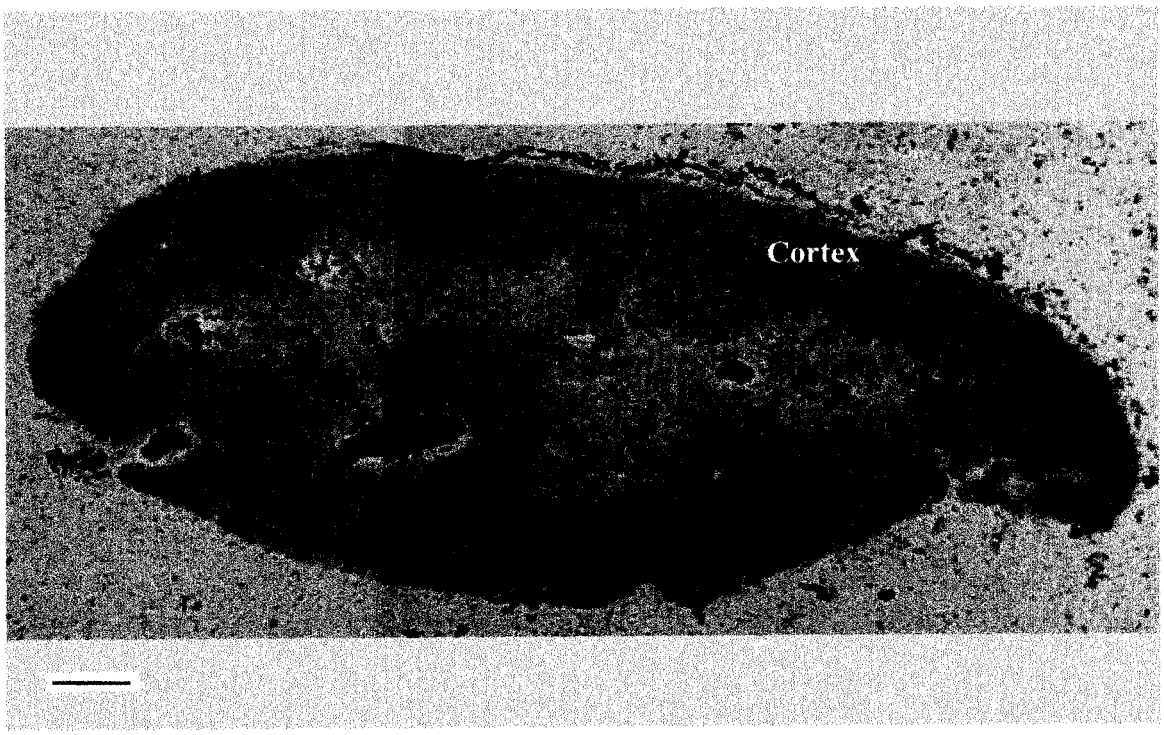

Figure 1.2a. Sema4A antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 µm.

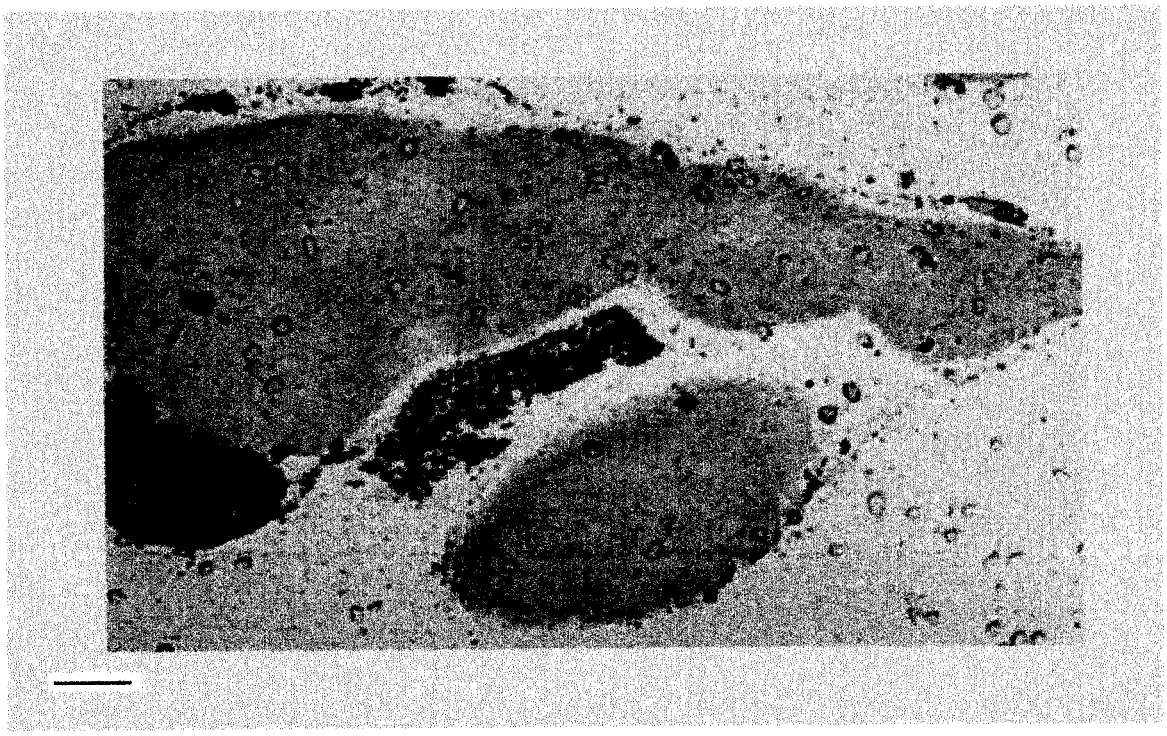

Figure 1.2b. Sema4A sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 µm.

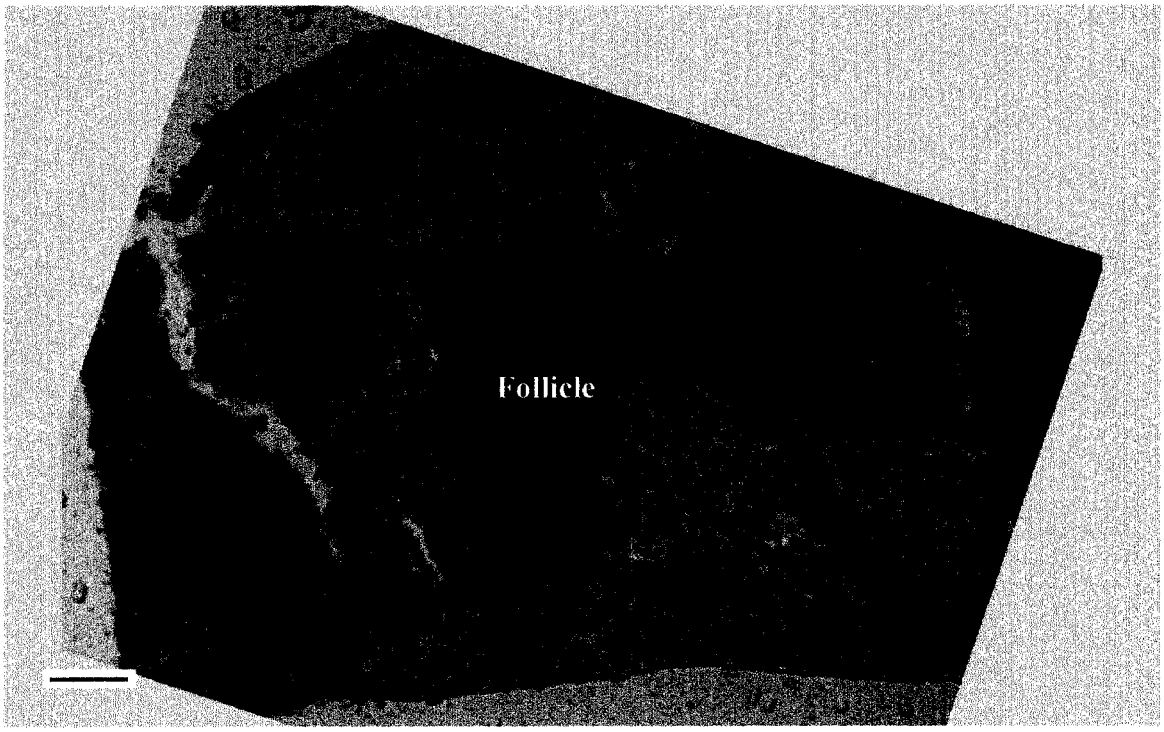

Figure 1.3a. Sema4A antisense probe. Spleen, 16 weeks. Follicular expression. Scale bar, 250 μm.

Figure 1.3b. Sema4A sense probe. Spleen, 16 weeks. No signal. Scale bar, 250 μm.

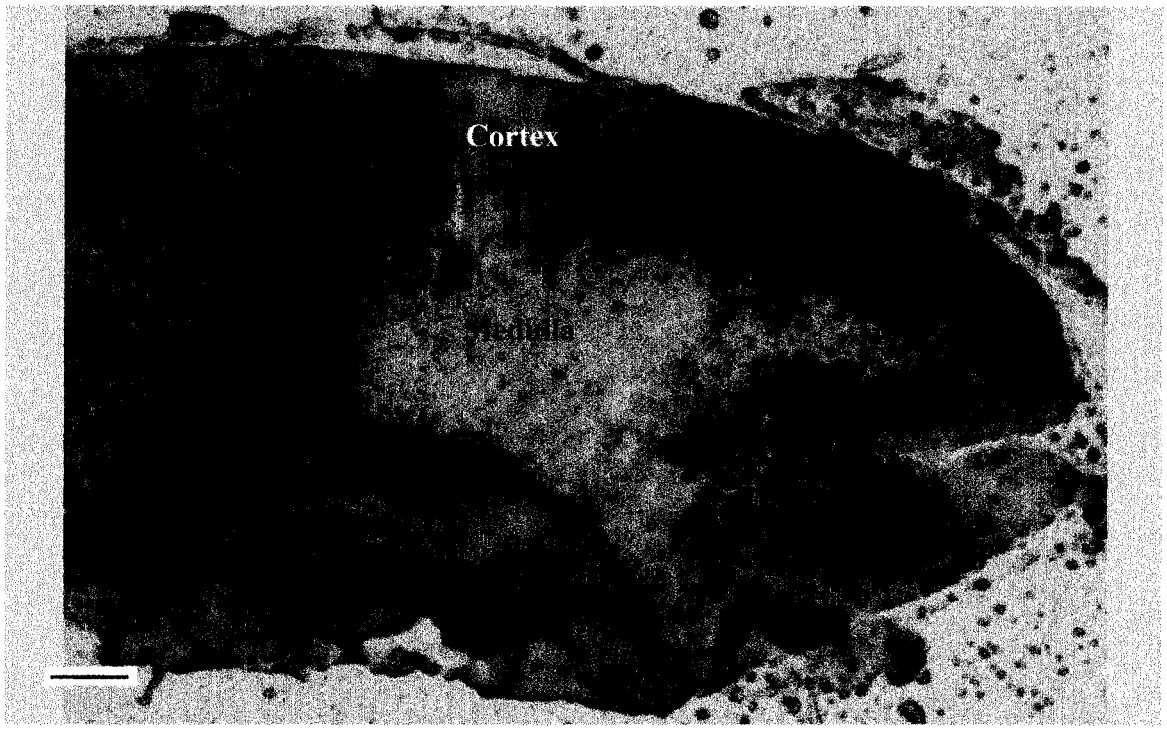

Figure 1.4a. Sema4A antisense probe. Thymus, 6 weeks. Cortical expression. Scale bar, 400 µm.

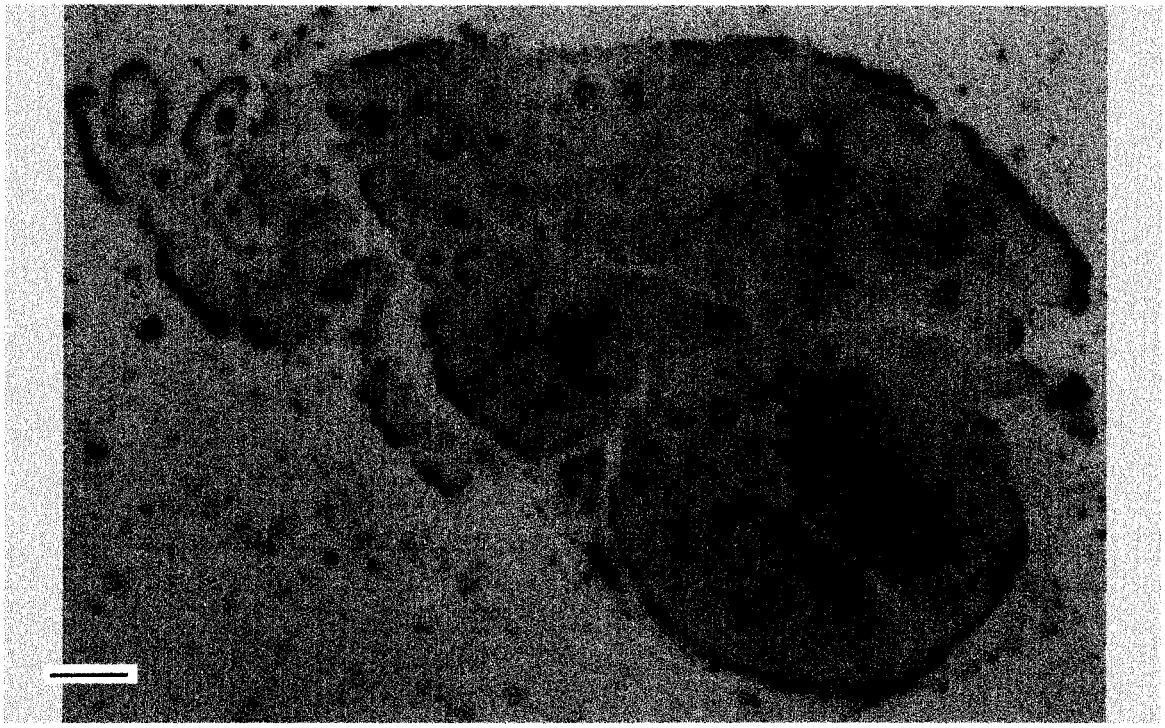

Figure 1.4b. Sema4A sense probe. Thymus, 6 weeks. No signal. Scale bar, 400 µm.

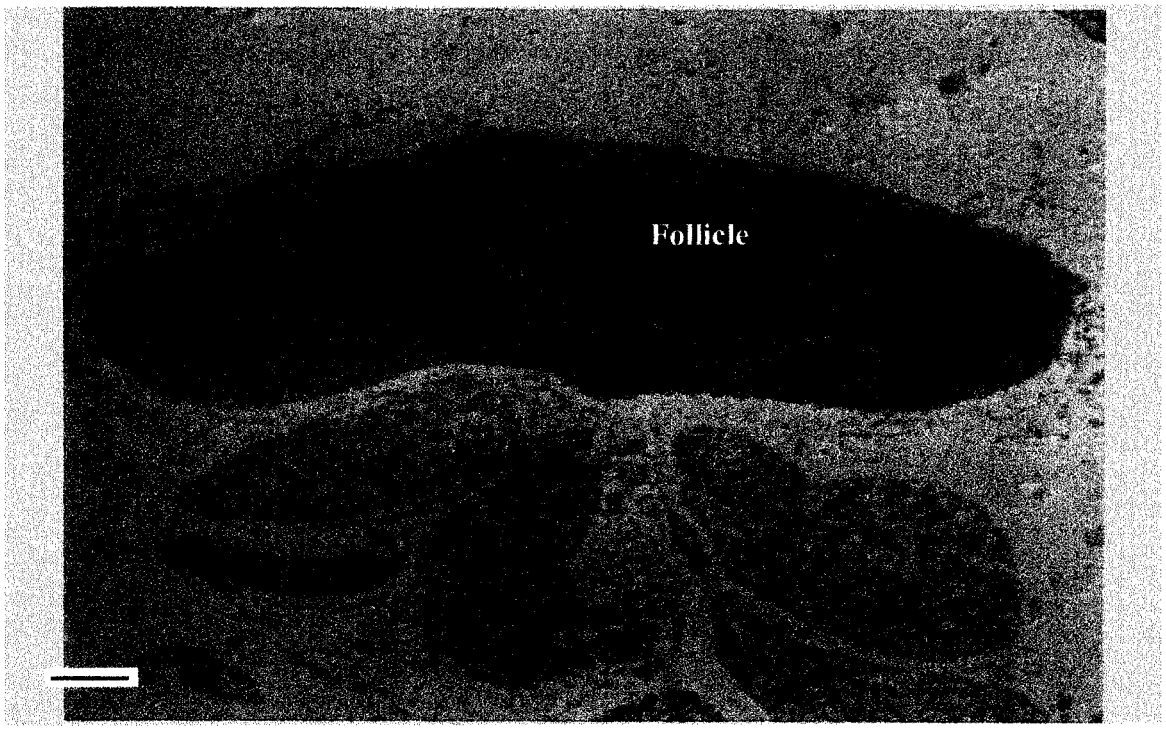

Figure 1.5a. Sema4A antisense probe. Lymph node, 6 weeks. Follicular expression. Scale bar, 400 µm.

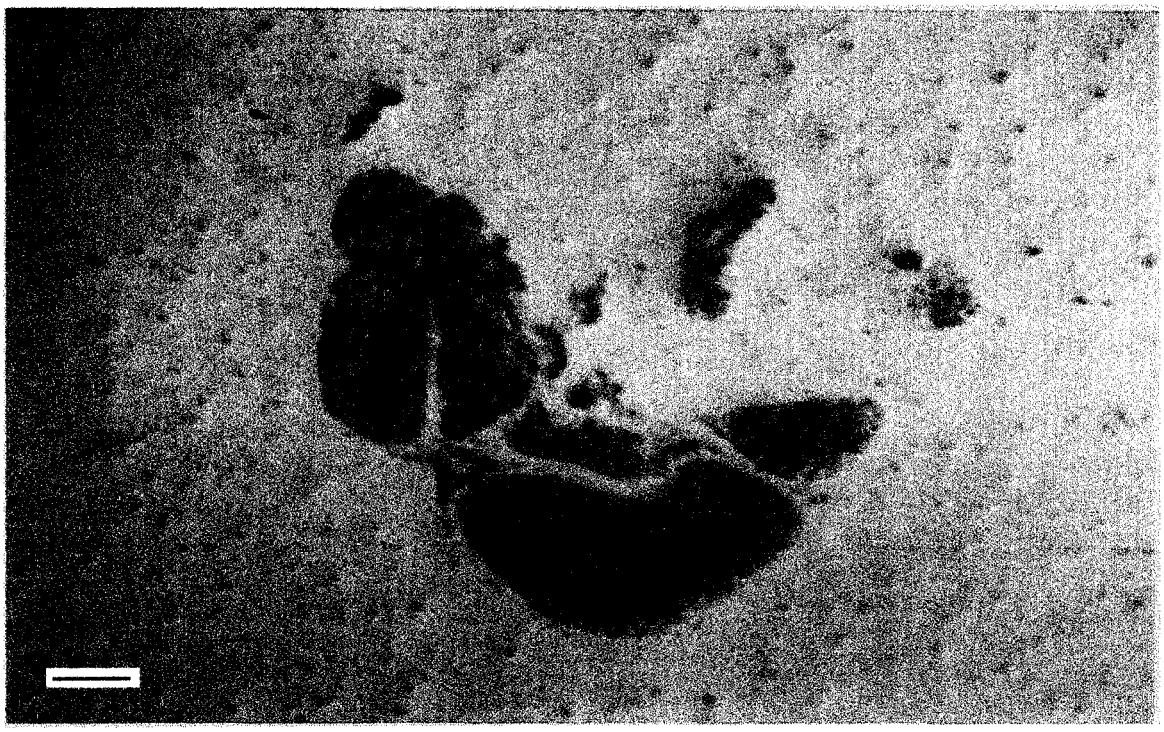

Figure 1.5b. Sema4A sense probe. Lymph node, 6 weeks. No signal. Scale bar, 250 µm

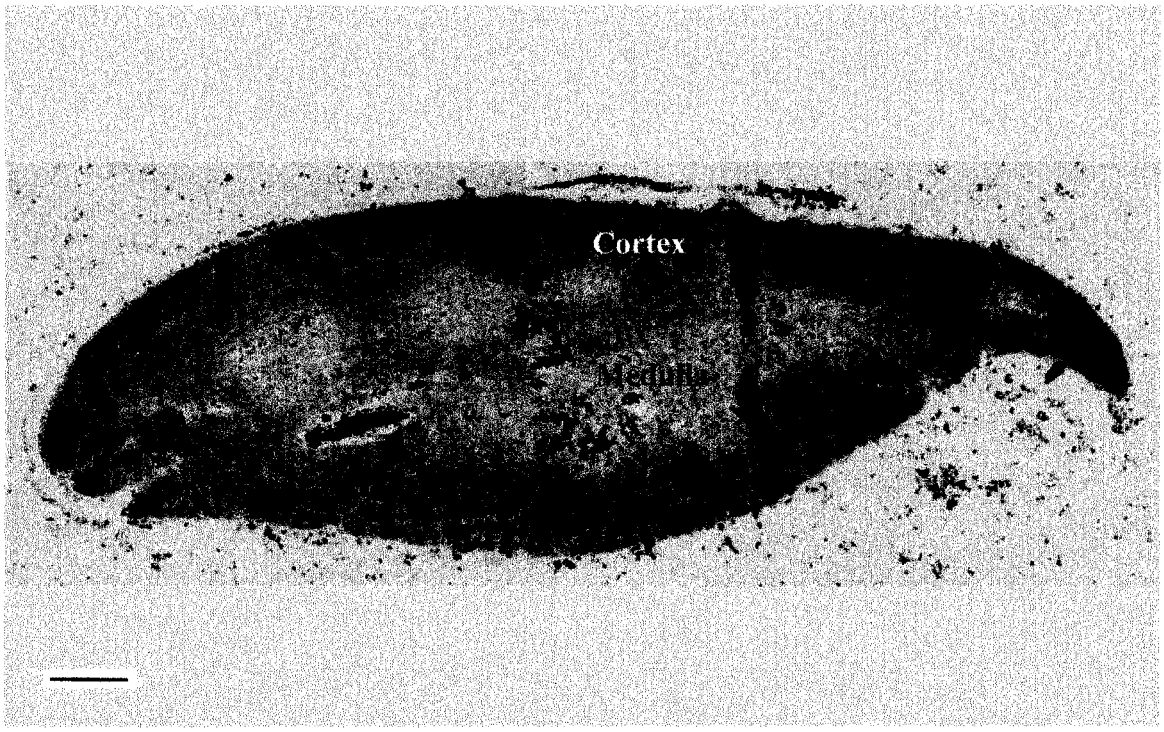

Figure 2.1a. Sema4D antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 μm.

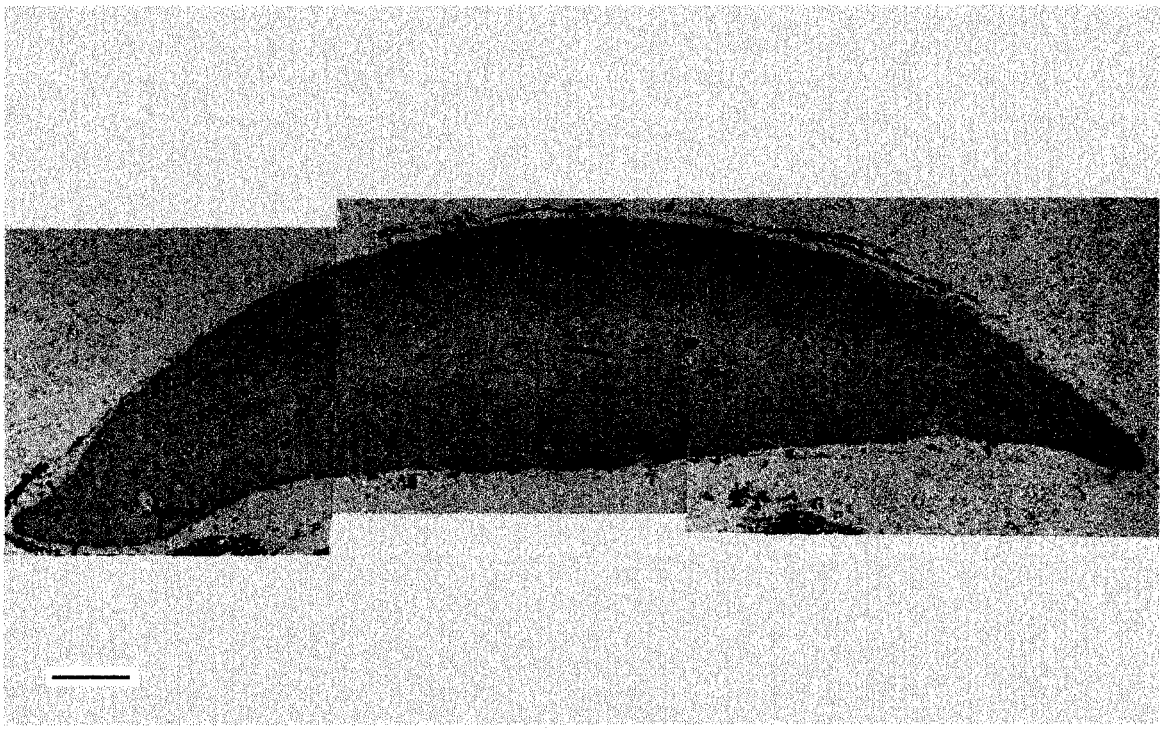

Figure 2.1b. Sema4D sense probe. Thymus, 16 weeks. No signal. Scale bar, 400 μm.

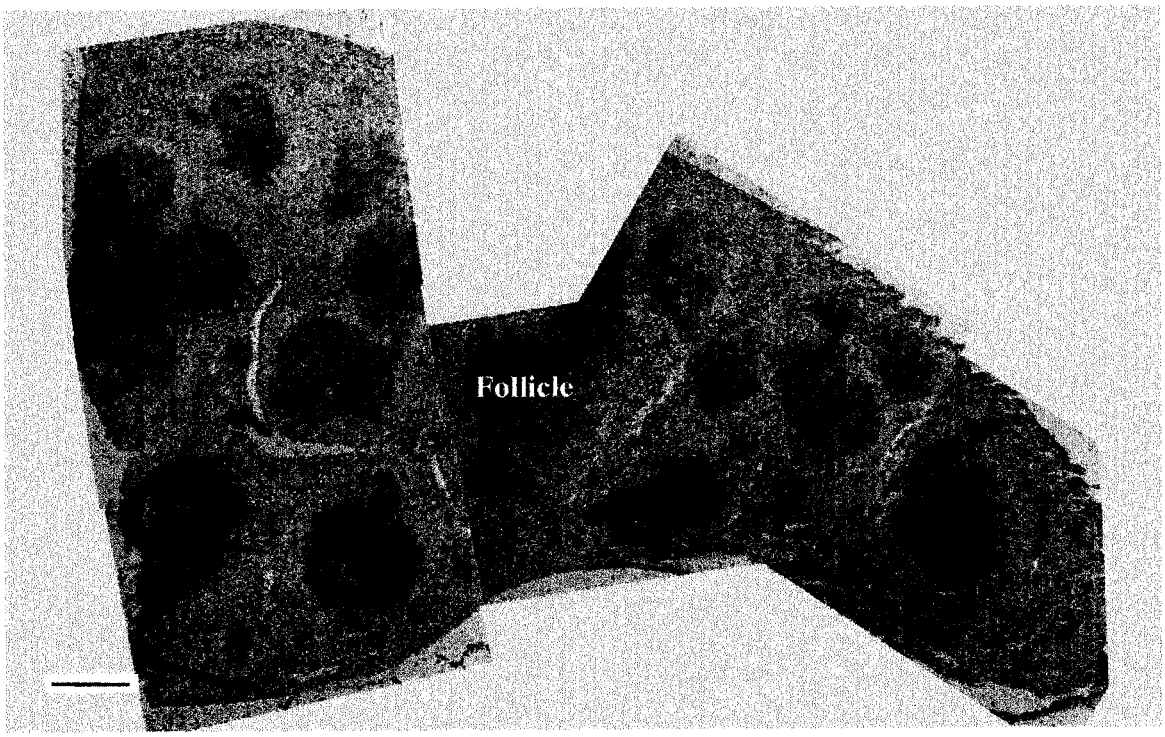

Figure 2.2a. Sema4D antisense probe. Spleen, 16 weeks. Follicular expression. Scale bar, 250 µm.

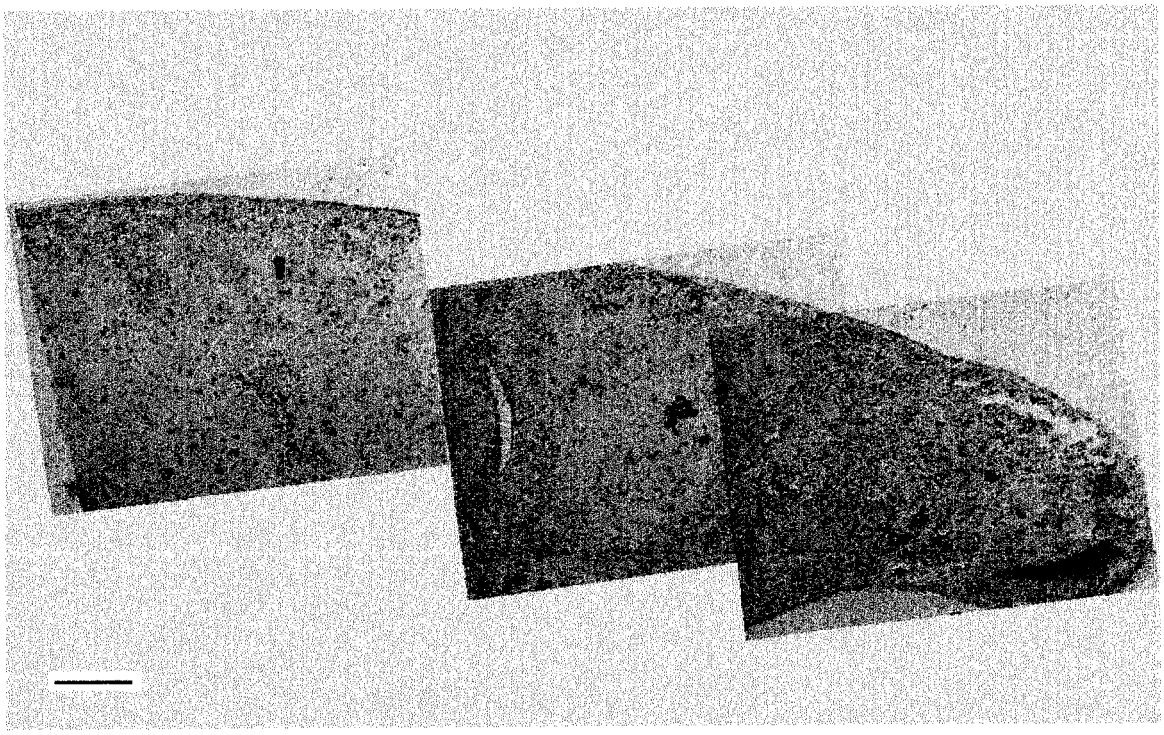

Figure 2.2b. Sema4D sense probe. Spleen, 16 weeks. No signal. Scale bar, 250 µm.

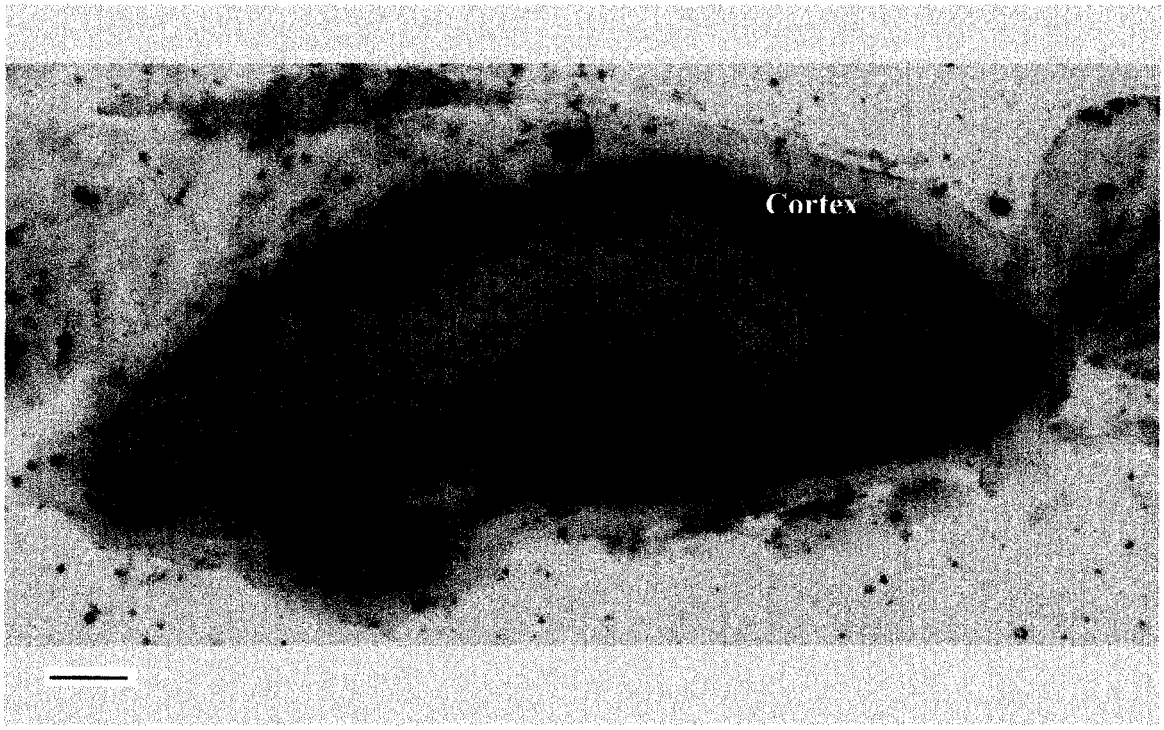

Figure 2.3a. Sema4D antisense probe. Thymus, 6 weeks. Cortical expression. Scale bar, 250 µm.

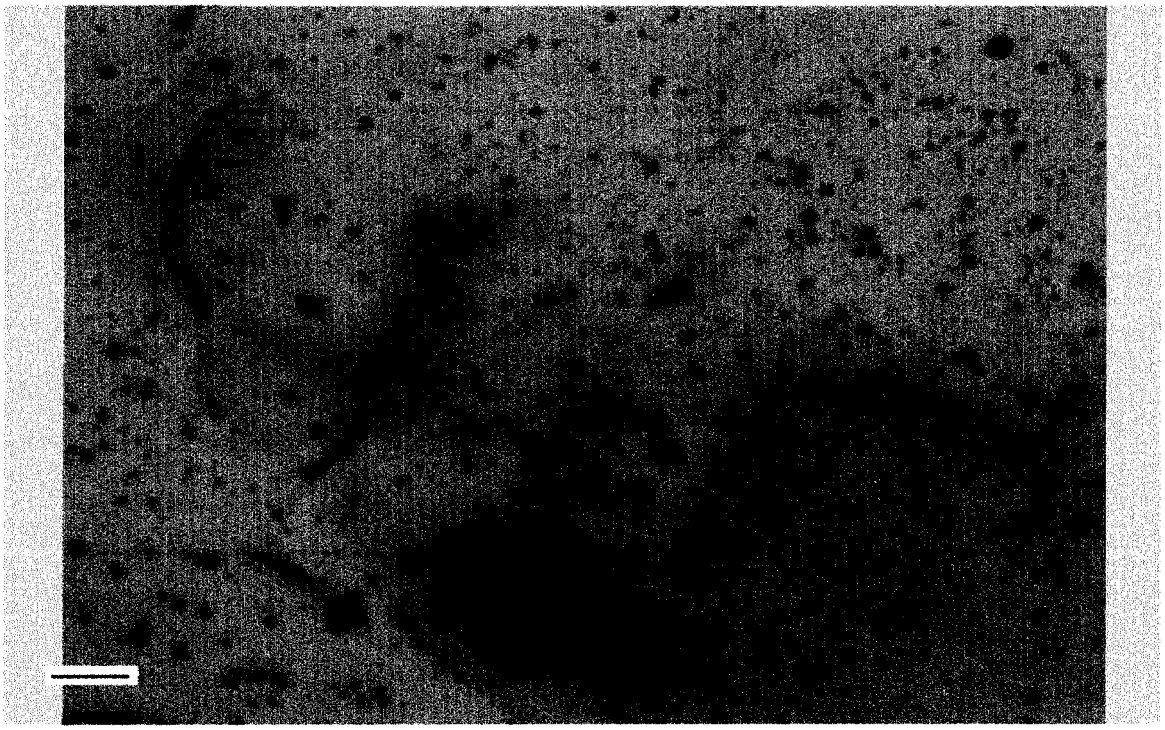

Figure 2.3b. Sema4D sense probe. Thymus, 6 weeks. No signal. Scale bar, 400 µm.

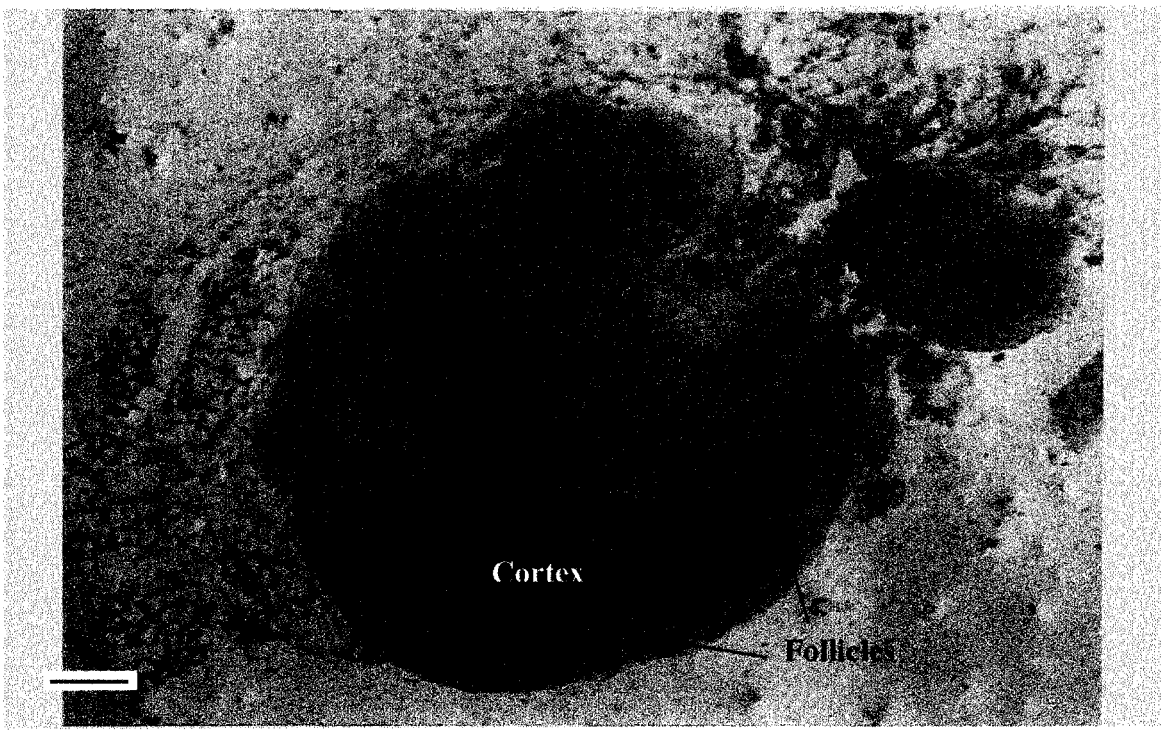

Figure 2.4a. Sema4D antisense probe. Lymph node, 6 weeks. Cortical expression. Scale bar, 400 µm.

Figure 2.4b. Sema4D sense probe. Lymph node, 6 weeks. No signal. Scale bar, 400 µm.

Figure 2.4c. Sema4D antisense probe. Lymph node, 6 weeks. Expression around efferent lymphatic vessel on HEV. Scale bar, 2000 μm.

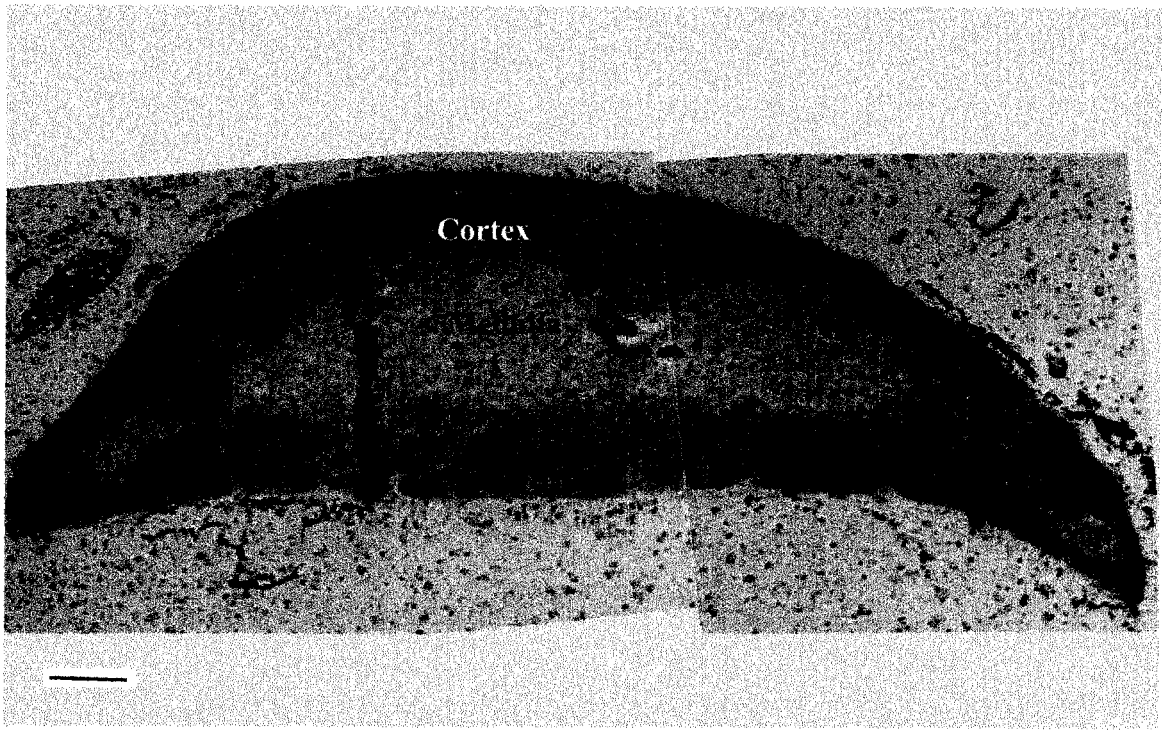

Figure 3.1a. Sema4F antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 μm.

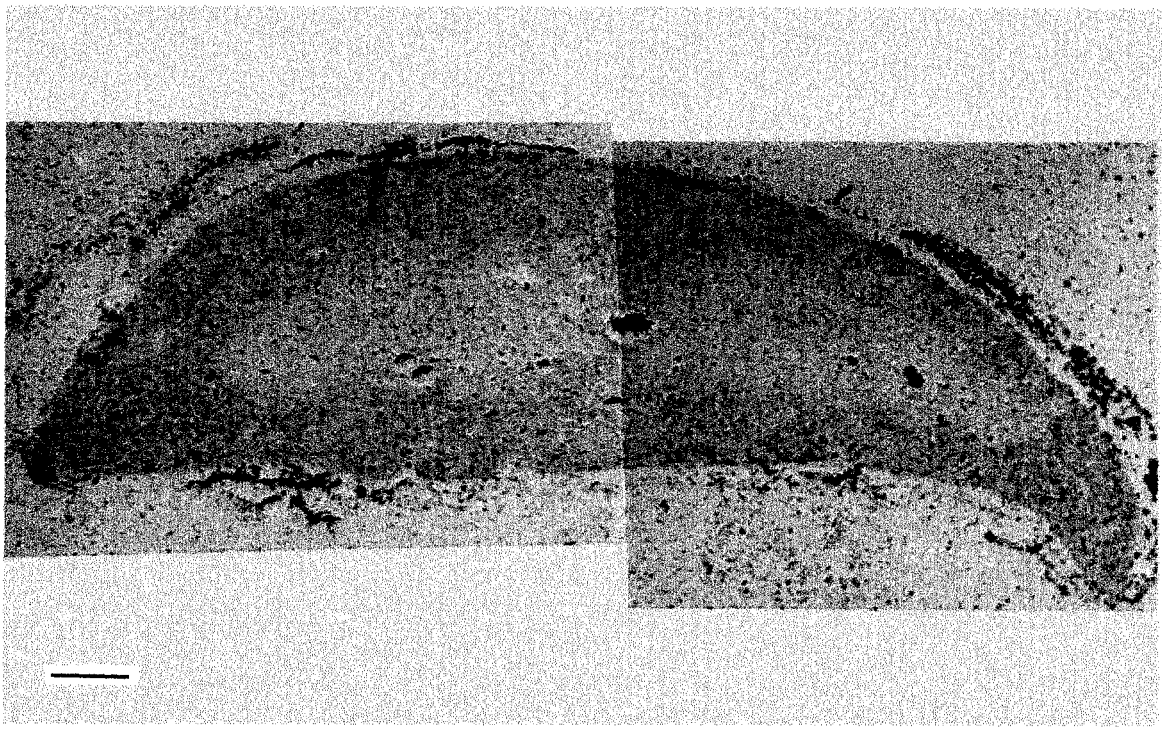

Figure 3.1b. Sema4F sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 μm.

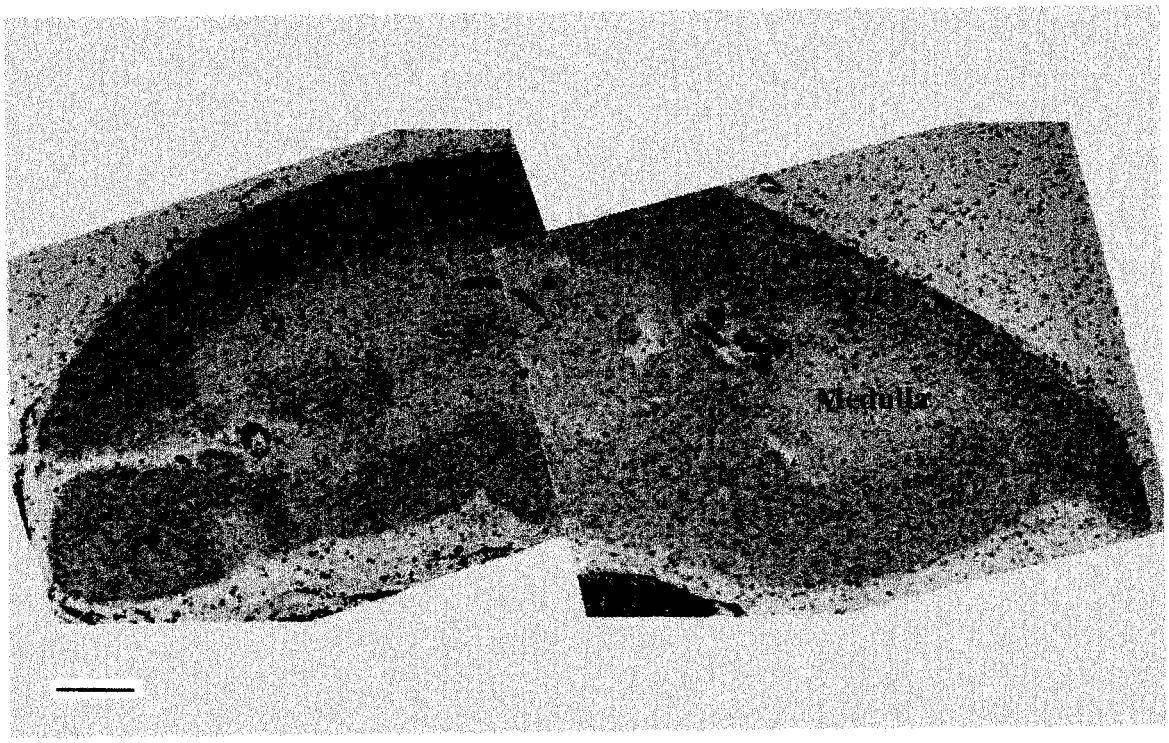

Figure 3.2a. Sema4F antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 μm.

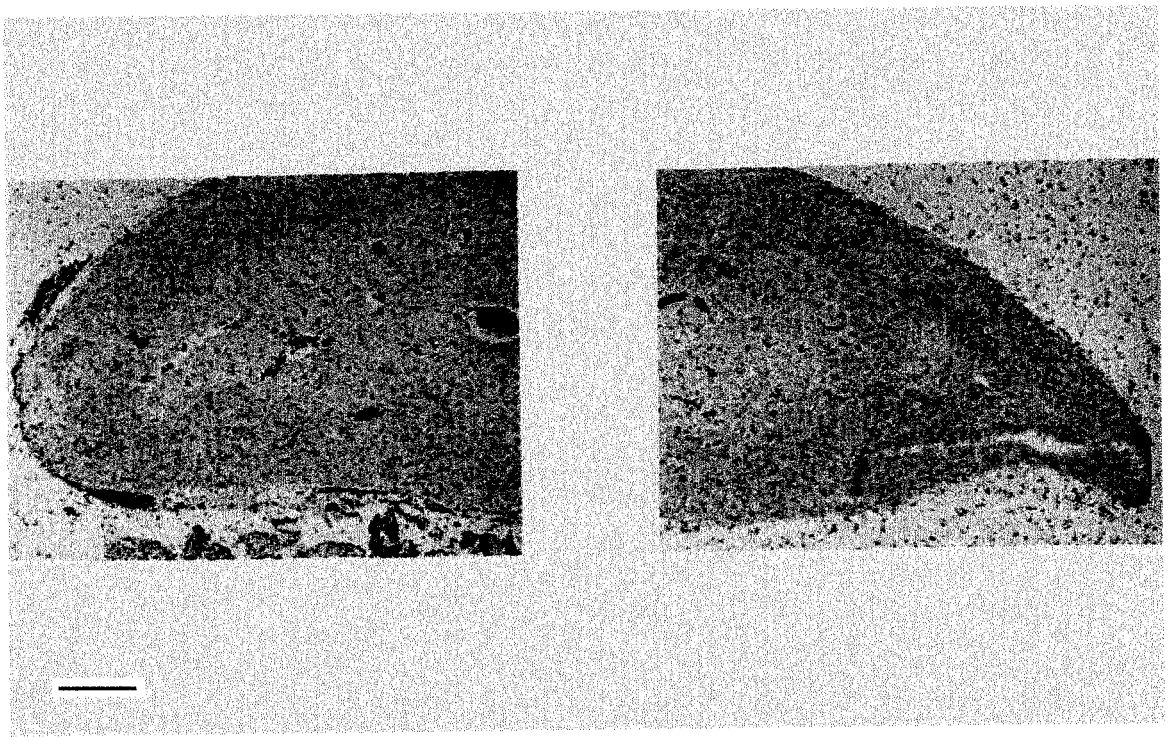

Figure 3.2b. Sema4F sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 μm.

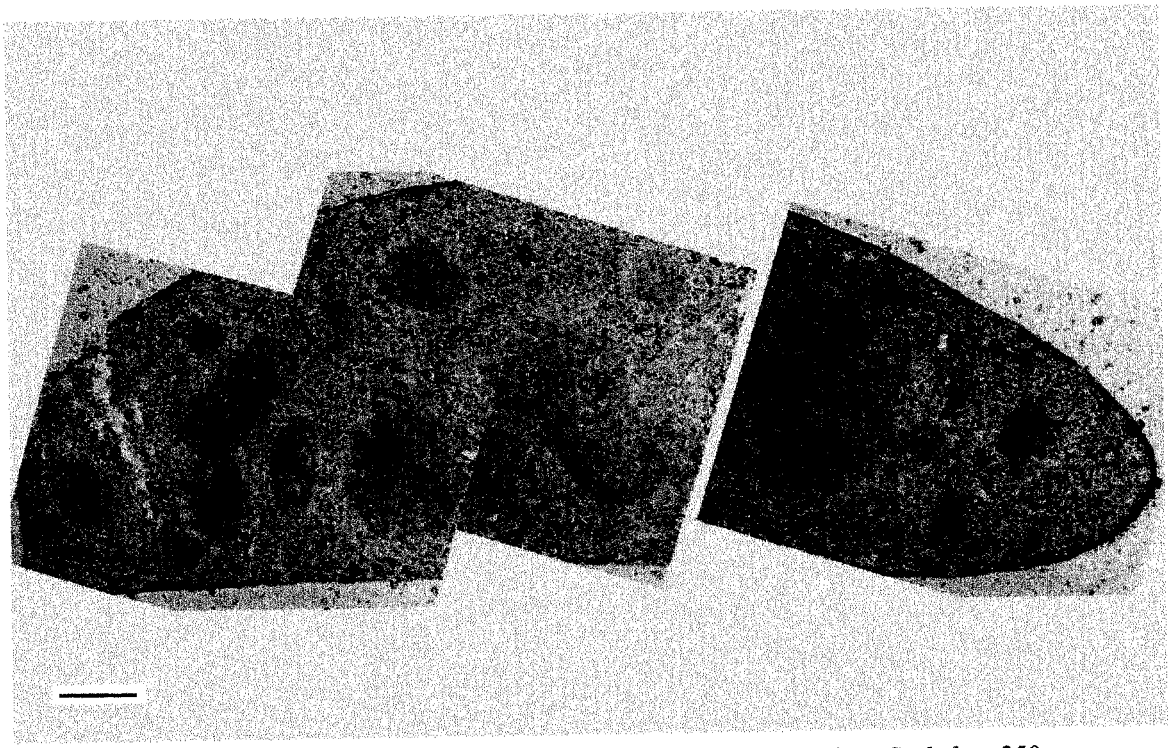

Figure 3.3a. Sema4F antisense probe. Spleen, 16 weeks. Follicular expression. Scale bar, 250 µm.

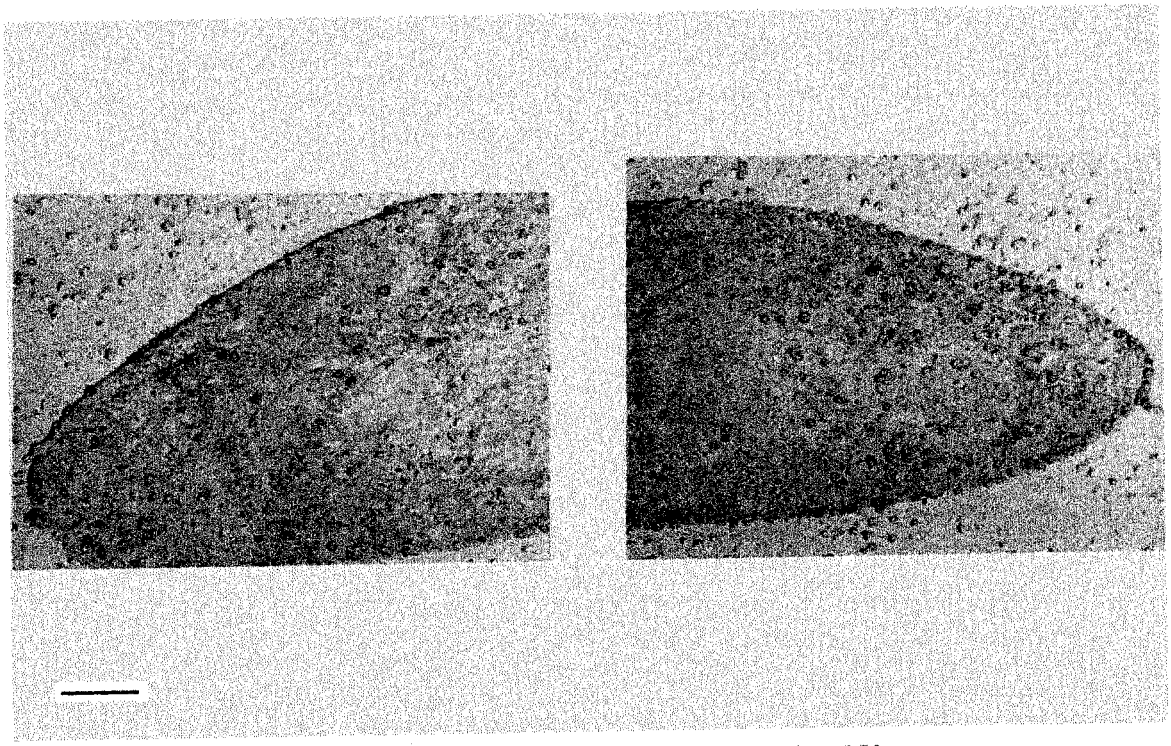

Figure 3.3b. Sema4F sense probe. Spleen, 16 weeks. No signal. Scale bar, 250 µm.

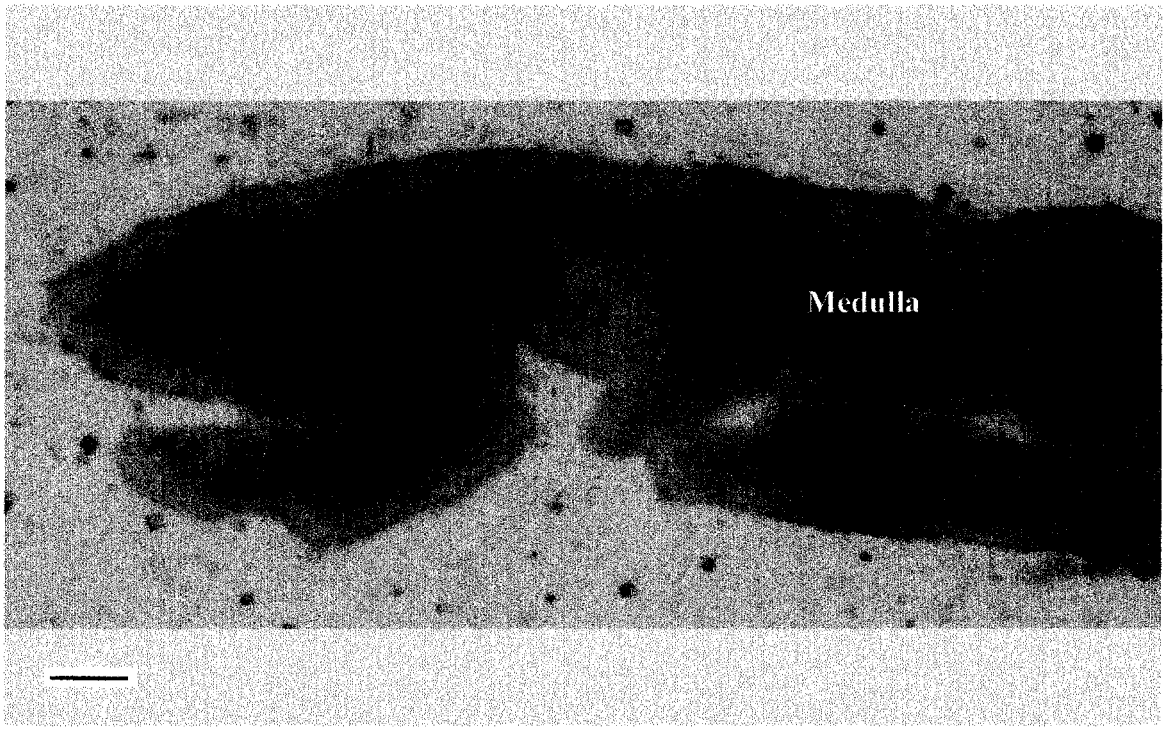

Figure 3.4a. Sema4F antisense probe. Thymus, 6 weeks. Medullary expression. Scale bar, 400 μm.

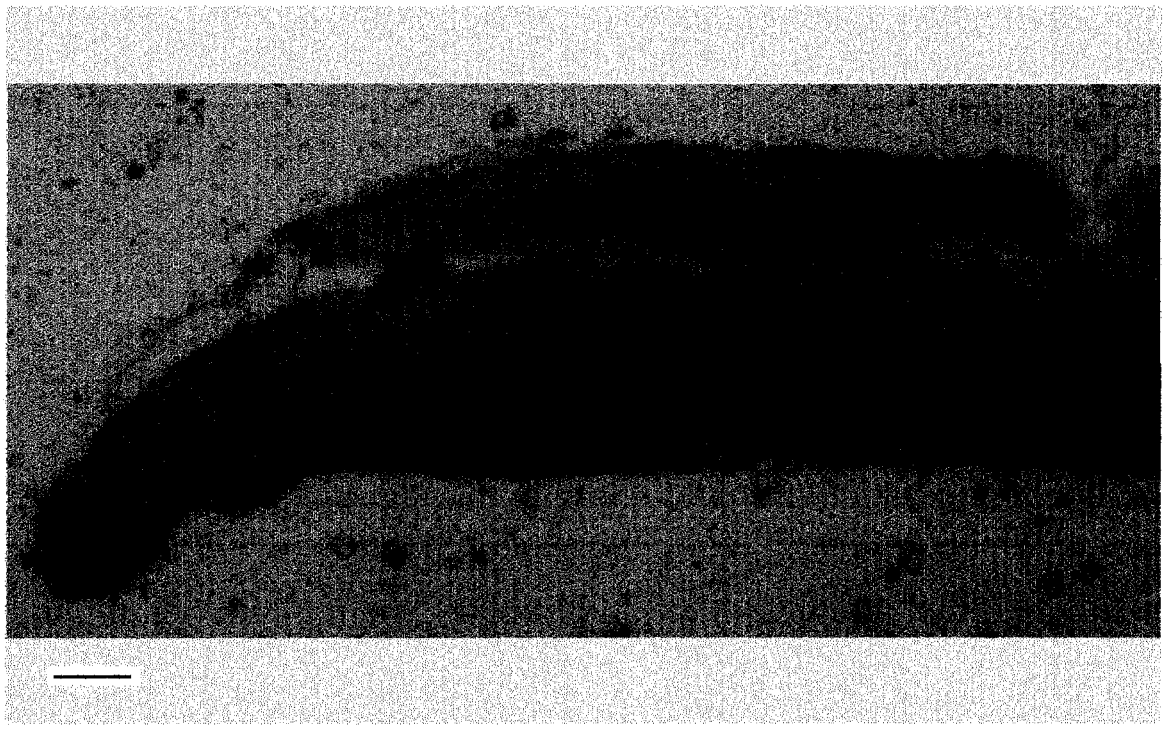

Figure 3.4b. Sema4F sense probe. Thymus, 6 weeks. No signal. Scale bar, 400 μm.

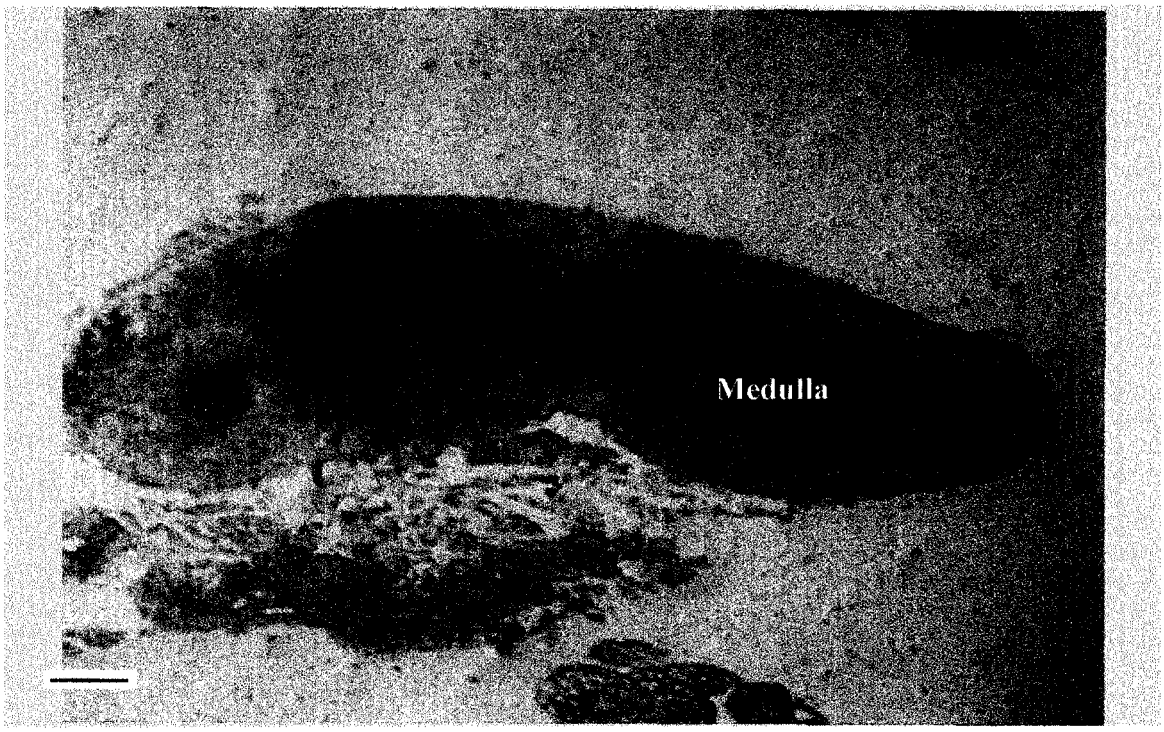

Figure 3.5a. Sema4F antisense probe. Lymph node, 6 weeks. Medullary expression. Scale bar, 250 µm.

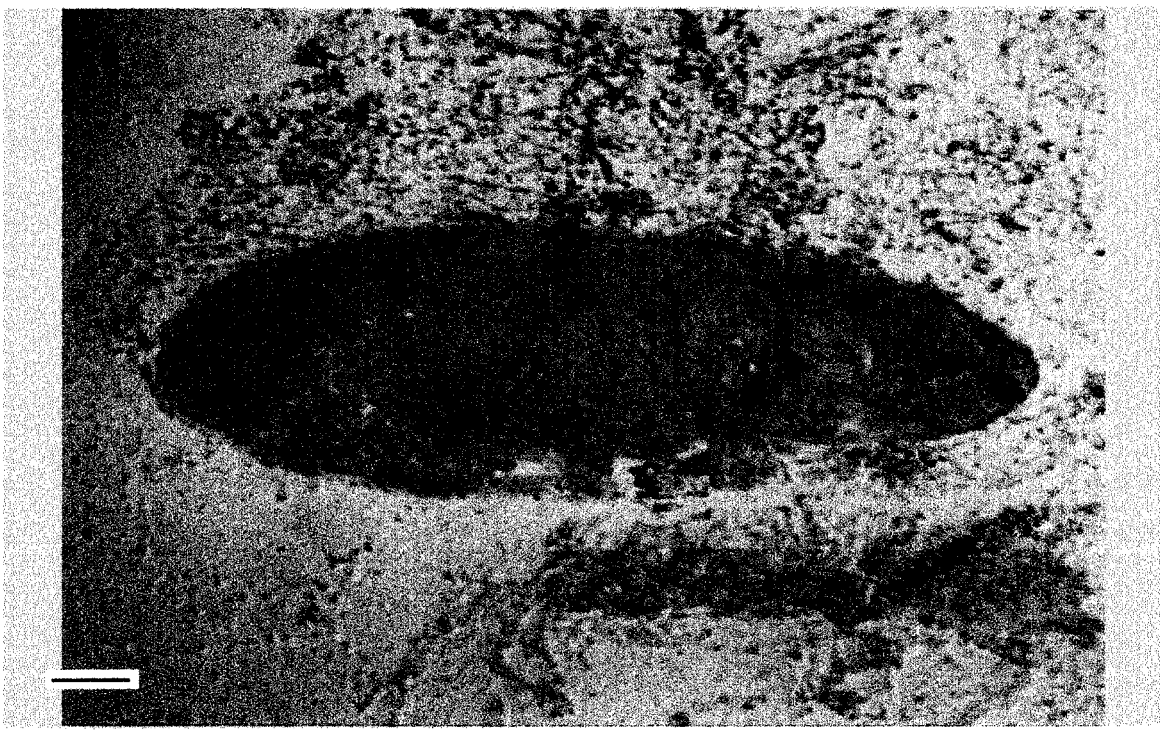

Figure 3.5b. Sema4F sense probe. Lymph node, 6 weeks. No signal. Scale bar, 250 µm.

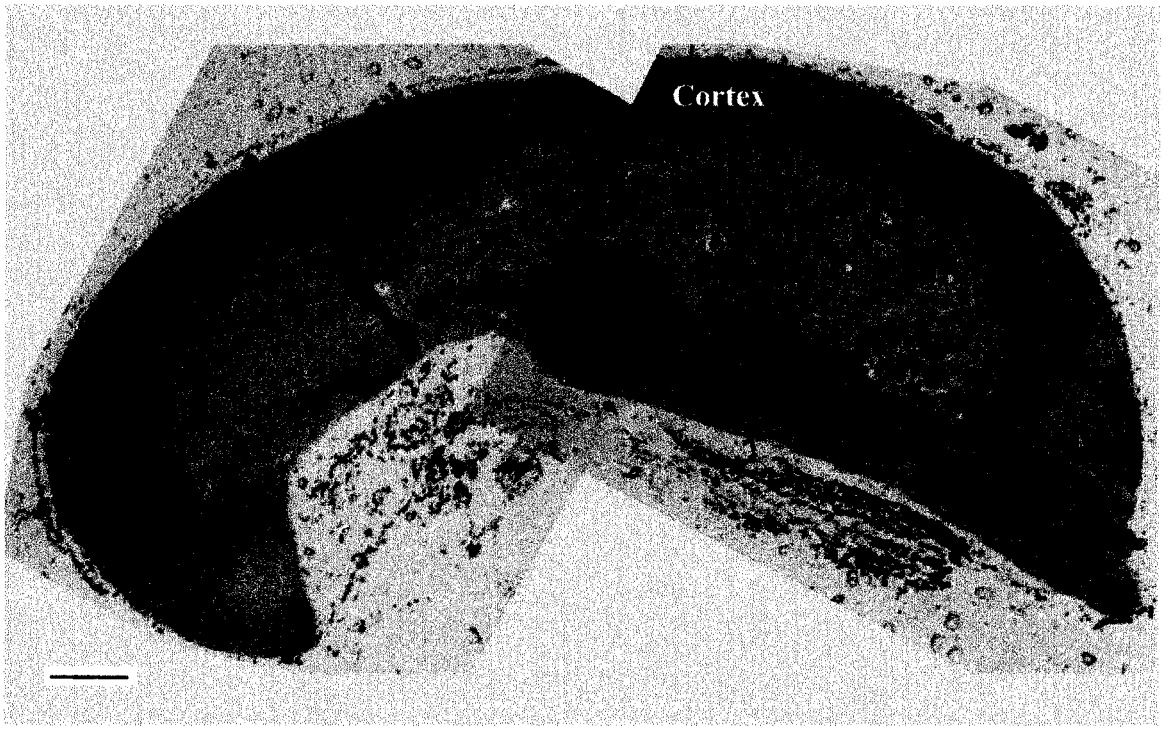

Figure 4.1a. Sema7A antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 µm.

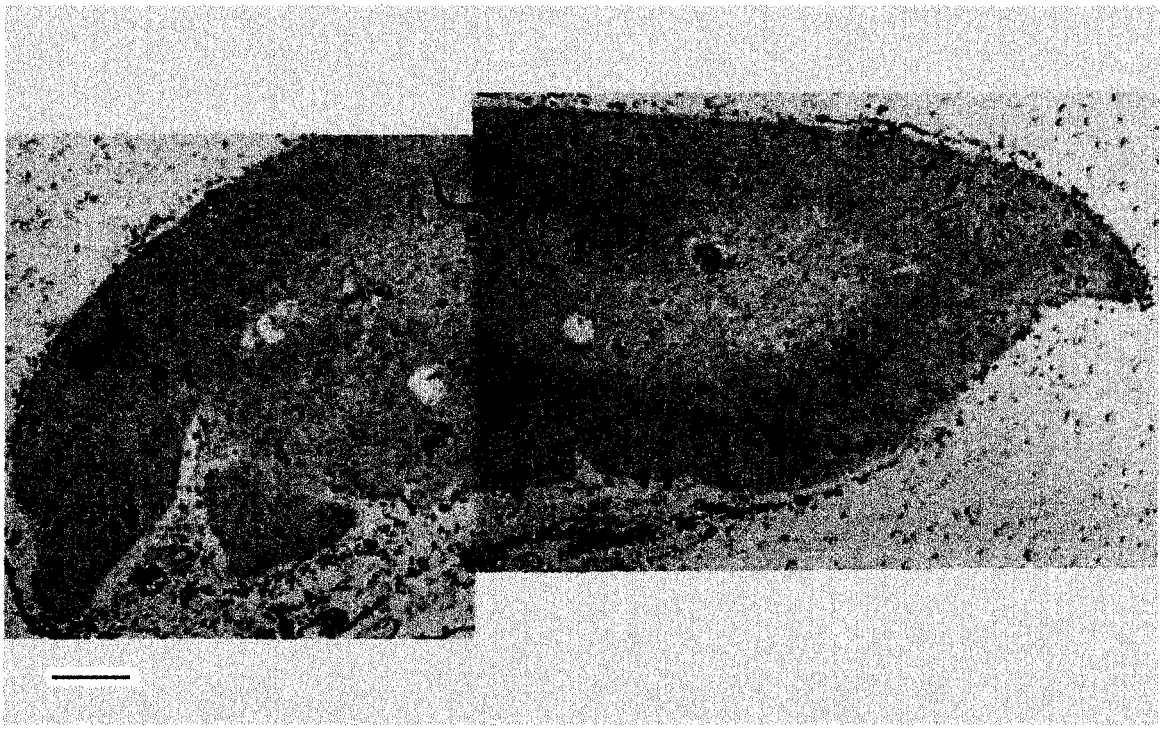

Figure 4.1b. Sema7A sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 µm.

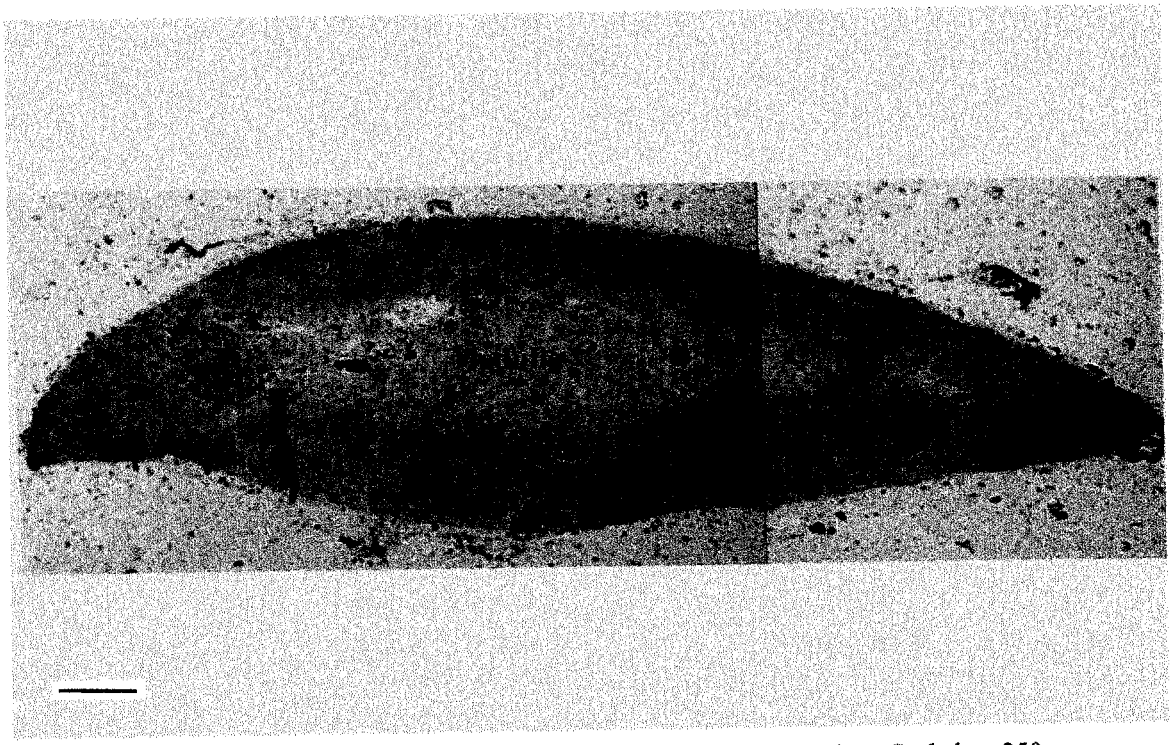

Figure 4.2a. Sema7A antisense probe. Thymus, 16 weeks. Cortical expression. Scale bar, 250 µm.

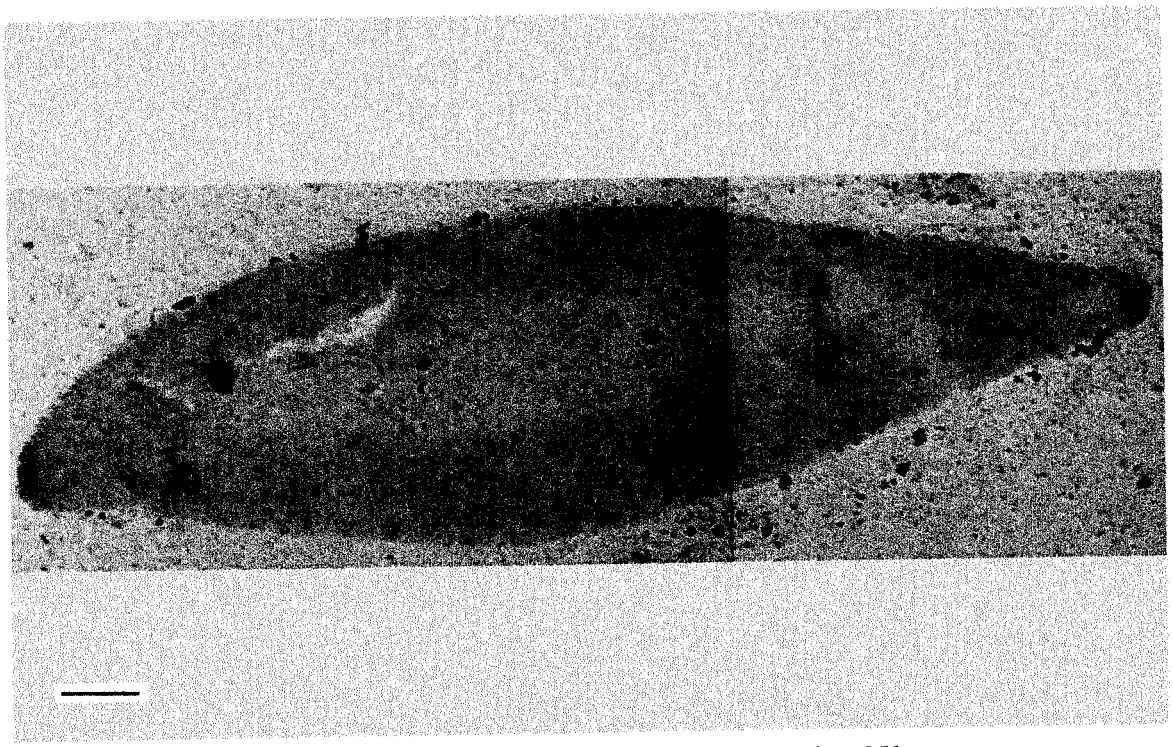

Figure 4.2b. Sema7A sense probe. Thymus, 16 weeks. No signal. Scale bar, 250 µm.

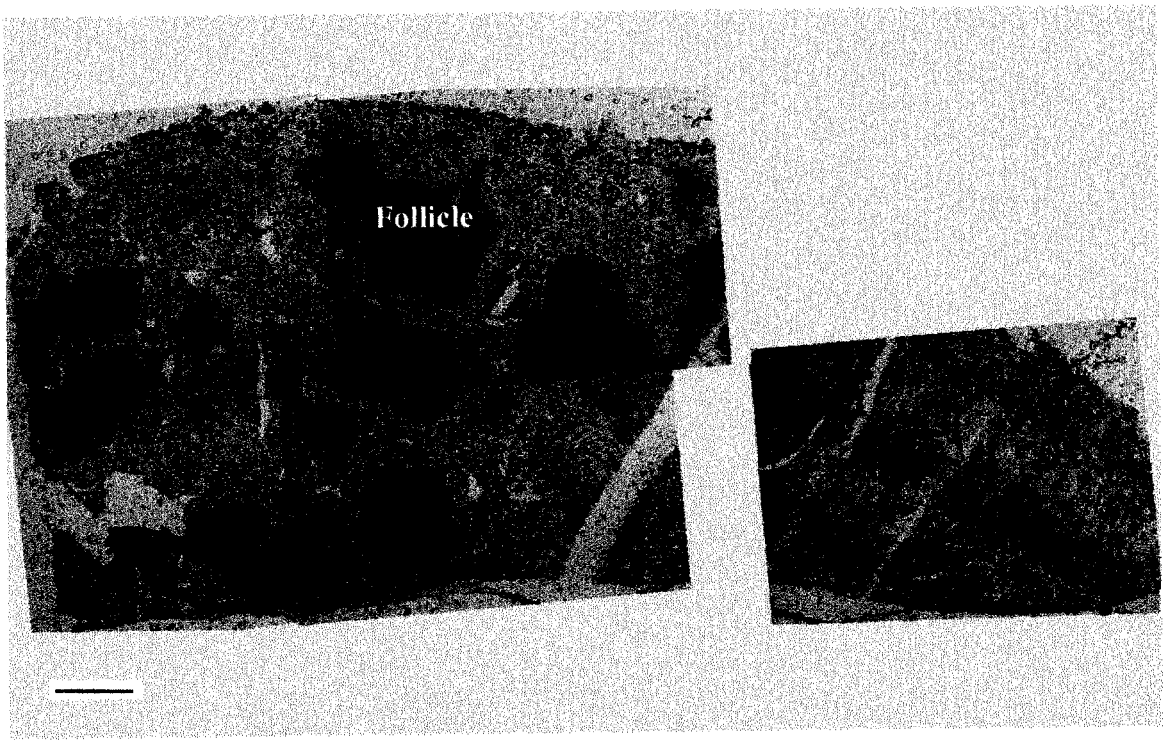

Figure 4.3a. Sema7A antisense probe. Spleen, 16 weeks. Follicular expression. Scale bar, 250 μm.

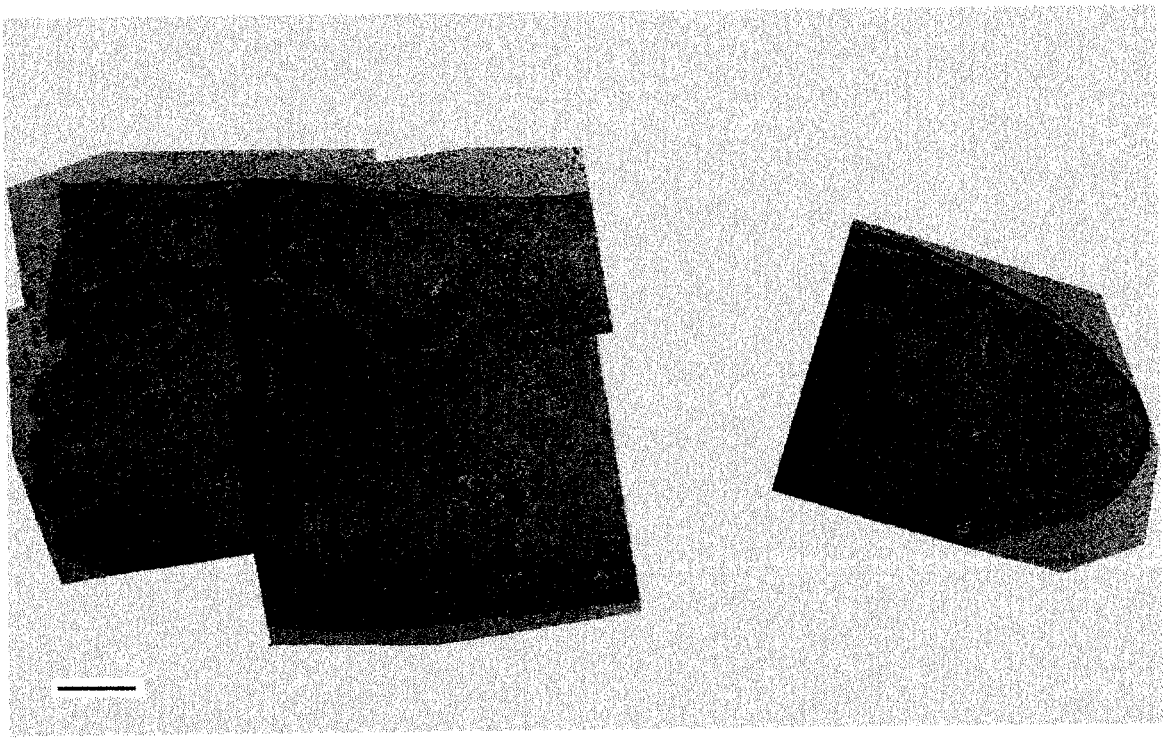

Figure 4.3b. Sema7A sense probe. Spleen, 16 weeks. No signal. Scale bar, 250 μm.

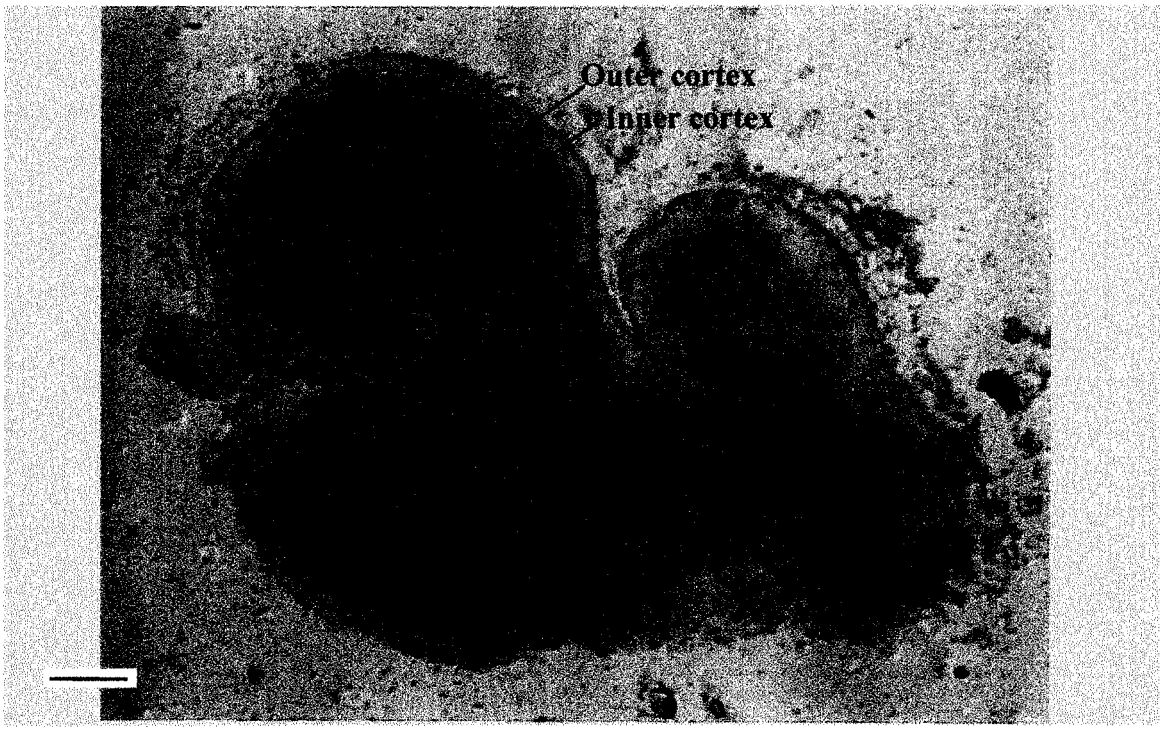

Figure 4.4a. Sema7A antisense probe. Thymus, 6 weeks. Inner cortical expression. Scale bar, 250 µm.

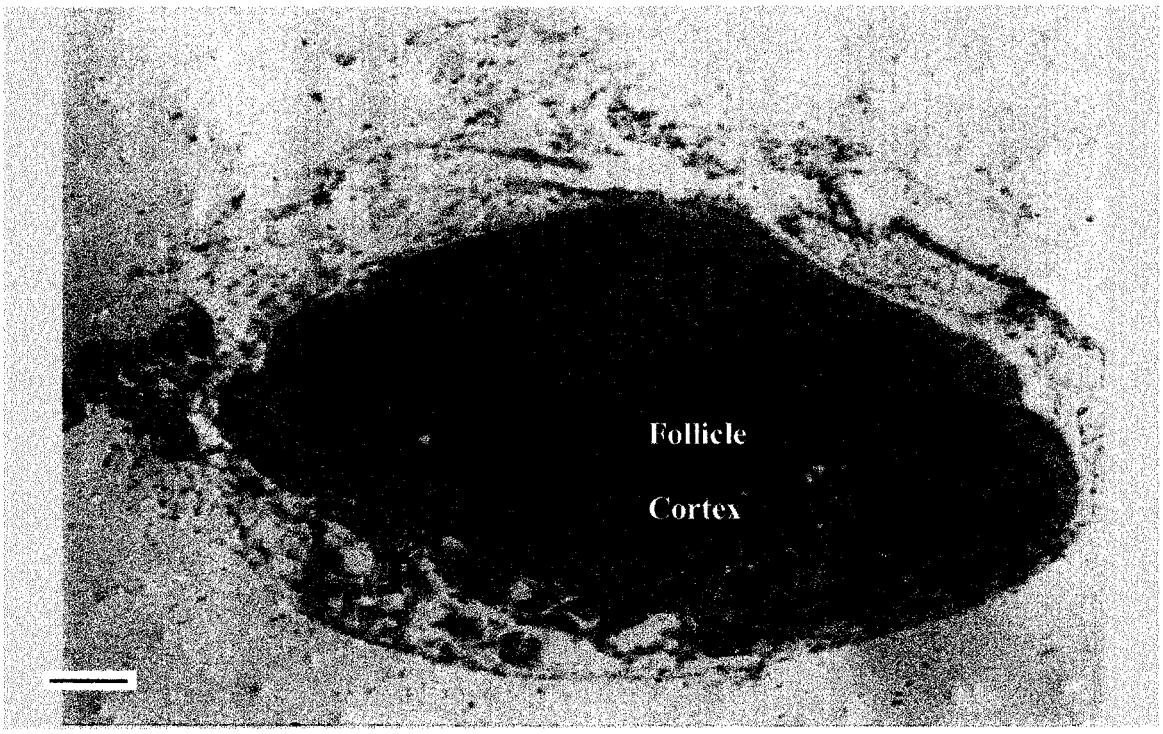

Figure 4.5a. Sema7A antisense probe. Lymph node, 6 weeks. Follicular expression. Scale bar, 400 µm.

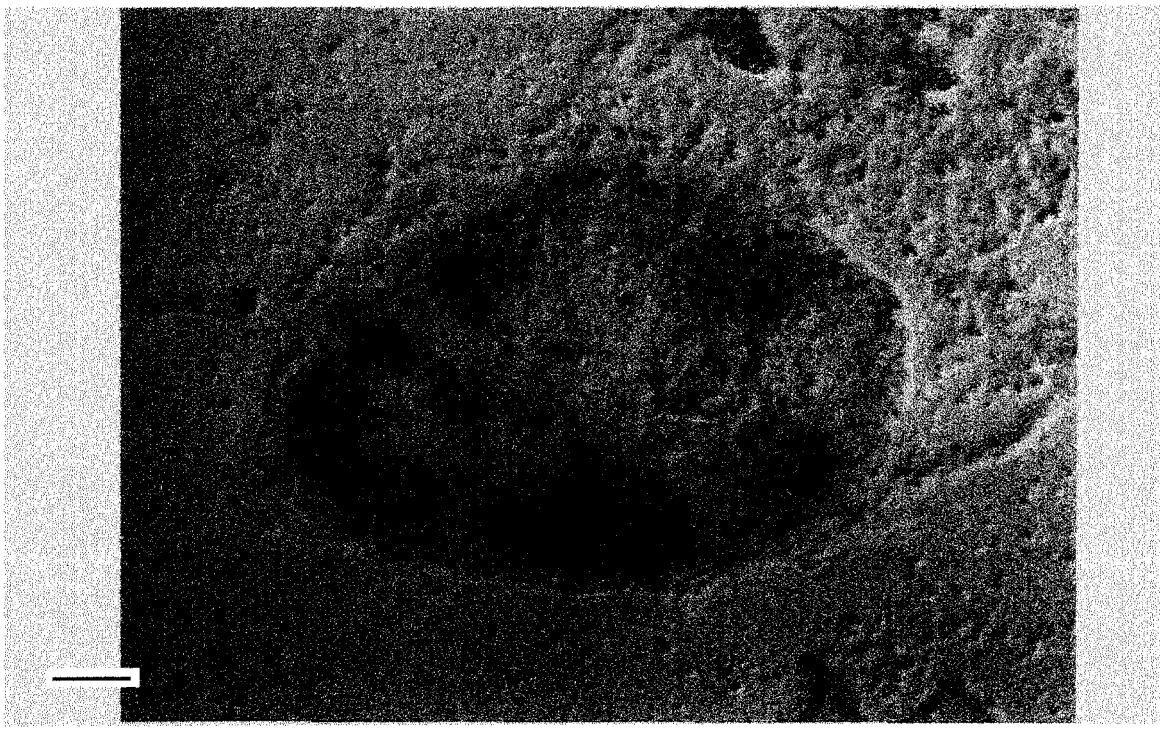

Figure 4.5b. Sema7A sense probe. Lymph node, 6 weeks. No signal. Scale bar, 400 µm.

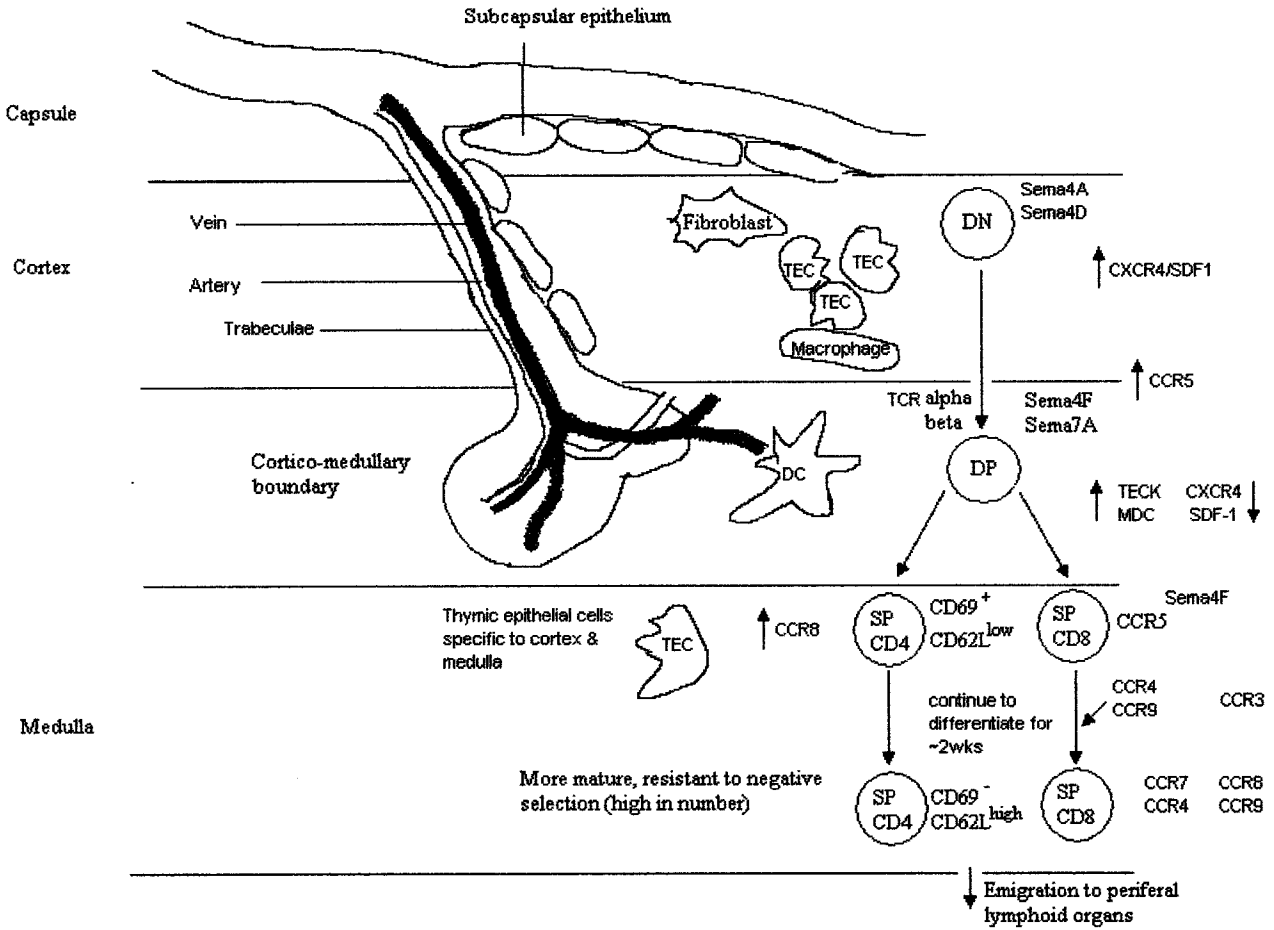

Figure 5. Thymus architecture with various chemokines and semaphorins 4A, 4D, 4F, 7A expression as determined in our work

DISCUSSION

Semaphorins 4A, 4D, 4F, and 7A expression was observed in thymus, spleen, and lymph nodes in 6- and 16-week old mice using DIG-labeled RNA probes. The expression patterns we have found for semaphorins 4A, 4D, 4F, and 7A in our experiments suggest a role in the developmental migration of the T and B lymphocytes within thymus, spleen, and lymph nodes. The migration of lymphocyte subsets to specific tissue microenvironments is known to be regulated by the differentially expressed adhesion molecules and chemokines.[24, 25, 42, 43]

Semaphorin expression could be influenced by several phenomena. Semaphorins might be working with chemokines to guide precursor cells to the diverse microanatomical sites in bone marrow and thymus to ensure proper progenitor cell development. Semaphorins in the selective microenvironments of thymus, spleen, and lymph nodes might antagonize the chemokine effect on lymphocytes thereby restricting their movement to certain regions of the tissue. Alternatively, semaphorins could be responsible for the reduced sensitivity to chemokines thereby helping the lymphocytes to move to a different region within a tissue. Finally, semaphorins could be working with chemokines to attract the lymphocytes to regions within lymphoid organs. To understand the specific roles that semaphorins may be playing in guiding developing leukocytes, it is necessary to understand the migration pattern of developing lymphocytes through the lymphoid organs in mice.

Thymus

In the mature fully vascularized thymus (Figure 5) subcapsular thymocytes

migrate to mid-cortical, cortico-medullarym, and finally medullary regions.[48, 49] This migration is associated with the development of thymocytes from immature precursors to mature T lymphocytes, which eventually leave the thymus to populate peripheral lymphoid organs. After the T cell progenitors enter the thymic parenchyma, early DN cells move towards the cortical sub-capsular region. The SDF-1/CXCR4 ligand/receptor pair may direct the DN cells to the outer cortex or promote their association with sub cortical stromal elements after arrival.[29, 30] Loss of expression of CXCR4 signaling facilitates the movement of maturing thymocytes away from the outer cortex. Our results show that Sema4A and Sema4D are expressed in the outer cortex. Presence of these two semaphorins in the outer cortex could be responsible for the decrease in the expression of CXCR4 or for the repulsive signals to the DN thymocytes, or for the reduced sensitivity to CXCR4 thereby facilitating the DN thymocytes to move towards the inner cortex following the attractive cues by CCR5. CCR5 expression increases at the DN to DP transition. Positive selection and DN to DP transition is accompanied by marked changes in chemokine receptor expression and function[27] (Figure 5).

Following positive selection, thymocytes migrate across the cortico-medullary boundary where they come in contact with increased numbers of bone marrow-derived dendritic cells and medullary epithelial cells. At this stage semaphorins along with chemokines might be facilitating association of the DP cells with APC or directing thymocytes to associate with medullary stromal cells that deliver signals for further maturation. It has been suggested that the loss of responsiveness to SDF-1 may facilitate the departure of positively selected DP thymocytes from the cortex.[29-31] Sema7A is

shown by our work to be expressed in the inner cortex. Mine et al[66] later found dominant expression of Sema7A on the DP subset of T cells. They also noted that approximately 50% of the DP subsets expressed Sema7A and its expression on the other subsets ($CD4^{bright}$ $CD8^{dull}$, CD4 SP or CD8 SP and DN) was almost negative.[66] T cells at the stage of thymic selection that are $CD34^{+}$ $CD117^{+}$ preferentially expressed Sema7A. Taken together these results suggest that Sema7A expression is coincident with cell proliferation or activation during T cell development at the stage of positive selection in thymus.[66]

Sema7A expression by the DP T cells might directly or indirectly be responsible for the loss of responsiveness to SDF-1 thereby facilitating the movement of DP T cells towards the medulla or Sema7A could be playing a role in association of DP T cells with antigen-presenting cells. DP cells acquire responsiveness to MDC as they mature to $CD69^{+}$ DP cells and it continues with progression through $CD4^{+}8^{low}$ intermediates, to immature $CD69^{+}CD62L^{low}$CD4 SP cells.[29, 64, 65] After entering the medulla, SP thymocytes continue to differentiate from immature $CD69^{+}CD62L^{low}$ stage to a more mature $CD69^{-}CD62L^{high}$ stage.[32, 34] The more mature SP cells are resistant to negative selection and they increase in number before they emigrate to the periphery.[32, 33] Sema4F, expressed in the medulla of the 6-week old thymus, could be sending repulsive signals to mature SP T cells, thereby facilitating their movement through postcapillary venules. The recent finding that viral semaphorin homolog A39R inhibits primary mouse dendritic cells from migrating to various chemokines *in vitro* supports our hypothesis that semaphorins play an important role in the regulation of leukocyte movement.[63]

Thymus architecture undergoes a great change with age in mice. The thymus reaches its maximal size at puberty and from then on decreases in size, with a significant decrease in both cortical and medullary cell populations, and increase in the total fat content of the organ. The cortex and medulla no longer have their clear seperation. This explains the variation in the expression patterns in 16-week old mice.

Lymph Node

Naive B cells, T cells, and dendritic cells enter the lymph node through HEV in the paracortex where we have shown Sema4D expression. Expression of Sema4D in HEV could suggest a role in physiological activation of lymphocytes in HEV for adhesiveness and its expression in paracortex could be explained by the presence of naive T cells which were shown by others to express Sema4D at high levels.[46] Upon their entry into the lymph node B cells, T cells, and dendritic cells are attracted by a gradient of chemokines to specific localizations within the lymph node both during homeostasis and immune response.[69, 76-78] B cells in particular move to follicles under the influence of chemokine gradients. Expression of Sema4A and Sema7A in follicles shown by our experiments could be explained by the presence of B cells in the follicles, since Sema4A is expressed by resting B cells.[13] Naive B cells become activated by antigen and primed T cells, at the edge of the T zone and follicles. Activated B cells then migrate to follicles, form germinal centers and undergo affinity maturation or migrate to medullary cords, differentiate into plasmablasts and then into plasma cells.[68, 79] Sema4F expression in the medulla, could be related to B cell differentiation or could be because of its expression by activated B cells. Many of the plasma cells generated in medullary cords die *in situ*, but

the remaining plasmablasts emigrate from the lymph node and settle in different lymphoid and non-lymphoid organs including bone marrow.[80, 81]

Spleen

All the four semaphorins studied in our project were expressed in B cell follicles in the white matter of spleen. Secondary follicles have mature B cells in the germinal center, immature B cells in the mantle and marginal zones. So, it could be said that Sema4A, Sema4D, Sema4F, and Sema7A expressed in follicles of 16-week old mice are involved in B cell maturation or are responsible for B cell localization to the follicles. More studies could be conducted in mice at different ages followed by flow cytometric analysis investigating which B cell subsets express which semaphorins.[41]

Conclusion

The hypothesis is that Sema4A, 4D, 4F, and 7A might act as chemotropic agents along with chemokines to regulate the movement of T cells and B cells during their maturation. For instance, the tissues and the regions of tissues that are avoided by a particular T cell subtype during development are expected to express the semaphorin that inhibits or repels that T cell subtype specifically. If a semaphorin attracts a T cell subtype then, that semaphorin is expected to be found in tissues and in regions of tissues that interact with that particular T cell subtype. The data obtained from our research along with published literature on the functions of the semaphorins 4A, 4D, 4F, and 7A in the immune system support our hypothesis.

Future Scope

Important questions that remain unresolved include the effect of age on the expression patterns of semaphorins in the lymphoid tissues and the effect on the migration of various lymphocytes at different stages of differentiation both in the presence and absence of chemokines specific for each cell type and differentiation.

Developmental northerns of embryonic and neonatal lymphoid tissues from mice could help in understanding the regulation of semaphorin expression with age. These experiments would help understand whether semaphorin expression was age related and would provide further insight into semaphorin functions.

Flow cytometric analysis of various subsets of lymphocytes at different maturation stages will expand our findings and provide more information as which T cell subtype in the thymocyte population is exactly expressing which semaphorin or semaphorin receptor. This could help in further understanding the role of semaphorins in influencing the migration of lymphocytes during their development and their possible interactions with other cells leading to the activation of lymphocytes.

Cell migration assays could be performed *in vitro* to determine the nature of the semaphorin effect on thymocytes at different developmental stages. Assays could be done with semaphorins alone and with semaphorins and various combinations of chemokines known to affect thymocyte movement and maturation. It would be important also to examine expression of semaphorin receptors other than the plexins and neuropilins.

Identification of semaphorin expression (or loss of expression) in adult animals during wound healing, cancer progression, (during vascular recruitment and metastasis) and various viral infections could prove to be very informative in understanding the etiology of these processes and in the design of therapies directed toward the semaphorin signaling system.

Finally, knock out or conditional knock out mice models could be designed for Sema4A, 4F, and 7A to improve our understanding of consequences of loss of semaphorin expression, *in vivo*, which to date has only been determined for Sema3A.

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APPENDIX 1: *Sequencing Results*

Primer sequence is underlined, restriction sites linked to primers are in red font, probe sequence is in **bold** font, SP_6 promoter is in green font.

Sema4A

CCANCGGGCCCTCTAGATGCATGCTCGAGCGGCCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTT**GGA**
AGCTTATCAAGATGGGCAACAGGAGGGAATCTTGGAGTTCAATGAAGGTACAGGCAGGGCTGAAGGCAAAG
GTCCCACAGGCATAGAGGTGAGTAGCATTGTAAGAGACCAGGACTCGAATGAAGTTGAAACACTGTGTCTC
ATTGCTCTTCTTCTTAAAGGCACATTCGGTCTTTTTTCTCTCACTGGCTGGCCAGGGTATCATGTTCTTTA
GCCTTGGGATTCCTGGGTTCTGGATATTCAAGGCCAGGACGGCCTCTCGAGCCCCCACATAGAGAGTGTTG
CCATCGTCACTCANGAGCAGCGTGTCAAAGTCTCGGAGGCCTTTTTGTGAATTCCCAAGCCGAATTCCAGC
ACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTTGAGTATTCTATAGT
GTCACCTAAATAGCTTGGCGTAATCATGGCATAGCTGGTTCCTGTGTGAAATTGGTATCCGCTCACAATTC
ACACAACATACCAACCGGAACATAAAGTGTAAAGCCTGGGGTGCTNATGAGTGAGCTACTCACATTAATGC
GTTGCGCTCACTGCCGCTTTNCAATCNGGAAACCTGCGTGCCACTGCATTAATGAATCGGCCACCCCCCGG
GGAAAAGGCGGTTGCNTNTGGGCGCTCTTC

Sema4F

CGAATNGGGCCCTCTAGATGCATGCTCGAGCGGCCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTT**GGA**
AGCTTGCTGGAAACTGGACACATCAATAACCCCGCACTTCGGATCAAAAGCGAAGGTGCCACACGTGAGGAG
GTGAGAGGCATTGGCAATGGCGAGAATCTGGATAAAATTGTGACATTCGTCCTCTTTCTTGCCTTTCTTCCT
ACAGTTCTGTCTGTGAGTCTCGGGCACCATCCAGTCGATCCTTCGAGGCTTTTCCCCAGAGAAGGGCAGGGT
TAAAGCGAAGATGCTGTCCCGTGCACCGACGTAAAGTGTGTGAGAAGCAGGATCCACCAGGAGAGCAGAGTA
ATTGTACCGTATGAGGGGCCGCAAACCGGGTGAGATAGGAGTCAGCGAATTCCCAAGCCGAATTCCAACACA
CTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTCCAAACTTGATGCATAGCTTGAGTATTCTATAGTGTCAC
CTAAATAGCTTGGCGTAATCATGGNCATAGCTGGTTCCTGTGTGAAATTGGTATNCGCTCACAATTNCACAC
AACATACGAACCCGGGAACATNAAGTGTAAAANCTGGGGTGCCTNATGAGTGACTNACTCACATTNATTGCG
TTGCGCTCACTGGCCGCTTTTCAATCNGGNAAACTGTCGTGCCACTGCATTAATGAATCGGCCACCCCCCGG
GGAAAAAGCGG

Sema4D

CNNNNNNNNNCNNNNCNAATTCGACTCGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGG
AATTCGGCTTGATCACGTCAGCAAAGACGAAGCTAGGCTCGTTCAGCCACGGGATGGCATACTCCGTCCTC
AAGGGACTGTGGGAAGAGTTTCGAGAGATGATGGGTTCACTGCCCAAGAAATTATAGGACGTCCCAGAGTA
GAGCTCGCCCCCAACCATGACTGATGTGTAGCTGTGGGCGGGGTCGAAGGGGCATCTTCCTTTGCCATCTT
CACTTTTCCCCAGAAACTTGAAGGATGTCAAGTTCAGGTGGTCACAGGTGGGCTGGAACGCATTGGTCCCA
CACACATAGAGGGAAGTGCTGCTTAGTGGCTGTAGTACTCGAATGTAGTTTAGGCATTCCGTCTGCTTAAG
CCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATA
GTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAA
TTCCACACAACATACGAGCCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCAC
ATTAATTGCGTTGCGCTCACTGGCCGCTTTTCAGTCNGGAAACCTGTCGTGCCACTGCATTAATGAATCGG
CAACCCCCGGGAAAGGCGGGTTGCGTATTGGGCGCTNTTCCCTTTCTNGCTNACTGACTCCTGCCTTGGNC
GTTCGGNTGCGGNGAGCGGNATCAACTTCACTTAAAGNNGGAATACGGTTTTCNCAAAATCAGGGGATAAC
CCAGGAAAAAACATGTGAACAAAGGCCACCAAANGGCNGAACCCTAAAAAGGCCCGTTTCT

Sema7A

TNNANNCNANTTCGACTCGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGGCTT
TCCCGTTGTATTCCTGCTTCCGGATGGTAGAGTACACTTCATCTCCTTCAAACAGAACCAGGGAGTTCTCAT
CCGGGCTGAAGGGGGCATAGCCTTTCATCTCACCAAGTGACATCACCACACTGTCATTCACCAAGTTCCAGC
AGCTGGGCTTCCGGGCATTGGTGCCGCAGACCAGCAGCCCATTACCCCGCCTCTCTAGAAGAGTGATGTAAT
TCTCACAGTCCTGTTTGTCCTGACAGGACCCCTTTGTGGAGCCGATGTTCACCGTGCGCACAGAGGCATTCT

TGCCCTCGGGGAAGTTGAAGTGGTAGACCTTGCCACGAAGCCGAATTCTGCAGATATCCATCACACTGGCGG
CCGCTCGACCTGCAGGCATGCAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTC
ATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTG
TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGGCCGCTTTTCAGTC
GGGAAACCTGTCGTGCCACTGCATTAATGAATCGGCCAACNCCCGGGGAGAGGCGGGTTGCGTATTGGCGCT
TTNCGTTTCTCGNTACTGACTCGTTGNGCTCGGTCGTTCGGTTNGGNGAGCGGTTTAACTTACTCAAAGGNG
GAATACGGTTTTCACANAATCAGGGATAACNCAGGAAAAACATGTGACNAAAGGCNNCAAAAGGCCCGAACC
GNAAAAGGCCCGTGCTGGGGTTTTCATAGGTCCCCCCCTGNCAN

APPENDIX 2: *Reference Sequences*

Nucleotide sequences of Semaphorins 4A, 4D, 4F and 7A with annotations indicating the start codon, the stop codon, the primer sequence, and the probe sequence: Start and stop codons are in blue font, primer sequence is underlined, probe sequence is in **bold** font.

Sema4A

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1     CTCGGACGCC TGGGTTAGGG GTCTGTACTG CTGGGGAACC ATCTGGTGAC CATCTCAGGC
61    TGACCATGGC CCTACCATCC CTGGGCCAGG ACTCATGGAG TCTCCTGCGT GTTTTTTTCT
121   TCCAACTCTT CCTGCTGCCA TCACTGCCAC CTGCTTCTGG GACTGGTGGT CAGGGGCCCA
181   TGCCCAGAGT CAAATACCAT GCTGGAGACG GGCACAGGGC CCTCAGCTTC TTCCAACAAA
241   AAGGCCTCCG AGACTTTGAC ACGCTGCTCC TGAGTGACGA TGGCAACACT CTCTATGTGG
301   GGGCTCGAGA GACCGTCCTG GCCTTGAATA TCCAGAACCC AGGAATCCCA AGGCTAAAGA
361   ACATGATACC CTGGCCAGCC AGTGAGAGAA AAAAGACCGA ATGTGCCTTT AAGAAGAAGA
421   GCAATGAGAC ACAGTGTTTC AACTTCATTC GAGTCCTGGT CTCTTACAAT GCTACTCACC
481   TCTATGCCTG TGGGACCTTT GCCTTCAGCC CTGCCTGTAC CTTCATTGAA CTCCAAGATT
541   CCCTCCTGTT GCCCATCTTG ATAGACAAGG TCATGGACGG GAAGGGCCAA AGCCCTTTGA
601   CCCTGTTCAC AAGCACACAA GCTGTCTTGG TCGATGGGAT GCTTTATTCC GGCACCATGA
661   ACAACTTCCT GGGCAGCGAG CCCATCCTGA TGCGGACACT GGGATCCCAT CCTGTTCTCA
721   AGACTGACAT CTTCTTACGC TGGCTGCACG CGGATGCCTC CTTCGTGGCA GCCATTCCAT
781   CCACCCAGGT CGTCTATTTC TTCTTTGAGG AGACAGCCAG CGAGTTTGAC TTCTTTGAAG
841   AGCTGTATAT ATCCAGGGTG GCTCAAGTCT GCAAGAACGA CGTGGGCGGT GAAAAGCTGC
901   TGCAGAAGAA GTGGACCACC TTCCTCAAAG CCCAGTTGCT CTGCGCTCAG CCAGGGCAGC
961   TGCCATTCAA CATCATCCGC CACGCGGTCC TGCTGCCCGC CGATTCTCCC TCTGTTTCCC
1021  GCATCTACGC AGTCTTTACC TCCCAGTGGC AGGTTGGCGG GACCAGGAGC TCAGCAGTCT
1081  GTGCCTTCTC TCTCACGGAC ATTGAGCGAG TCTTTAAAGG GAAGTACAAG GAGCTGAACA
1141  AGGAGACCTC CCGCTGGACC ACTTACCGGG GCTCAGAGGT CAGCCCGAGG CCAGGCAGTT
1201  GCTCCATGGG CCCCTCCTCT GACAAAGCCT TGACCTTCAT GAAGGACCAT TTTCTGATGG
1261  ATGAGCACGT GGTAGGAACA CCCCTGCTGG TGAAGTCTGG TGTGGAGTAC ACACGGCTTG
1321  CTGTGGAGTC AGCTCGGGGC CTTGATGGGA GCAGCCATGT GGTCATGTAT CTGGGTACCT
1381  CCACGGGTCC CCTGCACAAG GCTGTGGTGC CTCAGGACAG CAGTGCTTAT CTCGTGGAGG
1441  AGATTCAGCT GAGCCCTGAC TCTGAGCCTG TTCGAAACCT GCAGCTGGCC CCCGCCCAGG
1501  GTGCAGTGTT TGCAGGCTTC TCTGGAGGCA TCTGGAGAGT TCCCAGGGCC AATTGCAGTG
1561  TCTACGAGAG CTGTGTGGAC TGTGTGCTTG CCAGGGACCC TCACTGTGCC TGGGACCCTG
1621  AATCAAGACT CTGCAGCCTT CTGTCTGGCT CTACCAAGCC TTGGAAGCAG GACATGGAAC
1681  GCGGCAACCC GGAGTGGGTA TGCACCCGTG GCCCCATGGC CAGGAGCCCC CGGCGTCAGA
1741  GCCCCCCTCA ACTAATTAAA GAAGTCCTGA CAGTCCCCAA CTCCATCCTG GAGCTGCGCT
1801  GCCCCCACCT GTCAGCACTG GCCTCTTACC ACTGGAGTCA TGGCCGAGCC AAAATCTCAG
1861  AAGCCTCTGC TACCGTCTAC AATGGCTCCC TCTTGCTGCT GCCGCAGGAT GGTGTCGGGG
1921  GCCTCTACCA GTGTGTGGCG ACTGAGAACG GCTACTCATA CCCTGTGGTC TCCTATTGGG
1981  TAGACAGCCA GGACCAGCCC CTGGCGCTGG ACCCTGAGCT GGCGGGCGTT CCCCGTGAGC
2041  GTGTGCAGGT CCCGCTGACC AGGGTCGGAG GCGGAGCTTC CATGGCTGCC CAGCGGTCCT
2101  ACTGGCCCCA TTTTCTCATC GTTACCGTCC TCCTGGCCAT CGTGCTCCTG GGAGTGCTCA
2161  CTCTCCTCCT CGCTTCCCCA CTGGGGGCGC TGCGGGCTCG GGGTAAGGTT CAGGGCTGTG
2221  GGATGCTGCC CCCCAGGGAA AAGGCTCCAC TGAGCAGGGA CCAGCACCTC CAGCCCTCCA
2281  AGGACCACAG GACCTCTGCC AGTGACGTAG ATGCCGACAA CAACCATCTG GGCGCCGAAG
2341  TGGCTTAAAC AGGGACACAG ATCCGCAGCT GAGCAGAGCA AGCCACTGGC CTTGTTGGCT
2401  ATGCCAGGCA CAGTGCCACT CTGACCAGGG TAGGAGGCTC TCCTGCTAAC GTGTGTCACC
2461  TACAGCACCC AGTAGGTCCT CCCCTGTGGG ACTCTCTTCT GCAAGCACAT TGGGCTGTCT
2521  CCATACCTGT ACTTGTGCTG TGACAGGAAG AGCCAGACAG GTTTCTTTGA TTTTGATTGA
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2581 CCCAAGAGCC CTGCCTGTAA CAAACGTGCT CCAGGAGACC ATGAAAGGTG TGGCTGTCTG
2641 GGATTCTGTG GTGACAAACC TAAGCATCCG AGCAAGCTGG GGCTATTCCT GCAAACTCCA
2701 TCCTGAACGC TGTCACTCTA GAAGCAGCTG CTGCTTTGAA CACCAGCCCA CCCTCCTTCC
2761 CAAGAGTCTC TATGGAGTTG GCCCCTTGTG TTTCCTTTAC CAGTCGGGCC ATACTGTTTG
2821 GGAAGTCATC TCTGAAGTCT AACCACCTTC CTTCTTGGTT CAGTTTGGAC AGATTGTTAT
2881 TATTGTCTCT GCCCTGGCTA GAATGGGGGC ATAATCTGAG CCTTGTTCCC TTGTCCAGTG
2941 TGGCTGACCC TTGACCTCTT CCTTCCTCCT CCCTTTGTTT TGGGATTCAG AAAACTGCTT
3001 GTCACAGACA ATTTATTTTT TATTAAAAAA GATATAAGCT TTAAAG

Sema4D

1 GAATTCGGCA CGAGGCCATC CATGTGTGCC CGTTGCTGAA GGCCTCGGTG GCCCCTGCCC
61 ATGAGGATGT GTGCCCCCGT TAGGGGGCTG TTCTTGGCCC TGGTGGTAGT GTTGAGAACC
121 GCGGTGGCAT TTGCACCTGT GCCTCGGCTC ACCTGGGAAC ATGGAGAGGT AGGTCTGGTG
181 CAGTTTCACA AGCCAGGCAT CTTTAACTAC TCGGCCTTGC TGATGAGTGA GGACAAAGAC
241 ACTCTGTATG TAGGCGCCCG GGAAGCAGTC TTTGCAGTGA ATGCGCTGAA CATCTCTGAG
301 AAGCAACATG AGGTATATTG GAAGGTCTCT GAAGACAAAA AATCCAAGTG TGCAGAGAAG
361 GGGAAATCA**A AGCAGACGGA ATGCCTAAAC TACATTCGAG TACTACAGCC ACTAAGCAGC**
421 **ACTTCCCTCT ATGTGTGTGG GACCAATGCG TTCCAGCCCA CCTGTGACCA CCTGAACTTG**
481 **ACATCCTTCA AGTTTCTGGG GAAAAGTGAA GATGGCAAAG GAAGATGCCC CTTCGACCCC**
541 **GCCCACAGCT ACACATCAGT CATGGTTGGG GGCGAGCTCT ACTCTGGGAC GTCCTATAAT**
601 **TTCTTGGGCA GTGAACCCAT CATCTCTCGA AACTCTTCCC ACAGTCCCTT GAGGACGGAG**
661 **TATGCCATCC CGTGGCTGAA CGAGCCTAGC TTCGTCTTTG CTGACGTGAT CCAG**AAAAGC
721 CCAGATGGTC CGGAGGGTGA AGATGACAAG GTCTACTTCT TTTTTACGGA GGTATCCGTG
781 GAGTACGAAT TCGTCTTCAA GTTGATGATC CCGCGAGTTG CCAGGGTGTG CAAGGGCGAC
841 CAGGGCGGCC TGCGGACTTT GCAAAAAAAG TGGACCTCCT TCCTAAAGGC CAGGCTGATC
901 TGCTCCAAGC CAGACAGTGG CCTGGTCTTC AACATACTTC AGGATGTGTT TGTGCTGAGG
961 GCCCCGGGCC TCAAGGAGCC TGTGTTCTAT GCGGTCTTCA CCCCACAGCT GAACAATGTG
1021 GGTCTGTCAG CGGTGTGCGC CTACACACTG GCCACGGTGG AGGCAGTCTT CTCCCGTGGA
1081 AAGTACATGC AGAGTGCCAC AGTGGAGCAG TCTCACACCA AGTGGGTGCG CTACAATGGC
1141 CCAGTGCCCA CTCCCCGACC TGGAGCGTGT ATCGACAGTG AGGCCCGGGC AGCCAACTAC
1201 ACCAGCTCCT TGAATCTCCC AGACAAAACA CTGCAGTTTG TAAAAGACCA CCCTTTGATG
1261 GATGACTCAG TGACCCCGAT AGACAACAGA CCCAAGCTGA TCAAAAAAGA TGTAAACTAC
1321 ACCCAGATAG TGGTAGACAG GACCCAGGCC CTGGATGGGA CTTTCTACGA CGTCATGTTC
1381 ATCAGCACAG ACCGGGGAGC TCTGCATAAA GCAGTCATCC TTACAAAAGA GGTGCATGTC
1441 ATCGAGGAGA CCCAACTCTT CCGGGACTTT GAACCGGTCC TAACTCTGCT GCTATCGTCA
1501 AAGAAGGGGA GGAAGTTTGT CTATGCAGGC TCCAACTCTG GAGTGGTCCA AGCGCCCCTG
1561 GCATTCTGCG AAAAGCACGG TAGCTGTGAA GACTGTGTGT TAGCACGGGA CCCCTACTGT
1621 GCCTGGAGCC CAGCCATCAA GGCCTGTGTT ACCCTGCACC AGGAAGAGGC CTCCAGCAGG
1681 GGCTGGATTC AGGACATGAG CGGTGACACA TCCTCATGCC TGGATAAGAG TAAAGAAAGT
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1801 AACCTAGCCC GGGTGGTATG GAAGTTCCAG AATGGCGAGT TGAAGGCCGC AAGTCCCAAG
1861 TACGGCTTTG TGGGCAGGAA GCACCTGCTC ATCTTCAACC TGTCGGACGG AGACAGCGGC
1921 GTGTACCAGT GCCTGTCAGA GGAAAGGGTG AGGAATAAAA CGGTCTCCCA GCTGCTGGCC
1981 AAGCACGTTC TGGAAGTGAA GATGGTACCT CGGACCCCCC CCTCACCTAC CTCAGAGGAT
2041 GTTCAGACAG AAGGTAGTAA GATCACATCC AAAATGCCGG TTGGATCTAC CCAGGGGTCC
2101 TCTCCCCCTA CCCCGGCTCT GTGGGCAACC TCCCCCAGAG CCGCCACCCT ACCTCCCAAG
2161 TCCTCCTCCG GCACATCCTG TGAACCAAAG ATGGTCATCA ACACGGTCCC CCAGCTCCAC
2221 TCAGAGAAGA CGGTGTATCT CAAGTCCAGT GACAACCGCC TGCTCATGTC TCTCCTCCTC
2281 TTCATCTTTG TCCTCTTCCT CTGCCTCTTT TCCTACAACT GCTACAAGGG CTACCTGCCC
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2521 ATCACCAGCA AAGTCCCCAC GGATCGTGAG GACTCGCAAC GGATCGATGA ACTCTCTGCC
2581 CGGGACAAAC CGTTTGATGT CAAGTGTGAA CTGAAGTTTG CAGATTCGGA TGCTGACGGG
2641 GACTGAGGCC AGCGTGTCCC AGCCCATGCC CCTCTGTCTT CGTGGAGAGT GTTGTGTTGA
2701 GCCCATTCAG TAGCCGAGTC TTGTCACTCT GTGCCAGCCT CAGTCCTGTG TCCCCTTTTT
2761 CTCTGGTTT

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1     ATGCTGGCCA GGGCCGAGCG GCCCCGCCCG GGCCCCCGGC CGCCTCCGGT CTCTCTCTTC
61    CCGCCGCCGT CGTCGCTGCT GCTGCTGCTG CTGGCGATGC TGAGCGCCCC GGTGTGCGGC
121   CGCGTCCCCC GCTCGGTGCC CAGAACCTCG CTGCCCATCT CCGAGGCTGA CTCCTATCTC
181   ACCCGGTTTG CGGCCCCTCA TACGTACAAT TACTCTGCTC TCCTGGTGGA TCCTGCTTCT
241   CACACACTTT ACGTCGGTGC ACGGGACAGC ATCTTCGCTT TAACCCTGCC CTTCTCTGGG
301   GAAAAGCCTC GAAGGATCGA CTGGATGGTG CCCGAGACTC ACAGACAGAA CTGTAGGAAG
361   AAAGGCAAGA AAGAGGACGA ATGTCACAAT TTTATCCAGA TTCTCGCCAT TGCCAATGCC
421   TCTCACCTCC TCACGTGTGG CACCTTCGCT TTTGATCCGA AGTGCGGGGT TATTGATGTG
481   TCCAGTTTCC AGCAGGTTGA AAGACTTGAG AGTGGCCGGG GGAAATGTCC TTTTGAGCCA
541   GCTCAACGGT CAGCAGCTGT AATGGCTGGG GGCGTCCTCT ACACCGCCAC TGTGAAAAAC
601   TTCCTGGGGA CAGAGCCGAT TATCTCCCGA GCTGTGGGTC GAGCTGAGGA CTGGATTCGA
661   ACAGAGACCT TGTCATCCTG GCTTAATGCC CCAGCCTTTG TCGCAGCTAT GGTCCTGAGC
721   CCGGCTGAGT GGGGGGATGA AGATGGAGAC GATGAAATCT TCTTTTTCTT CACGGAGACC
781   TCCCGAGTCT TGGACTCATA TGAGCGCATC AAGGTCCCAC GAGTGGCCCG TGTGTGTGCG
841   GGGGACCTTG GGGGCCGGAA GACCCTTCAG CAGAGATGGA CGACGTTTCT AAAGGCTGAC
901   CTGCTGTGTC CAGGGCCCGA GCATGGAAGG GCCTCGGGGG TTCTGCAGGA TATGACAGAG
961   CTTCGACCTC AGCCTGGCGC GGGGACCCCC CTCTTTTATG GCATCTTTTC CTCCCAGTGG
1021  GAAGGAGCCG CCATTTCTGC TGTGTGTGCC TTCCGACCCC AAGACATCCG GGCAGTGCTG
1081  AATGGTCCCT TTAGAGAGCT AAAACATGAC TGCAACAGGG GACTACCTGT CATGGACAAC
1141  GAGGTGCCCC AGCCCAGACC TGGAGAGTGC ATCACCAACA ACATGAAGTT CCAGCAGTTT
1201  GGATCCTCAC TCTCCCTGCC AGACCGTGTG CTCACCTTTA TCAGAGACCA CCCGCTCATG
1261  GACAGGCCCG TGTTTCCAGC TGATGGCCGC CCCCTGCTGG TCACTACGGA TACAGCCTAT
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1441  CTGGAGGATC TGGCCTTGTT CCCGGAAACA CAGCCGGTTG AAAGCATGAA ATTGTACCAT
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1801  TCCTGTGTGT GGCACCAGCC CAGTGGAGTG ACTTCGCTCA CCCCACGAAG GGATGGGCTA
1861  GAGGTGGTGG TGACCCCAGG GGCCATGGGG GCTTATGCTT GCGAGTGTCA GGAGGGTGGA
1921  GCCGCCCGTG TGGTGGCTGC TTATAGCTTG GTGTGGGGCA GCCAGAGGGG GCCCGCAAAC
1981  CGGGCCCACA CCGTTGTGGG GGCTGGGCTG GTTGGCTTTT TCCTGGGTGT TCTAGCAGCA
2041  TCTCTCACTC TCCTCCTGAT TGGTCGCCGT CAGCAGCGGC GGCGACAGAG GGAGCTTCTA
2101  GCTAGAGACA AGGTGGGCTT AGATCTGGGG GCTCCACCTT CTGGGACCAC AAGCTATAGC
2161  CAAGACCCTC CCTCTCCTTC ACCTGAAGAT GAACGGCTGC CCCTGGCCCT GGGTAAGCGG
2221  GGCAGTGGTT TTGGTGGCTT CCCTCCACCC TTCCTGCTGG ATTCTTGCCC GAGCCCAGCC
2281  CACATCCGGC TCACTGGGGC TCCTCTGGCC ACGTGCGATG AGACGTCTAT CTAA
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1     GCGGGATGAC GCCTCCTCCT CCCGGACGTG CCGCCCCCAG CGCACCGCGC GCCCGCGTCC
61    TCAGCCTGCC GGCTCGGTTC GGGCTCCCGC TGCGGCTGCG GCTTCTGCTG GTGTTCTGGG
121   TGGCCGCCGC CTCCGCCCAA GGCCACTCGA GGAGCGGACC CCGCATCTCC GCCGTCTGGA
181   AAGGGCAGGA CCATGTGGAC TTTAGCCAGC CTGAGCCACA CACCGTGCTT TTCCATGAGC
241   CGGGCAGCTT CTCTGTCTGG GTGGGTGGAC GTGGCAAGGT CTACCACTTC AACTTCCCCG
301   AGGGCAAGAA TGCCTCTGTG CGCACGGTGA ACATCGGCTC CACAAAGGGG TCCTGTCAGG
361   ACAAACAGGA CTGTGGGAAT TACATCACTC TTCTAGAAAG GCGGGGTAAT GGGCTGCTGG
421   TCTGTGGCAC CAATGCCCGG AAGCCCAGCT GCTGGAACTT GGTGAATGAC AGTGTGGTGA
481   TGTCACTTGG TGAGATGAAA GGCTATGCCC CCTTCAGCCC GGATGAGAAC TCCCTGGTTC
541   TGTTTGAAGG AGATGAAGTG TACTCTACCA TCCGGAAGCA GGAATACAAC GGGAAGATCC
601   CTCGGTTTCG ACGCATTCGG GGCGAGAGTG AACTGTACAC AAGTGATACA GTCATGCAGA
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781   GAGTAGCCCA GTTGTGCAGG GGGGACCAGG GTGGTGAGAG TTCGTTGTCT GTCTCCAAGT
841   GGAACACCTT CCTGAAAGCC ATGTTGGTCT GCAGCGATGC AGCCACCAAC AGGAACTTCA
901   ATCGGCTGCA AGATGTCTTC CTGCTCCCTG ACCCCAGTGG CCAGTGGAGA GATACCAGGG
961   TCTATGGCGT TTTCTCCAAC CCCTGGAACT ACTCAGCTGT CTGCGTGTAT TCGCTTGGTG
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1261 GAGAGACCTT CCATGTGCTT TATCTAACCA CAGACAGGGG CACCATTCAC AAGGTGGTGG
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1381 GTGCAGCGGC CATCCAGGCT ATATCATTGG ATGCTGACCG GCGGAAGCTC TATGTGACCT
1441 CCCAGTGGGA AGTGAGCCAG GTACCCCTGG ACATGTGTGA GGTCTACAGC GGGGGCTGCC
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1681 GATACTACCT GACCTGCCCC ATGGAGTCCC GCCACGCCAC TTACTTATGG CGCCATGAGG
1741 AGAATGTGGA ACAGAGCTGT GAGCCAGGCC ACCAAAGCCC TAGCTGCATC CTGTTCATTG
1801 AGAACCTCAC GGCCCGTCAG TATGGCCACT ACCGCTGCGA GGCCCAAGAG GGCTCCTACC
1861 TCCGTGAGGC TCAACACTGG GAGCTGCTGC CAGAGGACAG AGCACTGGCC GAACAACTAA
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1981 TTGGTCTGCT GGTTCACTAG GGCTTCTAGG AGCTGGGGGC GCCCCAGGCT TCTGTAGCCC
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2161 GGCGGCTGCT GGGGCCCAGG TGGGGTAGGG ACAGTGAGGG ACTATAGAAG GAAGGCGCCG
2221 ACTGTGAAGC TGGGCCATCA GCAACTCAAG ACTTTATCTT CTCGAGAATA TTTTTTCGAA
2281 ATCTCACACT TGACTTCATG CAGTGATGCA CCTGGCCTGA GAGCCCATCA GCCTGGCAGT
2341 GGGCTTTGGG AAGGGGAGCT GGGACCCCCC ATCTCTGGAC CTTGGGGCTG AGTCCTTTGC
2401 GTCCTTCCCT GTTCCACTGC TGGGTGGCCA GTGTCCCTGC AGACCCGGGC TGCTCCTCTG
2461 TACCTCCCTC ACAGCCTGGG TCCCACCGGA CAGCGCCTTG CATGTTTATT GAAGGATGTT
2521 TGCTTTCCGG ACGGAAGGGC GGGAAAAGCT CTATTTTTAT GTTAGGCTTA TTTCATGTAT
2581 AGCTACTTCT GACTGCATCT GTATGCAT
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APPENDIX 3: *IACUC Approval*

San Jose State University
Institutional Animal Care and Use Committee

LETTER OF OFFICIAL PROTOCOL REVIEW

Date: June 4, 2001

Dear Dr. Matthes,

The animal care and use portion of your research proposal indicated below was reviewed by the Institutional Animal Care and Use Committee (IACUC). The status of your proposal is as follows:

Principle Investigator(s): David J. Matthes, Sreedevi Chalasani
Protocol #: 771
Title: Semaphorins as Modulators of Lymphocyte Migration: an in situ Analysis of Semaphorin Expression in Mouse Lymphocyte Organs.

The application was approved without modification by the IACUC.

Approval date: June 4, 2001 * Expiration Date: May 30, 2004

The IACUC must be informed in writing of any proposed changes to the approved protocol outline and approval must be granted in writing by the IACUC before any change is instituted. If you wish to continue the approved outline beyond the expiration date above, it is recommended that you resubmit a renewal application by April 2004.

The protocol number (#771) may only be used by the principle investigator and participants included on the approved application form. The protocol number will be required to order or transfer animals or tissues for the study (maximum of 100 mice), and on grant and contract proposals to fund the project. All animal orders or transfers are to be placed through the University Animal Care office.

If you have any questions, feel free to contact me at extension 4-4929.

Larry Young

Larry Young, RVT, LATg
Staff to the IACUC

Cc: UAC Office

APPENDIX 4: ***List of all the Reagents/Products/Kits Used in this Research Project***

Name of the Reagent/product/kit	**Name of the Supplier**	**Catalogue Number**
RNAqueous-Midi kit	Ambion	1911
RetroScript kit	Ambion	1710
TA cloning kit	Invitrogen	KNM2000-01, K2050-01
pGEM vector	Promega	P2271
Qiagen Maxi Prep kits with Qiagen-tip 500	Qiagen Inc.	10063
DIG-RNA labeling kit (SP_6/T_7)	Roche Applied Science	1175025
RNA Quantitation kit	Molecular Probes Inc.	R11490
Terg-a-zyme	Alconox Inc.	1304
DEPC	Sigma	D-5758
RNase Zap	Sigma	R-2020
APES	Sigma	A-3648
Na_2HPO_4 $7H_2O$	Sigma	S-9390
NaH_2PO_4 H_2O	Sigma	S-9638
NaCl	Sigma	S-3014
NaOH	Sigma	S-0899
Paraformaldehyde	Sigma	P-6148
Xylene	Sigma	X-2377
Proteinase K	Sigma	P-2308
Formamide	Sigma	F-9037
Denhardt's solution	Sigma	D-2532
Ecoli tRNA	Sigma	R-1753

Herring testis DNA	Sigma	D-6898
Glass coverslips (24 x 50)	Sigma	C-8181
SSC	Sigma	S-6639
Tris	Sigma	T-6066
Sheep serum	Sigma	S-2382
Monoclonal anti-DIG AP-conjugate Ig fraction of mouse ascites fluid	Sigma	A-1054
$MgCl_2$	Sigma	M-1028
NBT/BCIP	Sigma	B-3804
500 ml filters	Nalgene Labware	127-0045