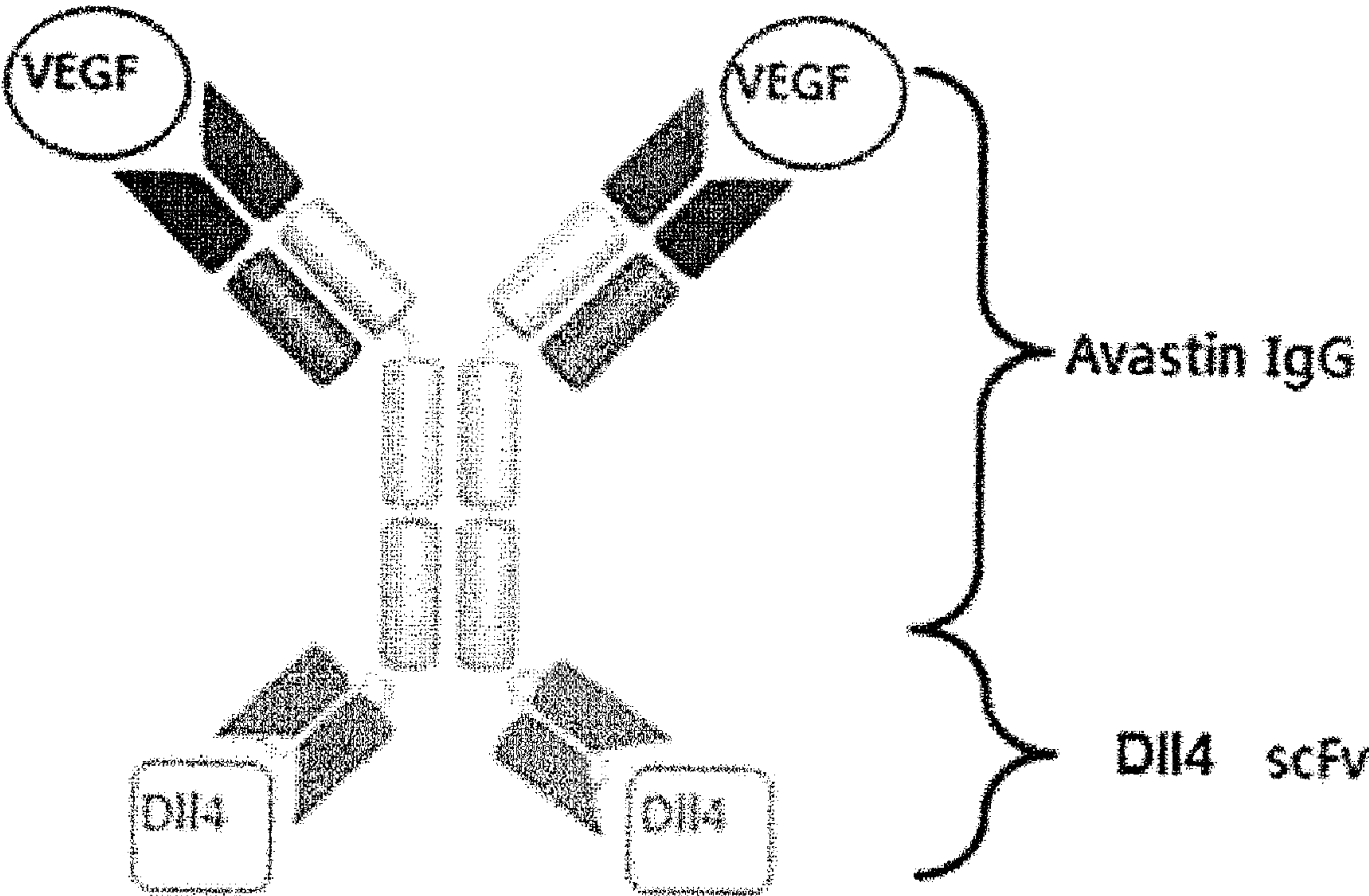




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(54) Titre : NOUVELLES PROTEINES A DOUBLE CIBLE SE LIANT SPECIFIQUEMENT A DLL4 ET VEGF ET SON UTILISATION

(54) Title: NOVEL DUAL-TARGETING PROTEIN BINDING SPECIFICALLY TO DLL4 AND VEGF AND USE THEREOF



(57) Abrégé/Abstract:
The present invention relates to a dual-targeted protein comprising: a novel protein specifically binding to delta like ligand 4 (DLL4); and an antibody specifically binding to a vascular endothelial cell growth factor (VEGF).

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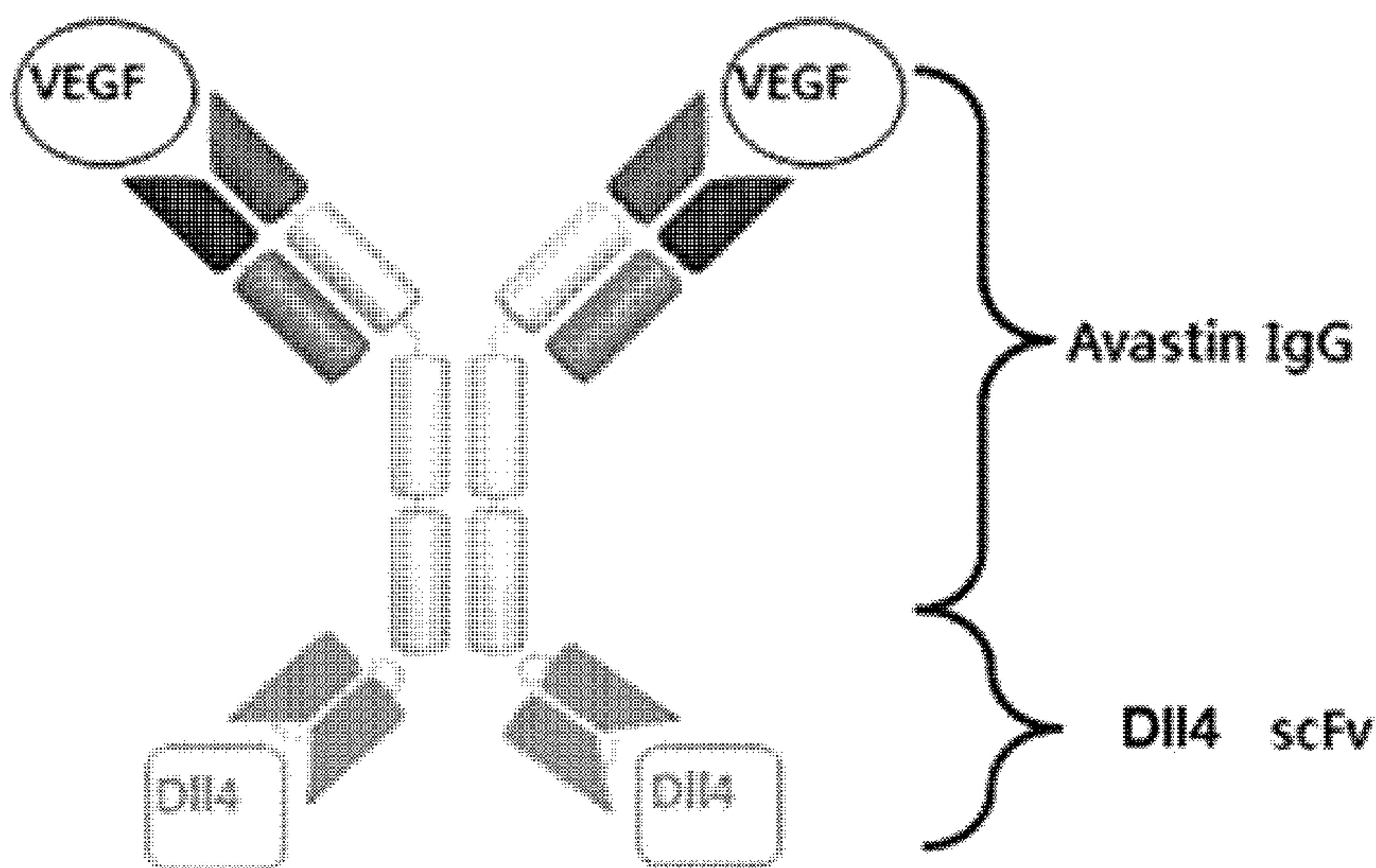
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(54) Title: NOVEL DUAL-TARGETED PROTEIN SPECIFICALLY BINDING TO DLL4 AND VEGF, AND USE THEREOF

(54) 발명의 명칭 : DLL4 와 VEGF 에 특이적으로 결합하는 신규 이중표적 단백질 및 이의 용도



(57) Abstract: The present invention relates to a dual-targeted protein comprising: a novel protein specifically binding to delta like ligand 4 (DLL4); and an antibody specifically binding to a vascular endothelial cell growth factor (VEGF).

(57) 요약서: 본 발명은 신규한 델타 유사 리간드 4(Delta like ligand 4, DLL4)에 특이적으로 결합하는 단백질 및 혈관 내피 세포 성장 인자(Vascular endothelial cell growth factor, VEGF)에 특이적으로 결합하는 항체를 포함하는 이중표적 단백질에 관한 것이다.

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**NOVEL DUAL-TARGETING PROTEIN BINDING SPECIFICALLY TO DLL4
AND VEGF AND USE THEREOF**

TECHNICAL FIELD

5 The present invention relates to a novel dual-targeting protein comprising: a protein that binds specifically to delta-like ligand 4 (DLL4) and an antibody that binds specifically to vascular endothelial cell growth factor (VEGF).

10

BACKGROUND ART

It has been reported that Notch signaling is an evolutionarily highly conserved in vertebrate and invertebrate animals and plays a very pivotal role in
15 determining the fate of cells in the initial stage of development. Notch signaling is known as a major pathway that regulates the differentiation of neural cells, intraocular cells, lymphocytes, muscular cells, hematocytes and the like and is also involved in the development of blood
20 vessels. Mammals have four Notch receptors (Notch 1, 2, 3 and 4), and each of Notch receptors is synthesized as a protein having a size of 300-350 kDa and cleaved at the S1 site by furin-like convertase in the Golgi to form a heterodimer on the cell surface. In addition, four Notch

ligands (jagged-1/2 and delta-like ligand (DLL) 1/3/4) were found in mammals.

Activated Notch signaling is known to induce tumorigenesis in various tumor models. When the activated Notch NICD was expressed in rat hematopoietic cells, T-cell leukemia/lymphomas occurred, and activated Notch 1 was found in about 50% of T-ALL (T-cell acute lymphoblastic leukemia). In addition, in the case of breast cancer, Notch 4 receptor was found to be overexpressed in rats (Czech II) introduced with MMTV (mouse mammary tumor virus), and the occurrence of a mammary gland tumor in these rats has been reported. It has been reported that Notch receptors and ligands and Notch signaling targets are activated in various cancers such as cervical cancer, lung cancer, pancreatic cancer, ovarian cancer, breast cancer and prostate cancer. It is known that Notch 1 receptor is associated with worse prognosis on breast cancer patients and associated with the metastasis of prostate cancer.

Delta-like ligand 4 (DLL4) (hereinafter referred to as "DLL4") is one of delta-class ligands that bind to Notch proteins which are overexpressed in vascular endothelial cells. It is known as a major factor that regulates angiogenesis. DLL4 particularly binds to Notch 1 or Notch 4 receptor which is overexpressed in vascular endothelial cells. It is known that DLL4 is highly overexpressed in cancer blood

vessels, although it is also expressed in normal blood vessels. Angiogenesis refers to the mechanism by which new blood vessels are formed from the pre-existing blood vessels. Particularly, in tumors, angiogenesis is caused by angiogenic
5 factors such as VEGF (vascular endothelial growth factor) in order to supply oxygen and nutrients to the hypoxia area of cancer tissue. It is known that angiogenesis in tumors plays an important role not only in the growth of the tumor, but also in the metastasis of the tumor. When Notch signaling by
10 DLL4 in tumors is blocked, angiogenesis cannot be easily controlled, and thus the growth of the tumors can be inhibited. In addition, when Notch signaling by DLL4 is inhibited, autoimmune disease can be treated by increasing the number of regulatory T cells (Treg) (US Patent
15 Publication No. 2011-0189200). For these reasons, DLL4 becomes a new target in the treatment of cancers and autoimmune diseases.

Meanwhile, as an anticancer antibody drug for inhibiting angiogenesis, Avastin® (Genentech/Roche) that targets VEGF was
20 approved by the FDA in 2004 and has been largely successful as an anticancer therapeutic agent. However, recent clinical model and preclinical animal model studies have indicated that all solid tumors do not respond to VEGF inhibitors, and have also reported a number of cases in which some tumors
25 treated with VEGF inhibitors in the initial stage show

resistance after a certain time. In addition, study results have been reported which indicate that the administration of VEGF inhibitors converts cancer cells into cancer cells that are more aggressive and easily metastasize. Such study reports have propelled research and development of novel anticancer targets that overcome Avastin resistance or that have efficacy superior to that of Avastin. Among such novel anticancer targets, proteins that are involved in the DLL4/Notch signaling pathway are attracting attention. According to the study results reported to date, it is expected that, because the VEGF/VEGFR signaling pathway and the DLL4/Notch signaling pathway influence angiogenesis by different mechanisms, stronger synergistic anticancer effects can be obtained when the two signaling pathways are all inhibited.

DISCLOSURE OF INVENTION

TECHNICAL PROBLEM

The present inventors have made extensive efforts to develop a dual-targeting protein which can bind specifically to human-derived DLL4 and VEGF to effectively inhibit the DLL4/Notch and VEGF/VEGFR signaling pathways and can minimize the risk of immunogenicity. As a result, the present inventors have constructed a novel human monoclonal antibody binding specifically to human VEGF, which is a dual-targeting

protein wherein a novel ScFv (single-chain variable fragment) that binds specifically to human DLL4 is connected to the C-terminal region of a protein similar to IgG -type Avastin, and have found that such a dual-targeting protein effectively
5 inhibits not only the interaction between VEGF and VEGF receptor, but also the interaction between DLL4 and Notch protein, and thus exhibits excellent anticancer effects, thereby completing the present invention.

10

TECHNICAL SOLUTION

It is an object of the present invention is to provide a dual-targeting protein comprising: a protein binding specifically to DLL4, which recognizes a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and
15 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21; and an antibody binding specifically to VEGF (vascular endothelial growth factor).

Another object of the present invention is to provide a
20 polynucleotide encoding the above-described dual-targeting protein, an expression vector comprising the polynucleotide, and a transformant comprising the expression vector.

Still another object of the present invention is to provide a method for producing the dual-targeting protein.

Yet another object of the present invention is to provide a composition comprising the above-described dual-targeting protein.

5 A further object of the present invention is to provide a pharmaceutical composition for treating cancer, which comprises the above-described dual-targeting protein.

A still further object of the present invention is to provide a composition for diagnosing cancer, which comprises the above-described dual-targeting protein.

10 A yet further object of the present invention is to provide a method for diagnosing cancer using the above-described dual-targeting protein.

Another further object of the present invention is to provide a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21.

Another still further object of the present invention is to provide a monoclonal antibody binding specifically to DLL4, 20 which recognizes the above-described conformational epitope.

Another yet further object of the present invention is to provide a polynucleotide encoding the monoclonal antibody, an expression vector comprising the polynucleotide, and a transformant comprising the expression vector.

Another yet further object of the present invention is to provide a method for treating cancer, which comprises a step of administering the above-described dual-targeting protein to a subject suspected of having cancer.

5

ADVANTAGEOUS EFFECTS

The dual-targeting protein according to the present invention can treat cancer by binding to both VEGF and DLL4, and exhibits excellent binding affinity and anticancer effects because it comprises a novel protein that binds specifically to DLL4. Thus, it can be widely used in the fields of cancer treatment and diagnosis.

10

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1a and 1b show the structure of a dual-targeting protein capable of binding to both DLL4 and VEGF.

15

FIG. 2a shows the results obtained by expressing a dual-targeting protein, which can bind to both DLL4 and VEGF, in CHO cells, purifying the expressed protein, and analyzing the purified protein by SDS-PAGE.

20

FIG. 2b shows the results obtained by expressing a dual-targeting protein, which can bind to both DLL4 and VEGF, in CHO cells, purifying the expressed protein, and analyzing the purified protein by SEC-HPLC chromatography.

FIG. 3 shows the results of an enzyme-linked immunosorbent assay (ELISA) performed to examine the abilities of the dual-targeting protein to bind to DLL4 and VEGF.

5 FIG. 4a shows the results of a Biacore assay performed to measure the equilibrium dissociation constant (KD) of the dual-targeting protein for DLL4, an antigen that is targeted by the dual-targeting protein.

FIG. 4b shows the results of a Biacore assay performed
10 to measure the equilibrium dissociation constant (KD) of the dual-targeting protein for VEGF, an antigen that is targeted by the dual-targeting protein.

FIG. 5 shows the results of an ELISA performed to measure the abilities of the dual-targeting protein to
15 neutralize DLL4 and VEGF.

FIG. 6 shows that human DLL4 and MLCK2 antibody form a complex in the presence or absence of a cross-linker.

FIG. 7 shows a model in which a fragment consisting of amino acid residues 58-65 [FRVCLKHF]) of the amino acid
20 sequence of DLL4 represented by SEQ ID NO: 21 and a fragment represented by SEQ ID NO: 22 constitute a continuous molecular surface on a human DLL4 C2 domain (amino acid residues 27-174).

FIG. 8 shows the results of Western blotting performed
25 to examine the binding affinities of mutant proteins encoding

a deletion fragment of the extracellular domain of each of wild-type and DLL4.

FIG. 9a shows that, when treatment with the VEGF-targeting antibody Avastin was performed, the proliferation
5 of vascular endothelial cells was inhibited in a concentration-dependent manner regardless of the presence or absence of DLL4.

FIG. 9b shows that, when treatment with an antibody against DLL4 alone was performed, the proliferation of
10 vascular endothelial cells appeared only in an experimental group with DLL4 in a manner dependent on the concentration of the anti-DLL4 antibody.

FIG. 9c shows that, when treatment with the dual-targeting protein was performed, an experimental group
15 without DLL4 showed a proliferation inhibitory effect similar to that of treatment with the Avastin antibody (black bars), and an experimental group with DLL4 showed a reduction in the vascular proliferation inhibitory effect compared to Avastin (white bars).

20 FIG. 10 shows the results of Western blot analysis, which indicate that the dual-targeting protein that binds to DLL4 and VEGF exhibits an activity of inhibiting the DLL4/Notch and VEGF/VEGFR signaling pathways in human umbilical vein endothelial cells (HUVECs).

FIG. 11 shows that the dual-targeting protein that binds to DLL4 and VEGF has a stronger anticancer effect than Avastin in an Avastin-resistant human SCH gastric cancer xenograft model constructed in nude mice.

5 FIG. 12 shows that the dual-targeting protein that binds to DLL4 and VEGF has a stronger anticancer effect than Avastin in an Avastin-resistant human A549 lung cancer xenograft model constructed in nude mice.

10 BEST MODE FOR CARRYING OUT THE INVENTION

In one aspect, the present invention provides a dual-targeting protein comprising: a protein binding specifically to DLL4, which recognizes a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in
15 the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21; and an antibody binding specifically to VEGF (vascular endothelial growth factor).

As used herein, the term "dual-targeting protein" refers to a protein capable of binding to two different antigens
20 (target proteins). Specifically, the dual-targeting protein does not naturally occur and is preferably produced by a genetic engineering method or any other method.

For the purpose of the present invention, the dual-targeting protein can bind to both VEGF that is overexpressed
25 in cancer cells and DLL4 that is expressed in endothelial

cells. The dual-targeting protein may be in the form of an antibody. The term "dual-targeting protein", as used herein, may be used interchangeably with the term "dual-targeting antibody", "bispecific antibody" or "bispecific antibody protein". Preferably, the dual-targeting protein of the present invention may target VEGF and DLL4 as antigens. The form of the dual-targeting protein according to the present invention includes a dual-targeting protein form wherein an IgG-type antibody that binds specifically to VEGF and a protein that binds specifically to DLL4 are connected to each other by a linker, but is not specifically limited thereto. The structure of the dual-targeting protein according to the present invention is as schematically shown in FIG. 1a.

Specifically, the dual-targeting protein of the present invention may comprise a heavy-chain amino acid sequence represented by SEQ ID NO: 1 and a light-chain amino acid sequence represented by SEQ ID NO: 20, but is not limited thereto.

As used herein, the term "antibody" refers to a protein molecule which comprises an immunoglobulin molecule immunologically reactive with a particular antigen, and which serves as a receptor that specifically recognizes an antigen. The term may include all polyclonal antibodies, monoclonal antibodies, full-length antibodies, and antibody fragments. In addition, the term may include forms produced by the

genetic engineering, such as chimeric antibodies (e.g., humanized murine antibodies) and heterogeneous antibodies (e.g., bispecific antibodies). A full-length antibody has two full-length light chains and two full-length heavy chains, in which each of the light chains is linked to the heavy chain by a disulfide bond. The full-length antibody may comprise IgA, IgD, IgE, IgM and IgG, and subtypes of IgG include IgG1, IgG2, IgG3 and IgG4. In addition, the term antibody may include bivalent molecules, diabodies, triabodies, and tetrabodies. Specifically, the antibodies that bind specifically to VEGF may be IgG type.

In the present invention, the dual-targeting protein may be a form wherein an immunoglobulin G (IgG)-type antibody that binds specifically to VEGF (vascular endothelial growth factor) and a full-length antibody, Fab', F(ab')₂, Fab, Fv, rIgG or scFv-type protein that binds specifically to DLL4 (delta-like ligand 4) are connected to each other by a linker.

Typically, an immunoglobulin and scFv have heavy chains and light chains, and each heavy and light chain contains a constant region and a variable region (the regions are also known as domains). Light and heavy chain variable regions contain four framework regions and three hypervariable regions, also called "complementarity-determining regions" (hereinafter referred to as "CDRs"). The CDRs are primarily responsible for binding to an epitope of an antigen. The

CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located.

5 The dual-targeting protein of the present invention, which comprises a protein that binds specifically to DLL4 and an antibody that binds specifically to VEGF, shows a strong affinity for human-derived DLL4 and VEGF, effectively inhibits the binding of DLL4-expressing cells (e.g., cancer
10 cells or vascular endothelial cells) to Notch 1 or Notch 4 receptor, and also inhibits an angiogenic process in which vascular endothelial cells expressing VEGF receptor are activated by VEGF that is overexpressed in cancer cells. Thus, the dual-targeting protein of the present invention can
15 exhibit a stronger therapeutic effect in the treatment of diseases such as cancer.

 The VEGF-specific binding antibody and DLL4-specific binding protein of the dual-targeting protein according to the present invention can maintain their specific binding,
20 and particularly, can simultaneously inhibit two targets (antigens). Thus, the antibody and the protein can be more effective than a protein or antibody that binds to and inhibits a single target, and these can simultaneously inhibit two signals.

As used herein, the term "antibody fragments" refers to fragments having the ability to bind to antigens, and includes antigen-binding forms of antibodies, for example, Fab', F(ab')₂, Fab, Fv, rIgG and scFv. In particular, the
5 term "antibody fragments" include scFv (single-chain variable fragment), and particularly, include bivalent molecules or diabodies, triabodies, and tetrabodies.

As used herein, the term "scFv (single-chain variable fragment)" refers to the minimum antibody fragment that
10 contains a complete antigen-recognition and antigen-binding site and comprises antibody VH and VL domains, in which the domains may be present in a single polypeptide chain.

As used herein, the phrase "dual-targeting protein comprising: a protein binding specifically to DLL4, which
15 recognizes a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21; and an antibody binding specifically to VEGF (vascular endothelial growth factor)" may include any
20 dual-targeting protein that can simultaneously inhibit two signaling pathways in which DLL4 and VEGF are involved. The VEGF-specific binding antibody and DLL4-specific binding protein of the dual-targeting protein may be in the form of full-length antibodies and antibody fragments as described
25 above.

As used herein, the phrase "protein binding specifically to DLL4, which recognizes a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21 refers to a protein binding specifically to a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21. The protein means a protein that can exhibit a cancer therapeutic effect by inhibiting the growth of cancer. The protein can bind to the epitope with high affinity, and can function to neutralize DLL4 activity. The protein can block the binding of DLL4 to Notch receptor, and inhibit DLL4-mediated signaling. A protein binding specifically to a conformational epitope of DLL4 comprising amino acid sequences SEQ ID NOs: 21 and 22 may be specifically in the form of full-length antibodies, Fab', F(ab')₂, Fab, Fv, rIgG, or scFv(Single-chain variable fragment).

The protein binding specifically to a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21, specifically, the protein that binds specifically to DLL4 may comprise: a heavy-chain variable region comprising heavy-chain CDR1

represented by SEQ ID NO: 2, heavy-chain CDR2 represented by
SEQ ID NO: 3, and heavy-chain CDR3 represented by SEQ ID NO:
4; and a light-chain variable region comprising light-chain
CDR1 represented by SEQ ID NO: 5, light-chain CDR2
5 represented by SEQ ID NO: 6, and light-chain CDR3 represented
by SEQ ID NO: 7.

More specifically, the heavy chain may comprise a heavy-
chain amino acid sequence represented by SEQ ID NO: 8, and
the light chain may comprise a light-chain amino acid
10 sequence represented by SEQ ID NO: 9. However, the protein
may also be any protein that comprises the above-described
CDR sequences and can bind specifically to DLL4 to exhibit a
cancer therapeutic effect. The heavy chain and the light
chain may be connected to each other by a linker.

15 In addition, the DLL4-specific binding protein of the
dual-targeting protein of the present invention can bind
specifically not only to human DLL4, but also to mouse DLL4,
and can inhibit the interaction between DLL4 and Notch
protein.

20 In an example of the present invention, the epitope of
the DLL4-specific binding antibody of the dual-targeting
protein of the present invention, which has an excellent
biological activity of inhibiting DLL4 and VEGF, was
identified. Specifically, in the present invention, it was
25 found that the antibody binds to the continuous molecular

surface of DLL4, which consists of amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of DLL4. Thus, amino acid residues 58th to 65th (SEQ ID NO: 22) and/or 110th to 115th (SEQ ID NO: 23) in the amino acid sequence of
5 DLL4 can be the epitope of the DLL4-specific binding antibody according to the present invention. More specifically, a molecular surface region of DLL4, which consists of SEQ ID NOs: 22 and 23, may be a conformational epitope.

As used herein, the term "delta-like ligand 4 (DLL4)"
10 refers to one of delta-class ligands binding to Notch receptors and preferably refers to a protein binding to Notch 1 or Notch 2, but is not limited thereto. DLL4 may be any mammalian DLL4, but is preferably human or mouse DLL4. It is known that DLL4 is overexpressed in various tumor cells
15 including tumor vasculatures and promotes the growth of cancer by increasing the number of abnormal vasculatures in xenograft models.

Thus, the dual-targeting protein of the present invention, which comprises a protein binding specifically to
20 a conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21, can be effectively used to treat cancer by inhibiting the function of DLL4. Information about DLL4 can
25 be obtained from known databases, including GenBank of the

National Institutes of Health, and may be, for example, information of DLL4 which is GenBank Accession Number NM_019074.3 (Gene ID: 54567 and NCBI Reference Sequence: NM_019074.3). The DLL4 may comprise the amino acid sequence
5 of SEQ ID NO: 21.

As used herein, the term "Notch receptor" refers to a protein that mediates Notch signaling, and may be used interchangeably with Notch. The Notch receptor may be any protein that mediates Notch signaling. Preferably, the Notch
10 receptor may be Notch 1 or Notch 4 receptor, but is not limited thereto.

As used herein, the phrase "inhibiting the interaction between human delta-like ligand 4 (DLL4) and Notch receptor" means that the DLL4-specific binding protein of the present
15 invention binds to DLL4 to inhibit the interaction between DLL4 and Notch receptor. Preferably, the phrase means that the dual-targeting protein specific for the conformational epitope of DLL4, which comprises amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of the DLL4
20 (delta-like ligand 4) protein represented by SEQ ID NO: 21, binds to DLL4 to inhibit the interaction between DLL4 and Notch 1 or Notch 4 receptor, but is not limited thereto. When the dual-targeting protein of the present invention binds specifically to the conformational epitope of DLL4,
25 which comprises amino acid residues 58th to 65th and 110th to

115th in the amino acid sequence of the DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21, it prevents Notch receptors from being structurally changed by the binding of DLL4 thereto. Thus, it prevents the hydrolysis of Notch proteins to inhibit Notch signaling. It is known that when DLL4 binds to Notch receptor in tumors, it increases the size of blood vessels and activates the signaling between vascular endothelial cells or Notch signaling between cancer cells and vascular endothelial cells, thereby taking a role in the proliferation and metastasis of tumors.

Thus, when Notch signaling by DLL4 in tumors is inhibited, angiogenesis cannot be easily controlled, and thus the growth of tumors can be inhibited. In addition, when DLL4 is blocked, the loss of lateral inhibition in cells at the end of an angiogenic site appears to cause excessive sprouting, resulting in a decrease in angiogenic reactions having low productivity, and perfusion for supplying oxygen can be reduced to induce hypoxia around tumors, resulting in anti-tumor effects even against tumors showing resistance to anti-VEGF therapy.

Accordingly, the dual-targeting protein of the present invention, which comprises the DLL4-specific binding protein that effectively inhibits the interaction between DLL4 and Notch, can be effectively used for the treatment of cancer.

As used herein, the phrase "antibody that binds specifically to VEGF" or "VEGF-specific binding antibody" includes all antibodies that bind specifically to the antigen VEGF in tumor cells. Specifically, the antibody may be 5 Bevacizumab(Avastin®), a therapeutic antibody that targets VEGF, but is not limited thereto. Such antibodies that bind specifically to VEGF may include full-length antibodies or antibody fragments as described above, and may be IgG antibodies, but are not limited thereto. VEGF is a ligand 10 playing an important role in angiogenesis, and when VEGF is inhibited, no angiogenesis will occur, and thus cancer can be treated. Bevacizumab (Avastin®, Genentech) approved by the US FDA is a therapeutic antibody that can be stably used.

The antibody binding specifically to VEGF, specifically, 15 may comprise: a heavy-chain variable region comprising heavy-chain CDR1 represented by SEQ ID NO: 10, heavy-chain CDR2 represented by SEQ ID NO: 11, and heavy-chain CDR3 represented by SEQ ID NO: 12; and a light-chain variable region comprising light-chain CDR1 represented by SEQ ID NO: 20 13, light-chain CDR2 represented by SEQ ID NO: 14, and light-chain CDR3 represented by SEQ ID NO: 15. More specifically, the antibody binding specifically to VEGF may comprise a heavy-chain variable region amino acid sequence represented by SEQ ID NO: 16 and a light-chain variable region amino acid 25 sequence represented by SEQ ID NO: 17. However, the antibody

may also be any antibody that comprises the above-described CDR sequences and can bind specifically to VEGF to exhibit a cancer therapeutic effect.

5 The VEGF-specific binding antibody of the dual-targeting protein according to the present invention can bind specifically to VEGF that is overexpressed in tumor cells, and thus can concentrate the dual-targeting protein of the present invention on tumor cells expressing VEGF. Also, it can exhibit anticancer activity by binding to VEGF.

10 As used herein, the term "vascular endothelial growth factor (VEGF)" refers to a kind of growth factor that enhances the growth activity of vascular endothelial cells and is secreted by various kinds of cells, including macrophages, smooth muscle cells and tumor cells. VEGF plays
15 an important role in fetal angiogenesis, and also functions to induce angiogenesis in order to supply oxygen to tumor tissue in which rapid growth and metabolism occur. Pathways in which VEGF protein and its receptor are involved have been studied as target signaling pathways of anticancer agents in
20 adults.

In addition, the VEGF-binding site of the dual-targeting protein means inhibiting the interaction between human VEGF and VEGF receptor. Specifically, it means that the dual-targeting protein specific for VEGF binds to VEGF to inhibit

the interaction between VEGF and VEGFR-2, but is not limited thereto.

For the purpose of the present invention, the VEGF receptor may be any protein that binds to mammalian VEGF.

5 Specifically, it may be a protein that binds to human VEGF.

When the interaction between VEGF and VEGF receptor is inhibited by the VEGF-specific dual-targeting protein of the present invention, VEGF/VEGF signaling by the binding of VEGF to VEGF receptor will be inhibited. It is known that when
10 VEGF and VEGF receptor in tumors bind to each other, VEGF/VEGF receptor signaling in stromal/endothelial cells of cancer tissue is activated to strongly inhibit angiogenesis, unlike the mechanism of the DLL4/Notch signaling pathway, to reduce the number of blood vessels and weaken a vascular
15 function in tumors, thereby inhibiting cancer proliferation and metastasis.

Thus, the dual-targeting protein of the present invention, which is specific for DLL4 and VEGF, shows the ability to inhibit angiogenesis in cancer tissue by a
20 different mechanism, and thus can be used as a therapeutic agent having better anticancer activity.

Specifically, the double-targeting protein may be a form in which the protein that binds specifically to DLL4 and the IgG (immunoglobulin G)-type antibody that binds specifically
25 to VEGF are connected to each other by a linker.

As used herein, the term "linker" refers to any moiety which can connect two different fusion partners (e.g., biological polymers) by use of a hydrogen bond, electrostatic interaction, van der Waals force, a disulfide bond, a salt
5 bridge, hydrophobic interaction, a covalent bond, etc. Specifically, the linker may have at least one cysteine residue which can participate in at least one disulfide bond under physiological conditions or other standard peptide conditions (e.g., conditions for purifying or storing
10 peptides). In addition to connecting the fusion partners, the linker may serve as a spacer and provide a space between the fusion partners or as a hinge to provide flexibility or rigidity for the conjugate. The linker may be a peptidyl linker or a non-peptidyl linker. Direct connection between
15 the fusion partners via a peptide bond or a disulfide bond is within the scope of the role of the linker.

In the present invention, the linker may preferably be a polypeptide which can connect the DLL4-specific binding protein to the VEGF-specific binding antibody, but is not
20 specifically limited thereto. More preferably, the linker may be a peptidyl linker which can connect the C-terminus of the Fc region of the VEGF-specific binding antibody to the DLL4-specific binding protein. More preferably, the linker may be a peptidyl linker comprising an amino acid sequence
25 consisting of three repeats of a GGGGS motif. The GGGGS

motif may be repeated 1-10 times. Most preferably, the linker may comprise an amino acid sequence of SEQ ID NO: 18 or an amino acid sequence encoded by a polynucleotide sequence of SEQ ID NO: 19.

5 Linker peptide (SEQ ID NO: 18): GGGGSGGGGSGGGGS

Linker polynucleotide (SEQ ID NO: 19):
GGTGGAGGTGGCAGCGGTGGTGGCGGCAGTC CCGGTGGCGGCTCC

As used herein, the term "non-peptide linker" refers to a biocompatible linker consisting of at least two repeating
10 units which may be connected to each other by any non-peptidyl covalent bond.

Examples of the non-peptide linker that is used in the present invention include polyethylene glycol (PEG) homopolymers, polypropylene glycol homopolymers, ethylene
15 glycol-propylene glycol copolymers, polyoxyethylated polyol, polyvinyl alcohols, polysaccharides, dextran, polyvinyl ethyl ether, biodegradable polymers, lipid polymers, chitins, hyaluronic acid, and combinations thereof. Preferably, the non-peptidyl linker may be a polyethylene glycol homopolymer.
20 Derivatives that have already been known in the art or can be readily prepared on the technical level of the art are within the scope of the present invention. More preferably, the non-peptidyl linker may be a polyethylene glycol homopolymer having a molecular weight of from 1 to 5 kDa. Most
25 preferably, it may be a linker having a molecular weight of

3.4 kDa and containing aldehyde groups at both ends, which can connect VEGF-specific binding antibody to the DLL4-specific binding protein. Particularly, aldehyde functional groups at both ends are effective in minimizing non-specific
5 reactions.

Regions that are connected directly or indirectly via the linker include Fc fragments, Fab', F(ab')₂, Fab, Fv and the like, but are not specifically limited thereto. The dual-targeting protein may be: a form in which the whole or
10 part of the DLL4-specific binding protein is connected to the whole or part of VEGF-specific binding antibody; or a form in which the whole or part of the DLL4-specific binding protein is connected to the whole or part of VEGF-specific binding antibody by a peptidyl linker; or a combination thereof, but
15 the dual-targeting protein is not limited thereto.

In addition, The dual-targeting protein may be: a form in which the whole or part of the DLL4-specific binding protein is connected to the whole or part of a heavy chain of VEGF-specific binding antibody by a peptidyl linker; a form
20 in which the whole or part of the DLL4-specific binding protein is connected to the whole or part of a light chain of VEGF-specific binding antibody by a peptidyl linker; or a combination thereof.

In an example of the present invention, the dual-
25 targeting protein Avastin-DLL4 BsAb that binds specifically

to DLL4 and VEGF was constructed by connecting the C-terminus of the heavy-chain region of IgG-type Avastin to an scFv-type DLL4-binding protein by a linker to prepare a dual-targeting protein-encoding polynucleotide, inserting the polynucleotide into a vector, introducing the vector into animal cells, and isolating an Avastin-DLL4-binding dual-targeting protein from the cells. The dual-targeting protein molecule has a structure in which an Avastin IgG antibody molecule is connected to a DLL4-binding scFv by a linker (FIG. 1). The Avastin-DLL4-binding dual-targeting protein expressed in animal cells was isolated, and the expression and purity thereof were measured (FIGS. 2a and 2b). In addition, it was found that the Avastin-DLL4-binding dual-targeting protein binds specifically to the targets VEGF and DLL4 (FIG. 3). In addition, it was shown that the binding affinity of the dual-targeting protein for each of the antigens was similar to that of a control antibody. Specifically, the dual-targeting protein showed a KD value of 30 nM for human DLL4 and a KD value of 0.126 nM for human VEGF (Tables 2 and 3). Moreover, it was shown that the signaling pathway caused by each of the binding vascular endothelial cell DLL4 and human Notch 1 receptor and the binding of VEGF to VEGF receptor was effectively inhibited by treatment with the dual-targeting protein (FIG. 10). Such results suggest that the dual-targeting protein of the present invention, which is specific

for DLL4 and VEGF, can efficiently block the binding of DLL4 to Notch receptor and the binding of VEGF to VEGF receptor, thereby providing an anticancer effect. The anticancer effect of the dual-targeting protein in Avastin-resistant
5 human SCH gastric cancer and A549 lung cancer xenograft models was found (FIGS. 11 and 12).

In another aspect, the present invention provides a polynucleotide encoding the dual-targeting protein, an expression vector comprising the polynucleotide, and a
10 transformant introduced with the expression vector.

An expression vector comprising a polynucleotide encoding the dual-targeting protein according to the present invention is not specifically limited, but may be a vector capable of replicating and/or expressing the polynucleotide
15 in eukaryotic or prokaryotic cells, including mammalian cells (e.g., human, monkey, rabbit, rat, hamster or mouse cells), plant cells, yeast cells, insect cells and bacterial cells (e.g., *E.coli*). Preferably, it may be a vector, which comprises at least one selective marker and is operably
20 linked to a suitable promoter so that the polynucleotide can be expressed in a host cell. More preferably, the vector may comprise the polynucleotide introduced into a phage, plasmid, cosmid, mini-chromosome, virus or retrovirus vector.

The expression vector comprising the polynucleotide
25 encoding the dual-targeting protein may be either an

expression vector comprising each polynucleotide encoding the heavy chain or light chain of the dual-targeting protein or an expression vector comprising all the polynucleotides encoding the heavy chain and light chain of the dual-targeting protein.

Cells into which the expression vector of the present invention is to be introduced to form transformants include bacterial cells such as *E. coli*, *Streptomyces* and *Salmonella typhimurium*; yeast cells; fungal cells such as *Pichia pastoris*; insect cells such as *Drosophila* or *Spodoptera Sf9* cells; animal cells such as Chinese hamster ovary (CHO) cells, SP2/0 (mouse myeloma), human lymphoblastoid, COS, NSO (mouse myeloma), 293T, Bowes melanoma cells, HT-1080, BHK (baby hamster kidney cells), HEK (human embryonic kidney cells), PERC.6 (human retinal cells), and the like; and plant cells. In an example of the present invention, CHO-S cells were used as host cells.

As used herein, the term "introduction" refers to the delivery of the vector comprising the polynucleotide encoding the dual-targeting protein into a host cell. This introduction may be performed by various methods known in the art, including calcium phosphate-DNA coprecipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome-mediated transfection, liposome fusion, lipofection and

protoplast fusion. Also, transfection means delivering a desired material into a cell by means of infection using viral particles. In addition, the vector may be introduced into a host cell by gene bombardment. In the present
5 invention, introduction may be used interchangeably with transfection.

In still another aspect, the present invention provides a method for producing the dual-targeting protein.

Preferably, the method for producing the dual-targeting
10 protein may be a method for producing a dual-targeting protein comprising a protein that binds specifically to DLL4 and an antibody that binds specifically to VEGF (vascular endothelial growth factor), the method comprising the steps of: (a) culturing the transformant to produce a dual-
15 targeting protein; and (b) recovering the dual-targeting protein produced in step (a).

More preferably, the method for producing the dual-targeting protein may be a method comprising the steps of:
(a) preparing a polynucleotide encoding an antibody that
20 binds specifically to VEGF, and a polynucleotide encoding a protein which binds specifically to DLL4 and which comprises: a heavy-chain variable region comprising heavy-chain CDR1 represented by SEQ ID NO: 2, heavy-chain CDR2 represented by SEQ ID NO: 3, and heavy-chain CDR3 represented by SEQ ID NO:
25 4; and a light-chain variable region comprising light-chain

CDR1 represented by SEQ ID NO: 5, light-chain CDR2 represented by SEQ ID NO: 6, and light-chain CDR3 represented by SEQ ID NO: 7; (b) connecting the 3' end of the Fc region-encoding polynucleotide portion of the polynucleotide encoding the VEGF-specific binding antibody, prepared in step (a), to the 5' end of the polynucleotide encoding the DLL4-specific binding protein by a linker, thereby obtaining a polynucleotide encoding the dual-targeting protein; (c) cloning the dual-targeting protein-encoding polynucleotide of step (b) to prepare an expression vector; (d) introducing the expression vector of step (c) into a host cell to obtain a transformant, and culturing the transformant; and (e) recovering the dual-targeting protein from the transformant of step (d).

15 In addition, the method for producing the dual-targeting protein may be a method comprising the steps of: (a) preparing a polynucleotide encoding an antibody that binds specifically to VEGF, and a polynucleotide encoding a protein which binds specifically to DLL4 and which comprises: a heavy-chain variable region comprising heavy-chain CDR1 represented by SEQ ID NO: 2, heavy-chain CDR2 represented by SEQ ID NO: 3, and heavy-chain CDR3 represented by SEQ ID NO: 4; and a light-chain variable region comprising light-chain CDR1 represented by SEQ ID NO: 5, light-chain CDR2 represented by SEQ ID NO: 6, and light-chain CDR3 represented

by SEQ ID NO: 7; (b) cloning the polynucleotide of step (a) to prepare an expression vector; (c) introducing the expression vector of step (b) into a host cell to obtain a transformant, and culturing the transformant; and (d)
5 recovering the VEGF-specific binding antibody and the DLL4-specific binding protein from the transformant of step (c), and connecting the C-terminus of the Fc region of the VEGF-specific binding antibody to the N-terminus of the DLL4-specific binding protein by a linker.

10 The dual-targeting protein of the present invention can be produced by a known recombination technique or biochemical method, and the antibody may be introduced into a suitable host cell and recovered from the culture medium of the transformant.

15 Specifically, the dual-targeting protein may be isolated by a known isolation method. For example, the dual-targeting protein may be suitably isolated from the culture medium by a conventional purification procedure such as protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis,
20 dialysis, or affinity chromatography, but is not limited thereto.

In yet another aspect, the present invention provides a composition comprising the dual-targeting protein.

In a further aspect, the present invention provides a pharmaceutical composition for treating cancer, which comprises the dual-targeting protein.

The dual-targeting protein can bind to both DLL4 and
5 VEGF to inhibit the binding of DLL4 and VEGF to Notch and VEGF receptor, thereby inhibiting the growth of cancer. The DLL4/Notch receptor and the VEGF/VEGF receptor are as described above. When the composition of the present invention, which comprises the dual-targeting protein that
10 binds specifically to DLL4 and VEGF, is administered *in vivo*, it can inhibit the development, proliferation or metastasis of cancer or prevent the progression of cancer, thereby treating cancer.

As used herein, the term "cancer" includes all the kinds
15 of cancers without limitations, but examples of the cancer may include esophageal cancer, stomach cancer, large intestine cancer, rectal cancer, oral cancer, pharynx cancer, larynx cancer, lung cancer, colon cancer, breast cancer, uterine cervical cancer, endometrial cancer, ovarian cancer,
20 prostate cancer, testis cancer, bladder cancer, renal cancer, liver cancer, pancreatic cancer, bone cancer, connective tissue cancer, skin cancer, brain cancer, thyroid cancer, leukemia, Hodgkin's disease, lymphoma, and multiple myeloid blood cancer. As used herein, the term "treatment" refers to

all actions that restore or beneficially change the symptoms of cancer by administering the composition.

In addition, the pharmaceutical composition of the present invention may further comprise a pharmaceutically
5 acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not impair the biological activity and characteristics of an administered compound without irritating an organism. As a
10 pharmaceutically acceptable carrier in a composition that is formulated as a liquid solution, a sterile and biocompatible carrier is used. The pharmaceutically acceptable carrier may be physiological saline, sterile water, Ringer's solution, buffered saline, albumin injection solution, dextrose
15 solution, maltodextrin solution, glycerol, ethanol, or a mixture of two or more thereof. In addition, the composition of the present invention may, if necessary, comprise other conventional additives, including antioxidants, buffers, and bacteriostatic agents. Further, the composition of the
20 present invention may be formulated as injectable forms such as aqueous solutions, suspensions or emulsions with the aid of diluents, dispersants, surfactants, binders and lubricants. In addition, the composition according to the present invention may be formulated in the form of pills, capsules,
25 granules, or tablets.

The pharmaceutical composition of the present invention may be formulated in various manners such as an oral or parenteral formulation. For formulations, commonly used diluents or excipients such as fillers, expanders, binders, wetting agents, disintegrants and surfactants, etc., are used. A pharmaceutical composition comprising the compound according to the present invention is formulated using diluents or excipients, such as fillers, extenders, binders, wetting agents, disintegrants or surfactants, which are commonly used. Solid Formulations for oral administration include tablets, pills, powders, granules, capsules, etc. Such Formulations are prepared by mixing the compound of present invention with at least one excipient, such as starch, calcium carbonate, sucrose, lactose, gelatin, etc. In addition to simple expedients, lubricants such as magnesium stearate, talc, etc. may also be added. Liquid Formulations for oral administration, such as suspensions, internal solutions, emulsions, syrups, etc., may include simple diluents, e.g., water and liquid paraffin, as well as various excipients, e.g., wetting agents, s, sweeteners, aromatics, preservatives, etc. Formulations for parenteral administration include sterilized aqueous solutions, non-aqueous solvents, suspensions, emulsions, lyophilized agents, suppositories, etc. Non-aqueous solvents and suspensions may be prepared using propylene glycol, polyethylene glycol,

vegetable oils such as olive oil, or injectable esters such as ethylolate. As a base for suppositories, Witepsol, Macrogol, Tween 61™, cacao fat, laurin fat, glycerogelatin, etc. may be used.

5 The pharmaceutical composition may have any one formulation selected from the group consisting of tablets, pills, powders, granules, capsules, suspensions, internal solutions, emulsions, syrups, sterilized aqueous solutions, non-aqueous solvents, lyophilized agents, and suppositories.

10 The pharmaceutical composition of the present invention is administered in a pharmaceutically effective amount.

As used herein, the term "pharmaceutically effective amount" refers to an amount sufficient to treat diseases at a reasonable benefit/risk ratio applicable to any medical treatment. The effective dosage level of the composition may be determined depending on the subject's type, the disease severity, the subject's age and sex, the type of the disease, the activity of the drug, sensitivity to the drug, the time of administration, the route of administration, excretion rate, the duration of treatment, factors including drugs used in combination with the composition, and other factors known in the medical field. The pharmaceutical composition of the present invention may be administered individually or in combination with other therapeutic agents, and may be administered sequentially or simultaneously with conventional

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therapeutic agents. The composition can be administered in a single or multiple dosage form. It is important to administer the composition in the minimum amount that can exhibit the maximum effect without causing side effects, in
5 view of all the above-described factors, and this amount can be easily determined by a person skilled in the art.

In an example of the present invention, it was found that the dual-targeting protein of the present invention could bind to both VEGF and DLL4 (FIGS. 3, 4a and 4b), could
10 neutralize DLL4 (FIG. 5), and exhibited an anticancer effect in Avastin-resistant human SCH gastric cancer and A549 lung cancer xenograft models (FIGS. 11 and 12), indicating that the dual-targeting protein can be used as an active ingredient in compositions for treating cancer.

15 In still another aspect, the present invention provides a method of treating cancer using a pharmaceutical composition comprising the dual-targeting protein. The method may comprise administering a pharmaceutically effective amount of the pharmaceutical composition.

20 The dual-targeting protein and the pharmaceutically effective amount are as described above.

The method of treating cancer may comprise administering a pharmaceutical composition comprising the dual-targeting protein together with a pharmaceutically acceptable carrier
25 to a subject having cancer or suspected of having cancer.

Herein, the pharmaceutically acceptable carrier and the cancer are as described above. Examples of the subject include mammals, including cattle, pigs, sheep, chickens, dogs, and humans. The subject may be any subject in which
5 cancer is to be treated by administration of the composition of the present invention.

In this case, the composition may be administered in the form of liquid, powder, aerosol, capsule, enteric-coated tablet, or suppository. The composition of the present
10 invention can be administered intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally, orally, topically, intranasally, intrapulmonarily or intrarectally, but is not limited thereto. However, because the peptide is digested when administered orally, the active
15 ingredient in the composition for oral administration is required to be coated or formulated so as to be protected from degradation in the stomach. In addition, the pharmaceutical composition may be administered by any device by which the active ingredient may be delivered to target
20 cells.

In a still further aspect, the present invention provides a composition for diagnosing cancer, which comprises the dual-targeting protein.

The dual-targeting protein and the cancer are as
25 described above.

As used herein, the term "diagnosing" means detecting the presence or feature of a pathological condition. For the purpose of the present invention, the term "diagnosing" means detecting the onset of cancer.

5 The composition for diagnosing cancer according to the present invention can be used as follows. The level of VEGF or DLL4 protein on a sample isolated from a subject suspected of having cancer is measured using the dual-targeting protein, and the subject is determined to have cancer, if the measured
10 level of VEGF or DLL4 is higher than that in a normal control sample.

To this end, analysis methods for measuring the amount of the protein include, but are not limited to, immunoblotting (Western blotting), ELISA (Enzyme Linked
15 Immunosorbent Assay), radioimmunoassay (RIA), radioimmunodiffusion, Ouchterlony immunodiffusion, rocket immunoelectrophoresis, immunohistostaining, immunoprecipitation assay, complement fixation assay, FACS, and protein chip assay. The levels of VEGF or DLL4 protein
20 in a normal control sample and a subject suspected of having cancer can be compared with each other through the analysis methods, and thus the onset of cancer of a patient suspected of having cancer can be diagnosed actually.

The composition for diagnosing cancer according to the
25 present invention may further comprise, in addition to the

dual-targeting protein, those known in the art which are required to perform the method for measuring the level of the protein.

In a yet further aspect, the present invention provides
5 a method for diagnosing cancer, comprising the steps of: (a) measuring the level of VEGF or DLL4 protein in a sample, isolated from a subject suspected of having cancer, using the dual-targeting protein; and (b) determining that the subject has cancer, if the level of VEGF or DLL4 protein measured in
10 step (a) is higher than that in a normal control sample.

Herein, the dual-targeting protein, the cancer, the subject, the diagnosing, and the method (step) of measuring the level of the protein, are as described above.

As used herein, the term "sample" is meant to include
15 whole blood, serum, blood, plasma, saliva, urine, phlegm, lymph, cerebrospinal fluid, and interstitial fluid, in which there is a difference in the expression level of VEGF or DLL4 in a cancer patient, but is not limited thereto.

In another further, the present invention provides a
20 conformational epitope of DLL4 comprising amino acid residues 58th to 65th and 110th to 115th in the amino acid sequence of a DLL4 (delta-like ligand 4) protein represented by SEQ ID NO: 21.

In an example of the present invention, amino acid
25 residues in DLL4 of SEQ ID NO: 21, which are cross-linked,

were identified by a cross-linking reaction and mass spectrometry. As shown in FIG. 7, it was found that two fragments, an amino acid sequence consisting of amino acid residues 58th to 65th [FRVCLKHF], and an amino acid sequence
5 consisting of amino acid residues 110th to 115th (SEQ ID NO: 23), constituted a continuous molecular surface, thereby forming the epitope of DLL4.

In another still further aspect, the present invention provides a monoclonal antibody binding specifically to DLL4,
10 which recognizes the conformational epitope.

Specifically, the monoclonal antibody may comprises: a heavy-chain variable region comprising heavy-chain CDR1 represented by SEQ ID NO: 2, heavy-chain CDR2 represented by SEQ ID NO: 3, and heavy-chain CDR3 represented by SEQ ID NO:
15 4; and a light-chain variable region comprising light-chain CDR1 represented by SEQ ID NO: 5, light-chain CDR2 represented by SEQ ID NO: 6, and light-chain CDR3 represented by SEQ ID NO: 7. More specifically, the heavy chain may comprise a heavy-chain variable region amino acid sequence
20 represented by SEQ ID NO: 8, and the light chain variable region may comprise a light-chain amino acid sequence represented by SEQ ID NO: 9.

In another yet further aspect, the present invention provides a polynucleotide encoding the monoclonal antibody,

an expression vector comprising the polynucleotide, and a transformant introduced with the expression vector.

Herein, the DLL4, the monoclonal antibody, the vector, the transformant, etc., of the present invention are as
5 described above.

In another yet further aspect, the present invention provides a method for treating cancer, which comprises a step of administering the dual-targeting protein to a subject suspected of having cancer.

10 The subject is a subject in need of the prevention or treatment of cancer, and may be selected from mammals, including humans, cattle, horses, sheep, pigs, goats, camels, antelopes, dogs and cats in need of the treatment of cancer and symptoms similar thereto, but is not limited thereto.

15 As used herein, the term "administration" means introducing the pharmaceutical composition of the present invention into a patient by any suitable method. The pharmaceutical composition of the present invention may be administered by various oral or parenteral routes, as long as
20 it can reach a desired tissue.

The cancer treatment method of the present invention includes administering the dual-targeting protein or the pharmaceutical composition comprising the dual-targeting protein in a therapeutically effective amount. It is
25 apparent to those skilled in the art that the suitable total

daily dose of the composition can be determined by an attending physician or veterinarian within the scope of sound medical judgment. In addition, the composition may be administered one time or several times within the preferred
5 range of its effective amount. In view of the purpose of the present invention, However, the specific therapeutically effective amount for any particular patient will depend upon various factors including the type and extent of response to be achieved, specific compositions according to whether other
10 agents are used therewith or not, the patient's age, body weight, health condition, sex and diet, the time and route of administration, the secretion rate of the composition, the duration of treatment, and other drugs used in combination or coincident with the composition, and other similar factors
15 well-known in the medical field.

EXAMPLES

Hereinafter, the present invention will be described in further detail with reference to examples. It will be
20 obvious to a person having ordinary skill in the art that these examples are for illustrative purposes only and are not to be construed to limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

25

Example 1: Preparation of Anti-DLL4/VEGF Dual-Targeting Protein

Example 1-1: Preparation of DLL4 Antigen

As the extracellular domain antigen of human DLL4, human
5 DLL4 protein (Cat: 1506-D4/CF) purchased from R&D System was used. The DLL4 antigen protein comprises amino acid residues 27 to 524 of the amino acid sequence of DLL4 (Accession No. Q9NR61). The C-terminus of the protein has a 10-His tag.

An antigen corresponding to a specific region of the
10 extracellular domain of DLL4 was prepared. This specific region comprises amino acid residues 27 to 251. This region contains a motif called "DSL (delta/Serrate)/lag-2)" domain known to bind to Notch 1 receptor. A mammalian expression vector plasmid comprising a CMV promoter upstream of a
15 polynucleotide encoding a deletion fragment (amino acid residues 27 to 251) of the extracellular domain of DLL4 fused with Fc protein was prepared using a standard recombinant DNA technique. An additional construct encoding the deletion fragment of DLL4, which is a chimera of human DLL4 fused with
20 Fc protein, was prepared using a general recombinant DNA technique. The prepared constructs were transiently transfected into HEK 293E cells to express recombinant fusion proteins comprising amino acid residues 27 to 251 of the amino acid sequence of human DLL4 fused with Fc protein. To
25 recover the antigen protein, conditioning media were

collected every 72 hours, and this process was repeated four times. The antigen protein was purified from the conditioning medium by protein A affinity chromatography.

5 Example 1-2: Preparation of Library Phage

2.7 x 10¹⁰ human scFv (single-chain variable fragment) library cells having diversity were cultured in a medium (3 l) containing 17 g of 2X YT CM [Tryptone (CONDA, 1612.00), 10 g of yeast extract (CONDA, 1702.00), 5 g of NaCl (Sigma, S7653-5 kg), 34 µg/ml of chloramphenicol (Sigma, C0857)], 2% glucose (Sigma, G5400) and 5 mM MgCl₂ (Sigma, M2393) at 37°C for 2-3 hours (OD₆₀₀=0.5-0.7), after which the cells were infected with helper phage and cultured in 2X YT CMK medium (2X YT CM, 70 µg/ml of kanamycin (Sigma, K1876), 1 mM IPTG (ELPISBIO, IPTG025)) at 30°C for 16 hours. The cultured cells were centrifuged (4500 rpm, 15 min, 4°C), and the supernatant was added to and dissolved in 4% PEG (Fluka, 81253) 6000 and 3% NaCl (Sigma, S7653), and then incubated on ice for 1 hour. Next, the solution was centrifuged (8000 rpm, 20 min, 4°C), and the pellets were added to and dissolved in PBS and then centrifuged (12000 rpm, 10 min, 4°C). The supernatant comprising library phage was placed in a fresh tube and stored at 4°C.

25 Example 1-3: Panning by Phage Display

To screen an anti-DLL4 antibody that binds to human DLL4, panning of human DLL4 antigen was performed for 3 rounds.

10 $\mu\text{g/mL}$ of a solution of recombinant human DLL4 (R&D System) was added to an immunotube, and the protein was
5 adsorbed onto the surface of the immunotube overnight at 4°C , and then a solution of 1% bovine serum albumin was added to the immunotube to protect the surface not adsorbed with DLL4. After the immunotube was evacuated, 10^{12} CFU of antibody phage library dispersed in 1% bovine serum albumin was added
10 thereto to bind to the antigen. Non-specifically bound phage was washed out with PBS-T (phosphate buffered saline - 0.05% Tween 20™) solution, and then the remaining antigen-specific phage antibody was recovered using 100 mM triethylamine solution.

15 The recovered phage was neutralized with 1M tris buffer (pH 7.4) and infected into *E. coli* ER2537 at 37°C for 1 hour, and the infected *E. coli* cells were plated on carbenicillin-containing LB (Luria-Bertani) agar medium and cultured overnight at 37°C . On the next day, the cultured *E. coli*
20 cells were suspended in 4 mL of SB (superbroth)-carbenicillin medium, and 15% glycerol was added thereto. A portion of the suspension was stored at -80°C , and 50 μl of the remainder was cultured in SB-carbenicillin medium containing 2% glucose at 37°C .

When the absorbance of the culture medium reached 0.6 at 600 nm (OD₆₀₀), the culture medium was removed by centrifugation, and the remaining material was suspended again in 20 mL of SB-carbenicillin medium, and 10¹² PFU of VCSM13 helper phage was added thereto, followed by incubation at 37°C with slow stirring. On the next day, the culture medium was collected by centrifugation, and precipitated in 4% polyethylene glycol 8000 (PEG8000) and 3% sodium chloride (NaCl) at 4°C for 30 minutes, followed by centrifugation. The supernatant was removed, and the precipitated phage was suspended in 1 mL of PBS. The above-described panning process was repeated using the suspended phage as a library, thereby amplifying/concentrating antigen-specific clones.

In order to screen an antibody that binds to the Notch 1-binding site of human DLL4 protein, cross-panning of human DLL4 protein and a deletion fragment (amino acid residues 27 to 251) corresponding to a specific region of human DLL4 was performed for rounds. Then, cells were plated and cultured on LB-carbenicillin agar media containing an antibody gene to obtain single colonies, which were then inoculated and incubated in 400 μ l of SB-carbenicillin medium, after which the expression of scFv-type protein in the periplasm of *E. coli* was induced by adding IPTG. The *E. coli* cells were suspended in TES solution (Tris, EDTA, sucrose) and allowed to stand at 4°C for 1 hour. Then, the suspension was

centrifuged to extract the periplasm, which was then used to examine the binding between the recombinant human DLL4 antigen and scFv by an ELISA technique.

The bound scFv was detected using a horseradish peroxidase (HRP)-anti-HA antibody and a tetramethylbenzidine (TMB) substrate. The detected antigen-specific antibody clones were sequenced. The results of sequencing of the screened scFv are shown in Table 1 below.

Table 1

	Amino Acid Sequences	CDR1	CDR2	CDR3
V _H	EVQLLESGGGLVQPGGSLRL SCAASGFTFSDYAMSWVRQA PGKCLEWVSWIYSGSGNKYY ADSVKGRFTISRDN SKNTLY LQMNSLRAEDTAVYYCARAD WPFDYWGQGTLVTVSS (SEQ ID NO:8)	GFTFSDY AMS (SEQ ID NO:2)	WIYSGSG NKYYAD SVKG (SEQ ID NO:3)	ADWPF Y (SEQ ID NO:4)
V _L	QSVLTQPPSASGTPGQRVTI SCTGSSSNIGSNDVTWYQQL PGTAPKLLIYADSKRPSGVP DRFSGSKSGTSASLAISGLR SEDEADYYCGTWDYSL SAYV FGCGTKLTVL (SEQ ID NO:9)	TGSSSNIG SNDVT (SEQ ID NO:5)	ADSKRPS (SEQ ID NO:6)	GTWDYS LSAYV (SEQ ID NO:7)

The anti-DLL4 antibody having the above sequence was named "MLCK-2".

Example 1-4: Preparation of Dual-Targeting Antibody
5 (Bispecific Antibody) That Targets DLL4 and VEGF

The human DLL4-binding scFv-type antibody prepared in Example 1-3 was connected to an Avastin IgG type antibody by use of a linker, thereby preparing a dual-targeting protein expression vector that can also bind to human VEGF (FIG. 1b).

10 The prepared dual-targeting protein has a heavy-chain amino acid sequence (VEGF-DLL4 BsAb heavy chain) of SEQ ID NO: 1 and a light-chain amino acid sequence of SEQ ID NO: 20. The heavy chain comprises a heavy-chain variable region comprising heavy-chain CDR1 represented by SEQ ID NO: 2,
15 heavy-chain CDR2 represented by SEQ ID NO: 3, and heavy-chain CDR3 represented by SEQ ID NO: 4; and a light-chain variable region comprising light-chain CDR1 represented by SEQ ID NO: 5, light-chain CDR2 represented by SEQ ID NO: 6, and light-chain CDR3 represented by SEQ ID NO: 7.

20 To produce an antibody in CHO cells using the dual-targeting protein expression vector, the gene of interest was transfected into animal cells using a polymer for increasing intracellular gene delivery efficiency, and the cells were cultured in a 500-ml Erlenmeyer flask (Corning Costar) at a
25 volume of 200 ml per bottle to a total volume of 1l. 1l of a

mixture of RPMI medium (Invitrogen Corp.) containing ultra-low-IgG fetal bovine serum (Invitrogen Corp.) and CHO cell culture medium was incubated in an incubator (Sanyo) for 4 days, thereby producing a recombinant protein. The cell
5 culture medium was collected and centrifuged to separate the supernatant containing the recombinant protein from the suspended cells, and the supernatant was filtered once through a 0.22- μ m vacuum filter (Millipore).

For antibody purification, the Avastin-DLL4 BsAb dual-
10 targeting antibody was first purified from the culture medium using a recombinant protein-A Sepharose column (Hitrap MabSelect Sure, 5 mL, GE healthcare). Specifically, the filtered culture medium was loaded on the recombinant protein-A Sepharose column. The column was washed with a 20-
15 fold volume of 50 mM Tris-Cl (pH7.5), 100 mM NaCl buffer, and washed with a 10-fold volume of 50 mM Na-citrate buffer (pH5.0) to remove impurities. The antibody was eluted with 5 mM Na-citrate 10 mM NaCl buffer (pH3.4) and neutralized with 1M Tris-HCl buffer (pH 8.0).

20 For second purification, an aggregation of the Avastin-DLL4 BsAb dual-targeting antibody was removed using HiLoad TM 26/60 Superdex 200 Prep grade GL (GE Healthcare). The column was equilibrated with a 2-fold volume of 50 mM Na-phosphate buffer (pH6.0), 20 mM L-Arg, and then the purified Avastin-

DLL4 BsAb dual-targeting antibody was allowed to run through the column to separate it according to size.

The fractions purified using the column were analyzed by SDS-PAGE (FIG. 2), and the positive fractions were concentrated by centrifugation using an Amicon Ultra (30,000 MWCO, Millipore) concentrator. Using the same concentrator, buffer replacement with phosphate buffer and concentration were performed. Finally, the antibody was sterile-filtered through a syringe filter having a pore size of 0.22 μ m, and the absorbance (A_{280}) thereof was measured to determine the antibody concentration.

Example 2: Analysis of Binding Affinities of Dual-Targeting Protein for DLL4 and VEGF by ELISA

The binding affinities of the dual-targeting proteins for DLL4 and VEGF were assessed using an ELISA-based solution competition assay. Specifically, a 96-well plate (Nunc-Immuno Plate, NUNC, Rochester, NY) was coated with 50 ng/ml of hVEGF (R&D Systems, cat: 293-VE) and 200 ng/ml of rhDLL4 (R&D Systems, cat: 1506-D4/CF) in an amount of 100 μ l per well at 4 °C overnight, and non-specific binding sites were blocked with BSA (bovine serum albumin) for 2 hours. The antibody on the 96-well microtiter plate was diluted 1/5-fold from 128 nM and 64 nM, and 100 μ l of each of the dilutions was added to each well of the plate coated with hDLL4 and hVEGF proteins.

Then, the plate was incubated for 2 hours, and washed five times with 0.05% tween 20-containing PBS. In order to detect the plate-bound antibody, an HRP-conjugated anti-Fab antibody (Pierce, cat: 31414)) was diluted at a ratio of 1:40,000, 5 transferred to the washed 96-well microtiter plate, and then allowed to react at 37°C for 1 hour. After the reaction, color development was performed using a colorimetric substrate (3,3',5,5'-tetramethylbenzidine; Sigma-Aldrich). The enzymatic reaction was stopped using 0.5 mol/l of 10 sulfuric acid. The absorbance at 450 nm was measured using SpectraMax 190™ (molecular device).

As can be seen in FIG. 3, it was shown that the dual-targeting protein of the present invention did bind specifically to its targets (VEGF and DLL4).

15

Example 3: Assay for Equilibrium Dissociation Constant (KD) of DLL4/VEGF Dual-Targeting Protein for DLL4 and VEGF

The dual-targeting protein (bispecific antibody) purified in Example 1 was named "Avastin-DLL4 BsAb", and the 20 affinities of the purified antibody for the antigens were analyzed as follows. To examine the difference in the binding affinities of the Avastin-DLL4 BsAb dual-targeting antibody for DLL4 and VEGF, a BIACORE assay was performed.

Specifically, Biacore T200 was used in SPR analysis and 25 HBS-EP (10 mM HEPES, pH7.4, 150 mM NaCl, 3 mM EDTA, 0.15%

surfactant P20) was used as a running buffer. Surface preparation was done by using a surface preparation target immobilization tool of a wizard program (condition: 25°C, 5 $\mu\text{l}/\text{min}$). Ligands (hVEGF and hDLL4) were diluted in 10 mM sodium acetate buffer (pH 4.5) to final concentrations of 5 $\mu\text{g}/\text{ml}$ and 4 $\mu\text{g}/\text{ml}$, respectively, and then immobilized to the surface of CM5 chip by a target immobilization level for each test group. In the immobilization process, two flow cells were included as one set wherein the first and third flow cells were set as a blank, the second flow cell has hVEGF immobilized to the surface thereof, and the fourth flow cell was set as hDLL4 in the present experiment. The first and third flow cells acted as a reference to account for experimental variability due to nonspecific bindings and buffer effects, and in the analysis, subtracted RU values (Fc2-Fc1, and Fc4-Fc3) were used as experimental results. The Avastin-DLL4 BsAb dual-targeting antibody that binds to hVEGF and hDLL4 was diluted in a running buffer to a final molar concentration of 100 nM, serially diluted 1/2 times, and each of the 5 dilutions was analyzed. The sample to be analyzed was prepared to have high purity and high concentration, enough to be diluted more than 100 times at minimum, thereby minimizing buffer effect. All analysis was done by using a wizard program, screening duplicates for each sample and a regeneration step was included in between each

analysis step, so that the standard of experiment remains constant.

The experimental results were analyzed by Biaevaluation software version 4.0. At this time, to determine the RU values (Fc2-Fc1 and Fc4-Fc3), the baseline was set to zero, the value measured at a buffer injection part (analyte, 0 nM) was subtracted from a whole sensorgram. Then, the resulting RU value was analyzed by a Bivalent binding model to determine a binding affinity. The factors to be analyzed include k_a ($M^{-1}s^{-1}$), k_d (s^{-1}), and K_D (M). To be specific, k_a is an association constant demonstrating a binding affinity (recognition), and k_d is a dissociation constant demonstrating stability.

Table 2 below shows the results of analyzing the binding affinity of the dual-targeting protein for hVEGF, and Table 3 below shows the results of analyzing the binding affinity of the dual-targeting protein for hDLL4.

Table 2

Antibody	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$	K_D
Avastin-DLL4 BsAb	1.34E04	1.68E-06	1.26E-10

Table 3

Antibody	$K_a(M^{-1}s^{-1})$	$K_d(s^{-1})$	K_D
Avastin-DLL4 BsAb	1.94E04	5.87E-04	3.02E-08

As can be seen in Tables 2 and 3 above, equilibrium dissociation constant K_D (M) was calculated by dividing k_d with k_a (k_d/k_a). The results of analysis of the binding affinity for hVEGF indicated that the K_D value was about 0.126 nM which is similar to the equilibrium dissociation constant of Avastin IgG (FIG. 4a and Table 2), and the results of analysis of the binding affinity for hDLL4 indicated that the K_D value was about 30 nM (FIG. 4b and Table 3). This suggests that the binding affinity of the dual-targeting protein of the present invention for each of the antigens is maintained at a high level without interference.

Example 4: Assay for Neutralization Effect of DLL4/VEGF Dual-Targeting Protein

The neutralization effect of the Avastin-DLL4 BsAb dual-targeting antibody was assessed using an ELISA-based solution competition assay. Specifically, each well of a 96-well microtiter plate (Nunc-Immuno Plate, NUNC, Rochester, NY) was coated with 100 μ l of 500 ng/ml of hNotch-1-hFc protein (R&D Systems) (diluted in PBS) at 4°C overnight, and then treated with BSA for 2 hours to block non-specific binding sites.

The Avastin-DLL4 BsAb dual-targeting antibody (purified protein) on the 96-well microtiter plate was premixed with serial dilutions of antigen protein (human DLL4-His, 600 ng/ml) at an antibody concentration ranging from 0 nM to 140

nM. The antigen/antibody mixture was incubated for 30 minutes, and then transferred to a microtiter plate precoated with the DLL4 receptor hNotch-1 protein (50 ng/well) in order to measure free antibody. Then, the plate was incubated for 2 hours and washed five times with 0.05% tween 20-containing PBS. In order to detect the DLL4 antigen bound to the plate, an HRP-conjugated His anti-mouse IgG polyclonal antibody reagent (Roche applied science) was diluted at a ratio of 1:800, and the washed microtiter plate was treated with the diluted antibody reagent, and then allowed to react at 37°C for 1 hour. Then, color development was performed using a colorimetric substrate (3,3',5,5'-tetramethylbenzidine; Sigma-Aldrich Co.), and the enzymatic reaction was stopped using 0.5 mol/l of sulfuric acid. The absorbance at 450 nm was measured, and the results of the measurement are shown in FIG. 5. The amount of antibody required to achieve a 50% decrease in human DLL4-His bound to plate-coated Notch 1-hFc protein (IC₅₀) is shown in Table 4 below.

Table 4

Clone	IC ₅₀ (nM)
VEGF-DLL4 BsAb	1.12

As can be seen in Table 4 above, the dual-targeting protein of the present invention showed a low IC₅₀ value of 1.12 nM for DLL4, suggesting that it has DLL4 inhibitory activity comparable to that of the anti-DLL4 antibody alone.

Example 5: Epitope Mapping by Cross-Linking Reaction and Mass Spectrometry

In order to identify a conformational epitope, which consists of a plurality of discontinuous sequences but conformationally forms a single molecular surface, a technique of determining the positions of cross-linking reactions by cross-linking reactions and mass spectrometry was used.

Example 5-1: Formation of Cross-Linked Complex

The antigen protein human delta-like ligand 4 (human DLL4, hDLL4, R&D Systems) and the MLCK2 of Example 1-3 were mixed with each other at a molar ratio of 2:1, and then a K200 cross-linker (CovalX AG) was added thereto at a final concentration of 0.2 mg/ml. The mixture was allowed to react at room temperature for 3 hours to form an antigen-antibody complex, and then the molecular weight of the reaction product was analyzed using an Ultraflex II MALDI ToF spectrometer (Bruker Daltonics). As shown in FIG. 6, it could be seen that when the cross-linker was used, 1:1 and 2:1 complexes between human DLL4 and MLCK2 antibody were formed, unlike a control experiment in which no cross-linker was used. However, it could be seen that when human DLL4 or MLCK2 antibody alone was allowed to react with the cross-

linker, any multimer or complex was not detected, suggesting that the formation of the human DLL4/MLCK2 antibody complex results from a specific reaction between DLL4 and MLCK2.

5 Example 5-2: Formation of Fragments by Protease

In order to identify cross-linked peptide fragments, d0-DSS (disuccinimidyl suberate) and d12-DSS were mixed with each other at a ratio of 1:1 and dissolved in DMF to make 2 mg/ml of a solution. The solution was added to a 2:1 mixture
10 of DLL4 and MLCK2 to a final concentration of 0.2 mg/ml and subjected to a cross-linking reaction at room temperature for 3 hours. The reaction product was modified by reduction and alkylation using DTT (dithiothreitol) and iodoacetamide for effective degradation, and was fragmented using a protease
15 such as trypsin, α -chymotrypsin or ASP-N protease. The produced fragments were analyzed by a Ultimate 3000 nano-liquid chromatography system (Dionex) and an LTQ Orbitrap XL mass spectrometer (Thermo), and the obtained mass spectrometry data were analyzed by Xquest (version 2.0)
20 software and Stavrox (version 2.1) software to detect cross-linked peptide pairs. As a result, as shown in Table 5 below, peptide pairs formed by cross-linking between hDLL4 and MLCK2 could be detected.

Positions on human DLL4, at which a cross-linking
25 reaction occurred, were amino acid residues 59, 63, 64 and

110 of the amino acid sequence of human DLL4. Two fragments, which are an amino acid sequence consisting of amino acid residues 58th to 65th [FRVCLKHF] (SEQ ID NO: 22) and an amino acid sequence consisting of amino acid residues 110-115th [TWPGTGTF] (SEQ ID NO: 23), constitute a continuous molecular surface on a human DLL4 C2 domain (27-174) model as shown in FIG. 7. Thus, the two sequences could be determined to be the epitope of human DLL4 for MLCK2 antibody.

Table 5

Sequence	Protein 1	Protein 2	Seq. Protein 1	Seq. Protein 2	Id- Score	nAA1	nAA2	Id on Xquest	Id on Stavro x
ADSVKG RF- TWPGTGTF -a3-b1	MLCK 2 HC	hDLL4	60-67	110- 115	13.62	62	110	yes	yes
ADSKRP SGVPDR F- FRVCLK HF-a12- b2	MLCK 2 LC	hDLL4	50-62	58-65	11.59	62	59	yes	yes
ADSKRP SGVPDR F- FRVCLK HF-a3-b7	MLCK 2 LC	hDLL4	50-62	58-65	9.47	53	64	yes	yes
ADSKRP	MLCK	hDLL4	50-62	58-65	6.33	53	59	yes	yes

SGVPDR F- FRVCLK HF-a3-b2	2 LC								
ADSKRP SGVPDR F- FRVCLK HF-a3-b6	MLCK 2 LC	hDLL4	50-62	58-65	6.15	53	63	yes	yes
ADSKRP SGVPDR F- FRVCLK HF-a4-b2	MLCK 2 LC	hDLL4	50-62	58-65	5.04	54	59	yes	yes
ADSKRP SGVPDR F- FRVCLK HF-a12- b7	MLCK 2 LC	hDLL4	50-62	58-65	3.87	62	64	yes	yes

Example 6: Examination of Epitope Map by Western Blotting

An alanine substitution mutant panel of human DLL4 was prepared as follows, in which each of the amino acid residues at positions 64 (histidine), 65 (phenylalanine) and 69 (valine) in the amino acid sequence of the extracellular protein region of human DLL4 was substituted with alanine.

As an expression vector for the alanine substitution mutants, the vector used in the preparation of the antigen corresponding to the specific region of the extracellular domain of DLL4 as described in Example 1-1 was used.

5 Specifically, the vector comprises a gene corresponding to amino acid residues 27 to 251 of the amino acid sequence of the specific region of human DLL4, and this region contains a motif called "DSL (delta/serrate)/lag-2)" which is known to bind to Notch 1 receptor.

10 Using a standard recombinant DNA technique, a mammalian expression plasmid vector comprising a CMV promoter upstream of a polynucleotide encoding a deletion fragment (amino acid residues 27 to 251) of the extracellular domain of DLL4 fused with Fc protein was prepared. To substitute each of amino
15 acid residues 64, 65 and 69 in the vector with alanine, a recombinant DNA technique (QuikChange Site-Directed Mutagenesis, Agilent) was used, and the mutants were transfected into HEK293E animal cells using Lipofectamine 2000 (Invitrogen) and incubated for 4 days, after which the
20 expression medium was recovered. As a control, a protein encoding a deletion fragment (amino acid residues 27 to 251) of the extracellular domain of wild-type DLL4 was used.

The mutant expression media incubated for 4 days were centrifuged at 1000 rpm at room temperature for 10 minutes to
25 remove the suspended material, and then filtered through a

0.45- μ m syringe. For Western blotting analysis, the level of the protein in the mutant expression media were quantified using Octet[®] system (ForteBio) so that a uniform amount of the mutant would be loaded on SDS gel. Next, 20 μ l of each of the mutant expression media was loaded on each of two Novex 4-12% Bis/Tris gels, and subjected to gel electrophoresis using MOPS buffer at 140 V for 50 minutes. As a control, a protein encoding a deletion fragment (amino acid residues 27 to 251) of the extracellular domain of wild-type DLL4 was used. After completion of the electrophoresis, the protein band was transferred to a polyvinylidene difluoride membrane. A total of two processes were performed. In one process, in order to examine whether uniform amounts of the mutant and wild-type proteins were loaded when the deletion fragment (amino acid residues 27 to 251) of the extracellular domain of DLL4 was loaded on SDS gel, an HRP-conjugated anti-human Fc antibody (1:10000) (Pierce Cat: 31413) was bound to the transferred membrane, and then the membrane was washed three times with PBS-T. In the other method, in order to examine the binding affinities of MLCK2 antibody to the mutants, MLCK2 antibody (1 μ g/mL) was first bound to the transferred membrane, the membrane was washed three times with PBS-T, and then an HRP-conjugated anti-human Fab antibody (1:10000) was bound to the membrane, followed by washing three times with PBS-T. Next, Amersham ECL Western blotting detection reagent

(GE Healthcare) was applied to the membrane, and signal detection was performed using ImageQuant LAS 4000 (GE Healthcare).

As shown in FIG. 8, the results of the Western blotting analysis indicated that the mutant proteins encoding the deletion fragment (amino acid residues 27 to 251) of the extracellular domain of wild-type and DLL4 were loaded in a uniform amount. In addition, when the binding affinities of MLCK2 antibody for the mutants were examined, it could be seen that, for the amino acid mutant at position 64, the binding affinity of MLCK2 antibody was lost, and for the amino acid mutant at position 65, the binding affinity of MLCK2 antibody significantly decreased. In addition, it was shown that the amino acid mutant at position 69 did not influence the binding affinity of MLCK2 antibody.

Example 7: Analysis of the Effect of DLL4/VEGF Dual-Targeting Antibody on Proliferation of Human Umbilical Vein Endothelial Cells (HUVECs)

In order to analyze the effect of the dual-targeting antibody, which binds to DLL4 and VEGF, on the proliferation of human umbilical vein endothelial cells (HUVECs), human umbilical vein endothelial cells (HUVECs) purchased from Lonza were used in this experiment.

For culture of HUVECs, T-flask (Nunc) was coated with PBS buffer (Gibco) containing 1% gelatin (Sigma) at a room temperature for 4-6 hours, followed by washing with PBS. EBM-2 containing EGM-2 Single Quot (Lonza) was used as a culture medium, the density of a cell culture was maintained below 80%, and the cells were cultured at 37°C in a 5% CO₂ incubator. The cells before passage 6 were used for this experiment. An HUVEC proliferation assay was done in the following manner. First, to prepare a hDLL4-coated plate, one day before performing the experiment, rhDLL4 (R&D Systems) was diluted in a carbonate buffer to a final concentration of 1 mg/ml in a 96-well plate (BD), and 100 µl of the diluted rhDLL4 was inoculated into each well, and the plate was incubated at 4°C overnight. In addition, HUVECs were cultured in EBM-2 minimal medium supplemented with 0.1% FBS for 24 hours to minimize the serum effect. On the first day of experiment, each well of the rhDLL4-coated plate was washed twice with PBS, and for each test group, each of hVEGF (50 ng/mL) and antibodies (Avastin: 20 mg/mL; anti-DLL4 antibody alone: 20 mg/mL; Avastin-DLL4 BsAb dual-targeting antibody: 26 mg/mL) was diluted with the EBM-2 minimal medium, and then added to each well in triplicate, followed by incubation at a room temperature for 20 minutes. The HUVECs starved for 24 hours were dissociated into single cells, and diluted to 4×10^3 cells/well with EBM-2 minimal medium. The

diluted cells were inoculated into the well treated with the antibody and were incubated in a 5% CO₂ incubator at 37°C for 96 hours. After completion of cell proliferation, 10 µl of cell counting kit-8 (CCK-8, Dojino) was added to each well and the plate was incubated in a 5% CO₂ incubator at 37°C for 5 hours. Using SpectraMax 190 (Molecular Devices), the absorption of the sample at 450 nm was measured and the levels of cell proliferation were compared among different test groups.

As shown in all the figures (PBS-treated group) of FIG. 9, when DLL4/Notch signaling is activated, the proliferation of vascular endothelial cells will be inhibited by about 30%, on the contrary to the case in which the proliferation of vascular endothelial cells is activated by VEGF. In the *in vivo* mechanisms, as described above, it is known that the VEGF antibody inhibits angiogenesis of tumors to thereby inhibit the growth and metastasis of tumors, whereas the DLL4 antibody induces the excessive production of abnormal blood vessels (inactive blood vessels) in tumors to thereby inhibit the growth of tumors. It can be said that the results in FIG. 9 reflect the different angiogenic mechanisms of VEGF and DLL4 *in vitro*.

As can be seen in FIG. 9a, when VEGF and its receptor, and the VEGFR signaling pathway, which play an important role in the proliferation of vascular endothelial cells, are

treated with the VEGF-targeting antibody (Avastin), the proliferation of vascular endothelial cells was inhibited in a concentration-dependent manner regardless of the presence or absence of DLL4. However, as shown in FIG. 9b showing
5 experimental results obtained by treatment with the DLL4-targeting antibody alone, in the experimental group in which no DLL4 was present, the concentration of the antibody had no significant effect on the proliferation of vascular endothelial cells, and in the experimental group in which
10 DLL4 was present, the proliferation of vascular endothelial cells occurred again in a manner dependent on the concentration of the DLL4-targeting antibody. In the case in which treatment with the dual-targeting protein was performed, in the experimental group in which no DLL4 was present, the
15 dual-targeting protein showed a proliferation inhibitory effect similar to that of treatment with the Avastin antibody (FIG. 9c; black bars), but in the experimental group in which DLL4 was present, the proliferation inhibitory effect of the dual-targeting protein was reduced (FIGS. 9a and 9c; white
20 bars).

From the fact that the group treated with the DLL4-targeting antibody did not show a proliferation inhibitory effect comparable to that of treatment with the VEGF-targeting antibody alone, it can be seen that the dual-

targeting antibody of the present invention effectively inhibit both the VEGF and DLL4 signaling pathways.

Example 8: Analysis of Inhibitory Activities of
5 **DLL4/VEGF Dual-Targeting Antibody on DLL4/Notch and**
VEGF/VEGFR Signaling Pathways

In order to examine the inhibitory activities of the dual-targeting antibody, which binds to DLL4 and VEGF, on the DLL4/Notch and VEGF/VEGFR signaling pathways, HUVECs were
10 used according to the same method as used in Example 4. Specifically, one day before performing the experiment, a recombinant human DLL4 (rhDLL4, R&D Systems) was diluted with carbonate buffer to a final concentration of 1 mg/ml, and then 1 ml/well of the diluted rhDLL4 was added to a 6-well
15 plate (BD) and incubated at 4°C overnight. For a control group that was not treated with rhDLL4, 1 ml/well of carbonate buffer was only added to the plate and incubated at 4°C overnight. On the next day, the DLL4-coated plate was taken from a 4°C refrigerator and washed once with PBS, and 1
20 ml of EGM-2 medium was added to each well of the plate. Then, each of antibodies (Avastin: 20 mg/mL; DBZ: 0.08 mM; DLL4-targeting antibody alone: 20 mg/mL; Oncomed DLL4-targeting antibody alone: 20 mg/mL; Avastin-DLL4 BsAb dual-targeting antibody: 26 mg/mL) was added to each well. The final volume
25 of medium in each well was 2 ml and the volume of antibody

added was twice the volume of the medium. The plate was incubated for 20 minutes at room temperature. During antibody treatment, the 75T plate containing HUVECs in passages #2 to #5 was taken, and the medium was removed from the plate, and then the cells were dissociated into single cells. Through centrifugation, HUVECs were washed and resuspended in a fresh EGM-2 medium. After counting the cells, the cells were diluted to 5×10^5 cells/ml, and 1ml of the cells were inoculated into each well and incubated in a 5% CO₂ incubator at 37°C for one day. After culturing HUVECs for one day, the medium was removed from each well, and the cells were washed once with PBS and treated with 2 ml of EBM-2 minimal medium including 0.2% FBS. Also, each well was treated with each of the same concentration of the antibodies (Avastin: 20 mg/mL; DBZ: 0.08 mM; DLL4-targeting antibody alone: 20 mg/mL; Oncomed DLL4-targeting antibody alone: 20 mg/mL; Avastin-DLL4 BsAb dual-targeting antibody: 26 mg/mL) which were treated the day before, and the cells were incubated at 37°C in a 5% CO₂ incubator for one day. Then, each well containing the HUVECs treated with each antibody was treated with 100 ng/ml of hVEGF (R&D Systems) and incubated at 37°C in a 5% CO₂ incubator for 5 minutes. Then the plate was taken out and the medium was removed quickly. The cells were washed once with PBS, and 150 µl of a cell lysis buffer (1% NP-40, 20mM Tris, 137mM NaCl, 10%

Glycerol, 2mM EDTA, 1mM Sodium orthovanabate, 1x Protease & phosphatase inhibitor cocktail) was added to each well, and the plate was shaken to spread the lysis buffer.

Subsequently, the plate was put on ice, and HUVECs were
5 collected from each well using a scraper and put into a 1.5 ml tube and allowed to stand on ice. Every 5 minutes, the 1.5 ml tube containing the cells was taken from ice, vortexed three times, and put on ice again for cell lysis. Then, the sample was centrifuged at 4°C and 14000 rpm for 10 minutes,
10 and the isolated supernatant was transferred to a fresh tube and weighted. For SDS-PAGE analysis, the supernatant was added to 5x SDS sample buffer and boiled at 100°C for 10 minutes, followed by SDS-PAGE analysis. At this time, the prepared protein samples were run through 4% to 12% bis-TRIS
15 gel, and separated according to their size, and the separated proteins were Western-blotted with the following antibodies (FIG. 10): NICD (Cell signaling), P-ERK (Cell signaling), ERK (Cell signaling), VEGFR2 (Cell signaling), P-VEGFR2 (Cell signaling), and Actin (Santa Cruz).

20 As shown in FIG. 10, the dual-targeting antibody of the present invention could inhibit the DLL4/Notch and VEGF/VEGFR signaling pathways to the levels similar to those achieved by the DLL4-targeting antibody alone and the VEGF-targeting antibody alone.

25

Example 9: Analysis of Anticancer Activity of Dual-Targeting Antibody in Avastin-Resistant Human SCH Gastric Cancer Xenograft Model

As reported in literature, human SCH human gastric
5 cancer cells have resistance to Avastin. Thus, an experiment on the effect of the dual-targeting antibody was performed in a nude mouse xenograft model with SCH cells.

Specifically, Avastin-resistant SCH gastric cancer cells were inoculated into female nude mice, and when the tumor
10 size reached an average of 200 mm³, each of antibodies was administered to the mice once a week to confirm the *in vivo* anticancer activity of the dual-targeting antibody of the present invention (FIG. 11). In this *in vivo* experiment on the nude mouse xenograft model, the bispecific antibody
15 Avastin-mouse DLL4 surrogate dual-targeting protein that binds to the mouse DLL4 epitope (DSL domain) equal to the human DLL4 epitope (DSL domain) was administered instead of the Avastin-DLL4 dual-targeting antibody that targets human DLL4 in order to demonstrate the excellent anticancer effect
20 of the dual-targeting antibody.

As shown in FIG. 11, the results of the *in vivo* experiment indicated that the dual-targeting protein of the present invention has a significantly increased anticancer effect against the Avastin-resistant gastric cancer cells.

25

Example 10: Analysis of Anticancer Activity of Dual-Targeting Antibody in Avastin-Resistant Human A549 Lung Cancer Xenograft Model

A549 cells were inoculated into nude mice which were
5 then treated with Avastin (2.5 mg/kg/week) for 3 months,
thereby obtaining Avastin-resistant A549 cancer cells whose
tumor grows without reducing its size even after treatment
with Avastin. The tumor was detached, and then the Avastin-
resistant A549 cells were incubated *ex vivo* in order to
10 analyze the effect of the dual-targeting antibody.

Specifically, Avastin-resistant A549 lung cancer cells
were inoculated into female nude mice, and when the tumor
size reached an average of 200 mm³, each of antibodies was
administered to the mice twice a week to confirm the *in vivo*
15 anticancer activity of the dual-targeting antibody of the
present invention (FIG. 12). In this *in vivo* experiment
using the Avastin-resistant A549 cells, the bispecific
antibody Avastin-mouse DLL4 surrogate dual-targeting protein
that binds to the mouse DLL4 epitope equal to the human DLL4
20 epitope was administered instead of the Avastin-DLL4 dual-
targeting antibody that targets human DLL4 in order to
demonstrate the excellent anticancer effect of the dual-
targeting antibody.

As shown in FIG. 12, the results of the *in vivo*
25 experiment indicated that the dual-targeting protein of the

present invention has a significantly increased anticancer effect against the Avastin-resistant lung cancer cells.

From the foregoing, it will be understood by those skilled in the art to which the present invention pertains
5 that the present invention can be carried out in other concrete embodiments without changing the technical spirit or essential feature thereof. In this regard, it should be understood that the aforementioned examples are of illustrative in all aspects but not is limited. The scope of
10 the present invention should be construed to include the meaning and scope of the appended claims, and all the alterations and modified forms which are derived from the equivalent concept thereof, rather than the detailed description.

15

CLAIMS

1. A dual-targeting protein comprising: a protein specifically binding to DLL4 (delta-like ligand 4), which recognizes a conformational epitope of DLL4 comprising FRVCLKHF as shown in SEQ ID NO:22 and TWPGETF as shown in SEQ ID NO:23 in the amino acid sequences of DLL4 protein represented by SEQ ID NO:21, and an antibody specifically binding to VEGF (Vascular endothelial growth factor);

wherein the protein specifically binding to DLL4 comprises:

a heavy chain variable region comprising heavy chain CDR1 comprising an amino acid sequence represented by SEQ ID NO:2, heavy chain CDR2 comprising an amino acid sequence represented by SEQ ID NO:3, and heavy chain CDR3 comprising an amino acid sequence represented by SEQ ID NO:4, and a light chain variable region comprising light chain CDR1 comprising an amino acid sequence represented by SEQ ID NO:5, light chain CDR2 comprising an amino acid sequence represented by SEQ ID NO:6, and light chain CDR3 comprising an amino acid sequence represented by SEQ ID NO:7.

2. The dual-targeting protein of claim 1, wherein the double-targeting protein is a form in which the protein that binds specifically to DLL4 and an IgG (immunoglobulin G)-type antibody that binds specifically to VEGF are connected to each other by a linker.

3. The dual-targeting protein of claim 2, wherein the linker is a peptidyl linker or a non-peptide linker.

4. The dual-targeting protein of claim 3, wherein the peptide linker comprises an amino acid sequence represented by SEQ ID NO:18.

5. The dual-targeting protein of claim 1, wherein the protein binding specifically to DLL4 comprises a heavy-chain amino acid sequence represented by SEQ ID NO: 8 and a light chain amino acid sequence represented by SEQ ID NO: 9.

6. The dual-targeting protein of claim 1, wherein the antibody specifically binding to VEGF comprises: a heavy chain variable region comprising heavy chain CDR1 comprising an amino acid sequence represented by SEQ ID NO:10, heavy chain CDR2 comprising an amino acid sequence represented by SEQ ID NO:11, and heavy chain CDR3 comprising an amino acid sequence represented by SEQ ID NO:12, and
a light chain variable region comprising light chain CDR1 comprising an amino acid sequence represented by SEQ ID NO:13, light chain CDR2 comprising an amino acid sequence represented by SEQ ID NO:14, and light chain CDR3 comprising an amino acid sequence represented by SEQ ID NO:15.
7. The dual-targeting protein of claim 1, wherein the antibody specifically binding to VEGF comprises a heavy chain variable region comprising an amino acid sequence represented by SEQ ID NO:16 and a light chain variable region comprising an amino acid sequence represented by SEQ ID NO:17.
8. The dual-targeting protein of claim 7, wherein the antibody binding specifically to VEGF is Bevacizumab.
9. The dual-targeting protein of claim 1, wherein the dual-targeting protein comprises a heavy chain variable region comprising an amino acid sequence represented by SEQ ID NO:1 and a light chain variable region comprising an amino acid sequence represented by SEQ ID NO:20.
10. The dual-targeting protein of claim 1, wherein the protein binding specifically to DLL4 is in the form of a full-length antibody, Fab', F(ab')₂, Fab, Fv, rIgG, or scFv (Single chain variable fragment).
11. A pharmaceutical composition for treating cancer comprising the dual-targeting protein of any one of claims 1 to 10 and a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11, wherein the cancer is selected from the 18022 group consisting of esophageal cancer, stomach cancer, large intestine cancer, rectal cancer, oral cancer, pharynx cancer, larynx cancer, lung cancer, colon cancer, breast cancer, uterine cervical cancer, endometrial cancer, ovarian cancer, prostate cancer, testis cancer, bladder cancer, renal cancer, liver cancer, pancreatic cancer, bone cancer, connective tissue cancer, skin cancer, brain cancer, thyroid cancer, leukemia, Hodgkin's disease, lymphoma, and multiple myeloid blood cancer.

13. Use of the dual-targeting protein of any one of claims 1 to 10 for diagnosing cancer.

14. A method for diagnosing cancer, comprising the steps of: (a) measuring the level of VEGF (vascular endothelial growth factor) or DLL4 protein in a sample, isolated from a subject suspected of having cancer, using the dual-targeting protein of any one of claims 1 to 10; and (b) determining that the subject has cancer, if the level of VEGF or DLL4 protein measured in step (a) is higher than that in a normal control sample.

15. Use of the dual-targeting protein of any one of claims 1 to 10 for treating cancer.

Drawings

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Fig. 1a

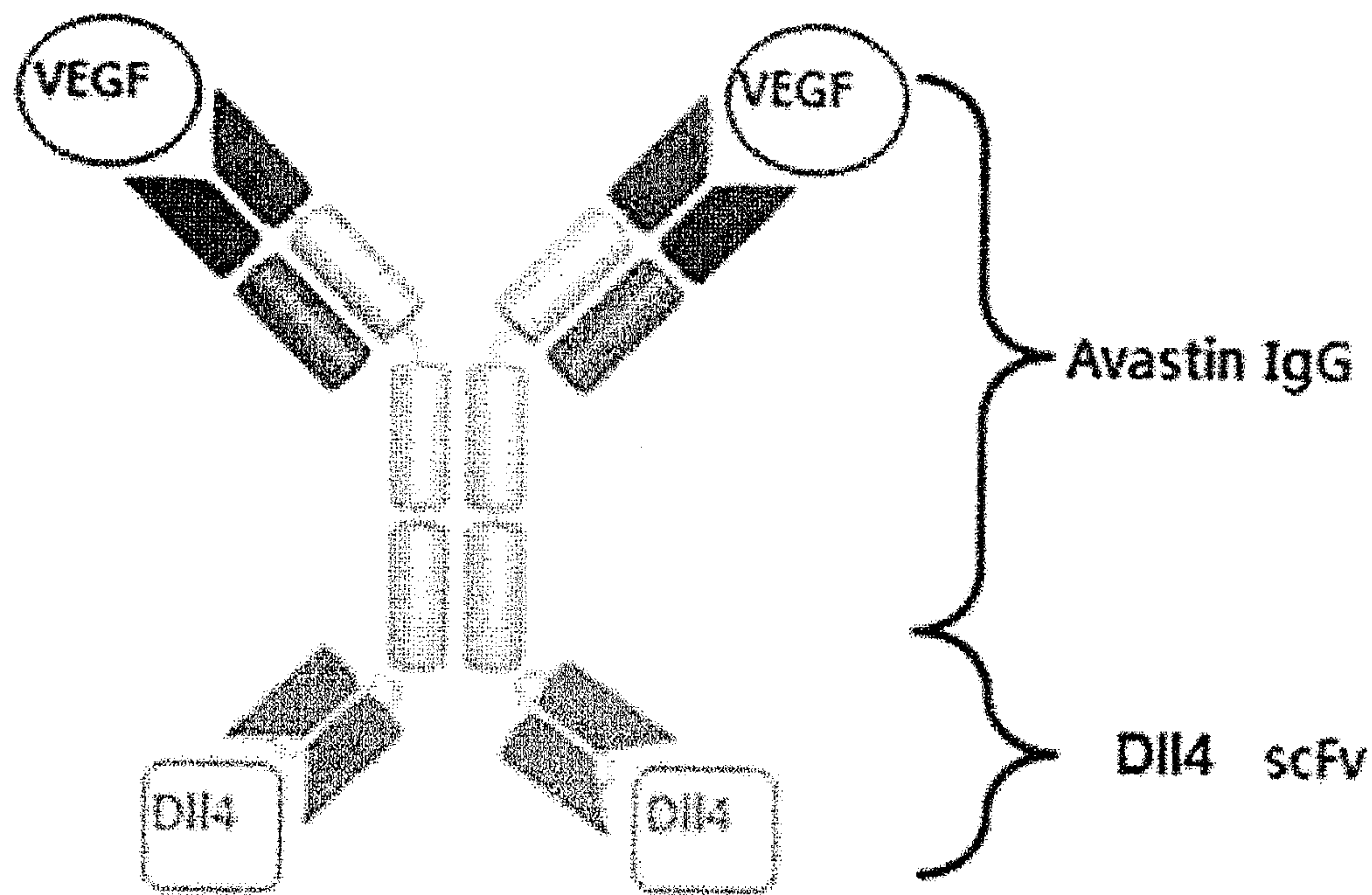
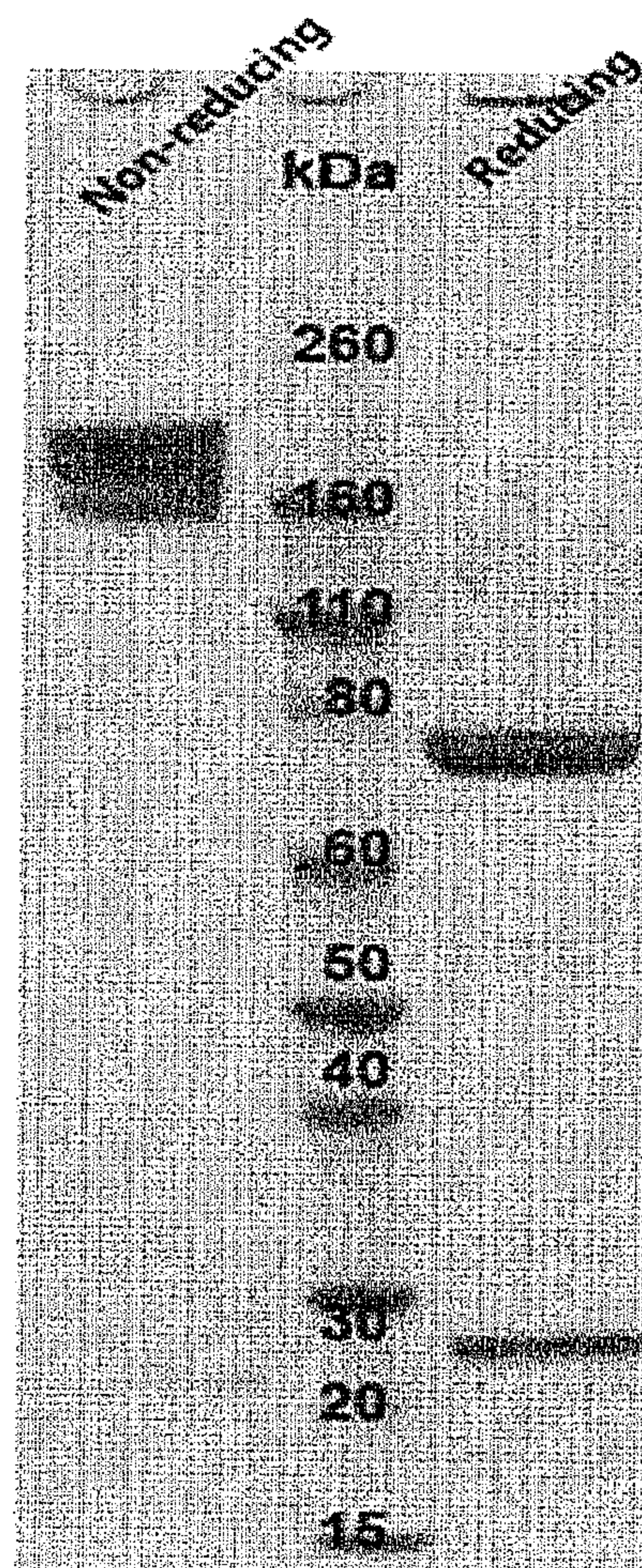


Fig. 1b

		DII4 scFv			
VEGF-DII4 BsAb Heavy chain	VEGF Heavy chain (VH-CH1-CH2-CH3)	Linker-1	DII4 VL	Linker-2	DII4 VH
VEGF-DII4 BsAb Light chain	VEGF Light chain (VL-CL)				

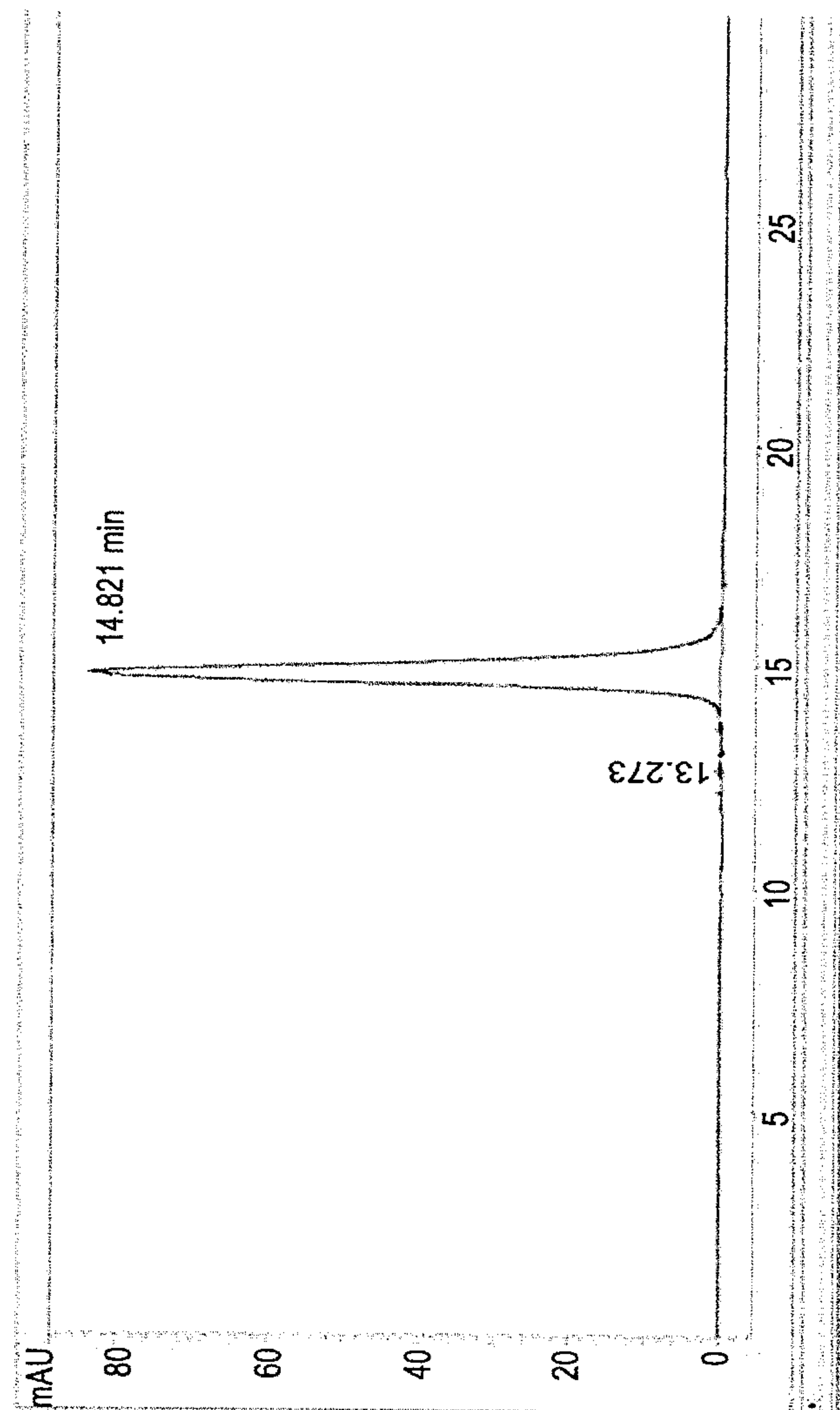
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Fig. 2a



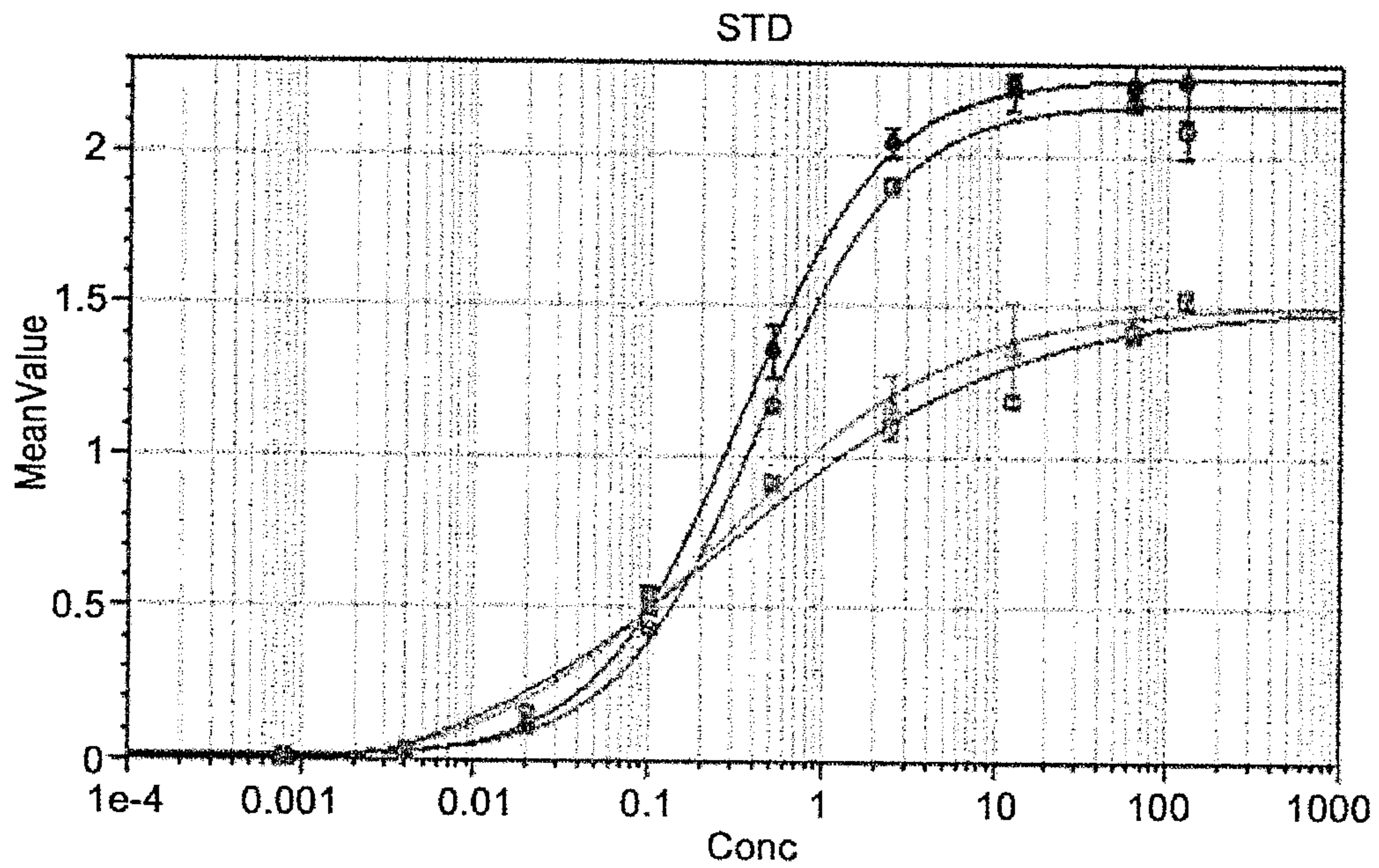
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Fig. 2b



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Fig. 3



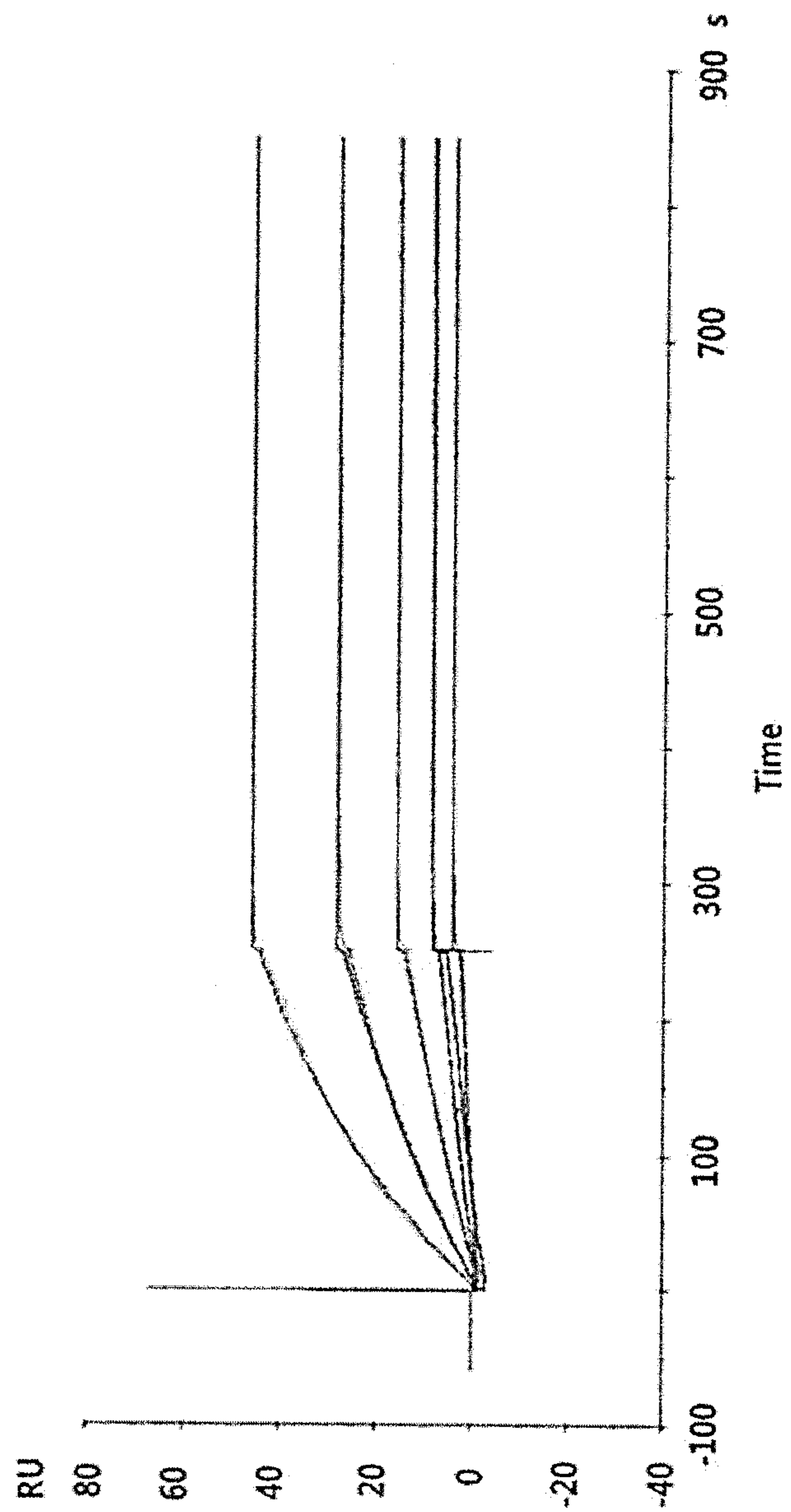
4-P Fit: $y = (A - D) / (1 + (x/C)^B) + D$

	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>R²</u>
● STD (VEGF-Transient: Conc vs MeanValue)	0.00268	1.05	0.337	2.26	1
○ Plot#1 (VEGF-(GS pool) cell line: Concentration ...	0.0144	1.07	0.43	2.17	0.997
□ Plot#2 (DLL4-Transient: Concentration vs Mean...	-0.126	0.506	0.264	1.51	0.987
△ Plot#3 (DLL4-(GS pool) cell line: Concentration v...	-0.0656	0.623	0.261	1.51	0.997

Curve Fit Option - Fixed Weight Value

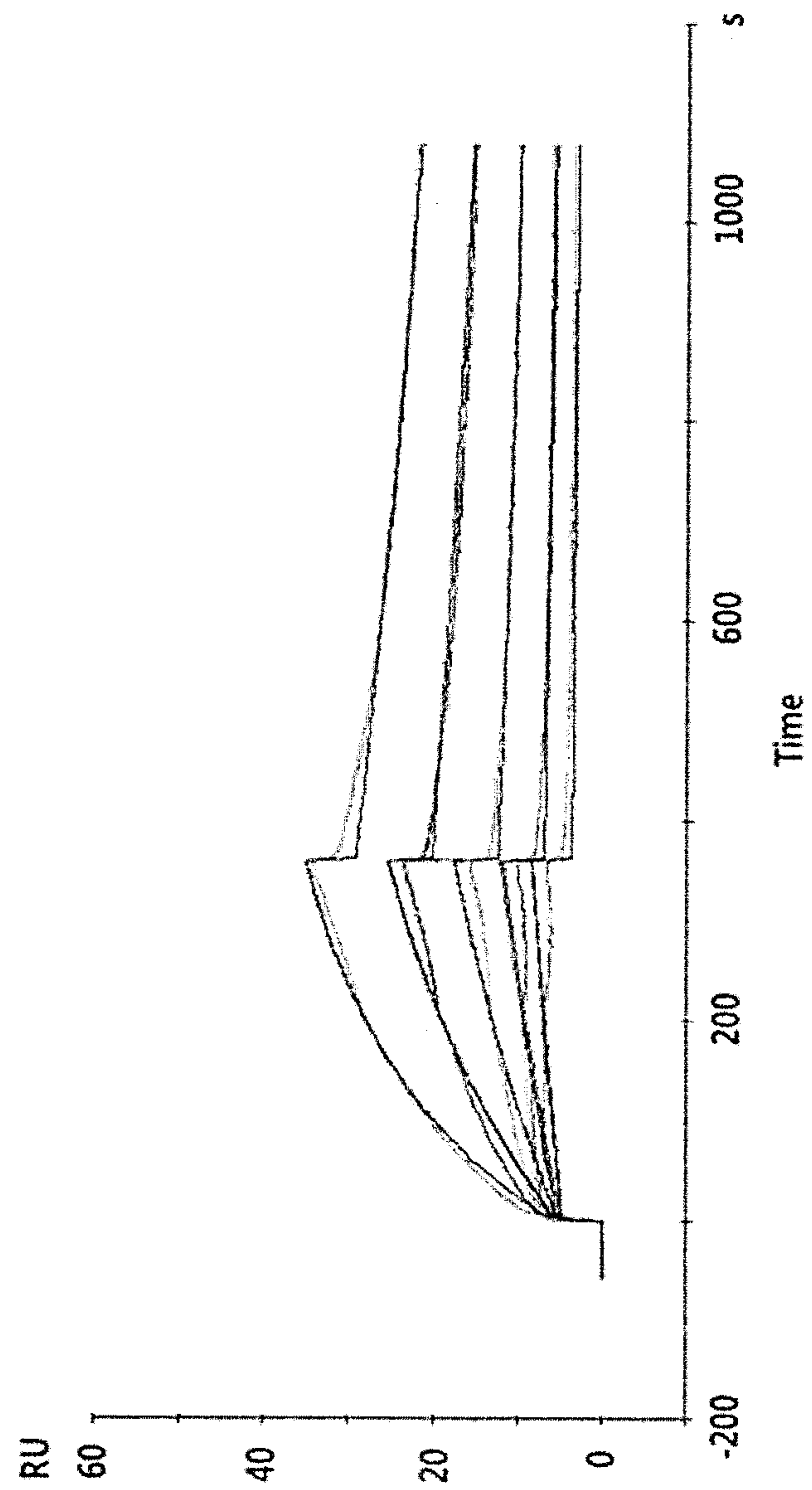
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Fig. 4a



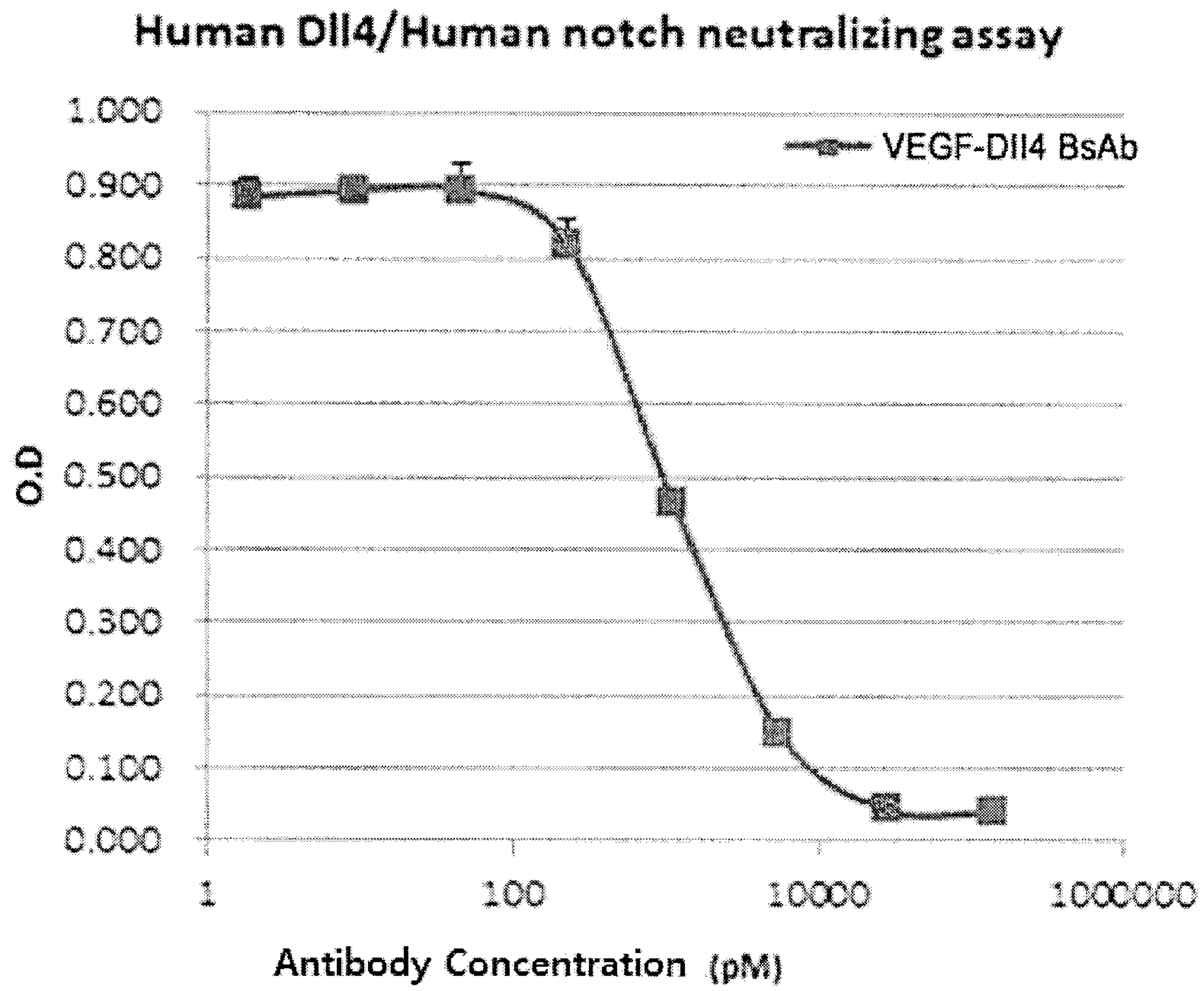
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Fig. 4b



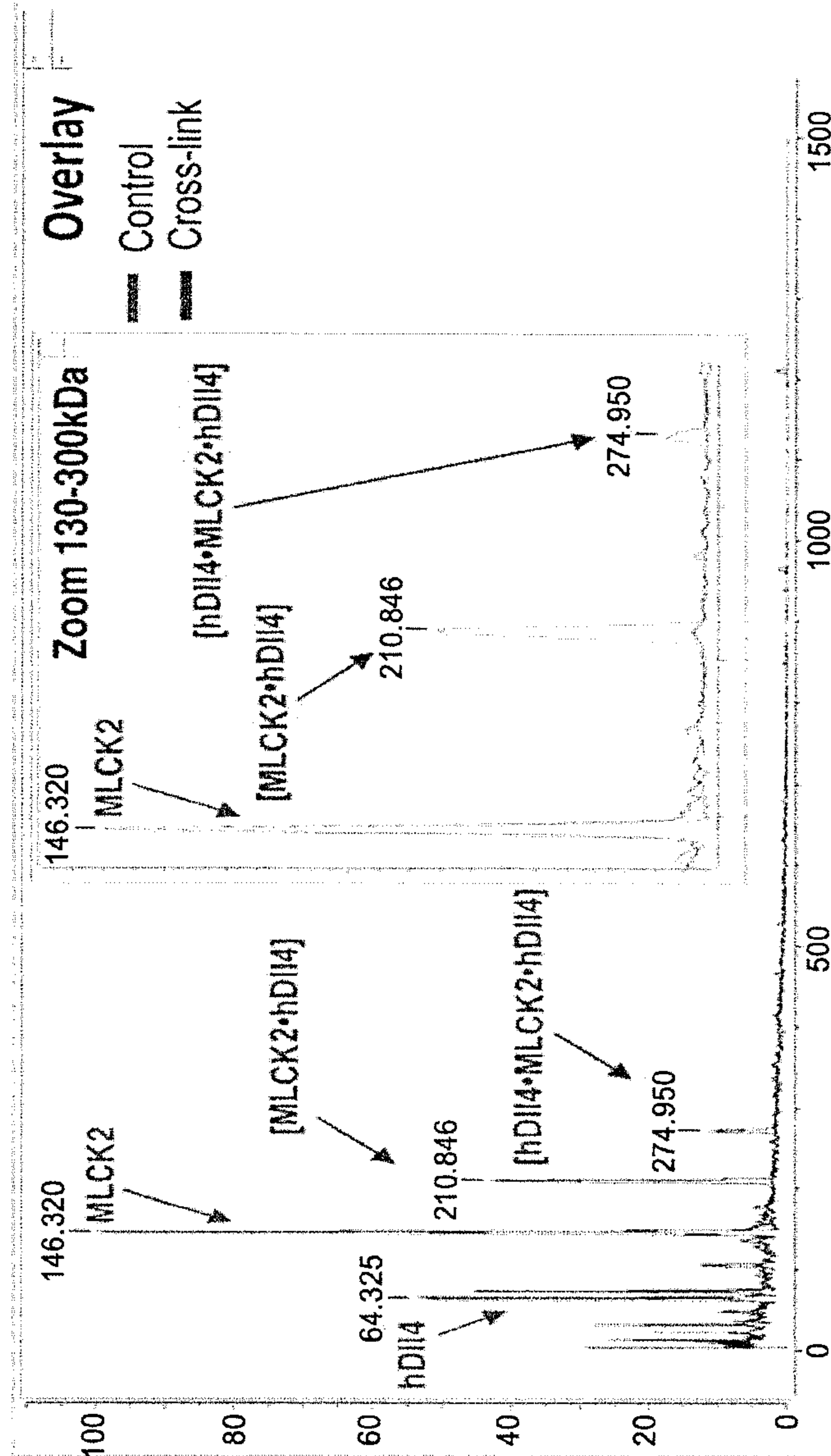
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Fig. 5



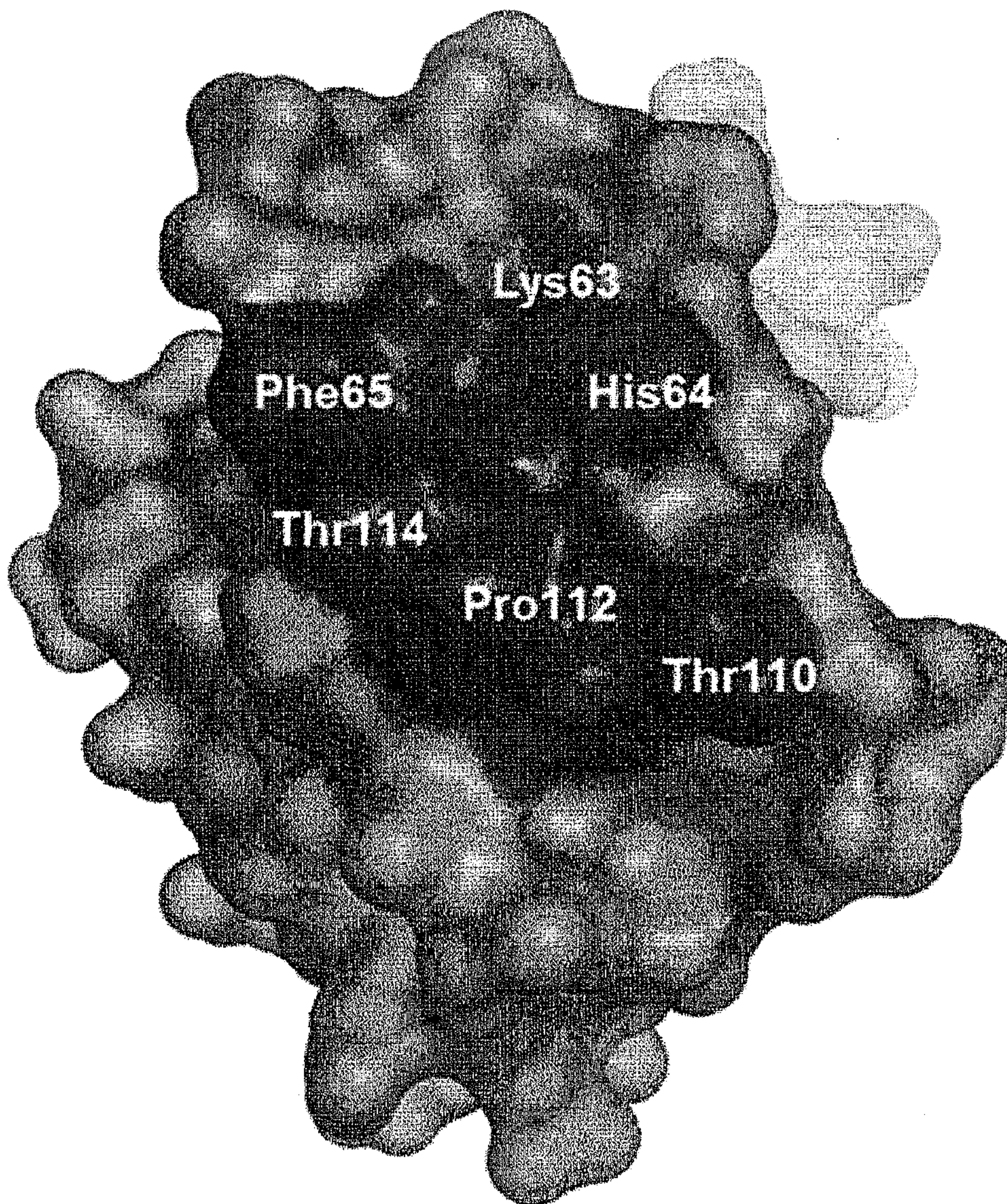
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Fig. 6



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Fig. 7



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Fig. 8

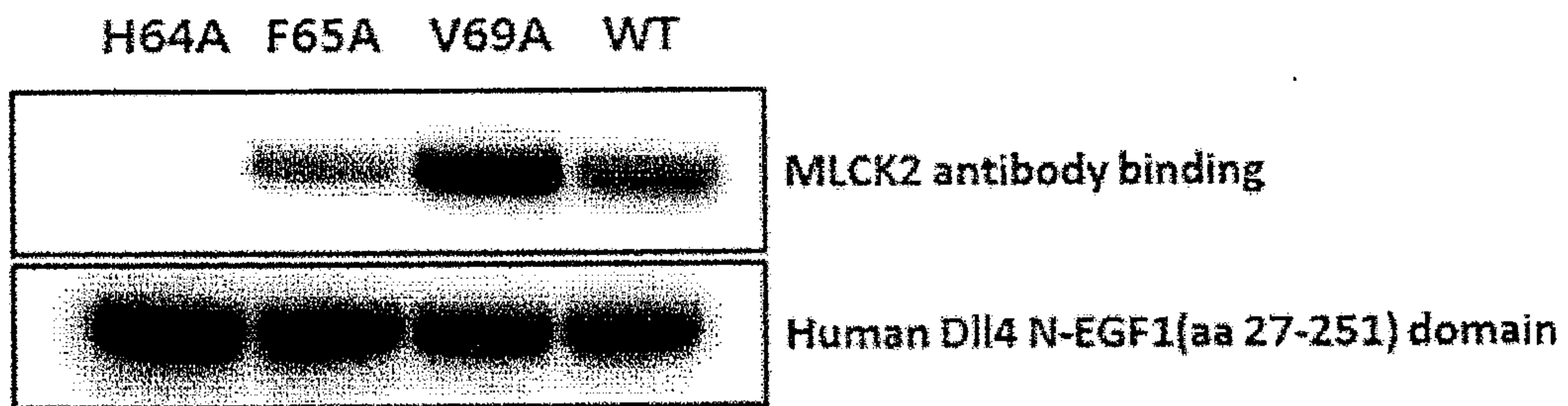
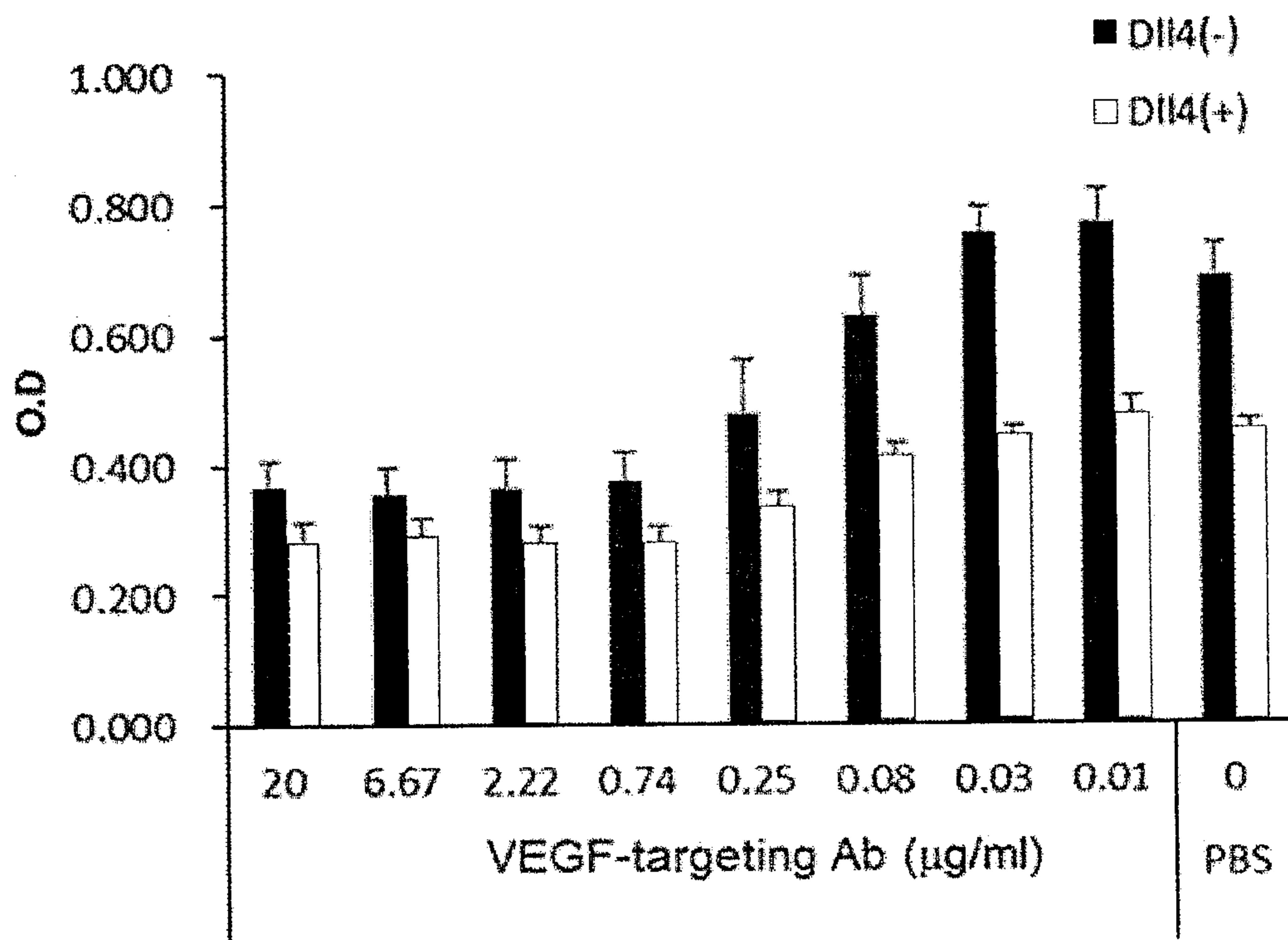


Fig.9a



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Fig. 9b

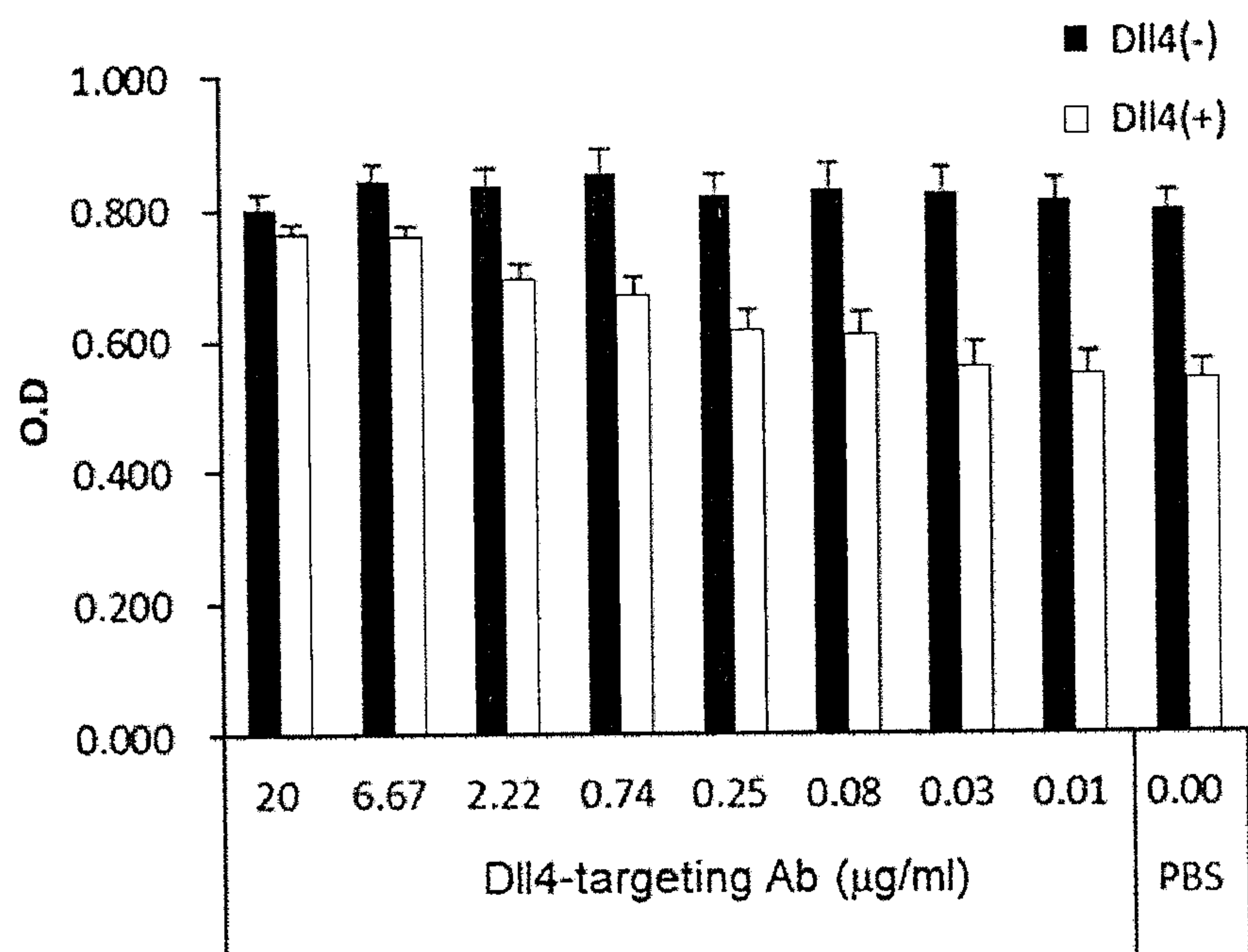
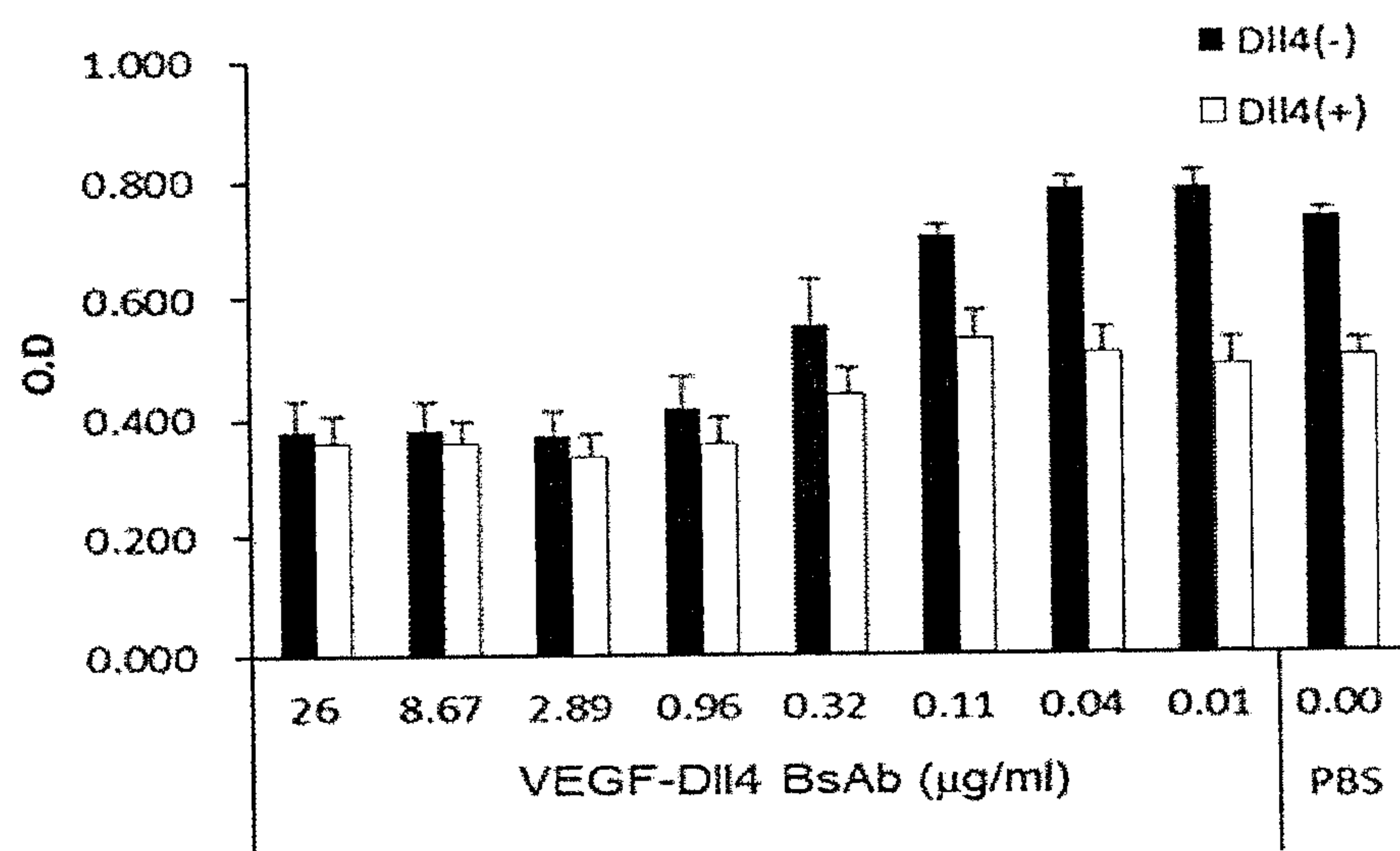


Fig. 9c



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Fig. 10

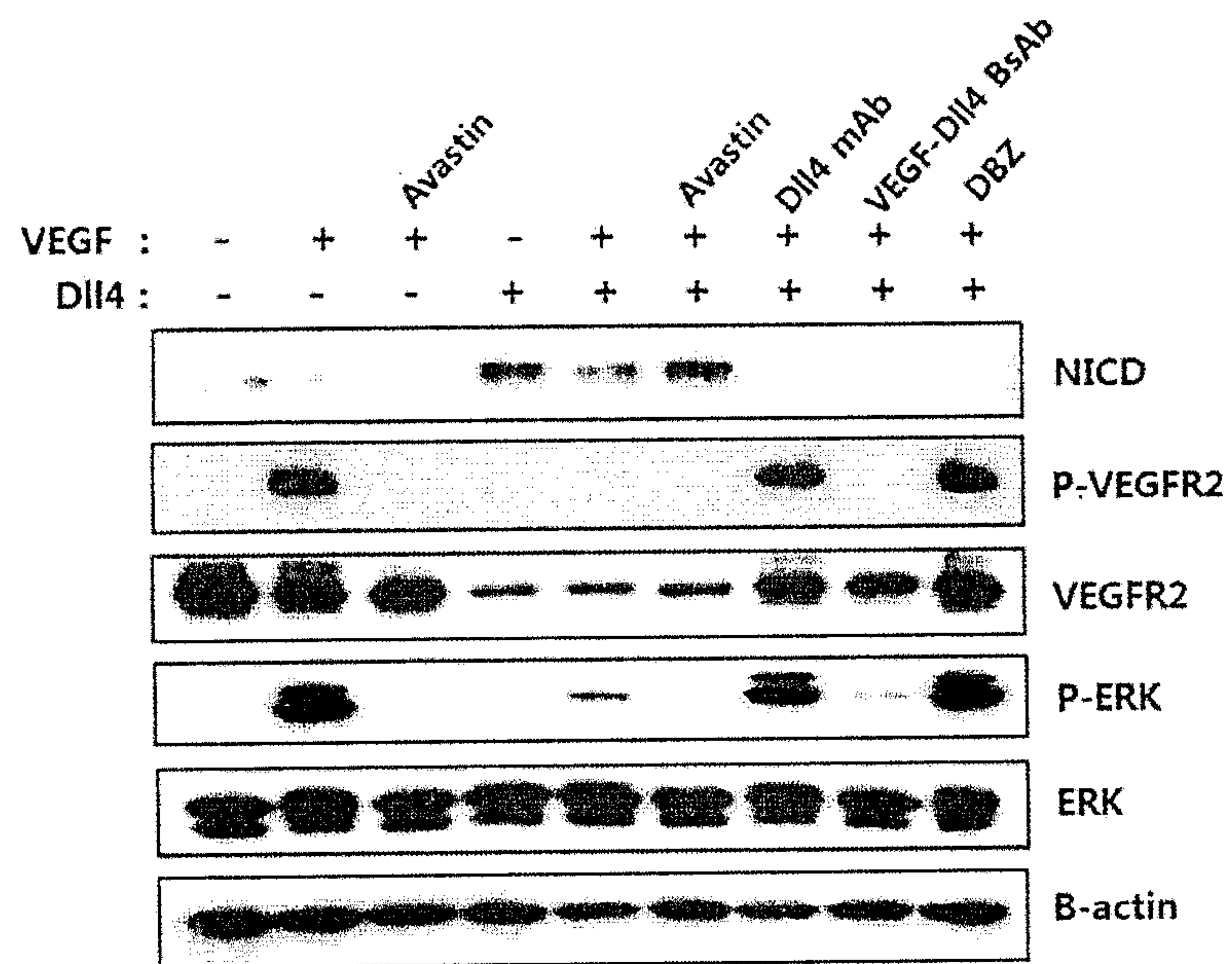
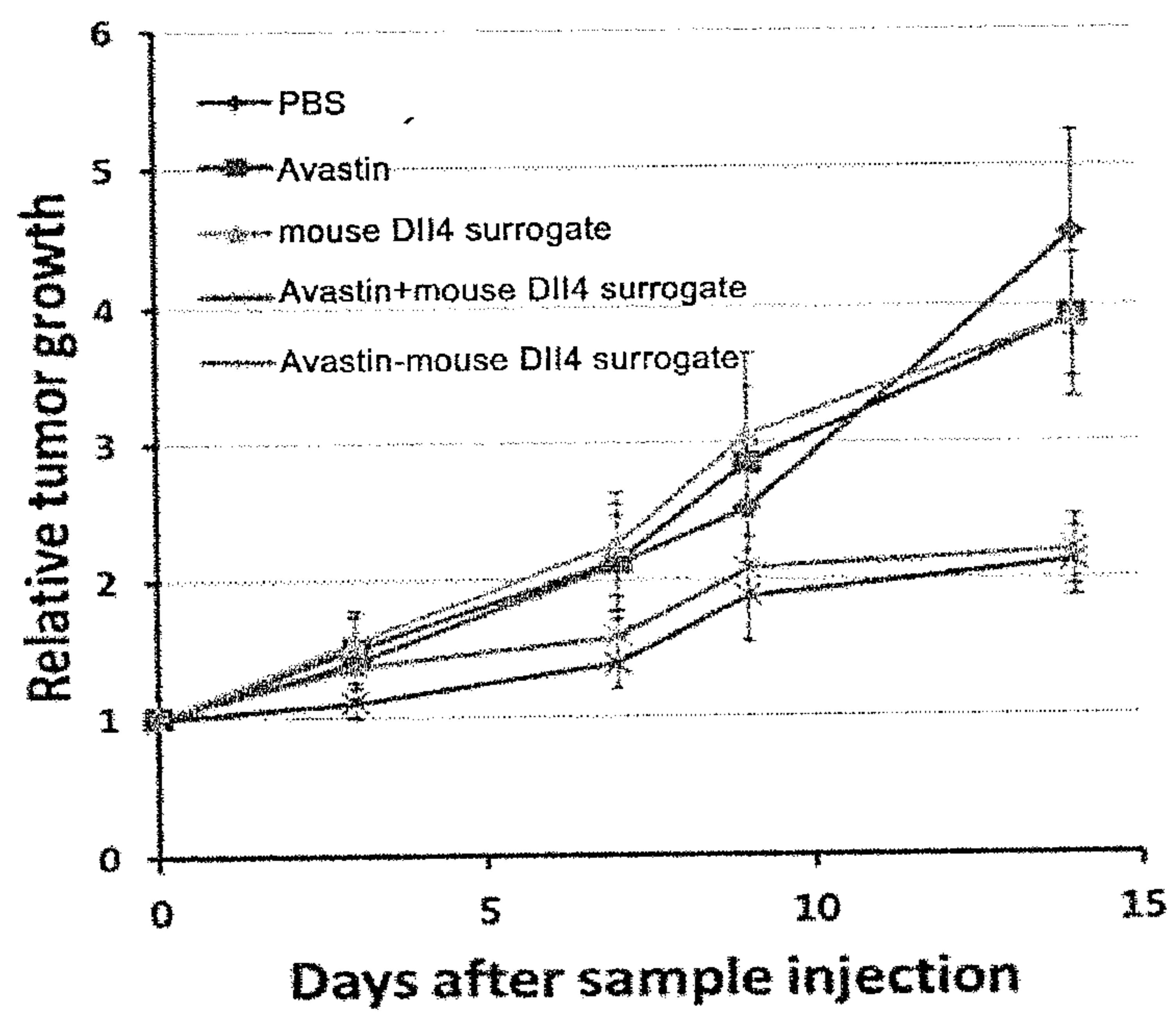


Fig. 11



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Fig. 12

