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(57) **Abrégé/Abstract:**

Anti-FLT4 antibodies, especially monoclonal anti-FLT4 antibodies, which are useful as a specific marker for endothelial cells of lymphatic vessels and HEVs, as a diagnostic tool for detecting changes in lymphatic tissue, especially in lymphatic vessels and HEVs in disease states, such as lymphangioma, metastatic lymph nodes and inflammatory, infectious and immunological disease.

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<p>(21) International Application Number: PCT/FI95/00337</p> <p>(22) International Filing Date: 9 June 1995 (09.06.95)</p> <p>(30) Priority Data: 257,754 9 June 1994 (09.06.94) US</p> <p>(71)(72) Applicants and Inventors: ALITALO, Kari [FI/FI]; Nyyrikintie 4 A, FIN-02100 Espoo (FI). KAIPAINEN, Arja [FI/FI]; Messeniuksenkatu 7 A 9, FIN-00250 Helsinki (FI). KORHONEN, Jaana [FI/FI]; Agricolankatu 7 C 68, FIN-00530 Helsinki (FI). MUSTONEN, Tuija [FI/FI]; Pih- lajatie 8 A 2, FIN-00270 Helsinki (FI). PAJUSOLA, Katri [FI/FI]; Kasteholmantie 4 A 8, FIN-00900 Helsinki (FI). MATIKAINEN, Marja-Terttu [FI/FI]; Lankkistentanhua 12, FIN-23100 Mynämäki (FI). KÄRNANI, Päivi [FI/FI]; Mullintie 10 B 62, FIN-20300 Turku (FI).</p> <p>(74) Agent: ORION CORPORATION; Orion-Farmos, Research & Development, Patent Service, P.O. Box 65, FIN-02101 Espoo (FI).</p>	<p>(81) Designated States: AM, AT, AU, BG, BR, BY, CA, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KG, KP, KR, KZ, LT, LU, LV, MD, MX, NO, NZ, PL, PT, RO, RU, SE, SG, SI, SK, TJ, UA, US, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p style="text-align: center; font-size: 2em; font-weight: bold;">2192235</p>	
<p>(54) Title: FLT4 RECEPTOR TYROSINE KINASE AND ITS USE IN DIAGNOSIS AND THERAPY</p>		
<p>(57) Abstract</p> <p>Anti-FLT4 antibodies, especially monoclonal anti-FLT4 antibodies, which are useful as a specific marker for endothelial cells of lymphatic vessels and HEVs, as a diagnostic tool for detecting changes in lymphatic tissue, especially in lymphatic vessels and HEVs in disease states, such as lymphangioma, metastatic lymph nodes and inflammatory, infectious and immunological disease.</p>		

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FLT4 RECEPTOR TYROSINE KINASE AND ITS USE IN DIAGNOSIS AND THERAPY**FIELD OF THE INVENTION**

The present invention relates generally to receptor tyrosine kinases, nucleic acid probes and antibodies specifically recognizing such receptors, and the use of such probes and antibodies for identifying lymphatic vessels and high endothelial venules (HEV) in animal and human tissues and lymphatic endothelial cells in culture. More specifically the present invention is directed to FLT4, a receptor tyrosine kinase, to nucleic acid probes recognizing FLT4, to antibodies specific to FLT4 and to methods for identifying FLT4 expression in lymphatic vessels and ultimately diagnosing and treating disease states in animals and humans involving changes in lymphatic tissue, such as inflammatory, infectious and immunological diseases, metastatic lymph nodes and lymphangiomas.

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BACKGROUND OF THE INVENTION

The physiology of the vascular system, embryonic vasculogenesis and angiogenesis, blood clotting, wound healing and reproduction, as well as several diseases, involve the vascular endothelium lining the blood vessels. The development of the vascular tree occurs through angiogenesis and, according to some theories, the formation of the lymphatic system starts shortly after arterial and venous development by sprouting from veins (1, 2).

After the fetal period endothelial cells proliferate very slowly, except during angiogenesis associated with neovascularization. Growth factors stimulating angiogenesis exert their effects via specific endothelial cell surface receptor tyrosine kinases.

The protein product of the FLT4 receptor tyrosine kinase cDNA, cloned from a human erythroleukemia cell line is N-glycosylated and contains seven immunoglobulin-like loops in its extracellular domain. The cytoplasmic tyrosine kinase domain of FLT4 is about 80 % identical at the amino acid

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level with the corresponding domains of FLT1 and KDR and about 60 % identical with the receptors for platelet-derived growth factor, colony stimulating factor-1, stem cell factor and the FLT3 receptor (3).

Although the biological function of FLT4 are as yet unknown, its
5 restricted expression pattern indicated that its functions may involve the vascular endothelium. Our previous results revealed FLT4 mRNA expression in endothelial cells in developing vessels of several fetal organs as disclosed by Kaipainen et al., in *J. Exp. Med.* 178: 2077-2088, 1993. A comparison of
10 FLT4, FLT1, and KDR/FLK-1 receptor mRNA signals showed overlapping, but distinct expression patterns in the tissues studied (4). These data suggested that the receptor tyrosine kinases encoded by this gene family may have distinct functions in the regulation of the growth and/or
5 differentiation of blood vessels.

A major function of the lymphatic system is to provide fluid return from
15 tissues and transport many extravascular substances back to the blood. In addition, during the process of maturation, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatic vessels, and return to the blood through the thoracic duct. Specialized
20 venules, high endothelial venules, (HEVs) bind lymphocytes again and cause their extravasation into tissues. The lymphatic vessels and especially the lymph nodes thus play an important role in immunology and they are also sites of development of metastasis of different tumors.

Since the turn of the 20th century, three different theories concerning
the embryonic origin of the lymphatic system have been presented.
25 However, prior to the present invention, lymphatic vessels have been difficult to identify, because there are no specific markers available for them.

Lymphatic vessels are most commonly studied with the aid of lympho-
graphy. In lymphography, X-ray contrast medium is injected directly into a
lymphatic vessel. That contrast medium is distributed along the efferent
30 drainage vessels of the lymphatic system. The contrast medium is collected in lymph nodes, where it stays for up to half a year, during which time X-ray analyses allow the follow-up of lymph node size and architecture. This

diagnostics is especially important in cancer patients with metastases in the lymph nodes and in lymphatic malignancies, such as lymphoma

SUMMARY OF THE INVENTION

The present invention is directed to FLT4 peptides and other constructs and to the use of FLT4 as a specific marker for lymphatic endothelial cells.

More precisely, the present invention is directed to an isolated monoclonal antibody that is specific for the extracellular domain of Flt4 receptor tyrosine kinase.

The invention is also directed to nucleic acid probes and antibodies specifically recognizing FLT4, especially to monoclonal antibodies, and compositions containing such antibodies. Further disclosed in the present application is the use of such monoclonal antibodies for diagnostic purposes for detecting and measuring the amount of FLT4 receptors in tissues, especially in lymphatic tissues and in lymphatic endothelial cells.

In a preferred embodiment, the invention provides monoclonal antibodies specifically recognizing the FLT4 receptor. More specifically this invention provides a monoclonal antibody designated 9D9F9. The hybridoma cell line which produces monoclonal antibody 9D9F9 is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) under the provisions of the Budapest Treaty (DSM accession number ACC2210).

Monoclonal antibodies labelled with a detectable marker are also provided. As used herein, the term detectable marker encompasses any detectable marker known to those skilled in the art. However, in a preferred embodiment of this invention, the detectable marker is selected from the group consisting of radioisotopes, fluorochromes, dyes, enzymes and biotin. For the purpose of this invention suitable radioisotopes include, but are not limited to ^{125}I and ^{131}I .

Monoclonal antibodies of the present invention may also be used in a

3a

More particularly, the present invention provides a method for detecting lymphatic vessels in a tissue sample, comprising:

a) contacting an isolated monoclonal antibody with the tissue sample suspected of containing lymphatic vessels, said isolated monoclonal antibody being specific for the extracellular domain of FLT4 receptor tyrosine kinase and further comprising a detectable label; and

b) detecting said antibody bound to said tissue sample.

10 The present invention also provides a method for identifying changes in lymphatic vessels and high endothelial venules (HEVs) characterized by FLT4 expression, comprising the steps of:

a) contacting a tissue sample from a patient suspected of having a disease characterized by changes in lymphatic vessels and HEVs with an isolated monoclonal antibody that is specific for the extracellular domain FLT4 reception tyrosine kinase and that comprises a detectable label; and

b) detecting the presence of said antibody in said tissue sample.

The present invention also provided a method for diagnosing a disease characterized by changes in lymphatic vessels and high endothelial venules (HEVs) comprising:

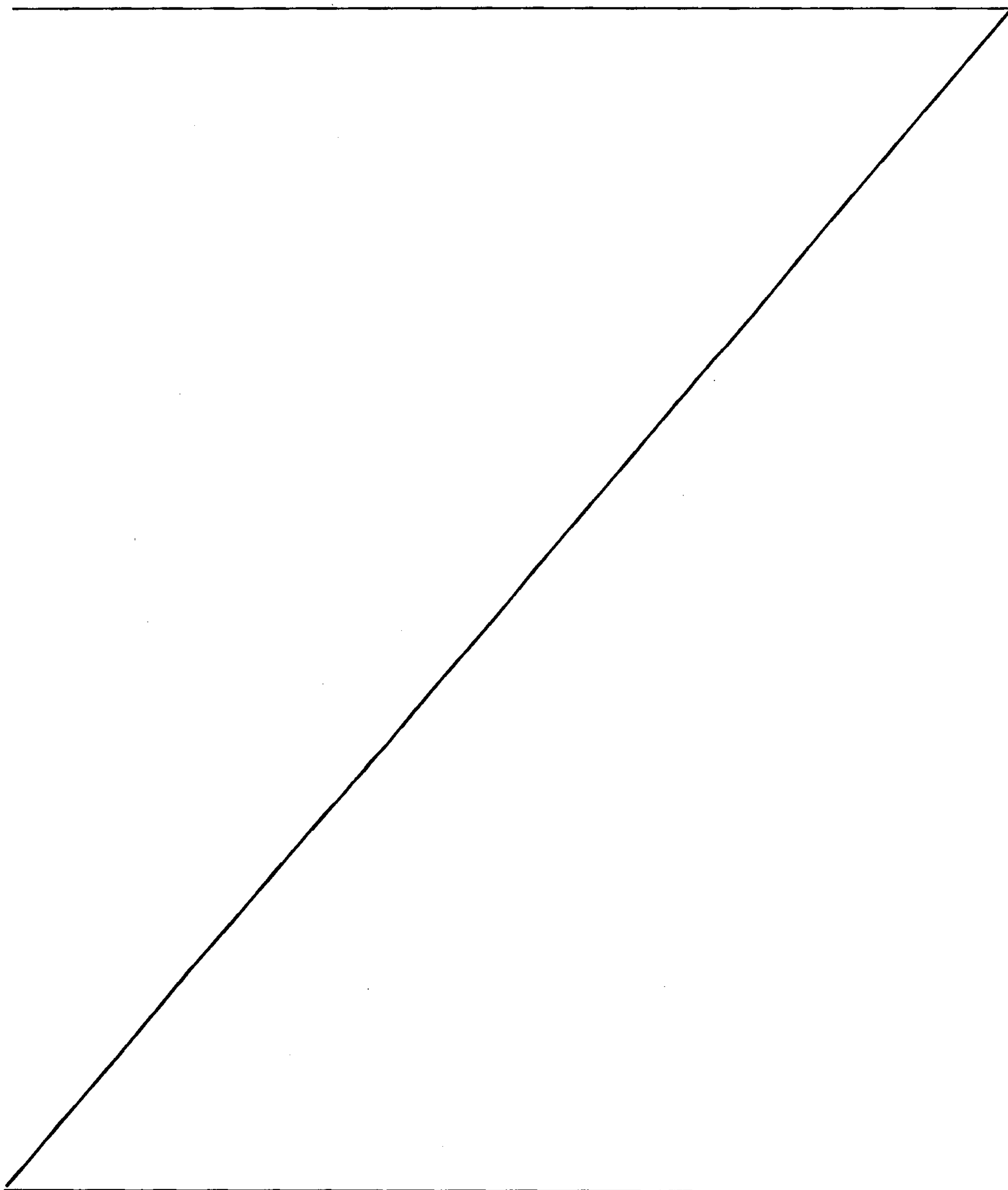
20 a. contacting a tissue sample from a patient suspected of having a disease characterized by changes in lymphatic vessels and HEVs with the isolated an isolated monoclonal antibody that is specific for the extracellular domain FLT4 reception tyrosine kinase and that comprises a detectable label; and

b. detecting the presence of said antibody in said tissue sample.

Also, the present invention concerns the use of a monoclonal antibody against the extracellular domain of FLT4 receptor tyrosine kinase for the preparation of a diagnostic composition for imaging lymphatic vessels, lymph nodes or high endothelial venules.

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Furthermore, the present invention concerns the use of a monoclonal antibody against the extracellular domain of FLT4 receptor tyrosine kinase for the preparation of a diagnostic composition for detecting lymphatic vessels, lymph nodes or high endothelial venules.



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Another aspect of the present invention thus relates to a method of determining the presence of FLT4-receptors in a cell sample, comprising the steps of:

- 5 (a) exposing a cell sample to a monoclonal antibody of the present invention;
- (b) detecting the binding of said monoclonal antibody to FLT4-receptors.

The exposure of a cell mixture to monoclonal antibodies of the invention can be in solution, as is the case for fluorescence-activated cell sorting, or it can be on solid tissue specimens, such as biopsy material, or it
10 can be with the monoclonal antibody immobilized on a solid support, as is the case with column chromatography or direct immune adherence. The mixture of cells that is to be exposed to the monoclonal antibody can be any solution of blood cells or tissue cells. Preferably, the cell mixture is from normal or pathological tissue containing or suspected to contain lymphatic
15 endothelial cells. After exposure of the cell mixture to the monoclonal antibody, those cells with FLT4 -receptors will bind to the monoclonal antibody to form an antibody-FLT4 -receptor complex. The presence of the antibody-FLT4 -receptor complex, and therefore FLT4 receptors, can be detected by methods known in the art. These methods include
20 immunohistochemical methods standard in the art, such as immunofluorescence, FACS analysis, ELISA, IRMA (a sandwich type of immunochemistry assay), immunohisto-chemistry, RIA using ¹²⁵I-label and autoradiography.

The present invention also provides monoclonal antibodies
25 conjugated to an imageable agent. As used herein, the term imageable agent includes, but is not limited to, radioisotopes. A preferred radioisotope is 99m-technetium.

In a specific embodiment, the invention is directed to a method for monitoring lymphatic vessels and their endothelial cells in tissue samples
30 and in organisms. The present invention further provides clinical detection methods describing the state of lymphatic tissue, and especially lymphatic vessels (inflammation, infection, traumas, growth, neoplasia etc.) and methods for detecting lymphatic vessels and thus lymphatic vascularization in an organism.

More specifically the present invention provides a method for detecting and identifying lymphatic changes characterized by FLT4 expression in connection to metastatic cancers, inflammatory, infectious and immunological conditions, which method comprises the steps of

5 (a) obtaining a tissue and/or body fluid sample suspected of lymphatic changes, and

(b) contacting said sample with a FLT4-specific monoclonal antibody under conditions suitable for forming a complex between the monoclonal antibody and the antigen, and

10 (c) detecting the presence of any complex formed.

A tissue which may be detected by this method is any normal, precancerous or cancerous solid tumor tissue with FLT4-containing lymphatic cells or cells which express the FLT4-receptor. In one embodiment of the present invention, the monoclonal antibody is labelled with a
15 detectable marker as described herein. Methods of the invention are useful for detecting and differentiating various forms of cancer, especially metastases in the lymph nodes and other lymphatic malignancies, such as lymphomas, as well as lymphangiomas.

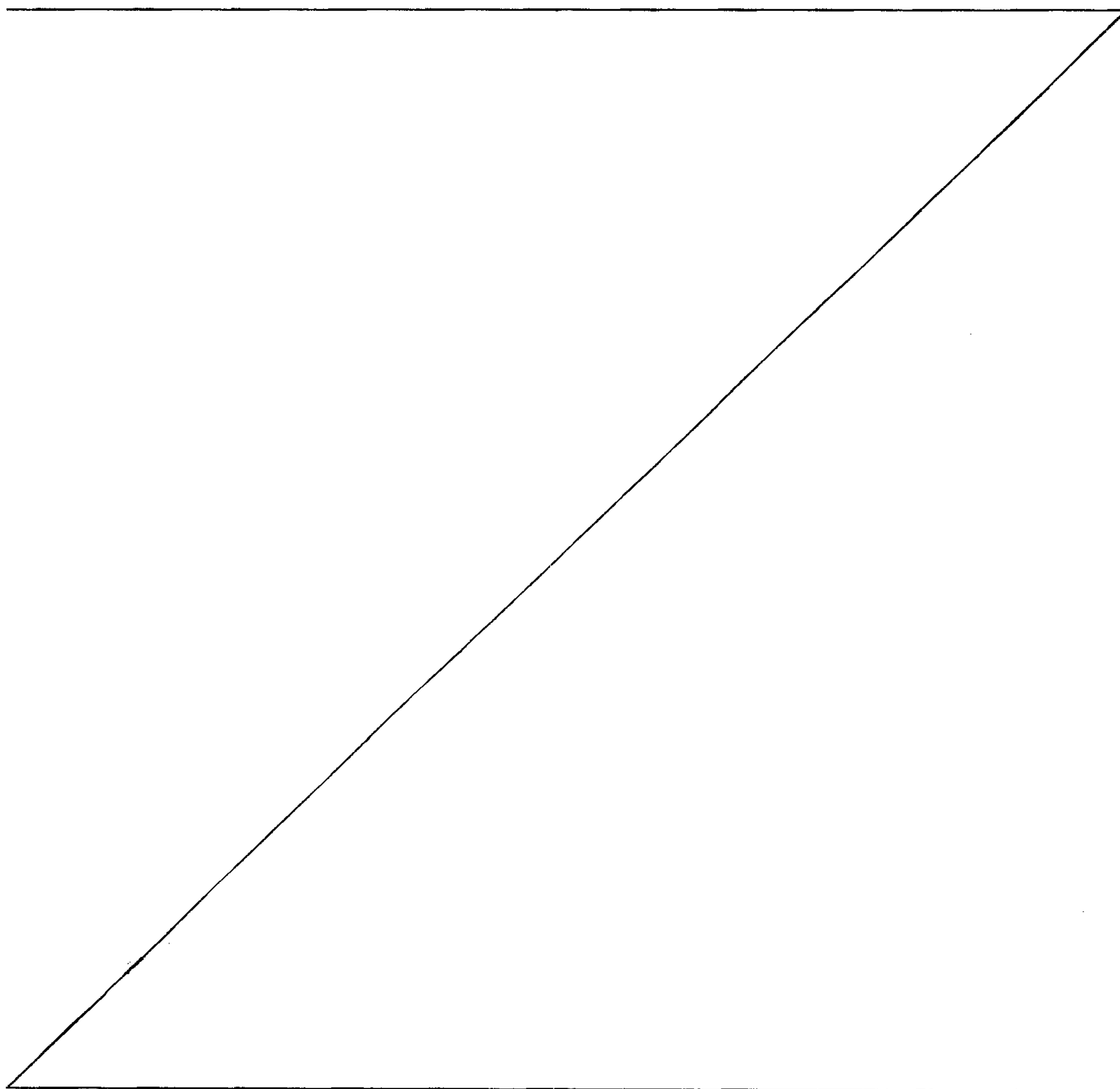
A method of imaging the presence of lymphatic vessels, high
20 endothelial venules or lymph nodes in human patients, is also provided by this invention. This method comprises administration of labelled antibodies and detection by imaging at sites where FLT4 expressing cells are present, in lymphatic vessels or lymph nodes.

The invention is further directed to a method of stimulating or
25 antagonizing the function of FTL4 in lymphatic vascularization and in inflammatory, infectious and immunological conditions, said method comprising inhibiting the FLT4-mediated lymphatic vascularization by providing amounts of a FLT4-binding compound sufficient to block the FLT4 endothelial cell sites participating in such reaction, especially where FLT4
30 function is associated with a disease such as metastatic cancers, lymphomas, inflammation (chronic or acute), infections and immunological diseases.

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The invention is also directed to the use of a monoclonal antibody against the extracellular domain of Flt4 receptor tyrosine kinase for the preparation of a diagnostic composition for imaging or detecting lymphatic vessels, lymph nodes or high endothelial venules.

The invention is further directed to the use of a monoclonal antibody against the extracellular domain of Flt4 receptor tyrosine kinase for the preparation of a pharmaceutical composition for inhibiting the FLT4-mediated lymphatic vascularization which is associated with a disease selected from the group consisting of metastatic cancers, lymphomas, lymphangiomas, chronic
10 inflammation, acute inflammation, infections and immunological diseases.



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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Expression of FLT4 mRNA in mouse tissues A. Hybridization of polyadenylated RNA isolated from the indicated tissues of adult mice. The size of the FLT4 mRNA band is given in kilobases. B. RNase protection analysis of RNA isolated from mouse embryos of various gestational ages (E8-E18) and from a newborn mouse (1 day). Sample E8+P contains also the placenta. The size of the probe and the protected FLT4 fragment are given in nt; β -actin was used as a control.

Figure 2. Expression of FLT4 mRNA in a 7.5-, 8.5- and 11.5-day p.c. embryos. Darkfield and brightfield photomicrographs of *in situ* autoradiograms are shown. No expression of FLT4 mRNA could be detected in a 7.5-day embryo (A). The FLT4 expression of an 8.5-day p.c. mouse embryo is shown in (B) and (C). Arrows point to FLT4 positive cells in the endothelium of posterior cardinal vein (cv), in the allantois (al) in (B) and in angioblasts (ab) of the head mesenchyme in (C). In a 8,5-day p.c. placenta FLT4 transcripts can be seen in endothelial cells of venous lacunae (vl) (D). Panels E and F show a comparison of FLT4 and Tie hybridization signals in 11.5-day p.c. embryos. The region of the developing dorsal aorta and metanephros (mn) is shown (20x). Note that the dorsal aorta is negative for FLT4, but positive for Tie mRNA, whereas both probes hybridize with the endothelium of the subendocardial vein (sv). Also, the FLT4 probe gives a signal from the metanephric vein (v), whereas Tie mostly hybridizes with the metanephric capillaries (c, arrows). da: dorsal aorta, ng: neural groove. Scale bar: 30 μ m.

Figure 3. FLT4 mRNA expression in a 12.5-day p.c. embryo. A sagittal section through the axillar plane is shown. Note that FLT4 mRNA is prominent in dilated vessels of the axilla (ax), in a plexus-like pattern in the periorbital (po) region, in the paravertebral tissue (arrowheads) and in the subcutaneous (sc) tissue. b: brain, li: liver. Scale bar: 5 μ m.

Figure 4. FLT4 in 14 and 16.5 day p.c. embryos. Panels A and B show bright and darkfield images of a midsagittal section. po: periorbital region, lj: lower jaw, ne: neck region, sc: subcutis, mt, mesenterium, ao, aorta. dt: thoracic duct. (C) shows a transverse section of a 16.5 day embryo in

hematoxylin-eosin staining. th: thymus, tr: trachea, e: esophagus, ca: carotid artery, ba: brachiocephalic artery, dt: thoracic duct. (D) shows a higher magnification (40x) of the region of ductus thoracicus; the autoradiographic grains can be seen over the endothelial cells. Also, the small vessel (v) in the upper part of the photograph is positive. Scale bar: 10 μ m (A-C), 1 μ m (D).

Figure 5. Comparison of FLT4 and Tie mRNA expression in cultured endothelial cells. Northern blot analysis of polyadenylated RNA from human foreskin microvascular (MV), femoral vein (VE), aortic (AO) and umbilical vein (HU) endothelial cells. For a comparison, the hybridization signal of the Tie receptor tyrosine kinase mRNA is shown. The bands resulting from the unspecific binding of the probe to the ribosomal RNA are marked with asterixes.

Figure 6. FLT4 in adult human lymphatic vessels of the mesenterium (A,B), lung (C,D), and tonsil (E,F). Note that only the lymphatic vessels in A and C give a FLT4 signal, whereas the veins, capillaries and the arteries are negative for FLT4 mRNA. In the tonsil, the signal is found in the endothelia of some HEVs. Scale bar: 200 μ m.

Figure 7. FLT4 mRNA in normal (A, B) and metastatic (C, D) lymph node and in lymphangioma (E-G). Arrowheads mark the lymphatic sinuses and HEVs, which are FLT4 positive. A comparison of FLT4 and von Willebrand factor signals shows both in the lymphatic endothelium, but only von Willebrand factor signal in the capillary (c) and venous (v) endothelia. Scale bars: 10 μ m (A-D) and 100 μ m (E-G).

Figure 8. FLT4 expression in fetal mesenterial vessels detected by immunoperoxidase staining. Sections were stained with affinity-purified anti-FLT4 antibodies (A), with antigen-blocked antiserum (B) and with preimmune serum (C) and with antiserum specific against the factor VIII-related antigen (D). Note that staining is confined to some, but not all vessels (v). Scale bar, 0.05 mm.

DETAILED DESCRIPTION OF THE INVENTION

Recognizing the importance of identifying changes in lymphatic tissues, especially in lymph nodes in connection to metastatic cancers and immunological disease states, the present inventors have shown that FLT4 is a specific marker that detects lymphatic vessel endothelium and therefore useful as a marker for lymphatic changes in pathological states in man.

The present inventors have earlier shown that the expression pattern of FLT4 in comparison to FLT1 and KDR differs greatly in tissues of 18-week-old human fetuses (4). In order to understand the role of FLT4 during development, the inventors cloned partial cDNAs for mouse FLT4. Using these probes in *in situ* hybridization, FLT4 mRNA expression during mouse development was analysed and it was found that FLT4 is expressed during vasculogenesis and angiogenesis of the lymphatic system. The relevance of these findings was also confirmed in normal and pathological human adult tissues, as FLT4 was found in lymphatic endothelial cells of human adult tissues both in normal and pathological conditions, as well as in some high endothelial venules (HEVs).

The cloning of mouse FLT4 cDNA fragments showed that their deduced amino acid sequence is almost identical with the corresponding human sequence (amino acid identity about 96 % in both segments studied). Further evidence for the identity of the mouse FLT4 cDNA was obtained from Northern hybridization where probes from both species yielded the typical 5.8 kb mRNA signal from mouse tissues. Analysis of RNA isolated from various tissues of adult mice showed FLT4 expression in the liver, lung, heart, spleen and kidney, with no or very little hybridization in the brain and testes. This pattern is similar to the pattern reported earlier by Galland et al. (5). The results of RNase protection suggested that the FLT4 gene is needed during mouse development, starting from 8.5 day p.c. embryos, and the relative expression levels appeared quite stable.

For the *in situ* hybridization two fragments of mouse FLT4 cDNA were selected, which encode sequences of the extracellular domain. This allowed a clear distinction of the hybridization pattern from the related FLK-1 and FLT-

1 receptor patterns, which show only a very low degree of sequence identity
with FLT4 in the extracellular region (6, 7, 8, 9). FLT4, similarly to the FLK-1,
FLT-1, Tie and Tek endothelial receptor tyrosine kinase genes was not
expressed in 7.5 day p.c. embryos. In a 8.5-day p.c. embryo the strongest
5 FLT4 signals were localised in the allantois, the angioblasts of head
mesenchyme and the cardinal vein. In contrast, the the dorsal aorta,
endocardium of the heart and angioblasts of the yolk sac were negative,
unlike for Tie, Tek, FLK-1 and FLT-1, Tie and Tek (10, 8). The restriction of
FLT4 expression to the venous system was even more clear in samples from
10 11.5 day mouse embryos; where the Tie mRNA was expressed also in
arteries. In 12.5-day p.c. embryos the FLT4 signal decorated developing
venous and presumptive lymphatic endothelia, but unlike for the endothelial
Tie receptor tyrosine kinase, arterial endothelia were negative. During later
stages of development FLT4 mRNA became restricted to vascular plexuses
15 devoid of blood cells, representing developing lymphatic vessels. Only the
lymphatic endothelium and some high endothelial venules expressed FLT4
mRNA in adult human tissues. Increased expression occurred in lymphatic
sinuses and high endothelial venules in metastatic lymph nodes and in
lymphangioma.

20 Due to difficulties in the interpretation of data from mouse embryos,
human endothelia were studied, because the lymphatic system is much
better defined in humans. Also, cells established from various endothelia
could be studied in cell culture to see if the specificity of FLT4 expression
persists in *in vitro* conditions. Endothelial cells lines are known to lose
25 differentiated features upon in vitro culture. Therefore, it was not unexpected
that they were negative for FLT4. Cultured aortic endothelial cells were also
devoid of FLT4 mRNA. However, signals were obtained from human
endothelial cells grown from the microvasculature and from femoral and
umbilical veins. Thus, at least some of the specificity of FLT4 expression was
30 retained in cell culture.

In situ hybridization analysis of adult human tissues confirmed the
restriction of FLT4 to the lymphatic system seen in the developing mouse
embryos. FLT4 expression was seen in the lymphatic endothelia and in the
sinuses of human lymph nodes. Interestingly, also some of the HEVs, which
35 have a cuboidal endothelium, shown to function in the trafficking of

leukocytes to the lymph nodes, were FLT4 positive. Furthermore, a parallel hybridization analysis showed that FLT4 mRNA levels were enhanced in these structures in metastatic as compared to normal lymph nodes. FLT4 was also very prominent in lymphangiomas, which are benign tumours composed of connective tissue stroma and growing, endothelial-lined lymphatic channels. FLT4 mRNA was restricted to the lymphatic endothelium of these tumors and absent from their arteries, veins and capillaries. In the human lung we were able to identify lymphatic structures, which were the only FLT4 positive vessels in this tissue.

10 The foregoing results suggest that FLT4 is a novel marker for lymphatic vessels and some high endothelial venules in human adult tissues. They also support the theory on the venous origin of lymphatic vessels. FLT4, as a growth factor receptor, may be involved in the differentiation and functions of these vessels.

15 These results, combined with the FLT4-binding compounds according to the present invention, allows a selective labelling of lymphatic endothelium, especially by using antibodies of the present invention coupled to radioactive, electron-dense or other reporter substances, which can be visualized. It may be possible to inject into the lymphatic system substances, containing FLT4 receptor internalization-inducing monoclonal antibodies, and thereby transport predefined molecules into the lymphatic endothelium. Also, it may be possible to use such the FLT4-binding compounds according to the invention for the detection of high endothelial venules, especially activated HEVs, which express enhanced levels of the FLT4 receptor. To our knowledge, no such specific markers are currently available for lymphatic endothelium.

25 The following examples are given merely to illustrate the present invention and not in any way to limit its scope.

EXAMPLES

EXAMPLE 1

Cloning of mouse FLT4 cDNA probes

Approximately 10^6 plaques from a iFIX®II genomic library from 129SV
5 mice (Stratagene) was screened with the S2.5 human FLT4 receptor cDNA
fragment covering the extracellular domain (3). A 2.5 kb Bam HI fragment
was subcloned from a positive plaque and sequenced from both ends. From
this subclone, polymerase chain reaction was used to amplify and clone into
the pBluescript*KSII+/- vector (Stratagene) an exon fragment covering
10 nucleotides 1745-2049 of the mouse FLT4 cDNA sequence (9).

A second fragment covering nucleotides 1-192 was similarly cloned.

EXAMPLE 2

Analysis of FLT4 mRNA in mouse tissues

Total RNA was isolated from developing embryos (8-18 days p.c. and
15 one day old mice) according to Chomczynski et al. (11). The sample from 8
day p.c. embryos included also the placenta.

For RNase protection analysis, RNA probe was generated from the
linearized FLT4 plasmid obtained according to Example 1 using [³²P]-UTP
and T7 polymerase for the antisense orientation. The β-actin probe used
20 corresponds to nucleotides 1188-1279 of the published mouse β-actin
sequence (12). After purification in a 6% polyacrylamide/7M urea gel, the
labelled transcripts were hybridized to 30 μg of total RNA overnight at 52 °C.
Unhybridized RNA was digested with RNase A (10 U/ml) and T1 (1 μg/ml) at
37 °C, pH 7.5 for 1 h. The RNases were inactivated by proteinase K
25 digestion at 37 °C for 15 min and the samples were analysed in a 6%
polyacrylamide/7M urea gel.

The pattern of expression of FLT4 analysed in this experiment showed
that very weak mRNA signals were obtained from lung, liver, heart, kidney,
skeletal muscle and spleen, whereas testis and brain were apparently

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without specific signal (Fig. 1A). Analysis of a series of RNAs collected during different phases of mouse development by RNase protection assay showed that the FLT4 mRNA was expressed throughout embryogenesis from day 8 p.c. to newborn mice without great variations in signal intensity (Fig. 1B).

EXAMPLE 3

In situ hybridization for FLT4 in mouse embryos

To better assign FLT4 transcripts to cells and tissues, sections of 7.5 and 8.5 day p.c. mouse embryos were hybridized with labelled FLT4 RNAs. Mouse embryos were derived from matings of CBA and NMRI mice. Pregnant mice were killed by cervical dislocation and the embryos were either immediately frozen or transferred via phosphate buffered saline into 4% paraformaldehyde. The embryos and isolated mouse organs were fixed for 18 h at 4°C, dehydrated, embedded in paraffin and cut into 6 µm sections.

RNA probes (antisense and sense) of 192 and 349 nucleotides (see Example 1) were generated from linearized plasmids using [³⁵S]-UTP. *In situ* hybridization of sections was performed according to Wilkinson et al. (13, 14), with the following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax, 2) 6 µm sections were cut, placed on a layer of diethyl pyrocarbonate-treated water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine, 3) alkaline hydrolysis of the probes was omitted, and 4) the high stringency wash was for 80 min at 65°C in a solution containing 30 mM DTT and 1 x SSC. The sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. The slides were exposed for 14 days, developed and stained with hematoxylin. Control hybridizations with sense strand and RNase A-treated sections did not give a specific signal above background.

As shown in Figures 2A and B, FLT4 mRNA was not expressed in 7.5 day p.c. mouse embryos, but bright signals were detected in the posterior cardinal vein (cv) on day 8.5 of development. In contrast, the developing heart (data not shown) and dorsal aorta (da) were FLT4-negative. In the extraembryonic tissues FLT4 was prominently expressed in the allantois (al

in panel B), whereas developing blood islands of the yolk sac were negative (data not shown). On the other hand, angioblasts (ab) of the head mesenchyme were strongly FLT4 positive (C). In the developing placenta FLT4 signal was first seen in peripheral sinusoidal veins (data not shown). In
5 9.5 day p.c. placenta the endothelium of venous lacunae (vl in D) and the giant cells partially fused to the Reichert's membrane (data not shown) expressed FLT4 mRNA.

Thus, although FLT4 expression was very prominent in the earliest endothelial cell precursors, the angioblasts, it appeared to be restricted only
10 to certain vessels of 8.5 day p.c. embryos. The Tie receptor is known to be expressed in all endothelial cells of developing mouse embryos and thus provides a marker for these cells. Notably, in contrast to the Tie probe, the FLT4 probe hybridized very weakly if at all with arterial endothelia of 11.5 day
15 p.c. embryos, e.g. with the endothelium of the developing dorsal aorta (da in Fig. 2 E,F) or the carotic arteries (data not shown). Instead, FLT4 signal was much more prominent in the developing veins. For example, FLT4 signal was detected veins surrounding the developing metanephros (v, sv in E), while the Tie probe predominantly recognized capillaries (c) within the metanephros (F).

20 As can be seen from Figure 3, FLT4 mRNA is distributed in several regions of a 12.5 day p.c. mouse embryo, being particularly prominent in the dilated vessel of the axillar region (ax). A similar FLT4 positive vessel structure was seen in the mid-sagittal section in the jugular area (data not shown). A plexus-like pattern of FLT4 expressing vessels appeared in the
25 periorbital region (po) and surrounding the developing vertebrae (vb). Also, just beneath the developing skin, a FLT4-positive vascular network was evident (sc). Weaker capillary signals were obtained from several regions, including the developing brain (b). FLT4 mRNA could also be detected in
30 small vessels of the neck region, of the developing snout and at the base of the developing tongue as well as in the tail region (data not shown). Besides, the liver (li) was strongly positive for FLT4 mRNA in a spotlike pattern.

During further development, FLT4 RNA appeared to become more restricted to certain vessels of the embryo. A 14.5 day p.c. embryo shows

nicely this restricted pattern of expression (Fig. 4 A,B). In the midsagittal section of Figure 4 the most prominent FLT4 signal is seen along the developing vertebral column in its anterior part. This signal was considered to originate from endothelial cells of the thoracic duct (dt), which is the largest lymphatic vessel formed at this time of development. In contrast, the dorsal aorta (da) and inferior vena cava (vc) were negative. Dilated vessels in the mesenteric region were also strongly positive for FLT4. Furthermore, as in the 12.5 day p.c. embryos, vessel networks along anatomical boundaries in the periorbital (po), lower jaw (lj) as well as in the neck (ne) region contained FLT4 positive endothelia. Similar structures were present in the pericardial space and throughout the subcutaneous (sc) tissue. Notably, in contrast to FLT4 negative vessels, all FLT4 positive vessels were devoid of blood cells in their lumen. These expression patterns suggest that FLT4 becomes confined to the endothelia of lymphatic vessels at this time of development. An additional site where we observed FLT4 expression, was in the sinusoids of the developing bone marrow (bm).

Photographs of a transverse section of the upper thorax of a 16.5 day p.c. embryo hybridized with the FLT4 probe are shown in panels C and D of Figure 4. The section shown in C has been stained with hematoxylin-eosin to visualize the different types of vessels in this area. These include the carotic and brachiocephalic arteries (ca, ba), the vena cava (vc) and the thoracic duct, which is smaller in size and lacks surrounding muscular and connective tissue (arrow). A magnification of the region of thoracic duct is shown in panel D, where the FLT4 autoradiographic grains can be seen. Endothelial cells of the thoracic duct as well as a small vessel (v) in the vicinity hybridize with the FLT4 probe.

EXAMPLE 4

Analysis of FLT4 mRNA in cultured endothelial cells

The *in situ* hybridization results described in Example 3, showed that FLT4 is expressed in venous endothelial cells and later in lymphatic vessels and some venous endothelial cells, but not in arterial endothelia. In order to see if such regulation was maintained *in vitro*, we studied cultured endothelial cells using Northern blotting and hybridization analysis.

Endothelial cells from human aorta, femoral vein, umbilical vein, and from foreskin microvessels were isolated, cultured and characterized as previously described by Van Hinsberg, (15, 16). They were used at confluent density after five to eight passages (split ratio 1:3) for the isolation of polyadenylated RNA.

The endothelial cell lines EA-hy926, BCE and LEII did not express FLT4 (data not shown). However, cultured human microvascular, venous and umbilical vein endothelial cells were positive for the FLT4-specific 5.8 and 4.5 kb mRNAs, whereas the aortic endothelial cells were negative (Fig. 5). In contrast, another endothelial receptor tyrosine kinase gene, Tie, was expressed as a 4.4 kb mRNA in all endothelial cell types studied.

EXAMPLE 5

FLT4 mRNA in in adult human tissues

The results obtained in Example 3 indicated that the FLT4 mRNA becomes largely confined to the endothelium of lymphatic vessels during development. Because of the potential significance of this finding in humans, we also studied FLT4 in adult human tissues using a human FLT4 probe. The human FLT4 probe used was an EcoRI-SphI fragment covering base pairs 1-595 of the cDNA (3). The von Willebrand factor probe was an EcoRI-HindIII fragment covering base pairs 1-2334 (17).

We used routinely fixed material sent for histopathological diagnosis. Normal lung tissue was obtained from a resection of the left inferior lung lobe affected by epidermoid cancer. Mesenterium and mesenterial lymph nodes were obtained from a patient having a colonic adenocarcinoma. A normal lymph node adjacent to the salivary gland was enucleated because of its abnormal size. The tonsils from two patients and the two appendixes had no diagnostic changes. Two lymphangiomyomas and three cystic lymphangiomas were studied with similar results.

For human tissues, which were routine samples fixed with 10% formalin for histopathological diagnosis, the normal *in situ* protocol gave just background, whereas microwave treatment instead of proteinaase K enabled specific hybridization (18, 19).

In the mesenterium, lung and appendix lymphatic endothelia (lv) gave FLT4 signals, while veins (v), arteries (a) and capillaries (c) were negative (Fig. 6A-D and data not shown). To study whether FLT4 is expressed in the HEVs, the tonsils were studied. Indeed, in the tonsils, FLT4 specific
5 autoradiographic grains were detected in some HEVs (E, F).

EXAMPLE 6

Analysis of FLT4 mRNA in normal and metastatic lymph node and in lymphangioma

A portion of a human mesenterial lymph node (see Example 5) was
10 analysed for FLT4 expression. The results are shown in Figure 7.

FLT4 is expressed in the lymphatic sinuses (ls) and afferent and efferent lymphatic vessels (data not shown). The same pattern is seen in a lymph node containing adenocarcinoma metastases (C, D). Some HEVs in both normal and metastatic lymph node were also positive. In panel E, FLT4
15 expression is shown in a cystic lymphangioma (compare with the hematoxylin-eosin stained section in F). Notably, the specificity of FLT4 to lymphatic endothelia is evident from the comparison with the *in situ* signals for von Willebrandt factor in all blood vessels (F).

EXAMPLE 7

20 Localization of FLT4 in fetal endothelial cells

An FLT4 cDNA fragment encoding the 40 carboxy terminal amino acids of the short form was cloned as a 657 bp *EcoRI*-fragment into the pGEX-1IT bacterial expression vector (Pharmacia) in frame with the glutathione-S-transferase coding region. The resulting GST-FLT4 fusion
25 protein was produced in *E.coli* and purified by affinity chromatography using a glutathione-Sepharose* 4B column. The purified protein was lyophilized, dissolved in PBS, mixed with Freund's adjuvant and used for immunization of rabbits. Antisera were used after the fourth booster immunization.

Tissues from 17 and 20-week-old human fetuses were obtained from
30 legal abortions induced with prostaglandins. The study was approved by the Ethical Committee of the Helsinki University Central Hospital. The

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gestational age was estimated from the fetal foot length. The fetal tissues were embedded in Tissue-Tek* (Miles), frozen immediately and stored at -70 °C.

5 Anti-FLT4 antiserum was cross-absorbed to a GST-Sepharose column to remove anti-GST-antibodies and then purified by GST-FLT4 affinity chromatography. Several 6 µm-thick cryostat sections of the tissues were fixed with acetone and treated with 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. After washing, the sections were incubated with 5% normal swine serum. Sections were then incubated with antibodies
10 against FLT4, washed and bound antibodies were detected with peroxidase-conjugated swine anti-rabbit IgG followed by staining for peroxidase activity using 0.2% 3,3-diaminobenzidine (Amersham) as a substrate. The sections were counterstained in Meyer's hematoxylin.

15 Anti-FLT4 immunoperoxidase staining of human fetal mesenterium showed FLT4 protein in the endothelium of several vessels (Fig. 4A), while control stainings with antigen-blocked anti-FLT4 antibodies (B) and preimmune sera (C) were negative. For comparison, Figure 4D shows results of staining using an antiserum against the factor VIII-related antigen, which is specific for vascular endothelial cells.

20 EXAMPLE 8

Production of monoclonal antibodies against FLT4

Fusion I:

Four months old Balb/c male mice were immunized by intraperitoneal injection of the recombinantly produced FLT4 protein (see Example 7) in
25 concentrated medium (150 µg/mouse), emulsified with Freund's complete adjuvant. Booster injections of 150 µg were given at three to four week intervals and a final booster (10 µg FLT4 in PBS administered intraperitoneally) was given after another three-week interval. Four days
30 after the final booster dose, the mice were sacrificed and mouse splenic lymphoid cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

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The fused cells were harvested in 96-well culture plates (NUNC) in Ex-Cell 320 medium (SERALAB) containing 20% fetal calf serum and HAT supplement (hypoxanthine-aminopterin-thymidine; GIBCO, 043-01060H; diluted 50-fold). Cells were cultured at +37°C, in a 5% CO₂ atmosphere.

5 After 10 days, HAT-supplemented medium was changed to HT-supplemented cell culture medium (GIBCO; 043-01065H, diluted 50-fold). HT medium is identical to HAT medium, but lacks aminopterin.

In three weeks specific antibody production was determined by the antigen-specific immunofluorometric assay, IFMA, described in Example 10

10 The master clones were cloned by limited dilutions as described by Staszewski et al., Yale Journal of Biology and Medicine, 57:865-868 (1984). Positive clones were expanded onto 24-well tissue culture plates (NUNC), recloned, and re-tested by the same method. Positive clones were tested by fluorescence-activated cell sorting (FACS).

15 The stable clones secreted immunoglobulins belonging to the IgG1 class, except one, which produced Ig probably belonging to class IgA. The subclass of monoclonal antibody was determined using rat monoclonal antibody to mouse subclass as biotin conjugate (SEROTEC) in IFMA.

20 Balb/c mice were used to produce monoclonal antibodies in ascites fluid. The hybridomas described above were intraperitoneally injected into mice after pretreatment of the animals with pristane (2,6,10,14-tetramethylpentadecane 98%, ALDRICH-CHEMIE D7924 Steinheim, Cat.No. T 2,280-2). 0.5 ml of pristane (i.v.) was injected about two weeks prior to the hybridoma cells. The amount of cells injected were approximately 7.5 to 9 x
25 10⁶ per mouse. Ascites was collected 10 to 14 days after injection of the hybridomas.

Fusion II:

Two months old Balb/c mice (female) were immunized by intraperitoneal injection of the recombinantly produced FLT-4 protein (see
30 Example 7) (20 µg/mouse), emulsified with Freund's complete adjuvant. Booster injections of 20 µg were given at three to four week intervals and a final booster (10 µg FLT-4 in PBS administered i.v.) was given after another three-week interval. Four days after the final booster dose, the mice were

sacrificed and mouse splenic lymphoid cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

The fused cells were harvested in 96-well culture plates (FALCON) in OptiMEM^{*} 1 (with Glutamax^{*} 1, 51985-026, GIBCO BRL) medium containing
5 20 % fetal calf serum and HAT supplement (hypoxanthine-aminopterin-thymidine; GIBCO BRL 21060-017; diluted 1:50 fold). Cells were cultured at +37 °C, in a 5% CO₂ atmosphere. After 10 days, HAT-supplemented medium was changed to HT-supplemented cell culture medium (GIBCO BRL; 41065-012, diluted 1:50-fold). HT-medium is identical to HAT-medium, but
10 lacks aminopterin.

In three weeks specific antibody production was determined by the antigen-specific ImmunoFluoroMetric Assay (IFMA) described in Example 9. The master clones were cloned by limited dilutions as described by Staszewski et al., Yale Journal of Biology and Medicine, 57:865-868 (1984).
15 Positive clones were expanded onto 24-well tissue culture plates (FALCON), recloned, and re-tested by the same method. Positive clones were tested by fluorescence-activated cell sorting (FACS).

The 2E11 and 6B2 clones secreted immunoglobulins belonging to the IgG₁ class, 2B12 clones produced Ig belonging to subclass IgM . The mouse subclass IgG₁ was determined using rat monoclonal antibody against mouse subclass heavy chain as biotin conjugate (SEROTEC) in IFMA and the mouse subclass IgM was determined with Mouse Monoclonal Antibody Isotyping Kit (Dipstick Format) (19663-012, Life Technologies Inc.).
20

EXAMPLE 9

25 Specificity of monoclonal antibodies against FLT4

Fusion I antibodies:

The extracellular domain of FLT4 described in Example 7, was labelled according to Mukkala et al., in *Anal.Biochem.* 176(2):319-325, 1989 , with the following modification: a 250 times molar excess of isothiocyanate
30 DTTA-Eu (N1 chelate, WALLAC, Finland) was added to the FLT4 solution (0.5 mg/ml in PBS) and the pH was adjusted to about 9 by adding 0.5 mol/L

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sodium carbonate buffer, pH 9.8. The labelling was performed overnight at +4°C. Unbound label was removed using PD-10 (PHARMACIA, Sweden) with TSA buffer (50 mmol/L Tris-HCl, pH 7.8 containing 0.15 mol/l NaCl) as eluent.

5 After purification, 1 mg/ml bovine serum albumin (BSA) was added to the labelled FLT4 and the label was stored at +4°C. The number of europium ions incorporated per FLT4 molecule was 1.9, as determined by measuring the fluorescence in a ratio to that of known EuCl₃ standards (Hemmilä et al., *Anal.Biochem.*, 137:335-343, 1984).

10 The antibodies produced in Example 8, were screened using a Sandwich type immunofluorometric assay using microtitration strip wells (NUNC, polysorb) coated with rabbit anti-mouse Ig (Z 259, DAKOPATTS). The precoated wells were washed once by Platewash* 1296-024 (WALLAC) with DELFIA wash solution. The DELFIA assay buffer was used as a dilution
15 buffer for cell culture supernatants and for serum of the splenectomized mouse (at dilutions between 1:1000 to 1: 100 000) used as positive control in the preliminary screening assay.

 An overnight incubation at +4°C (or alternatively for 2 hours at room temperature) was begun by shaking on a Plateshake shaker (1296-001,
20 WALLAC) for 5 min followed by washing four times with wash solution as described above.

 The europium-labelled FLT4 was added at a dilution of 1:500 in 100 µl of the assay buffer. After 5 min on a Plateshake shaker and one hour incubation at RT the strips were washed as described above.

25 Enhancement solution (DELFIA) was added at 200 µl/well. The plates were then shaken for 5 min on a Plateshake shaker and the intensity of fluorescence was measured by ARCUS*1230 (WALLAC) for 10-15 min. (Lövgren et al., In: Collins W.P. (Ed) *Alternative Immunoassays*, John Wiley & Sons Ltd, 1985; pp. 203-216).

30 The resulting monoclonal antibodies against FLT4 and corresponding FACS results are summarized in Table 2.

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TABLE 2

Mab clones	LTR% ^{a)}	NEO% ^{b)}	DELFLIA-counts
1B1	67,3	1	20625
1B1D11	75	1,2	19694
1B1F8	76,1	1,4	18580
4F6	69,9	1,2	23229
4F6B8G12	75	0,3	24374
4F6B8H11	75,9	0,3	28281
4F6B8E12	74,8	0,4	27097
4F6B8G10	75,3	0,4	26063
9D9	45,1	0,75	17316
9D9D10	71,7	2,3	18230
9D9F9	73	1,8	11904
9D9G6	74,3	2,9	16743
9D9G7	70,7	1,3	17009
10E4	24,2	1,4	39202
10E4B10E12	32,3	0,3	42490
10E4B10G10	36,5	0,3	54815
10E4B10F12	45,6	0,4	43909
10E4B10G12	45,7	0,5	35576
11G2	30,2	1,6	11304
11G2D12	74,4	1,5	14660
11G2G9	74,2	0,9	10283
11G2H7	74,4	2,1	25382

a) FACS results with LTR transfected cells

b) FACS results with NEO cells (control)

- 5 One clone, designated anti-FLT4 9D9F9 was found to stably secrete monoclonal antibody which was determined to be of immunoglobulin class IgG1 by IFMA. Hybridoma 9d9f9 was deposited with the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures and Viruses, Mascheroder Weg 1b, 3300 Braunschweig, Germany, 10 March 23, 1995, and given accession No. ACC2210.

Fusion II antibodies:

The extracellular domain of FLT-4 described in Example 7, was labelled according to Mukkala et al., in Anal.Biochem. 176(2): 319-325, 1989, with the following modification: a 250 times molar excess of isothiocyanate
5 DTTA-Eu (N1 chelate, Wallac, Finland) was added to the FLT-4 solution (0.5 mg/ml in PBS) and the pH was adjusted to about 9 by adding 0.5 mol/L sodium carbonate buffer, pH 9.8. The labelling was performed overnight at +4°C. Unbound label was removed using PD-10 (PHARMACIA) with TSA buffer (50 mmol/L Tris-HCl, pH 7.8 containing 0.15 mol/L NaCl) as eluent.

10 After purification, 1 mg/ml bovine serum albumin (BSA) was added to the labelled FLT-4 and the label was stored at +4°C. The number of europium ions incorporated per FLT-4 molecule was 1.9, as determined by measuring the fluorescence in a ratio to that of known EuCl₃ standards (Hemmil et al., Anal.Biochem., 137: 335-343, 1984).

15 The antibodies produced in Example 8, were screened using a FLT-4 specific IFMA using microtitration wells (Nunc, Polysorb^{*}) coated with rabbit antimouse Ig (Z 259, DAKO). The precoated wells were washed once with wash solution (Wallac) by using DELFIA^{*} Plate wash.

The DELFIA assay buffer was used as dilution buffer for cell culture
20 supernatants (dilution 1:2 in preliminary screening) and for serum of the splenectomized mouse (dilutions 1:1 000 to 1:100 000) which was used as positive control. As standard the purified antiFLT-4 9D9F9 (mouse subclass IgG1) was used at concentrations between 1.0 ng/ml and 250 ng/ml. Samples were first shaken at room temperature for five minutes on Plate
25 shake (Wallac) and then incubated approx. 18 hours at +4°C. The frames were first washed four times, then the Eu-labelled FLT-4 (1:2000, in 100 µl assay buffer) was added and finally the frames were incubated for one hour at room temperature. After washing as described the enhancement solution (200 µl/well, Wallac) was added and the frames were shaken for 5 minutes
30 on Plate shake. The intensity of fluorescence was measured by ARCUS-1230 (Wallac).

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The resulting monoclonal antibodies against FLT-4 and corresponding results are summarized in Table 3.

A standard curve for quantitation of antiFLT-4 antibodies was made by using affinity purified antiFLT-4 9D9F9. The linear range reached from 1.0
5 ng/ml to 250 ng/ml.

Cell lysate of NIH 3T3 cells cotransfected with pLTRFLT4 construct expressing full-length FLT4 on the surface was electrophoresed in 6.5% SDS-PAGE, proteins were transferred onto nitrocellulose nitrate membrane (0.45 μ m, SCHLEICHER & SCHUELL) and immunoblotted with Mab cell
10 culture supernatants (1:10, 50 mmol/L TRIS - 40 mmol/L glycine buffer containing methanol 4%, SDS 0.04%). The specificity of Mab was detected using incubation with HRP-conjugated rabbit antimouse Ig (P 161, DAKO, diluted 1:1000 in 20 mmol/L TRIS buffer pH 7.5 containing 150 mmol/L
15 saline, 5% milk powder) and ECL (Enhanced chemiluminescence, AMERSHAM).

TABLE 3.

	Mab clones	LTR %a)	NEOb)	approx.Mab prod. ng/ml/10 ⁶ cells	WB
20	2B12E10	39.5	6.0	440	+
	2E11D11	44.6	8.8	110	+
	2E11F9	49.5	4.5	100	+
	2E11F12	46.0	4.1	180	+
25	2E11G8	41.2	7.8	160	+
	6B2E12	NF	NF	1390	+
	6B2F8	NF	NF	470	+
	6B2G6	NF	NF	630	+
30	6B2H5	NF	NF	740	+
	6B2H8	NF	NF	1800	+

a) FACS results with LTR transfected cells

b) FACS results with NEO cells (control)

35 NF not functioning in FACS


c) quantitation of Mab production based on affinity purified antiFLT 9D9F9 antibody used as standard

As is evident from the foregoing, antibodies according to the present invention are useful in the diagnosis and identification of lymphatic vessels, lymphatic endothelial cells, high endothelial venules, lymphangiomas, metastatic lymph nodes and other disease states of the lymphatic system, the
5 detection and monitoring of metastatic spread, in the stimulation and inhibition of endothelial cells of lymphatic vessels and high endothelial venules, in the introduction of molecules selectively into endothelial cells and in the imaging of lymphatic vessels and their disease states. Other uses of the presently-claimed subject matter are apparent to the skilled artisan.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>13 - 17</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-38124 Braunschweig Germany	
Date of deposit 1995-03-23	Accession Number DSM ACC2210
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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(ii) TITLE OF INVENTION: FLT4 RECEPTOR TYROSINE KINASE AND ITS USE
IN DIAGNOSIS AND THERAPY

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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CCACGCGCAG CGGCCGGAG ATG CAG CGG GGC GCC GCG CTG TGC CTG CGA CTG
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Trp Leu Cys Leu Gly Leu Leu Asp Gly Leu Val Ser Gly Tyr Ser Met
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GCT TGG CCA GGA GCT CAG GAG GCG CCA GCC ACC GGA GAC AAG GAC AGC
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GAG GAC ACG GGG GTG GTG CGA GAC TGC GAG GGC ACA GAC GCC AGG CCC
 292
 Glu Asp Thr Gly Val Val Arg Asp Cys Glu Gly Thr Asp Ala Arg Pro
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TAC TGC AAG GTG TTG CTG CTG CAC GAG GTA CAT GCC AAC GAC ACA GGC
 340
 Tyr Cys Lys Val Leu Leu Leu His Glu Val His Ala Asn Asp Thr Gly
 95 100 105

AGC TAC GTC TGC TAC TAC AAG TAC ATC AAG GCA CGC ATC GAG GGC ACC
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 Ser Tyr Val Cys Tyr Tyr Lys Tyr Ile Lys Ala Arg Ile Glu Gly Thr
 110 115 120

ACG GCC GCC AGC TCC TAC GTG TTC GTG AGA GAC TTT GAG CAG CCA TTC
 436
 Thr Ala Ala Ser Ser Tyr Val Phe Val Arg Asp Phe Glu Gln Pro Phe
 125 130 135

ATC AAC AAG CCT GAC ACG CTC TTG GTC AAC AGG AAG GAC GCC ATG TGG
 484
 Ile Asn Lys Pro Asp Thr Leu Leu Val Asn Arg Lys Asp Ala Met Trp
 140 145 150 155

GTG CCC TGT CTG GTG TCC ATC CCC GGC CTC AAT GTC ACG CTG CGC TCG
 532
 Val Pro Cys Leu Val Ser Ile Pro Gly Leu Asn Val Thr Leu Arg Ser
 160 165 170

CAA AGC TCG GTG CTG TGG CCA GAC GGG CAG GAG GTG GTG TGG GAT GAC
 580
 Gln Ser Ser Val Leu Trp Pro Asp Gly Gln Glu Val Val Trp Asp Asp
 175 180 185

CGG CGG GGC ATG CTC GTG TCC ACG CCA CTG CTG CAC GAT GCC CTG TAC
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 Arg Arg Gly Met Leu Val Ser Thr Pro Leu Leu His Asp Ala Leu Tyr
 190 195 200

CTG CAG TGC GAG ACC ACC TGG GGA GAC CAG GAC TTC CTT TCC AAC CCC
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TTC CTG GTG CAC ATC ACA GGC AAC GAG CTC TAT GAC ATC CAG CTG TTG
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 Phe Leu Val His Ile Thr Gly Asn Glu Leu Tyr Asp Ile Gln Leu Leu
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CCC AGG AAG TCG CTG GAG CTG CTG GTA GGG GAG AAG CTG GTC CTG AAC
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 916
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 285 290 295

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GAC GGC TTC ACC ATC GAA TCC AAG CCA TCC GAG GAG CTA CTA GAG GGC
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 Asp Gly Phe Thr Ile Glu Ser Lys Pro Ser Glu Glu Leu Leu Glu Gly
 560 565 570

CAG CCG GTG CTC CTG AGC TGC CAA GCC GAC AGC TAC AAG TAC GAG CAT
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 Gln Pro Val Leu Leu Ser Cys Gln Ala Asp Ser Tyr Lys Tyr Glu His
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 1876
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 1924
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 1972
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 640 645 650

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 655 660 665

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 670 675 680

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 2116
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 685 690 695

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 2164
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 700 705 710 715

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AAG CTG AGC ATC CAG CGC GTG CGC GAG GAG GAT GCG GGA CGC TAT CTG
 2260
 Lys Leu Ser Ile Gln Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu
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 Cys Ser Val Cys Asn Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val
 750 755 760

GCC GTG GAA GGC TCC GAG GAT AAG GGC AGC ATG GAG ATC GTG ATC CTT
 2356
 Ala Val Glu Gly Ser Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu
 765 770 775

GTC GGT ACC GGC GTC ATC GCT GTC TTC TTC TGG GTC CTC CTC CTC CTC
 2404
 Val Gly Thr Gly Val Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu
 780 785 790 795

ATC TTC TGT AAC ATG AGG AGG CCG GCC CAC GCA GAC ATC AAG ACG GGC
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 Ile Phe Cys Asn Met Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly
 800 805 810

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 Tyr Leu Ser Ile Ile Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln
 815 820 825

TGC GAA TAC CTG TCC TAC GAT GCC AGC CAG TGG GAA TTC CCC CGA GAG
 2548
 Cys Glu Tyr Leu Ser Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu
 830 835 840

CGG CTG CAC CTG GGG AGA GTG CTC GGC TAC GGC GCC TTC GGG AAG GTG
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GTG GAA GCC TCC GCT TTC GGC ATC CAC AAG GGC AGC AGC TGT GAC ACC
 2644
 Val Glu Ala Ser Ala Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr
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 2692
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 2740
 Ala Leu Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly Asn His Leu
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WHAT IS CLAIMED IS:

1. A method for detecting lymphatic vessels in a tissue sample, comprising:
 - a) contacting an isolated monoclonal antibody with the tissue sample suspected of containing lymphatic vessels, said isolated monoclonal antibody being specific for the extracellular domain of FLT4 receptor tyrosine kinase and further comprising a detectable label; and
 - b) detecting said antibody bound to said tissue sample.
- 10 2. A method for identifying changes in lymphatic vessels and high endothelial venules (HEVs) characterized by FLT4 expression, comprising the steps of:
 - a) contacting a tissue sample from a patient suspected of having a disease characterized by changes in lymphatic vessels and HEVs with an isolated monoclonal antibody that is specific for the extracellular domain FLT4 reception tyrosine kinase and that comprises a detectable label; and
 - b) detecting the presence of said antibody in said tissue sample.
- 20 3. A method for diagnosing a disease characterized by changes in lymphatic vessels and high endothelial venules (HEVs) comprising:
 - a) contacting a tissue sample from a patient suspected of having a disease characterized by changes in lymphatic vessels and HEVs with the isolated an isolated monoclonal antibody that is specific for the extracellular domain FLT4 reception tyrosine kinase and that comprises a detectable label; and
 - b) detecting the presence of said antibody in said tissue sample.
4. The method of any one of claims 1 to 3, wherein the antibody is anti-FLT4 monoclonal antibody 9D9F9.

5. The method according to any one of claims 1 to 4, wherein the tissue sample comprises a lymph node.

6. Use of a monoclonal antibody against the extracellular domain of FLT4 receptor tyrosine kinase for the preparation of a diagnostic composition for imaging lymphatic vessels, lymph nodes or high endothelial venules.

7. Use of a monoclonal antibody against the extracellular domain of FLT4 receptor tyrosine kinase for the preparation of a diagnostic composition for detecting lymphatic vessels, lymph nodes or high endothelial venules.

8. The use of claim 7, for the preparation of a diagnostic
10 composition for detecting a lymph node tissue.

9. Use of a monoclonal antibody against the extracellular domain of FLT4 receptor tyrosine kinase for the preparation of a pharmaceutical composition for inhibiting the FLT4-mediated lymphatic vascularization which is associated with a disease selected from the group consisting of metastatic cancers, lymphomas, lymphangiomas, chronic inflammation, acute inflammation, infections and immunological diseases.

10. The use of any one of claims 6 to 9, wherein the antibody further comprises a detectable label.

11. The use of any one of claims 6 to 9, wherein the detectable
20 label is selected from the group consisting of radioisotopes, fluorochromes, dyes, enzymes and biotin.

12. The use of any one of claims 6 to 11, wherein the antibody is anti-FLT4 monoclonal antibody 9D9F9.

13. The use of claim 12, wherein the antibody is produced by a hybridoma cell deposited as DSM Accession No. ACC 2210.

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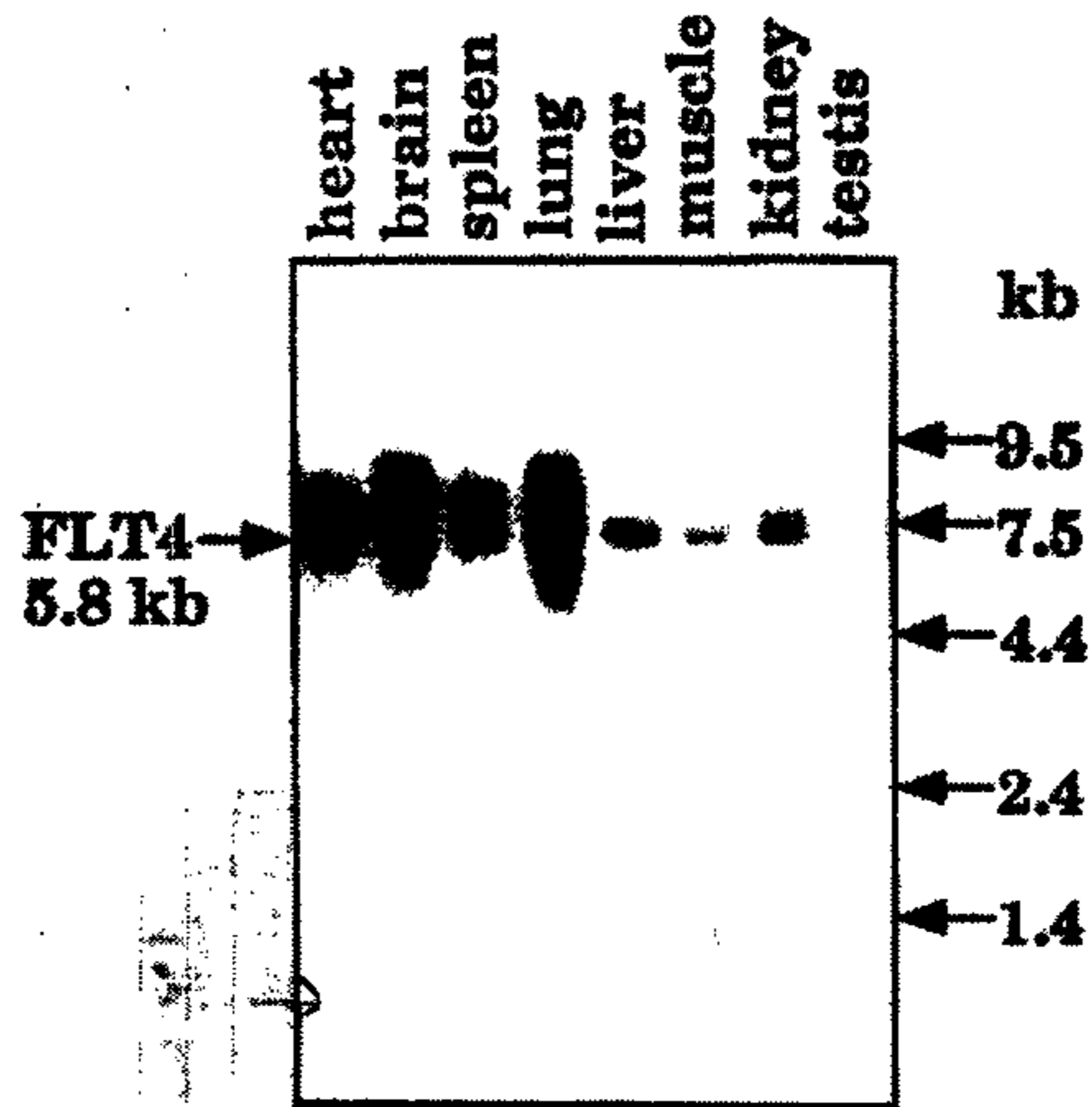


Fig. 1A

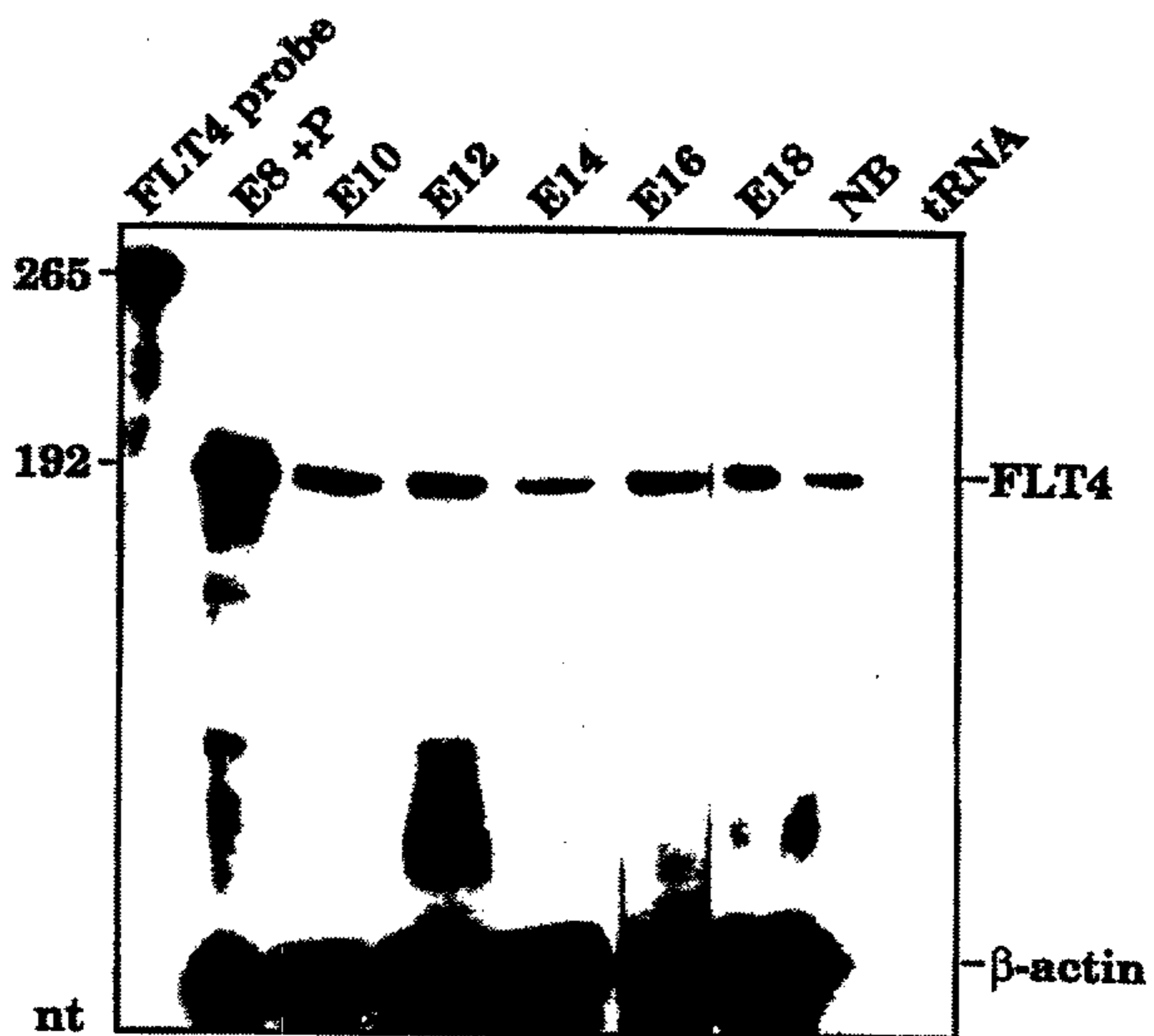


Fig. 1B

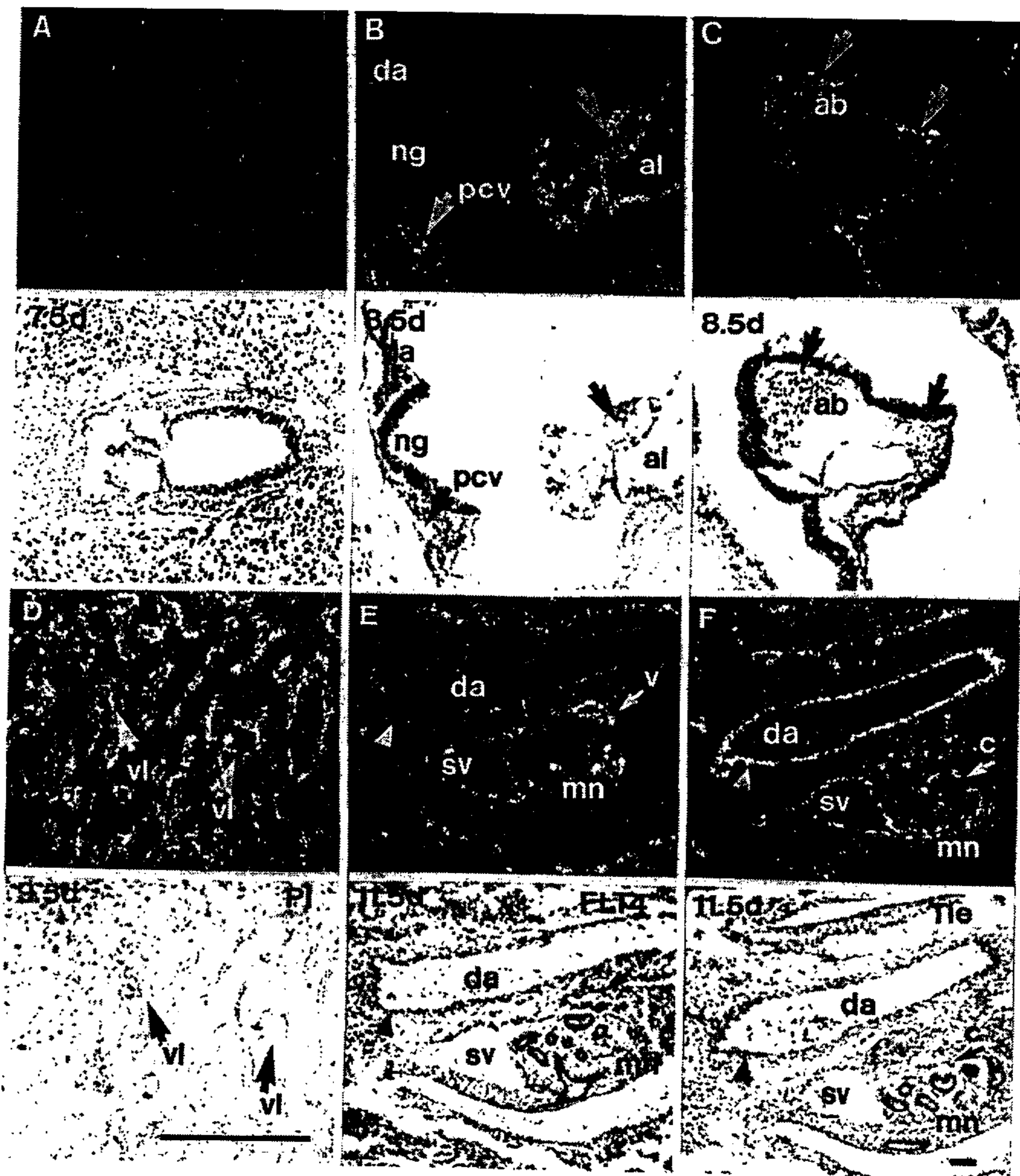


Fig. 2

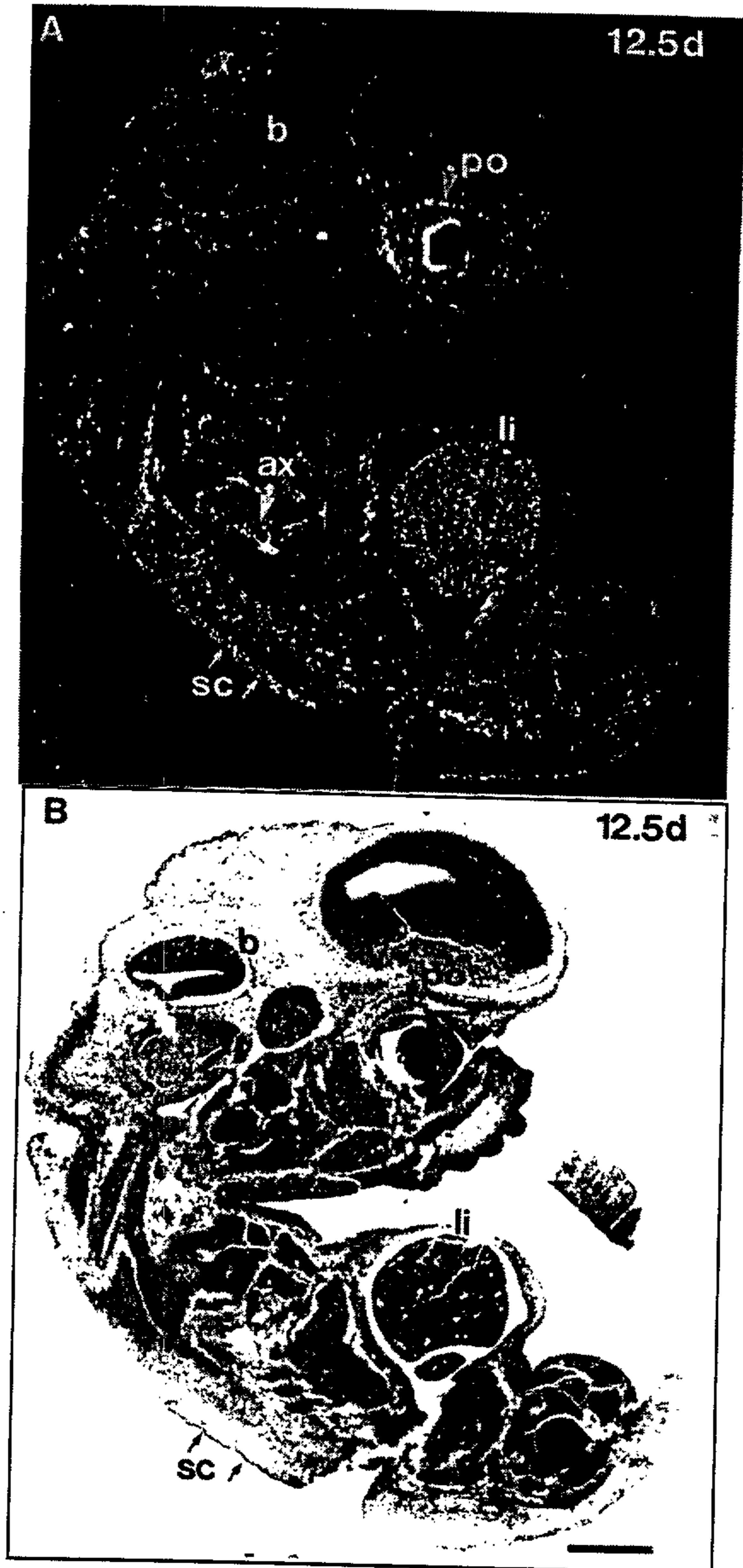


Fig. 3
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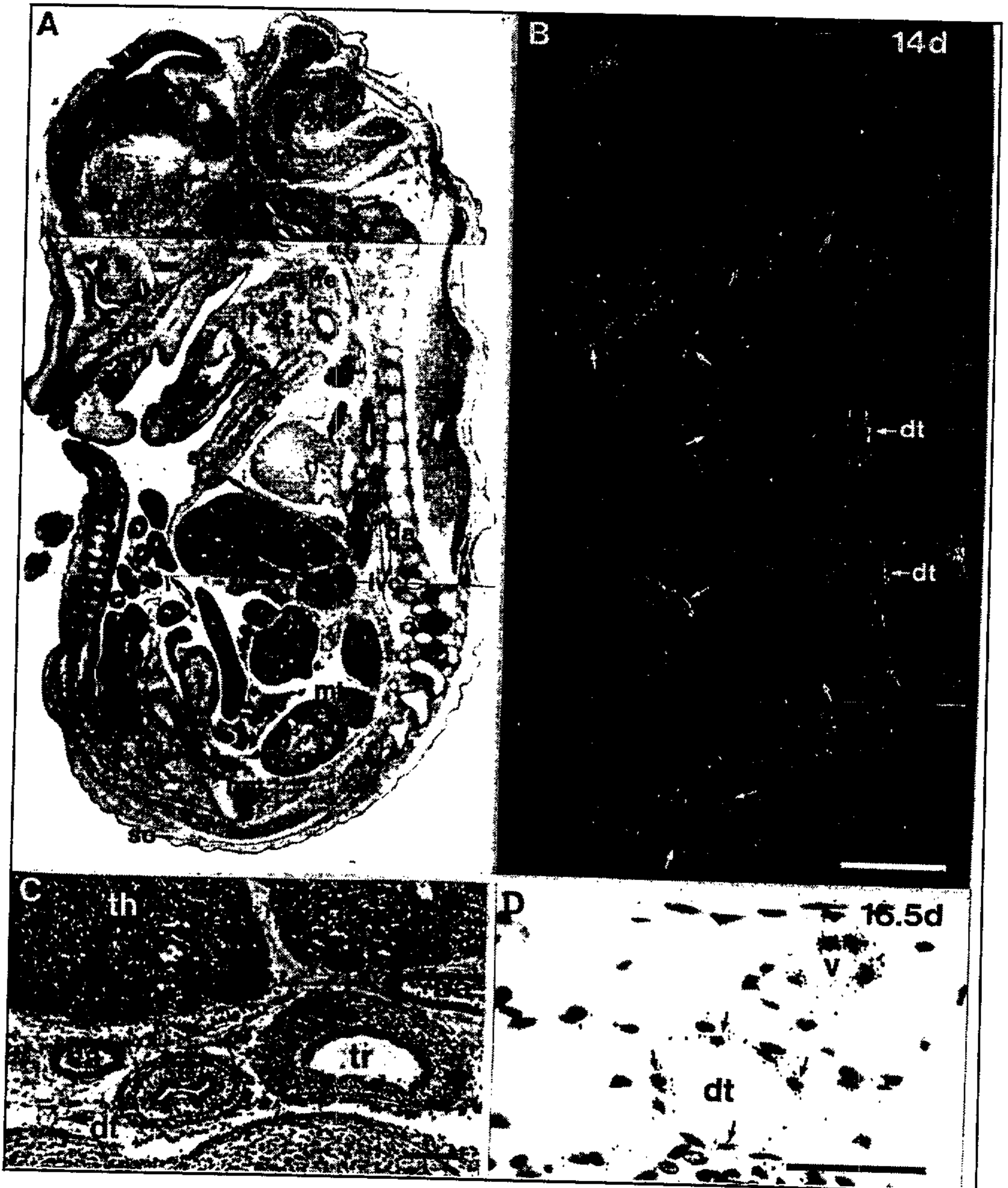


Fig. 4

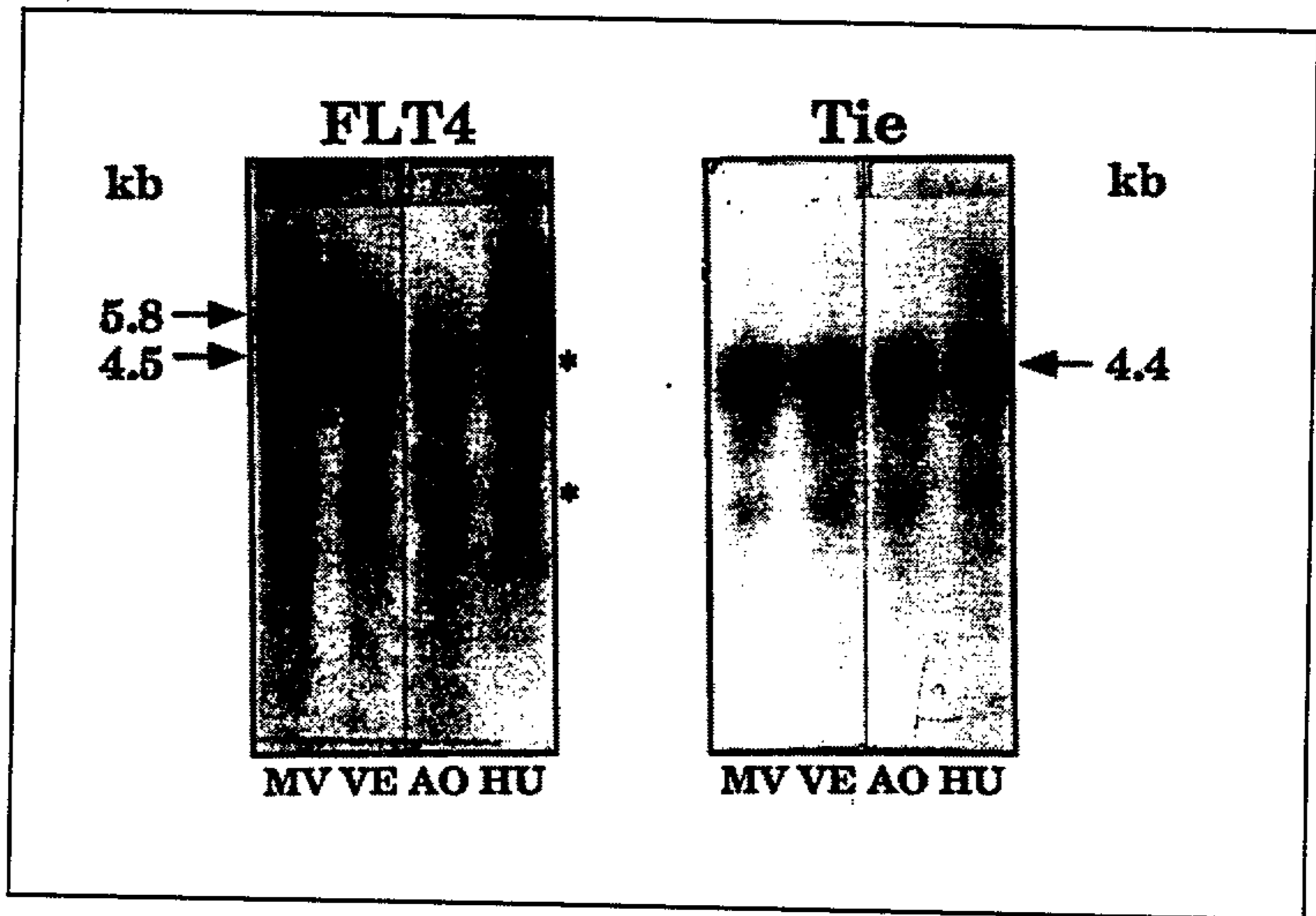


Fig. 5

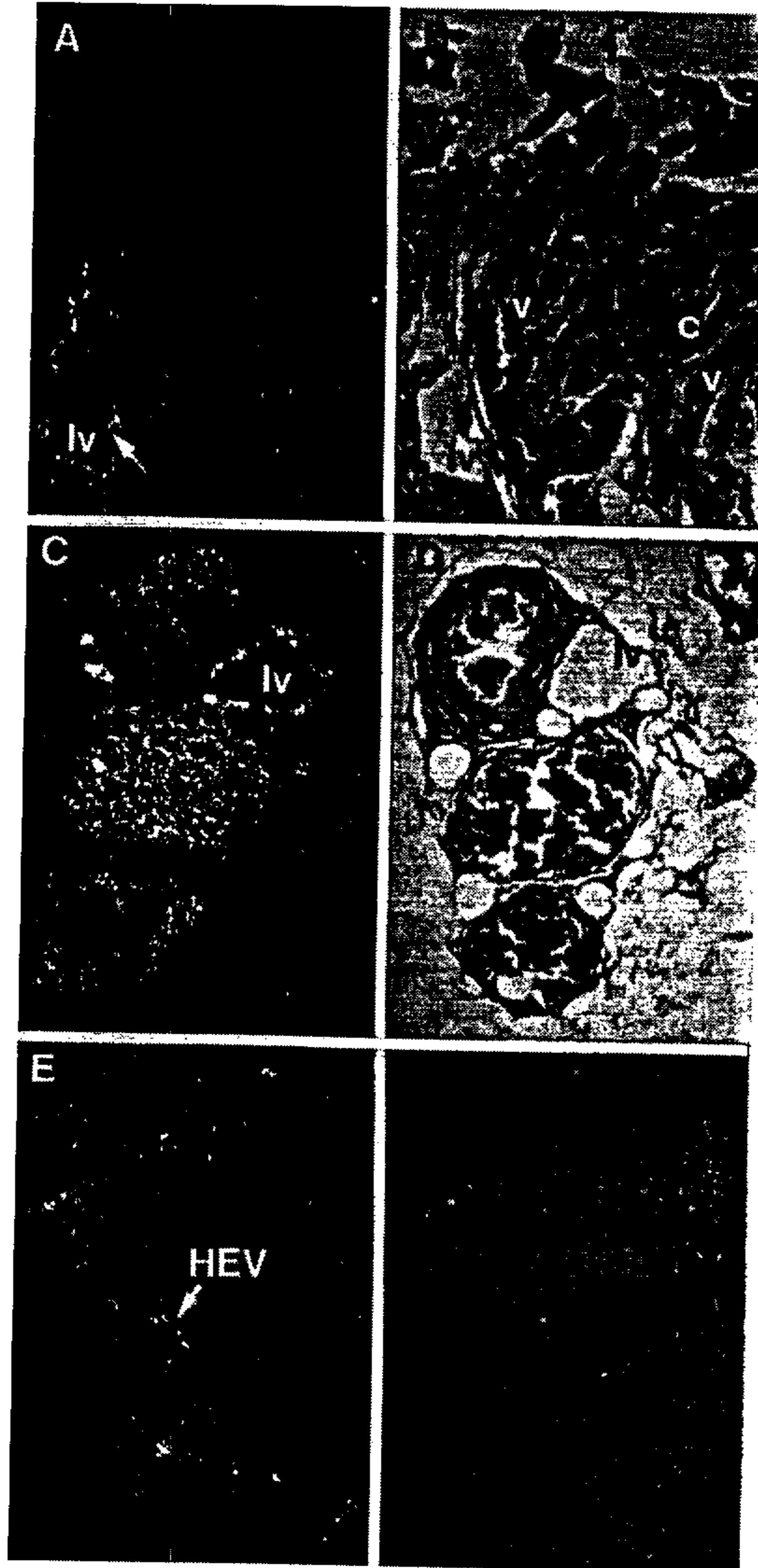


Fig. 6

SUBSTITUTE SHEET

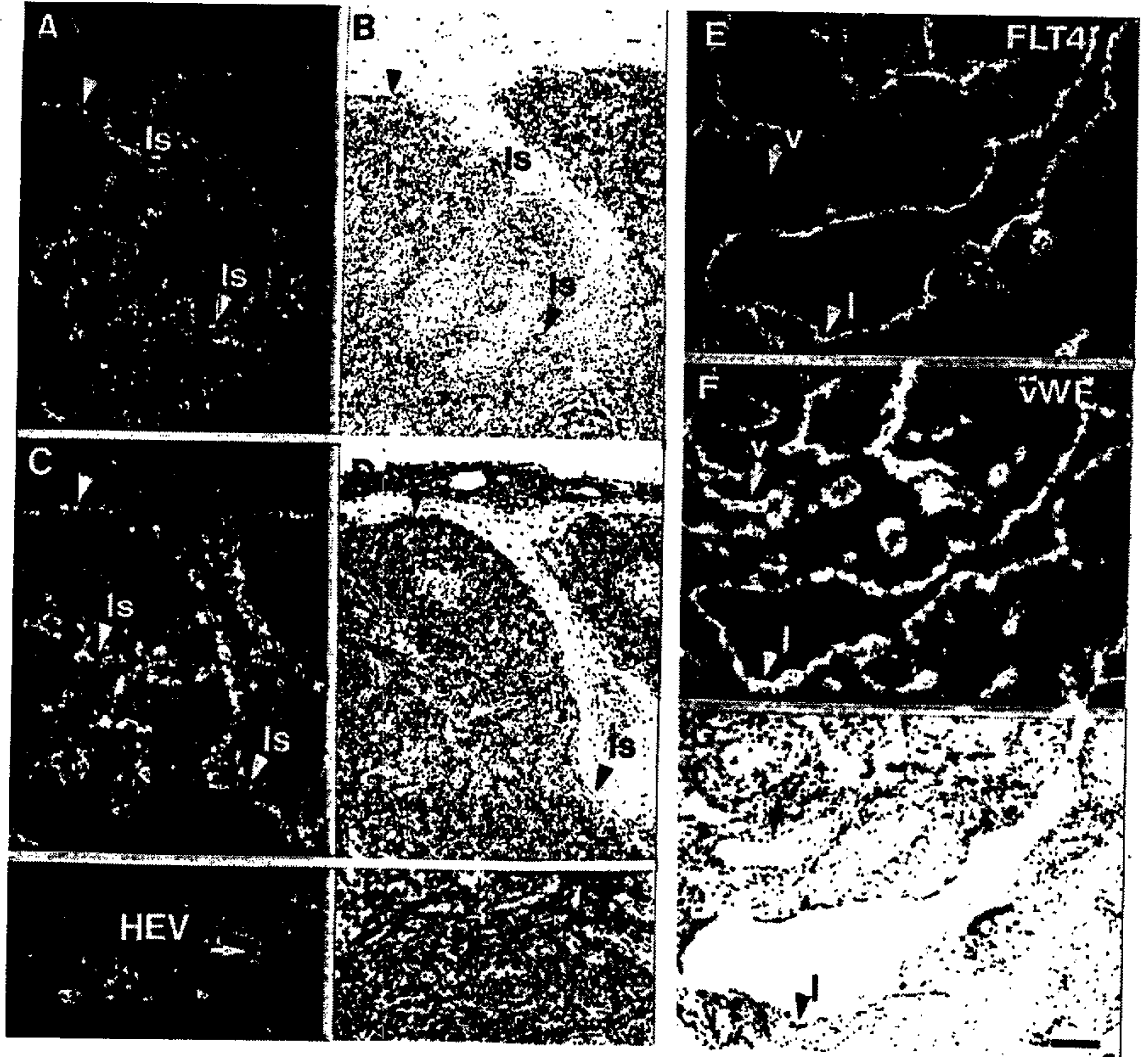


Fig. 7



Fig. 8