

ANTI-EPHA4 ANTIBODY

FIELD OF THE INVENTION

[0001]

The present invention relates to an antibody binding to EphA4.

BACKGROUND OF THE INVENTION

[0002]

EphA4 is a member of the receptor tyrosine kinase family. Ephrin type A and type B are known as ligands of EphA4. Upon binding of EphA4 to its ligand ephrin, deadhesion signals are induced. EphA4 is expressed in motor neurons and regulates correct axonal guidance through ephrin expressed in non-projective regions of the motor neurons in the spinal cord during a neural network formation stage.

[0003]

Previous studies suggest that the functional inhibition of EphA4 is an effective therapeutic procedure for neurodegenerative diseases such as amyotrophic lateral sclerosis (hereinafter, also referred to as "ALS") and Alzheimer's disease, and spinal cord injury.

[0004]

The EphA4 gene has been reported to adjust the phenotype of ALS (Patent Literature 1; and Non-Patent Literature 1). Genetic defect of EphA4 or antagonism by EphA4-Fc or the like has been found to promote axonal elongation or functional recovery at the time of spinal cord injury in mice or rats (Non-Patent Literature 2; and Non-Patent Literature 3).

KYL peptide and compound 1 are known as existing EphA4 signaling inhibitors (Patent Literature 1; Non-Patent Literature 1; and Non-Patent Literature 2). However, there has been no report on an antibody having neutralizing activity.

PRIOR ART

[0005]

Patent Literature 1: WO2012/156351 A1

[0006]

Non-Patent Literature 1: Van Hoecke et al., Nature Medicine, vol. 18: 1418-1422, 2012

Non-Patent Literature 2: Goldschmit et al., PLoS one, vol. 6: e24636, 2011

Non-Patent Literature 3: Spanevello et al., Journal of Neurotrauma, vol. 30: 1023-1034, 2013

SUMMARY OF INVENTION

[0007]

An object of the present invention is to provide an anti-EphA4 antibody or an EphA4-binding fragment thereof which is capable of binding to EphA4 and inhibiting the binding between EphA4 and its ligand, and a pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof as an active ingredient.

[0008]

The present inventors have conducted diligent studies to attain the object and consequently completed the present invention by obtaining an anti-EphA4 antibody capable of binding to EphA4 and inhibiting the binding between EphA4 and its ligand.

Specifically, in one embodiment, the present invention relates to the following inventions.

[0009]

(1) An anti-EphA4 antibody or an EphA4-binding fragment thereof, comprising

(a) CDR-H1 comprising the amino acid sequence represented by SEQ ID NO: 26 or SEQ ID NO: 27;

(b) CDR-H2 comprising the amino acid sequence represented by SEQ ID NO: 28 or SEQ ID NO: 29;

(c) CDR-H3 comprising the amino acid sequence represented by SEQ ID NO:

30;

(d) CDR-L1 comprising the amino acid sequence represented by SEQ ID NO: 31;

(e) CDR-L2 comprising the amino acid sequence represented by SEQ ID NO: 32;

and

(f) CDR-L3 comprising the amino acid sequence represented by SEQ ID NO: 33.

[0010]

(2) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (1), wherein

the antibody or the EphA4-binding fragment thereof is humanized.

[0011]

(3) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (1) or (2), wherein

the antibody or the EphA4-binding fragment thereof specifically binds to EphA4 and inhibits the binding between EphA4 and ephrin.

[0012]

(4) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of (1) to (3), wherein

the antibody or the EphA4-binding fragment thereof comprises a heavy chain and a light chain, and

the constant region of the heavy chain and the constant region of the light chain each comprise a human antibody-derived sequence.

[0013]

(5) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (4), wherein

the constant region of the heavy chain is derived from human IgG.

[0014]

(6) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (5), wherein

the human IgG is human IgG₁ or human IgG₂.

[0015]

(7) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of (4) to (6), wherein

the constant region of the light chain is derived from human Igk.

[0016]

(8) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of (1) to (7), wherein

the EphA4-binding fragment is selected from the group consisting of Fab, Fab', F(ab')₂, and Fv.

[0017]

(9) The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (8), wherein

the EphA4-binding fragment is F(ab')₂.

[0018]

(10) A pharmaceutical composition comprising an anti-EphA4 antibody or an EphA4-binding fragment thereof according to any one of (1) to (9).

[0019]

(11) The pharmaceutical composition according to (10) further comprising a pharmaceutically acceptable carrier.

[0020]

(12) The pharmaceutical composition according to (10) or (11), wherein the pharmaceutical composition is used for the treatment of amyotrophic lateral sclerosis (ALS).

[0021]

In another embodiment, the present invention also relates to the following inventions.

(1') An anti-EphA4 antibody or an EphA4-binding fragment thereof, comprising a heavy chain and a light chain, wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, 68, 70, 72, 74 or 76, or an amino acid sequence derived from said sequence by substitution, addition, and/or deletion of one or more amino acids,

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78, 80, 82 or 84, or an amino acid sequence derived from said sequence by the substitution, addition, and/or deletion of one or more amino acids, and

the anti-EphA4 antibody or the EphA4-binding fragment thereof specifically binds to EphA4 and inhibits the binding between EphA4 and ephrin.

[0022]

(2') An anti-EphA4 antibody or an EphA4-binding fragment thereof, comprising a heavy chain and a light chain, wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, 68, 70, 72, 74 or 76, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78, 80, 82 or 84.

[0023]

(3') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78.

[0024]

(4') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 68, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78.

[0025]

(5') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 70, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78.

[0026]

(6') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 72, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78.

[0027]

(7') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 74, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78.

[0028]

(8') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 76, and

a variable region of the light chain comprises the amino acid sequence

represented by SEQ ID NO: 78.

[0029]

(9') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0030]

(10') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 68, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0031]

(11') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 70, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0032]

(12') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 72, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0033]

(13') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 74, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0034]

(14') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 76, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 80.

[0035]

(15') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0036]

(16') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 68, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0037]

(17') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 70, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0038]

(18') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 72, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0039]

(19') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 74, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0040]

(20') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 76, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 82.

[0041]

(21') An anti-EphA4 antibody or an EphA4-binding fragment thereof

according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0042]

(22') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 68, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0043]

(23') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 70, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0044]

(24') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 72, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0045]

(25') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 74, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0046]

(26') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to (1') or (2'), wherein

a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 76, and

a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 84.

[0047]

(27') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to any one of (1') to (26'), wherein

the antibody or the EphA4-binding fragment thereof specifically binds to EphA4 and inhibits the binding between EphA4 and ephrin.

[0048]

(28') An anti-EphA4 antibody or an EphA4-binding fragment thereof according to any one of (1') to (27'), wherein

the constant region of the heavy chain and the constant region of the light chain each comprise a human antibody-derived sequence.

[0049]

(29') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (28'), wherein

the constant region of the heavy chain is derived from human IgG.

[0050]

(30') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (29'), wherein

the human IgG is human IgG consisting of human IgG₂ or a combination of

human IgG₁ and human IgG₂.

[0051]

(31') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (30'), wherein

the human IgG is human IgG₂.

[0052]

(32') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (31'), wherein

the human IgG₂ has a C131S, C219S, V234A and/or G237A mutation under Eu numbering, and does not have a lysine residue at the carboxy terminal.

[0053]

(33') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (32'), wherein

the human IgG₂ comprises the amino acid sequence represented by SEQ ID NO: 62.

[0054]

(34') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (30'), wherein

the human IgG is human IgG consisting of a combination of human IgG₁ and human IgG₂.

[0055]

(35') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (34'), wherein

in the human IgG consisting of a combination of human IgG₁ and human IgG₂, a CH1 region and a hinge region are human IgG₁, and a CH2 region and a CH3 region are human IgG₂.

[0056]

(36') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (35'), wherein

the human IgG consisting of a combination of human IgG₁ and human IgG₂ has a V234A and/or a G237A mutation under Eu numbering, and does not have a lysine residue at the carboxy terminal.

[0057]

(37') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (36'), wherein

the human IgG consisting of a combination of human IgG₁ and human IgG₂ comprises the amino acid sequence represented by SEQ ID NO: 60.

[0058]

(38') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of (28') to (37'), wherein

the constant region of the light chain is derived from human Igk.

[0059]

(39') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of (1') to (38'), wherein

the EphA4-binding fragment is selected from the group consisting of Fab, Fab', F(ab')₂, and Fv.

[0060]

(40') The anti-EphA4 antibody or the EphA4-binding fragment thereof according to (39'), wherein

the EphA4-binding fragment is F(ab')₂.

[0061]

(41') A pharmaceutical composition comprising an anti-EphA4 antibody or an EphA4-binding fragment thereof according to any one of (1') to (40').

[0062]

(42') The pharmaceutical composition according to (41') further comprising a pharmaceutically acceptable carrier.

[0063]

(43') The pharmaceutical composition according to (41') or (42'), wherein the pharmaceutical composition is used for the treatment of amyotrophic lateral sclerosis (ALS).

[0064]

One of or any combination of two or more of the aspects of the present invention mentioned above is also included in the scope of the present invention.

[0065]

The present invention provides an anti-EphA4 antibody or an EphA4-binding fragment thereof which is capable of binding to EphA4 and inhibiting the binding between EphA4 and its ligand, and a pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof as an active ingredient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0066]

FIG. 1 shows the binding affinity of an anti-EphA4 monoclonal antibody (antibody A) for human EphA4 and mouse EphA4.

FIG. 2 shows inhibition of the binding of mouse EphA4 to mouse Ephrin A1 and mouse Ephrin B2 by the anti-EphA4 monoclonal antibody (antibody A), KYL peptide, and compound 1.

FIG. 3 shows inhibition of the binding of human EphA4 to human Ephrin A5 and human Ephrin B3 by the anti-EphA4 monoclonal antibody (antibody A), and KYL peptide.

FIG. 4A shows the binding affinity of antibody A-IgG (antibody A) and antibody A-Fab for mouse EphA4.

FIG. 4B shows the binding affinity of antibody A-IgG (antibody A) and antibody A-F(ab')₂ for mouse EphA4.

FIG. 4C shows the binding affinity of antibody A-IgG (antibody A) and antibody A-Fab for human EphA4.

FIG. 4D shows the binding affinity of antibody A-IgG (antibody A) and antibody

A-F(ab')₂ for human EphA4.

FIG. 5 shows the inhibition of the binding between mouse EphA4 and mouse Ephrin B2 by antibody A-IgG (antibody A), antibody A-F(ab')₂, antibody A-Fab, and KYL peptide.

FIG. 6 shows the binding specificity of antibody A for human Eph receptor (FIG.6A) and mouse Eph receptor (FIG.6B).

FIG. 7 shows the binding activity of antibody A against mouse, rat, monkey, and human EphA4.

FIG. 8 shows that antibody A suppresses, in a concentration-dependent manner, EphA4 autophosphorylation induced by Ephrin A1 in hippocampal neurons. The pY in FIG.8 exhibit phosphorylated EphA4.

FIG. 9 shows that antibody A suppresses, in a concentration-dependent manner, growth cone collapse induced by Ephrin A1 in hippocampal neurons.

FIG. 10 shows that antibody A suppresses EphA4 autophosphorylation induced by Ephrin A1 in the mouse newborn brain. The pY in FIG.10 exhibit phosphorylated EphA4.

FIG. 11 shows a schematic view of an evaluation system carried out in Example 13.

FIG. 12 shows that antibody A protects motor neurons in *in vitro* ALS models using mouse ES cells.

FIG. 13 shows a schematic view of an evaluation system carried out in Example 14.

FIG. 14 shows that antibody A protects motor neurons in *in vitro* ALS models using human iPS cells.

FIG. 15 shows the amino acids of EphA4 Ligand-Binding Domain (EphA4-LBD) on the abscissa and the structural region of Fab on the ordinate. The black bits depict the points of intersection of combinations having an interaction. A plurality of bits presenting for one amino acid correspond to the types of the interaction (hydrogen bond, surface contact, etc.). An amino acid having a larger number of bits means that the

amino acid binds to Fab with diverse interactions.

FIG. 16 shows the surface structure of EphA4 Ligand-Binding Domain (EphA4-LBD). In FIG. 16, the dark color regions correspond to Fab-binding regions. In this figure, the names and the residue numbers of amino acids contained in the binding regions are shown at the corresponding positions, and the H chain and L chain CDRs of Fab to be bound are indicated by ribbon models.

DETAILED DESCRIPTION OF THE INVENTION

[0067]

The present invention relates to an anti-EphA4 antibody which binds to EphA4.

The anti-EphA4 antibody used in the present invention is an antibody that can recognize and bind to EphA4. As mentioned below, the antibody may be an intact antibody or may be an antigen-binding fragment thereof or a synthetic antibody (e.g., a recombinant antibody, a chimeric antibody, and a humanized antibody) as long as it has binding affinity for EphA4. In the present invention, it can be understood that EphA4 refers to human-, mouse-, rat-, or monkey-derived EphA4. The human-, mouse-, rat-, or monkey-derived EphA4 can be obtained from a public database in which sequence information is registered, such as GenBank provided by National Center for Biotechnology Information (USA). Alternatively, primers are designed on the basis of nucleotide sequence information on EphA4 of an animal species closely related thereto, and sequence information on the EphA4 gene can be obtained by cloning from RNA extracted from the desired animal species. For example, nucleotide sequence information on human, mouse, rat, or monkey EphA4 is registered under GenBank Accession Nos. NM_004438.4, NM_007936.3, NM_001162411.1, and NM_001260870, respectively, on the database.

[0068]

In one aspect of the present invention, EphA4 comprises the amino acid sequence represented by SEQ ID NO: 1 or an amino acid sequence derived from the amino acid sequence by the substitution, addition, and/or deletion of one or more amino

acids, or the amino acid sequence represented by SEQ ID NO: 3 or an amino acid sequence derived from the amino acid sequence by the substitution, addition, and/or deletion of one or more amino acids. In the present invention, the term "or more" used as to EphA4 is not limited as long as the resulting sequence maintains functional characteristics equivalent to the original sequence. The term "or more" is 2 to 100, for example, 2 to 90, 2 to 80, 2 to 70, 2 to 60, 2 to 50, 2 to 40, 2 to 30, 2 to 20, 2 to 10, or 2 to 5 or is within 10%, for example, within 9%, within 8%, within 7%, within 6%, or within 5% of the number of amino acids in the amino acid sequence.

[0069]

In one aspect of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof is an antibody specifically binding to EphA4. The term "specific binding" is a term well known to those skilled in the art, and a method for determining the specific binding of an antibody or an antigen-binding fragment thereof to an antigen or an epitope is also well known. In one embodiment of the present invention, it is understood that the "specific binding" means that the anti-EphA4 antibody or the EphA4-binding fragment thereof is capable of binding to EphA4 through immunological reaction more rapidly and/or for a duration of a longer time with larger binding affinity and larger binding activity as compared with its binding to other target molecules. In this context, the specific binding to other targets of an antibody or an antigen-binding fragment thereof specifically binding to one target is not excluded. In another embodiment of the present invention, the "specific binding" can be indicated by an antibody having KD of at least approximately 10^{-7} M, at least approximately 10^{-8} M, at least approximately 10^{-9} M, at least approximately 10^{-10} M, at least approximately 10^{-11} M, or at least approximately 10^{-12} M or greater for EphA4. In a further alternative embodiment of the present invention, it is understood that the "specific binding" is binding to EphA4 through immunological reaction, but not substantially binding to other subclasses and subtypes of Eph receptors.

[0070]

In one aspect of the present invention, the anti-EphA4 antibody or the EphA4-

binding fragment thereof of the present invention is an antibody binding to the extracellular region of EphA4. The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be, for example, an antibody or an antigen-binding fragment that comprises the amino acid sequence represented by SEQ ID NO: 2 or an amino acid sequence derived from the amino acid sequence by the substitution, addition, and/or deletion of one or more amino acids, or comprises the amino acid sequence represented by SEQ ID NO: 4 or an amino acid sequence derived from the amino acid sequence by the substitution, addition, and/or deletion of one or more amino acids, and binds to any site in the EphA4 extracellular region. In the present invention, the term "or more" used as to the extracellular region of EphA4 is, but is not limited to, 2 to 50, for example, 2 to 45, 2 to 40, 2 to 35, 2 to 30, 2 to 25, 2 to 20, 2 to 15, 2 to 10, or 2 to 5, or within 10%, for example, within 9%, within 8%, within 7%, within 6%, or within 5% of the number of amino acids in the amino acid sequence.

[0071]

In one aspect of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof can specifically bind to EphA4 and inhibit the binding between EphA4 and ephrin.

[0072]

In one embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof can specifically bind to at least one of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding thereof to their ligands. In a preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof can specifically bind to two or more of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding thereof to their ligands. In another preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof can specifically bind to all of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding thereof to their ligands.

[0073]

A method generally known to those skilled in the art can be used as a method for measuring the antigen-binding properties (e.g., binding affinity and interspecies cross-reactivity) of the antibody or the antigen-binding fragment thereof. For example, the binding affinity can be measured by use of Biacore(R) biosensor, KinExA biosensor, scintillation proximity assay, ELISA, ORIGEN immunoassay (IGEN International), flow cytometry, fluorescence quenching, fluorescence transfer, yeast display, and/or immunostaining, though the method is not limited thereto. The neutralizing activity of the antibody or the antigen-binding fragment thereof against the binding between EphA4 and its ligand can be measured by use of Biacore(R) biosensor, ELISA, and/or flow cytometry, though the method is not limited thereto.

[0074]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be any of a monoclonal antibody, a polyclonal antibody, and an EphA4-binding fragment thereof as long as it binds to EphA4, preferably, specifically binds to EphA4.

[0075]

In the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be of any class such as IgG, IgA or IgM (or subclass thereof) and is not limited by a particular class. Immunoglobulins are classified into different classes depending on the antibody amino acid sequences of their heavy chain (also called H chain) constant regions. There are five main immunoglobulin classes: IgA, IgD, IgE, IgG, and IgM, some of which can be further divided into subclasses (isotypes) of, for example, IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant regions corresponding to different classes of immunoglobulins are respectively called α , δ , ϵ , γ , and μ . The light chain (also called L chain) types of antibodies are λ and κ chains.

[0076]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be an IgG antibody and may be, for example, an IgG₁ antibody or an IgG₂

antibody. Also, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be a monomer, a dimer, or a multimer in some cases.

[0077]

In one aspect of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be a combination of IgG antibodies derived from different subclasses, such as IgG antibody consisting of a combination of IgG₁ antibody and IgG₂ antibody.

[0078]

In the present specification, the antigen-binding fragment of the antibody is not particularly limited as long as the antigen-binding fragment is a functional and structural fragment of the antibody and maintains binding activity against the antigen to which the antibody can bind. Examples of the antigen-binding fragment of the antibody include, but are not limited to, Fab, Fab', F(ab')₂, Fv, and single-chain Fv (scFv), their variants, fusion proteins comprising an antibody moiety, and other modified structures of immunoglobulin molecules comprising an antigen recognition site. In one aspect, the binding fragment of the antibody of the present invention is F(ab')₂.

[0079]

The antigen-binding fragment of the antibody can be obtained, for example, via the protein digestion of the whole antibody with a protease such as papain or pepsin, or may be produced directly by recombinant host cells (e.g., eukaryotes such as yeast cells, plant cells, insect cells, or mammalian cells, or prokaryotes such as *E. coli*). For example, Fab'-SH fragments can be recovered directly from *E. coli* and chemically bonded to form a F(ab')₂ fragment. Alternatively, F(ab')₂ may be formed using leucine zipper GCN4, which promotes the assembly of F(ab')₂ molecules. In the case of producing scFv by a chemical synthesis technique, an automatic synthesizer can be used. In the case of producing scFv by a gene recombination technique, an appropriate plasmid containing a polynucleotide encoding scFv can be transferred to appropriate host cells (e.g., eukaryotes such as yeast cells, plant cells, insect cells, or mammalian cells, or prokaryotes such as *E. coli*). The polynucleotide encoding scFv of interest may be

prepared by a well known operation such as polynucleotide ligation. The resulting scFv may be isolated by use of a standard protein purification technique known in the art.

[0080]

In the present invention, the variable region of the antibody may mean a variable region of an antibody light chain and/or a variable region of an antibody heavy chain, and the constant region of the antibody may mean a constant region of an antibody light chain and/or a constant region of an antibody heavy chain. The heavy chain variable region and the light chain variable region are each composed of four framework regions (FRs) connected via three CDRs also known as hypervariable regions. The CDRs in each chain are held in close proximity by FRs and contribute, together with CDRs in the other chain, to the formation of the antigen-binding site of the antibody. Examples of techniques for determining CDRs include, but are not limited to: (1) an approach based on cross-species sequence variability (e.g., Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th ed., 1991, National Institutes of Health, Bethesda MD); and (2) an approach based on the crystallographic study of an antigen-antibody complex (Al-lazikani et al., 1997 *J. Molec. Biol.* 273: 927-948). These approaches or other approaches may be used in combination. The constant region of the heavy chain is composed of three domains, i.e., CH1, CH2 and CH3, and a hinge region, and they are positioned from the amino terminus (N-terminus) to carboxy terminus (C-terminus) in order of CH1, a hinge region, CH2 and CH3. The constant region of the light chain is composed of one domain CL.

[0081]

In the present invention, the monoclonal antibody may mean an antibody that is obtained from a population of substantially homogeneous antibodies. Specifically, individual antibodies contained in the population are identical except for natural mutants that might be present to some extent. The monoclonal antibody is directed to a single antigen site and is very specific. Moreover, in contrast to a typical polyclonal antibody targeting different antigens or different epitopes, each monoclonal antibody targets a single epitope in an antigen. The modifier "monoclonal" denotes the

characteristics of the antibody that is obtained from a population of substantially homogeneous antibodies, and should not be restrictively interpreted as requiring the production of the antibody by a particular method.

[0082]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be a chimeric antibody, a humanized antibody, a human antibody, a nonhuman mammal (e.g., monkey, mouse, rat, rabbit, bovine, horse, or goat) antibody, or an EphA4-binding fragment thereof. The chimeric antibody is, for example, an antibody comprising the variable regions of a nonhuman (e.g., mouse or rat) antibody joined to the constant regions of a human antibody, and may refer to, for example, an antibody having nonhuman antibody-derived variable regions and human antibody-derived constant regions. The humanized antibody is, for example, an antibody comprising the hypervariable regions (also referred to as complementarity-determining regions (CDRs)) of a nonhuman antibody introduced in a human antibody, and may refer to, for example, an antibody having nonhuman antibody-derived CDRs and the other antibody regions derived from a human antibody. However, in the present invention, the distinction between the chimeric antibody and the humanized antibody is not necessarily required to be clear, and the antibody may be in a form that may be regarded as both of the chimeric antibody and the humanized antibody. A preferred aspect of the humanized antibody according to the present invention is an antibody having rodent antibody-derived CDRs and the other antibody regions derived from a human antibody, particularly preferably an antibody having mouse antibody-derived CDRs and the other antibody regions derived from a human antibody. The humanization can be performed by use of a CDR grafting method (Kontermann and Dubel, *Antibody Engineering*, Springer Lab Manual (2001); and Tsurushita et al., *Methods* 36: 69-83 (2005)) and can also be performed by a method known in the art (see e.g., Jones et al., *Nature* 321: 522-525 (1986); Riechmann et al., *Nature* 332: 323-327 (1988); and Verhoeyen et al., *Science* 239: 1534-1536 (1988)) which involves replacing CDR sequences for the corresponding sequences of a human antibody. The humanized antibody is typically a human

antibody, some CDR residues and, optionally, some FR residues of which are replaced with residues derived from the analogous sites of a nonhuman antibody.

[0083]

For reducing antigenicity, it can be important to select the use of human variable regions in both of the light chain and the heavy chain in the preparation of the humanized antibody. According to a "best-fit" method, the whole library of known human FR sequences is screened for the sequences of variable regions of a rodent antibody. Next, human sequences most similar to the rodent sequences are accepted as human FRs of the humanized antibody. See, for example, Sims et al., *J. Immunol.* 151: 2296-2308 (1993) and Chothia et al., *J. Mol. Biol.* 196: 901-917 (1987). In another method, particular frameworks derived from common sequences of all human antibodies as to particular light chain or heavy chain subgroups are used. The same frameworks can be used for some different humanized antibodies. See, for example, Carter et al., *Proc. Natl. Acad. Set USA* 89: 4285-4289 (1992) and Presta et al., *J. Immunol.* 151: 2623-2632 (1993).

[0084]

Moreover, it is generally desirable that the humanized antibody should maintain high binding affinity for the antigen and other preferred biological properties. In order to attain this goal, according to one method, the humanized antibody is prepared by the step of analyzing parent sequences and various conceptual humanized products using three-dimensional models of the parent sequences and humanized sequences. In general, a three-dimensional immunoglobulin model can be utilized and is known to those skilled in the art. A computer program that illustrates and indicates potential three-dimensional conformations of selected candidate immunoglobulin sequences can be utilized. These indications can be studied to analyze the possible roles of residues in the functions of the candidate immunoglobulin sequences, i.e., to analyze residues that influence the ability of the candidate immunoglobulins to bind to the antigen. By this method, FR residues can be selected from a recipient sequence and an import sequence and combined so as to achieve desirable antibody characteristics such as enhanced

binding affinity for one or more target antigens (e.g., EphA4 or a fragment thereof).

[0085]

Needless to say, the antibody of the present invention also includes an antibody derived from the chimeric antibody or the humanized antibody exemplified above by appropriate engineering (e.g., the modification of the antibody or the partial substitution, addition, and/or deletion of the amino acid sequence of the antibody) such that the antibody maintains its functions (or a function is imparted to the antibody or a function of the antibody is improved). More specifically, an antibody lacking lysine (Lys) positioned at the carboxy terminus (C-terminus) of the heavy chain by an artificial method such as genetic engineering in order to reduce the heterogeneity of antibodies produced by antibody-producing cells is also included in the scope of the present invention. Also, an antibody having a modified amino acid sequences in the constant region for modifying an effector function of antibody, such as an antibody in which valine (Val) at the position 234 of human IgG₂ antibody under Eu numbering has been substituted with alanine (Ala), and glycine (Gly) at the position 237 has been substituted with alanine (Ala) so as to reduce the activity of antibody-dependent cell-mediated cytotoxicity (ADCC) and/or of antibody-dependent cell-mediated phagocytosis (ADCP) is also included in the scope of the present invention. Furthermore, a bispecific antibody (Kontermann (2012), mAbs 4, 182-97) which has, along with an antibody-binding region having CDR sequences of the antibody of the present invention, an antigen-binding region which binds to another antigen, is also included in the scope of the present invention.

[0086]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be modified, if desired. The modification of the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be a modification that changes (a) the three-dimensional structure of an amino acid sequence in a modification region, such as sheet or helix conformation; (b) the electric charge or hydrophobic status of the molecule at a target site; or (c) the effects of a modification on the maintenance of

side chain volume, or may be a modification by which these changes are not clearly observed.

[0087]

The modification of the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be achieved by, for example, the substitution, deletion, and/or addition of a constituent amino acid residue(s).

[0088]

In the present specification, the amino acid is used in the broadest sense thereof and includes not only natural amino acids, for example, serine (Ser), asparagine (Asn), valine (Val), leucine (Leu), isoleucine (Ile), alanine (Ala), tyrosine (Tyr), glycine (Gly), lysine (Lys), arginine (Arg), histidine (His), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), threonine (Thr), cysteine (Cys), methionine (Met), phenylalanine (Phe), tryptophan (Trp), and proline (Pro) but non-natural amino acids such as amino acid variants and derivatives. Those skilled in the art naturally understand, by taking this wide definition into consideration, that examples of the amino acid in the present specification include: L-amino acids; D-amino acids; chemically modified amino acids such as amino acid variants and amino acid derivatives; amino acids, such as norleucine, β -alanine, and ornithine, which do not serve as materials constituting proteins *in vivo*; and chemically synthesized compounds having the characteristics of amino acids generally known to those skilled in the art. Examples of the non-natural amino acids include α -methylamino acids (α -methylalanine, etc.), D-amino acids (D-aspartic acid, D-glutamic acid, etc.), histidine-like amino acids (2-amino-histidine, β -hydroxy-histidine, homohistidine, α -fluoromethyl-histidine, α -methyl-histidine, etc.), amino acids having extra methylene in their side chains ("homo" amino acids), and amino acids in which a carboxylic acid functional group in the side chain is replaced with a sulfonic acid group (cysteic acid, etc.).

[0089]

Naturally occurring amino acid residues can be classified into, for example, the following groups based on general side chain characteristics:

- (1) hydrophobic residues: Met, Ala, Val, Leu, and Ile;
- (2) neutral hydrophilic residues: Cys, Ser, and Thr;
- (3) acidic residues: Asp and Glu;
- (4) basic residues: Asn, Gln, His, Lys, and Arg;
- (5) residues influencing chain orientation: Gly and Pro; and
- (6) aromatic residues: Trp, Tyr, and Phe.

[0090]

The non-conservative substitution of an amino acid sequence constituting the antibody or the antigen-binding fragment thereof may be performed by replacing an amino acid belonging to one of these groups with an amino acid belonging to any of the other groups. More conservative substitution may be performed by replacing an amino acid belonging to one of these groups with another amino acid belonging to the same group thereas. Likewise, the deletion or the substitution in an amino acid sequence may be appropriately performed.

[0091]

The modification of amino acid(s) constituting the antibody or the antigen-binding fragment thereof may be, for example, a posttranslational modification such as glycosylation with a sugar, acetylation, or phosphorylation. The antibody may be glycosylated at a conserved position in its constant region. The glycosylation of the antibody is usually of N-linked or O-linked type. The N-linked glycosylation means the binding of a carbohydrate moiety to the side chain of an asparagine residue. Tripeptide sequences asparagine-X-serine, asparagine-X-threonine, and asparagine-X-cysteine (wherein X is any amino acid other than proline) are recognition sequences for enzymatically adding a carbohydrate moiety to the asparagine side chain. Any of these tripeptide sequences are present in the antibody or the antigen-binding fragment thereof so that a potential glycosylation site is present. The O-linked glycosylation may be the binding of N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid (e.g., serine or threonine), or may be the binding thereof to 5-hydroxyproline or 5-hydroxylysine in some cases. Those skilled in the art can appropriately select the

glycosylation conditions (in the case of performing the glycosylation by use of a biological approach, for example, host cells and the type and pH of a cell medium) according to the purpose.

[0092]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be further modified by using other modification methods alone or in combination on the basis of the technical common sense generally known to those skilled in the art.

[0093]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be produced by a method well known to those skilled in the art. For example, a hybridoma producing the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be used to produce an antibody, or a gene encoding the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be integrated into an expression vector, which can then be transferred to *E. coli* cells, monkey COS cells, Chinese hamster ovary (CHO) cells, or the like to produce an antibody. The gene encoding the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention preferably has DNA encoding a signal sequence and more preferably has DNA encoding a signal sequence at each of the 5' ends of DNA encoding the heavy chain variable region and DNA encoding the light chain variable region. The signal sequence is amino acid residues located at the N-terminus of a protein, which are required for a secretory protein or an integral membrane protein to pass through the lipid bilayer after being synthesized on the ribosome. The signal sequence according to the present invention is not particularly limited as long as the sequence has this function. Examples of the signal sequence that may be contained in the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention include signal sequences derived from a human, a mouse, a rat, a rabbit, a donkey, a goat, a horse, a chicken, a dog, a cat, a yeast, and the like. A specific aspect of the signal sequence includes a peptide comprising the amino acid sequence represented by SEQ ID

NO: 10 or SEQ ID NO: 55 as the signal sequence for the heavy chain, and a peptide comprising the amino acid sequence represented by SEQ ID NO: 12 or SEQ ID NO: 58 as the signal sequence for the light chain. The amino acid sequence represented by SEQ ID NO: 10, the amino acid sequence represented by SEQ ID NO: 55, the amino acid sequence represented by SEQ ID NO: 12 or the amino acid sequence represented by SEQ ID NO: 58 may have the substitution, addition, and/or deletion of one or more (e.g., 2, 3, 4, or 5) amino acids as long as the resulting sequence is functionally equivalent thereto. [0094]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be isolated or purified according to a method generally known to those skilled in the art. In this context, the term "isolated" or "purified" means being artificially isolated or purified from a natural state. When a naturally occurring molecule or composition is altered or removed from its original environment, or both, the molecule or the composition is "isolated" or "purified". Examples of the isolation or purification method include electrophoretic, molecular biological, immunological, and chromatographic approaches and specifically include, but are not limited to, ion-exchange chromatography, hydrophobic chromatography, reverse-phase HPLC chromatography, and isoelectric focusing electrophoresis. [0095]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof has the following CDRs:

(a) CDR-H1 comprising the amino acid sequence represented by SEQ ID NO: 26 or SEQ ID NO: 27;

(b) CDR-H2 comprising the amino acid sequence represented by SEQ ID NO: 28 or SEQ ID NO: 29;

(c) CDR-H3 comprising the amino acid sequence represented by SEQ ID NO: 30;

(d) CDR-L1 comprising the amino acid sequence represented by SEQ ID NO: 31;

(e) CDR-L2 comprising the amino acid sequence represented by SEQ ID NO: 32;

and

(f) CDR-L3 comprising the amino acid sequence represented by SEQ ID NO: 33.

In one embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof is a humanized antibody or a chimeric antibody, preferably a humanized antibody.

[0096]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof has the following CDRs:

(a) CDR-H1 comprising the amino acid sequence represented by SEQ ID NO: 26;

(b) CDR-H2 comprising the amino acid sequence represented by SEQ ID NO: 28;

(c) CDR-H3 comprising the amino acid sequence represented by SEQ ID NO: 30;

(d) CDR-L1 comprising the amino acid sequence represented by SEQ ID NO: 31;

(e) CDR-L2 comprising the amino acid sequence represented by SEQ ID NO: 32;

and

(f) CDR-L3 comprising the amino acid sequence represented by SEQ ID NO: 33.

[0097]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof has the following CDRs:

(a) CDR-H1 comprising the amino acid sequence represented by SEQ ID NO: 27;

(b) CDR-H2 comprising the amino acid sequence represented by SEQ ID NO: 29;

(c) CDR-H3 comprising the amino acid sequence represented by SEQ ID NO: 30;

(d) CDR-L1 comprising the amino acid sequence represented by SEQ ID NO: 31;

(e) CDR-L2 comprising the amino acid sequence represented by SEQ ID NO: 32;

and

(f) CDR-L3 comprising the amino acid sequence represented by SEQ ID NO: 33.

[0098]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof comprises a heavy chain and a light chain, a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 11 or an amino acid sequence derived from the sequence by the substitution, addition, and/or deletion of one or more amino acids, and a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 13 or an amino acid sequence derived from the sequence by the substitution, addition, and/or deletion of one or more amino acids.

[0099]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof comprises a heavy chain and a light chain, wherein a variable region of the heavy chain comprises the amino acid sequence represented by SEQ ID NO: 66, 68, 70, 72, 74 or 76, or an amino acid sequence derived from said sequence by substitution, addition, and/or deletion of one or more amino acids, and a variable region of the light chain comprises the amino acid sequence represented by SEQ ID NO: 78, 80, 82 or 84, or an amino acid sequence derived from said sequence by the substitution, addition, and/or deletion of one or more amino acids.

[0100]

In this context, the term "or more" used as to the heavy chain variable region or the light chain variable region in the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention is not limited as long as it maintains binding affinity for EphA4 and inhibits the binding between EphA4 and ephrin. The term "or more" is 2 to 15, more preferably 2 to 10, for example, 9, 8, 7, 6, 5, 4, 3, or 2 or is within 10%, for example, within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, or within 1% of the number of amino acids in the amino acid sequence.

[0101]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, the heavy chain variable region comprises the amino acid sequence represented by of SEQ ID NO: 11, and the light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 13.

[0102]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 66, 68, 70, 72, 74 or 76, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, 80, 82 or 84.

[0103]

In an alternative preferred embodiment of the present invention, the anti-EphA4 antibody or the EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 66, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 68, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 70, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 72, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 74, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 76, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 78.

[0104]

In an alternative preferred embodiment of the present invention, an anti-EphA4 antibody or an EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 66, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 68, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 70, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 72, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 74, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 76, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 80.

[0105]

In an alternative preferred embodiment of the present invention, an anti-EphA4 antibody or an EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 66, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 68, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 70, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 72, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 74, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 76, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 82.

[0106]

In an alternative preferred embodiment of the present invention, an anti-EphA4 antibody or an EphA4-binding fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 66, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84, or

said heavy chain variable region comprises the amino acid sequence represented

by SEQ ID NO: 68, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 70, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 72, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 74, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84, or

said heavy chain variable region comprises the amino acid sequence represented by SEQ ID NO: 76, and said light chain variable region comprises the amino acid sequence represented by SEQ ID NO: 84.

[0107]

In a specific embodiment of the present invention, an anti-EphA4 antibody or an EphA4-binding fragment thereof has a constant region of human IgG₂. Preferably, said human IgG₂ constant region has at least one amino acid mutation selected from C131S, C219S, V234A and G237A (EU numbering). In one embodiment, said human IgG₂ constant region has a combination of C131S and C219S amino acid mutations. In one embodiment, said human IgG₂ constant region has a combination of V234A and G237A amino acid mutations. In another embodiment, said human IgG₂ constant region has all amino acid mutations of C131S, C219S, V234A and G237A. In a further alternative embodiment, said human IgG₂ constant region does not have a lysine residue at the C-terminus.

[0108]

In a preferred embodiment of the present invention, human IgG₂ constant region comprise the amino acid sequence represented by SEQ ID NO: 62.

[0109]

In a specific embodiment of the present invention, an anti-EphA4 antibody or an EphA4-binding fragment thereof has human IgG constant region consisting of a combination of human IgG₁ and human IgG₂. Preferably, in said human IgG constant region, CH1 and a hinge region are human IgG₁, and CH2 and CH3 are human IgG₂. In one embodiment, said human IgG constant region has V234A or G237A (Eu numbering) amino acid mutation. In another aspect, said human IgG constant region has V234A and G237A amino acid mutations. In a further alternative aspect, said human IgG constant region does not have a lysine residue at the C-terminus.

[0110]

In a preferred embodiment of the present invention, the human IgG constant region consisting of a combination of human IgG₁ and human IgG₂ comprises the amino acid sequence represented by SEQ ID NO: 60.

[0111]

In one aspect, the present invention relates to a pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention.

[0112]

The pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may further comprise a pharmaceutically acceptable carrier, excipient, and/or stabilizer in the form of an aqueous or dry preparation. Examples of the acceptable carrier, excipient and/or stabilizer include: physiological saline; buffer solutions of phosphate, citrate, or other organic acids; antioxidants including ascorbic acid; low-molecular-weight polypeptides; proteins (e.g., serum albumin, gelatin, and immunoglobulins); hydrophilic polymers such as polyvinylpyrrolidone; amino acids; monosaccharides, disaccharides, and other carbohydrates, including glucose, mannose, and dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol and sorbitol; salt-forming counterions such as sodium; and nonionic surfactants such as TWEEN (trademark), PLURONICS (trademark), and PEG.

[0113]

The pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be enclosed in, for example, a microcapsule, a colloidal drug delivery system (e.g., a liposome, an albumin microsphere, a microemulsion, a nanoparticle, or a nanocapsule), or a microemulsion. When the sustained-release administration of the antibody is desired for a preparation having release properties suitable for any disease in need of the administration of the antibody, the antibody may be intended to be microencapsulated. Examples of the sustained-release matrix include polyester, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) and poly(vinyl alcohol)), polylactic acids, copolymers of L-glutamic acid and γ -ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers (microspheres for injection constituted by a lactic acid-glycolic acid copolymer and leuprolide acetate) such as LUPRON DEPOT (trademark), and poly-D-(-)-3-hydroxybutyric acid.

[0114]

In one aspect, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can inhibit the binding between EphA4 and its ligand. Therefore, the pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be useful in the treatment of ALS. Specifically, an alternative aspect of the present invention encompasses a method for treating ALS, comprising the step of administering a therapeutic effective amount of the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention to a subject. An alternative aspect of the present invention encompasses use of the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention for producing a therapeutic drug for ALS. An alternative aspect of the present invention encompasses the anti-EphA4 antibody or the EphA4-binding fragment thereof for use in a method for treating ALS.

[0115]

The anti-EphA4 antibody or the EphA4-binding fragment thereof of the present

invention can be used alone or in combination with an additional drug or composition in the treatment method. For example, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention may be administered at the same time or at different times with an additional drug. Such combination therapy includes combined administration (two or more drugs are contained in the same preparation or separate preparations) and separated administration (e.g., concurrent or continuous). In the case of separately administering two or more drugs, the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention can be administered prior to or following the concomitant treatment method.

[0116]

The subject for administering the pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention is not limited, and the pharmaceutical composition of the present invention can be used for, for example, a human or a nonhuman mammal (a monkey, a mouse, a rat, a rabbit, cattle, a horse, a goat, etc.).

[0117]

The method for administering the pharmaceutical composition comprising the anti-EphA4 antibody or the EphA4-binding fragment thereof of the present invention to the subject (administration route, dose, the number of doses per day, the timing of administration, etc.) is not limited and can be appropriately determined by those skilled in the art (e.g., a physician) according to the health condition of the subject, the severity of the disease, the type of a drug to be used in combination therewith, etc.

[0118]

Those skilled in the art should understand that the present invention may be carried out by any one of or appropriate combination of two or more of all aspects described in the present specification unless a technical contradiction arises. Further, those skilled in the art should understand that the present invention can be preferably carried out by an appropriate combination of all preferable or advantageous aspects described in the present specification unless a technical contradiction arises.

[0119]

Literatures cited in the present specification should be interpreted as being clearly incorporated herein by reference in their entirety. Those skilled in the art can understand related contents disclosed in these literatures by reference as a part of the present specification without departing from the spirits and scope of the present invention according to the context of the present specification.

[0120]

Literatures cited in the present specification are provided merely for the purpose of disclosing related techniques before the filing date of the present application. It should not be understood that the present inventors admit to having no right preceding such disclosure due to the prior inventions or any other reasons. All statements of these literatures are based on information which has been available by the present applicant, and there is no admission that the contents of these statements are accurate.

[0121]

The terms in the present specification are used for illustrating particular embodiments and are not intended to limit the invention.

[0122]

The term "comprise" used in the present specification means that described items (members, steps, factors, numbers, etc.) are present and the presence of the other items (members, steps, factors, numbers, etc.) is not excluded therefrom, unless the context evidently requires different interpretation. The term "consist of" encompasses aspects described by the terms "consist of" and/or "consist essentially of".

[0123]

The term "neutralizing activity" used in the present specification means the activity of inhibiting the binding between EphA4 and its ligand and/or the activity of inhibiting signal transduction or the molecular expression response or functional change of cells induced by the binding between EphA4 and its ligand in the living body of a human.

[0124]

All terms (including technical terms and scientific terms) used herein have the same meanings as those understood in a broad sense by those skilled in the art to which the present invention belongs, unless otherwise defined. The terms used herein should be interpreted as having meanings consistent with meanings in the present specification and related technical fields and should not be interpreted in an idealized or excessively formal sense, unless otherwise defined.

[0125]

Terms such as "first" or "second" are used for expressing various factors. However, these factors are understood to be not limited by these terms themselves. These terms are used merely for differentiating one factor from the other factors. For example, the first factor may be described as the second factor, and vice versa, without departing from the scope of the present invention.

[0126]

In the present specification, it should be understood that numerical values used for indicating component contents, numerical ranges, etc., are modified with the term "approximately" unless otherwise specified. For example, "4°C" is interpreted as meaning "approximately 4°C" unless otherwise specified. Those skilled in the art can naturally understand the extent thereof rationally according to the technical common sense and the context of the present specification.

[0127]

It should be understood that each aspect indicated in a singular form used in the present specification and claims may be in a plural form, and vice versa, unless the context evidently requires different interpretation and unless a technical contradiction arises.

[0128]

Hereinafter, the present invention will be described in more detail with reference to Examples. However, the present invention can be embodied by various aspects and is not intended to be limited by Examples described herein. Those skilled in the art in the related field can implement the present invention by various

modifications, additions, deletions, substitutions, etc., without altering the spirit or scope of the present invention.

Examples

[0129]

Example 1: Preparation of anti-mouse EphA4 monoclonal antibody

Preparation of mouse anti-mouse EphA4 monoclonal antibody

In order to prepare a monoclonal antibody binding to mouse EphA4 (GenBank Accession No. NP_031962.2, SEQ ID NO: 1), a protein of a mouse EphA4 extracellular region (positions 20 to 547) (SEQ ID NO: 2) fused with secreted alkaline phosphatase (SEAP) and histidine tag (hereinafter, referred to as "mouse EphA4 extracellular region-SEAP-His protein", SEQ ID NO: 43) was prepared by the following steps.

[0130]

First, a DNA sequence encoding the signal sequence (SEQ ID NO: 42) and the extracellular region (SEQ ID NO: 2) of mouse EphA4 was amplified by RT-PCR using mouse brain-derived total RNA and cloned into the SalI/NotI site of a pENTR1A vector (Invitrogen/Life Technologies) having a DNA sequence encoding SEAP and histidine tag. Next, the DNA sequence encoding the signal sequence and the extracellular region of mouse EphA4, SEAP, and histidine tag was transferred to a pcDNA3.1_rfcB vector through LR reaction using Gateway System (Invitrogen/Life Technologies) to construct a pcDNA3.1-mouse EphA4 extracellular region-SEAP-His expression vector. HEK293EBNA cells (Invitrogen/Life Technologies) were transfected with the constructed pcDNA3.1-mouse EphA4 extracellular region-SEAP-His expression vector using TransIT-LT1 (TAKARA). After incubation (5% CO₂, 37°C) for 6 days, the culture supernatant was recovered. From the recovered culture supernatant, the mouse EphA4 extracellular region-SEAP-His protein (SEQ ID NO: 43) was purified using Protino column (MACHEREY-NAGEL).

[0131]

20 µg of the mouse EphA4 extracellular region-SEAP-His protein was mixed with the same amount of TiterMax Gold adjuvant (TiterMax USA) or GERBU adjuvant

(GERBU Biotechnik), and the mixture was subcutaneously injected to the footpad of a Balb/c mouse. Then, at days 3, 7, and 10, the mouse EphA4 extracellular region-SEAP-His protein was administered in the same way as above. In this operation, the TiterMax Gold adjuvant (TiterMax USA) was used only for day 10, and the GERBU adjuvant (GERBU Biotechnik) was used for days 3, 7, and 10. At day 13, the mouse was sacrificed, and the peripheral lymph node was recovered to prepare lymph node cells. The prepared lymph node cells and P3U1 myeloma cells (kindly provided by Kyoto University) were fused at a ratio of 5:1 in the presence of GenomONE-CF (Ishihara Sangyo Kaisha). The fused cells were cultured in a 96-well plastic plate. After incubation (5% CO₂, 37°C) for 7 days, the culture supernatant was recovered.

[0132]

The obtained culture supernatant was used to pick up a well having reactivity with mouse, rat, and human EphA4 and inhibitory activity against the binding between mouse EphA4 and mouse Ephrin A1.

[0133]

The reactivity with mouse, rat, and human EphA4 was evaluated by ELISA using a protein of the extracellular region of mouse EphA4, the extracellular region (positions 20 to 547) of rat EphA4 (GenBank Accession No. NP_001155883.1), or the extracellular region (positions 20 to 547) (SEQ ID NO: 4) of human EphA4 (GenBank Accession No. NP_004429.1, SEQ ID NO: 3) fused with a human IgG1 Fc region and histidine tag (hereinafter, referred to as "mouse EphA4 extracellular region-Fc-His protein", "rat EphA4 extracellular region-Fc-His protein", or "human EphA4 extracellular region-Fc-His protein", respectively).

[0134]

The mouse, rat, or human EphA4 extracellular region-Fc-His protein was prepared by the following steps. First, a pcDNA3.1-mouse, rat, or human EphA4 extracellular region-Fc-His expression vector was constructed. First, a DNA sequence encoding the signal sequence and the extracellular region of mouse, rat, or human EphA4 was amplified by RT-PCR using mouse, rat, or human brain-derived total RNA

and cloned into the Sall/NotI site of a pENTR1A vector (Invitrogen/Life Technologies) having a DNA sequence encoding Fc and histidine tag. Next, the DNA sequence encoding the signal sequence and the extracellular region of mouse, rat, or human EphA4, Fc, and histidine tag was transferred to a pcDNA3.1_rfcB vector through LR reaction using Gateway System (Invitrogen/Life Technologies) to construct a pcDNA3.1-mouse, rat, or human EphA4 extracellular region-Fc-His expression vector. HEK293EBNA cells (Invitrogen/Life Technologies) were transfected with each of the constructed expression vectors using TransIT-LT1 (TAKARA). After incubation (5% CO₂, 37°C) for 6 days, each culture supernatant was recovered.

[0135]

ELISA using the mouse, rat, or human EphA4 extracellular region-Fc-His protein was conducted according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-human IgG antibody (Jackson ImmunoResearch Laboratories). After incubation overnight at 4°C, each well was blocked with 1 × BlockAce (Sumitomo Dainippon Pharma) at room temperature for 1 hour. After washing with 0.02% Tween 20/PBS (Nacalai Tesque) three times, the culture supernatant containing the mouse, rat, or human EphA4 extracellular region-Fc-His protein was added (final concentration: 1 nM) to each well and incubated at room temperature for 1 hour. After washing three times, the culture supernatant of the fused cells was added to each well. After incubation at room temperature for 1 hour and subsequent washing three times, a horseradish peroxidase-labeled anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 5 to 20 minutes. An equal amount of a reaction stopping solution (2N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (PerkinElmer).

[0136]

The inhibitory activity against the binding between mouse EphA4 and mouse

Ephrin A1 was evaluated according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-alkaline phosphatase antibody (Seradyn). After incubation overnight at 4°C, each well was blocked with 1 × BlockAce (Sumitomo Dainippon Pharma) at room temperature for 1 hour. After washing with 0.02% Tween 20/PBS (Nacalai Tesque) three times, the mouse EphA4 extracellular region-SEAP-His protein was added (final concentration: 10 nM) to each well and incubated at room temperature for 1 hour. After washing three times, Ephrin A1-Fc chimera (R&D Systems, final concentration: 20 nM) and the culture supernatant of the fused cells were added to each well. After incubation at room temperature for 1 hour and subsequent washing three times, a horseradish peroxidase-labeled anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 5 to 20 minutes. An equal amount of a reaction stopping solution (2N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (PerkinElmer).

[0137]

From the well picked up through these steps, a hybridoma was cloned by the limiting dilution method. Finally, a hybridoma clone expressing a mouse anti-EphA4 antibody having reaction activity against mouse, rat, and human EphA4 and having inhibitory activity against the binding between mouse EphA4 and mouse Ephrin A1 was obtained.

[0138]

The obtained hybridoma clone was cultured, and the anti-EphA4 antibody (antibody A) was purified from the culture supernatant using protein A (GE Healthcare). The isotype of antibody A was determined using a monoclonal antibody isotyping kit (Serotec) and was IgG1 for the heavy chain and κ for the light chain.

[0139]

Sequence analysis of antibody A

DNA sequences encoding the heavy chain and light chain signal sequences and variable regions of antibody A were amplified by 5'-RACE (5'-rapid amplification of cDNA ends). Total RNA was prepared from the hybridoma using TRIZOL (Invitrogen/Life Technologies) and treated with DNase (Qiagen N.V., RNase free DNase set). Double-stranded cDNA was prepared from the total RNA using a cDNA synthesis kit (TAKARA). 5' adaptor obtained by the annealing of oligo DNA ad29S (ACATCACTCCGT) (SEQ ID NO: 5) and oligo DNA ad29AS (ACGGAGTGATGTCCGTCGACGTATCTCTGCGTTGATACTTCAGCGTAGCT) (SEQ ID NO: 6) was added to the cDNA. The obtained cDNA was amplified using 5' forward primer (5'-PCR4 primer, AGCTACGCTGAAGTATCAACGCAGAG) (SEQ ID NO: 7) and 3' reverse primer (GCCAGTGGATAGACTGATGG (SEQ ID NO: 8) was used for the amplification of the mouse IgG heavy chain gene, and GATGGATACAGTTGGTGCAGC (SEQ ID NO: 9) was used for the amplification of the mouse Igκ light chain gene). The amplified cDNA was inserted to a pCR2.1 vector (Invitrogen/Life Technologies). The gene sequence of antibody A was analyzed using ABI3130XL. As for the amino acid sequence encoded by the gene sequence of antibody A identified by this analysis, the heavy chain signal sequence is shown in SEQ ID NO: 10; the heavy chain variable region is shown in SEQ ID NO: 11; the light chain signal sequence is shown in SEQ ID NO: 12; and the light chain variable region is shown in SEQ ID NO: 13. As for the nucleotide sequence encoding the gene sequence of antibody A, the heavy chain signal sequence is shown in SEQ ID NO: 14, the heavy chain variable region is shown in SEQ ID NO: 15; the light chain signal sequence is shown in SEQ ID NO: 16; and the light chain variable region is shown in SEQ ID NO: 17.

[0140]

The full-length sequences of the heavy chain and the light chain of antibody A were obtained by the following steps. Total RNA was prepared from the hybridoma using TRIZOL (Invitrogen/Life Technologies) and treated with DNase (Qiagen N.V., RNase free DNase set). cDNA was prepared from the total RNA using a cDNA synthesis kit (TAKARA). The obtained cDNA was used as a template in PCR to amplify

the gene sequences encoding the antibody A heavy chain and light chain using 5' forward primer (GCGAAGCTTGCCGCCACCATGGCTTGGGTGTGGACCTTGC (SEQ ID NO: 18) was used for the amplification of the heavy chain gene, and GCGAAGCTTGCCGCCACCATGAGTGTGCCCACTCAGGTCC (SEQ ID NO: 19) was used for the amplification of the light chain gene) and 3' reverse primer (GCGGAATTCATCATTTACCAGGAGAGTGGGAGAGGC (SEQ ID NO: 20) was used for the amplification of the heavy chain gene, and CGCGAATTCACTAACAACCTCATTCTGTTGAAGCTCTTGAC (SEQ ID NO: 21) was used for the amplification of the light chain gene). The amplification products were cloned into pEE6.4 and pEE12.4 vectors (Lonza), respectively. These gene sequences were analyzed using ABI3130XL. As for the amino acid sequence encoded by the gene sequence of antibody A identified by this analysis, the heavy chain constant region is shown in SEQ ID NO: 22, and the light chain constant region is shown in SEQ ID NO: 23. As for the nucleotide sequence encoding the gene sequence of antibody A, the heavy chain constant region is shown in SEQ ID NO: 24, and the light chain constant region is shown in SEQ ID NO: 25.

[0141]

CDRs of antibody A were determined by numbering the amino acid sequence of antibody A using Abysis software (UCL) according to the Kabat numbering system and identifying the CDRs according to the Kabat definition or the AbM definition method on the basis of the numbers. The CDR amino acid sequences and nucleotide sequences of antibody A are shown in Tables 1 and 2, respectively.

[0142]

[Table 1]

Table 1 Amino acid sequences of CDRs of antibody A

Name	Sequence
Heavy chain CDR 1 (Kabat definition)	DYSMH (SEQ ID NO: 26)
Heavy chain CDR 1 (AbM definition)	GYTFTDYSMH (SEQ ID NO: 27)
Heavy chain CDR 2 (Kabat definition)	WINTETGEPTYADDFKG (SEQ ID NO: 28)
Heavy chain CDR 2 (AbM definition)	WINTETGEPT (SEQ ID NO: 29)
Heavy chain CDR 3	IPLYYYGSRYWYFDV (SEQ ID NO: 30)
Light chain CDR 1	RASENIYRNLA (SEQ ID NO: 31)
Light chain CDR 2	AATNLAD (SEQ ID NO: 32)
Light chain CDR 3	QHFWGTPWT (SEQ ID NO: 33)

[0143]

[Table 2]

Table 2 Nucleic acid sequences of CDRs of antibody A

Name	Sequence
Heavy chain CDR 1 (Kabat definition)	GACTATTCAATGCAC (SEQ ID NO: 34)
Heavy chain CDR 1 (AbM definition)	GGTTATACCTTCACAGACTATTCAATGCAC (SEQ ID NO: 35)
Heavy chain CDR 2 (Kabat definition)	TGGATAAACTGAGACTGGTGAGCCAACATATGCAGATGACTTCAAGGGA (SEQ ID NO: 36)
Heavy chain CDR 2 (AbM definition)	TGGATAAACTGAGACTGGTGAGCCAACA (SEQ ID NO: 37)
Heavy chain CDR 3	ATCCCCCTCTATTACTACGGTAGTAGGTACTGGTACTTCGATGTC (SEQ ID NO: 38)
Light chain CDR 1	CGAGCAAGTGAGAAATTTACAGAAATTTAGCA (SEQ ID NO: 39)
Light chain CDR 2	GCTGCAACAACTTAGCAGAT (SEQ ID NO: 40)
Light chain CDR 3	CAACATTTTGGGGTACTCCGTGGACG (SEQ ID NO: 41)

[0144]

Example 2: Binding affinity of anti-EphA4 monoclonal antibody for mouse and human EphA4

The binding affinity of antibody A obtained in Example 1 for mouse and human EphA4 was determined by surface plasmon resonance (SPR) using Biacore A100 (GE Healthcare). First, a rat anti-mouse IgG₁ antibody produced with a conventional procedure by immunizing rat with a mouse IgG₁ antibody was immobilized on sensor chip CM5. The immobilization of the rat anti-mouse IgG₁ antibody on sensor chip CM5 was performed by the amine coupling method using N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Ethanolamine was used in blocking (the sensor chip and the reagents for immobilization were all manufactured by GE Healthcare). The antibody was diluted with a buffer solution for immobilization (10 mM sodium acetate, pH 4.5) into 1 to 2 µg/mL and immobilized on the sensor chip according to the protocol attached to Biacore A100. Antibody A was diluted with a running buffer solution HBS-EP (GE Healthcare, BR-1001-88), injected onto a flow cell for 120 seconds, and captured (amount of the antibody captured: approximately 70 to 100 RU). Subsequently, mouse or human EphA4 extracellular region-SEAP-His serially diluted in the range of 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0 nM using HBS-EP was added to the sensor chip for 120 seconds. Binding reaction curves were sequentially observed at the time of the addition (association phase, for 120 sec) and after the completion of the addition (dissociation phase, for 900 sec). After the completion of each observation, the sensor chip was regenerated by the addition of 3 M MgCl₂ (Wako Pure Chemical Industries, Ltd.) (for 30 sec). The obtained binding reaction curves were subjected to fitting analysis with 1:1 binding models using software BIA evaluation attached to the system to calculate the binding affinity ($KD = k_d / k_a$) for mouse and human EphA4.

[0145]

The binding affinity (KD) of antibody A for mouse and human EphA4 was 7.29×10^{-10} M and 6.61×10^{-10} M, respectively (FIG. 1). Other binding parameters for mouse and human EphA4 were almost equivalent. Accordingly, antibody A is considered to have equivalent binding affinity for mouse and human EphA4.

[0146]

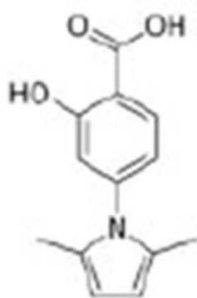
Example 3: Mouse EphA4-mouse ligand binding inhibitory activity of anti-EphA4 monoclonal antibody

Antibody A obtained in Example 1 was evaluated for its inhibitory activity against the binding between mouse EphA4 and its mouse ligand according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-alkaline phosphatase antibody (Thermo Fisher Scientific). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Thermo Fisher Scientific) three times, the mouse EphA4 extracellular region-SEAP-His protein obtained by the method of Example 1 was added (final concentration: 10 nM) to each well and incubated at room temperature for 1 hour. After washing three times, ligands and serially diluted antibody A (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 nM) or a known EphA4 inhibitor KYL peptide (KYLPLYWPVLSSL, 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 µM, its synthesis was outsourced to Hokkaido System Science) or compound 1 (0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 µM, Formula 1, Matrix Scientific) were added to each well. The ligands used were mouse Ephrin A1-Fc chimera (R&D Systems, final concentration: 20 nM) and mouse Ephrin B2-Fc chimera (R&D Systems, final concentration: 0.6 nM). After incubation at room temperature for 1 hour and subsequent washing three times, a horseradish peroxidase-labeled anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 2 minutes. An equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (Molecular Devices).

[0147]

Formula 1

[Formula 1]



Antibody A suppressed the binding between mouse EphA4 and its mouse ligand in a concentration-dependent manner with IC_{50} values of approximately 1.2 and 1.2 nM for binding to mouse Ephrin A1 and Ephrin B2, respectively. The IC_{50} values of the existing EphA4 inhibitor KYL peptide were approximately 1.3 and 1.3 μ M for binding to mouse Ephrin A1 and Ephrin B2, respectively (FIG. 2). Compound 1 had weaker activity, and no concentration dependence was found. Accordingly, antibody A was found to inhibit the binding between mouse EphA4 and mouse ligand with the activity 1,000 or more times stronger than that of the existing EphA4 inhibitor KYL peptide. [0148]

Example 4: Human EphA4-human ligand binding inhibitory activity of anti-EphA4 monoclonal antibody

Antibody A obtained in Example 1 was evaluated for its inhibitory activity against the binding between human EphA4 and its human ligand according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-alkaline phosphatase antibody (Thermo Fisher Scientific). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Thermo Fisher Scientific) three times, the human EphA4 extracellular region-SEAP-His protein obtained by the method of Example 1 was added (final concentration: 10 nM) to each well and incubated at room temperature for 1 hour. After washing three times, ligands and serially diluted antibody A (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) or an EphA4 inhibitor KYL peptide (KYLPYWPVLSSL, 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 μ M, its synthesis was outsourced to Toray Research Center)

were added to each well. The ligands used were biotinylated human Ephrin A5-Fc chimera (R&D Systems, final concentration: 0.7 nM) and biotinylated human Ephrin B3-Fc chimera (R&D Systems, final concentration: 2.3 nM). After incubation at room temperature for 1 hour and subsequent washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 2 minutes. An equal amount of a reaction stopping solution (1 N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (Molecular Devices or PerkinElmer).

[0149]

Antibody A suppressed the binding between human EphA4 and its human ligand in a concentration-dependent manner with IC₅₀ values of approximately 2.7 and 1.9 nM for binding to human Ephrin A5 and Ephrin B3, respectively. The IC₅₀ values of the EphA4 inhibitor KYL peptide were approximately 6.1 and 1.6 μM for binding to human Ephrin A5 and Ephrin B3, respectively (FIG. 3). Accordingly, antibody A was also found to strongly inhibit the binding between human EphA4 and its human ligand.

[0150]

Example 5: Binding affinity of EphA4-binding fragment for mouse and human EphA4

First, a Fab fragment and a F(ab')₂ fragment of antibody A (hereinafter, referred to as antibody A-Fab and antibody A-F(ab')₂, respectively) were prepared as EphA4-binding fragments.

[0151]

The preparation of antibody A-Fab was performed according to the following steps. In a 1.5-mL tube (Eppendorf), antibody A (4.36 mg/mL, 1 mL), 10 mM L-cysteine (Wako Pure Chemical Industries), 1 mM EDTA (Gibco), and 2.18 μg/mL papain (Sigma-Aldrich) were mixed and incubated at 37°C for 12 hours. Iodoacetamide (Wako Pure Chemical Industries) was added at a final concentration of 50 mM to the tube after the incubation. After the termination of the reaction, the antibody solution was dialyzed

against PBS (Sigma-Aldrich). To the antibody solution, an equal amount of 0.1 M Tris (Sigma-Aldrich)-HCl/5 M NaCl (pH 8.0, Wako Pure Chemical Industries) was added, followed by purification using rProtein A FF resin. A 2-mL tube was filled with 800 μ L of rProtein A FF, which was then equilibrated by the addition of 3.5 C.V. each of ultrapure water and a binding buffer (0.1 M Tris (Sigma-Aldrich)-HCl/3 M NaCl (pH 8.0, Wako Pure Chemical Industries)) in this order. The antibody solution supplemented with an equal amount of 0.1 M Tris (Sigma-Aldrich)-HCl/5 M NaCl (Wako Pure Chemical Industries) (pH 8.0) was injected to the column. A solution eluted from the column (flow-through fraction) was recovered and added again to the column. This operation was repeated three times. Then, the flow-through fraction of the final run was recovered. Washing was repeated twice by the addition of 2.5 mL of a binding buffer. The flow-through and washing fractions were dialyzed against PBS to obtain antibody A-Fab.

[0152]

The preparation of antibody A-F(ab')₂ was performed according to the following steps. Antibody A was dialyzed against a 0.2 M acetate buffer (pH 4.0, Wako Pure Chemical Industries) overnight at 4°C. The dialyzed solution was recovered and filtered through a 0.22- μ m filter (Merck Millipore), and then quantified, and the antibody concentration was adjusted to 4.0 mg/mL. Pepsin (Sigma-Aldrich) was brought back to room temperature and adjusted to 2.0 mg/mL using a 0.2 M acetate buffer (pH 4.0, Wako Pure Chemical Industries). In a 1.5-mL tube (Eppendorf), antibody A (4.0 mg/mL, 800 μ L), the pepsin solution (2.0 mg/mL, 16 μ L), and a 0.2 M acetate buffer (pH 4.0, Wako Pure Chemical Industries) were mixed at 64 μ L/tube and incubated at 37°C for 15 hours. The reaction was terminated by the addition of 2 M Tris-base (Sigma-Aldrich) at 112 μ L/tube to the tube after the incubation. Then, the molecular species was confirmed by SDS-PAGE. After the termination of the reaction, the antibody solution was dialyzed against 100 mM Tris-HCl (pH 8.0). Subsequently, antibody A-F(ab')₂ was purified using rProtein A FF (GE Healthcare, 17-1279-02). A 5-mL tube was filled with 1 mL of rProtein A resin, which was then equilibrated by the addition of 3.5 C.V. each of

ultrapure water and a binding buffer in this order. The dialyzed antibody solution equilibrated by the addition of an equal amount of 0.1 M Tris (Sigma-Aldrich)-HCl/5 M NaCl (pH 8.0, Wako Pure Chemical Industries) was injected to the column. A flow-through fraction was recovered and added again to the column. This operation was repeated three times. Then, the flow-through fraction of the final run was recovered. Then, washing was repeated twice by the addition of 5 mL of a binding buffer. In order to remove unreacted IgG, etc., elution was performed with 0.1 M citrate (pH 3.0, Wako Pure Chemical Industries). Then, the molecular species was confirmed by SDS-PAGE. The flow-through and washing fractions were dialyzed against PBS to obtain antibody A-F(ab')₂.

[0153]

Next, the binding affinity of antibody A-Fab and antibody A-F(ab')₂ for mouse and human EphA4 was determined by surface plasmon resonance (SPR) using Biacore T200 (GE Healthcare). Antibody A obtained in Example 1 was used as a control for the comparison of each fragment. First, an anti-His tag antibody was immobilized on sensor chip CM5. The immobilization of the anti-His tag antibody on sensor chip CM5 was performed by the amine coupling method using N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Ethanolamine was used in blocking (the sensor chip and the reagents for immobilization were all manufactured by GE Healthcare). The antibody was diluted with a buffer solution for immobilization (10 mM sodium acetate, pH 4.5) into 3 µg/mL and immobilized on the sensor chip according to the protocol attached to Biacore T200.

[0154]

Mouse or human EphA4 extracellular region-SEAP-His protein was diluted with a running buffer solution HBS-EP (GE Healthcare, BR-1001-88), injected onto a flow cell for 120 seconds, and captured (amount of the protein captured: approximately 10 to 20 RU). Subsequently, antibody A (50, 16.7, 5.6, 1.9, 0.6, and 0 nM), antibody A-Fab (500, 166.7, 55.6, 18.5, 6.2, and 0 nM), or antibody A-F(ab')₂ (50, 16.7, 5.6, 1.9, 0.6, and 0 nM) serially diluted using HBS-EP was added to the sensor chip for 120 seconds.

Binding reaction curves were sequentially observed at the time of the addition (association phase, for 120 sec) and after the completion of the addition (dissociation phase, for 900 sec). After the completion of each observation, the sensor chip was regenerated by the addition of 3 M MgCl_2 (Wako Pure Chemical Industries) (for 30 sec). The obtained binding reaction curves were subjected to fitting analysis with 1:1 binding models using software BIA evaluation attached to the system to calculate the binding affinity ($K_D = k_d / k_a$) for mouse and human EphA4.

[0155]

The binding affinity (K_D) of antibody A-Fab for mouse and human EphA4 was 4.51×10^{-8} M and 4.04×10^{-8} M, respectively (FIGs. 4A and 4C). On the other hand, the binding affinity (K_D) of antibody A-F(ab')₂ for mouse and human EphA4 was 2.29×10^{-11} M and 5.30×10^{-11} M, respectively (FIGs. 4B and 4D).

[0156]

Example 6: Mouse EphA4-mouse ligand binding inhibitory activity of EphA4-binding fragment

Antibody A-Fab and antibody A-F(ab')₂ obtained in Example 5 were evaluated for their inhibitory activity against the binding between EphA4 and its ligand according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-alkaline phosphatase antibody (Thermo Fisher Scientific). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Thermo Fisher Scientific) three times, the mouse EphA4 extracellular region-SEAP-His protein obtained by the method of Example 1 was added (final concentration: 10 nM) to each well and incubated at room temperature for 1 hour. After washing three times, a ligand and serially diluted antibody A (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM), antibody A-Fab (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM), antibody A-F(ab')₂ (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM), or the EphA4 inhibitor KYL peptide (KYL PYWPVLSSL, 0, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 μM , its synthesis was

outsourced to Toray Research Center) were added to each well. The ligand used was biotinylated mouse Ephrin B2-Fc chimera (R&D Systems, final concentration: 2.5 nM). After incubation at room temperature for 1 hour and subsequent washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 2 minutes. An equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (Molecular Devices or PerkinElmer).

[0157]

The IC₅₀ values of antibody A (Antibody A-IgG), antibody A-Fab, antibody A-F(ab')₂, and KYL peptide were 2.6 (or 3.6) nM, 438.5 nM, 2.9 nM, and 5.293 μM, respectively (FIG. 5). Antibody A and antibody A-F(ab')₂ had 1000 or more times the activity of the KYL peptide, and antibody A-Fab also had 10 or more times the activity of KYL peptide.

[0158]

Example 7: Selectivity of anti-EphA4 monoclonal antibody for human Eph receptor

According to the method described in Example 1, a DNA sequence encoding the signal sequence and the extracellular region of each human Eph receptor (EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) was amplified by RT-PCR using tissue-derived total RNA and cloned into a pENTR1A vector (Invitrogen/Life Technologies) having a DNA sequence encoding a human IgG1 Fc region and histidine tag. Next, the DNA sequence encoding the signal sequence and the extracellular region of each human Eph receptor, Fc, and histidine tag was transferred to a pcDNA3.1_rfcB vector through LR reaction using Gateway System (Invitrogen/Life Technologies) to construct a vector for the expression of a protein of the extracellular region of each human Eph receptor fused with the human IgG1 Fc region and the His tag (referred to as "Eph receptor extracellular region-Fc-His protein") (this

vector is referred to as "Eph receptor extracellular region-Fc-His protein expression vector").

[0159]

Next, HEK293EBNA cells (Life Technologies) were inoculated to a 10-cm dish (Falcon) and cultured at 37°C for 1 day. The HEK293EBNA cells were transfected with each human Eph receptor extracellular region-Fc-His protein expression vector obtained above using TransIT-LT1 (TAKARA). After incubation (5% CO₂, 37°C) for 4 days, the culture supernatant was recovered and centrifuged at 1500 rpm at room temperature for 5 minutes. The centrifugation supernatant was filtered through a 0.22-μm filter (Merck Millipore), and Hepes (Dojindo Laboratories) and sodium azide (Wako Pure Chemical Industries) were added thereto at final concentrations of 20 mM and 0.02%, respectively.

[0160]

Antibody A was evaluated for its binding activity against each human Eph receptor according to the following steps.

Each well of a 96-well plate (Nunc) was coated with a donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Nacalai Tesque) three times, each human Eph receptor extracellular region-Fc-His protein was disseminated (final concentration: 1 nM) to each well and incubated at room temperature for 1 hour. After washing three times, a human IgG solution (100 μg/mL, Mitsubishi Pharma) and antibody A (10 μg/mL) were added to each well and incubated at room temperature for 1 hour. A horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well. After confirmation of moderate color development, an equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (PerkinElmer).

Antibody A specifically had reaction activity only with human EphA4 among the members of the human Eph receptor family (FIG. 6A).

[0161]

Example 8: Selectivity of anti-EphA4 monoclonal antibody for mouse Eph receptor

According to the method described in Example 1, a DNA sequence encoding the signal sequence and the extracellular region of each mouse Eph receptor (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) was amplified by RT-PCR using tissue-derived total RNA and cloned into a pENTR1A vector (Invitrogen/Life Technologies) having a DNA sequence encoding a human IgG1 Fc region and histidine tag. Next, the DNA sequence encoding the signal sequence and the extracellular region of each mouse Eph receptor (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6), Fc, and histidine tag was transferred to a pcDNA3.1_rfcB vector through LR reaction using Gateway System (Invitrogen/Life Technologies) to construct each mouse Eph receptor extracellular region-Fc-His protein expression vector. For the construction of a mouse EphA2 extracellular region-Fc-His protein expression vector, a DNA sequence encoding the signal sequence and the extracellular region of mouse EphA2 was amplified by RT-PCR using tissue-derived total RNA and cloned into a pcDNA3.1 vector having a DNA sequence encoding Fc and histidine tag to construct a mouse EphA2 extracellular region-Fc-His protein expression vector.

[0162]

Next, HEK293EBNA cells (Life Technologies) were inoculated to a 10-cm dish (Falcon or BD Biosciences) and cultured at 37°C for 1 day. The HEK293EBNA cells were transfected with the mouse EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, or EphB6 extracellular region-Fc-His protein expression vector obtained as described above using TransIT-LT1 (TAKARA). After incubation (5% CO₂, 37°C) for 4 days, the culture supernatant was recovered and centrifuged at 1500 rpm at room temperature for 5 minutes. The centrifugation supernatant was filtered through a 0.22-μm filter (Merck Millipore), and Hepes (Dojindo

Laboratories) and sodium azide (Wako Pure Chemical Industries) were added thereto at final concentrations of 20 mM and 0.02%, respectively.

[0163]

Antibody A was evaluated for its binding activity against each mouse Eph receptor according to the following steps.

Each well of a 96-well plate (Nunc) was coated with a donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Thermo Fisher Scientific) three times, each mouse Eph receptor extracellular region-Fc-His protein was inoculated (final concentration: 1 nM) to each well and incubated at room temperature for 1 hour. After washing three times, a human IgG solution (100 µg/mL, Sigma-Aldrich) and antibody A (10 µg/mL) were added to each well and incubated at room temperature for 1 hour. A horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well. After confirmation of moderate color development, an equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (PerkinElmer).

Antibody A specifically had reaction activity only with mouse EphA4 among the members of the mouse Eph receptor family (FIG. 6B).

[0164]

Example 9: Reactivity of anti-EphA4 monoclonal antibody with mouse, rat, monkey, and human EphA4

Mouse, rat, monkey, and human EphA4 extracellular region-Fc-His proteins were prepared according to the following steps. First, according to the method described in Example 1, a monkey EphA4 extracellular region-Fc-His protein expression vector was constructed. The amino acid sequence of monkey EphA4 used in the vector

construction is shown in SEQ ID NO: 44, and its extracellular region is shown in SEQ ID NO: 45. Next, HEK293EBNA cells (Life Technologies) were inoculated to a 10-cm dish (Falcon) and cultured at 37°C for 1 day. The HEK293EBNA cells were transfected with the monkey EphA4 extracellular region-Fc-His protein expression vector or the mouse EphA4, rat EphA4, or human EphA4 extracellular region-Fc-His protein expression vector described in Example 1 using TransIT-LT1 (TAKARA). After incubation (5% CO₂, 37°C) for 4 days, the culture supernatant was recovered and centrifuged at 1500 rpm at room temperature for 5 minutes. The centrifugation supernatant was filtered through a 0.22-μm filter (Merck Millipore), and Hepes (Dojindo Laboratories) and sodium azide (Wako Pure Chemical Industries) were added thereto at final concentrations of 20 mM and 0.02%, respectively.

[0165]

Antibody A was evaluated for its binding activity against various Eph receptors according to the following steps.

Each well of a 96-well plate (Nunc) was coated with a donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (Sumitomo Dainippon Pharma) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Nacalai Tesque) three times, the mouse, rat, monkey, or human EphA4 extracellular region-Fc-His protein was disseminated (final concentration: 1 nM) to each well and incubated at room temperature for 1 hour. After washing three times, a human IgG solution (100 μg/mL, Mitsubishi Pharma) and antibody A (0, 0.00128, 0.0064, 0.032, 0.16, 0.8, 4, and 20 μg/mL) were added to each well and incubated at room temperature for 1 hour. A horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well. After confirmation of moderate color development, an equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader

(PerkinElmer).

[0166]

Antibody A had equivalent reaction activity against all of mouse, rat, monkey, and human EphA4 (FIG. 7).

[0167]

Example 10: Inhibitory effect of anti-EphA4 monoclonal antibody on ligand-induced EphA4 autophosphorylation in hippocampal neurons

Rat hippocampal neurons were prepared according to the following steps. A fetus was removed from an 18-day pregnant rat (Charles River Laboratories Japan), and its head was incised to isolate the brain. A hippocampal region was excised under a stereoscopic microscope, then placed in a digestion solution (137 mM NaCl (Wako Pure Chemical Industries), 5 mM KCl (Wako Pure Chemical Industries), 7 mM Na₂HPO₄ (Wako Pure Chemical Industries), 25 mM Hepes (Dojindo Laboratories), 0.5 mg/ml DNase (Sigma-Aldrich), and 0.25% trypsin (Life Technologies)), and shaken at 37°C for 10 minutes. The solution was removed, and 20% fetal bovine serum/Hanks buffer solution (Sigma-Aldrich) was added to the hippocampal tissues. The solution was removed, and the hippocampal tissues were washed with a Hanks buffer solution twice and then pipetted in a Hanks buffer solution to prepare a cell suspension. The cells were inoculated to a 6-well dish (Falcon) coated with poly-L-lysine containing a culture solution (Neurobasal medium (Life Technologies), 1 × B-27 supplement (Life Technologies), and 0.5 mM L-glutamine (Life Technologies)).

[0168]

The evaluation of EphA4 autophosphorylation inhibitory activity using the hippocampal neurons was conducted according to the following steps. The rat hippocampal neurons inoculated to a 6-well dish (Falcon) were treated with mouse Ephrin A1-Fc chimera (R&D Systems, final concentration: 10 nM), antibody A (0, 1, 10, 100, and 1000 nM) or KYL peptide (KYL PYWPVLSSL, an EphA4 inhibitor, its synthesis was outsourced to Hokkaido System Science, 0, 0.01, 0.1, 1, 10, and 100 μM), and washed with cold-PBS (Wako Pure Chemical Industries) 45 minutes later. A lysis buffer (20

mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (Wako Pure Chemical Industries), 1 × protease inhibitor (Nacalai Tesque), and 1 × phosphatase inhibitor (Nacalai Tesque)) was added thereto to recover the cells. After mixing at 4°C for 15 minutes, the supernatant was recovered by refrigerated centrifugation at 15000 rpm at 4°C for 15 minutes. A rabbit anti-EphA4 polyclonal antibody (Medical & Biological Laboratories) was added to the supernatant and reacted for 90 minutes. Then, protein G beads (GE Healthcare) were added thereto and further reacted for 30 minutes. The supernatant was removed by refrigerated centrifugation at 3000 rpm at 4°C for 1 minute, followed by the addition of 1 mL of a lysis buffer. This operation was performed three times. Then, 2 × SDS sample buffer was added to each sample, which was then boiled for 10 minutes. This sample was used in SDS-PAGE and Western blotting using an anti-phosphorylated tyrosine antibody (Santa Cruz Biotechnology). Western blotting using an anti-EphA4 monoclonal antibody (Abnova) was further performed, and band intensity was quantified to calculate a value of phosphorylated EphA4/total EphA4. The anti-EphA4 monoclonal antibody (Abnova), whose immunogen is a synthetic peptide for a C-terminal region of human EphA4, is recognized as an antibody lacking neutralizing activity against human EphA4 having a N-terminal extracellular region.

[0169]

Antibody A and KYL peptide (an EphA4 inhibitor) suppressed, in a concentration-dependent manner, EphA4 autophosphorylation induced by mouse EphrinA1 in the hippocampal neurons, and the IC₅₀ values were 24.2nM and 9.91μM, respectively (FIG. 8). These results demonstrated that antibody A antagonizes EphA4/ephrin signaling, as with KYL peptide, in cell systems.

[0170]

Example 11: Inhibitory effect of anti-EphA4 monoclonal antibody on ligand-induced growth cone collapse in hippocampal neurons

Rat hippocampal neurons were prepared as described in Example 10 above. The cells were inoculated to a 96-well plate (Greiner Bio-One) coated with poly-L-lysine containing culture solution.

[0171]

The growth cone collapse assay using the hippocampal neurons was conducted according to the following steps. The rat hippocampal neurons of culture day 2 inoculated to the 96-well dish (Greiner Bio-One) were treated with PBS (Wako Pure Chemical Industries), antibody A (0.1, 0.3, and 1 μ M), or an EphA4 inhibitor KYL peptide (KYLPYWPVLSSL, 10, 30, and 100 μ M, its synthesis was outsourced to Toray Research Center) for 15 minutes and then treated with goat anti-human Fc γ fragment IgG1 antibody (Jackson ImmunoResearch Laboratories)-preclustered mouse Ephrin A1-Fc chimera (R&D Systems, final concentration: 1 μ g/mL) (ratio: 1:5) for 30 minutes. Then, the culture solution was removed, and 2% PFA (Wako Pure Chemical Industries)/4% sucrose (Wako Pure Chemical Industries)/PBS was added, and left standing for 20 minutes to fix the cells. The solution was removed, and the cells were washed with PBS three times, followed by the addition of 0.25% Triton X-100 (Wako Pure Chemical Industries)/PBS for cell penetration treatment for 15 minutes. The solution was removed, and blocked for 1 hour by the addition of 2% BSA (Sigma-Aldrich)/0.25% Triton X-100/Opti-MEM (Life technologies) and then reacted with an anti-Tau-1 antibody (Merck Millipore) and an anti-MAP-2 antibody (Merck Millipore) for 2 hours. The primary antibody solution was removed, and was washed with PBS three times and reacted with a secondary antibody and Alexa Fluor 546 Phalloidin (Molecular Probes) for 1 hour. The secondary antibody solution was removed, and was washed with PBS three times, and then were enclosed by the addition of SlowFade Gold Antifade Reagents (Molecular Probes) and observed under BIOREVO (Keyence). Neurons forming growth cone were counted in 30 fields of view per sample to calculate the proportion of the number of neurons causing growth cone collapse.

[0172]

Antibody A and the EphA4 inhibitor KYL peptide suppressed, in a concentration-dependent manner, growth cone collapse induced by mouse Ephrin A1 in the hippocampal neurons (FIG. 9). Accordingly, it is shown that antibody A functionally inhibits EphA4, as with KYL peptide, in cell systems.

[0173]

Example 12: Ligand antagonistic effect of anti-EphA4 monoclonal antibody in vivo

The *in vivo* competition assay using mouse newborns was conducted according to the following steps. PBS (Wako Pure Chemical Industries), antibody A, or a control antibody (mouse anti-dinitrophenol antibody) produced with a conventional procedure by immunizing rat with dinitrophenol was subcutaneously administered at a dose of 300 mg/kg (30 mL/kg) to each 8-day-old mouse (Charles River Laboratories Japan). After 24 hours, the scalp was incised under 4% isoflurane (Intervet) anesthesia, and mouse Ephrin A1-Fc chimera (300 pmol/head, R&D Systems) or PBS (Wako Pure Chemical Industries) was administered into the lateral ventricle. 1 hour thereafter, the mouse was euthanized by decapitation, followed by the extirpation of the cerebral hemisphere. The collected cerebral hemisphere was placed in a filter tube loaded in a tube for recovery of BioMasher(R) I (Nippi). After insertion of a crusher rod, the cerebral hemisphere was homogenized by refrigerated centrifugation at 15000 rpm at 4°C for 2 minutes. The homogenate was suspended in TNE buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 × protease inhibitor (Nacalai Tesque), 1 × phosphatase inhibitor (Nacalai Tesque)). 3 × SDS sample buffer was added to a portion of the homogenate, which was then boiled for 10 minutes, followed by protein quantification. This sample was used in SDS-PAGE and Western blotting using a donkey anti-human IgG (H+L) antibody (Jackson ImmunoResearch Laboratories), an anti-EphA4 monoclonal antibody (Abnova), and an anti-actin antibody (Sigma-Aldrich). The remaining homogenate was subjected to protein quantification and then dispensed in an amount corresponding to 3 mg of protein, and 1% Triton X-100 (Wako Pure Chemical Industries) and 0.1% SDS (Nacalai Tesque) were added thereto. After mixing at 4°C for 15 minutes, the supernatant was recovered by refrigerated centrifugation at 15000 rpm at 4°C for 15 minutes. 3 × SDS sample buffer was added to a portion of the supernatant, which was then boiled for 10 minutes to prepare an input sample. This sample was used in SDS-PAGE and Western blotting using an anti-EphA4 monoclonal antibody (Abnova) and an anti-actin antibody (Sigma-Aldrich). A rabbit anti-EphA4 polyclonal antibody (Santa Cruz Biotechnology) was

added to the remaining supernatant and reacted for 60 minutes. Then, protein G beads (GE Healthcare) were added thereto and further reacted for 30 minutes. The supernatant was removed by refrigerated centrifugation at 3000 rpm at 4°C for 2 minutes, followed by the addition of 0.5 mL of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (Wako Pure Chemical Industries), 0.1% SDS (Nacalai Tesque), 1 × protease inhibitor (Nacalai Tesque), 1 × phosphatase inhibitor (Nacalai Tesque)). This operation was performed three times. Then, 1 × SDS sample buffer was added to the beads, which were then boiled for 10 minutes to prepare a bead sample. This sample was used in SDS-PAGE and Western blotting using an anti-phosphorylated tyrosine antibody (Santa Cruz Biotechnology). Western blotting using an anti-EphA4 monoclonal antibody (Abnova) was further performed, and band intensity was quantified to calculate a value of phosphorylated EphA4/total EphA4. The anti-EphA4 monoclonal antibody (Abnova), whose immunogen is a synthetic peptide for a C-terminal region of human EphA4, is recognized as an antibody lacking neutralizing activity against human EphA4 having a N-terminal extracellular region.

[0174]

The administration of mouse Ephrin A1 into the lateral ventricle of the mouse newborn induced EphA4 autophosphorylation in a cerebral hemisphere. Antibody A suppressed mouse EphrinA1-induced EphA4 autophosphorylation by 66% (FIG. 10). Accordingly, it is shown that antibody A also inhibits the binding between EphA4 and its ligand *in vivo*.

[0175]

Example 13: Motor neuron protective effect of anti-EphA4 monoclonal antibody in *in vitro* ALS model derived from mouse ES cells

Mouse ES cells were maintained and cultured according to the following steps. Mouse ES cells (129×1/SvJ) cryopreserved at -80°C were thawed in a thermostat bath and then diluted with a mouse ES cell culture medium (KnockOut(TM) DMEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 0.1 mM β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% nonessential

amino acids (Invitrogen), 100 units/mL penicillin-100 µg/mL streptomycin (Invitrogen), and 1000 units/mL ESGRO(R) leukemia inhibitory factor (Merck Millipore)) warmed to 37°C. Each cell suspension was centrifuged (1500 rpm, 3 min, room temperature), followed by the removal of the supernatant. The cells were suspended in a fresh medium, then transferred to a culture dish with feeder cells inoculated in advance, and maintained and cultured in a CO₂ incubator (5% CO₂, 37°C).

[0176]

Astrocytes were established from a mouse newborn and maintained and cultured according to the following steps. A two-day-old wild-type mouse newborn (C57BL/6JJmsSlc (Japan SLC)) and a hybrid mouse newborn of a wild-type mouse and a variant human SOD1 (G93A) Tg-(B6.Cg-Tg(SOD1_G93A)1Gur/J (Jackson ImmunoResearch Laboratories)) mouse were each euthanized by inhalation anesthesia with isoflurane (Intervet) or decapitation. Then, the cerebral cortex was isolated from each mouse and dispersed by treatment with 0.25% trypsin-EDTA (Invitrogen) at 37°C for 15 minutes. After the enzymatic treatment, the cells were diluted with 4 mL of Dulbecco's Modified Eagle Medium (Gibco) containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen) (10% FBS-DMEM) to terminate the enzymatic digestion. Then, impurities other than single cells were subject to filtration using a cell strainer (BD Biosciences), and the cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated, diluted with 4 mL of fresh 10% FBS-DMEM, inoculated to a 60-mm culture dish on an individual basis, and cultured at 37°C. Two days after the inoculation, the medium was aspirated and replaced by the addition of 4 mL of fresh 10% FBS-DMEM. After reaching confluency, a culture supernatant containing non-adherent cells was recovered and subjected to genotyping of variant human SOD1 (G93A). 2 mL of PBS (Wako Pure Chemical Industries) was newly added to the supernatant and aspirated again. 1 mL of 0.25% trypsin-EDTA was added to the cells and incubated at 37°C for 3 minutes. The enzymatic treatment was terminated with 3 mL of 10% FBS-DMEM, and the cells were centrifuged at 1500 rpm for 3 minutes. After the centrifugation, the medium was aspirated, and 6 mL of fresh 10% FBS-DMEM was

added to the cells, which were each inoculated to a 100-mm culture dish and subcultured (passage number: 2). After reaching confluency, the medium was aspirated, and 3 mL of PBS (Wako Pure Chemical Industries) was then added to the cells and aspirated again. 2 mL of 0.25% trypsin-EDTA was added to the cells and incubated at 37°C for 3 minutes. The enzymatic treatment was terminated with 4 mL of 10% FBS-DMEM, and the cells were centrifuged at 1500 rpm for 3 minutes. After the centrifugation, the medium was aspirated, and 12 mL of fresh 10% FBS-DMEM was added to the cells, then 6 mL of which were each inoculated to a 100-mm culture dish and subcultured (passage number: 3). The medium of the astrocytes at a passage number of 3 was aspirated, and 2 mL of PBS (Wako Pure Chemical Industries) was added to the cells and aspirated again. 2 mL of 0.25% trypsin-EDTA was added to the cells and incubated at 37°C for 3 minutes. The enzymatic treatment was terminated by the addition of 4 mL of 10% FBS-DMEM. The suspension was recovered, centrifuged at 1500 rpm for 3 minutes, diluted with Cell banker (Nippon Zenyaku Kogyo), and cryopreserved at -80°C until subjected to a test. When subjected to a test, each cryopreserved cell suspension was thawed in a thermostat bath and then diluted with 10% FBS-DMEM warmed to 37°C. After centrifugation (1500 rpm, 3 min, room temperature) of each cell suspension, the supernatant was removed, and the cells were suspended in a fresh medium, then inoculated to an 8-well chamber (ibidi), and maintained and cultured in a CO₂ incubator (5% CO₂, 37°C).

[0177]

The genotyping of variant human SOD1 (G93A) was conducted using REDEExtract-N-Amp(TM) Tissue PCR kit (Sigma-Aldrich). In the step of maintaining and culturing the mouse ES cells, the culture supernatant containing non-adherent cells of variant human SOD1 (G93A)-expressing astrocytes recovered during the subculture was recovered into a 1.5-mL tube and centrifuged at 1500 rpm for 3 minutes. After the centrifugation, the supernatant was aspirated, and the cells were washed by the addition of 1 mL of PBS and centrifuged again, followed by the aspiration of PBS. 50 µL of an extraction solution and 12.5 µL of a tissue preparation solution were mixed and added to each sample. After mixing, the mixture was transferred to a polymerase chain

reaction (PCR) tube, followed by genome extraction at a cycle of 55°C for 10 minutes → 95°C for 3 minutes → 4°C ∞ using GeneAmp(R) PCR system 9700 (Applied Biosystems(R)). Then, the reaction solution was neutralized by the addition of 50 µL of neutralization solution B.

[0178]

The extracted genome was used in genomic PCR according to the composition shown in Table 3. The primer sequences used in the PCR are shown in Table 4. Each resulting PCR product was electrophoresed on a 1% agarose gel at 100 V for 20 minutes. Samples with two bands of a 324-bp internal standard and the 236-bp variant human SOD1 (G93A) detected were identified as variant human SOD1 (G93A)-expressing astrocytes.

[0179]

[Table 3]

Table 3 Genomic PCR Mixture

Reagent name	Liquid volume
Template:	1 µ L
R e d m i x :	5 µ L
Primer 1 (100 µ m o l / L):	0.05 µ L
Primer 2 (100 µ m o l / L):	0.05 µ L
Primer 3 (100 µ m o l / L):	0.05 µ L
Primer 4 (100 µ m o l / L):	0.05 µ L
Distilled water	3.8 µ L
Total:	10 µ L

Red mix = REDExtract-N-Amp PCR reaction mix

[0180]

[Table 4]

Table 4 Nucleotide sequences of primers

Primer 1	Variant human <i>SOD1</i> (G93A)	CATCAGCCCTAATCCATCTGA
Primer 2	Variant human <i>SOD1</i> (G93A)	CGCGACTAACAATCAAAGTGA
Primer 3	Internal standard	CTAGGCCACAGAATTGAAAGATCT
Primer 4	Internal standard	GTAGGTGGAAATTCTAGCATCATCC

[0181]

The motor neuron protective effect in the *in vitro* ALS models was evaluated according to the following steps. The cultured and maintained mouse ES cells were treated with 0.25% trypsin/0.05% EDTA solution (Gibco) to dissociate the cells from the culture dish. The cells were recovered by centrifugation, and a suspension was then prepared and inoculated at 1.2×10^5 cells/mL to a low adsorptive 12-well plate (Nunc) (day 0). Floating culture of aggregates was performed for 2 days in a DFK medium (advanced DMEM/F-12 (Invitrogen):Neurobasal (Invitrogen) [1:1] medium containing 5% KnockOut serum replacement (Invitrogen), 2 mM L-glutamine, 100 units/mL penicillin-100 µg/mL streptomycin, and 0.1 mM β-mercaptoethanol). At day 2, the DFK medium was replaced with a DFK medium containing 1 µM retinoic acid (Sigma-Aldrich) and 2 µM purmorphamine (Stemgent). Then, medium replacement was performed at a frequency of once every two days. The differentiation into motor neurons was induced by culture for 5 days (days 3 to 7).

[0182]

At day 7, the cell masses of differentiated motor neurons were dispersed in a cell dispersion solution Accumax (MS Tech) and prepared into a suspension having a cell density of 5.5×10^5 cells/mL. The suspension was inoculated at 200 µL/well to the 8-well chamber containing the mouse-derived wild-type astrocytes or the variant human SOD1 (G93A)-expressing astrocytes cultured and maintained in advance, and the resulting cocultured cells of the astrocytes and the motor neurons were used in evaluation.

[0183]

The number of motor neurons observed by the coculture of the wild-type astrocytes and the motor neurons was used as a control. For the drug-treated group, the variant human SOD1 (G93A)-expressing astrocytes and the motor neurons were cocultured under a condition involving vehicle addition (IgG and 0.1% ultrapure water), antibody A (10, 30, and 100 nM), EphA4-Fc (R&D Systems, 3, 10, and 30 nM), or KYL peptide (Toray Research Center, 1, 3, and 10 μ M). After culture for 2 days at 37°C in a 5% CO₂ environment under each condition, the motor neurons were immunocytochemically stained with an anti-rabbit ISL1 antibody (Abcam) and Hoechst 33342 (Molecular Probes). ISL1/Hoechst 33342-copositive cells per unit area were counted as live motor neurons, and the survival rate of the motor neurons was calculated as a percentage (%) with respect to the control. FIG. 11 shows a simple schematic view showing the steps of the evaluation system.

[0184]

The survival rate of the motor neurons was significantly reduced in the variant human SOD1 (G93A)-expressing astrocyte/mouse ES cell derived-motor neuron coculture (40-50%). The antibody A suppressed, in a concentration-dependent manner, mouse ES cell derived-motor neuron death induced by the variant human SOD1 (G93A)-expressing astrocytes (FIG. 12). The treatment with KYL peptide or EphA4-Fc, as with antibody A, was confirmed to have a motor neuron protective effect in this experimental system, showing that antibody A promotes the survival of mouse ES cell-derived motor neurons by inhibiting EphA4/ephrin signaling in this in vitro ALS model.

[0185]

Example 14: Motor neuron protective effect of anti-EphA4 monoclonal antibody in *in vitro* ALS model derived from human iPS cells

Human iPS cells were maintained and cultured according to the following steps. Human iPS cells (201B7) cryopreserved in liquid nitrogen using Stem cell banker (TAKARA) were taken out of the gas phase of liquid nitrogen and immediately suspended and thawed in 5 mL of a human iPS cell culture medium (Essential 8, Thermo Fisher

Scientific) prewarmed to 37°C. The cell suspension was recovered into a 15-mL conical tube (Falcon) and centrifuged (1000 rpm, 5 min, room temperature), followed by the removal of the supernatant. The cells were suspended in a fresh medium and then disseminated to a ϕ 60 mm cell culture dish (Falcon BD) coated with 0.5 μ g/cm² Human recombinant vitronectin (Invitrogen) in advance. 10 μ M Y-27632 (Wako Pure Chemical Industries) was added thereto, and the cells were maintained and cultured in a CO₂ incubator (5% CO₂, 37°C). Medium replacement was performed every day, and the cells were subjected to the experiment when reaching confluency.

[0186]

The motor neuron protective effect in the *in vitro* ALS models was evaluated according to the following steps. The culture medium of the maintained and cultured human iPS cells was aspirated, and the cells were washed with 2 mL of PBS (Wako Pure Chemical Industries). After aspiration of PBS, 500 μ L of 0.5 mM EDTA was added to the cells, which were then incubated for 2 to 3 minutes in a CO₂ incubator (5% CO₂, 37°C) (the cells were confirmed under a microscope every 30 seconds, and the incubation was discontinued when the intercellular association became weak). The EDTA reaction was terminated by suspension in 5 mL of a human iPS cell culture medium, and the cells were recovered into a 15-mL conical tube. The cells were centrifuged at 1000 rpm at room temperature for 5 minutes, and the supernatant was aspirated. The cell suspension containing human iPS cell masses was inoculated in an amount of approximately 1/10 per well to a low-adhesion 6-well cell culture plate (Nunc) and cultured in a CO₂ incubator (5% CO₂, 37°C) using a DFK medium (advanced DMEM/F-12 (Invitrogen):Neurobasal medium (Invitrogen) [1:1] medium containing 2% B27 supplement, 5% KnockOut serum replacement (Invitrogen), 2 mmol/L L-glutamine, 100 units/mL penicillin-100 μ g/mL streptomycin, and 0.1 mmol/L β -mercaptoethanol) supplemented with 2 μ M SB431542 (Sigma-Aldrich), 300 nM LDN193189 (Sigma-Aldrich), and 3 μ M CHIR99021 (Sigma-Aldrich). Medium replacement was performed every 2 days by the following method. First, a human iPS cell differentiated cell aggregates (SFEBs) was recovered on a medium basis into a 15-mL conical tube and left

standing at ordinary temperature for 5 minutes to precipitate the cell masses. This supernatant was aspirated, and a fresh DFK medium and 2 μ M SB431542 (Sigma-Aldrich), 300 nM LDN193189 (Sigma-Aldrich), 3 μ M CHIR99021 (Sigma-Aldrich) were added, and then brought back to the original well for medium replacement. At culture day 8, the SFEBs were recovered on a medium basis into a 15-mL conical tube and left standing at ordinary temperature for 5 minutes to precipitate the SFEBs. This supernatant was aspirated, and a fresh DFK medium and then 0.1 μ M retinoic acid (Sigma-Aldrich) and 0.5 μ M purmorphamine (Miltenyi Biotec) were added, and then brought back to the original well and cultured in a CO₂ incubator (5% CO₂, 37°C). Medium replacement was performed every 2 days. At culture day 12, the SFEBs were recovered on a medium basis into a 15-mL conical tube and left standing at ordinary temperature for 5 minutes to precipitate the SFEBs. The supernatant was aspirated, and 500 μ L of Accumax (MS TechnoSystems) was added to the cells, which were then pipetted several times and then incubated for 5 minutes in a CO₂ incubator (37°C, 5% CO₂). The cells were taken out of the incubator, suspended in 5 mL of a DFK medium, and pipetted several times to disperse the cell masses. The cell suspension was dissociated into single cells by filtration through a cell strainer (Falcon). Then, the number of cells was counted using a counting chamber. The cell suspension was recovered into another 15-mL conical tube and centrifuged at 1000 rpm at room temperature for 5 minutes. A suspension having a cell density of 5.5×10^5 cells/mL was prepared with a motor neuron culture medium (advanced DMEM/F-12 (Invitrogen):Neurobasal medium (Invitrogen) [1:1] medium containing 2% B27 Supplement, 1% horse serum, 2 mmol/L L-glutamine, 100 units/mL penicillin-100 μ g/mL streptomycin, and 0.1 mmol/L β -mercaptoethanol) and inoculated at 200 μ L/well to an 8-well chamber containing mouse-derived wild-type astrocytes or variant human SOD1 (G93A)-expressing astrocytes inoculated at 8×10^4 cells/well in advance. The resulting cocultured cells of the astrocytes and the motor neurons were used in evaluation (the establishment, freezing, thawing, inoculation, and maintenance and culture of the wild-type and human variant SOD1 (G93A)-expressing astrocytes were performed in the

same way as in Example 13). The number of motor neurons observed by the coculture of the wild-type astrocytes and the motor neurons was used as a control. For the drug-treated group, the variant human SOD1 (G93A)-expressing astrocytes and the motor neurons were cocultured under a condition involving vehicle addition (IgG and 0.1% ultrapure water), antibody A (10, 30, and 100 nM), the EphA4 inhibitor KYL peptide (KYLPYWPVLSSL, 1, 3, and 10 μ M, its synthesis was outsourced to Toray Research Center), or EphA4-Fc (3, 10, and 30 nM, R&D systems). After culture for 2 days at 37°C in a 5% CO₂ environment under each condition, the motoneurons were immunocytochemically stained with an anti-ISL1 antibody (obtained from Developmental Studies Hybridoma Bank) and Hoechst 33342 (Molecular Probes). ISL1/Hoechst 33342-copositive cells per well were counted as live motor neurons, and the survival rate of the motor neurons was calculated as % with respect to the control. FIG. 13 shows a simple schematic view showing the steps of the evaluation system.

[0187]

The survival rate of the motor neurons was significantly reduced (approximately 50%) in the variant human SOD1 (G93A)-expressing astrocyte/human iPS cell-derived motor neuron coculture, as with the assay system using mouse ES cells. The antibody A suppressed, in a concentration-dependent manner, human iPS cell-derived motor neuron death induced by the variant human SOD1 (G93A)-expressing astrocytes (FIG. 14). The treatment with KYL peptide or EphA4-Fc, as with antibody A, was confirmed to have a human iPS cell-derived motor neuron protective effect in this experimental system. Accordingly, it is shown that antibody A also promotes the survival of motor neuron by inhibiting the binding between EphA4 and its ligand in human cells.

[0188]

Example 15: Epitope mapping of EphA4 Ligand-Binding Domain (EphA4-LBD) by X-ray crystallography

In order to prepare a complex of antibody A-Fab prepared in Example 5 and an antigen EphA4-LBD, EphA4-LBD was prepared (Qin H. et al., J. Biol. Chem., 283:

29473-29484 (2008)). 1.33 $\cdot$ mol (950 $\cdot$ M, 1.4 ml) of EphA4-LBD and 0.9 $\cdot$ mol (150 $\cdot$ M, 6 ml) of antibody A-Fab were mixed such that EphA4-LBD had approximately 1.5 times the molar ratio of antibody A-Fab. The mixture was incubated on ice for 30 minutes. Next, the mixed solution was applied to HILOAD 26/60 Superdex 75 prep grade (GE Healthcare), followed by elution with a buffer solution for chromatography (25 mM Tris/HCl (pH 7.5), 100 mM NaCl). Fractions containing the complex were analyzed by SDS PAGE, and highly pure fractions were collected and concentrated into 34 mg/ml. This concentrate was used in crystallization.

[0189]

The crystallization of the complex was performed by the sitting drop vapor diffusion method using an automatic crystallization apparatus Hydra II Plus One system (Matrix Technologies). The plate used was MRC-2 (Molecular Dimensions). The composition of a reservoir solution was 100 mM Tris/HCl (pH 7.5 to 8.5) and 30% polyethylene glycol 400. This reservoir solution and the complex solution described above were mixed at a volume ratio of 1:1 to prepare crystallization droplets. The prepared crystallization plate was left standing at 20°C.

[0190]

As a result of performing crystallization under the conditions described above, crystals having a space group of P212121, lattice constant a of 70.0 angstroms, lattice constant b of 82.3 angstroms, and lattice constant c of 216.0 angstroms were obtained. Diffraction data at 2.1 angstroms was obtained by the incidence of synchrotron X-ray (1.0 angstroms) to the obtained crystals. The diffraction data was processed with HKL2000 (HKL Research Inc.), and its phase determination was performed by the molecular replacement method. The molecular replacement method employed a program PHASER (version 2.5.0, McCoy A.J. et al., J. Appl. Cryst. 40: 658-674 (2007)) contained in CCP4 Software Suite (Collaborative computational project number 4, [CCP4] version 6.5.0, Acta Cryst. D 67: 235-242 (2011)). The search models used in the molecular replacement method were the crystal structure (PDBID:3CKH) of EphA4-LBD and the crystal structure of Fab of a different antibody determined in the past by

the present inventors. A molecular model appropriate for an electron density obtained from the determined phase was constructed using a program COOT (Emsley P. et al., Acta Cryst. D 60: 2126-2132 (2004)) and subjected to structure refinement using a program REFMAC (Murshudov G.N., Acta Cryst. D 53: 240-255 (1997)).

In this way, the complex crystal structure having a resolution of 2.1 angstroms was obtained by structure calculation ($R = 0.234$, $R_{\text{free}} = 0.288$).

[0191]

The obtained crystal structure of the Fab/EphA4-LBD complex was analyzed using an interaction detection tool installed in a computational chemical system MOE 2011.10 (Chemical Computing Group Inc.) to identify amino acid residues on EphA4-LBD directly interacting with Fab (FIG. 15). Standard settings of MOE were used as detection protocol. The identified amino acid residues were Ser58, Met60, Gln71, Val72, Cys73, Thr104, Arg106, Gln156, Asp161, Arg162, Ile163, Cys191, and Ile192. FIG. 16 shows the surface structure of EphA4-LBD prepared using Maestro (version 10.6, Schrodinger). As a result, the present inventors concluded that regions having these amino acid residues are Fab-binding regions of EphA4-LBD.

[0192]

Example 16: Preparation of humanized antibody of antibody A

Preparation of humanized anti-human EphA4 antibody

Variable regions of each humanized antibody were designed. On the basis of high homology to the framework regions (FRs) of antibody A, human antibody light chain FRs IGKV1-NL1*01 (SEQ ID NO: 50) or IGKV3D-15*01 (SEQ ID NO: 51) and JK1 (SEQ ID NO: 52) and heavy chain FRs IGHV7-4-1*02 (SEQ ID NO: 53) and JH6 (SEQ ID NO: 54) were selected as humanized antibody FRs. Then, FR amino acids interacting with CDR amino acids were predicted using the 3D structural prediction model of mouse antibody A and used in grafting with CDRs (SEQ ID NOs: 26 to 30, and 31 to 33). In light of the enhancement of EphA4 phosphorylation and the ADCC activity, a human IgG₂ heavy chain constant region (SEQ ID NO: 62) which had C131S, C219S, V234A, and G237A mutations and lacked a C-terminal lysine residue, or a heavy chain constant

region (SEQ ID NO: 60) which contained human IgG₁-derived CH1 and hinge region and human IgG₂-derived CH2 and CH3 having V234A and G237A mutations and lacking a C-terminal lysine residue, was used as a heavy chain constant region. Human Igκ light chain constant region (SEQ ID NO: 64) was used as a light chain constant region. HK1 (SEQ ID NO: 72), HK2 (SEQ ID NO: 74), and HK4 (SEQ ID NO: 76) were designed as humanized antibody heavy chain variable regions carrying grafted CDRs (SEQ ID NOs: 26, 28, and 30) determined by the Kabat definition method. HA1 (SEQ ID NO: 66), HA2 (SEQ ID NO: 68), and HA4 (SEQ ID NO: 70) were designed as humanized antibody heavy chain variable regions carrying grafted CDRs (SEQ ID NOs: 27, 29, and 30) determined by the AbM definition method. L1-4 (SEQ ID NO: 78), L1-5 (SEQ ID NO: 80), and L1-6 (SEQ ID NO: 82) were designed as humanized antibody light chain variable regions using IGKV1-NL1*01 and JK1. L2-4 (SEQ ID NO: 84) was designed as a humanized antibody light chain variable region using IGKV3D-15*01 and JK1. Incidentally, in the design of the heavy chain constant regions, EphA4 phosphorylation was confirmed in the same way as that described in Example 10.

[0193]

A gene sequence encoding the amino acid sequence of HK1, HK2, or HK4 was synthesized by converting the amino acid sequence of heavy chain CDRs (SEQ ID NOs: 26, 28, and 30) of antibody A grafted in IGHV7-4-1*02 (SEQ ID NO: 53) and JH6 (SEQ ID NO: 54) with a signal sequence (SEQ ID NO: 55) further added to the N terminus, to a gene sequence by GenScript USA Inc., and prepared by PCR mutagenesis (HK1: SEQ ID NO: 73, HK2: SEQ ID NO: 75, HK4: SEQ ID NO: 77, signal sequence: SEQ ID NO: 57). A gene sequence encoding the amino acid sequence of HA1, HA2, or HA4 was synthesized by converting the amino acid sequence of heavy chain CDRs (SEQ ID NOs: 27, 29, and 30) of antibody A grafted in IGHV7-4-1*02 (SEQ ID NO: 53) and JH6 (SEQ ID NO: 54) with a signal sequence (SEQ ID NO: 55) further added to the N terminus, to a gene sequence by GenScript USA Inc., and prepared by PCR mutagenesis (HA1: SEQ ID NO: 67, HA2: SEQ ID NO: 69, HA4: SEQ ID NO: 71, signal sequence: SEQ ID NO: 56). The genes encoding these humanized heavy chain variable regions and signal

sequences were inserted to expression vectors (pcDNA3.4) containing a gene sequence (SEQ ID NO: 63) encoding the human IgG₂ constant region (SEQ ID NO: 62) which had C131S, C219S, V234A, and G237A mutations and lacked a C-terminal lysine residue, or expression vectors (pcDNA3.4) containing a gene sequence (SEQ ID NO: 61) encoding the constant region (SEQ ID NO: 60) which contained human IgG₁-derived CH1 and hinge and human IgG₂-derived CH2 and CH3 having V234A and G237A mutations. A gene sequence encoding the amino acid sequence of L1-4, L1-5, or L1-6 was synthesized by converting the amino acid sequence of light chain CDRs (SEQ ID NOs: 31 to 33) of antibody A grafted in IGKV1-NL1*01 (SEQ ID NO: 50) and JK1 (SEQ ID NO: 52) with a signal sequence (SEQ ID NO: 58) further added to the N terminus, to a gene sequence by GenScript USA Inc., and prepared by PCR mutagenesis (L1-4: SEQ ID NO: 79, L1-5: SEQ ID NO: 81, L1-6: SEQ ID NO: 83, signal sequence: SEQ ID NO: 59). A gene sequence encoding the amino acid sequence of L2-4 was synthesized by converting the amino acid sequence of light chain CDRs (SEQ ID NOs: 31 to 33) of antibody A grafted in IGKV3D-15*01 (SEQ ID NO: 51) and JK1 (SEQ ID NO: 52) with a signal sequence (SEQ ID NO: 58) further added to the N-terminus, to a gene sequence by GenScript USA Inc., and prepared by PCR mutagenesis (L2-4: SEQ ID NO: 85, signal sequence: SEQ ID NO: 59). The genes encoding these humanized light chain variable regions and signal sequences were inserted to expression vectors (pcDNA3.4) containing a gene sequence (SEQ ID NO: 65) encoding the human Igk constant region (SEQ ID NO: 64). In this context, the term "C131S" refers to a mutation that substitutes cysteine at Eu numbering position 131 with serine. The term "C219S" refers to a mutation that substitutes cysteine at Eu numbering position 219 with serine. The term "V234A" refers to a mutation that substitutes valine at Eu numbering position 234 with alanine. The term "G237A" refers to a mutation that substitutes glycine at Eu numbering position 237 with alanine. In this context, the CH1 refers to a region from Eu numbering positions 118 to 215 in the human IgG constant region. The hinge refers to a region from Eu numbering positions 216 to 230 in the human IgG constant region. The CH2 refers to a region from Eu numbering positions 231 to 340 in the human IgG constant region.

The CH3 refers to a region from Eu numbering positions 341 to 446 in the human IgG constant region. In order to produce these humanized antibodies, the expression vectors described above were used in combination as shown in Table 5 using Expi293 expression system (Gibco/Thermo Fisher Scientific) to transfect Expi293F cells (Gibco/Thermo Fisher Scientific). Each supernatant was recovered and purified using protein A (GE Healthcare).

[0194]

[Table 5]

Humanized antibody No.	L chain			H chain				
	Variable region			Variable region			Constant region	
	Name	Amino acid sequence (SEQ ID NO)	Nucleic acid sequence (SEQ ID NO)	Name	Amino acid sequence (SEQ ID NO)	Nucleic acid sequence (SEQ ID NO)	Amino acid sequence (SEQ ID NO)	Nucleic acid sequence (SEQ ID NO)
75	L1-4	78	79	HA1	66	67	60	61
76	L1-4	78	79	HA2	68	69		
67	L1-4	78	79	HA4	70	71		
77	L1-4	78	79	HK1	72	73		
78	L1-4	78	79	HK2	74	75		
69	L1-4	78	79	HK4	76	77		
81	L1-5	80	81	HA1	66	67		
82	L1-5	80	81	HA2	68	69		
83	L1-5	80	81	HA4	70	71		
84	L1-5	80	81	HK1	72	73		
85	L1-5	80	81	HK2	74	75		
86	L1-5	80	81	HK4	76	77		
87	L1-6	82	83	HA1	66	67		
88	L1-6	82	83	HA2	68	69		
89	L1-6	82	83	HA4	70	71		
90	L1-6	82	83	HK1	72	73		
91	L1-6	82	83	HK2	74	75		
92	L1-6	82	83	HK4	76	77		
93	L2-4	84	85	HA1	66	67		
94	L2-4	84	85	HA2	68	69		
71	L2-4	84	85	HA4	70	71		
95	L2-4	84	85	HK1	72	73		
96	L2-4	84	85	HK2	74	75		
73	L2-4	84	85	HK4	76	77		
139	L1-4	78	79	HA1	66	67	62	63
140	L1-4	78	79	HA2	68	69		
138	L1-4	78	79	HA4	70	71		
141	L1-4	78	79	HK1	72	73		
142	L1-4	78	79	HK2	74	75		
143	L1-4	78	79	HK4	76	77		
152	L1-5	80	81	HA1	66	67		
153	L1-5	80	81	HA2	68	69		
151	L1-5	80	81	HA4	70	71		
154	L1-5	80	81	HK1	72	73		
145	L1-5	80	81	HK2	74	75		
155	L1-5	80	81	HK4	76	77		
157	L1-6	82	83	HA1	66	67		
158	L1-6	82	83	HA2	68	69		
156	L1-6	82	83	HA4	70	71		
144	L1-6	82	83	HK1	72	73		
159	L1-6	82	83	HK2	74	75		
160	L1-6	82	83	HK4	76	77		
133	L2-4	84	85	HA1	66	67		
134	L2-4	84	85	HA2	68	69		
132	L2-4	84	85	HA4	70	71		
135	L2-4	84	85	HK1	72	73		
136	L2-4	84	85	HK2	74	75		
137	L2-4	84	85	HK4	76	77		

[0195]

Example 17: Affinity of anti-EphA4 monoclonal humanized antibody for human EphA4

The binding affinity of each anti-EphA4 monoclonal humanized antibody obtained in Example 16 for human EphA4 was determined by surface plasmon resonance (SPR) using Biacore T200 (GE Healthcare). First, for the assay of antibody A, a rat anti-mouse IgG₁ antibody produced with a conventional method by immunizing rat with mouse IgG₁ antibody was immobilized on sensor chip CM5. The immobilization of the rat anti-mouse IgG₁ antibody on sensor chip CM5 was performed by the amine coupling method using N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Ethanolamine was used in blocking (the sensor chip and the reagents for immobilization were all manufactured by GE Healthcare). The antibody was diluted with a buffer solution for immobilization (10 mM sodium acetate, pH 4.5) and immobilized on the sensor chip according to the protocol attached to Biacore T200. For the assay of each humanized monoclonal antibody, a protein A chip (GE Healthcare, 29-1383-03) was used. Antibody A or the humanized monoclonal antibody was diluted with a running buffer solution HBS-EP (GE Healthcare), injected onto only flow cell 2 for 120 seconds, and captured (captured amount: approximately 30 to 60 RU). Subsequently, human EphA4 extracellular region-SEAP-His protein serially diluted in the range of 50, 16.7, 5.6, 1.9, and 0.6 nM using HBS-EP was sequentially added from the lower toward higher concentration sides without regeneration operation. Binding reaction curves were sequentially observed at the time of the addition (association phase, for 120 sec) and after the completion of the addition (dissociation phase, for 900 sec). After the completion of each observation, the sensor chip was regenerated by the addition of 3 M MgCl₂ (for 60 sec) or 10 mM Glycine-HCl pH 1.5 (for 30 sec). The obtained binding reaction curves were subjected to fitting analysis with 1:1 binding models using software BIA evaluation attached to the system to calculate the affinity ($KD = k_d / k_a$) for human EphA4 (Table 6).

[0196]

All of the humanized antibodies described in Table 5 were found to exhibit affinity substantially equivalent to their parent antibody A (Table 6).

[0197]

[Table 6]

humanized antibody No.	k_a 1/Ms	k_d 1/s	K_D M	R_{max1} RU	χ^2 RU ²
75	3.6E+05	4.2E-04	1.2E-09	29	0.17
76	3.3E+05	3.4E-04	1.0E-09	29	0.18
67	3.3E+05	3.5E-04	1.1E-09	28	0.15
77	4.1E+05	5.8E-04	1.4E-09	27	0.20
78	3.4E+05	3.9E-04	1.1E-09	30	0.17
69	3.4E+05	3.7E-04	1.1E-09	29	0.19
81	3.4E+05	5.4E-04	1.6E-09	24	0.12
82	2.9E+05	4.6E-04	1.6E-09	24	0.10
83	2.9E+05	5.4E-04	1.9E-09	20	0.06
84	3.5E+05	5.8E-04	1.7E-09	25	0.13
85	3.1E+05	5.0E-04	1.6E-09	26	0.12
86	3.1E+05	4.6E-04	1.5E-09	26	0.13
87	3.5E+05	5.1E-04	1.5E-09	27	0.12
88	3.0E+05	4.2E-04	1.4E-09	26	0.13
89	3.1E+05	4.1E-04	1.3E-09	24	0.13
90	3.6E+05	5.5E-04	1.5E-09	27	0.14
91	3.3E+05	4.4E-04	1.3E-09	25	0.13
92	3.3E+05	4.3E-04	1.3E-09	27	0.16
93	3.1E+05	2.4E-04	7.7E-10	24	0.10
94	2.6E+05	2.4E-04	9.5E-10	25	0.06
71	2.8E+05	2.3E-04	8.2E-10	25	0.09
95	3.0E+05	3.1E-04	1.0E-09	27	0.08
96	2.7E+05	3.1E-04	1.1E-09	28	0.07
73	2.9E+05	2.7E-04	9.3E-10	27	0.09
139	3.7E+05	5.3E-04	1.4E-09	29	0.15
140	3.2E+05	4.3E-04	1.4E-09	29	0.12
138	3.3E+05	4.3E-04	1.3E-09	29	0.12
141	3.9E+05	6.2E-04	1.6E-09	26	0.14
142	3.4E+05	5.0E-04	1.5E-09	28	0.12
143	3.5E+05	5.1E-04	1.5E-09	26	0.12
152	4.0E+05	8.2E-04	2.0E-09	23	0.09
153	2.8E+05	6.0E-04	2.1E-09	24	0.07
151	3.2E+05	5.3E-04	1.7E-09	26	0.13
154	3.9E+05	8.5E-04	2.2E-09	24	0.10
145	3.3E+05	6.5E-04	2.0E-09	27	0.11
155	3.3E+05	6.4E-04	1.9E-09	27	0.11
157	3.5E+05	6.8E-04	2.0E-09	24	0.09
158	3.1E+05	6.1E-04	1.9E-09	24	0.06
156	3.1E+05	5.6E-04	1.8E-09	24	0.08
144	4.0E+05	7.8E-04	2.0E-09	23	0.09
159	3.1E+05	6.1E-04	2.0E-09	22	0.07
160	3.2E+05	6.1E-04	1.9E-09	25	0.06
133	3.0E+05	3.2E-04	1.1E-09	26	0.07
134	2.6E+05	3.2E-04	1.2E-09	24	0.06
132	2.7E+05	3.1E-04	1.2E-09	26	0.06
135	3.2E+05	4.0E-04	1.3E-09	26	0.08
136	2.9E+05	3.9E-04	1.4E-09	25	0.07
137	2.9E+05	3.8E-04	1.3E-09	27	0.09
antibody A	4.2E+05	2.5E-04	6.0E-10	37	0.19

[0198]

Example 18: Human EphA4-human ligand binding inhibitory activity of anti-EphA4 monoclonal humanized antibody

Each anti-EphA4 monoclonal humanized antibody obtained in Example 16 was evaluated for its inhibitory activity against the binding between human EphA4 and its

human ligand according to the following steps. Each well of a 96-well plate (Nunc) was coated with an anti-alkaline phosphatase antibody (Thermo Fisher Scientific). After incubation overnight at 4°C, each well was blocked with 1% BlockAce (DS Pharma Biomedical) at room temperature for 1 hour. After washing with 0.05% Tween 20/PBS (Thermo Fisher Scientific) three times, the human EphA4 extracellular region-SEAP-His protein obtained by the method of Example 2 was added (final concentration: 10 nM) to each well and incubated at room temperature for 1 hour. After washing three times, a ligand and each serially diluted humanized antibody of antibody A (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) were added to each well. The ligand used was biotinylated human Ephrin A5-Fc chimera (R&D Systems, final concentration: 0.7 nM). After incubation at room temperature for 1 hour and subsequent washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added thereto and incubated at room temperature for 1 hour. After washing three times, a TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich) solution was added to each well and incubated at room temperature for 2 minutes. An equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (PerkinElmer).

[0199]

All of the humanized antibodies described in Table 5 were found to exhibit inhibitory activity substantially equivalent to their parent antibody A (Table 7).

[0200]

[Table 7]

humanized antibody No.	IC50 nM
75	2.9
76	2.9
67	4.4
77	3.5
78	2.8
69	3.1
81	4.0
82	4.1
83	3.7
84	4.8
85	4.0
86	4.0
87	3.0
88	3.6
89	4.3
90	4.5
91	4.9
92	3.5
93	2.5
94	2.7
71	2.5
95	3.1
96	3.7
73	2.4
139	3.3
140	2.4
138	2.9
141	3.0
142	3.7
143	5.3
152	2.0
153	2.3
151	2.0
154	2.5
145	3.7
155	2.4
157	2.6
158	2.8
156	2.5
144	3.6
159	2.6
160	2.2
133	3.0
134	3.0
132	3.1
135	2.5
136	2.5
137	2.5

[0201]

Example 19: Selectivity of anti-EphA4 monoclonal humanized antibody for human Eph receptor

According to the method described in Example 7, a DNA sequence encoding the signal sequence and the extracellular region of each human Eph receptor (EphA1, EphA2,

EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) was amplified by RT-PCR using tissue-derived total RNA and cloned into a pENTR1A vector (Invitrogen/Life Technologies) having a DNA sequence encoding SEAP protein and histidine tag. Next, the DNA sequence encoding the signal sequence and the extracellular region of each human Eph receptor, SEAP protein, and histidine tag was transferred to a pcDNA3.1_rfcB vector through LR reaction using Gateway System (Invitrogen/Life Technologies) to construct a vector for the expression of a protein of the extracellular region of each human Eph receptor fused with the SEAP protein and the His tag (referred to as "Eph receptor extracellular region-SEAP-His protein") (this vector is referred to as "Eph receptor extracellular region-SEAP-His protein expression vector"). [0202]

Next, each human Eph receptor extracellular region-SEAP-His protein expression vector was transferred to Expi293F cells (Gibco/Thermo Fisher Scientific) using Expi293 expression system (Gibco/Thermo Fisher Scientific). After incubation (5% CO₂, 37°C) for 5 days, the culture supernatant was recovered and centrifuged at 1500 rpm at room temperature for 5 minutes. The centrifugation supernatant was filtered through a 0.45-μm filter (Merck Millipore).

Each anti-EphA4 monoclonal humanized antibody obtained in Example 16 was evaluated for its binding activity against each human Eph receptor according to the following steps.

Each well of a 96-well plate (Nunc) was coated with a rabbit anti-6-His antibody (Bethyl Laboratories). Each well was blocked at room temperature for 1 hour and then incubated overnight with 1% BlockAce (DS Pharma Biomedical) at 4°C. After washing with 0.05% Tween 20/PBS (Nacalai Tesque) three times, the SEAP-His protein of human EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, or EphB6 was added (final concentration: 1 nM) to each well and incubated at room temperature for 1 hour. After washing three times, the humanized antibody was added to each well and incubated at room temperature for 1 hour. After washing three times, a horseradish peroxidase-labeled donkey anti-mouse IgG antibody

(Jackson ImmunoResearch Laboratories) was added thereto and incubated at room temperature for 1 hour. After washing three times, TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories (KPL)) was added to each well. After confirmation of moderate color development, an equal amount of a reaction stopping solution (1N H₂SO₄, Wako Pure Chemical Industries) was added to each well. The absorbance at 450 nm was read using a microplate reader (Thermo Fisher Scientific).
[0203]

The percentages with respect to the absorbance at 450 nm for human EphA4 (hEphA4-SEAP-His) as 100% were summarized in Table 8. As a result, all of the humanized antibodies described in Table 5 were found to specifically bind to human EphA4, as with their parent antibody A (Table 8).

[0204]

[Table 8]

humanized antibody	SEAP-His	hEphA1-SEAP-	hEphA2-SEAP-	hEphA3-SEAP-	hEphA4-SEAP-	hEphA5-SEAP-	hEphA6-SEAP-	hEphA7-SEAP-	hEphA8-SEAP-	hEphA10-SEAP-	hEphB1-SEAP-	hEphB2-SEAP-	hEphB3-SEAP-	hEphB4-SEAP-	hEphB6-SEAP-
75	4.4	4.7	4.6	4.8	100	4.7	8.3	4.7	4.8	4.4	4.4	4.5	4.7	4.6	4.7
76	5.5	5.1	4.8	4.9	100	5.3	7.9	4.8	5.5	4.7	4.9	4.9	5.0	5.2	5.2
77	5.0	4.6	4.6	4.8	100	4.8	7.3	4.7	4.7	4.6	4.6	4.7	4.9	4.9	4.7
77	4.7	4.5	4.5	4.5	100	4.4	6.5	4.3	4.8	4.5	4.6	4.5	4.6	4.5	4.5
78	5.3	6.6	4.9	5.1	100	5.1	10.0	5.0	5.4	5.2	5.0	5.1	5.2	5.1	5.2
69	6.1	5.7	5.5	5.4	100	5.6	9.5	5.3	5.5	5.4	5.6	5.5	5.9	5.5	5.6
81	5.0	4.4	4.8	5.0	100	4.9	8.8	5.2	5.2	4.8	4.9	5.0	4.9	5.0	5.0
82	5.9	5.4	5.4	5.4	100	6.5	8.7	5.4	5.8	5.4	5.4	5.6	5.6	5.7	5.5
83	6.5	5.7	5.8	5.7	100	5.8	7.5	5.5	6.0	5.6	5.6	5.6	5.7	5.7	5.8
84	5.1	5.2	4.9	4.9	100	5.0	6.0	4.8	5.3	5.1	5.2	4.9	4.8	5.4	4.9
85	5.3	5.0	5.1	5.1	100	5.0	6.2	4.8	5.5	5.0	5.0	5.1	5.2	5.2	5.2
86	6.1	5.9	5.7	5.8	100	5.7	8.6	5.6	5.7	5.6	5.7	5.7	5.9	5.8	5.7
87	5.5	5.3	5.4	5.5	100	5.4	6.4	5.3	5.7	5.2	5.3	5.4	5.5	5.5	5.3
88	6.1	5.8	5.8	6.0	100	5.9	7.5	6.0	6.2	5.8	6.1	6.0	6.3	6.1	6.1
89	5.8	5.5	5.6	5.8	100	5.8	8.4	5.4	5.7	5.7	5.7	5.7	5.7	5.8	4.8
90	5.2	5.1	5.2	5.2	100	5.1	7.8	4.9	5.4	5.1	4.9	5.9	5.1	5.0	5.2
91	6.4	5.8	6.1	6.6	100	6.4	8.8	5.6	6.2	8.5	6.6	7.0	7.0	6.2	6.3
92	6.5	7.0	6.9	6.5	100	6.8	9.6	6.4	6.7	6.6	6.9	6.6	6.9	6.6	6.5
93	5.0	5.0	5.1	5.1	100	4.9	7.1	4.9	5.0	4.9	5.0	5.0	5.1	5.0	4.9
94	6.7	6.5	6.4	6.8	100	6.5	8.6	6.1	7.0	6.4	6.7	7.0	6.7	6.5	6.4
71	5.6	5.6	5.7	5.6	100	5.6	9.2	5.2	5.3	5.3	5.4	5.5	5.5	5.5	5.3
95	4.4	4.5	4.2	4.8	100	4.6	7.0	4.4	4.4	4.5	4.6	4.7	4.6	4.6	4.7
96	5.5	5.6	5.3	5.5	100	6.0	9.8	5.3	5.4	5.3	5.6	5.5	5.6	5.5	5.5
73	5.8	6.0	5.7	5.8	100	6.0	7.4	5.6	5.8	5.6	5.7	5.6	5.8	5.7	5.6
139	5.7	5.7	5.9	5.7	100	5.7	10.0	5.5	6.0	5.5	5.5	5.7	5.6	5.7	5.9
140	5.4	5.5	5.3	5.5	100	5.4	10.8	5.2	5.4	5.2	5.3	5.3	5.4	5.4	5.2
138	5.1	5.2	5.0	4.9	100	5.1	10.2	4.9	5.4	5.0	5.1	5.2	5.1	5.2	5.0
141	5.0	5.3	5.2	5.3	100	5.3	9.0	5.3	5.5	5.2	5.4	5.2	5.2	5.4	5.4
142	5.8	5.6	5.6	5.5	100	5.7	9.7	5.5	5.7	5.4	5.6	5.6	5.6	5.7	5.6
143	6.3	6.0	5.7	5.6	100	6.1	9.9	5.8	6.0	5.2	5.9	5.8	5.9	5.9	5.6
152	5.4	5.8	5.8	5.8	100	5.8	8.3	5.7	6.0	5.8	5.9	5.8	6.0	5.9	5.9
153	5.9	6.3	6.0	6.1	100	6.1	8.7	6.2	6.1	6.0	6.0	5.9	5.9	6.0	6.2
151	6.8	7.1	6.9	6.9	100	7.0	9.9	6.8	6.9	6.6	6.8	7.0	7.3	7.4	7.1
154	6.3	6.4	6.2	6.2	100	6.3	8.4	6.3	5.5	5.9	6.3	6.2	6.2	6.4	6.4
145	6.4	6.7	6.5	6.6	100	6.6	8.6	6.3	6.6	6.5	6.5	6.7	6.7	6.7	6.6
155	5.8	5.5	5.5	5.6	100	5.6	7.2	5.6	5.5	5.4	5.4	5.5	5.5	5.7	5.6
157	6.1	6.2	6.3	6.4	100	6.1	9.0	6.0	6.0	6.1	6.1	6.2	6.4	6.5	6.3
158	6.3	6.5	6.7	6.5	100	6.7	10.0	6.3	6.4	6.4	6.2	6.3	6.5	6.5	6.3
156	5.4	5.7	5.5	5.7	100	5.7	8.9	5.6	5.5	5.3	5.4	5.5	5.6	5.6	5.7
144	5.7	5.6	5.6	5.7	100	5.6	8.1	5.6	5.8	5.7	5.7	5.7	5.8	5.7	5.9
159	5.3	5.4	5.4	5.4	100	5.5	7.6	5.5	5.5	5.3	5.3	5.4	5.5	5.4	5.5
160	6.3	6.5	6.1	6.2	100	6.5	8.7	6.1	5.4	5.0	6.1	6.2	6.2	6.3	6.2
133	4.7	4.9	4.7	5.0	100	4.7	8.8	4.6	4.7	4.7	4.6	4.6	4.7	4.9	4.8
134	5.1	5.1	5.0	5.0	100	5.2	9.6	5.1	5.7	5.0	5.1	5.1	5.2	5.1	5.2
132	4.9	4.8	4.8	4.8	100	4.7	9.7	4.5	4.7	4.7	4.8	4.7	4.7	4.8	4.7
135	5.3	5.2	5.0	5.0	100	5.0	7.0	5.2	5.0	5.1	5.1	5.0	5.0	5.1	5.3
136	5.2	5.3	5.1	5.2	100	5.2	7.2	5.4	5.4	5.2	5.2	5.3	5.5	5.2	5.5
137	5.9	6.1	5.4	5.6	100	5.7	7.1	5.8	5.9	5.6	5.8	5.6	5.7	5.7	5.7

[0205]

Example 20: Motor neuron protective effect of anti-EphA4 monoclonal humanized antibody in *in vitro* ALS model derived from human iPS cells

Human iPS cells are maintained and cultured according to the following steps. Human iPS cells (201B7) cryopreserved in liquid nitrogen using Stem cell banker (TAKARA) are taken out of the gas phase of liquid nitrogen and immediately suspended and thawed in 5 mL of a human iPS cell culture medium (Essential 8, Thermo Fisher Scientific) prewarmed to 37°C. The cell suspension is recovered into a 15-mL conical tube (Falcon) and centrifuged (1000 rpm, 5 min, room temperature), followed by the removal of the supernatant. The cells are suspended in a fresh medium and then disseminated to a ϕ 60 mm cell culture dish (Falcon BD) coated with 0.5 μ g/cm² Human recombinant vitronectin (Invitrogen) in advance. 10 μ M of Y-27632 (Wako Pure Chemical Industries) is added thereto, and the cells are maintained and cultured in a CO₂ incubator (5% CO₂, 37°C). Medium replacement is performed every day, and the cells are subjected to the experiment when reaching confluency.

[0206]

The motor neuron protective effect of the anti-EphA4 monoclonal humanized antibody obtained in Example 16 in the *in vitro* ALS models is evaluated according to the following steps. The culture medium of the maintained and cultured human iPS cells is aspirated, and the cells are washed with 2 mL of PBS (Wako Pure Chemical Industries). After aspiration of PBS, 500 μ L of 0.5 mM EDTA is added to the cells, which are then incubated for 2 to 3 minutes in a CO₂ incubator (5% CO₂, 37°C) (the cells are confirmed under a microscope every 30 seconds, and the incubation is discontinued when the intercellular association became weak). The EDTA reaction is terminated by suspension in 5 mL of a human iPS cell culture medium, and the cells are recovered into a 15-mL conical tube. The cells are centrifuged at 1000 rpm at room temperature for 5 minutes, and the supernatant is aspirated. The cell suspension containing human iPS cell masses is inoculated in an amount of approximately 1/10 per well to a low-adhesion 6-well cell culture plate (Nunclon Sphere, Nunc) and cultured in a CO₂ incubator (5% CO₂, 37°C) using a DFK medium (advanced DMEM/F-12 (Invitrogen):Neurobasal

medium (Invitrogen) [1:1] medium containing 2% B27 supplement, 5% KnockOut serum replacement (Invitrogen), 2 mmol/L L-glutamine, 100 units/mL penicillin-100 µg/mL streptomycin, and 0.1 mmol/L β-mercaptoethanol) supplemented with 2 µM SB431542 (Sigma-Aldrich), 300 nM LDN193189 (Sigma-Aldrich), and 3 µM CHIR99021 (Sigma-Aldrich). Medium replacement is performed every 2 days by the following method. First, a human iPS cell differentiated cell aggregates (SFEBS) is recovered on a medium basis into a 15-mL conical tube and left standing at ordinary temperature for 5 minutes to precipitate the cell masses. This supernatant is aspirated, and a fresh DFK medium and 2 µM SB431542 (Sigma-Aldrich), 300 nM LDN193189 (Sigma-Aldrich), 3 µM CHIR99021 (Sigma-Aldrich) are added, and then brought back to the original well for medium replacement. At culture day 8, the SFEBS are recovered on a medium basis into a 15-mL conical tube and left standing at ordinary temperature for 5 minutes to precipitate the SFEBS. This supernatant is aspirated, and a fresh DFK medium and then 0.1 µM retinoic acid (Sigma-Aldrich) and 0.5 µM purmorphamine (Milenyi Biotec) are added, and then brought back to the original well and cultured in a CO₂ incubator (5% CO₂, 37°C). Medium replacement is performed every 2 days. At culture day 12, the SFEBS are recovered on a medium basis into a 15-mL conical tube and left standing at ordinary temperature for 5 minutes to precipitate the SFEBS. The supernatant is aspirated, and 500 µL of Accumax (MS TechnoSystems) is added to the cells, which are then pipetted several times and then incubated for 5 minutes in a CO₂ incubator (5% CO₂, 37°C). The cells are taken out of the incubator, suspended in 5 mL of a DFK medium, and pipetted several times to disperse the cell masses. The cell suspension is dissociated into single cells by filtration through a cell strainer (Falcon). Then, the number of cells is counted using a counting chamber. The cell suspension is recovered into another 15-mL conical tube and centrifuged at 1000 rpm at room temperature for 5 minutes. A suspension having a cell density of 5.5×10^5 cells/mL is prepared with a motoneuron culture medium (advanced DMEM/F-12 (Invitrogen):Neurobasal medium (Invitrogen) [1:1] medium containing 2% B27 Supplement, 1% horse serum, 2 mmol/L L-glutamine, 100 units/mL penicillin-100 µg/mL streptomycin, and 0.1 mmol/L β-

mercaptoethanol) and inoculated at 200 μ L/well to an 8-well chamber containing mouse-derived wild-type astrocytes or variant human SOD1 (G93A)-expressing astrocytes inoculated at 8×10^4 cells/well in advance. The resulting cocultured cells of the astrocytes and the motor neurons are used in evaluation (the establishment, freezing, thawing, inoculation, and maintenance and culture of the wild-type and human variant SOD1 (G93A)-expressing astrocytes are performed in the same way as in Example 13). The number of motor neurons observed by the coculture of the wild-type astrocytes and the motor neurons is used as a control. For the drug-treated group, the variant human SOD1 (G93A)-expressing astrocytes and the motor neurons are cocultured under a condition involving vehicle addition (IgG and 0.1% ultrapure water) and the humanized antibody. After culture for 2 days at 37°C in a 5% CO₂ environment under each condition, the motor neurons are immunocytochemically stained with an anti-ISL1 antibody (obtained from Developmental Studies Hybridoma Bank) and Hoechst 33342 (Molecular Probes). ISL1/Hoechst 33342-copositive cells per well are counted as live motor neurons, and the survival rate of the motor neurons is calculated as % with respect to the control.

[0207]

The survival rate of the motor neurons is significantly reduced in the variant human SOD1 (G93A)-expressing astrocyte/human iPS cell-derived motor neuron coculture. The humanized antibody described in Table 5 suppressed, in a concentration-dependent manner, human iPS cell-derived motor neuron death induced by the variant human SOD1 (G93A)-expressing astrocytes. Accordingly, it is shown that the anti-EphA4 monoclonal humanized antibody promotes the survival of motor neurons in human cells.

Industrial Applicability

[0208]

The present invention can provide an anti-EphA4 antibody or an EphA4-binding fragment thereof which is capable of binding to EphA4 and inhibiting the binding between EphA4 and its ligand, and a pharmaceutical composition comprising

the anti-EphA4 antibody or the EphA4-binding fragment thereof as an active ingredient. The antibody or the pharmaceutical composition according to the present invention can be useful in the treatment of diseases caused by the binding between EphA4 and its ligand, for example, ALS.

What is claimed is:

1. An anti-EphA4 antibody or an EphA4-binding fragment thereof, comprising
 - (a) CDR-H1 comprising the amino acid sequence represented by SEQ ID NO: 26 or SEQ ID NO: 27;
 - (b) CDR-H2 comprising the amino acid sequence represented by SEQ ID NO: 28 or SEQ ID NO: 29;
 - (c) CDR-H3 comprising the amino acid sequence represented by SEQ ID NO: 30;
 - (d) CDR-L1 comprising the amino acid sequence represented by SEQ ID NO: 31;
 - (e) CDR-L2 comprising the amino acid sequence represented by SEQ ID NO: 32;and
 - (f) CDR-L3 comprising the amino acid sequence represented by SEQ ID NO: 33.
2. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to claim 1, wherein
the antibody or the EphA4-binding fragment thereof is humanized.
3. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to claim 1 or 2, wherein
the antibody or the EphA4-binding fragment thereof specifically binds to EphA4 and inhibits the binding between EphA4 and ephrin.
4. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of claims 1 to 3, wherein
the antibody or the EphA4-binding fragment thereof comprises a heavy chain and a light chain, and
a constant region of the heavy chain and a constant region of the light chain each comprise a human antibody-derived sequence.

5. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to claim 4, wherein
the constant region of the heavy chain is derived from human IgG.
6. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to claim 5, wherein
the human IgG is human IgG₁ or human IgG₂.
7. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of claims 4 to 6, wherein
the constant region of the light chain is derived from human Igκ.
8. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to any one of claims 1 to 7, wherein
the EphA4-binding fragment is selected from the group consisting of Fab, Fab', F(ab')₂, and Fv.
9. The anti-EphA4 antibody or the EphA4-binding fragment thereof according to claim 8, wherein
the EphA4-binding fragment is F(ab')₂.
10. A pharmaceutical composition comprising
an anti-EphA4 antibody or an EphA4-binding fragment thereof according to any one of claims 1 to 9.
11. The pharmaceutical composition according to claim 10 further comprising
a pharmaceutically acceptable carrier.

12. The pharmaceutical composition according to claim 10 or 11, wherein
the pharmaceutical composition is used for the treatment of amyotrophic lateral
sclerosis (ALS).