ANTI-ROBO4 ANTIBODY

The present invention relates to an antibody having an anti-angiogenesis activity. More specifically, the present invention relates to an antibody against ROBO4 and a pharmaceutical composition containing the antibody.

[Background Art]

Roundabout homolog 4 (ROBO4), a protein of 110 kDa in molecular weight, has a single-pass transmembrane structure (Non Patent Literature 1) and is known to suppress angiogenesis through the binding to a known angiogenesis suppressor Slit homolog 2 (Slit2) (Patent Literature 1 and Non Patent Literature 2). Slit2 has been reported to suppress the migration of HUVEC promoted by a vascular endothelial growth factor (VEGF) and has also been reported to suppress the promotion of cell migration by VEGF or bFGF for ROBO4 gene-transfected vascular endothelial cells (hereinafter, the vascular endothelial cell is also referred to as "EC") compared with empty vector-transfected EC (Patent Literature 1 and Non Patent Literatures 2 to 4).

Moreover, it has been reported that the suppressive effect of Slit2 on the promotion of cell migration, promotion of lumen formation, or increase in permeability by VEGF is observed in EC derived from a wild-type mouse or EC transfected with control siRNA, but not observed in EC derived from a ROBO4 gene-knockout mouse or EC transfected with siRNA to knock-down the ROBO4 gene (Patent Literature 2 and Non Patent Literatures 4 to 6). Furthermore, it has been reported that Slit2 suppresses, via ROBO4, angiogenesis or increase in vascular permeability in mouse models with laser-induced choroidal neovascularization or oxygen-induced retinopathy, which are animal disease models with exudative age-related macular degeneration or diabetic retinopathy (Patent Literature 2 and Non Patent Literature 4).

In spite of these findings, there are also reports showing that ROBO4 does not bind to Slit2 (Non Patent Literature 9 and Patent Literature 5). It has also been reported as to its functions that ROBO4 participates in the promotion of angiogenesis rather than the suppression of angiogenesis, because the migration or lumen formation of ROBO4 gene-knockout EC is inhibited (Non Patent Literatures 10 and 11).

In clinical practice, ROBO4 has been reported to be highly expressed in intratumoral vessels in liver metastasis from colon cancer, ganglioglioma, bladder cancer, breast cancer, metastatic melanoma, kidney cancer, lung cancer, liver cancer, or colon cancer (Patent Literature 3 and Non Patent Literatures 1, 3, and 7). Moreover, ROBO4 has been reported to be also expressed in blood vessels in the fibrovascular membranes of proliferative diabetic retinopathy patients (Non Patent Literature 8). As such, ROBO4 is expressed in vascular endothelial cells, particularly, endothelial cells in blood vessels newly formed in a pathological condition. This may suggest a pathological angiogenesis resulting from the high expression of ROBO4, but may also suggest the compensatory expression of ROBO4 for suppressing pathological angiogenesis.

As described above, ROBO4 is involved in an anti-angiogenesis effect. Thus, an antibody against ROBO4 and a functional fragment thereof are presumably useful in the treatment of a disease involving angiogenesis. However, it is uncertain whether either an agonistic or antagonistic antibody against ROBO4 suppresses or promotes angiogenesis.

Antibodies described in EP Patent No. 1,565,491 (Patent Literature 4) and WO2008/100805 (Patent Literature 5) are known as the antibody against ROBO4 (hereinafter, referred to as an "anti-ROBO4 antibody"). But none of these antibodies does not show a suppressive or inhibitory effect on angiogenesis *in vivo*.

[Citation List]

[Patent Literature]

[Patent Literature 1] WO2004/003163

[Patent Literature 2] WO2008/073441

[Patent Literature 3] WO2002/036771

[Patent Literature 4] European Patent No. 1,565,491

[Patent Literature 5] WO2008/100805

[Non Patent Literature]

[Non Patent Literature 1] Genomics, 2002, vol. 79, p. 547-552

[Non Patent Literature 2] Developmental Biology, 2003, vol. 261, p. 251-267

[Non Patent Literature 3] Biochemical and Biophysical Research Communications, 2005, vol. 332, p. 533-541

[Non Patent Literature 4] Nature Medicine, 2008, No. 14, p. 448-453

[Non Patent Literature 5] Science Translational Medicine, 2010, vol. 2, p. 23ra19

[Non Patent Literature 6] Proceedings of the National Academy of Sciences, 2010, vol. 107, p. 10520-10525

[Non Patent Literature 7] Oncology Reports, 2006, vol. 15, p.1437-1443

[Non Patent Literature 8] Molecular Vision, 2009, vol. 15, p. 1057-1069

[Non Patent Literature 9] The FASEB Journal, 2005, vol. 19, p. 121-123

[Non Patent Literature 10] BMC Cell Biology, 2008, vol. 9, p. 61-72

[Non Patent Literature 11] The FASEB Journal, 2009, vol. 23, p. 513-522

[Summary of Invention]

[Problems to be Solved by the Invention]

An object of the present invention is to provide an antibody against ROBO4.

A further object of the present invention is to provide a pharmaceutical composition containing an anti-ROBO4 antibody having an anti-angiogenesis effect, etc.

A further object of the present invention is to provide a method for producing the antibody.

A further object of the present invention is to provide a method for suppressing angiogenesis using the antibody, etc.

[Means for Solving the Problems]

The present inventors have conducted diligent studies to attain the objects and consequently successfully constructed a screening system that detects the activation of the downstream signal of ROBO4. Moreover, the present inventors have used the screening system to successfully obtain a novel anti-ROBO4 monoclonal antibody that activates the downstream signal of ROBO4, has a suppressive activity against cell migration induced by various angiogenic factors such as VEGF, bFGF, HGF, PDGF-BB and SDF-1 in ROBO4-expressing EC, and exhibits an anti-angiogenesis effect even in *in-vivo* models. In this way, the present invention has been completed.

Specifically, the present invention relates to:

(1) an antibody having properties described in the following (I) to (III) or a functional fragment thereof:

(I) binding to the ROBO4 protein, preferably with a K_D value of 1×10^{-8} or lower, specifically preferred 5×10^{-9} or lower;

(II) suppressing or inhibiting vascular endothelial cell migration in the absence of a cross-linking antibody *in vitro*; and

(III) suppressing or inhibiting angiogenesis *in vivo*;

(2) the antibody or the functional fragment thereof according to (1), wherein the ROBO4 protein is the human ROBO4 protein;

(3) the antibody or the functional fragment thereof according to (1), wherein the ROBO4 protein is a protein consisting of an amino acid sequence of amino acid Nos. 1 to 1007 of SEQ ID NO: 2 ;

(4) the antibody or the functional fragment thereof according to (1), wherein the ROBO4 protein is a protein consisting of an amino acid sequence of amino acid Nos. 46 to 1007 of SEQ ID NO: 2 ;

(5) the antibody or the functional fragment thereof according to (3) or (4), wherein the antibody or the functional fragment thereof binds to a site consisting of an amino acid sequence of amino acid Nos. 132 to 209 of SEQ ID NO: 2;

(6) the antibody or the functional fragment thereof according to (1), wherein the antibody or the functional fragment thereof is a monoclonal antibody or a functional fragment thereof;

(7) the antibody or the functional fragment thereof according to any one of (1) to (6), wherein the antibody consists of a heavy chain comprising CDRH1 consisting of the amino acid sequence represented by SEQ ID NO: 44 (Figure 25), CDRH2 consisting of the amino acid sequence represented by SEQ ID NO: 46 (Figure 26) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 46 by the substitution of one amino acid, and CDRH3 consisting of the amino acid sequence represented by SEQ ID NO: 48 (Figure 27), and a light chain comprising CDRL1 consisting of the amino acid sequence represented by SEQ ID NO: 50 (Figure 28) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 50 by the substitution of one to three amino acid(s), CDRL2 consisting of the amino acid sequence represented by SEQ ID NO: 52 (Figure 29), and CDRL3 consisting of the amino acid sequence represented by SEQ ID NO: 54 (Figure 30);

(8) the antibody or the functional fragment thereof according to any one of (1) to (7), wherein the antibody consists of a heavy chain comprising CDRH1 consisting of the amino acid sequence represented by SEQ ID NO: 44 (Figure 25), CDRH2 consisting of the amino acid sequence represented by SEQ ID NO: 46 (Figure 26) or the amino acid sequence represented by SEQ ID NO: 68 (Figure 44), and CDRH3 consisting of the amino acid sequence represented by SEQ ID NO: 48 (Figure 27), and a light chain comprising CDRL1 consisting of the amino acid sequence represented by SEQ ID NO: 50 (Figure 28) or the amino acid sequence represented by SEQ ID NO: 70 (Figure 46), CDRL2 consisting of the amino acid sequence represented by SEQ ID NO: 52 (Figure

29), and CDRL3 consisting of the amino acid sequence represented by SEQ ID NO: 54 (Figure 30);

(9) the antibody or the functional fragment thereof according to any one of (1) to (7), wherein the antibody comprises a heavy chain variable region consisting of the amino acid sequence represented by SEQ ID NO: 31 (Figure 16), and a light chain variable region consisting of the amino acid sequence represented by SEQ ID NO: 33 (Figure 18);

(10) the antibody or the functional fragment thereof according to any one of (1) to (8), wherein the antibody comprises any one heavy chain variable region selected from the following a) to d) and a light chain variable region selected from e) and f):

a) a heavy chain (hMAb1-H1-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32),

b) a heavy chain (hMAb1-H2-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34),

c) a heavy chain (hMAb1-H3-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) , and

d) a heavy chain (hMAb1-H4-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) ; and

e) a light chain (hMAb1-L1-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) , and

f) a light chain (hMAb1-L2-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) ;

(11) the antibody or the functional fragment thereof according to any one of (1) to (8), wherein the antibody comprises any one of the following combinations 1) to 6) of a heavy chain variable region and a light chain variable region:

1) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light

chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40),

2) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42),

3) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) ;

4) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ,

5) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) , and

6) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ,

(12) the antibody or the functional fragment thereof according to any one of (1) to (11), wherein the antibody or the functional fragment thereof is a chimeric antibody or a functional fragment thereof;

(13) the antibody or the functional fragment thereof according to any one of (1) to (11), wherein the antibody or the functional fragment thereof is a humanized antibody or a functional fragment thereof;

(14) the antibody or the functional fragment thereof according to any one of (1) to (13), wherein the antibody comprises a human IgG1 or human IgG2 heavy chain constant region;

(15) the antibody or the functional fragment thereof according to any one of (1) to (6), wherein the antibody or the functional fragment thereof binds to a site on an antigen recognized by any one antibody according to (7) to (14);

(16) the antibody or the functional fragment thereof according to any one of (1) to (6), wherein the antibody or the functional fragment thereof competes with any one antibody according to (7) to (14) for the binding to ROBO4 protein;

(17) the antibody according to any one of (1) to (8), wherein the antibody comprises any one of the following combinations 1) to 6) of a heavy chain and a light chain:

1) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-1140),

2) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) (H-1143),

3) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) (H-2143);

4) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-2140),

5) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 56 (Figure 32) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-1040), and

6) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 60 (Figure 36) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-2040),

(18) an antibody comprising a modified form of the heavy chain of any one antibody according to (17) , wherein said modified form lacks one to several carboxyl-terminal amino acid(s) of said heavy chain, preferably one to eight carboxyl-terminal amino acid(s) of said heavy chain, more preferably one to two carboxyl-terminal amino acid(s) of said heavy chain;

(19) the antibody according to any one of (1) to (6), wherein the antibody is 95% or more identical in the amino acid sequence to any one antibody according to (17) ; has a K_D value of 1×10^{-8} or lower for a human ROBO4; suppresses or inhibits vascular endothelial cell migration in the absence of a cross-linking antibody *in vitro*; and suppresses or inhibits angiogenesis *in vivo*.;

(20) the antibody or the functional fragment thereof according to (15), wherein the antibody or the functional fragment thereof is a human antibody or a functional fragment thereof;

(21) a nucleotide sequence selected from the group consisting of the following (I) to (III):

(I) a nucleotide sequence comprising a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of (1) to (20);

(II) a nucleotide sequence consisting of a nucleotide sequence comprising a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of (1) to (20); and

(III) a nucleotide sequence consisting of a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of (1) to (20);

(22) a recombinant vector containing an insert of a nucleotidesequence according to (21);

(23) a recombinant cell containing a nucleotide according to (21) or a recombinant vector according to (22) introduced therein;

(24) a cell producing an antibody according to any one of (1) to (20), wherein the cell is preferably a mammalian cell, more preferably a CHO cell and even more preferably a CHOK1SV;

(25) a method for producing an antibody or a functional fragment thereof according to any one of (1) to (20), comprising the following steps (I) and (II):

(I) culturing a cell according to (23) or (24); and

(II) collecting the antibody or the functional fragment thereof according to any one of (1) to (20) from the culture obtained in the step (I);

(26) an antibody or a functional fragment thereof produced by a production method according to (25);

(27) a modified form of an antibody or a functional fragment thereof according to any one of (1) to (20) and (26);

(28) a pharmaceutical composition comprising an antibody or a functional fragment thereof according to any one of (1) to (20) and (26) or a modified form according to (27) as an active ingredient;

(29) the pharmaceutical composition according to (28), wherein the pharmaceutical composition is an agent for treating or preventing an angiogenic disease;

(30) the pharmaceutical composition according to (28), wherein the angiogenic disease is exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity;

(31) the pharmaceutical composition according to (28), wherein the angiogenic disease is exudative age-related macular degeneration, diabetic retinopathy, macular edema, retrolental fibroplasia, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, or immune rejection of a corneal tissue transplant;

(32) an angiogenesis inhibitor comprising an antibody or a functional fragment thereof according to any one of (1) to (20) and (26) or a modified form according to (27) as an active ingredient;

(33) a method for treating or preventing an angiogenic disease comprising administering to a subject in need thereof an effective amont of an antibody or a functional fragment thereof according to any one of (1) to (20) and (26) or a modified form according to (27), or the composition according to any one of (28) to (31), preferably wherein the angiogenic disease is the angiogenic disease in an individual having the expressed ROBO4 protein; and

(34) the pharmaceutical composition according to any one of claims 28 to 31, wherein said composition is used in combination with a further therapeutic or prophylactic agent,

preferably wherein said agent is an anti-angiogenesis drug, anti-inflammatory drug, and/or an anticancer drug.

[Advantageous Effects of Invention]

According to the present invention, a therapeutic agent or the like for an angiogenic disease containing an antibody that binds to ROBO4 and has an anti-angiogenesis effect can be obtained.

[Brief Description of Drawings]

[Figure 1] Figure 1 is a diagram showing the presence or absence of change in reporter activity against NF-κB, GAS, ISRE, IL-8 promoter, or TCF as response elements in the transient expression of human ROBO4 in HEK293 cells. The error bar in the diagram represents standard deviation (n = 3).

[Figure 2] Figure 2 is a diagram showing the presence or absence of change in IL-8 promoter activity in the transient expression of full-length human ROBO4 or an intracellular region deletion variant of human ROBO4 in HEK293 cells. The error bar in the diagram represents standard deviation (n = 5 or 10).

[Figure 3] Figure 3 is a diagram showing change in IL-8 promoter activity in human ROBO4-transfected HEK293 cells caused by an anti-ROBO4 antibody MAb1. The error bar in the diagram represents standard deviation (n = 3).

[Figure 4] Figure 4 is a diagram showing change in IL-8 promoter activity in human ROBO4-transfected HEK293 cells caused by an anti-ROBO4 antibody MAb2, MAb3, or MAb4. The error bar in the diagram represents standard deviation (n = 3).

[Figure 5] Figure 5 is a diagram showing change in the migratory capacity of HUVEC in the presence of VEGF or bFGF caused by the anti-ROBO4 antibody MAb1. The error bar in the diagram represents standard deviation (n = 3 or 4).

[Figure 6] Figure 6 is a diagram showing change in the migratory capacity of HUVEC in the presence of bFGF caused by the anti-ROBO4 antibody MAb2, MAb3, or MAb4. The error bar in the diagram represents standard deviation (n = 4).

[Figure 7] Figure 7 is a diagram showing the presence or absence of the binding activity of the anti-ROBO4 antibody MAb1 against human ROBO4, mouse ROBO4, rat ROBO4, or cynomolgus monkey ROBO4.

[Figure 8] Figure 8 is a diagram showing the presence or absence of the binding activity of the anti-ROBO4 antibody MAb1 against human ROBO1, human ROBO2, or human ROBO3. The upper boxes show the results about MAb1, and the lower boxes show the results about a positive control antibody with which the expression of these proteins on cell surface was confirmed.

[Figure 9] Figure 9 is a diagram showing the presence or absence of the binding activity of the anti-ROBO4 antibody MAb1 against an extracellular region/domain deletion variant of human ROBO4. The upper boxes show the results about MAb1, and the lower boxes show the results about an anti-FLAG antibody with which the expression of these proteins on cell surface was confirmed.

[Figure 10] Figure 10 is a diagram showing change in angiogenesis in laser-induced choroidal neovascularization models caused by the anti-ROBO4 antibody MAb1. The error bar in the diagram represents standard deviation (n = 4 eyes), and ■ or □ shows the results of each eye.

[Figure 11] Figure 11 is a diagram showing change in IL-8 promoter activity in human ROBO4-transfected HEK293 cells caused by an anti-ROBO4 chimeric antibody cMAb1-1 or cMAb1-2. The error bar in the diagram represents standard deviation (n = 3).

[Figure 12] Figure 12 is a diagram showing change in the migratory capacity of HUVEC in the presence of bFGF caused by the anti-ROBO4 chimeric antibody

cMAb1-1 or cMAb1-2. The error bar in the diagram represents standard deviation (n = 4).

[Figure 13] Figure 13 shows cDNA encoding full-length human ROBO4 (SEQ ID NO: 1).

[Figure 14] Figure 14 shows the full-length amino acid sequence of human ROBO4 (SEQ ID NO: 2).

[Figure 15] Figure 15 shows the nucleotide sequence of cDNA encoding the heavy chain variable region of MAb1 (SEQ ID NO: 30).

[Figure 16] Figure 16 shows the amino acid sequence of the MAb1 heavy chain variable region (SEQ ID NO: 31).

[Figure 17] Figure 17 shows the nucleotide sequence of cDNA encoding the light chain variable region of MAb1 (SEQ ID NO: 32).

[Figure 18] Figure 18 shows the amino acid sequence of the MAb1 light chain variable region (SEQ ID NO: 33).

[Figure 19] Figure 19 shows the nucleotide sequence of cDNA encoding the light chain of cMAb1 (SEQ ID NO: 37).

[Figure 20] Figure 20 shows the amino acid sequence of the cMAb1 light chain (SEQ ID NO: 38).

[Figure 21] Figure 21 shows the nucleotide sequence of cDNA encoding the heavy chain of cMAb1-1 (SEQ ID NO: 39).

[Figure 22] Figure 22 shows the amino acid sequence of the cMAb1-1 heavy chain (SEQ ID NO: 40).

[Figure 23] Figure 23 shows the nucleotide sequence of cDNA encoding the heavy chain of cMAb1-2 (SEQ ID NO: 41).

[Figure 24] Figure 24 shows the amino acid sequence of the cMAb1-2 heavy chain (SEQ ID NO: 42).

[Figure 25] Figure 25 shows the amino acid sequence of the heavy chain CDRH1 of MAb1 (SEQ ID NO: 44).

[Figure 26] Figure 26 shows the amino acid sequence of the heavy chain CDRH2 of MAb1 (SEQ ID NO: 46).

[Figure 27] Figure 27 shows the amino acid sequence of the heavy chain CDRH3 of MAb1 (SEQ ID NO: 48).

[Figure 28] Figure 28 shows the amino acid sequence of the light chain CDRL1 of MAb1 (SEQ ID NO: 50).

[Figure 29] Figure 29 shows the amino acid sequence of the light chain CDRL2 of MAb1 (SEQ ID NO: 52).

[Figure 30] Figure 30 shows the amino acid sequence of the light chain CDRL3 of MAb1 (SEQ ID NO: 54).

[Figure 31] Figure 31 shows the nucleotide sequence of cDNA encoding a hMAb1-H1-type heavy chain (SEQ ID NO: 55).

[Figure 32] Figure 32 shows the amino acid sequence of the hMAb1-H1-type heavy chain (SEQ ID NO: 56).

[Figure 33] Figure 33 shows the nucleotide sequence of cDNA encoding a hMAb1-H2-type heavy chain (SEQ ID NO: 57).

[Figure 34] Figure 34 shows the amino acid sequence of the hMAb1-H2-type heavy chain (SEQ ID NO: 58).

[Figure 35] Figure 35 shows the nucleotide sequence of cDNA encoding a hMAb1-H3-type heavy chain (SEQ ID NO: 59).

[Figure 36] Figure 36 shows the amino acid sequence of the hMAb1-H3-type heavy chain (SEQ ID NO: 60).

[Figure 37] Figure 37 shows the nucleotide sequence of cDNA encoding a hMAb1-H4-type heavy chain (SEQ ID NO: 61).

[Figure 38] Figure 38 shows the amino acid sequence of the hMAb1-H4-type heavy chain (SEQ ID NO: 62).

[Figure 39] Figure 39 shows the nucleotide sequence of cDNA encoding a hMAb1-L1-type light chain (SEQ ID NO: 63).

[Figure 40] Figure 40 shows the amino acid sequence of the hMAb1-L1-type light chain (SEQ ID NO: 64).

[Figure 41] Figure 41 shows the nucleotide sequence of cDNA encoding a hMAb1-L2-type light chain (SEQ ID NO: 65).

[Figure 42] Figure 42 shows the amino acid sequence of the hMAb1-L2-type light chain (SEQ ID NO: 66).

[Figure 43] Figure 43 shows the amino acid sequence of CDRH1 of the hMAb1-H2- or hMAb1-H4-type heavy chain (SEQ ID NO: 67).

[Figure 44] Figure 44 shows the amino acid sequence of CDRH2 of the hMAb1-H2- or hMAb1-H4-type heavy chain (SEQ ID NO: 68).

[Figure 45] Figure 45 shows the amino acid sequence of CDRH3 of the hMAb1-H2- or hMAb1-H4-type heavy chain (SEQ ID NO: 69).

[Figure 46] Figure 46 shows the amino acid sequence of CDRL1 of the hMAb1-L2-type light chain (SEQ ID NO: 70).

[Figure 47] Figure 47 shows the amino acid sequence of CDRL2 of the hMAb1-L2-type light chain (SEQ ID NO: 71).

[Figure 48] Figure 48 shows the amino acid sequence of CDRL3 of the hMAb1-L2-type light chain (SEQ ID NO: 72).

[Figure 49] Figure 49 is a diagram showing change in IL-8 promoter activity in human ROBO4-transfected HEK293 cells caused by H-1040, H-1143, H-1140, H-2040, H-2143, or H-2140.

[Figure 50] Figure 50 is a diagram showing change in the migratory capacity of HUVEC in the presence of bFGF caused by H-1143, H-2140, or H-2143. The error bar in the diagram represents standard deviation (n = 4).

[Figure 51] Figure 51 is a diagram showing the cross-species reactivity of H-1143.

[Figure 52] Figure 52 is a diagram showing the cross-species reactivity of H-2140.

[Figure 53] Figure 53 is a diagram showing the cross-species reactivity of H-2143.

[Figure 54] Figure 54 is a diagram showing the binding specificity of H-1143, H-2140, or H-2143.

[Figure 55] Figure 55 is a diagram showing change in angiogenesis in laser-induced choroidal neovascularization models caused by H-2143. The error bar in the diagram represents standard deviation (n = 3-4 eyes).

[Mode for Carrying Out the Invention]

1. Definition

In the present invention, "gene" means nucleotide(s) or nucleotide sequence comprising a nucleotide sequence encoding the amino acids of a protein, or its complementary strand. The "gene" is meant to include, for example, a polynucleotide, an oligonucleotide, DNA, mRNA, cDNA, and cRNA as the nucleotide sequence comprising a nucleotide sequence encoding the amino acids of a protein, or its complementary strand. Such a gene is a single-stranded, double-stranded, or triple or more stranded nucleotide sequence, and the "gene" is also meant to include an associate of DNA and RNA strands, a mixture of ribonucleotides (RNAs) and deoxyribonucleotides (DNAs) on one nucleotide strand, and a double-stranded or triple or more stranded nucleotide sequence comprising such a nucleotide strand. Examples of the "ROBO4 gene" of the present invention can include DNA, mRNA, cDNA, and cRNA comprising a nucleotide sequence encoding the amino acid sequence of the ROBO4 protein.

In the present invention, the term "nucleotide(s)" or "nucleotide sequence" has the same meaning as in a "nucleic acid" and is also meant to include, for example, DNA, RNA, a probe, an oligonucleotide, a polynucleotide, and a primer. Such a nucleotide sequence is a single-stranded, double-stranded or triple or more stranded nucleotide, and the "nucleotide" sequence is also meant to include an associate of DNA and RNA strands, a mixture of ribonucleotides (RNAs) and deoxyribonucleotides (DNAs) on one nucleotide strand, and an associate of two strands or three or more strands comprising such a nucleotide strand.

In the present invention, the terms "polypeptide", "peptide", and "protein" have the same meaning.

In the present invention, the "antigen" is also used as the meaning of an "immunogen".

In the present invention, the "cell" also includes various cells derived from animal individuals, subcultured cells, primary cultured cells, cell lines, recombinant cells, and the like.

In the present invention, an antibody recognizing the ROBO4 protein is also referred to as an "anti-ROBO4 antibody". The "anti-ROBO4 antibody" includes an anti-ROBO4 chimeric antibody, an anti-ROBO4 humanized antibody, an anti-ROBO4 human antibody, and the like.

In the present invention, the "functional fragment of the antibody" means an antibody fragment that exerts at least one of the functions, e.g., the binding affinity (K_D value) of the original antibody. Examples of the "functional fragment of the antibody" can include, but not limited to, Fab, F(ab')2, scFv, Fab', single-chain immunoglobulin, and the like. Such a functional fragment of the antibody may be obtained by the treatment of a full-length molecule of the antibody protein with an enzyme such as papain or pepsin or may be a recombinant protein produced in an

appropriate host cell using a recombinant gene. Preferred "functional fragments" also have at least one of the biological activities of the original antibody.

Further, in the context of the present invention, a nucleotide sequence encoding "the partial amino acid sequence" of the heavy or light chain is or includes a nucleotide sequence encoding a "functional fragment of the antibody" as defined herein above.

In the present invention, the "site" to which an antibody binds, i.e., the "site" recognized by an antibody, means a partial peptide or partial conformation on an antigen bound or recognized by the antibody. In the present invention, such a site is also called an epitope or an antibody-binding site. Examples of the site on the ROBO4 protein bound or recognized by the anti-ROBO4 antibody of the present invention can include a partial peptide or partial conformation on the ROBO4 protein.

The heavy and light chains of an antibody molecule are known to each have three complementarity determining regions (CDRs). The complementarity determining regions are also called hypervariable domains. They are located in the variable regions of the antibody heavy and light chains. These sites have a particularly highly variable primary structure and are usually separated at three positions on the respective primary structures of heavy and light chain polypeptide strands. In the present invention, the complementarity determining regions of the antibody are referred to as CDRH1, CDRH2, and CDRH3 from the amino terminus of the heavy chain amino acid sequence as to the complementarity determining regions of the heavy chain and as CDRL1, CDRL2, and CDRL3 from the amino terminus of the light chain amino acid sequence as to the complementarity determining regions of the light chain. These sites are proximal to each other on the three-dimensional structure and determine specificity for the antigen to be bound.

In the present invention, the "antibody mutant" means a polypeptide that has an amino acid sequence derived from the amino acid sequence of the original antibody by the substitution, deletion, addition, and/or insertion (hereinafter, collectively referred to

as "mutation") of amino acid(s) and binds to the ROBO4 protein of the present invention. The number of the mutated amino acids in such an antibody mutant is 1 to 2, 1 to 3, 1 to 4, 1 to 5, 1 to 6, 1 to 7, 1 to 8, 1 to 9, 1 to 10, 1 to 12, 1 to 15, 1 to 20, 1 to 25, 1 to 30, 1 to 40, or 1 to 50. Such an antibody mutant is also encompassed by the "antibody" of the present invention.

In the present invention, the term "several" in "1 to several" refers to 2 to 10, preferably 2 to 8, more preferably 2.

Examples of activities or properties exerted by the antibody of the present invention can include biological activities or physicochemical properties and can specifically include various biological activities, a binding activity against an antigen or an epitope, stability during production or storage, and thermal stability.

In the present invention, the phrase "hybridizing under stringent conditions" means hybridization under conditions involving hybridization at 65°C in a solution containing 5 × SSC, followed by washing at 65°C for 20 minutes in an aqueous solution containing 2 × SSC-0.1% SDS, at 65°C for 20 minutes in an aqueous solution containing 0.5 × SSC-0.1% SDS, and at 65°C for 20 minutes in an aqueous solution containing 0.2 × SSC-0.1% SDS, or hybridization under conditions equivalent thereto. SSC means an aqueous solution of 150 mM NaCl-15 mM sodium citrate, and n × SSC means SSC with an n-fold concentration.

2. ROBO4 protein

In the present specification, the terms "ROBO4" and "ROBO4 protein" are used as the same meaning.

(2-1) Property

The ROBO4 protein of the present invention has the following properties:

(i) ROBO4 has a molecular weight of approximately 110 kDa and a single-pass transmembrane structure and is a receptor protein of the SLIT2 protein involved in

angiogenesis. Any ROBO4 protein of the present invention can be found in a form liberated from a membrane such as a cell membrane and may be in a form bound to a membrane such as a cell membrane. In this context, the molecular weight means an apparent molecular weight under the non-reducing conditions of SDS-PAGE. The N-terminal extracellular region of ROBO4 contains two immunoglobulin-like domains (hereinafter, referred to as "Ig-like domains") and two fibronectin type III domains, while its C-terminal intracellular region contains a protein-rich region. In the present specification, these two immunoglobulin-like domains are referred to as Ig-like domain 1 and Ig-like domain 2, respectively, from the amino terminus. The human ROBO4 protein consists of an amino acid sequence of amino acid Nos. 28 to 1007 of SEQ ID NO: 2 . Amino acid Nos. 1 to 27 of SEQ ID NO: 2 represent a secretory signal; amino acid Nos. 28 to 467 thereof represent an extracellular region; amino acid Nos. 46 to 131 thereof represent Ig-like domain 1; amino acid Nos. 137 to 224 thereof represent Ig-like domain 2; amino acid Nos. 252 to 340 thereof represent fibronectin type III domain 1; amino acid Nos. 347 to 438 thereof represent fibronectin type III domain 2; amino acid Nos. 468 to 490 thereof represent a region in the cell membrane; and amino acid Nos. 491 to 1007 thereof represent an intracellular region.

(ii) ROBO4 has an anti-angiogenesis effect. In the present invention, the term "anti-angiogenesis" means that the molecule directly or indirectly suppresses and/or inhibits angiogenesis by itself, in collaboration with another factor, or as an associate with another factor. The anti-angiogenesis effect can be evaluated, for example, with a suppressive effect on increase in vascular permeability, cell migration promoting activity, or lumen formation activity by VEGF as an index.

(iii) ROBO4 comprises an amino acid sequence described in any one of the following (a) to (e) (hereinafter, referred to as a "ROBO4 amino acid sequence"), consists of an amino acid sequence comprising the ROBO4 amino acid sequence, or consists of the ROBO4 amino acid sequence:

(a) the amino acid sequence represented by SEQ ID NO: 2 (Figure 14) ;

(b) the amino acid sequence of a polypeptide that exhibits 80% or more, 82% or more, 84% or more, 86% or more, 88% or more, 90% or more, 92% or more, 94% or more, 96% or more, 98% or more, or 99% or more sequence identity to the amino acid sequence represented by SEQ ID NO: 2 (Figure 14) and exhibits an anti-angiogenesis effect;

(c) the amino acid sequence of a polypeptide that comprises an amino acid sequence represented by SEQ ID NO: 2 (Figure 14) having a substitution, deletion, addition, or insertion of 1 to 50, 1 to 45, 1 to 40, 1 to 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 8, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 or 2, or 1 amino acid(s) and suppresses angiogenesis;

(d) the amino acid sequence of a polypeptide that comprises an amino acid sequence of SEQ ID NO: 2 having the deletion of amino acid Nos. 1 to 45 or 1 to 131 and suppresses angiogenesis; and

(e) the amino acid sequence of a polypeptide that is encoded by the nucleotide sequence of a nucleotide hybridizing under stringent conditions to a nucleotide having a nucleotide sequence complementary to a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 2 (Figure 14) and suppresses angiogenesis.

The ROBO4 protein may be present as the whole or a portion of a homo or hetero oligo associate constituted of two or more subunits.

The amino acid sequence and/or other properties of the ROBO4 protein may be neither the same nor homogeneous in an individual, a tissue, a body fluid, a cell, a ROBO4 protein-containing fraction, a purified or partially purified ROBO4 protein preparation, or the like, or among a plurality of individuals, tissues, cells, ROBO4 protein-containing fractions, or ROBO4 protein preparations. One individual, tissue, body fluid, cell, ROBO4 protein-containing fraction, purified or partially purified ROBO4 protein preparation, or the like may contain plural types of ROBO4 proteins differing in amino acid sequence and/or property. Alternatively, a plurality of

individuals, tissues, cells, ROBO4 protein-containing fractions, or ROBO4 protein preparations may differ in the amino acid sequence and/or other properties of the ROBO4 protein. Even such proteins differing in amino acid sequence and/or properties from each other are all encompassed by the "ROBO4 protein" of the present invention as long as they possess the properties described above in (i) to (iii).

(iv) The ROBO4 protein of the present invention can be obtained from the tissue of a vertebrate, preferably a mammal, more preferably a rodent such as a mouse or a rat or a human, even more preferably tissues of a human or a mouse, cells derived from such a tissue, cultures of such cells, and the like. Such a tissue and cells are not particularly limited as long as they contain the ROBO4 protein. Examples thereof can include joint tissues, blood, lymph, thymus glands, spleens, and cells derived from any of them. Preferable tissues and cells are tissues and cells derived from animals or patients having angiogenesis. However, the origin of the ROBO4 protein of the present invention is not limited to those described above, and the ROBO4 protein of the present invention is also meant to include even ROBO4 proteins derived from other animal species, other tissues, other cells, or the like as long as they possess the properties described above in (i) to (iii).

The ROBO4 protein of the present invention may be any of native and recombinant proteins. The ROBO4 protein is also meant to include fusion products with another peptide or protein such as a carrier or a tag. The ROBO4 protein is further meant to include forms provided with chemical modification including the addition of a polymer such as PEG and/or with biological modification including sugar chain modification. Moreover, the ROBO4 protein of the present invention is meant to include a ROBO4 protein fragment. A ROBO4 protein fragment possessing the property described above in (ii) is called a functional fragment of the ROBO4 protein.

(2-2) ROBO4 gene

The ROBO4 gene of the present invention comprises a nucleotide sequence described in any one of the following (a) to (c) (hereinafter, referred to as a "ROBO4 gene sequence"), consists of a nucleotide sequence comprising the ROBO4 gene sequence, or consists of the ROBO4 gene sequence:

(a) the nucleotide sequence represented by SEQ ID NO: 1 (Figure 13) ;

(b) a nucleotide sequence that hybridizes under stringent conditions to a nucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO: 1 (Figure 13) and encodes the amino acid sequence of a polypeptide suppressing angiogenesis; and

(c) a nucleotide sequence that comprises a nucleotide sequence represented by SEQ ID NO: 1 (Figure 13) having a substitution, deletion, addition, or insertion of 1 to 150, 1 to 140, 1 to 130, 1 to 120, 1 to 110, 1 to 100, 1 to 90, 1 to 80, 1 to 70, 1 to 60, 1 to 50, 1 to 45, 1 to 40, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 8, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 or 2, or 1 base(s) and encodes the amino acid sequence of a polypeptide suppressing angiogenesis.

The ROBO4 gene is overexpressed in blood vessels in fibrovascular membranes or intratumoral vessels of patients with a disease accompanied by angiogenesis, for example, proliferative diabetic retinopathy. In addition, the ROBO4 gene seems to be overexpressed in tissue or blood fractions derived from patients affected with a disease considered to involve angiogenesis, such as exudative age-related macular degeneration, macular edema, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity, or from model animals of these diseases.

The expression and expression level of the ROBO4 gene may be assayed with any of a ROBO4 gene transcription product and the ROBO4 protein as an index and can

be determined by RT-PCR, Northern blot hybridization, or the like for the upper index and by immunoassay (e.g., enzyme-linked immuno-sorbent assay; hereinafter, referred to as "ELISA") or the like for the latter index.

(2-3) Preparation of protein

The ROBO4 protein of the present invention can be purified or isolated from animal tissues (including body fluids), cells derived from the tissues, or cultures of the cells and prepared by gene recombination, *in-vitro* translation, chemical synthesis, etc.

(2-3-1) Purification or isolation of native ROBO4

The native ROBO4 protein can be purified or isolated from, for example, tissues (including body fluids, cells, etc.) derived from patients or non-human animals affected with an angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity, cells derived from the tissues, or cultures of the cells. Such non-human animals also include model animals of these diseases. Animals subjected to model preparation are not particularly limited as long as they are vertebrates. The animals are preferably mammals, more preferably rodents such as mice or rats, even more preferably mice or rats. The tissues and cells of such patients or model animals are not particularly limited as long as they contain the ROBO4 protein. Examples thereof can include joint tissues, blood, lymph, thymus glands, spleens, and cells derived from any of them. Preferable tissues and cells are derived from patients or model animals having angiogenesis or exhibiting similar symptoms. However, the origin of the ROBO4 protein of the present invention is not limited to those described above, and the

ROBO4 protein of the present invention may be derived from other animal species, other tissues, other cells, or the like.

Purification or isolation from such tissues, cells, cell cultures, or the like can be performed by the combination of approaches well known by those skilled in the art, such as fractionation and chromatography. Such approaches include, but not limited to, salting out, gel filtration, ion-exchange chromatography, affinity chromatography, hydrophobic chromatography, normal-phase or reverse-phase chromatography, and the like. An affinity gel cross-linked with an anti-ROBO4 monoclonal antibody can be prepared and loaded to a column to thereby prepare a column for affinity chromatography. A crude or partially purified fraction containing the ROBO4 protein is added to such a column. Subsequently, non-specific adsorbed matter is removed with sterilized phosphate-buffered saline (PBS), and a buffer solution for elution can then be added thereto to thereby selectively collect the ROBO4 protein. The solution containing the ROBO4 protein can be subjected to gel filtration or to buffer replacement and/or concentration using a concentrator such as Centriprep.

(2-3-2) Preparation of recombinant ROBO4 protein

The ROBO4 protein of the present invention can also be prepared in a recombinant form. Specifically, host cells are transfected with a gene encoding the amino acid sequence of the ROBO4 protein or a ROBO4 protein fragment, and the ROBO4 protein can be collected from cultures of the cells. For example, the ROBO4 gene or its fragment is inserted into an expression vector. Subsequently, prokaryotic or eukaryotic host cells are transfected with the resulting recombinant vector, and the obtained recombinant cells can be incubated to thereby express the ROBO4 protein. An expression pattern known in the art, such as secretion expression, intracellular expression of soluble forms, or an inclusion body method can be used. Also, the ROBO4 protein can be expressed not only as a molecule having the same amino

terminus (N-terminus) and/or carboxy terminus (C-terminus) as native ones, but also as a fusion protein with a secretory signal, an intracellular localization signal, a tag for affinity purification, or a partner peptide. The ROBO4 protein can be purified or isolated from such recombinant cell cultures by the appropriate combination of operations such as fractionation and chromatography described in (2-3-1) Purification or isolation of native ROBO4 protein.

The ROBO4 gene or its fragment can be prepared by a method well known by those skilled in the art.

Examples thereof can include: polymerase chain reaction (hereinafter, referred to as "PCR"; Saiki, R.K., et al., Science (1988) 239, p. 487-489) with a ROBO4 cDNA expression library as a template using one set of primers capable of specifically amplifying the sequence; reverse transcription PCR (hereinafter, referred to as "RT-PCR") with an mRNA fraction for ROBO4 expression as a template using a primer capable of reverse-transcribing the sequence and one set of primers capable of specifically amplifying the sequence; expression cloning using immunoassay; and cDNA cloning using the partial amino acid sequence of a purified ROBO4 protein.

(2-3-3) *In-vitro* translation

The ROBO4 protein of the present invention can also be prepared by *in-vitro* translation. Such a translation method is not particularly limited as long as it is a method using a cell-free translation system involving enzymes necessary for transcription and translation, substrates, and energy substances. Examples thereof can include a method using Rapid Translation System (RTS) manufactured by Roche Diagnostics.

(2-3-4) Chemical synthesis

The ROBO4 protein of the present invention can also be prepared by chemical synthesis. Examples of the chemical synthesis method can include solid-phase peptide synthesis methods such as Fmoc synthesis and Boc synthesis methods.

3. Anti-ROBO4 antibody

(3-1) Type of anti-ROBO4 antibody

The antibody of the present invention may be any of monoclonal and polyclonal antibodies. Examples of the monoclonal antibody of the present invention can include a non-human animal-derived antibody (non-human animal antibody), a human-derived antibody (human antibody), a chimeric antibody, and a humanized antibody, preferably a chimeric antibody, a humanized antibody, and a human-derived antibody (human antibody), more preferably a humanized antibody, and a human-derived antibody (human antibody).

Examples of the non-human animal antibody can include antibodies derived from vertebrates such as mammals and birds. Examples of the mammal-derived antibody can include rodent-derived antibodies such as mouse antibodies and rat antibodies. Examples of the bird-derived antibody can include chicken antibodies.

Examples of the chimeric antibody can include, but not limited to, an antibody comprising non-human animal antibody-derived variable regions bound with human antibody (human immunoglobulin) constant regions. Examples of the non-human animal antibody-derived variable regions can include heavy and light chain variable regions derived from MAb1 described later.

Examples of the humanized antibody can include, but are not limited to, a human antibody (human immunoglobulin variable regions) grafted with CDRs in the variable regions of a non-human animal antibody, a human antibody grafted with the CDRs as well as with partial sequences of framework regions of a non-human animal antibody, and an antibody having human antibody amino acid(s) replaced for one or two or more

non-human animal antibody-derived amino acid(s) in any of these humanized antibodies. Examples of the CDRs in the variable regions of a non-human animal antibody can include CDRH1 to 3 in the heavy chain variable region and CDRL1 to 3 in the light chain variable region derived from MAb1 described later.

The human antibody is not particularly limited as long as it is an antibody recognizing the antigen of the present invention. Examples thereof can include a human antibody binding to the same site as that bound by an antibody having the antibody CDRs of the present invention, and a human antibody binding to the same site on ROBO4 as that bound by MAb1 described above.

The antibody according to the present invention may be an antibody constituted of sites derived from a plurality of different antibodies. Examples thereof can include an antibody comprising heavy and/or light chains exchanged among a plurality of different antibodies, an antibody comprising full-length heavy and/or light chains exchanged thereamong, an antibody comprising variable or constant regions exchanged thereamong, and an antibody comprising all or some CDRs exchanged thereamong. The heavy and light chain variable regions of the chimeric antibody may be derived from different antibodies of the present invention. CDRH1 to 3 and CDRL1 to 3 in the heavy and light chain variable regions of the humanized antibody may be derived from two or more different antibodies of the present invention. CDRH1 to 3 and CDRL1 to 3 in the heavy and light chain variable regions of the human antibody may be the combination of CDRs carried by two or more different antibodies of the present invention.

The isotype of the monoclonal antibody of the present invention is not particularly limited, and examples thereof can include IgG such as IgG1, IgG2, IgG3, and IgG4, IgM, IgA such as IgA1 and IgA2, IgD, and IgE and can preferably include IgG and IgM, more preferably IgG2. The isotype and subclass of the monoclonal antibody can be determined by, for example, an Ouchterlony test, ELISA, radio

immunoassay (hereinafter, referred to as "RIA"). A commercially available kit for identification (Mouse Typer Kit manufactured by Bio-Rad Laboratories, Inc., RAT MONOCLONAL ANTIBODY ISOTYPING TEST KIT manufactured by AbD Serotec, etc.) may be used.

(3-2) Binding specificity of anti-ROBO4 antibody

The antibody of the present invention recognizes the ROBO4 protein. In other words, the antibody of the present invention binds to the ROBO4 protein. Such an antibody is referred to as an "anti-ROBO4 antibody". Moreover, the preferable antibody of the present invention specifically recognizes the ROBO4 protein. In other words, the preferable antibody of the present invention specifically binds to the ROBO4 protein. Furthermore, the more preferable antibody of the present invention specifically binds to an Ig-like domain carried by the ROBO4 protein. Examples of such an Ig-like domain can include Ig-like domain 1 and Ig-like domain 2. The more preferable antibody of the present invention recognizes a region consisting of an amino acid sequence of amino acid Nos. 132 to 209 of SEQ ID NO: 2 . The antibody of the present invention binds to a human ROBO4 protein, a monkey, preferably cynomolgus monkey ROBO4 protein, and a rabbit ROBO4 protein, but does not bind to mouse and rat ROBO4 proteins (Cross-species reactivity in Example 4)-3 and Example 11)-4).

.

In the present invention, the "specific recognition", i.e., "specific binding", means binding which is not non-specific adsorption. Examples of criteria for determination on whether binding is specific or not can include a dissociation constant (hereinafter, referred to as "K_D"). The preferable antibody of the present invention has a K_D value of 1×10^{-5} or lower, 5×10^{-6} or lower, 2×10^{-6} or lower, or 1×10^{-6} or lower, more preferably 5×10^{-7} or lower, 2×10^{-7} or lower, or 1×10^{-7} or lower, even more preferably 5×10^{-8} or lower, 2×10^{-8} or lower, or 1×10^{-8} or lower, further more

preferably 5×10^{-9} or lower, 2×10^{-9} or lower, or 1×10^{-9} or lower, most preferably 5×10^{-10} or lower, 2×10^{-10} or lower, or 1×10^{-10} or lower for the ROBO4 protein. More specifically, the preferable antibody of the present invention has a K_D value of 2×10^{-8} or lower, more preferably 1×10^{-8} or lower, even more preferably 5×10^{-9} or lower for the ROBO4 protein.

In the present invention, the binding of the antibody to the antigen can be assayed or determined by ELISA, RIA, surface plasmon resonance (hereinafter, referred to as "SPR") analysis, or the like. Examples of equipment used in the SPR analysis can include BIAcore(TM) (manufactured by GE Healthcare Bio-Sciences Crop.), ProteOn(TM) (manufactured by Bio-Rad Laboratories, Inc.), SPR-Navi(TM) (manufactured by BioNavis Oy Ltd.), Spreeta(TM) (manufactured by Texas Instruments Inc.), SPRi-PlexII(TM) (manufactured by Horiba, Ltd.), and Autolab SPR(TM) (manufactured by Metrohm). The binding of the antibody to the antigen expressed on cell surface can be assayed by flow cytometry or the like.

(3-3) *In-vitro* anti-angiogenesis activity of anti-ROBO4 antibody

The antibody of the present invention has an anti-angiogenesis activity in the absence of a cross-linking antibody *in vitro*. It is known that certain antibodies do not exhibit a pharmacological activity in the absence of the cross-linking antibody *in vitro*, but exhibit a pharmacological activity in the absence of the cross-linking antibody *in vivo* (Cancer Cell (2011), 19, p. 101-113). This is probably because leukocytes are found *in vivo* to express Fcγ receptor having the same functions as those of the cross-linking antibody (Nature (2008), 8, p. 34-47); thus, the antibodies exhibit a pharmacological activity through crosslink in the presence of leukocytes even without the cross-linking antibody. In actual organisms, however, the number of leukocytes in lesions differs among individuals (Cancer Res (2011), 71, 5670-5677), presumably resulting in, among individuals, the different effects of the antibodies exhibiting a

pharmacological activity dependent on crosslink induced by leucocytes. The antibody of the present invention exhibits an excellent anti-angiogenesis activity even in the absence of a cross-linking antibody *in vitro*. Thus, the antibody of the present invention can also have an anti-angiogenesis effect independent of the number of leukocytes *in vivo* and is thus pharmaceutically suitable.

The anti-angiogenesis activity means the activity of suppressing vascular endothelial cell growth, migration, lumen formation, etc. The *in-vitro* anti-angiogenesis activity can be evaluated by a vascular permeability, vascular endothelial cell migration, or lumen formation test.

For example, the vascular permeability test for such evaluation can involve inoculating a normal human umbilical vein endothelial cell (HUVEC) to the upper layer of Boyden Chamber having a pore size of 1 μm to form a single layer and then measuring the amount of FITC-labeled dextran or the like permeating through the cell layer. The amount of FITC-labeled dextran permeating through the cell layer may be measured using, for example, In Vitro Vascular Permeability Assay (Cat. ECM640, manufactured by Millipore Corp.). When the antibody added at a concentration of 5 μg/mL or lower exhibits the effect of suppressing the amount of FITC-labeled dextran permeating through the cell layer, this antibody can be evaluated as having a suppressive effect on vascular permeability and having an anti-angiogenesis activity. The antibody of the present invention exhibits a suppressive activity against vascular permeability at a concentration of preferably 5 μg/mL or lower, more preferably 1 μg/mL or lower, particularly preferably 0.5 μg/mL or lower, under the measurement conditions described above.

The cell migration test for such evaluation can involve inoculating HUVEC to the upper layer of Boyden Chamber having a pore size of 3 to 8 μm, adding a medium containing an endothelial cell migration enhancer such as VEGF to the lower layer, and measuring the number of cells migrating to the lower layer. When the antibody

exhibits the effect of decreasing the number of migrating HUVEC cells, this antibody can be evaluated as having a suppressive effect on vascular endothelial cell migration and having an anti-angiogenesis activity. The number of migrating cells may be measured using, for example, vascular endothelial cell migration assay system (Cat. 354143, manufactured by BD Biosciences). The antibody of the present invention exhibits a suppressive activity against cell migration at a concentration of preferably 5μg/mL or lower, more preferably 1 μg/mL or lower, particularly preferably 0.5 μg/mL or lower, under the measurement conditions described above.

The lumen formation test for such evaluation can involve inoculating HUVEC to a cell culture container coated with Matrigel and measuring the number of branch points, tube length, or the like, of a lumen structure formed by HUVEC on the Matrigel. When the antibody exhibits the effect of decreasing the number of branch points or tube length of the lumen structure, this antibody can evaluated as having a suppressive effect on lumen formation and having an anti-angiogenesis activity. The number of branch points or tube length of the lumen structure may be measured using, for example, vascular endothelial cell tube formation assay system (Cat. 354149, manufactured by BD Biosciences). The antibody of the present invention exhibits a suppressive activity against lumen formation at a concentration of preferably 5 μg/mL or lower, more preferably 1 μg/mL or lower, particularly preferably 0.5 μg/mL or lower, under the measurement conditions described above.

Such an assay system, however, is not limited to these tests as long as it is capable of assaying angiogenesis and its suppression induced by the ROBO4 protein.

The cross-linking antibody means an antibody that binds to the Fc region of the antibody of the present invention and acts to cross-link two or more antibody molecules of the present invention. For example, when the Fc region of the antibody of the present invention is derived from a mouse, the cross-linking antibody refers to an antibody that binds to the mouse Fc region and associates two or more antibody

molecules of the present invention through the binding of these two antibody molecules of the present invention at two binding sites, respectively, of the cross-linking antibody.

The phrase "having an anti-angiogenesis activity in the absence of a cross-linking antibody" means that the antibody exhibits an anti-angiogenesis effect in an evaluation system relating to angiogenesis suppression, for example, the evaluation system described above, even without coexisting with a cross-linking antibody.

The phrase "having an anti-angiogenesis activity in the presence of a cross-linking antibody" means that the antibody does not exhibit an anti-angiogenesis activity in the absence of a cross-linking antibody in an evaluation system relating to angiogenesis, for example, the anti-angiogenesis activity evaluation system described above, but exhibits the anti-angiogenesis activity when coexisting with one or more cross-linking antibody molecule(s), preferably two or more cross-linking antibody molecules, with respect to one antibody molecule of the present invention.

(3-4) *In-vivo* suppressive or inhibitory activity of anti-ROBO4 antibody against angiogenesis

The antibody of the present invention suppresses or inhibits angiogenesis *in vivo*. The *in-vivo* suppressive or inhibitory activity against angiogenesis can be evaluated with animal disease models according to a standard method. For example, laser-induced choroidal neovascularization models described later in Example 4)-6 are widely used as disease models of angiogenesis and can be used in evaluation with the amount of blood vessels newly formed as a score. Also in the case of patients, for example, tumor samples are collected by biopsy from tumor patients before and after administration of the antibody of the present invention, and the vascular densities of their intratumoral vessels can be measured by immunohistochemical analysis (IHC) to score the amount of blood vessels newly formed.

(3-5) Activation of downstream signal by anti-ROBO4 antibody

The anti-ROBO4 antibody of the present invention may be subjected to an evaluation system using a cell line or primary cultured cells that exhibit some induced response to the ROBO4 protein. Examples of such a cell line can include a mouse vascular endothelial cell line (ATCC NO. CRL-2779). Examples of such primary cultured cells can include mouse vascular endothelial cells and human vascular endothelial cells.

The antibody of the present invention is an agonistic antibody against ROBO4. Specifically, the antibody of the present invention binds to ROBO4 and activates the downstream signal of ROBO4. Thus, the anti-angiogenesis effect of the antibody of the present invention may be evaluated with the activation of the ROBO4 downstream signal as an index. Examples of the ROBO4 downstream signal can include an IL-8 promoter activity. The IL-8 promoter activity was drastically increased in cells expressing full-length human ROBO4 compared with cells expressing no human ROBO4 and was hardly observed in cells expressing intracellular domain-deleted human ROBO4. Thus, the increase in IL-8 promoter activity demonstrated that the activation of the ROBO4 signal was detected (Example 3). The IL-8 promoter activity can be evaluated, for example, by the addition of the anti-ROBO4 antibody or the co-addition of the anti-ROBO4 antibody and a cross-linking antibody to cells transfected with a reporter vector having an IL-8 promoter sequence insert and a human ROBO4 expression plasmid, followed by the determination of the reporter activity.

(3-6) Anti-ROBO4 mouse monoclonal antibody and the chimeric antibody

MAb1 is an anti-ROBO4 mouse monoclonal antibody obtained by a method described in Example 2.

The nucleotide sequence of cDNA encoding the heavy chain variable region of MAb1 is shown in SEQ ID NO: 30 (Figure 15) , and its amino acid sequence is shown

in SEQ ID NO: 31 (Figure 16). The amino acid sequence of CDRH1 is shown in SEQ ID NO: 44 (Figure 25); the amino acid sequence of CDRH2 is shown in SEQ ID NO: 46 (Figure 26); and the amino acid sequence of CDRH3 is shown in SEQ ID NO: 48 (Figure 27). The nucleotide sequence of cDNA encoding the light chain variable region of MAb1 is shown in SEQ ID NO: 32 (Figure 17) , and its amino acid sequence is shown in SEQ ID NO: 33 (Figure 18). The amino acid sequence of CDRL1 is shown in SEQ ID NO: 50 (Figure 28); the amino acid sequence of CDRL2 is shown in SEQ ID NO: 52 (Figure 29); and the amino acid sequence of CDRL3 is shown in SEQ ID NO: 54 (Figure 30).

The antibody mutant of the present invention, preferably, exhibits reduced sensitivity to protein degradation or oxidation, an improved biological activity, an improved ability to bind to the antigen, or physicochemical or functional properties imparted thereto, or the like. Examples of such an antibody mutant can include an antibody having an amino acid sequence derived from the amino acid sequence of the original antibody by conservative amino acid substitution. The conservative amino acid substitution is substitution that occurs in an amino acid group related to amino acid side chains.

Preferable amino acid groups are as follows: an acidic group involving aspartic acid and glutamic acid; a basic group involving lysine, arginine, and histidine; a nonpolar group involving alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan; and an uncharged polar family involving glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine. Other preferable amino acid groups are as follows: an aliphatic hydroxy group involving serine and threonine; an amide-containing group involving asparagine and glutamine; an aliphatic group involving alanine, valine, leucine, and isoleucine; and an aromatic group involving phenylalanine, tryptophan, and tyrosine. Such amino acid substitution in the

antibody mutant is preferably performed without reducing the antigen binding activity of the original antibody.

An antibody mutant having an amino acid sequence derived from the amino acid sequence of MAb1 of the present invention by conservative amino acid substitution as well as a mouse antibody, rat antibody, chimeric antibody, humanized antibody, human antibody, or the like comprising a CDR amino acid sequence derived from the amino acid sequence of any of MAb1-derived CDRH1 to 3 and CDRL1 to 3 by conservative amino acid mutation is also encompassed by the present invention.

The constant regions of the antibody of the present invention are not particularly limited. Preferably, those derived from a human antibody are used in the antibody of the present invention for the treatment or prevention of a disease in a human. Examples of the heavy chain constant region of the human antibody can include Cγ1, Cγ2, Cγ3, Cγ4, Cμ, Cδ, Cα1, Cα2, and Cε. Examples of the light chain constant region of the human antibody can include Cκ and Cλ.

A nucleotide sequence encoding the secretory signal-containing light chain of cMAb1-1 exemplified as the mouse-human IgG1-type chimeric antibody of the present invention and its amino acid sequence as well as a nucleotide sequence encoding the heavy chain thereof and its amino acid sequence are shown in SEQ ID NOs: 37, 38, 39, and 40 (Figures 19, 20, 21, and 22), respectively, . Likewise, a nucleotide sequence encoding the secretory signal-containing light chain of cMAb1-2 exemplified as the mouse-human IgG2-type chimeric antibody of the present invention and its amino acid sequence as well as a nucleotide sequence encoding the heavy chain thereof and its amino acid sequence are shown in SEQ ID NOs: 37, 38, 41, and 42 (Figures 19, 20, 23, and 24), respectively.

(3-7) Functional fragment of anti-ROBO4 antibody

According to one aspect, the present invention provides a functional fragment of the anti-ROBO4 antibody of the present invention. The functional fragment of the antibody means a fragment maintaining at least a portion of the functions of the antibody, or a modified form thereof described later in (3-10). Examples of such functions of the antibody can generally include an antigen binding activity, an antigen activity-regulating activity, an antibody-dependent cytotoxic activity, and a complement-dependent cytotoxic activity. Examples of the functions of the anti-ROBO4 antibody of the present invention can include a ROBO4 protein binding activity, an anti-angiogenesis activity, and a ROBO4 downstream signal-activating effect. More specifically, any functional fragment having all or some of the above-described activities (3-3) to (3-5) exhibited by the antibody against ROBO4 of the present invention is included in the functional fragment of the antibody of the present invention.

The functional fragment of the antibody is not particularly limited as long as it is a fragment of the antibody maintaining at least a portion of the activities of the antibody, or a modified form thereof. Examples thereof can include, but not limited to, Fab, F(ab')2, Fv, single-chain Fv (scFv) comprising heavy and light chain Fvs linked via an appropriate linker, diabodies, linear antibodies, polyspecific antibodies formed from antibody fragments, and Fab', which is a monovalent fragment of antibody variable regions obtained by the treatment of F(ab')2 under reducing conditions. A molecule containing a moiety other than the fragment of the antibody of the present invention, as in scFv carrying the linker moiety, is also encompassed in the meaning of the functional fragment of the antibody of the present invention.

A molecule that is derived from the antibody protein by the deletion of 1 to several or more amino acid(s) at its amino terminus and/or carboxy terminus and maintains at least a portion of the functions of the antibody is also encompassed in the meaning of the functional fragment of the antibody of the present invention.

The antibody of the present invention or the functional fragment thereof may be a polyspecific antibody having specificity for at least 2 types of different antigens. The polyspecific antibody is not limited to a bispecific antibody, which binds to 2 types of different antigens, and an antibody having specificity for 3 or more types of different antigens is also encompassed in the meaning of the "polyspecific antibody" of the present invention.

The polyspecific antibody of the present invention may be a full-length antibody or a functional fragment thereof (e.g., bispecific F(ab')2 antibody). The bispecific antibody can also be prepared by binding the heavy and light chains (HL pairs) of two types of antibodies. The bispecific antibody can also be obtained by fusing two or more types of monoclonal antibody-producing hybridomas to prepare bispecific antibody-producing fusion cells (Millstein et al., Nature (1983) 305, p. 537-539). The polyspecific antibody can also be prepared in the same way as above.

According to one aspect, the antibody of the present invention is a single-chain antibody (single-chain Fv; hereinafter, referred to as "scFv"). The scFv is obtained by linking the heavy and light chain variable regions of the antibody via a polypeptide linker (Pluckthun, The Pharmacology of Monoclonal Antibodies, 113, ed Rosenburg and Moore, Springer Verlag, New York, p.269-315 (1994), Nature Biotechnology (2005), 23, p.1126-1136). Moreover, bi-scFv comprising two scFvs linked via a polypeptide linker can be used as a bispecific antibody. Furthermore, multi-scFv comprising three or more scFvs can also be used as a polyspecific antibody.

The present invention includes a single-chain immunoglobulin comprising full-length heavy and light chain sequences of the antibody linked via an appropriate linker (Lee, H-S, et. al., Molecular Immunology (1999) 36, p.61-71; Shirrmann, T. et. al., mAbs (2010), 2, (1) p.1-4). Such a single-chain immunoglobulin can be dimerized to thereby maintain a structure and activities similar to those of the antibody, which is originally a tetramer. Also, the antibody of the present invention may be an antibody

that has a single heavy chain variable region and has no light chain sequence. Such an antibody is called a single domain antibody (sdAb) or a nanobody and has been reported to maintain the ability to bind to the antigen (Muyldemans S. et. al., Protein Eng. (1994) 7 (9), 1129-35, Hamers-Casterman C. et. al., Nature (1993) 363 (6428) 446-8). These antibodies are also encompassed in the meaning of the functional fragment of the antibody according to the present invention.

(3-8) Anti-human ROBO4 humanized antibody (hereinafter "anti-ROBO4 humanized antibody")

According to one aspect, the present invention provides a humanized antibody or a functional fragment thereof. The anti-ROBO4 humanized antibody of the present invention or the functional fragment thereof has an anti-angiogenesis activity and, preferably, has an anti-angiogenesis activity *in vivo*. Preferably, the humanized antibody or the functional fragment thereof specifically binds to the ROBO4 protein. Moreover, the humanized antibody or the functional fragment thereof is an agonistic antibody against ROBO4 and activates its downstream signal. Furthermore, the humanized antibody or the functional fragment thereof suppresses or inhibits vascular endothelial cell migration in the absence of a cross-linking antibody *in vitro*.

Examples of the humanized antibody of the present invention can include a human-derived antibody having MAb1 complementarity determining regions (CDRs) replaced with the CDRs of a non-human animal antibody (see Nature (1986) 321, p. 522-525), and a human antibody grafted with the CDR sequences and with some amino acid residues of framework regions by a CDR grafting method (International Publication No. WO90/07861). Furthermore, a variant derived from the humanized antibody by the substitution of 1 to 3 amino acid residues in each CDR with other amino acid residues is also included in the antibody of the present invention as long as the variant has all or some of the activities (3-3) to (3-5).

Preferred examples of the anti-ROBO4 humanized antibody of the present invention or the functional fragment thereof can include an antibody that consists of a heavy chain having a variable region comprising CDRH1 consisting of the amino acid sequence represented by SEQ ID NO: 44 (Figure 25) , CDRH2 consisting of the amino acid sequence represented by SEQ ID NO: 46 (Figure 26) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 46 by the substitution of one amino acid, and CDRH3 consisting of the amino acid sequence represented by SEQ ID NO: 48 (Figure 27) , and a light chain having a variable region comprising CDRL1 consisting of the amino acid sequence represented by SEQ ID NO: 50 (Figure 28) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 50 by the substitution of 1 to 3 amino acid(s), CDRL2 consisting of the amino acid sequence represented by SEQ ID NO: 52 (Figure 29) , and CDRL3 consisting of the amino acid sequence represented by SEQ ID NO: 54 (Figure 30) , and recognizes the ROBO4 protein of the present invention, and a fraction of the antibody maintaining the ROBO4 protein binding activity of the antibody.

Examples of the amino acid substitution in CDRH2 can include the substitution of the amino acid represented by amino acid No. 4 of SEQ ID NO: 46 in CDRH2. Specifically, an asparagine, amino acid No.4 of SEQ ID NO: 46 , can be replaced with a glutamine. The amino acid to be substituted therefor is not limited as long as the resulting antibody has all or some of the activities (3-3) to (3-5) exhibited by the antibody against ROBO4 of the present invention.

Examples of the amino acid substitution in CDRL1 can include the substitution of any 1 to 3, preferably 3, of the amino acids represented by amino acid Nos. 9, 11, and 13 of SEQ ID NO: 50 in CDRL1. Specifically, a serine (amino acid No.9), a glycine (amino acid No.11) and a threonine (amino acid No.13) of SEQ ID NO: 50 can be replaced with an amino acid selected from a glutamic acid, a lysine and a leucine,

preferably with a glutamic acid, a lysine and a leucine respectively. The amino acid(s) to be substituted therefor is not limited as long as the resulting antibody has all or some of the activities (3-3) to (3-5) exhibited by the antibody against ROBO4 of the present invention.

An asparagine residue in peptides or a protein is reported to easily undergo deamidation in some conditions (Gerger et al: The Journal of Biological Chemistry Vol.262 No.2, 785-794, 1987), therefore the amino acid replacement in CDRs described above can increase the stability of the humanized antibodies of the present invention.

Examples of the heavy chain variable region of the more preferred humanized antibody having these CDRHs can include an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32) , wherein CDRH1,CDRH2 and CDRH3 are represented by amino acid Nos. 50 to 54, 69 to 85 and 118 to 126 of SEQ ID NO: 56 (Figure 32), respectively, an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) , wherein CDRH1,CDRH2 and CDRH3 are represented by amino acid Nos. 50 to 54, 69 to 85 and 118 to 126 of SEQ ID NO: 58 (Figure 34), respectively, an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) , wherein CDRH1,CDRH2 and CDRH3 are represented by amino acid Nos. 50 to 54, 69 to 85 and 118 to 126 of SEQ ID NO: 60 (Figure 36), respectively, and an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) , wherein CDRH1,CDRH2 and CDRH3 are represented by amino acid Nos. 50 to 54, 69 to 85 and 118 to 126 of SEQ ID NO: 60 (Figure 38). Examples of the light chain variable region of the more preferred humanized antibody having these CDRLs can include an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) , wherein CDRL1,CDRL2 and CDRL3 are represented by amino acid Nos. 44 to 59, 75 to 81 and 114 to 122 of SEQ ID NO: 64 (Figure 40), respectively, and an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) , wherein

CDRL1,CDRL2 and CDRL3 are represented by amino acid Nos. 44 to 59, 75 to 81 and 114 to 122 of SEQ ID NO: 66 (Figure 42), respectively.

Examples of more preferable combinations of the heavy chain variable region and the light chain variable region of the more preferred humanized antibodies can include: a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ; a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) ; a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) ; a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ; a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ; and a humanized antibody comprising a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40).

Even more preferred examples of the full-length humanized antibody comprising the more preferred combination of the heavy chain variable region and the light chain variable region can include: a humanized antibody (H-1140) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) ; a humanized antibody (H-1143) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) ; a humanized antibody (H-2143) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) ; a humanized antibody (H-2140) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) ; a humanized antibody (H-1040) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 56 (Figure 32) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) ; and a humanized antibody (H-2040) comprising a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 60 (Figure 36) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) .

The most preferable antibodies of the present invention are H-1140, H-1143, H-2140 and H-2143.

H-1140 has properties of 1) binding specifically to human ROBO4 and not to ROBO1, ROBO2, and ROBO3, 2)having a K_D value of 3.9nM for human ROBO4, 3) maintaining affinity to human ROBO4 under 40°C for 4weeks, 4) inhibiting HUVEC migration induced by one or more angiogenic factors selected from VEGF, bFGF, HGF, PDGF-BB and SDF-1, specifically VEGF or bFGF, 5) inhibiting angiogenesis in vivo, and 6) low immunogenicity in ISPRI Web-based Immunogenicity Screening (EpiVax, Inc).

H-1143 has properties of 1) binding specifically to human ROBO4 and not to ROBO1, ROBO2, and ROBO3, 2)having a K_D value of 3.5nM for human ROBO4, 3) maintaining affinity to human ROBO4 under 40°C for 4weeks, 4) inhibiting HUVEC migration induced by one or more angiogenic factors selected from VEGF, bFGF, HGF, PDGF-BB and SDF-1, specifically VEGF and bFGF, 5) inhibiting angiogenesis in vivo, and 6) low immunogenicity in EpiScreen™ immunogenicity testing (Antitope Ltd.)

H-2140 has properties of 1) binding specifically to human ROBO4 and not to ROBO1, ROBO2, and ROBO3, 2) having a K_D value of 1.8nM for human ROBO4, 3) maintaining affinity to human ROBO4 under 40°C for 4weeks, 4) inhibiting HUVEC migration induced by various angiogenic factors such as VEGF, bFGF, HGF, PDGF-BB and SDF-1, specifically VEGF and bFGF, 5) inhibiting angiogenesis in vivo, 6) low immunogenicity in EpiScreen™ immunogenicity testing (Antitope Ltd.)

H-2143 has properties of 1) binding specifically to human ROBO4 and not to ROBO1, ROBO2, and ROBO3, 2) having a K_D value of 1.7nM for human ROBO4, 3) maintaining affinity to human ROBO4 under 40°C for 4weeks, 4) inhibiting HUVEC migration induced by various angiogenic factors such as VEGF, bFGF, HGF, PDGF-BB and SDF-1, specifically VEGF and bFGF, 5) inhibiting angiogenesis in vivo, 6) low immunogenicity in EpiScreen™ immunogenicity testing (Antitope Ltd.), and 7) showing no serious change in clinical sign, body weight, food consumption,

hematology, blood chemistry, pathology, electroretinography after single intravitreal injection (2.75mg/eye) to a Cynomolgus monkey.

An antibody comprising an amino acid sequence having 95% or more, preferably 97% or more, more preferably 99% or more identity to the amino acid sequence of the antibody such as H-1140, H-1143, H-2143, H-2140, H-1040 and H-2040 is also included in the antibody of the present invention as long as the antibody has all or some of the activities (3-3) to (3-5). Moreover, an antibody that has CDRs identical in amino acid sequence to the CDRs of the antibody comprising the combination of the heavy chain variable region and the light chain variable region or the antibody comprising the combination of the heavy chain and the light chain, and has an amino acid sequence other than the CDR amino acid sequence having 95% or more, preferably 97% or more, more preferably 99% or more identity thereto is also included in the antibody of the present invention as long as the antibody has all or some of the activities (3-3) to (3-5).

(3-9) Antibody binding to the same site

An "antibody binding to the same site" as that bound by the antibody provided by the present invention is also included in the antibody of the present invention. The "antibody binding to the same site" as that bound by a certain antibody means another antibody that binds to a site on an antigen molecule recognized by the antibody. If a second antibody binds to a partial peptide or a partial three-dimensional structure on an antigen molecule bound by a first antibody, the first and second antibodies can be determined to bind to the same site. Moreover, the first and second antibodies can be determined to bind to the same site by confirming that the second antibody competes with the first antibody for binding to the antigen, i.e., the second antibody interferes with the binding of the first antibody to the antigen, even if the peptide sequence or three-dimensional structure of the specific binding site is not determined. Furthermore,

when the first and second antibodies bind to the same site and the first antibody has an effect characteristic of one aspect of the antibody of the present invention, such as an anti-angiogenesis activity, the second antibody also has an exceedingly high probability of having the same activity thereas. Thus, if a second anti-ROBO4 antibody binds to the site bound by a first anti-ROBO4 antibody, the first and second antibodies can be determined to bind to the same site on the ROBO4 protein. Moreover, the first and second antibodies can be determined to be antibodies binding to the same site on the ROBO4 protein by confirming that the second anti-ROBO4 antibody competes with the first anti-ROBO4 antibody for binding to the ROBO4 protein.

An antibody binding to a site on the ROBO4 protein recognized by MAb1 of the present invention is also included in the present invention.

The antibody-binding site can be determined by a method well known by those skilled in the art, such as immunoassay. For example, a series of peptides are prepared by the appropriate C-terminal or N-terminal truncation of the amino acid sequence of the antigen, and the reactivity of the antibody thereto is studied to roughly determine a recognition site. Then, shorter peptides are synthesized, and the reactivity of the antibody to these peptides can be studied to thereby determine the binding site. The antigen fragment peptides can be prepared using a technique such as gene recombination or peptide synthesis.

When the antibody binds to or recognizes the partial conformation of the antigen, the binding site for the antibody can be determined by identifying amino acid residues on the antigen adjacent to the antibody using an X-ray structural analysis.

(3-10) Modified form of anti-ROBO4 antibody or functional fragment thereof

The present invention provides a modified form of the antibody or the functional fragment thereof. The modified form of the antibody of the present invention or the functional fragment thereof means an antibody of the present invention or a functional

fragment thereof provided with chemical or biological modification. The chemically modified form includes a form having an amino acid skeleton conjugated with a chemical moiety, a form having a chemically modified N-linked or O-linked carbohydrate chain, and the like. Said chemical moiety or form can be toxic or cytotoxic.

The biologically modified form includes a form that has undergone post-translational modification (e.g., N-linked or O-linked glycosylation, N-terminal or C-terminal processing, deamidation, isomerization of aspartic acid, or oxidation of methionine), a form containing a methionine residue added to the N-terminus by expression using prokaryotic host cells, and the like. Such a modified form is also meant to include a form labeled to permit detection or isolation of the antibody or the antigen of the present invention, for example, an enzyme-labeled form, a fluorescently labeled form, or an affinity-labeled form. Such a modified form of the antibody of the present invention or the functional fragment thereof is useful in improvement in the stability or blood retention of the original antibody of the present invention or functional fragment thereof, reduction in antigenicity, detection or isolation of the antibody or the antigen, etc.

Examples of the chemical moiety contained in the chemically modified form can include water-soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, and polyvinyl alcohol.

Examples of the biologically modified form can include a form modified by enzymatic treatment, cell treatment, or the like, a form fused with another peptide, such as a tag, added by gene recombination, and a form prepared from host cells expressing an endogenous or exogenous sugar chain-modifying enzyme.

Such a modification may be made at an arbitrary position or the desired position in the antibody or the functional fragment thereof. Alternatively, the same or two or more different modifications may be made at one or two or more positions therein.

In the present invention, the "modified form of the antibody fragment" is also meant to include even a "fragment of the modified form of the antibody".

For example, occasionally, an antibody produced by cultured mammalian cells is known to lack a carboxyl-terminal lysine residue in its heavy chain (Journal of Chromatography A, 705: 129-134 (1995)). It is also known that occasionally 2 carboxyl-terminal amino acid residues (i.e., glycine and lysine) of a heavy chain are missing and that a proline residue newly located at the carboxyl terminus is amidated (Analytical Biochemistry, 360: 75-83 (2007)). Such lack or modification in these heavy chain sequences, however, affects neither the ability of the antibody to bind to its antigen nor the effector functions (complement activation, antibody-dependent cytotoxicity, etc.) of the antibody. Thus, an antibody having the modification and a functional fragment of the antibody are also included in the antibody of the present invention. Examples of such an antibody can include a deletion mutant derived from the antibody of the present invention by the deletion or lack of 1 or 2 amino acid(s) in the carboxyl terminus of the heavy chain, and the deletion mutant having an amidated residue (e.g., an amidated proline residue at the carboxyl-terminal site of the heavy chain). However, the deletion mutant ofthe antibody according to the present invention is not limited to the types described above as long as the deletion mutant maintains the ability to bind to the antigen and all or some of the activities (3-3) to (3-5). Two heavy chains constituting the antibody according to the present invention may be composed of any one type of heavy chain selected from the group consisting of the full-length heavy chains and the heavy chains of the deletion mutant or may be composed of the combination of any two types selected therefrom. The quantitative ratio of the deletion variant heavy chain(s) is susceptible to, for example, the type of cultured mammalian cells producing the antibody according to the present invention, and the culture conditions of the cells. Examples of such deletion variant heavy chains as the main components of the antibody according to the present invention can include two heavy chains, both of which lack one carboxyl-terminal amino acid residue. All of

these deletion variants are encompassed in the antibody variant, the functional fragment of the antibody, or the modified form thereof according to the present invention.

4. Method for producing antibody

(4-1) Method using hybridoma

In order to prepare the anti-ROBO4 antibody of the present invention, anti-ROBO4 antibody-producing cells are isolated from the spleens of animals immunized with the ROBO4 protein according to the method of Kohler and Milstein (Kohler and Milstein, Nature (1975) 256, p.495-497, Kennet, R. ed., Monoclonal Antibody, p.365-367, Prenum Press, N. Y. (1980)). The cells are fused with myeloma cells to thereby establish hybridomas, and monoclonal antibodies can be obtained from cultures of these hybridomas.

(4-1-1) Preparation of antigen

The antigen for the preparation of the anti-ROBO4 antibody can be obtained according to, for example, a native or recombinant ROBO4 protein preparation method described in other paragraphs of the present specification. Examples of the antigen that may be thus prepared can include the ROBO4 protein or a ROBO4 protein fragment comprising a partial sequence with at least 6 consecutive amino acids thereof, and their derivatives further comprising an arbitrary amino acid sequence or carrier added thereto (hereinafter, collectively referred to as a "ROBO4 antigen").

The recombinant ROBO4 antigen can be prepared by transfecting host cells with a gene comprising a nucleotide sequence encoding the amino acid sequence of the ROBO4 antigen, and collecting the antigen from cultures of the cells. The native ROBO4 antigen can be purified or isolated from, for example, human or rodent tissues with angiogenesis, cells derived from the tissues, or cultures of the cells. A ROBO4 antigen obtained in a cell-free *in-vitro* translation system from a gene comprising a

nucleotide sequence encoding the amino acid sequence of the ROBO4 antigen is also included in the "ROBO4 antigen" of the present invention.

(4-1-2) Production of anti-ROBO4 monoclonal antibody

The monoclonal antibody is typically produced by the following steps:

(a) preparing an antigen,

(b) preparing antibody-producing cells,

(c) preparing myeloma cells (hereinafter, referred to as "myelomas"),

(d) fusing the antibody-producing cells with the myelomas,

(e) screening for a hybridoma group producing the antibody of interest, and

(f) obtaining single cell clones (cloning).

This production method further involves (g) a step of culturing the hybridomas, a step of raising hybridoma-transplanted animals, etc., (h) a step of assaying or determining the biological activity of the monoclonal antibody, etc., if necessary.

Hereinafter, the method for preparing the monoclonal antibody will be described in detail with reference to these steps. However, the method for preparing the antibody is not limited to them, and, for example, antibody-producing cells other than spleen cells and myelomas may be used.

(a) Step of preparing antigen

An antigen can be prepared according to the ROBO4 protein preparation method described above in (2-3).

(b) Step of preparing antibody-producing cells

The antigen obtained in step (a) is mixed with an adjuvant such as a Freund's complete or incomplete adjuvant or potassium aluminum sulfate, and laboratory animals are immunized with the resulting immunogen. Any laboratory animal used in a hybridoma preparation method known in the art can be used without limitations. Specifically, for example, mice, rats, goats, sheep, cattle, or horses can be used. From

the viewpoint of readily available myeloma cells to be fused with isolated antibody-producing cells, etc., the animals to be immunized are preferably mice or rats.

Mouse and rat strains actually used are not particularly limited. In the case of mice, for example, A, AKR, BALB/c, BALB/cAnNCrj, BDP, BA, CE, C3H, 57BL, C57BL, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, R III, SJL, SWR, WB, 129 can be used. In the case of rats, for example, Wistar, Low, Lewis, Sprague-Dawley, ACI, BN, or Fischer can be used.

These mice and rats can be available from laboratory animal breeders/distributors, for example, CLEA Japan, Inc. or Charles River Laboratories Japan Inc.

Of them, a BALB/c mouse strain or Wistar and Low rat strains are particularly preferable as animals to be immunized in consideration of fusion compatibility with the myeloma cells described later.

Also in consideration of the homology between human and mouse antigens, mice whose biological mechanism to remove autoantibodies has been reduced, i.e., autoimmune disease mice, is also preferably used.

In this context, these mice or rats are preferably 5 to 12 weeks old, more preferably 6 to 8 weeks old at the time of immunization.

The animals can be immunized with the ROBO4 protein using, for example, the method of Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher Spigfield, Illinois (1964).

Examples of antibody titer determination methods can include, but not limited to, immunoassay such as RIA and ELISA.

Antibody-producing cells derived from spleen cells or lymphocytes separated from the immunized animals can be prepared according to a method known in the art, for example, the method of Kohler et al., Nature (1975) 256, p.495,; Kohler et al., Eur. J.

Immnol. (1977) 6, p.511,; Milstein et al., Nature (1977), 266, p.550,; Walsh, Nature, (1977) 266, p.495,).

In the case of spleen cells, a general method can be adopted, which involves chopping the spleens, filtering cells through a stainless mesh, and then floating the resulting cells in an Eagle's minimum essential medium (MEM) or the like to separate antibody-producing cells.

(c) Step of preparing myelomas

The myeloma cells used in cell fusion are not particularly limited and can be selected appropriately for use from cell lines known in the art. An HGPRT (hypoxanthine-guanine phosphoribosyl transferase)-deficient line, i.e., mouse-derived X63-Ag8 (X63), NS1-ANS/1 (NS1), P3X63-Ag8.Ul (P3Ul), X63-Ag8.653 (X63.653), SP2/0-Ag14 (SP2/0), MPC11-45.6TG1.7 (45.6TG), FO, S149/5XXO, BU.1 or the like, rat-derived 210.RSY3.Ag.1.2.3 (Y3) or the like, or human-derived U266AR (SKO-007), GM1500-GTG-A12 (GM1500), UC729-6, LICR-LOW-HMy2 (HMy2), 8226AR/NIP4-1 (NP41), or the like, whose screening procedures have already been established, is preferably used in consideration of convenience in the selection of hybridomas from fusion cells. These HGPRT-deficient lines can be available from, for example, American Type Culture Collection (ATCC).

These cell lines are subcultured in an appropriate medium, for example, an 8-azaguanine medium [RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, and fetal calf serum (hereinafter, referred to as "FCS") and further supplemented with 8-azaguanine], an Iscove's modified Dulbecco's medium (hereinafter, referred to as "IMDM"), or a Dulbecco's modified Eagle medium (hereinafter, referred to as "DMEM") and subcultured 3 to 4 days before cell fusion in a normal medium [e.g., ASF104 medium (manufactured by Ajinomoto Co., Inc.) containing 10% FCS] to secure the number of cells equal to or larger than 2×10^7 cells on the day of cell fusion.

(d) Step of fusing the antibody-producing cells with the myeloma cells

The antibody-producing cells can be fused with the myeloma cells under conditions that prevent cell viability from being exceedingly reduced, according to a method known in the art (Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher Spigfield, Illinois (1964) etc.). For example, a chemical method which involves mixing antibody-producing cells with myeloma cells in a high-concentration solution of a polymer such as polyethylene glycol, or a physical method using electric stimulation can be used.

(e) Step of screening for a hybridoma group producing the antibody of interest

A selection method for the hybridomas obtained by cell fusion is not particularly limited, and HAT (hypoxanthine-aminopterin-thymidine) selection method (Kohler et al., Nature (1975) 256, p.495; Milstein et al., Nature (1977) 266, p.550) is typically used. This method is effective for obtaining hybridomas using an HGPRT-deficient myeloma cell line, which cannot survive in the presence of aminopterin. Specifically, unfused cells and hybridomas can be cultured in a HAT medium to thereby allow only hybridomas resistant to aminopterin to selectively remain and grow.

(f) Step of obtaining single cell clones (cloning)

The hybridomas can be cloned using a method known in the art, for example, a methylcellulose, soft agarose, or limiting dilution method (see e.g., Barbara, B. M. and Stanley, M. S.: Selected Methods in Cellular Immunology, W. H. Freeman and Company, San Francisco (1980)). The limiting dilution method is preferable.

(g) Step of culturing the hybridomas and step of raising hybridoma-transplanted animals

The selected hybridomas can be cultured to thereby produce monoclonal antibodies. Preferably, the desired hybridomas are cloned and then subjected to antibody production.

The monoclonal antibody produced by this hybridoma can be collected from cultures of the hybridoma. Moreover, a recombinant antibody can also be collected from cultures of cells transfected with the monoclonal antibody gene. Furthermore, the hybridomas can be injected intraperitoneally to mice of the same strain (e.g., BALB/cAnNCrj described above) or Nu/Nu mice and allowed to grow. Then, the monoclonal antibody can also be collected from their ascites.

(h) Step of assaying or determining the biological activity of the monoclonal antibody

Various biological tests can be selected and applied thereto according to the purpose.

(4-2) Cell immunization method

Cells expressing the native ROBO4 protein, cells expressing the recombinant ROBO4 protein or its fragment, or the like can be used as immunogens to thereby prepare an anti-ROBO4 antibody by the hybridoma method described above.

Examples of the cells expressing the native ROBO4 protein can include cells derived from patients affected with an angiogenic disease such as proliferative diabetic retinopathy or tumor, and cells derived from the tissues of these patients. Such cells are preferably vascular endothelial cells, but not limited to them. These ROBO4 protein-expressing cells are used in an amount of 1×10^5 to 1×10^9 cells, preferably 1×10^6 to 1×10^8 cells, more preferably 0.5 to 2×10^7 cells, even more preferably 1×10^7 cells, in one immunization. The number of cells subjected to immunization can be changed according to the expression level of the ROBO4 protein. The immunogens are generally administered intraperitoneally and may be administered through an intradermal route or the like. The method described in (4-1-2) can be applied to the hybridoma preparation approach.

(4-3) Gene recombination and host cells

In order to prepare the antibody of the present invention, host cells are transfected with a nucleotide comprising a nucleotide sequence encoding the amino acid sequence of its heavy chain (heavy chain nucleotide) and a nucleotide comprising a nucleotide sequence encoding the amino acid sequence of its light chain (light chain nucleotide), or with a vector containing an insert of the heavy chain nucleotide and a vector containing an insert of the light chain nucleotide, and then cultured, and the antibody can be collected from the cultures. The heavy and light chain nucleotides may be inserted in one vector.

Prokaryotic or eukaryotic cells can be used as host cells. When eukaryotic cells are used as hosts, animal cells, plant cells, or eukaryotic microbes can be used.

Examples of the animal cells can include mammal-derived cells, i.e., monkey-derived COS cells (Gluzman, Y. Cell (1981) 23, p. 175-182, ATCC CRL-1650), mouse fibroblast NIH3T3 (ATCC No.CRL-1658), mouse NS0 cell lines (ECACC), Chinese hamster ovary cells (CHO cells, ATCC CCL-61), dihydrofolate reductase-deficient lines thereof (CHOdhfr-: Urlaub, G. and Chasin, L. A. Proc. Natl. Acad. Sci. U. S. A. (1980) 77, p.4126-4220), CHOK1SV developed by Lonza Biologics, cells derived from birds such as chickens, and cells derived from insects.

Also, host cells of the present invention include cells that may produce an antibody protein, wherein the structure of a sugar chain attached to the antibody protein is modified, wherein a biological activity of the antibody with the modification is preferably enhanced compared to the antibody without the modification. Example of such host cells of the present invention includes CHO cells that may produce an antibody protein having complex N-glycoside-linked sugar chains bound to the Fc region of the antibody, wherein among the total complex N-glycoside-linked sugar chains bound to the Fc region of the antibody, the ratio of a sugar chain in which fucose is not bound to N-acetylglucosamine in the reducing end in the sugar chain is 20% or more (WO2000/61739, WO2002/31140).

Examples of the eukaryotic microbes can include yeasts. Examples of the prokaryotic cells can include *E. coli* and *Bacillus subtilis*.

Mammal-derived cells are preferably used, CHO cells are more preferably used, and CHOK1SV are even more preferably used as host cells for producing the anti-ROBO4 antibodies of the present invention.

A signal peptide for the secretion of the antibody of the present invention (monoclonal antibodies derived from various animal species, rat antibody, mouse antibody, chimeric antibody, humanized antibody, human antibody, etc.) is not limited to the secretory signal of an antibody of the same species, the same type, and the same subtype as the antibody or to the secretory signal of the antibody itself. Any secretory signal of an antibody of different type or subtype therefrom or any secretory signal of a protein derived from a different eukaryotic species therefrom or a prokaryotic species can be selected and used.

(4-4) Methods for designing and preparing a humanized antibody

Examples of the humanized antibody can include, but not limited to, a human-derived antibody having CDRs replaced with the CDRs of a non-human animal antibody (see Nature (1986) 321, p. 522-525), a human antibody grafted with the CDR sequences and with some amino acid residues of framework regions by a CDR grafting method (see WO90/07861 and US6972323), and an antibody having human antibody amino acid(s) replaced for one or two or more non-human animal antibody-derived amino acid(s) in any of these humanized antibodies.

(4-5) Method for preparing a human antibody

Further examples of the antibody of the present invention can include a human antibody. The anti-ROBO4 human antibody means an anti-ROBO4 antibody consisting of the amino acid sequence of a human-derived antibody. The anti-ROBO4

human antibody can be obtained by a method using human antibody-producing mice carrying a human genomic DNA fragment comprising human antibody heavy chain- and light chain-encoding genes (see Tomizuka, K. et al., Nature Genetics (1997) 16, p.133-143,; Kuroiwa, Y. et. al., Nuc. Acids Res. (1998) 26, p.3447-3448; Yoshida, H. et. al., Animal Cell Technology: Basic and Applied Aspects vol. 10, p.69-73 (Kitagawa, Y., Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999.; Tomizuka, K. et. al., Proc. Natl. Acad. Sci. USA (2000) 97, p.722-727 etc.).

Specifically, such human antibody-producing animals can be prepared by disrupting the endogenous immunoglobulin heavy and light chain gene loci of non-human mammals and instead introducing thereto human immunoglobulin heavy and light chain gene loci via yeast artificial chromosome (YAC) vectors or the like. Alternatively, eukaryotic cells are transformed with heavy chain- and light chain-encoding cDNAs of such a human antibody, preferably with vectors comprising each of the cDNAs, by a gene recombination technique, and the transformed cells producing a recombinant human monoclonal antibody are cultured. This antibody can be obtained from the culture supernatant.

In this context, for example, eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes, or myelomas can be used as hosts.

Also, a method for obtaining a phage display-derived human antibody selected from a human antibody library (see Wormstone, I. M. et. al, Investigative Ophthalmology & Visual Science. (2002) 43 (7), p.2301-2308; Carmen, S. et. al., Briefings in Functional Genomics and Proteomics (2002), 1 (2), p.189-203; Siriwardena, D. et. al., Opthalmology (2002) 109 (3), p.427-431 etc.) is known.

For example, a phage display method (Nature Biotechnology (2005), 23, (9), p. 1105-1116) can be used, which involves allowing the variable regions of a human antibody to be expressed as a single-chain antibody (scFv) on phage surface and selecting a phage binding to the antigen.

The phage selected based on its binding to the antigen can be subjected to gene analysis to thereby determine DNA sequences encoding the variable regions of the human antibody binding to the antigen.

If the DNA sequence of scFv binding to the antigen is determined, an expression vector having this sequence is prepared and appropriate hosts can be transfected with the expression vector and allowed to express the human antibody (WO92/01047, WO92/20791, WO93/06213, WO93/11236, WO93/19172, WO95/01438, WO95/15388, Annu. Rev. Immunol (1994) 12, p.433-455, Nature Biotechnology (2005) 23 (9), p.1105-1116.

(4-6) Method for preparing functional fragments of an antibody

The method for preparing a single-chain antibody is well known in the art (see e.g., US. Patent Nos. 4,946,778, 5,260,203, 5,091,513, and 5,455,030). In this scFv, a heavy chain variable region and a light chain variable region are linked via a linker that prevents them from forming a conjugate, preferably a polypeptide linker (Huston, J.S. et al., Proc. Natl. Acad. Sci. U.S.A. (1988), 85, p. 5879-5883). The heavy chain variable region and the light chain variable region in scFv may be derived from the same antibody or may be derived from different antibodies.

For example, an arbitrary single-chain peptide consisting of 12 to 19 residues is used as the polypeptide linker that links these variable regions.

In order to obtain scFv-encoding DNA, of the sequences of DNA encoding the heavy chain or heavy chain variable region of the antibody and DNA encoding the light chain or light chain variable region thereof, each DNA portion encoding the whole or desired amino acid sequence is used as a template and amplified by PCR using a primer pair flanking both ends of the template. Subsequently, DNA encoding the polypeptide linker moiety is further amplified in combination with a primer pair flanking both ends thereof to link them to the heavy and light chains, respectively.

The scFv-encoding DNA can be used to thereby prepare, according to a routine method, an expression vector containing the DNA and host cells transformed with the expression vector. In addition, the host cells are cultured, and the scFv can be collected from the cultures according to a routine method.

Also in order to obtain other functional fragments of the antibody, a gene encoding each functional fragment is obtained according to the method described above, and cells are transfected with the gene. The functional fragment of interest can be collected from cultures of the cells.

The antibody of the present invention may be multimerized to thereby enhance its affinity for the antigen. The antibodies to be multimerized may be antibodies of one type or may be a plurality of antibodies recognizing a plurality of epitopes, respectively, or the same antigen. Examples of antibody multimerization methods can include the binding of two scFvs to an IgG CH3 domain, the binding thereof to streptavidin, and the introduction of a helix-turn-helix motif.

The antibody of the present invention may be a mixture of plural types of anti-ROBO4 antibodies differing in amino acid sequence, i.e., a polyclonal antibody. Examples of the polyclonal antibody can include a mixture of plural types of antibodies differing in a portion or the whole of the CDR set. Such a polyclonal antibody can be collected from cultures of mixed-cultured different antibody-producing cells (WO2004/061104). Moreover, separately prepared antibodies may be mixed. Furthermore, antiserum, which is one aspect of the polyclonal antibody, can be prepared by immunizing animals with the desired antigen and collecting serum from the animals according to a standard method.

Antibodies conjugated with various molecules such as polyethylene glycol (PEG) can also be used as modified forms of the antibody.

The antibody of the present invention may further be any of conjugates formed by these antibodies with other drugs (immunoconjugates). Examples of such an

antibody can include the antibody conjugated with a radioactive material or a compound having a pharmacological effect (Nature Biotechnology (2005) 23, p. 1137-1146).

(4-7) Purification of antibody

The obtained antibody can be purified into a homogeneous level. Usual protein separation and purification methods can be used for the separation and purification of the antibody.

The antibody can be separated and purified by appropriately selected or combined approaches, for example, chromatography columns, filters, ultrafiltration, salting out, dialysis, preparative polyacrylamide gel electrophoresis, and/or isoelectric focusing (Strategies for Protein Purification and Charcterization: A Laboratoy Course Manual, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)) but not limited to them.

Examples of chromatography include affinity chromatography, ion-exchange chromatography, hydrophobic chromatography, gel filtration, reverse-phase chromatography, and adsorption chromatography.

These chromatography approaches can be performed using liquid-phase chromatography such as HPLC or FPLC.

Examples of columns used in affinity chromatography can include protein A, protein G, and antigen columns.

Examples of the protein A column include Hyper D (manufactured by Pall Corp.), POROS (manufactured by Applied Biosystems, Inc.), and Sepharose F.F. (manufactured by GE Healthcare Bio-Sciences Crop.).

Also, the antibody may be purified based on its binding activity against the antigen using an antigen-immobilized carrier.

The present invention provides even a gene encoding the antibody of the present invention or the functional fragment thereof, or the modified form thereof, a recombinant vector containing an insert of the gene, a cell transfected with the gene or the vector, and a cell producing the antibody of the present invention.

An antibody or functional fragment thereof which is produced by any of the methods (4-1) to (4-6) can be included in the present invention.

5. Pharmaceutical composition

The present invention provides a pharmaceutical composition comprising the anti-ROBO4 antibody or the functional fragment thereof, or the modified form thereof.

The pharmaceutical composition of the present invention is useful in the treatment or prevention of a disease that shows angiogenesis as one of pathological findings during the course of onset, progression, and/or exacerbation and can be improved by the suppression of this angiogenesis or vascular permeability (hereinafter, this disease is referred to as an "angiogenic disease" for the sake of convenience). Examples of the angiogenic disease can include exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, and obesity. The pharmaceutical composition of the present invention is useful as an agent in the treatment or prevention of an angiogenic disease, preferably useful in the treatment or prevention of exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, retrolental fibroplasia, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, and immune rejection of a corneal tissue transplant, more preferably useful in the treatment or prevention of exudative age-related macular degeneration, diabetic

retinopathy, macular edema, benign or malignant tumor, retrolental fibroplasia, ocular neovascular disease, proliferative retinopathy and neovascular glaucoma.

In the present invention, the treatment and/or treatment of a disease includes, but not limited to, the prevention of onset of the disease, preferably the disease in an individual having the expressed ROBO4 protein, the suppression or inhibition of exacerbation or progression thereof, the alleviation of one or two or more symptoms exhibited by an individual affected with the disease, the suppression or remission of exacerbation or progression thereof, the treatment or prevention of a secondary disease, and the like.

The pharmaceutical composition of the present invention can contain a therapeutically or preventively effective amount of the anti-ROBO4 antibody or the functional fragment of the antibody and a pharmaceutically acceptable diluent, vehicle, solubilizer, emulsifier, preservative, and/or additive.

The "therapeutically or preventively effective amount" means an amount that exerts therapeutic or preventive effects on a particular disease by means of a particular dosage form and administration route.

The pharmaceutical composition of the present invention may contain materials for changing, maintaining, or retaining pH, osmotic pressure, viscosity, transparency, color, tonicity, sterility, or the stability, solubility, sustained release, absorbability, permeability, dosage form, strength, properties, shape, etc., of the composition or the antibody contained therein (hereinafter, referred to as "pharmaceutical materials"). The pharmaceutical materials are not particularly limited as long as they are pharmacologically acceptable materials. For example, no or low toxicity is a property preferably possessed by these pharmaceutical materials.

Examples of the pharmaceutical materials can include, but not limited to, the followings: amino acids such as glycine, alanine, glutamine, asparagine, histidine, arginine, and lysine; antimicrobial agents; antioxidants such as ascorbic acid, sodium

sulfate, and sodium bisulfite; buffers such as phosphate, citrate, or borate buffers, sodium bicarbonate, and Tris-HCl solutions; fillers such as mannitol and glycine; chelating agents such as ethylenediaminetetraacetic acid (EDTA); complexing agents such as caffeine, polyvinylpyrrolidine, β-cyclodextrin, and hydroxypropyl-β-cyclodextrin; bulking agents such as glucose, mannose, and dextrin; other hydrocarbons such as monosaccharides, disaccharides, glucose, mannose, and dextrin; coloring agents; corrigents; diluents; emulsifiers; hydrophilic polymers such as polyvinylpyrrolidine; low-molecular-weight polypeptides; salt-forming counterions; antiseptics such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, and hydrogen peroxide; solvents such as glycerin, propylene glycol, and polyethylene glycol; sugar alcohols such as mannitol and sorbitol; suspending agents; surfactants such as PEG, sorbitan ester, polysorbates such as polysorbate 20 and polysorbate 80, triton, tromethamine, lecithin, and cholesterol; stability enhancers such as sucrose and sorbitol; elasticity enhancers such as sodium chloride, potassium chloride, mannitol, and sorbitol; transport agents; diluents; excipients; and/or pharmaceutical additives.

The amount of these pharmaceutical materials added is 0.001 to 1000 times, preferably 0.01 to 100 times, more preferably 0.1 to 10 times the weight of the anti-ROBO4 antibody or the functional fragment thereof, or the modified form thereof.

A pharmaceutical composition containing an immunoliposome comprising the anti-ROBO4 antibody or the functional fragment thereof, or the modified form thereof encapsulated in a liposome or a modified antibody form comprising the antibody conjugated with a liposome (U.S. Patent No. 6214388, etc.) is also included in the pharmaceutical composition of the present invention.

The excipients or vehicles are not particularly limited as long as they are liquid or solid materials usually used in injectable water, saline, artificial cerebrospinal fluids,

and other preparations for oral or parenteral administration. Examples of the saline can include neutral saline and serum albumin-containing saline.

Examples of the buffers can include Tris buffers adjusted to bring about the final pH of the pharmaceutical composition to 7.0 to 8.5, acetate buffers adjusted to bring about the final pH thereof to 4.0 to 5.5, citrate buffers adjusted to bring about the final pH thereof to 5.0 to 8.0, and histidine buffers adjusted to bring about the final pH thereof to 5.0 to 8.0.

The pharmaceutical composition of the present invention is a solid, a liquid, a suspension, or the like. Another example of the pharmaceutical composition of the present invention can include freeze-dried preparations. The freeze-dried preparations can be formed using an excipient such as sucrose.

The administration route of the pharmaceutical composition of the present invention may be any of enteral administration, local administration, and parenteral administration and may be selected preferably according to the targeted disease. Specific examples thereof can include intravenous administration, intraarterial administration, intramuscular administration, intradermal administration, hypodermic administration, intraperitoneal administration, transdermal administration, intraosseous administration, and intraarticular administration. Also, intraocular administration can be used preferably for an ophthalmic angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, retrolental fibroplasia, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, or immune rejection of a corneal tissue transplant.

The recipe of the pharmaceutical composition can be determined according to the administration method, the binding affinity of the antibody for the ROBO4 protein, etc. The anti-ROBO4 antibody of the present invention or the functional fragment thereof, or the modified form thereof having higher affinity (lower K_D value) for the ROBO4 protein can exert its drug efficacy at a lower dose.

The dose of the anti-ROBO4 antibody of the present invention can be determined appropriately according to the species of an individual, the type of a disease, symptoms, sex, age, pre-existing conditions, the binding affinity of the antibody for the ROBO4 protein or its biological activity, and other factors. A dose of usually 0.01 to 1000 mg/kg, preferably 0.1 to 100 mg/kg, can be administered once every day to 180 days or twice or three or more times a day.

Examples of the form of the pharmaceutical composition can include injections (including freeze-dried preparations and drops), suppositories, transnasal absorption preparations, transdermal absorption preparations, sublingual formulations, capsules, tablets, ointments, granules, aerosols, pills, powders, suspensions, emulsions, eye drops, and biological implant formulations.

The pharmaceutical composition comprising the anti-ROBO4 antibody of the present invention or the functional fragment thereof, or the modified form thereof as an active ingredient may be used in combination with a further therapeutic or prophylactic agent. Examples of said agent include an anti-angiogenesis drug, anti-inflammatory drug, and/or an anticancer drug. For example, the anti-angiogenesis drug, anti-inflammatory drug, and/or anticancer drug is administered to a subject, and then, the pharmaceutical composition comprising the anti-ROBO4 antibody or the functional fragment of the antibody as an active ingredient is administered thereto. Alternatively, the pharmaceutical composition is administered to a subject, and then, the anti-angiogenesis drug, anti-inflammatory drug, and/or anticancer drug is administered thereto. Alternatively, the pharmaceutical composition may be administered to a subject simultaneously with the anti-angiogenesis drug, anti-inflammatory drug, and/or anticancer drug. Examples of the anti-angiogenesis drug can include ranibizumab.

The present invention provides even a method for treating or preventing an angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental

fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity, use of the antibody of the present invention for the preparation of a pharmaceutical composition for the treatment or prevention of the angiogenic disease, and use of the antibody of the present invention for the treatment or prevention of the angiogenic disease. A kit for treatment or prevention comprising the antibody of the present invention is also included in the present invention.

6. Composition for diagnosis

The present invention provides a composition for examination or diagnosis comprising the anti-ROBO4 antibody of the present invention or the functional fragment thereof, or the modified form thereof (hereinafter, collectively referred to as a "composition for diagnosis").

The composition for diagnosis of the present invention is useful in the examination or diagnosis of an angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity. The composition for diagnosis of the present invention is also useful in the examination or diagnosis of early angiogenesis or pre-angiogenesis symptoms, which do not satisfy the conventional diagnosis criteria, undiagnosed symptoms that evolve to angiogenesis, etc. In the present invention, the examination or the diagnosis includes, for example, the determination or testing of a risk of acquiring a disease, the determination of the presence or absence of a disease, the testing of the degree of progression or exacerbation, the testing or determination of the

effect of drug therapy using the pharmaceutical composition comprising the anti-ROBO4 antibody or the like, the testing or determination of the effect of therapy other than drug therapy, the testing of a risk of recurrence, and the determination of the presence or absence of recurrence. However, the examination or the diagnosis according to the present invention is not limited to them as long as it is usual examination or diagnosis.

When the ROBO4 protein is detected in a 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold or more amount, preferably 10-fold or more amount, in a sample derived from a test subject compared with a sample derived from a healthy individual, the test subject can be diagnosed as having an angiogenic disease or as being at a high risk of acquiring it. Moreover, when the serum concentration of the ROBO4 protein exceeds a particular reference value, the test subject is diagnosed as having an angiogenic disease or can be diagnosed as being at a high risk of acquiring it. The reference value is usually 0.01 to 10 ng/ml, preferably 0.1 to 1 ng/ml, more preferably 0.1 to 0.3 ng/ml.

Such a composition for diagnosis can contain a pH buffer, an osmoregulator, salts, a stabilizer, an antiseptic, a color developer, a sensitizer, an aggregation inhibitor, and the like.

The present invention provides even a method for examining or diagnosing an angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity, use of the antibody of the present invention for the preparation of a composition for the diagnosis of the angiogenic disease, and use of the antibody of the present invention for the examination or diagnosis of the angiogenic disease. A kit for

examination or diagnosis comprising the antibody of the present invention is also included in the present invention.

The examination or diagnosis method involving the antibody of the present invention is preferably sandwich ELISA. A usual detection method using antibodies, such as ELISA, RIA, ELISPOT (enzyme-linked immunospot) assay, dot blotting, an Ouchterlony test, or CIE (counterimmunoelectrophoresis), may be used. Antibodies applied to the sandwich ELISA assay system may be any combination of two antibodies that recognize ROBO4, but do not compete with each other. In addition to biotin, a labeling method that can be carried out in biochemical analysis, such as HRP, alkaline phosphatase, or FITC, can be used as a labeling method for the antibodies. A chromogenic substrate such as TMB (3, 3', 5, 5'-tetramethylbenzidine), BCIP (5-bromo-4-chloro-3-indolyl phosphate), ρ-NPP (ρ-nitrophenyl phosphate), OPD (o-Phenylenediamine), ABTS (3-Ethylbenzothiazoline-6-sulfonic acid), SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.), a fluorescent substrate such as QuantaBlu(TM) Fluorogenic Peroxidase Substrate (Thermo Fisher Scientific Inc.), and a chemiluminescent substrate can be used in detection using enzymatic labeling. Samples derived from human or non-human animals as well as artificially treated samples such as recombinant proteins can be subjected to this assay. Examples of test samples derived from organism individuals can include, but not limited to, blood, synovial fluids, ascites, lymph, cerebrospinal fluids, and tissue homogenate supernatants.

The sandwich ELISA kit for examination or diagnosis comprising the antibody of the present invention may contain a solution of ROBO4 protein standards, a coloring reagent, a buffer solution for dilution, an antibody for solid phase, antibody for detection, and a washing solution, and the like. The amount of the antibody bound to the antigen can be measured preferably using a method such as an absorbance, fluorescence, luminescence, or RI (radioisotope) method. An absorbance plate reader,

a fluorescence plate reader, a luminescence plate reader, an RI liquid scintillation counter, or the like is preferably used in the measurement.

[Examples]

Hereinafter, the present invention will be described in more detail with reference to Examples. However, the present invention is not intended to be limited to them.

In the Examples below, each operation for genetic engineering was performed by methods described in "Molecular Cloning" (Sambrook, J., Fritsch, E.F., and Maniatis, T., Cold Spring Harbor Laboratory Press, 1989) or methods described in other experimental manuals used by those skilled in the art, or performed according to the instructions of commercially available reagents or kits used, unless otherwise specified.

(Example 1) Preparation of expression vector

1)-1 Preparation of human ROBO4 expression vector

1)-1-1 Preparation of full-length human ROBO4 expression vector

Human ROBO4 cDNA was cleaved off with EcoRV and NotI from a plasmid (manufactured by Open Biosystems) comprising human ROBO4 cDNA (Accession No. BC039602) and incorporated between EcoRV and NotI of a pCI vector (manufactured by Promega Corp.) to prepare a full-length human ROBO4 expression vector (hereinafter, referred to as "pCI-hROBO4"). The sequence of the human ROBO4 gene cloned in this vector is shown in SEQ ID NO: 1 . Also, the amino acid sequence of human ROBO4 is shown in SEQ ID NO: 2 .

1)-1-2 Preparation of human ROBO4 extracellular region expression vector

cDNA encoding a human ROBO4 extracellular region polypeptide (consisting of an amino acid sequence represented by amino acid Nos. 1 to 461 of SEQ ID NO: 2 ;

hereinafter, abbreviated to "human ROBO4-ECD") was amplified through PCR reaction using a primer set:

primer 1F: 5'-aaaggtaccaccatgggctctggaggagacagcctcctg-3' and

primer 1R: 5'-aaagatatcctgctccagggtccagggaccatgctcact-3'

The obtained PCR product was cloned into a pEF6/V5-His-TOPO vector (manufactured by Life Technologies Corp.) (hereinafter, the resulting vector is abbreviated to "pEF6-ROBO4-ECD"; hereinafter, a recombinant protein expressed by "pEF6-ROBO4-ECD" is referred to as "rROBO4-ECD").

1)-1-3 Preparation of N-terminal FLAG-tagged full-length human ROBO4 and human ROBO4 extracellular region/domain deletion variant expression vectors

In order to construct vectors for expression of a protein comprising a region consisting of an amino acid sequence represented by amino acid Nos. 28 to 1007 of SEQ ID NO: 2 of human ROBO4 (in the diagram, this region is referred to as "hROBO4-28"), a region consisting of an amino acid sequence represented by amino acid Nos. 46 to 1007 thereof (in the diagram, this region is referred to as "hROBO4-46"), a region consisting of an amino acid sequence represented by amino acid Nos. 132 to 1007 thereof (in the diagram, this region is referred to as "hROBO4-132"), a region consisting of an amino acid sequence represented by amino acid Nos. 210 to 1007 thereof (in the diagram, this region is referred to as "hROBO4-210"), a region consisting of an amino acid sequence represented by amino acid Nos. 225 to 1007 thereof (in the diagram, this region is referred to as "hROBO4-225"), or a region consisting of an amino acid sequence represented by amino acid Nos. 341 to 1007 thereof (in the diagram, this region is referred to as "hROBO4-341") with the N-terminus tagged with FLAG, PCR reaction was performed with pCI-hROBO4 as a template using each primer set:

primer set for hROBO4-28 amplification:

primer 2F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataagcaggactccccgccccagatcctagtccac-3' and

primer 2R: 5'-gctagcggagtaatctacaggagaagcaccagccttg-3',

primer set for hROBO4-46 amplification:

primer 3F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataagcctggccctgccaggatgagctgccaag-3' and the primer 2R

primer set for hROBO4-132 amplification:

primer 4F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataaggtggctgtcctccgggaggatttccagatc-3' and the primer 2R,

primer set for hROBO4-210 amplification:

primer 5F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataagaccaacagcgcaggacatagggagagcc-3' and the primer 2R,

primer set for hROBO4-225 amplification:

primer 6F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataagatccaggagccccaggactacacggagcc-3' and the primer 2R,

primer set for hROBO4-341 amplification:

primer 7F: 5'-

ggtaccgccatgggctctggaggagacagcctcctcggcggcagaggttccctgcctctgctgctcctgctcatcatgggag

gcatggctgattacaaggatgacgacgataagaggctgccggaaaaagtgcccagtgcccca-3' and the primer

2R. The obtained PCR product was incorporated into a pCR4Blunt-TOPO vector (manufactured by Life Technologies Corp.) to prepare a cloning vector. From each cloning vector, the corresponding cDNA was cleaved off with KpnI and NheI and

incorporated between KpnI and NheI of a pCI vector to prepare an N-terminal FLAG-tagged full-length human ROBO4 expression vector and four human ROBO4 extracellular region/domain deletion variant expression vectors. The N-terminal FLAG-tagged full-length human ROBO4 vector consists of a nucleotide encoding the signal sequence of ROBO4 (amino acids of amino acid Nos. 1 to 27 of SEQ ID NO: 2) + FLAG sequence (DYKDDDDK) + ROBO4 (amino acids of amino acid Nos. 28 to 1007 of SEQ ID NO: 2) from the N-terminus. Hereinafter, the vector for expression of N-terminal FLAG-tagged full-length human ROBO4 is referred to as "pCI-FLAG-hROBO4-28". Of the human ROBO4 extracellular region/domain deletion variant expression vectors, for example, the vector for expression of ROBO4 consisting of amino acid Nos. 46 to 1007 of SEQ ID NO: 2 consists of a nucleotide encoding the signal sequence of ROBO4 (amino acids of amino acid Nos. 1 to 27 of SEQ ID NO: 2) + FLAG sequence (DYKDDDDK) + extracellular region-deleted ROBO4 (amino acids of amino acid Nos. 46 to 1007 of SEQ ID NO: 2). This vector is referred to as "pCI-hROBO4-46". Likewise, the vectors encoding ROBO4 having a partial deletion in the extracellular region of ROBO4 are referred to as "pCI-hROBO4-132", "pCI-hROBO4-210", "pCI-hROBO4-225", and "pCI-hROBO4-341", respectively.

The nucleotide sequence encoding the amino acid sequence of FLAG-tagged full-length human ROBO4 or each extracellular region/domain deletion variant of human ROBO4 cloned in the vector is shown in SEQ ID NO: 3, 5, 7, 9, 11, or 13 . Also, the amino acid sequence of the corresponding FLAG-tagged full-length human ROBO4 or extracellular region/domain deletion variant of human ROBO4 is shown in SEQ ID NO: 4, 6, 8, 10, 12, or 14 .

1)-1-4 Preparation of human ROBO4 intracellular region deletion variant expression vector

In order to construct a vector for expression of a protein comprising a region consisting of an amino acid sequence represented by amino acid Nos. 1 to 511 of SEQ ID NO: 2 of human ROBO4 (hereinafter, this region is referred to as "hROBO4-ΔC"), a stop codon was inserted immediately after a codon encoding the 511th amino acid of human ROBO4 with pCI-hROBO4 as a template using primer set for hROBO4-ΔC:
primer 8F: 5'-cagatataccagtgaggatgcctgaatcctaaaacacaggatggatc-3' and
primer 8R: 5'-gatccatcctgtgttttaggattcaggcatcctcactggtatatctg-3',
and QuikChange XL Site-Directed Mutagenesis Kit (manufactured by Agilent Technologies, Inc.) to prepare a hROBO4-ΔC expression vector (hereinafter, referred to as "pCI-hROBO4-ΔC").

1)-2 Preparation of mouse ROBO4 expression vector

PCR reaction was performed with Mouse Heart QUICK-Clone cDNA (manufactured by Takara Bio Inc.) as a template using a primer set:
primer 9F: 5'-ggtaccgccatgggacaaggagaggagccgagagcagccatg-3' and
primer 9R: 5'-gcggccgcggaggaatcaccagccttgggcacagcaccag-3'
The obtained PCR product was incorporated into a pCR-Blunt II-TOPO vector (manufactured by Life Technologies Corp.) to prepare a cloning vector comprising mouse ROBO4 cDNA. From the cloning vector, mouse ROBO4 cDNA was cleaved off with KpnI and NotI and incorporated between KpnI and NotI of a pCI vector to prepare a mouse ROBO4 expression vector (hereinafter, referred to as "pCI-mROBO4"). The sequence of an ORF site in the mouse ROBO4 gene cloned in this vector is shown in nucleotide Nos. 7 to 3051 of SEQ ID NO: 15 . Also, the amino acid sequence of mouse ROBO4 is shown in SEQ ID NO: 16 .

1)-3 Preparation of rat ROBO4 expression vector

PCR reaction was performed with Rat Spleen QUICK-Clone cDNA (manufactured by Takara Bio Inc.) as a template using a primer set:
primer 10F: 5'-ggtaccgccatgggacaaggagaggagctgagagcagcc-3' and
primer 10R: 5'-gcggccgcggaggaatcaccagccttgggcacaacacc-3'
The obtained PCR product was incorporated into a pCR4Blunt-TOPO vector to prepare a cloning vector comprising rat ROBO4 cDNA. From the cloning vector, rat ROBO4 cDNA was cleaved off with KpnI and NotI and incorporated between KpnI and NotI of a pCI vector to prepare a rat ROBO4 expression vector (hereinafter, referred to as "pCI-raROBO4"). The nucleotide sequence of rat ROBO4 cDNA is shown in SEQ ID NO: 17 . The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 18 .

1)-4 Preparation of N-terminal FLAG-tagged cynomolgus monkey ROBO4 expression vector

PCR reaction was performed with cDNA synthesized from cynomolgus monkey kidney total RNA as a template using a primer set:
primer 11F: 5'-ggtaccgccatgggctctggaggagaaagcctccggg-3' and
primer 11R: 5'-ggagtaatctacaggagaagcaccagccttg-3'
The obtained PCR product was incorporated into a pCR4Blunt-TOPO vector to prepare two types of cloning vectors comprising each cynomolgus monkey ROBO4 cDNA (hereinafter, referred to as cynoROBO4-1 or cynoROBO4-2) (hereinafter, these vectors are referred to as pCR-cynoROBO4-1 and pCR-cynoROBO4-2, respectively).

Next, PCR reaction was performed with pCR-cynoROBO4-1 or pCR-cynoROBO4-2 as a template using a primer set:
primer 12F: 5'-ggatccgccatgggctctggaggagaaagcctccg-3' and
primer 12R: 5'-gcggccgctcaggagtaatctacaggagaagcaccagccttg-3'

The obtained PCR product was incorporated intoa pCR4Blunt-TOPO vector to prepare a cloning vector comprising each cynomolgus monkey ROBO4 cDNA. From the cloning vector, the corresponding cynomolgus monkey ROBO4 cDNA was cleaved off with BamHI and NotI and incorporated between BamHI and NotI of a pCI vector to prepare two types of cynomolgus monkey ROBO4 expression vectors (hereinafter, referred to as pCI-cynoROBO4-1 and pCI-cynoROBO4-2, respectively).

Next, PCR reaction was performed with pCI-cynoROBO4-1 or pCI-cynoROBO4-2 as a template using a primer set:

primer 13F: 5'-

ggtaccgccatgggctctggaggagaaagcctccgaggctcccgggcttcccggcctctgctgctcctgctcatcatgggag gcatggctgattacaaggatgacgacgataagcaggactccccgccccagatcctagtccac-3'

and the primer 12R.

The obtained PCR product was incorporated into a pCR-TOPO vector (manufactured by Life Technologies Corp.) to prepare each cloning vector comprising N-terminal FLAG-tagged cynomolgus monkey ROBO4 cDNA. From the cloning vector, the corresponding cynomolgus monkey ROBO4 cDNA was cleaved off with KpnI and NotI and incorporated between KpnI and NotI of a pCI vector to prepare N-terminal FLAG-tagged cynomolgus monkey ROBO4 expression vectors (hereinafter, referred to as "pCI-FLAG-cynoROBO4-1" and "pCI-FLAG-cynoROBO4-2", respectively). The nucleotide sequence of cynomolgus monkey ROBO4 cDNA cloned in each of pCI-FLAG-cynoROBO4-1 and pCI-FLAG-cynoROBO4-2 is shown in SEQ ID NOs: 19 and 21, respectively, . The amino acid sequence encoded by each nucleotide sequence is shown in SEQ ID NOs: 20 and 22, respectively, .

1)-5 Preparation of N-terminal FLAG-tagged human ROBO1 expression vector

PCR reaction was performed with Human Heart QUICK-Clone cDNA (manufactured by Takara Bio Inc.) as a template using a primer set:

primer 13F: 5'-ggggacaagtttgtacaaaaaagcaggcttcaccatgattgcggagcccgctcacttttacctg-3'
and
primer 13R: 5'-ggggaccactttgtacaagaaagctgggtcgctttcagtttcctctaattcttc-3'
The obtained PCR product and a pDONR221 vector (manufactured by Life Technologies Corp.) were subjected to BP reaction to prepare a donor vector comprising human ROBO1 cDNA.

Next, the PCR reaction was performed with the donor vector as a template using a primer set:
primer 14F: 5'-
gcggccgcatgattgcggagcccgctcacttttacctgtttggattaatatgtctctgttcaggctcccgtcttgattacaaggatg
acgacgataagcgtcaggaagattttccacctcgcattgttg-3' and
primer 14R: 5'-gctagctcagctttcagtttcctctaattcttc-3'
The obtained PCR product was incorporated intoa pCR4Blunt-TOPO vector to prepare a cloning vector comprising N-terminal FLAG-tagged human ROBO1 cDNA. From the cloning vector, N-terminal FLAG-tagged human ROBO1 cDNA was cleaved off with NheI and NotI and incorporated between NheI and NotI of a pCI vector to prepare an expression vector (hereinafter, referred to as "pCI-FLAG-hROBO1"). The nucleotide sequence of N-terminal FLAG-tagged human ROBO1 cDNA is shown in SEQ ID NO: 23 . The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 24 .

1)-6 Preparation of human ROBO2 expression vector

PCR reaction was performed with Human Lung QUICK-Clone cDNA (manufactured by Takara Bio Inc.) as a template using a primer set:
primer 15F: 5'-gcggccgcatgagtctgctgatgtttacacaactactg-3' and
primer 15R: 5'-gctagcctataattcacctgtaaactgtccttgactgttg-3'

The obtained PCR product was incorporated to a pCR4Blunt-TOPO vector to prepare a cloning vector comprising human ROBO2 cDNA. From the cloning vector, human ROBO2 cDNA was cleaved off with NotI and NheI and incorporated between NotI and NheI of a pCI vector to prepare an expression vector (hereinafter, referred to as "pCI-hROBO2"). The nucleotide sequence of human ROBO2 cDNA is shown in SEQ ID NO: 25 . The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 26 .

1)-7 Preparation of human ROBO3 expression vector

PCR reaction was performed with human ROBO3/pENTR223.1 (manufactured by Open Biosystems) as a template using a primer set:
primer 16F: 5'-gcggccgcatgctgcgctacctgctgaaaacgctgctg-3' and
primer 16R: 5'-gctagctcatcttggttcctctcggcgtttctgtcc-3'
The obtained PCR product was incorporated to a pCR4Blunt-TOPO vector to prepare a cloning vector comprising human ROBO3 cDNA. From the cloning vector, human ROBO3 cDNA was cleaved off with NotI and NheI and incorporated between NotI and NheI of a pCI vector to prepare an expression vector (hereinafter, referred to as "pCI-hROBO3"). The sequence of an ORF site in the human ROBO3 gene cloned in this vector is shown in nucleotide Nos. 35 to 4192 of SEQ ID NO: 27 . Also, the amino acid sequence of human ROBO3 is shown in SEQ ID NO: 28 .

(Example 2) Preparation of monoclonal antibody

2)-1 Preparation of antigenic protein

In order to express rROBO4-ECD, FreeStyle 293-F cells (manufactured by Life Technologies Corp.) were transfected with pEF6-ROBO4-ECD using 293fectin (manufactured by Life Technologies Corp.) and cultured at 37°C for 6 days under 8% CO_2 conditions. After completion of the culture, the culture solution was collected by

centrifugation and used as a rROBO4-ECD purification stock. The obtained culture supernatant was dialyzed against 20 mM Tris-HCl, pH 7.5 using a dialysis tube having a molecular weight cutoff of 15000, filtered through a filter (0.45 μm), and then added to HiTrap 16/10 Q XL (manufactured by GE Healthcare Bio-Sciences Crop.) equilibrated with 20 mM Tris-HCl, pH 7.5. Elution was performed with a NaCl gradient (20 mM Tris-HCl, pH 7.5/0.2 M NaCl, 20 mM Tris-HCl, pH 7.5/1 M NaCl). A portion of the elution fraction was separated by SDS-polyacrylamide gel electrophoresis (hereinafter, abbreviated to "SDS-PAGE"). Then, the gel was subjected to Coomassie Brilliant Blue staining (hereinafter, abbreviated to "CBB staining") and detection by Western blotting to confirm a fraction containing rROBO4-ECD. Next, the fraction containing rROBO4-ECD was collected and added to HiLoad 16/60 Superdex 75 pg (manufactured by GE Healthcare Bio-Sciences Crop.) equilibrated with PBS. After elution with PBS, a portion of the elution fraction was separated by SDS-PAGE. Then, the gel was subjected to CBB staining and detection by Western blotting to confirm a fraction containing rROBO4-ECD. The fraction containing rROBO4-ECD was collected and used as an antigen for immunization and as an antigen for binding affinity assay. The protein concentration was measured using BCA Protein Assay Reagent (manufactured by Pierce Biotechnology, Inc.).

2)-2 Immunization

Six-week-old female BALB/c mice were used. At day 0, 50 μg of a mixture of rROBO4-ECD and a Freund's complete adjuvant was hypodermically or intradermally administered to each mouse. At days 7, 14, and 21, 50 μg of a mixture of rROBO4-ECD and a Freund's incomplete adjuvant was hypodermically or intradermally administered to the mouse. At day 38, 50 μg of rROBO4-ECD was intraperitoneally administered to the mouse. At day 42, the mouse lymph node or spleen was collected and used in hybridoma preparation.

2)-3 Hybridoma preparation

The lymph node cells or spleen cells and mouse myeloma SP2/0-ag14 cells were electrically fused using Hybrimune Hybridoma Production System (manufactured by Cyto Pulse Sciences, Inc.), diluted with ClonaCell-HY Selection Medium D (manufactured by StemCell Technologies Inc.), and cultured. Hybridoma colonies that appeared were collected to prepare monoclonal hybridomas. Each hybridoma colony collected was cultured, and the obtained hybridoma culture supernatant was screened for anti-ROBO4 antibody-producing hybridomas.

2)-4 Antibody screening

2)-4-1 Preparation of antigen gene-expressing cell for Cell-ELISA

HEK293 cells were adjusted to 7.5×10^5 cells/mL in a DMEM medium containing 10% FBS. The cells were transfected with pCI-hROBO4 or a negative control pCI-mock using Lipofectamine 2000 (manufactured by Life Technologies Corp.), dispensed at a concentration of 50 μL/well to a 96-well half area plate (manufactured by Corning Inc.), and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. The obtained transfected cells were used in Cell-ELISA with them adhering to each other.

2)-4-2 Preparation of antigen gene-expressing cell for flow cytometry analysis

HEK293T cells were inoculated at a concentration of 1.125×10^7 cells/flask to a 225-cm^2 flask (manufactured by Sumitomo Bakelite Co., Ltd.) and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. On the next day, the HEK293T cells were transfected with pCI-ROBO4 or a negative control pCI-mock using Lipofectamine 2000 and further cultured overnight at 37°C under 5% CO_2 conditions. On the next day, the expression vector-transfected HEK293T cells

were treated with TrypLE Express (manufactured by Life Technologies Corp.), washed with DMEM containing 10% FBS, and then suspended in PBS containing 5% FBS. The obtained cell suspension was used in flow cytometry analysis.

2)-5 Cell-ELISA

After removal of a supernatant from the expression vector-transfected HEK293 cells prepared in 2)-4-1, the hybridoma culture supernatant was added to each of the pCI-hROBO4- and pCI-mock-transfected HEK293 cells, and the cells were left standing at 4°C for 1 hour. The cells in each well were washed once with PBS containing 5% FBS. Then, Anti-Mouse IgG-Peroxidase antibody produced in goat (manufactured by Sigma-Aldrich Corp.) diluted 500-fold with PBS containing 5% FBS was added thereto, and the cells were left standing at 4°C for 1 hour. The cells in each well were washed 5 times with PBS containing 5% FBS. Then, an OPD coloring solution (o-phenylenediamine dihydrochloride (manufactured by Wako Pure Chemical Industries, Ltd.) and H_2O_2 dissolved at concentrations of 0.4 mg/mL and 0.6% (v/v), respectively, in an OPD dissolving solution (0.05 M trisodium citrate, 0.1 M disodium hydrogen phosphate dodecahydrate, pH 4.5)) was added thereto at a concentration of 25 μL/well. Color reaction was performed with intermittent stirring and stopped by addition of 1 M HCl at a concentration of 25 μL/well. Then, absorbance at 490 nm was measured using a plate reader (ENVISION; manufactured by Perkin Elmer, Inc.). In order to select hybridomas producing an antibody specifically binding to ROBO4 expressed on cell membrane surface, hybridomas in the culture supernatants exhibiting higher absorbance in the pCI-hROBO4-transfected HEK293 cells compared with the negative control pCI-mock-transfected HEK293 cells were selected as anti-ROBO4 antibody production-positive hybridomas.

2)-6 Flow cytometry analysis

The antibody produced by each hybridoma determined to be positive in 2)-5 Cell-ELISA was further confirmed to bind to ROBO4 by flow cytometry. The HEK293T cell suspension prepared in 2)-4-2 was centrifuged. After removal of the supernatant, the hybridoma culture supernatant was added to each of the pCI-hROBO4-transfected cells and the pCI-mock-transfected cells, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed twice with PBS containing 5% FBS. Then, Anti-Mouse IgG FITC conjugate (manufactured by Sigma-Aldrich Corp.) diluted 1000-fold with PBS containing 5% FBS or Anti-Rat IgG FITC conjugate (manufactured by Sigma-Aldrich Corp.) diluted 320-fold with PBS containing 5% FBS was added thereto, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed three times with PBS containing 5% FBS, then resuspended in PBS containing 5% FBS and 2 μg/mL 7-aminoactinomycin D (manufactured by Molecular Probes), and subjected to detection using a flow cytometer (FC500; manufactured by Beckman Coulter, Inc.). Data was analyzed using Flowjo (manufactured by TreeStar Inc.). 7-aminoactinomycin D-positive dead cells were excluded by gating, and the histogram of FITC fluorescence intensity was then prepared for live cells. Hybridomas producing a sample in which the histogram of the pCI-ROBO4-transfected HEK293T cells was shifted to a stronger fluorescence intensity region compared with the histogram of the negative control pCI-mock-transfected 293T cells were obtained as anti-ROBO4 antibody-producing hybridomas. Anti-ROBO4 antibodies produced by the obtained hybridomas were designated as MAb1, MAb2, MAb3, and MAb4, respectively.

2)-7 Isotyping of monoclonal antibody

The isotype of each monoclonal antibody was determined using Mouse monoclonal isotyping kit or Rat monoclonal isotyping kit (manufactured by AbD Serotec). The results were IgG1 (MAb1 and MAb2) and IgG2b (MAb3 and MAb4).

2)-8 Preparation of monoclonal antibody

Each monoclonal antibody was purified from a hybridoma culture supernatant (hereinafter, referred to as an "antibody purification stock").

The antibody purification stock was prepared as follows: 8 to 9×10^7 hybridomas were inoculated to a 1272-cm^2 flask (manufactured by Corning Inc.) and cultured in a hybridoma SFM medium (manufactured by Life Technologies Corp.) containing 20% Ultra-LoW IgG fetal bovine serum at 37°C for 4 days under 5% CO_2 conditions, and the supernatant was then collected.

The antibody was purified using Hitrap Protein G HP or Hitrap MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Crop.). For Hitrap Protein G HP, the antibody purification stock was added to a column and washed with a binding buffer (0.02 M sodium phosphate, pH 7.0), followed by elution with 0.1 M glycine, pH 2.7. By contrast, for Hitrap MabSelect SuRe, the antibody purification stock was added to a column and washed with PBS, followed by elution with 2 M arginine-HCl, pH 4.0. The eluted antibody solution was neutralized, and the buffer was then replaced by PBS. The concentration of the antibody purified with Hitrap Protein G HP was measured using BCA Protein Assay Reagent. Mouse IgG2a (manufactured by R&D systems, Inc.) was used as a standard for a calibration curve. Alternatively, the concentration of the antibody purified with Hitrap MabSelect SuRe was determined by the measurement of absorbance (O.D. 280 nm) in an eluate of the antibody bound to POROS G 20 μm Column PEEK, 4.6 mm × 50 mm, 0.83 mL (manufactured by Applied Biosystems, Inc.). Specifically, the antibody sample diluted with PBS was added to POROS G 20 μm equilibrated with an equilibration buffer (30.6 mM sodium dihydrogen phosphate dodecahydrate, 19.5 mM monopotassium phosphate, 0.15 M NaCl, pH 7.0). The column was washed with an equilibration buffer, and an antibody bound to the column was then eluted with an eluent (0.1% (v/v) HCl, 0.15 M NaCl). The peak area of

absorbance (O.D.280 nm) in the eluate was measured, and the concentration was calculated according to the following equation: Antibody sample concentration (mg/mL) = (Peak area of antibody sample) / (Peak area of standard (human IgG1)) × Concentration of standard (mg/mL) × Dilution ratio of sample.

(Example 3) Detection of activation of ROBO4 downstream signal

3)-1 Preparation of reporter vector comprising interleukin-8 (IL-8) promoter region as response element

PCR reaction was performed with IL-8 promoter region DNA as a template using a primer set:

primer 17F: 5'-ggtaccgataaggaacaaataggaag-3' and

primer 17R: 5'-gagctcagcttgtgtgctctgctgtc-3'

The obtained PCR product was incorporated to a pCR4Blunt-TOPO vector to prepare a cloning vector comprising IL-8 promoter region (-253 to -59) DNA. From the cloning vector, IL-8 promoter region (-253 to -59) DNA was cleaved off with KpnI and SacI and incorporated between KpnI and SacI of a pGL4.15 vector (manufactured by Promega Corp.) to prepare a reporter vector comprising the IL-8 promoter region as a response element. The nucleotide sequence of IL-8 promoter region (-253 to -59) DNA is shown in SEQ ID NO: 29 .

3)-2 Reporter vector comprising Nuclear Factor-κB (NF-κB), Interferon gamma activation sequence (GAS), Interferon stimulated response element (ISRE), Transfection grade T cell factor (TCF)
as response element

pGL4.32[luc2P/NF-κB-RE/Hygro] Vector (manufactured by Promega Corp.), pGAS-TA-Luc Vector (manufactured by Takara Bio Inc.), pISRE-TA-Luc Vector (manufactured by Takara Bio Inc.), and TOPflash (manufactured by Millipore Corp.)

were respectively used as reporter vectors comprising NF-κB, GAS, ISRE, or TCF as a response element. Alternatively, response sequence-free pTA-Luc Vector (manufactured by Takara Bio Inc.) was used as a negative control. pRL-TK Vector (manufactured by Takara Bio Inc.) was used as an internal control.

3)-3 Analysis of signal varying in cell transiently expressing human ROBO4

HEK293 cells were inoculated at a concentration of 2×10^4 cells/well to a 96-well plate (coated with collagen I; manufactured by Asahi Glass Co., Ltd.) and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. On the next day, the HEK293 cells were transfected with pCI-ROBO4, pCI-ROBO4-ΔC, or a negative control pCI-mock and each of the reporter vectors shown in 3)-1 and 3)-2 using FuGene6 Transfection Reagent, and further cultured overnight at 37°C under 5% CO_2 conditions. On the next day, the firefly luciferase and Renilla luciferase activities of each well were determined as luminescence intensity in a plate reader (Mithras; manufactured by Berthold Technologies GmbH & Co, KG) using Dual-Glo Luciferase Assay System (manufactured by Promega Corp.), and the reporter activity of each well was calculated according to the following equation: Reporter activity = Firefly luciferase activity-derived luminescence intensity / Renilla luciferase activity-derived luminescence intensity. As a result, only the IL-8 promoter activity was increased in the pCI-hROBO4-transfected cells compared with the negative control pCI-mock-transfected cells (Figure 1). Moreover, this increase in the IL-8 promoter activity detected in the pCI-hROBO4-transfected cells was drastically attenuated in the cells transfected with pCI-hROBO4-ΔC (intracellular region deletion mutant of hROBO4), demonstrating that the increase in the IL-8 promoter activity detected in the pCI-hROBO4-transfected cells requires the intracellular region of ROBO4 (Figure 2). Accordingly, the increase in the IL-8 promoter activity in the pCI-hROBO4-transfected cells demonstrated that the activation of the ROBO4 downstream signal was detected.

(Example 4) Properties of MAb1

4)-1 Activation of ROBO4 downstream signal by MAb1

The pCI-hROBO4-transfected cells or the pCI-mock-transfected cells prepared in 3)-3 were cultured overnight. On the next day, each anti-ROBO4 antibody (MAb1, MAb2, MAb3, or MAb4) or a negative control Mouse IgG1 (manufactured by R&D systems, Inc.) was added thereto at concentrations of 0, 0.3125, 1.25, 5, and 20 μg/mL or 0, 0.25, 1, 4, and 16 μg/mL, and the cells were cultured at 37°C for 5 hours under 5% CO_2 conditions. Then, the firefly luciferase and Renilla luciferase activities of each well were determined as luminescence intensity in a plate reader (Mithras) using Dual-Glo Luciferase Assay System (manufactured by Promega Corp.), and the reporter activity of each well was calculated according to the following equation: Reporter activity = Firefly luciferase activity-derived luminescence intensity / Renilla luciferase activity-derived luminescence intensity. As a result, the negative control mouse IgG did not influence the IL-8 promoter activity in the cells transiently expressing human ROBO4, whereas MAb1 increased the IL-8 promoter activity (Figure 3). As in MAb1, MAb2 also increased the IL-8 promoter activity, whereas MAb3 or MAb4 did not increase the IL-8 promoter activity (Figure 4). In the pCI-mock cells, MAb1 or MAb2 did not increase the IL-8 promoter activity. These results demonstrated that MAb1 activated the downstream signal of ROBO4 and not all antibodies against ROBO4 activated the downstream signal of ROBO4.

MAb3 and MAb4 that were confirmed not to increase the activity of the ROBO4 downstream signal were evaluated for promoter activity in the presence of cross-linking antibodies (AffiPure Goat Anti-Mouse IgG Fc Fragment Specific, Cat NO. 115-005-071, Jackson ImmunoResearch) (two cross-linking antibody molecules with respect to one molecule of MAb3 or MAb4). As a result, increase in the promoter activity was observed for both the antibodies.

4)-2 HUVEC migration test

HUVEC (manufactured by KURABO INDUSTRIES LTD.) was cultured overnight in HuMedia-EB2 (manufactured by KURABO INDUSTRIES LTD.) containing 0.1% BSA at 37°C under 5% CO_2 conditions and then adjusted to 4×10^5 cells/mL with HuMedia-EB2 containing 0.1% BSA. 0.25 mL of the cell suspension having a concentration of 4×10^5 cells/mL was added to the upper layer of a chamber in BD Falcon FluoroBlok 24 multi-well insert system (Pore size: 8 μm) having a gelatin-coated membrane. Then, HuMedia-EB2 containing 0.1% BSA and 10 ng/mL human VEGF165 (manufactured by PeproTech Inc.) or human bFGF (BD Biosciences) and 2 μg/mL Mouse IgG2a or each anti-ROBO4 antibody (MAb1, MAb2, MAb3, or MAb4) was added to the lower layer of the chamber. After incubation at 37°C for 2 to 3 hours under 5% CO_2 conditions, HUVEC that migrated to the lower layer was stained with HuMedia-EB2 containing 4 μg/mL Calcein-AM (manufactured by Life Technologies Corp.) for 15 minutes. Then, the fluorescence intensity (excitation wavelength/fluorescence wavelength: 485 nm/538 nm) of each well was measured using a plate reader (FlexStation; Molecular Devices, LLC.), and the amount of migrating cells in each well was calculated according to the following equation: Amount of migrating cells = Fluorescence intensity of HUVEC-supplemented well - Fluorescence intensity of HUVEC-unsupplemented well. As a result, MAb1 suppressed the migration of HUVEC induced by VEGF or bFGF (Figure 5). As in MAb1, MAb2 also suppressed the migration of HUVEC induced by bFGF, whereas MAb3 or MAb4 did not suppress the cell migration (Figure 6). These results demonstrated that the increase in the IL-8 promoter activity by the anti-ROBO4 antibody correlated with the suppressive activity against HUVEC migration.

4)-3 Cross-species reactivity

4)-3-1 Preparation of antigen gene-expressing cell

HEK293 cells were inoculated at a concentration of 1.5×10^6 cells/dish to a 60-mm dish (manufactured by Corning Inc.) and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. On the next day, the HEK293 cells were transfected with pCI-hROBO4, pCI-mROBO4, pCI-raROBO4, pCI-FLAG-cynoROBO4-1, pCI-FLAG-cynoROBO4-2, pCI-hROBO1, pCI-hROBO2, or pCI-hROBO3 using FuGENE6 Transfection Reagent and further cultured overnight at 37°C under 5% CO_2 conditions. On the next day, the expression vector-transfected cells were treated with TrypLE Express (manufactured by Life Technologies Corp.), washed with PBS containing 5% FBS, and then suspended in PBS containing 5% FBS. The obtained cell suspension was used in flow cytometry analysis.

4)-3-2 Flow cytometry analysis

Each cell suspension prepared in 4)-3-1 was centrifuged, and the supernatant was removed. Then, MAb1 or a negative control Mouse IgG2a was added at a concentration of 10 μg/mL to 2×10^5 expression vector-transfected cells, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed once with PBS containing 5% FBS. Then, Anti-Mouse IgG FITC conjugate diluted 1000-fold with PBS containing 5% FBS was added thereto, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed with three times with PBS containing 5% FBS, then resuspended in PBS containing 5% FBS, and subjected to detection using a flow cytometer (BD FACSCalibur). Data was analyzed using Flowjo. The histogram of FITC fluorescence intensity was prepared. The antibody was determined to bind in a cross-species manner when the histogram of MAb1 was shifted to a stronger fluorescence intensity region compared with the histogram of the negative control Mouse IgG2a. The results of cross-species reactivity study demonstrated that

MAb1 did not bind to mouse ROBO4 or rat ROBO4, but bound to human ROBO4 and cynomolgus monkey ROBO4 (Figure 7).

4)-4 Binding specificity

4)-4-1 Preparation of antigen gene-expressing cell

HEK293 cells were inoculated at a concentration of 1.2×10^6 cells/dish or 1.5×10^6 cells/dish to a 60-mm dish (manufactured by Corning Inc.) and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. On the next day, the HEK293 cells were transfected with pCI-hROBO4, pCI-FLAG-hROBO1, pCI-hROBO2, or pCI-hROBO3 using FuGENE6 Transfection Reagent and further cultured overnight at 37°C under 5% CO_2 conditions. On the next day, the expression vector-transfected cells were treated with TrypLE Express (manufactured by Life Technologies Corp.), washed with PBS containing 5% FBS, and then suspended in PBS containing 5% FBS. The obtained cell suspension was used in flow cytometry analysis.

4)-4-2 Flow cytometry analysis

Each cell suspension prepared in 4)-4-1 was centrifuged, and the supernatant was removed. Then, MAb1, a positive control Human ROBO4 Antibody (manufactured by R&D systems, Inc.), Monoclonal ANTI-FLAG M2 antibody produced in mouse (manufactured by Sigma-Aldrich Corp.), Human ROBO2 Antibody (manufactured by R&D systems, Inc.), or Monoclonal Anti-human ROBO3 Antibody (manufactured by R&D systems, Inc.), or a negative control Mouse IgG1 or Mouse IgG2a was added at a concentration of 10 μg/mL to 2×10^5 expression vector-transfected cells, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed once with PBS containing 5% FBS. Then, Anti-Mouse IgG FITC conjugate diluted 1000-fold with PBS containing 5% FBS was added thereto, and the resulting suspension was

left standing at 4°C for 1 hour. The cells were washed with three times with PBS containing 5% FBS, then resuspended in PBS containing 5% FBS, and subjected to detection using a flow cytometer (BD FACSCalibur). Data was analyzed using Flowjo. The histogram of FITC fluorescence intensity was prepared. The antibody was determined to bind in a specific manner when the histogram of MAb1 was shifted to a stronger fluorescence intensity region compared with the histogram of the negative control Mouse IgG1 or Mouse IgG2a. The results demonstrated that MAb1 did not bind to hROBO1, hROBO2, or hROBO3 and specifically bound to hROBO4 (Figure 8). In this context, hROBO4, hROBO1, hROBO2, and hROBO3 were each confirmed to be expressed on the cell membrane using the positive control antibody (Figure 8).

4)-5 Epitope determination

4)-5-1 Preparation of antigen gene-expressing cell

HEK293 cells were inoculated at a concentration of 1.5×10^6 cells/dish to a 60-mm dish (manufactured by Corning Inc.) and cultured overnight in a DMEM medium containing 10% FBS at 37°C under 5% CO_2 conditions. On the next day, the HEK293 cells were transfected with pCI-FLAG-hROBO4-28, pCI-FLAG-hROBO4-46, pCI-FLAG-hROBO4-132, pCI-FLAG-hROBO4-210, pCI-FLAG-hROBO4-225, pCI-FLAG-hROBO4-341 using FuGENE6 Transfection Reagent and further cultured overnight at 37°C under 5% CO_2 conditions. On the next day, the expression vector-transfected cells were treated with TrypLE Express (manufactured by Life Technologies Corp.), washed with PBS containing 5% FBS, and then suspended in PBS containing 5% FBS. The obtained cell suspension was used in flow cytometry analysis.

4)-5-2 Flow cytometry analysis

Each cell suspension prepared in 4)-5-1 was centrifuged, and the supernatant was removed. Then, MAb1, a positive control Monoclonal ANTI-FLAG M2 antibody

produced in mouse, or a negative control Mouse IgG2a was added at a concentration of 10 μg/mL to 2×10^5 expression vector-transfected cells, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed once with PBS containing 5% FBS. Then, Anti-Mouse IgG FITC conjugate diluted 1000-fold with PBS containing 5% FBS was added thereto, and the resulting suspension was left standing at 4°C for 1 hour. The cells were washed with three times with PBS containing 5% FBS, then resuspended in PBS containing 5% FBS, and subjected to detection using a flow cytometer (BD FACSCalibur; BD Biosciences). Data was analyzed using Flowjo. The histogram of FITC fluorescence intensity was prepared. The antibody was determined to bind to a cell when the histogram of MAb1 was shifted to a stronger fluorescence intensity region compared with the histogram of the negative control Mouse IgG2a. The results demonstrated that MAb1 bound to the pCI-FLAG-hROBO4-28-, pCI-FLAG-hROBO4-46-, or pCI-FLAG-hROBO4-132-transfected cell and did not bind to the pCI-FLAG-hROBO4-210-, pCI-FLAG-hROBO4-225-, or pCI-FLAG-hROBO4-341-transfected cell. Thus, MAb1 was shown to recognize the amino acid sequence of Nos. 132 to 209 in human ROBO4 shown in SEQ ID NO: 2 (Figure 9). In this context, each intracellular region/domain deletion variant was confirmed to be expressed on the cell membrane using the positive control anti-FLAG antibody (Figure 9).

4)-6 Drug efficacy evaluation in monkey models with laser-induced choroidal neovascularization

4)-6-1 Anesthesia

Medetomidine hydrochloride (manufactured by Nippon Zenyaku Kogyo Co., Ltd.) was intramuscularly injected at a dose of 0.04 mg/kg to each cynomolgus monkey. 15 minutes thereafter, ketamine hydrochloride (manufactured by Daiichi Sankyo Co., Ltd.) was intramuscularly injected thereto at a dose of 15 mg/kg.

4)-6-2 Model preparation

Each cynomolgus monkey anesthetized in 4)-6-1 was retained in a monkey chair. 4% Xylocaine eye drops (manufactured by AstraZeneca plc) were applied to both eyes for anesthetic/analgesic treatment of eye surface. 5 mg/mL tropicamide-5 mg/mL phenylephrine hydrochloride mixture eye drops (manufactured by Santen Pharmaceutical Co., Ltd.) were applied to the eyes for mydriasis. The macular region of the retina was thermally damaged by laser irradiation (quantity of heat irradiated: 350-500 mW, irradiation time: 0.1 seconds, spot size: 50 μm, the number of spots: 6 or 9 spots) using a green laser photocoagulator OcuLight GLx (manufactured by Iridex Corp.).

4)-6-3 Administration of test substance

At day 7 after the model preparation, a 33G Nanopass needle was inserted into the vitreous body from the conjunctiva, and 50 μL of vehicle or 13.2 mg/mL MAb1 was injected thereto over 2 minutes using a 100-μL Hamilton syringe via a PE20 polyethylene tube. The test was conducted on each group involving 4 eyes. After completion of the administration, 0.5% levofloxacin hydrate eye drops (manufactured by Santen Pharmaceutical Co., Ltd.) were applied to the eyes.

4)-6-4 Drug efficacy evaluation

At days 7, 14, and 21 after the model preparation, the ocular fundus was photographed by a routine method using a hybrid fundus camera CX-1 (manufactured by Canon Inc.) under anesthesia. Then, 10% fluorescein was intravenously injected thereto at a dose of 0.1 mL/Kg. After completion of the intravenous injection of fluorescein, fluorescent angiography was performed every 1 minute up to 6 minutes later. The image data was stored, and the area of a site at which fluorescein

accumulated was calculated using an image analyzer (WinRoof, manufactured by Mitani Corp.). The amount of blood vessels newly formed was calculated according to the following equation: Amount of blood vessels newly formed = Area of site at which fluorescein accumulated at day 21 after model preparation - Area of site at which fluorescein accumulated at day 7 after model preparation. As a result of comparing the amount of blood vessels newly formed between the vehicle-administered group and the MAb1-administered group, three out of the four eyes in the MAb1-administered group were confirmed to decrease the amount of blood vessels newly formed, though no difference was confirmed in the amount of blood vessels newly formed between the remaining one eye and the vehicle group. This means that the administration of MAb1 suppressed laser-induced choroidal neovascularization (Figure 10).

(Example 5) Cloning and sequencing of MAb1 cDNA

5)-1 Determination of N-terminal amino acid sequences of MAb1 heavy and light chains

In order to determine the N-terminal amino acid sequences of the heavy and light chains of MAb1, MAb1 purified in Example 2)-8 was separated by SDS-PAGE. The proteins thus separated in the gel were transferred from the gel to a Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Inc.), which was in turn washed with a washing buffer (25 mM NaCl, 10 mM sodium borate buffer, pH 8.0), then stained by dipping for 5 minutes in a staining solution (50% methanol, 20% acetic acid, 0.05% Coomassie Brilliant Blue), and then decolorized with 90% methanol. Band portions corresponding to the heavy chain (band with smaller mobility) and the light chain (band with larger mobility) visualized on the PVDF membrane were cleaved off. Their respective N-terminal amino acid sequences were identified according to the automatic Edman method (see Edman et al., (1967) Eur. J. Biochem. 1, 80) using Procise cLC protein sequencer Model 492cLC (Applied Biosystems, Inc.). As a result, the N-

terminal amino acid sequence of the band corresponding to the heavy chain of MAb1 was

EVQLVESGGGLVKPGGSLKL, and

the N-terminal amino acid sequence of the band corresponding to the light chain was DAVMTQTPLSLPVSL.

5)-2 Preparation of mRNA from MAb1-producing hybridoma

In order to clone heavy chain- and light chain-encoding cDNAs of MAb1, mRNA was prepared from the MAb1-producing hybridoma using mRNA Isolation kit (Roche Applied Science).

5)-3 Cloning and sequencing of MAb1 cDNA

Several oligonucleotide primers hybridizing to the 5'-terminal sequence of the coding region of the antibody gene and the stop codon-containing 3'-terminal sequence thereof, respectively, were synthesized with reference to the N-terminal amino acid sequences of the heavy and light chains determined based on the γ1 and κ isotypes of the MAb1 heavy and light chains, respectively (Examples 2)-7) and 5-1), and the antibody amino acid sequence database prepared by Kabat et al. (see Strausberg, R. L., et al. (2002) Proc. Natl. Acad. Sci. U. S. A. 99. 16899-16903, and Kabat, E. A., et al. (1991) in Sequences of Proteins of Immunological Interest Vol. I and II, U. S. Department of Health and Human Services). Heavy chain- and light chain-encoding cDNAs were amplified using the mRNA prepared in 5)-2 and TaKaRa One Step RNA PCR Kit (AMV) (Takara Bio Inc.). As a result, the heavy chain- and light chain-encoding cDNAs of the antibody were successfully amplified using the following primer set:

primer set for the heavy chain

LYHF6: 5'-cctcaccatgaactttgg-3'

G1EVR1: 5'-aagatatcttatttaccaggagagtgggagag-3'

primer set for the light chain

MK19EIF1: 5'-aagaattcatgaagttgcctgttagg-3'

KEVR1: 5'-aagatatcttaacactcattcctgttgaagct-3'

The heavy and light chain cDNAs amplified by PCR were separately cloned using pEF6/V5-His TOPO TA Expression Kit (Invitrogen Corp.). The nucleotide sequences of the cloned cDNAs encoding the respective variable regions of the heavy and light chains were determined using a gene sequencer ("ABI PRISM 3700 DNA Analyzer; Applied Biosystems" or "Applied Biosystems 3730xl Analyzer; Applied Biosystems"). The sequencing reaction was performed using GeneAmp 9700 (Applied Biosystems, Inc.).

The determined nucleotide sequence of cDNA encoding the heavy chain variable region of MAb1 is shown in SEQ ID NO: 30 , and its amino acid sequence is shown in SEQ ID NO: 31. The nucleotide sequence of cDNA encoding the light chain variable region of the mouse antibody MAb1 is shown in SEQ ID NO: 32 , and its amino acid sequence is shown in SEQ ID NO: 33.

(Example 6) Preparation of chimeric MAb1 (cMAb1)

6)-1 Preparation of expression vectors pCMA-LK, pCMA-G1, and pCMA-G2

6)-1-1 Construction of chimeric and humanized light chain expression vector pCMA-LK

A plasmid pcDNA3.3-TOPO/LacZ (Invitrogen Corp.) was digested with restriction enzymes XbaI and PmeI, and the obtained fragment of approximately 5.4 kb was ligated to a DNA fragment comprising a DNA sequence (SEQ ID NO: 34) encoding a human κ chain secretory signal and a human κ chain constant region using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to prepare pcDNA3.3/LK.

PCR was performed with pcDNA3.3/LK as a template using a primer set shown below. The obtained fragment of approximately 3.8 kb was phosphorylated and then self-ligated to construct, from pcDNA3.3/LK, a chimeric and humanized light chain expression vector pCMA-LK having a signal sequence-encoding sequence, a cloning site, and a human κ chain constant region-encoding sequence downstream of a CMV promoter.

Primer set

3. 3-F1: 5'-tataccgtcgacctctagctagagcttggc-3'

3. 3-R1: 5'-gctatggcagggcctgccgccccgacgttg-3'

6)-1-2 Construction of chimeric and humanized IgG1-type heavy chain expression vector pCMA-G1

pCMA-LK was digested with XbaI and PmeI to remove a sequence encoding a κ chain secretory signal and a human κ chain constant region. The resulting DNA fragment was ligated to a DNA fragment comprising a DNA sequence (SEQ ID NO: 35) encoding the amino acids of a human heavy chain signal sequence and a human IgG1 constant region using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to construct a chimeric and humanized IgG1-type heavy chain expression vector pCMA-G1 having a signal sequence-encoding sequence, a cloning site, and a human IgG1 heavy chain constant region-encoding sequence downstream of a CMV promoter.

6)-1-3 Construction of chimeric and humanized IgG2-type heavy chain expression vector pCMA-G2

pCMA-LK was digested with XbaI and PmeI to remove a sequence encoding a κ chain secretory signal and a human κ chain constant region. The resulting DNA fragment was ligated to a DNA fragment comprising a DNA sequence (SEQ ID NO: 36) encoding the amino acids of a human heavy chain signal sequence and a human

IgG2 constant region using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to construct a chimeric and humanized IgG2-type heavy chain expression vector pCMA-G2 having a signal sequence-encoding sequence, a cloning site, and a human IgG2 heavy chain constant region-encoding sequence downstream of a CMV promoter.

6)-2 Construction of chimeric MAb1 light chain expression vector

A site comprising cDNA encoding the light chain variable region was amplified with cDNA encoding the light chain variable region of MAb1 as a template using KOD-Plus- (TOYOBO CO., LTD.) and a primer set shown below, and inserted into a restriction enzyme BsiWI-cleaved site of the general-purpose vector pCMA-LK for chimeric and humanized antibody light chain expression using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to construct a chimeric MAb1 light chain expression vector. The obtained expression vector was designated as "pCMA-LK/MAb1 L". The nucleotide sequence encoding the chimeric MAb1 light chain is shown in SEQ ID NO: 37 , and its amino acid sequence is shown in SEQ ID NO: 38. Nucleotide Nos. 1 to 60 of SEQ ID NO: 37 represent the nucleotide sequence encoding the signal sequence. Nucleotide Nos. 61 to 402 thereof represent the nucleotide sequence encoding the variable region. Nucleotide Nos. 403 to 717 thereof represent the nucleotide sequence encoding the constant region. Amino acid Nos. 1 to 20 of SEQ ID NO: 38 represent the amino acids of the signal sequence. Amino acid Nos. 21 to 134 thereof represent the amino acids of the variable region. Amino acid Nos. 135 to 239 represent the amino acids of the constant region.

Primer set for the light chain

MAb1 LF: 5'-tctccggcgcgtacggcgatgctgtgatgacccaaactccactctcc-3'

MAb1 LR: 5'-ggaggggggcggccacagcccgtttgatttccagcttggtgcctcc-3'

6)-3 Construction of chimeric MAb1 IgG1-type heavy chain expression vector

A site comprising cDNA encoding the heavy chain variable region was amplified with cDNA encoding the heavy chain variable region of MAb1 as a template using KOD-Plus- (TOYOBO CO., LTD.) and a primer set shown below, and inserted into a restriction enzyme BsiWI-cleaved site of the chimeric and humanized IgG1-type heavy chain expression vector pCMA-G1 using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to construct a chimeric MAb1 IgG1-type heavy chain expression vector. The obtained expression vector was designated as "pCMA-G1/ MAb1 H". The nucleotide sequence encoding the chimeric MAb1 IgG1-type heavy chain is shown in SEQ ID NO: 39 , and its amino acid sequence is shown in SEQ ID NO: 40. Nucleotide Nos. 1 to 57 of SEQ ID NO: 39 represent the nucleotide sequence encoding the signal sequence. Nucleotide Nos. 58 to 411 thereof represent the nucleotide sequence encoding the variable region. Nucleotide Nos. 412 to 1401 thereof represent the nucleotide sequence encoding the constant region. Amino acid Nos. 1 to 19 of SEQ ID NO: 40 represent the amino acid sequence of the signal sequence. Amino acid Nos. 20 to 137 thereof represent the amino acid sequence of the variable region. Amino acid Nos. 138 to 467 represent the amino acid sequence of the constant region.

Primer set for the IgG1-type heavy chain

MAb1 HF: 5'-cagatgggtgctgagcgaagtgcagctggtggagtctgggggag-3'

MAb1 H1R: 5'-ttggtggaggctgagctgactgtgagagtggtgccgtggccccag-3'

6)-4 Construction of chimeric MAb1 IgG2-type heavy chain expression vector

A site comprising cDNA encoding the heavy chain variable region was amplified with cDNA encoding the heavy chain variable region of MAb1 as a template using KOD-Plus- (TOYOBO CO., LTD.) and a primer set shown below, and inserted into a restriction enzyme BsiWI-cleaved site of the chimeric and humanized IgG2-type heavy

chain expression vector pCMA-G2 using In-Fusion Advantage PCR cloning kit (Clontech Laboratories, Inc.) to construct a chimeric MAb1 IgG2-type heavy chain expression vector. The obtained expression vector was designated as "pCMA-G2/MAb1". The nucleotide sequence encoding the chimeric MAb1 IgG2-type heavy chain is shown in SEQ ID NO: 41 , and its amino acid sequence is shown in SEQ ID NO: 42.

Primer set for the IgG2-type heavy chain

MAb1 HF: 5'-cagatgggtgctgagcgaagtgcagctggtggagtctgggggag-3'

MAb1 2R: 5'-ttggtgctggctgagctgactgtgagagtggtgccgtggccccag-3'

(Example 7) Preparation of IgG1-type chimeric MAb1 antibody and IgG2-type chimeric MAb1 antibody

7)-1 Production of IgG1-type chimeric MAb1 antibody and IgG2-type chimeric MAb1 antibody

FreeStyle 293-F cells (Invitrogen Corp.) were subcultured and cultured according to the manual. 1.2×10^9 cells of FreeStyle 293-F cells (Invitrogen Corp.) at the logarithmic growth phase were inoculated to a 3L Fernbach Erlenmeyer Flask (Corning Inc.), diluted with FreeStyle 293 expression medium (Invitrogen Corp.) into 1.0×10^6 cells/mL, and then shake-cultured in an 8% CO_2 incubator at 90 rpm at 37°C for 1 hour. 3.6 mg of polyethyleneimine (Polysciences, Inc., #24765) was dissolved in 20 mL of Opti-Pro SFM medium (Invitrogen Corp.). Next, an H chain expression vector (0.4 mg) and an L chain expression vector (0.8 mg) prepared using PureLink HiPure Plasmid kit (Invitrogen Corp.) were suspended in 20 mL of Opti-Pro SFM medium (Invitrogen Corp.). 20 mL of the expression vector/Opti-Pro SFM mixture solution was added to 20 mL of the polyethyleneimine/Opti-Pro SFM mixture solution, and the mixture was gradually stirred, further left for 5 minutes, and then added to the FreeStyle 293-F cells. The cells were shake-cultured in an 8% CO_2 incubator at 90

rpm at 37°C for 7 days, and the obtained culture supernatant was filtered through Disposable Capsule Filter (Advantec, #CCS-045-E1H).

IgG1-type and IgG2-type chimeric MAb1 antibodies obtained by the combinations between pCMA-G1/MAb1 H and pCMA-LK/MAb1 L and between pCMA-G2/MAb1 H and pCMA-LK/MAb1 L are abbreviated to "cMAb1-1" and "cMAb1-2", respectively. The term "cMAb1" means both IgG1-type and IgG2-type chimeric MAb1 antibodies.

7)-2 Purification of cMAb1-1 and cMAb1-2

The culture supernatant obtained above in 7-1) was purified by rProtein A affinity chromatography (at 4-6°C). A buffer replacement step after the rProtein A affinity chromatography purification was carried out at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Crop., HiTrap column; volume 1 mL × 2 connected) equilibrated with PBS. The whole culture solution was placed in the column, and the column was then washed with PBS. Next, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0) to collect an antibody-containing fraction. The buffer in this fraction was replaced by PBS using a desalting column (manufactured by GE Healthcare Bio-Sciences Corp., HiTrap Desalting column; volume 5 mL × 2 connected). Finally, the fraction was concentrated with Centrifugal UF Filter Device VIVASPIN20 (molecular weight cutoff: 30 K, Sartorius, at 4°C) into an IgG concentration of 20.0 mg/mL or higher and used as a purification sample.

7)-3 Properties of cMAb1-1 and cMAb1-2

7)-3-1 Activation of ROBO4 downstream signal

This test was conducted according to the method of 4)-1 except that: Human IgG (manufactured by Sigma-Aldrich Corp.) was used as a negative control; and Human

IgG, cMAb1-1, or cMAb1-2 was added at a final concentration of 0.313 μg/mL to a medium. As a result, the negative control Human IgG did not influence the IL-8 promoter activity in the cells transiently expressing human ROBO4, whereas cMAb1-1 and cMAb1-2 increased the IL-8 promoter activity (Figure 11). These results demonstrated that cMAb1-1 or cMAb1-2 activated the downstream signal of ROBO4, as in MAb1.

7)-3-2 HUVEC migration test

This test was conducted according to the method of 4)-2 except that: Human IgG was used as a negative control; and Human IgG, cMAb1-1, or cMAb1-2 was added at a final concentration of 0.5 μg/mL to a medium. As a result, cMAb1-1 or cMAb1-2 suppressed the migration of HUVEC induced by bFGF (Figure 12). These results demonstrated that cMAb1-1 or cMAb1-2 suppressed the migration of HUVEC, as in MAb1.

(Example 8) Design of humanized antibody of mouse anti-human ROBO4 monoclonal antibody MAb1

8)-1 Design of humanized version of MAb1

8)-1-1 Molecular modeling of MAb1 variable region

The molecular modeling of the MAb1 variable regions was carried out by a generally known homology modeling method (Methods in Enzymology, 203, 121-153, (1991)). The primary sequences (available as three-dimensional structures induced from x-ray crystal structures) of human immunoglobulin variable regions registered in Protein Data Bank (Nuc. Acid Res. 35, D301-D303 (2007)) were compared with those of the MAb1 variable regions thus determined. As a result, 3FFD was selected because of its highest sequence homology to the MAb1 heavy chain variable region among antibodies deficient in framework. Also, 1T66 was selected because of its

highest sequence homology to the MAb1 light chain variable region. The three-dimensional structures of the framework regions were prepared by obtaining a "framework model" from the combination of the coordinates of 3FFD and 1T66 corresponding to the MAb1 heavy and light chains. As for the CDRs of MAb1, CDRL1, CDRL2, CDRL3, CDRH1, and CDRH2 were assigned to clusters 16A, 7A, 9A, 10A, and 10B, respectively, according to the classification of Thornton et al. (J. Mol. Biol., 263, 800-815, (1996)). CDRH3 was classified into k(7)B using the H3 rule (FEBS letter 399, 1-8 (1996)). Subsequently, the typical conformation of each CDR was incorporated into the framework model.

Finally, energy calculation for excluding disadvantageous interatomic contact was conducted to obtain a molecular model likely to be the MAb1 variable regions in terms of energy. These procedures were performed using a commercially available protein three-dimensional structure prediction program Prime and coordinate searching program MacroModel (Schrodinger, LLC).

8)-1-2 Design of humanized MAb1 amino acid sequence

Each humanized MAb1 antibody (hMAb1) was constructed by a generally known CDR grafting method (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). Two different acceptor antibodies were selected on the basis of the homology of amino acids in framework regions. The sequences of the MAb1 framework regions were compared with all human frameworks in the Kabat antibody amino acid sequence database (Nuc. Acid Res. 29, 205-206 (2001)). As a result, a B3 antibody was selected as an acceptor because of its 83% sequence homology to the framework regions. The amino acid residues of the B3 framework regions were aligned with those of MAb1 to identify the positions of different amino acids used. These positions of the residues were analyzed using the three-dimensional model of MAb1 constructed above. Then, donor residues to be grafted onto the acceptor were selected according to the criteria

given by Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). Some selected donor residues were integrated into the acceptor antibody to construct the sequence of humanized MAb1 as described in Examples below. Also, hMAb1 variants were constructed by the substitution of 1 to 3 amino acid residue(s) in each CDR of hMAb1 with other amino acid residues, as described in Examples below.

8)-2 Humanization of cMAb1 heavy chain

8)-2-1 hMAb1-H1-type heavy chain:

A humanized MAb1 heavy chain designed by replacing amino acid No. 32 (lysine) with glutamine, amino acid No. 38 (lysine) with arginine, amino acid No. 59 (threonine) with alanine, amino acid No. 61 (glutamic acid) with glycine, amino acid No. 63 (arginine) with glycine, amino acid No. 95 (glutamic acid) with lysine, amino acid No. 103 (serine) with asparagine, amino acid No. 107 (serine) with alanine, amino acid No. 112 (methionine) with valine, amino acid No. 114 (phenylalanine) with tyrosine, amino acid No. 129 (histidine) with glutamine, amino acid No. 132 (threonine) with leucine, and amino acid No. 133 (leucine) with valine, in the cMAb1-2 heavy chain represented by SEQ ID NO: 42 was designated as a "hMAb1-H1-type heavy chain".

The amino acid sequence of the hMAb1-H1-type heavy chain is shown in SEQ ID NO: 56 . A sequence consisting of amino acid residues 1 to 19, a sequence consisting of amino acid residues 20 to 137, and a sequence consisting of amino acid residues 138 to 463 in the amino acid sequence of SEQ ID NO: 56 correspond to a signal sequence, a heavy chain variable region, and a heavy chain constant region, respectively. A sequence consisting of amino acid residues 50 to 54, a sequence consisting of amino acid residues 69 to 85, and a sequence consisting of amino acid residues 118 to 126 in the amino acid sequence of SEQ ID NO: 56 correspond to a CDRH1 sequence, a CDRH2 sequence, and a CDRH3 sequence, respectively. A

nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 56 is shown in SEQ ID NO: 55. A sequence consisting of nucleotides 1 to 57, a sequence consisting of nucleotides 58 to 411, and a sequence consisting of nucleotides 412 to 1389 in the nucleotide sequence of SEQ ID NO: 55 encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 55 and the amino acid sequence of SEQ ID NO: 56 are also shown in Figures 31 and 32, respectively.

8)-2-2 hMAb1-H2-type heavy chain:

A humanized MAb1 heavy chain designed by replacing amino acid No. 32 (lysine) with glutamine, amino acid No. 38 (lysine) with arginine, amino acid No. 59 (threonine) with alanine, amino acid No. 61 (glutamic acid) with glycine, amino acid No. 63 (arginine) with glycine, amino acid No. 72 (asparagine) with glutamine, amino acid No. 95 (glutamic acid) with lysine, amino acid No. 103 (serine) with asparagine, amino acid No. 107 (serine) with alanine, amino acid No. 112 (methionine) with valine, amino acid No. 114 (phenylalanine) with tyrosine, amino acid No. 129 (histidine) with glutamine, amino acid No. 132 (threonine) with leucine, and amino acid No. 133 (leucine) with valine, in the cMAb1-2 heavy chain represented by SEQ ID NO: 42 was designated as a "hMAb1-H2-type heavy chain".

The amino acid sequence of the hMAb1-H2-type heavy chain is shown in SEQ ID NO: 58 . A sequence consisting of amino acid residues 1 to 19, a sequence consisting of amino acid residues 20 to 137, and a sequence consisting of amino acid residues 138 to 463 in the amino acid sequence of SEQ ID NO: 58 correspond to a signal sequence, a heavy chain variable region, and a heavy chain constant region, respectively. A sequence consisting of amino acid residues 50 to 54, a sequence consisting of amino acid residues 69 to 85, and a sequence consisting of amino acid residues 118 to 126 in the amino acid sequence of SEQ ID NO: 58 correspond to a

CDRH1 sequence, a CDRH2 sequence, and a CDRH3 sequence, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 58 is shown in SEQ ID NO: 57 . A sequence consisting of nucleotides 1 to 57, a sequence consisting of nucleotides 58 to 411, and a sequence consisting of nucleotides 412 to 1389 in the nucleotide sequence of SEQ ID NO: 57 encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 57 and the amino acid sequence of SEQ ID NO: 58 are also shown in Figures 33 and 34, respectively.

8)-2-3 hMAb1-H3-type heavy chain:

A humanized MAb1 heavy chain designed by replacing amino acid No. 32 (lysine) with glutamine, amino acid No. 38 (lysine) with arginine, amino acid No. 59 (threonine) with alanine, amino acid No. 61 (glutamic acid) with glycine, amino acid No. 95 (glutamic acid) with lysine, amino acid No. 103 (serine) with asparagine, amino acid No. 107 (serine) with alanine, amino acid No. 112 (methionine) with valine, amino acid No. 114 (phenylalanine) with tyrosine, amino acid No. 129 (histidine) with glutamine, amino acid No. 132 (threonine) with leucine, and amino acid No. 133 (leucine) with valine, in the cMAb1-2 heavy chain represented by SEQ ID NO: 42 was designated as a "hMAb1-H3-type heavy chain".

The amino acid sequence of the hMAb1-H3-type heavy chain is shown in SEQ ID NO: 60. A sequence consisting of amino acid residues 1 to 19, a sequence consisting of amino acid residues 20 to 137, and a sequence consisting of amino acid residues 138 to 463 in the amino acid sequence of SEQ ID NO: 60 correspond to a signal sequence, a heavy chain variable region, and a heavy chain constant region, respectively. A sequence consisting of amino acid residues 50 to 54, a sequence consisting of amino acid residues 69 to 85, and a sequence consisting of amino acid residues 118 to 126 in the amino acid sequence of SEQ ID NO: 60 correspond to a

CDRH1 sequence, a CDRH2 sequence, and a CDRH3 sequence, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 60 is shown in SEQ ID NO: 59. A sequence consisting of nucleotides 1 to 57, a sequence consisting of nucleotides 58 to 411, and a sequence consisting of nucleotides 412 to 1389 in the nucleotide sequence of SEQ ID NO: 59 encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 59 and the amino acid sequence of SEQ ID NO: 60 are also shown in Figures 35 and 36, respectively.

8)-2-4 hMAb1-H4-type heavy chain:

A humanized MAb1 heavy chain designed by replacing amino acid No. 32 (lysine) with glutamine, amino acid No. 38 (lysine) with arginine, amino acid No. 59 (threonine) with alanine, amino acid No. 61 (glutamic acid) with glycine, amino acid No. 72 (asparagine) with glutamine, amino acid No. 95 (glutamic acid) with lysine, amino acid No. 103 (serine) with asparagine, amino acid No. 107 (serine) with alanine, amino acid No. 112 (methionine) with valine, amino acid No. 114 (phenylalanine) with tyrosine, amino acid No. 129 (histidine) with glutamine, amino acid No. 132 (threonine) with leucine, and amino acid No. 133 (leucine) with valine, in the cMAb1-2 heavy chain represented by SEQ ID NO: 42 was designated as a "hMAb1-H4-type heavy chain".

The amino acid sequence of the hMAb1-H4-type heavy chain is shown in SEQ ID NO: 62 . A sequence consisting of amino acid residues 1 to 19, a sequence consisting of amino acid residues 20 to 137, and a sequence consisting of amino acid residues 138 to 463 in the amino acid sequence of SEQ ID NO: 62 correspond to a signal sequence, a heavy chain variable region, and a heavy chain constant region, respectively. A sequence consisting of amino

acid residues 50 to 54, a sequence consisting of amino acid residues 69 to 85, and a sequence consisting of amino acid residues 118 to 126 in the amino acid sequence of SEQ ID NO: 62 correspond to a CDRH1 sequence, a CDRH2 sequence, and a CDRH3 sequence, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 62 is shown in SEQ ID NO: 61 . A sequence consisting of nucleotides 1 to 57, a sequence consisting of nucleotides 58 to 411, and a sequence consisting of nucleotides 412 to 1389 in the nucleotide sequence of SEQ ID NO: 61 encode the signal sequence, the heavy chain variable region sequence, and the heavy chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 61 and the amino acid sequence of SEQ ID NO: 62 are also shown in Figures 37 and 38, respectively.

8)-3 Humanization of MAb1 light chain

8)-3-1 hMAb1-L1-type light chain:

A humanized MAb1 light chain designed by replacing amino acid No. 22 (alanine) with isoleucine, amino acid No. 27 (threonine) with serine, amino acid No. 34 (serine) with threonine, amino acid No. 37 (aspartic acid) with glutamic acid, amino acid No. 38 (glutamine) with proline, amino acid No. 62 (phenylalanine) with leucine, amino acid No. 84 (leucine) with proline, amino acid No. 108 (phenylalanine) with valine, amino acid No. 112 (phenylalanine) with tyrosine, amino acid No. 125 (glycine) with proline, amino acid No. 129 (leucine) with valine, amino acid No. 130 (glutamic acid) with aspartic acid, and amino acid No. 134 (alanine) with threonine, in the cMAb1 light chain represented by SEQ ID NO: 38 was designated as a "hMAb1-L1-type light chain".

The amino acid sequence of the hMAb1-L1-type light chain is shown in SEQ ID NO: 64. A sequence consisting of amino acid residues 1 to 20, a sequence consisting of amino acid residues 21 to 134, and a sequence consisting of amino acid residues 135

to 239 in the amino acid sequence of SEQ ID NO: 64 correspond to a signal sequence, a light chain variable region, and a light chain constant region, respectively. A sequence consisting of amino acid residues 44 to 59, a sequence consisting of amino acid residues 75 to 81, and a sequence consisting of amino acid residues 114 to 122 in the amino acid sequence of SEQ ID NO: 64 correspond to a CDRL1 sequence, a CDRL2 sequence, and a CDRL3 sequence, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 64 is shown in SEQ ID NO: 63 . A sequence consisting of nucleotides 1 to 60, a sequence consisting of nucleotides 61 to 402, and a sequence consisting of nucleotides 403 to 717 in the nucleotide sequence of SEQ ID NO: 63 encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 63 and the amino acid sequence of SEQ ID NO: 64 are also shown in Figures 39 and 40, respectively.

8)-3-2 hMAb1-L2-type light chain:

A humanized MAb1 light chain designed by replacing amino acid No. 22 (alanine) with isoleucine, amino acid No. 27 (threonine) with serine, amino acid No. 34 (serine) with threonine, amino acid No. 37 (aspartic acid) with glutamic acid, amino acid No. 38 (glutamine) with proline, amino acid No. 52 (serine) with glutamic acid, amino acid No. 54 (glycine) with lysine, amino acid No. 56 (threonine) with leucine, amino acid No. 62 (phenylalanine) with leucine, amino acid No. 84 (leucine) with proline, amino acid No. 108 (phenylalanine) with valine, amino acid No. 112 (phenylalanine) with tyrosine, amino acid No. 125 (glycine) with proline, amino acid No. 129 (leucine) with valine, amino acid No. 130 (glutamic acid) with aspartic acid, and amino acid No. 134 (alanine) with threonine, in the cMAb1 light chain represented by SEQ ID NO: 38 was designated as a "hMAb1-L2-type light chain".

The amino acid sequence of the hMAb1-L2-type light chain is shown in SEQ ID NO: 66 . A sequence consisting of amino acid residues 1 to 20, a sequence consisting of amino acid residues 21 to 134, and a sequence consisting of amino acid residues 135 to 239 in the amino acid sequence of SEQ ID NO: 66 correspond to a signal sequence, a light chain variable region, and a light chain constant region, respectively. A sequence consisting of amino acid residues 44 to 59, a sequence consisting of amino acid residues 75 to 81, and a sequence consisting of amino acid residues 114 to 122 in the amino acid sequence of SEQ ID NO: 66 correspond to a CDRL1 sequence, a CDRL2 sequence, and a CDRL3 sequence, respectively. A nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 66 is shown in SEQ ID NO: 65. A sequence consisting of nucleotides 1 to 60, a sequence consisting of nucleotides 61 to 402, and a sequence consisting of nucleotides 403 to 717 in the nucleotide sequence of SEQ ID NO: 65 encode the signal sequence, the light chain variable region sequence, and the light chain constant region sequence, respectively. The nucleotide sequence of SEQ ID NO: 65 and the amino acid sequence of SEQ ID NO: 66 are also shown in Figures 41 and 42, respectively.

(Example 9) Preparation of humanized MAb1

9)-1 Construction of humanized MAb1 heavy chain expression vector

9)-1-1 Construction of hMAb1-H1-type heavy chain expression vector

DNA comprising the gene encoding the hMAb1-H1-type heavy chain variable region represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 was synthesized (GeneArt artificial gene synthesis service) and cleaved with a restriction enzyme BlpI. The obtained DNA fragment was inserted to a restriction enzyme BlpI-cleaved site of the chimeric and humanized IgG2-type heavy chain expression vector (pCMA-G2) to construct a hMAb1-H1-type heavy chain expression vector. The obtained expression

vector was designated as "pCMA-G2/hMAb1-H1". The nucleotide sequence of the hMAb1-H1-type heavy chain is shown in SEQ ID NO: 55 .

9)-1-2 Construction of hMAb1-H2-type heavy chain expression vector

A hMAb1-H2-type heavy chain expression vector was constructed with the pCMA-G2/hMAb1-H1 constructed in 9)-1-1 as a template using a primer set shown below and QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Inc.). The obtained expression vector was designated as "pCMA-G1/hMAb1-H2". The nucleotide sequence of the hMAb1-H2-type heavy chain is shown in SEQ ID NO: 57 , and its amino acid sequence is shown in SEQ ID NO: 58.

Primer set:

H-N53Q-F: 5'-gggtggcaaccatcagccaaggcggcacctacacctac-3'

H-N53Q-R: 5'-gtaggtgtaggtgccgccttggctgatggttgccaccc-3'

9)-1-3 Construction of hMAb1-H3-type heavy chain expression vector

DNA comprising the gene encoding the hMAb1-H3-type heavy chain variable region represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 was synthesized (GeneArt artificial gene synthesis service) and cleaved with a restriction enzyme BlpI. The obtained DNA fragment was inserted to a restriction enzyme BlpI-cleaved site of the chimeric and humanized IgG2-type heavy chain expression vector (pCMA-G2) to construct a hMAb1-H3-type heavy chain expression vector. The obtained expression vector was designated as "pCMA-G2/hMAb1-H3". The nucleotide sequence of the hMAb1-H3-type heavy chain is shown in SEQ ID NO: 59 .

9)-1-4 Construction of hMAb1-H4-type heavy chain expression vector

A hMAb1-H4-type heavy chain expression vector was constructed with the pCMA-G2/hMAb1-H3 constructed in 9)-1-3 as a template using a primer set shown

below and QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Inc.). The obtained expression vector was designated as "pCMA-G1/hMAb1-H4". The nucleotide sequence of the hMAb1-H4-type heavy chain is shown in SEQ ID NO: 61 , and its amino acid sequence is shown in SEQ ID NO: 62.

Primer set:

H-N53Q-F: 5'-gggtggcaaccatcagccaaggcggcacctacacctac-3'

H-N53Q-R: 5'-gtaggtgtaggtgccgccttggctgatggttgccaccc-3'

9)-2 Construction of humanized MAb1 light chain expression vector

9)-2-1 Construction of hMAb1-L1-type light chain expression vector

DNA comprising the gene encoding the hMAb1-L1-type light chain variable region represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 was synthesized (GeneArt artificial gene synthesis service) and cleaved with a restriction enzyme BsiWI. The obtained DNA fragment was inserted to a restriction enzyme BsiWI-cleaved site of the general-purpose vector (pCMA-LK) for chimeric and humanized antibody light chain expression to construct a hMAb1-L1-type light chain expression vector. The obtained expression vector was designated as "pCMA-LK/hMAb1-L1". The nucleotide sequence of the hMAb1-L1-type light chain is shown in SEQ ID NO: 63 .

9)-2-2 Construction of hMAb1-L2-type light chain expression vector

DNA fragments were obtained by PCR with the pCMA-LK/hMAb1-L1 constructed in 9)-2-1 as a template using KOD-Plus- (TOYOBO CO., LTD.) and each of primer sets A and B, and linked by overlap extension PCR using primer set C to prepare DNA comprising a gene encoding the hMAb1-L2-type light chain. This DNA was cleaved with restriction enzymes XbaI and PmeI to obtain a DNA fragment, which was then inserted to a restriction enzyme XbaI/PmeI-cleaved site of the general-purpose vector (pCMA-LK) for chimeric and humanized antibody light chain expression to

construct a hMAb1-L2-type light chain expression vector. The obtained expression vector was designated as "pCMA-LK/hMAb1-L2". The nucleotide sequence of the hMAb1-L2-type light chain is shown in SEQ ID NO: 65 , and its amino acid sequence is shown in SEQ ID NO: 66.

Primer set A

L inf-F: 5'-gcctccggactctagagccaccatggtgctgcagacccaggtgttc-3'

L-EKL-R: 5'-caggtacaggttcttgttctcgttttccaggctctggctgcttctgcagc-3'

Primer set B

L-EKL-F: 5'-gaaaacgagaacaagaacctgtacctgaactggtatctgcagaagcccg-3'

L inf-R: 5'-tagcctcccccgtttaaacgggcccctaacactcccccctg-3'

Primer set C

L inf-F: 5'-gcctccggactctagagccaccatggtgctgcagacccaggtgttc-3'

L inf-R: 5'-tagcctcccccgtttaaacgggcccctaacactcccccctg-3'

(Example 10) Preparation of humanized MAb1

10-7-1) Production of humanized MAb1

FreeStyle 293-F cells (Invitrogen Corp.) were subcultured and cultured according to the manual.

1.2×10^9 cells of FreeStyle 293-F cells (Invitrogen Corp.) at the logarithmic growth phase were inoculated to 3L Fernbach Erlenmeyer Flask (Corning Inc.), diluted with FreeStyle 293 expression medium (Invitrogen Corp.) into 1.0×10^6 cells/mL, and then shake-cultured in an 8% CO_2 incubator at 90 rpm at 37°C for 1 hour. 3.6 mg of polyethyleneimine (Polysciences, Inc., #24765) was dissolved in 20 mL of Opti-Pro SFM medium (Invitrogen Corp.). Next, an H chain expression vector (0.4 mg) and an

L chain expression vector (0.8 mg) prepared using PureLink HiPure Plasmid kit (Invitrogen Corp.) were suspended in 20 mL of Opti-Pro SFM medium (Invitrogen Corp.). 20 mL of the expression vector/Opti-Pro SFM mixture solution was added to 20 mL of the polyethyleneimine/Opti-Pro SFM mixture solution, and the mixture was gradually stirred, further left for 5 minutes, and then added to the FreeStyle 293-F cells. The cells were shake-cultured in an 8% CO_2 incubator at 90 rpm at 37°C for 7 days, and the obtained culture supernatant was filtered through Disposable Capsule Filter (Advantec, #CCS-045-E1H), and purified in the same way as in 7)-2.

The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H1 and pCMA-LK/hMAb1-L1 was designated as "H-1040". The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H2 and pCMA-LK/hMAb1-L1 was designated as "H-1140". The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H2 and pCMA-LK/hMAb1-L2 was designated as "H-1143". The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H3 and pCMA-LK/hMAb1-L1 was designated as "H-2040". The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H4 and pCMA-LK/hMAb1-L1 was designated as "H-2140". The humanized MAb1 antibody obtained by the combination between pCMA-G2/hMAb1-H4 and pCMA-LK/hMAb1-L2 was designated as "H-2143".

(Example 11) Property of anti-ROBO4 humanized antibody

11)-1 Binding affinity

The dissociation constant between each antibody and rROBO4-ECD was determined with the antibody immobilized as a ligand and the antigen as an analyte using Biacore 3000 (manufactured by GE Healthcare Bio-Sciences Corp.). Approximately 80 RU of the antibody was bound via an anti-human IgG antibody

(manufactured by GE Healthcare Bio-Sciences Corp.) immobilized on a sensor chip CM5 (manufactured by GE Healthcare Bio-Sciences Corp.) by an amine coupling method. The running buffer used was PBS containing 0.05% Surfactant P20. Dilution series solutions (0.1-200 nM) of the antigen were added at a flow rate of 30 μL/min. for 300 seconds onto the antibody-bound chip. Subsequently, a dissociation phase was monitored for 300 seconds. 3 M $MgCl_2$ was added thereto as a regeneration solution at a flow rate of 10 μL/min. for 30 seconds. Data was analyzed using the 1:1 binding model of analysis software (BIAevaluation Software, version 4.1) to calculate an association rate constant kon, a dissociation rate constant koff, and a dissociation constant (K_D; K_D = koff/kon). As a result, the K_D value was 0.41 nM for H-1040, 3.5 nM for H-1143, 3.9 nM for H-1140, 0.40 nM for H-2040, 1.7 nM for H-2143, and 1.8 nM for H-2140.

11)-2 Activation of ROBO4 downstream signal

This test was conducted according to the method of 4)-1 except that: human IgG was used as a negative control; and Human IgG, H-1040, H-1140, H-1143, H-2040, H-2140, H-2143, or cMAb1-2 was added at a final concentration of 0.63 μg/mL to a medium. As a result, the negative control human IgG did not influence the IL-8 promoter activity in the cells transiently expressing human ROBO4, whereas all the anti-ROBO4 humanized antibodies increased the IL-8 promoter activity at a level equivalent to cMAb1-2 (Figure 49). These results demonstrated that all the anti-ROBO4 humanized antibodies activated the downstream signal of ROBO4, as in MAb1.

11)-3 HUVEC migration test

This test was conducted according to the method of 4)-2 except that: human IgG was used as a negative control; human IgG, H-1143, H-2140, or H-2143 was added at a final concentration of 0.5 μg/mL to a medium; and the fluorescence intensity (excitation

wavelength/fluorescent wavelength: 485 nm/538 nm) of each well was measured using a plate reader (EnVision: Perkin Elmer, Inc.). As a result, H-1143, H-2140, or H-2143 suppressed the migration of HUVEC induced by bFGF (Figure 50). Additionally, H-1140 showed the suppressive activity against HUVEC migration. These results demonstrated that H-1140, H-1143, H-2140, or H-2143 suppressed the migration of HUVEC.

11)-4 Cross-species reactivity

11)-4-1 Preparation of antigen gene-expressing cell

The cells were prepared according to the method of 4)-3-1.

11)-4-2 Flow cytometry analysis

This test was conducted according to the method of 4)-3-2 except that: human IgG was used as a negative control; FITC-AffiniPure Goat Anti-Human IgG, Fcγ Fragment Specific (manufactured by Jackson ImmunoResearch Laboratories, Inc.) was used as a secondary antibody; and a flow cytometer (FC500; manufactured by Beckman Coulter, Inc.) was used as a detector. As a result, H-1143, H-2140, or H-2143 did not bind to mouse ROBO4 or rat ROBO4, but was shown to bind to human ROBO4 and cynomolgus monkey ROBO4, as in the parent antibody MAb1 (Figures 51, 52, and 53). Additionally, H-1140 showed the same result as H-1143, H-2140 and H-2143.

11)-5 Binding specificity of H-1140, H-1143, H-2140, or H-2143

11)-5-1 Preparation of antigen gene-expressing cell

The cells were prepared according to the method of 4)-4-1 except that pCI-FLAG-hROBO4-28 was used as a human ROBO4 expression vector.

11)-5-2 Flow cytometry analysis

This test was conducted according to the method of 4)-4-2 except that: human IgG or mouse IgG2A was used as a negative control; Fluorescein-conjugated goat IgG fraction to mouse IgG (manufactured by Cappel Laboratories, Inc.) or FITC-AffiniPure Goat Anti-Human IgG, Fcγ Fragment Specific was used as a secondary antibody; and a flow cytometer (FC500) was used as a detector. As a result, H-1143, H-2140, or H-2143 did not bind to hROBO1, hROBO2, or hROBO3, but was shown to specifically bind to hROBO4, as in the parent antibody MAb1 (Figure 54). Additionally, H-1140 showed the same result as H-1143, H-2140 and H-2143. In this context, hROBO4, hROBO1, hROBO2, and hROBO3 were each confirmed to be expressed on the cell membrane using the positive control antibody (Figure 54).

11)-6 Drug efficacy evaluation in monkey models with laser-induced choroidal neovascularization

11)-6-1 Anesthesia

Medetomidine hydrochloride was intramuscularly injected at a dose of 0.08 mg/kg to each cynomolgus monkey. 15 minutes thereafter, ketamine hydrochloride was intramuscularly injected thereto at a dose of 15 mg/kg.

11)-6-2 Model preparation

Each cynomolgus monkey anesthetized in 11)-6-1 was retained on a stainless operating table in the supine position. 5 mg/mL tropicamide-5 mg/mL phenylephrine hydrochloride mixture eye drops were applied to the eyes for mydriasis. The macular region of the retina was thermally damaged by laser irradiation (quantity of heat irradiated: 500 mW, irradiation time: 0.1 seconds, spot size: 50 μm, the number of spots: 9 spots) using a green laser photocoagulator OcuLight GLx. After the operation, Cravit eye drops were added dropwise to the operated eyes.

11)-6-3 Administration of test substance

At day 7 after the model preparation, each cynomolgus monkey was anesthetized and then retained on a stainless operating table in the supine position. Then, PA IODO Ophthalmic and Eye washing Solution (Nitten Pharmaceutical Co., Ltd.) diluted 4-fold with sterile purified water was applied to the eyes to disinfect the external eyes. A 33G needle was inserted into the vitreous body from the conjunctiva, and saline or 0.05 mL of H-2143 adjusted to 1.1 mg/0.05 mL was injected thereto using a 1-mL syringe. After completion of the administration, Rinderon-A Ointment for Eye and Ear was applied to the conjunctiva using a swab and spread throughout the surfaces of the eyes by the opening and closing of the eyes.

11)-6-4 Drug efficacy evaluation

At days 7, 14, and 21 after the model preparation, the ocular fundus was photographed by a routine method using a hybrid fundus camera CX-1 under anesthesia. Then, fluorescein was intravenously injected thereto at a dose of 0.05 mL/Kg. After completion of the intravenous injection of fluorescein, fluorescent angiography was performed every 1 minute up to 10 minutes later, and image data was stored. From the image data, severity was classified at a scale of grades 1 to 5 based on the fluorescence intensity of each laser-irradiated site at which fluorescein accumulated according to the method of Zahn G et al. (Zahn G et al., Arch. Ophthalmol. 2009 127: 1329-1335). Then, the ratio of a site corresponding to grades 4 and 5 in the severity classification among all the laser-irradiated sites was calculated on the percentage basis to evaluate choroidal neovascularization. As a result of comparing choroidal neovascularization between the saline-administered group and the H-2143-administered group, the ratio of grades 4 and 5 increased in the saline-administered group with a lapse of time from the model preparation, whereas such increase was not observed in the H-2143-administered

group. This means that the administration of H-2143 suppressed laser-induced choroidal neovascularization (Figure 55).

[Industrial Applicability]

Use of an antibody provided by the present invention enables the treatment or prevention of an angiogenic disease such as exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity, and the examination or diagnosis of the angiogenic disease.

[Free Text of Sequence Listing]

SEQ ID NO: 1: Nucleotide sequence of full-length human ROBO4 cDNA (Figure 13)

SEQ ID NO: 2: Amino acid sequence of human ROBO4 (Figure 14)

SEQ ID NO: 3: Nucleotide sequence encoding the amino acid sequence of N-terminal FLAG-tagged full-length human ROBO4

SEQ ID NO: 4: Amino acid sequence of N-terminal FLAG-tagged full-length human ROBO4

SEQ ID NO: 5: Nucleotide sequence encoding the amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 46 to 1007 of SEQ ID NO: 2

SEQ ID NO: 6: Amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 46 to 1007 of SEQ ID NO: 2

SEQ ID NO: 7: Nucleotide sequence encoding the amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 132 to 1007 of SEQ ID NO: 2

SEQ ID NO: 8: Amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 132 to 1007 of SEQ ID NO: 2

SEQ ID NO: 9: Nucleotide sequence encoding the amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 210 to 1007 of SEQ ID NO: 2

SEQ ID NO: 10: Amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 210 to 1007 of SEQ ID NO: 2

SEQ ID NO: 11: Nucleotide sequence encoding the amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 225 to 1007 of SEQ ID NO: 2

SEQ ID NO: 12: Amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 225 to 1007 of SEQ ID NO: 2

SEQ ID NO: 13: Nucleotide sequence encoding the amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 341 to 1007 of SEQ ID NO: 2

SEQ ID NO: 14: Amino acid sequence of an N-terminal FLAG-tagged extracellular region/domain deletion variant of human ROBO4 consisting of an amino acid sequence of amino acid Nos. 341 to 1007 of SEQ ID NO: 2

SEQ ID NO: 15: Nucleotide sequence of mouse ROBO4 cDNA

SEQ ID NO: 16: Amino acid sequence of mouse ROBO4

SEQ ID NO: 17: Nucleotide sequence of rat ROBO4 cDNA

SEQ ID NO: 18: Amino acid sequence of rat ROBO4

SEQ ID NO: 19: Nucleotide sequence of monkey ROBO4 cDNA1

SEQ ID NO: 20: Monkey ROBO4 amino acid sequence 1

SEQ ID NO: 21: Nucleotide sequence of monkey ROBO4 cDNA2

SEQ ID NO: 22: Monkey ROBO4 amino acid sequence 2

SEQ ID NO: 23: Nucleotide sequence of human ROBO1 cDNA

SEQ ID NO: 24: Amino acid sequence of human ROBO1

SEQ ID NO: 25: Nucleotide sequence of human ROBO2 cDNA

SEQ ID NO: 26: Amino acid sequence of human ROBO2

SEQ ID NO: 27: Nucleotide sequence of human ROBO3 cDNA

SEQ ID NO: 28: Amino acid sequence of human ROBO4

SEQ ID NO: 29: Nucleotide sequence of an IL-8 promoter region

SEQ ID NO: 30: Nucleotide sequence of cDNA encoding MAb1 heavy chain variable region (Figure 15)

SEQ ID NO: 31: Amino acid sequence of MAb1 heavy chain variable region (Figure 16)

SEQ ID NO: 32: Nucleotide sequence of cDNA encoding MAb1 light chain variable region (Figure 17)

SEQ ID NO: 33: Amino acid sequence of MAb1 light chain variable region (Figure 18)

SEQ ID NO: 34: Nucleotide sequence comprising cDNA encoding the amino acids of human κ chain secretory signal and human κ chain constant region

SEQ ID NO: 35: Nucleotide sequence comprising cDNA encoding the amino acids of human heavy chain signal sequence and human IgG1 constant region

SEQ ID NO: 36: Nucleotide sequence comprising cDNA encoding the amino acids of human heavy chain signal sequence and human IgG2 constant region

SEQ ID NO: 37: Nucleotide sequence of cDNA encoding cMAb1 light chain (Figure 19)

SEQ ID NO: 38: Amino acid sequence of cMAb1 light chain (Figure 20)

SEQ ID NO: 39: Nucleotide sequence of cDNA encoding cMAb1-1 heavy chain (Figure 21)

SEQ ID NO: 40: Amino acid sequence of cMAb1-1 heavy chain (Figure 22)

SEQ ID NO: 41: Nucleotide sequence of cDNA encoding cMAb1-2 heavy chain (Figure 23)

SEQ ID NO: 42: Amino acid sequence of cMAb1-2 heavy chain (Figure 24)

SEQ ID NO: 43: Nucleotide sequence encoding the amino acid sequence of MAb1 heavy chain CDRH1

SEQ ID NO: 44: Amino acid sequence of MAb1 heavy chain CDRH1 (Figure 25)

SEQ ID NO: 45: Nucleotide sequence encoding the amino acid sequence of MAb1 heavy chain CDRH2

SEQ ID NO: 46: Amino acid sequence of MAb1 heavy chain CDRH2 (Figure 26)

SEQ ID NO: 47: Nucleotide sequence encoding the amino acid sequence of MAb1 heavy chain CDRH3

SEQ ID NO: 48: Amino acid sequence of MAb1 heavy chain CDRH3 (Figure 27)

SEQ ID NO: 49: Nucleotide sequence encoding the amino acid sequence of MAb1 light chain CDRL1

SEQ ID NO: 50: Amino acid sequence of MAb1 light chain CDRL1 (Figure 28)

SEQ ID NO: 51: Nucleotide sequence encoding the amino acid sequence of MAb1 light chain CDRL2

SEQ ID NO: 52: Amino acid sequence of MAb1 light chain CDRL2 (Figure 29)

SEQ ID NO: 53: Nucleotide sequence encoding the amino acid sequence of MAb1 light chain CDRL3

SEQ ID NO: 54: Amino acid sequence of MAb1 light chain CDRL3 (Figure 30)

SEQ ID NO: 55: Nucleotide sequence of cDNA encoding hMAb1-H1-type heavy chain (Figure 31)

SEQ ID NO: 56: Amino acid sequence of hMAb1-H1-type heavy chain (Figure 32)

SEQ ID NO: 57: Nucleotide sequence of cDNA encoding hMAb1-H2-type heavy chain (Figure 33)

SEQ ID NO: 58: Amino acid sequence of hMAb1-H2-type heavy chain (Figure 34)

SEQ ID NO: 59: Nucleotide sequence of cDNA encoding hMAb1-H3-type heavy chain (Figure 35)

SEQ ID NO: 60: Amino acid sequence of hMAb1-H3-type heavy chain (Figure 36)

SEQ ID NO: 61: Nucleotide sequence of cDNA encoding hMAb1-H4-type heavy chain (Figure 37)

SEQ ID NO: 62: Amino acid sequence of hMAb1-H4-type heavy chain (Figure 38)

SEQ ID NO: 63: Nucleotide sequence of cDNA encoding hMAb1-L1-type light chain (Figure 39)

SEQ ID NO: 64: Amino acid sequence of hMAb1-L1-type heavy chain (Figure 40)

SEQ ID NO: 65: Nucleotide sequence of cDNA encoding hMAb1-L2-type heavy chain (Figure 41)

SEQ ID NO: 66: Amino acid sequence of hMAb1-L2-type heavy chain (Figure 42)

SEQ ID NO: 67: CDRH1 of hMAb1-H2 or hMAb1-H4-type heavy chain (Figure 43)

SEQ ID NO: 68: CDRH2 of hMAb1-H2 or hMAb1-H4-type heavy chain (Figure 44)

SEQ ID NO: 69: CDRH3 of hMAb1-H2 or hMAb1-H4-type heavy chain (Figure 45)

SEQ ID NO: 70: CDRL1 of hMAb1-L2-type light chain (Figure 46)

SEQ ID NO: 71: CDRL2 of hMAb1-L2-type light chain (Figure 47)

SEQ ID NO: 72: CDRL3 of hMAb1-L2-type light chain (Figure 48)

[Document Name] Claims

[Claim 1]

An antibody having properties described in the following (I) to (III) or a functional fragment thereof:

(I) binding to the ROBO4 protein with a K_D value of 1×10^{-8} or lower;

(II) suppressing or inhibiting vascular endothelial cell migration in the absence of a cross-linking antibody *in vitro*; and

(III) suppressing or inhibiting angiogenesis *in vivo*.

[Claim 2]

The antibody or the functional fragment thereof according to claim 1, wherein the ROBO4 protein is the human ROBO4 protein.

[Claim 3]

The antibody or the functional fragment thereof according to claim 1, wherein the ROBO4 protein is a protein consisting of an amino acid sequence of amino acid Nos. 1 to 1007 of SEQ ID NO: 2 .

[Claim 4]

The antibody or the functional fragment thereof according to claim 1, wherein the ROBO4 protein is a protein consisting of an amino acid sequence of amino acid Nos. 46 to 1007 of SEQ ID NO: 2 .

[Claim 5]

The antibody or the functional fragment thereof according to claim 3 or 4, wherein the antibody or the functional fragment thereof binds to a site consisting of an amino acid sequence of amino acid Nos. 132 to 209 of SEQ ID NO: 2 .

[Claim 6]

The antibody or the functional fragment thereof according to claim 1, wherein the antibody or the functional fragment thereof is a monoclonal antibody or a functional fragment thereof.

[Claim 7]

The antibody or the functional fragment thereof according to any one of claims 1 to 6, wherein the antibody consists of a heavy chain comprising CDRH1 consisting of the amino acid sequence represented by SEQ ID NO: 44 (Figure 25) , CDRH2 consisting of the amino acid sequence represented by SEQ ID NO: 46 (Figure 26) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 46 by the substitution of one amino acid, and CDRH3 consisting of the amino acid sequence represented by SEQ ID NO: 48 (Figure 27) , and a light chain comprising CDRL1 consisting of the amino acid sequence represented by SEQ ID NO: 50 (Figure 28) or an amino acid sequence derived from the amino acid sequence of SEQ ID NO: 50 by the substitution of one to three amino acid(s), CDRL2 consisting of the amino acid sequence represented by SEQ ID NO: 52 (Figure 29) , and CDRL3 consisting of the amino acid sequence represented by SEQ ID NO: 54 (Figure 30) .

[Claim 8]

The antibody or the functional fragment thereof according to any one of claims 1 to 7, wherein the antibody consists of a heavy chain comprising CDRH1 consisting of the amino acid sequence represented by SEQ ID NO: 44 (Figure 25) , CDRH2 consisting of the amino acid sequence represented by SEQ ID NO: 46 (Figure 26) or the amino acid sequence represented by SEQ ID NO: 68 (Figure 44) , and CDRH3 consisting of the amino acid sequence represented by SEQ ID NO: 48 (Figure 27) , and a light chain comprising CDRL1 consisting of the amino acid sequence represented by SEQ ID NO: 50 (Figure 28) or the amino acid sequence represented by SEQ ID NO: 70 (Figure 46) , CDRL2 consisting of the amino acid sequence represented by SEQ ID NO: 52 (Figure 29) , and CDRL3 consisting of the amino acid sequence represented by SEQ ID NO: 54 (Figure 30) .

[Claim 9]

The antibody or the functional fragment thereof according to any one of claims 1 to 7, wherein the antibody comprises a heavy chain variable region consisting of the amino acid sequence represented by SEQ ID NO: 31 (Figure 16), and a light chain variable region consisting of the amino acid sequence represented by SEQ ID NO: 33 (Figure 18).

[Claim 10]

The antibody or the functional fragment thereof according to any one of claims 1 to 8, wherein the antibody comprises any one heavy chain variable region selected from the following a) to d) and a light chain variable region selected from e) and f):

a) a heavy chain (hMAb1-H1-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32) ,

b) a heavy chain (hMAb1-H2-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) ,

c) a heavy chain (hMAb1-H3-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) , and

d) a heavy chain (hMAb1-H4-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) ; and

e) a light chain (hMAb1-L1-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) , and

f) a light chain (hMAb1-L2-type) variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) .

[Claim 11]

The antibody or the functional fragment thereof according to any one of claims 1 to 8, wherein the antibody comprises any one of the following combinations 1) to 6) of a heavy chain variable region and a light chain variable region:

1) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light

chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64(Figure 40) ,

2) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 58 (Figure 34) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) ,

3) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 66 (Figure 42) .

4) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 62 (Figure 38) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ,

5) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 56 (Figure 32) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) , and

6) a heavy chain variable region consisting of an amino acid sequence represented by amino acid Nos. 20 to 137 of SEQ ID NO: 60 (Figure 36) and a light chain variable region consisting of an amino acid sequence represented by amino acid Nos. 21 to 134 of SEQ ID NO: 64 (Figure 40) ,

[Claim 12]

The antibody or the functional fragment thereof according to any one of claims 1 to 11, wherein the antibody or the functional fragment thereof is a chimeric antibody or a functional fragment thereof.

[Claim 13]

The antibody or the functional fragment thereof according to any one of claims 1 to 11, wherein the antibody or the functional fragment thereof is a humanized antibody or a functional fragment thereof.

[Claim 14]

The antibody or the functional fragment thereof according to any one of claims 1 to 13, wherein the antibody comprises a human IgG1 or human IgG2 heavy chain constant region.

[Claim 15]

The antibody or the functional fragment thereof according to any one of claims 1 to 6, wherein the antibody or the functional fragment thereof binds to a site on an antigen recognized by the antibody according to claims 7 to 14.

[Claim 16]

The antibody or the functional fragment thereof according to any one of claims 1 to 6, wherein the antibody or the functional fragment thereof competes with any one antibody according to claims 7 to 14 for the binding to ROBO4 protein.

[Claim 17]

The antibody according to any one of claims 1 to 8, wherein the antibody comprises any one of the following combinations 1) to 6) of a heavy chain and a light chain:

1) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-1140),

2) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 58 (Figure 34) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) (H-1143),

3) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 66 (Figure 42) (H-2143).

4) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 62 (Figure 38) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-2140),

5) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 56 (Figure 32) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-1040), and

6) a heavy chain consisting of an amino acid sequence represented by amino acid Nos. 20 to 463 of SEQ ID NO: 60 (Figure 36) and a light chain consisting of an amino acid sequence represented by amino acid Nos. 21 to 239 of SEQ ID NO: 64 (Figure 40) (H-2040).

[Claim 18]

An antibody comprising a modified form of a heavy chain of any one antibody according to claim 17, wherein said modified form lacks one to several carboxyl-terminal amino acid(s) of said heavy chain.

[Claim 19]

The antibody according to any one of claims 1 to 6, wherein the antibody is 95% or more identical in the amino acid sequence to any one antibody according to claim 17; has a K_D value of 1×10^{-8} or lower for a human ROBO4; suppresses or inhibits vascular endothelial cell migration in the absence of a cross-linking antibody *in vitro*; and suppresses or inhibits angiogenesis *in vivo*.

[Claim 20]

The antibody or the functional fragment thereof according to claim 15, wherein the antibody or the functional fragment thereof is a human antibody or a functional fragment thereof.

[Claim 21]

A nucleotide sequence selected from the group consisting of the following (I) to (III):

(I) a nucleotide sequence comprising a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of claims 1 to 20,

(II) a nucleotide sequence consisting of a nucleotide sequence comprising a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of claims 1 to 20, and

(III) a nucleotide sequence consisting of a nucleotide sequence encoding the partial or whole amino acid sequence of the heavy chain or light chain of an antibody according to any one of claims 1 to 20.

[Claim 22]

A recombinant vector containing an insert of a nucleotide sequence according to claim 21.

[Claim 23]

A recombinant cell containing a nucleotide sequence according to claim 21 or a recombinant vector according to claim 22 introduced therein.

[Claim 24]

A cell producing an antibody according to any one of claims 1 to 20.

[Claim 25]

A method for producing an antibody or a functional fragment thereof according to any one of claims 1 to 20, comprising the following steps (I) and (II):

(I) culturing a cell according to claim 23 or 24; and

(II) collecting the antibody or the functional fragment thereof according to any one of claims 1 to 20 from the culture obtained in the step (I).

[Claim 26]

An antibody or a functional fragment thereof produced by a production method according to claim 25.

[Claim 27]

A modified form of an antibody or a functional fragment thereof according to any one of claims 1 to 20 and 26.

[Claim 28]

A pharmaceutical composition comprising an antibody or a functional fragment thereof according to any one of claims 1 to 20 and 26 or a modified form according to claim 27 as an active ingredient.

[Claim 29]

The pharmaceutical composition according to claim 28, wherein the pharmaceutical composition is for use in treating or preventing an angiogenic disease.

[Claim 30]

The pharmaceutical composition for the use according to claim 29, wherein the angiogenic disease is exudative age-related macular degeneration, diabetic retinopathy, macular edema, benign or malignant tumor, atherosclerosis, retrolental fibroplasia, angioma, chronic inflammation, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, immune rejection of a corneal tissue transplant or other tissue transplants, rheumatoid arthritis, psoriasis, acute inflammation, sepsis, or obesity.

[Claim 31]

The pharmaceutical composition for the use according to claim 29, wherein the angiogenic disease is exudative age-related macular degeneration, diabetic retinopathy,

macular edema, retrolental fibroplasia, ocular neovascular disease, proliferative retinopathy, neovascular glaucoma, or immune rejection of a corneal tissue transplant.

[Claim 32]

An angiogenesis inhibitor comprising an antibody or a functional fragment thereof according to any one of claims 1 to 20 and 26 or a modified form according to claim 27 as an active ingredient.

[Claim 33]

A method for treating or preventing an angiogenic disease comprising administering to a subject in need thereof an effective amont of an antibody or a functional fragment thereof according to any one of claims 1 to 20 and 26 or a modified form according to claim 27, or the composition according to any one of claims 28 to 31.

[Claim 34]

The pharmaceutical composition according to any one of claims 28 to 31, wherein said composition is used in combination with a further therapeutic or prophylactic agent.