(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 July 2008 (31.07.2008)

PCT

(10) International Publication Number
WO 2008/091222 A1

(51) International Patent Classification:
A61K 39/395 (2006.01) *A61P 35/00* (2006.01)
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/SE2008/050099

(22) International Filing Date: 28 January 2008 (28.01.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/886,665 26 January 2007 (26.01.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

WO 2008/091222 A1

(54) Title: DLL4 SIGNALING INHIBITORS AND USES THEREOF

(57) Abstract: DLL4-binding antibodies, specifically antibodies preventing Notch signaling and internalization of DLL4, can: more efficiently than inhibitors only preventing DLL4-mediated Notch-signaling disrupt angiogenesis and pathological processes including tumor growth.

Title of the Invention

DLL4 Signaling Inhibitors and Uses Thereof

Field of the Invention

The invention relates to the field of angiogenesis. More specifically, the invention relates to antibodies inhibiting the Delta-like 4 (DLL4) pathway, which have an affect on angiogenesis-dependent diseases.

Background of the Invention

Developmental angiogenesis

Blood vessels are the body's infrastructure for the transportation of nutrients, oxygen and macromolecules to peripheral tissues and removal of carbon dioxide and waste products. For this reason, the cardio-vascular system is the first functional organ in the embryo, as it supports the development of the other organs. In a process termed vasculogenesis, the first vessels are assembled from scattered mesodermal cells and form the blood islands. These fuse to form the future large vessels and the first primitive blood vessel network (Risau, W. and Flamme, I., 1995, Annu Rev Cell Dev Biol 11, 73-91). The primitive networks grow and get remodeled through endothelial sprouting, splitting, growth and regression, collectively referred to as angiogenesis (Adams, R. H. and Alitalo, K., 2007, Nat Rev Mol Cell Biol 8, 464-78). The endothelial plexus early acquires a coating of mural cells (pericytes or smooth muscle cells) (Armulik, A., et al., 2005, Circ Res 97, 512-23). This is essential for proper vascular morphogenesis and vessel stability (Hellstrom, M., et al., 1999 Development 126, 3047-55. Hellstrom, M. et al., 2001, J Cell Biol 153, 543-53). Blood vessels develop not only to cover the tissues' need for oxygen and nutrients, they are also crucially involved in the signaling interplay between developing tissues (inductive signaling) (Red-Horse, K., J. et al., 2007, Dev Cell 12, 181-94).

Tumor angiogenesis

Although a tumor may initially take advantage of pre-existing blood vessels in the tissues where they develop, their growth will eventually make them dependent on the recruitment of new blood vessels. Tumor blood vessels are generally very abnormal and exhibit undirected sprouting, limited control of vessel size, and a low degree of functionality (Baluk, P., J. et al., 2005, Curr Opin Genet Dev 15, 102-11). This abnormality is also seen molecularly - angiogenic growth factors and endothelial markers are abnormally expressed (Seaman, S. et

al., 2007, Cancer Cell 11, 539-54). Many attempts have been made to exploit the therapeutic potential in angiogenesis inhibition, and in 2004 the first therapy targeted against angiogenesis was successfully launched for metastatic colorectal cancer (Hurwitz, H. et al., 2004, N Engl J Med 350, 2335-42). Bevacizumab (Avastin™), is a monoclonal antibody blocking Vascular Endothelial Growth Factor-A (VEGF-A) signaling (Ferrara, N., J. et al., 2004, Nat Rev Drug Discov 3, 391-400). Despite the fundamental importance of tumor angiogenesis, this process is still poorly understood and few drugs target this process. Therefore, there is a great need for new and improved drugs inhibiting angiogenesis.

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors and metastasis, atherosclerosis, retrolental fibroplasia, hemangiomas, chronic inflammation, intraocular neovascular diseases such as proliferative retinopathies, e.g., diabetic retinopathy, age-related macular degeneration (AMD), neovascular glaucoma, immune rejection of transplanted corneal tissue and other tissues, rheumatoid arthritis, and psoriasis. Folkman et al., J. Biol. Chem., 267:10931-10934 (1992); Klagsbrun et al., Annu. Rev. Physiol. 53:217-239 (1991); and Gamer A., "Vascular diseases" In: Pathobiology of Ocular Disease. A Dynamic Approach Garner A., Klintworth G K, eds., 2nd Edition (Marcel Dekker, NY, 1994), pp 1625-1710.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment for the growth and metastasis of the tumor. Folkman et al., Nature 339:58 (1989). The neovascularization allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. A tumor usually begins as a single aberrant cell which can proliferate only to a size of a few cubic millimeters due to the distance from available capillary beds, and it can stay `dormant` without further growth and dissemination for a long period of time. Some tumor cells then switch to the angiogenic phenotype to activate endothelial cells, which proliferate and mature into new capillary blood vessels. These newly formed blood vessels not only allow for continued growth of the primary tumor, but also for the dissemination and recolonization of metastatic tumor cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors. Weidner et al, N. Engl. J. Med 324:1-6 (1991); Horak et al., Lancet 340:1120-1124 (1992); Macchiarini et al, Lancet 340:145-146 (1992). The precise mechanisms that control the angiogenic switch are not well understood, but it is believed that

neovascularization of tumor mass results from the net balance of a multitude of angiogenesis stimulators and inhibitors (Folkman, 1995, Nat Med 1(1):27-31).

The endothelial tip cell.

The discovery of the endothelial tip cell in 2003 immediately improved our understanding of the angiogenic process (Gerhardt, H. et al., 2003, J Cell Biol 161, 1163-77). The tip cell is located at the very tip of the angiogenic sprout and has many similarities to the neuronal growth cone, as it has long filopodia extensions probing the environment for attractive and repulsive cues. It is also analogues to the Drosophila epithelial tip cell that leads the tubular branching morphogenesis during tracheal (lung) development (Samakovlis, C. et al., 1996, Development 122, 1395-407). Other characteristics of the endothelial tip cell include distinct gene expression, lack of lumen and low degree of proliferation, compared to its neighbors - the stalk cells. The tip cell is followed by several endothelial cells reffered to as endothelial stalk or trunk cells. The characterization of the endothelial tip cell was a significant advancement of the field, which allowed distinct roles of growth factors to be established for tip- and stalk cells in angiogenesis. It also paved the way for the understanding of Delta-like 4 (Dll4) signaling, which is now viewed as an anti-angiogenic target as promising as VEGF-A Thurston, G., J. et al., 2007, Nat Rev Cancer 7, 327-31).

Summary of the Invention

Data related to the present invention supports the novel concept that the simultaneous inhibition of DLL4-mediated Notch signaling *and* internalization of DLL4 disrupts angiogenesis more efficiently than an inhibitor that only inhibits DLL4-mediated Notch signaling. Moreover, the invention reveals that the simultaneous inhibition of DLL4-mediated Notch signaling *and* internalization of DLL4 more efficiently disrupts tumor angiogenesis and tumor growth than inhibitors that only inhibit DLL4-mediated Notch signaling. The present invention provides angiogenesis-modulating drugs in the form of mono-, bi- or multi-valent antibodies binding DLL4, either as single agents or as a part of combination treatments. Inhibitors designed to both prevent the DLL4-mediated Notch signaling and internalization of DLL4 provide a new strategy for disrupting angiogenesis and inhibiting tumor growth .

A drug that efficiently blocks the activities of DLL4 should target both the signaling via the Notch receptor pathway but should also prevent possible signaling triggered via internalization of the DLL4. As further described herein, DLL4 signaling and activity

correlates with internalization of DLL4. One set of supporting evidence comes from the analysis DLL4 localization during normal developmental angiogenesis in the mouse retina. There it is shown that the internalization of DLL4 correlates with area of the retina where active angiogenesis takes place.

The present invention is based in part on the discovery that vascular development is inhibited by treatment with an agent that simultaneously modulates Delta-like 4 (interchangeably termed "DLL4") activation of the Notch receptor pathway *and* internalization of DLL4. Treatment with a DLL4 antagonist resulted in increased endothelial cell (EC) proliferation, improper endothelial cell differentiation and improper arterial development in vasculature, including tumor vasculature. Strikingly, treatment with a blocking anti-DLL4 antibody not triggering internalization of DLL4 resulted in inhibition of dual bi-directional signaling of DLL4 and tumor growth in several different cancers. Accordingly, the invention provides methods, compositions, kits and articles of manufacture for modulating (e.g., promoting or inhibiting) processes involved in angiogenesis and for use in targeting pathological conditions associated with angiogenesis.

In one aspect, the invention provides methods for treating a tumor, a cancer, and/or a cell proliferative disorder comprising administering an effective amount of a DLL4 antagonist the simultaneously blocking both Notch signalling *and* internalisation of DLL4 to a subject in need of such treatment.

In one aspect, the invention provides methods for reducing, inhibiting, blocking, or preventing growth of a tumor or cancer, the methods comprising administering an effective amount of an anti-DLL4 antagonist the simultaneously blocking both Notch signalling and internalisation of DLL4 to a subject in need of such treatment.

In one aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of a DLL4 antagonist the simultaneously blocking both Notch signalling and internalisation of DLL4 (such as an anti-DLL4 antibody) to a subject in need of such treatment.

In one aspect, the invention provides methods for treating a pathological condition associated with angiogenesis comprising administering an effective amount of a DLL4 antagonist (such

as an anti-DLL4 antibody) the simultaneously blocking both Notch signalling *and* internalisation of DLL4 to a subject in need of such treatment. In some embodiments, the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder. In some embodiments, the pathological condition associated with angiogenesis is an intraocular neovascular disease.

In one aspect, the invention provides methods for stimulating endothelial cell proliferation comprising administering an effective amount of a DLL4 antagonist simultaneously blocking both Notch signalling *and* internalisation of DLL4 to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In one aspect, the invention provides methods for inhibiting endothelial cell differentiation comprising administering an effective amount of a DLL4 antagonist simultaneously blocking both Notch signalling and internalisation of DLL4 to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In another aspect, the invention provides a method of enhancing the efficacy of an anti-angiogenic agent treatment in a subject having a pathological condition associated with angiogenesis, comprising administering to the subject an effective amount of DLL4 antagonist simultaneously blocking both Notch signalling and internalisation of DLL4 in combination with the anti-angiogenic agent. Such a method will be useful in treating disorders, for example cancers or intraocular neovascular diseases, especially those diseases or stages of the disorders that responded poorly to a treatment with the anti-angiogenic agent alone. The anti-angiogenic agent can be any agent capable of reducing or inhibiting angiogenesis, including VEGF antagonists such as anti-VEGF antibody.

In one aspect, the invention provides methods comprising administration of an effective amount of a DLL4 antagonist (such as an anti-DLL4 antibody) simultaneously blocking both Notch signalling and internalisation of DLL4 in combination with and effective amount of another therapeutic agent (such as an anti-angiogenesis agent). For example, DLL4 antagonists are used in combinations with anti-cancer agent or an anti-angiogenic agent to treat various neoplastic or non-neoplastic conditions. In one embodiment, the neoplastic or

non-neoplastic condition is a pathological condition associated with angiogenesis. In some embodiments, the other therapeutic agent is an anti-angiogenic agent, an anti-neoplastic agent, and/or a chemotherapeutic agent.

The DLL4 antagonist can be administered serially or in combination with the other therapeutic agent that is effective for those purposes, either in the same composition or as separate compositions. The administration of the DLL4 antagonist and the other therapeutic agent (e.g., anti-cancer agent, anti-angiogenic agent) can be done simultaneously, e.g., as a single composition or as two or more distinct compositions, using the same or different administration routes. Alternatively, or additionally, the administration can be done sequentially, in any order. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, in any order. In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions. For example, the anti-cancer agent may be administered first, followed by the DLL4 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of an anti-angiogenic agent (such as an anti-VEGF antibody, such as bevacizumab). In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions.

In certain aspects, the invention provides a method of treating a disorder (such as a tumor, a cancer, and/or a cell proliferative disorder) by administering effective amounts of an antagonist of DLL4 simultaneously blocking both Notch signalling and internalisation of DLL4 and/or an angiogenesis inhibitor(s) and one or more chemotherapeutic agents. A variety of chemotherapeutic agents may be used in the combined treatment methods of the invention. An exemplary and non-limiting list of chemotherapeutic agents contemplated is provided herein under "Definitions." The administration of the DLL4 antagonist and the chemotherapeutic agent can be done simultaneously, e.g., as a single composition or as two or more distinct compositions, using the same or different administration routes. Alternatively, or additionally, the administration can be done sequentially, in any order. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, in any order. In certain embodiments, intervals ranging from minutes to days,

to weeks to months, can be present between the administrations of the two or more compositions. For example, the chemotherapeutic agent may be administered first, followed by the DLL4 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of a chemotherapeutic agent. In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions.

In one aspect, the invention provides use of a DLL4 antagonist simultaneously blocking both Notch signalling *and* internalisation of DLL4 in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a pathological condition associated with angiogenesis. In some embodiments, the disorder is a tumor, a cancer, and/or a cell proliferative disorder.

In one aspect, the invention provides methods for treating a disorder comprising administering an effective amount of a DLL4 agonist simultaneously blocking both Notch signalling *and* internalisation of DLL4 to a subject in need of such treatment. In some embodiments, the disorder is associated with expression and/or activity of the DLL4-Notch receptor pathways (such as increased activity of the DLL4-Notch receptor pathway). In some embodiments, the disorder is a disorder wherein angiogenesis, neovascularization and/or hypertrophy is desired, e.g. vascular trauma, wounds, lacerations, incisions, burns, ulcers (e.g., diabetic ulcers, pressure ulcers, haemophiliac ulcers, varicose ulcers), tissue growth, weight gain, peripheral arterial disease, induction of labor, hair growth, epidermolysis bullosa, retinal atrophy, bone fractures, bone spinal fusions, meniscal tears, etc. In some embodiments, the disorder is a disorder wherein inhibition of angiogenesis is desired. In some embodiments, the DLL4 agonist is DAPT or DBZ.

DLL4 antagonists and agonists are known in the art and some are described and exemplified herein. In some embodiments, the DLL4 antagonist is a molecule which binds to DLL4 and neutralizes, blocks, inhibits, abrogates, reduces or interferes with one or more aspects of DLL4-associated effect. In some embodiments, the DLL4 antagonist is a molecule which binds to Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and neutralizes, blocks, inhibits, abrogates, reduces or interferes with one or more aspects of DLL4-

associated effects. In some embodiments, the DLL4 antagonist is capable of promoting endothelial cell proliferation, inhibiting endothelial cell differentiation, inhibiting arterial development and/or reducing vascular perfusion. As is well-established in the art, endothelial cell proliferation, endothelial cell differentiation, arterial development and vascular function (such as vascular perfusion) can be assessed using any of a variety of assays (some of which are described and exemplified herein), and expressed in terms of a variety of quantitative values. In some embodiments, the ability of a DLL4 antagonist to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is assessed relative to level of endothelial cell proliferation, endothelial cell differentiation, arterial development and/or vascular function (such as vascular perfusion) in the absence of treatment with the DLL4 antagonist. In some embodiments, ability to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is determined in an in vitro assay (such as the HUVEC assay described herein). In some embodiments, ability to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is determined in an in vivo assay (such as the mouse retinal development assay described herein).

The DLL4 antagonist may be an anti-DLL4 antibody. In some embodiments, the anti-DLL4 antibody is a monoclonal antibody. In some embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is selected from the group consisting of a chimeric antibody, an affinity matured antibody, a humanized antibody, and a human antibody. In some embodiments, the antibody is an antibody fragment. In some embodiments, the antibody is a Fab, Fab', Fab'-SH, F(ab').sub.2, or scFv.

In one embodiment, the antibody is a chimeric antibody, for example, an antibody comprising antigen binding sequences from a non-human donor grafted to a heterologous non-human, human or humanized sequence (e.g., framework and/or constant domain sequences). In one embodiment, the non-human donor is a mouse. In one embodiment, an antigen binding sequence is synthetic, e.g. obtained by mutagenesis (e.g., phage display screening, etc.). In one embodiment, a chimeric antibody of the invention has murine V regions and human C region. In one embodiment, the murine light chain V region is fused to a human kappa light chain. In one embodiment, the murine heavy chain V region is fused to a human IgG1 C

region.

Humanized antibodies include those that have amino acid substitutions in the FR and affinity maturation variants with changes in the grafted CDRs. The substituted amino acids in the CDR or FR are not limited to those present in the donor or recipient antibody. In other embodiments, the antibodies of the invention further comprise changes in amino acid residues in the Fc region that lead to improved effector function including enhanced CDC and/or ADCC function and B-cell killing. Other antibodies of the invention include those having specific changes that improve stability. In other embodiments, the antibodies of the invention comprise changes in amino acid residues in the Fc region that lead to decreased effector function, e.g. decreased CDC and/or ADCC function and/or decreased B-cell killing.

In one aspect, the invention provides compositions comprising one or more DLL4 antagonist and a carrier. In one embodiment, the carrier is pharmaceutically acceptable. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody.

In one aspect, the invention provides a composition for use in treating a tumor, a cancer and/or a cell proliferative disorder comprising an effective amount of a DLL4 antagonist and a pharmaceutically acceptable carrier, wherein said use comprises simultaneous or sequential administration of an anti-angiogenesis agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody. In some embodiments, the anti-angiogenesis agent is an anti-VEGF antibody (such as bevacizumab).

In one aspect, the invention provides a composition for use in treating a tumor, a cancer and/or a cell proliferative disorder comprising an effective amount of a DLL4 antagonist and a pharmaceutically acceptable carrier, wherein said use comprises simultaneous or sequential administration of an anti-angiogenesis agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody preventing Notch signaling and DLL4 internalization. In some embodiments, the anti-angiogenesis agent is another anti-DLL4 antibody.

In one aspect, the invention provides a composition for use in treating a tumor, a cancer and/or a cell proliferative disorder comprising an effective amount of a DLL4 antagonist and a pharmaceutically acceptable carrier, wherein said use comprises simultaneous or sequential administration of an anti-cancer agent. In some embodiments, the DLL4 antagonist is an anti-

DLL4 antibody. In some embodiments, the anti-cancer agent is a chemotherapeutic agent. In some embodiments, the use further comprises simultaneous or sequential administration of an anti-angiogenesis agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody. In some embodiments, the anti-angiogenesis agent is an anti-VEGF antibody (such as bevacizumab).

In one aspect, the invention provides an article of manufacture comprising a container; and a composition contained within the container, wherein the composition comprises one or more DLL4 antagonists or DLL4 agonists.

In one aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more DLL4 antagonists or DLL4 agonists; and a second container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, the DLL4 antagonist is an anti-DLL4 antibody.

In another aspect, the present invention provides a method for preparing a composition comprising admixing a therapeutically effective amount of a DLL4 antagonist or DLL4 agonist with a pharmaceutically acceptable carrier.

Brief Description of the Drawing

Figure 1.

The images were captured from mouse postnatal day 5 retinas, stained according to "Example 5". The images in the figure a and a', b and b' etc. are the same except that the green and red channel are captured in a, b etc and only the red channel is captured in the a', b' etc. This allowed for clearer visualization of the internalized DLL4.

DLL4 (red) is generally present in the cell membrane of most of the capillary endothelial cells (green), **a'**. InternalizedDLL4 (arrows) recognized as being accumulated at certain non-membrane location (uneavenly distributed) was frequently observed in the endothelial cells at the vascular front in the mouse retina where active angiogenesis takes place, **a**. DLL4 is present in the cell membranes of endothelial cells in the vascular plexus that has matured, but is not internalized, **c** and **c'**. Internalized DLL4 is also observed in developing arterioles (arrows), **b** and **b'**, but not in veins, **d** and **d'**.

In summary, some DLL4 positive cells exhibited intracellular accumulation of DLL4 staining suggesting prior or present engagement in Notch activation. This correlated with sites where

active angiogenesis and vascular remodeling takes place. Almost all tip cells (48 out of 50 analyzed) showed intracellular accumulation of Dll4 staining. Interestingly, many stalk cells immediately in contact with the tip-cells (33/58) also showed intracellular accumulations of Dll4 staining, indicating active bi-directional Dll4 signaling between tip-cells and stalk cells at the sprouting front. DLL4 accumulation was similarly observed in arterioles, a known site of DLL4/Notch signaling but endothelial cells in the plexus behind the growing front, adjacent to arterioles or in veins showed no or minor intracellular accumulation of Dll4

Figure 2.

The amino acid sequence of human DLL4 protein

Figure 3.

The nucleotide sequence of human DLL4 cDNA

Figure 4.

The amino acid sequence of mouse DLL4 protein.

Figure 5.

The nucleotide sequence of mouse DLL4 cDNA.

Detailed Description

General Techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (2003)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

Definitions

The term "DLL4" (interchangeably termed "Delta-like 4"), as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) DLL4 polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated or secreted forms (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic

variants. The term "wild type DLL4" generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring DLL4 protein. The term "wild type DLL4 sequence" generally refers to an amino acid sequence found in a naturally occurring DLL4.

The term "Notch receptor" (interchangeably termed "Notch"), as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) Notch receptor polypeptide. Humans have four Notch receptors (Notch1, Notch 2, Notch3, and Notch4). As used herein, the term Notch receptor includes any one of or all four human Notch receptors. The term "native sequence" specifically encompasses naturally occurring truncated or secreted forms (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term "wild type Notch receptor" generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring Notch receptor protein. The term "wild type Notch receptor sequence" generally refers to an amino acid sequence found in a naturally occurring Notch receptor.

"DLL4 nucleic acid" is RNA or DNA that encodes a DLL4 polypeptide, as defined above, or which hybridizes to such DNA or RNA and remains stably bound to it under stringent hybridization conditions and is greater than about 10 nucleotides in length. Stringent conditions are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSO.sub.4 at 50.degree. C., (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinlypyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl.sub.2, 75 mM sodium citrate at 42.degree. C.

A "chimeric DLL4" molecule is a polypeptide comprising full-length DLL4 or one or more domains thereof fused or bonded to heterologous polypeptide. The chimeric DLL4 molecule will generally share at least one biological property in common with naturally occurring DLL4. An example of a chimeric DLL4 molecule is one that is epitope tagged for purification purposes. Another chimeric DLL4 molecule is a DLL4 immunoadhesin.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the

boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue.

Unless indicated otherwise, herein the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays as herein disclosed, for example.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-.LAMBDA. and .LAMBDA. allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, preferably one or more amino acid substitution(s). Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent

polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% homology therewith, more preferably at least about 95% homology therewith.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "antibody" and "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies (for e.g., full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies so long as they exhibit the desired biological activity) and may also include certain antibody fragments (as described in greater detail herein). An antibody can be human, humanized and/or affinity matured.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light

chains each comprise four FR regions, largely adopting a .beta.-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the .beta.-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab').sub.2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (.kappa.) and lambda (.lamda.), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called .alpha., .delta., .epsilon., .gamma., and .mu., respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody. Examples of antibody fragments include Fab, Fab', F(ab').sub.2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For e.g., such an antibody fragment may comprise on antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "hypervariable region", "HVR", or "HV", when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National

Institutes of Health, Bethesda, Md. (1991)). Chothia refers instead to the location ofthe structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below. TABLE-US-00002 Loop Kabat AbM Chothia Contact L1 L24-L34 L24-L34 L26-L32 L30-L36 L2 L50-L56 L50-L56 L50-L52 L46-L55 L3 L89-L97 L89-L97 L91-L96 L89-L96 H1 H31-H35B H26-H35B H26-H32 H30-H35B (Kabat Numbering) H1 H31-H35 H26-H35 H26-H32 H30-H35 (Chothia Numbering) H2 H50-H65 H50-H58 H53-H55 H47-H58 H3 H95-H102 H95-H102 H96-H101 H93-H101

Hypervariable regions may comprise "extended hypervariable regions" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102 or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat et al., supra for each of these definitions.

"Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a

monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., Nature, 256:495 (1975); Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., Nature, 352:624-628 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991); Sidhu et al., J. Mol. Biol. 338(2):299-310 (2004); Lee et al., J. Mol. Biol. 340(5):1073-1093 (2004); Fellouse, Proc. Nat. Acad. Sci. USA 101(34):12467-12472 (2004); and Lee et al. J. Immunol. Methods 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993); U.S. Pat. Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); U.S. Pat. No. 5,545,807; WO 1997/17852; U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., Bio/Technology, 10: 779-783 (1992); Lonberg et al., Nature, 368: 856-859 (1994); Morrison, Nature, 368: 812-813 (1994); Fishwild et al., Nature Biotechnology, 14: 845-851 (1996); Neuberger, Nature Biotechnology, 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol., 13: 65-93 (1995).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman

primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1:105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994).

"Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

An "antigen" is a predetermined antigen to which an antibody can selectively bind. The target antigen may be polypeptide, carbohydrate, nucleic acid, lipid, hapten or other naturally

occurring or synthetic compound. Preferably, the target antigen is a polypeptide.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. Proc Nat. Acad. Sci, USA 91:3809-3813 (1994); Schier et al. Gene 169:147-155 (1995); Yelton et al. J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al, J. Mol. Biol. 226:889-896 (1992).

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in

which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express Fc.gamma.RIII only, whereas monocytes express Fc.gamma.RI, Fc.gamma.RII and Fc.gamma.RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. Nos. 5,500,362 or 5,821,337 or Presta U.S. Pat. No. 6,737,056 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least Fc.gamma.RIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, e.g. from blood.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc.gamma.RI, Fc.gamma.RII, and Fc.gamma.RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc.gamma.RII receptors include Fc.gamma.RIIA (an "activating receptor") and Fc.gamma.RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc.gamma.RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc.gamma.RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991); Capel et al., Immunomethods 4:25-34 (1994); and de Haas

et al., J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)) and regulates homeostasis of immunoglobulins.

WO00/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. The content of that patent publication is specifically incorporated herein by reference. See, also, Shields et al. J. Biol. Chem. 9(2): 6591-6604 (2001).

Methods of measuring binding to FcRn are known (see, e.g., Ghetie 1997, Hinton 2004). Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates administered with the Fc variant polypeptides.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), maybe performed.

Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in U.S. Pat. No. 6,194,551B1 and WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie et al. J. Immunol. 164: 4178-4184 (2000).

The term "Fc region-comprising polypeptide" refers to a polypeptide, such as an antibody or immunoadhesin (see definitions below), which comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during purification of the polypeptide or by recombinant engineering the nucleic acid encoding the polypeptide. Accordingly, a composition comprising a polypeptide having an Fc region according to this invention can comprise polypeptides with K447, with all K447 removed, or a mixture of polypeptides with and without the K447 residue.

A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds. Preferred blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

A "disorder" or "disease" is any condition that would benefit from treatment with a substance/molecule or method of the invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include malignant and benign tumors; carcinoma, blastoma, and sarcoma.

The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer", "cancerous", "cell proliferative disorder", "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval

cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplastics include but are not limited those described above. Non-neoplastic disorders include but are not limited to undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/closed head injury/trauma), synovial inflammation, pannus formation in RA, myositis ossificans, hypertropic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies are used to delay development of a disease or disorder.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs and horses), primates, mice and rats.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

An "intraocular neovascular disease" is a disease characterized by ocular neovascularization. Examples of intraocular neovascular diseases include, but are not limited to, proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusions, including Central Retinal Vein Occlusion (CRVO), corneal neovascularization, retinal neovascularization, etc.

The "pathology" of a disease includes all phenomena that compromise the well-being of the patient. For cancer, this includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines

or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, etc.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN.TM., polyethylene glycol (PEG), and PLURONICS.TM..

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as a DLL4 polypeptide or antibody thereto) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The terms "VEGF" and "VEGF-A" are used interchangeably to refer to the 165-amino acid vascular endothelial cell growth factor and related 121-, 145-, 183-, 189-, and 206- amino acid vascular endothelial cell growth factors, as described by Leung et al. Science, 246:1306 (1989), Houck et al. Mol. Endocrin., 5:1806 (1991), and, Robinson & Stringer, Journal of Cell Science, 144(5):853-865 (2001), together with the naturally occurring allelic and processed forms thereof.

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding

fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases, and fusions proteins, e.g., VEGF-Trap (Regeneron), VEGF121-gelonin (Peregrine). VEGF antagonists also include antagonist variants of VEGF, antisense molecules directed to VEGF, RNA aptamers, and ribozymes against VEGF or VEGF receptors.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. The anti-VEGF antibody can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. See, e.g., U.S. Pat. Nos. 6,582,959, 6,703,020; WO98/45332; WO 96/30046; WO94/10202, WO2005/044853; ; EP 0666868B1; US Patent Applications 20030206899, 20030190317, 20030203409, 20050112126, 20050186208, and 20050112126; Popkov et al., Journal of Immunological Methods 288:149-164 (2004); and WO2005012359. An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF. The anti-VEGF antibody "Bevacizumab (BV)", also known as "rhuMAb VEGF" or "Avastin ", is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. Cancer Res. 57:4593-4599 (1997). It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of Bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated. Bevacizumab and other humanized anti-VEGF antibodies, including the anti-VEGF antibody fragment "ranibizumab", also known as "Lucentis.RTM.", are further described in U.S. Pat. No. 6,884,879 issued Feb. 26, 2005.

The term "biological activity" and "biologically active" with regard to a DLL4 polypeptide refer to physical/chemical properties and biological functions associated with DLL4. In some embodiments, DLL4 "biological activity" includes one or more of: binding a Notch receptor (eg, Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, and activating a Notch receptor downstream molecular signaling. In this context, the term "modulate" includes both promotion and inhibition.

A "DLL4 antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with the activities of a DLL4 including, for example, reduction or blocking of Notch receptor activation, reduction or blocking of Notch receptor downstream molecular signaling, disruption or blocking of Notch receptor binding to DLL4, and/or promotion of endothelial cell proliferation, and/or inhibition of endothelial cell differentiation, and/or inhibition of arterial differentiation. DLL4 antagonists include antibodies and antigen-binding fragments thereof, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. Antagonists also include small molecule inhibitors of a protein, and fusions proteins, receptor molecules and derivatives which bind specifically to protein thereby sequestering its binding to its target, antagonist variants of the protein, siRNA molecules directed to a protein, antisense molecules directed to a protein, RNA aptamers, and ribozymes against a protein. In some embodiments, the DLL4 antagonist is a molecule which binds to DLL4 and neutralizes, blocks, inhibits, abrogates, reduces or interferes with a biological activity of DLL4. In some embodiments, the DLL4 antagonist is a molecule which binds to Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and neutralizes, blocks, inhibits, abrogates, reduces or interferes with a biological activity of DLL4. In some embodiments, the DLL4 antagonist modulates one or more aspects of DLL4-associated effects, including but not limited to any one or more of reduction or blocking of Notch receptor activation, reduction or blocking of Notch receptor downstream molecular signaling, disruption or blocking of Notch receptor binding to DLL4, and/or promotion of endothelial cell proliferation, and/or inhibition of endothelial cell differentiation, and/or inhibition of arterial differentiation, and/or inhibition of tumor vascular perfusion, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with DLL4 expression and/or activity and/or treatment or prevention of a disorder associated with Notch receptor expression and/or activity.

The term "anti-neoplastic composition" refers to a composition useful in treating cancer comprising at least one active therapeutic agent, e.g., "anti-cancer agent". Examples of therapeutic agents (anti-cancer agents, also termed "anti-neoplastic agent" herein) include, but are limited to, e.g., chemotherapeutic agents, growth inhibitory agents, cytotoxic agents,

agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, toxins, and other-agents to treat cancer, e.g., anti-VEGF neutralizing antibody, VEGF antagonist, anti-HER-2, anti-CD20, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor, erlotinib, a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the ErbB2, ErbB3, ErbB4, or VEGF receptor(s), inhibitors for receptor tyrosine kinases for platet-derived growth factor (PDGF) and/or stem cell factor (SCF) (e.g., imatinib mesylate (Gleevec.RTM. Novartis)), TRAIL/Apo2L, and other bioactive and organic chemical agents, etc.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in Cancer Chemotherapy" Biochemical Society Transactions, 14, pp.375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prod rugs, sulfate-containing prod rugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, beta-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

An "angiogenic factor or agent" is a growth factor which stimulates the development of blood vessels, e.g., promotes angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis, etc. For example, angiogenic factors, include, but are not limited to, e.g., VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins), ephrins, ANGPTL3, DLL4, etc. It would also include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of its family, and TGF-.alpha. and TGF-.beta.. See, e.g., Klagsbrun and D'Amore, Annu. Rev. Physiol.,

53:217-39 (1991); Streit and Detmar, Oncogene, 22:3172-3179 (2003); Ferrara & Alitalo, Nature Medicine 5(12):1359-1364 (1999); Tonini et al., Oncogene, 22:6549-6556 (2003) (e.g., Table 1 listing angiogenic factors); and, Sato Int. J. Clin. Oncol., 8:200-206 (2003).

An "anti-angiogenesis agent" or "angiogenesis inhibitor" refers to a small molecular weight substance, a polynucleotide (including, e.g., an inhibitory RNA (RNAi or siRNA)), a polypeptide, an isolated protein, a recombinant protein, an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF, antibodies to VEGF receptors, small molecules that block VEGF receptor signaling (e.g., PTK787/ZK2284, SU6668, SUTENT.RTM./SU11248 (sunitinib malate), AMG706, or those described in, e.g., international patent application WO 2004/113304). Anti-angiogensis agents also include native angiogenesis inhibitors, e.g., angiostatin, endostatin, etc. See, e.g., Klagsbrun and D'Amore, Annu. Rev. Physiol., 53:217-39 (1991); Streit and Detmar, Oncogene, 22:3172-3179 (2003) (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo, Nature Medicine 5(12):1359-1364 (1999); Tonini et al., Oncogene, 22:6549-6556 (2003) (e.g., Table 2 listing antiangiogenic factors); and, Sato Int. J. Clin. Oncol., 8:200-206 (2003) (e.g., Table 1 lists Anti-angiogenic agents used in clinical trials).

Methods and Compositions of the Invention

The present invention is based in part on the discovery that vascular development is inhibited by treatment with an agent that modulates Delta-like 4 (interchangeably termed "DLL4") activation of the Notch receptor pathway and hampers DLL4 internalization to at least 60%, or at least 70%, preferably at least 80% and most preferably at least 90%. Unlike a typical (bi-valent) antibody that would crosslink and trigger internalization of DLL4, treatment with a DLL4 antagonist resulted in increased endothelial cell (EC) proliferation, improper endothelial cell differentiation and improper arterial development in vasculature, including tumor vasculature. Strikingly, treatment with an anti-DLL4 antibody resulted in inhibition of tumor growth in several different cancers. Without being bound by theory, it is believed that increased EC proliferation and impaired EC differentiation results in improper tumor vascular function, leading to inhibition of tumor growth. Accordingly, DLL4 antagonists are believed to demonstrate a broadly efficacious approach for the treatment of cancer.

Accordingly, the invention provides methods, compositions, kits and articles of manufacture for modulating (e.g., promoting or inhibiting) processes involved in angiogenesis and for use in targeting pathological conditions associated with angiogenesis, such as cancer.

It is contemplated that, according to the present invention, the DLL4 modulators and/or combinations of DLL4 modulators and other therapeutic agents can be used to treat various disorders.

Accordingly, the invention encompasses methods for inhibiting angiogenesis using an effective amount of a DLL4 antagonist (such as an anti-DLL4 antibody or a DLL4 immunoadhesin) to inhibit DLL4 activation of Notch receptors (such as Notch1, Notch2, Notch3, and/or Notch4). In another aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment. In some embodiments, the DLL4 antagonist is capable of promoting endothelial cell proliferation, inhibits endothelial cell differentiation, inhibits arterial development and/or reduces vascular perfusion. In another aspect, the invention provides methods for stimulating endothelial cell proliferation, inhibiting endothelial cell differentiation, inhibiting arterial development and/or inhibiting tumor vascular perfusion comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment.

Examples of neoplastic disorders to be treated with a DLL4 antagonist (such as an anti-DLL4 antibody) include, but are not limited to, those described herein under the terms "cancer" and "cancerous." Non-neoplastic conditions that are amenable to treatment with antagonists useful in the invention, but are not limited to, e.g., undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, edema from myocardial infarction, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, comeal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), comeal and other tissue

transplantation, chronic inflammation, lung inflammation, acute lung injury/ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/closed head injury/trauma), synovial inflammation, pannus formation in RA, myositis ossificans, hypertropic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), obesity, adipose tissue mass growth, hemophilic joints, hypertrophic scars, inhibition of hair growth, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion. Further examples of disorders to be treated with a DLL4 antagonist (such as an anti-DLL4 antibody) include an epithelial or cardiac disorder.

Modulators of DLL4, e.g., agonists or activators of DLL4, can be utilized for treatment of pathological disorders. In some embodiments, modulators of DLL4, e.g., agonists of DLL4, can be utilized in the treatment of pathological disorders where inhibition of angiogenesis is desired. Modulators of DLL4, e.g. DLL agonists, can also be used for treatment of pathological disorders where angiogenesis or neovascularization and/or hypertrophy is desired, which include, but are not limited to, e.g., vascular trauma, wounds, lacerations, incisions, burns, ulcers (e.g., diabetic ulcers, pressure ulcers, haemophiliac ulcers, varicose ulcers), tissue growth, weight gain, peripheral arterial disease, induction of labor, hair growth, epidermolysis bullosa, retinal atrophy, bone fractures, bone spinal fusions, meniscal tears, etc.

Combination Therapies

As indicated above, the invention provides combined therapies in which a DLL4 antagonist (such as an anti-DLL4 antibody) or a DLL4 agonist is administered with another therapy. For example, DLL4 antagonists are used in combinations with anti-cancer agent or an anti-angiogenic agent to treat various neoplastic or non-neoplastic conditions. In one embodiment, the neoplastic or non-neoplastic condition is characterized by pathological disorder associated with aberrant or undesired angiogenesis. The DLL4 antagonist can be administered serially or in combination with another agent that is effective for those

purposes, either in the same composition or as separate compositions. Alternatively, or additionally, multiple inhibitors of DLL4 can be administered, including e.g. one antibody only blocking DLL4-mediated Notch signaling and one antibody that inhibits both DLL4-mediated Notch signaling and internalization of DLL4.

The administration of the DLL4 antagonist (or DLL4 agonist) and the other therapeutic agent (e.g., anti-cancer agent, anti-angiogenic agent) can be done simultaneously, e.g., as a single composition or as two or more distinct compositions using the same or different administration routes. Alternatively, or additionally, the administration can be done sequentially, in any order. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, in any order.

In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions. For example, the anti-cancer agent may be administered first, followed by the DLL4 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of an anti-angiogenic agent (such as anti-VEGF). In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions.

The effective amounts of therapeutic agents administered in combination with a DLL4 antagonist (or DLL4 agonist) will be at the physician's or veterinarian's discretion. Dosage administration and adjustment is done to achieve maximal management of the conditions to be treated. The dose will additionally depend on such factors as the type of therapeutic agent to be used and the specific patient being treated. Suitable dosages for the anti-cancer agent are those presently used and can be lowered due to the combined action (synergy) of the anti-cancer agent and the DLL4 antagonist. In certain embodiments, the combination of the inhibitors potentiates the efficacy of a single inhibitor. The term "potentiate" refers to an improvement in the efficacy of a therapeutic agent at its common or approved dose. See also the section entitled Pharmaceutical Compositions herein.

Typically, the DLL4 antagonists and anti-cancer agents are suitable for the same or similar

diseases to block or reduce a pathological disorder such as a tumor, a cancer or a cell proliferative disorder. In one embodiment the anti-cancer agent is an anti-angiogenesis agent.

Antiangiogenic therapy in relationship to cancer is a cancer treatment strategy aimed at inhibiting the development of tumor blood vessels required for providing nutrients to support tumor growth. Because angiogenesis is involved in both primary tumor growth and metastasis, the antiangiogenic treatment provided by the invention is capable of inhibiting the neoplastic growth of tumor at the primary site as well as preventing metastasis of tumors at the secondary sites, therefore allowing attack of the tumors by other therapeutics.

Many anti-angiogenic agents have been identified and are known in the arts, including those listed herein, e.g., listed under Definitions, and by, e.g., Carmeliet and Jain, Nature 407:249-257 (2000); Ferrara et al., Nature Reviews:Drug Discovery, 3:391-400 (2004); and Sato Int. J. Clin. Oncol., 8:200-206 (2003). See also, US Patent Application US20030055006. In one embodiment, a DLL4 antagonist is used in combination with an anti-VEGF neutralizing antibody (or fragment) and/or another VEGF antagonist or a VEGF receptor antagonist including, but not limited to, for example, soluble VEGF receptor (e.g., VEGFR-1, VEGFR-2, VEGFR-3, neuropillins (e.g., NRP1, NRP2)) fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti-VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases (RTK), antisense strategies for VEGF, ribozymes against VEGF or VEGF receptors, antagonist variants of VEGF; and any combinations thereof. Alternatively, or additionally, two or more angiogenesis inhibitors may optionally be co-administered to the patient in addition to VEGF antagonist and other agent. In certain embodiment, one or more additional therapeutic agents, e.g., anti-cancer agents, can be administered in combination with DLL4 antagonist, the VEGF antagonist, and an anti-angiogenesis agent.

According to the invention combination treatment also envisages the combination with other types of cancer treatments such as radiation treatment.

DLL4

DLL4 is a transmembrane protein. The extracellular region contains 8 EGF-like repeats, as well as a DSL domain that is conserved among all Notch ligands and is necessary for receptor binding. The predicted protein also contains a transmembrane region, and a

cytoplasmic tail lacking any catalytic motifs. Human DLL4 protein is a 685 amino acid protein and contains the following regions: signal peptide (amino acids 1-25); MNNL (amino acids 26-92); DSL (amino acids 155-217); EGF-Like (amino acids 221-251); EGF-Like (amino acids 252-282); EGF-Like (amino acids 284-322); EGF-Like (amino acids 324-360); EGF-Like (amino acids 366-400); EGF-Like (amino acids 402-438); EGF-Like (amino acids 440-476); EGF-Like (amino acids 480-518); transmembrane (amino acids529-551); cytoplasmic domain (amino acids 552-685). DLL4 nucleic acid and amino acid sequences are known in the art and are further discussed herein. Nucleic acid sequence encoding the DLL4 can be designed using the amino acid sequence of the desired region of DLL4. Alternatively, the cDNA sequence (or fragments thereof) of DLL4 can be used. The accession number of human DLL4 is NM.sub.--019074, and the accession number of mouse DLL4 is NM.sub.--019454.

DLL4 binds the Notch receptors. The evolutionarily conserved Notch pathway is a key regulator of many developmental processes as well as postnatal self-renewing organ systems. From invertebrates to mammals, Notch signaling guides cells through a myriad of cell fate decisions and influences proliferation, differentiation and apoptosis (Miele and Osborne, 1999). The Notch family consists of structurally conserved cell surface receptors that are activated by membrane-bound ligands of the DSL gene family (named for Delta and Serrate from Drosophila and Lag-2 from C. elegans). Mammals have four receptors (Notch1, Notch2, Notch3, Notch4) and five ligands (Jag1, Jag2, DLL1, Dll3 and DLL4). Upon activation by ligands presented on neighboring cells, Notch receptors undergo successive proteolytic cleavages. This leads to the release of the Notch Intra-Cellular Domain (NICD), which translocates into the nucleus and forms a transcriptional complex with the DNA binding protein, RBP-Jk also know as CSL [for CBF1/Su(H)/Lag-1] and other transcriptional cofactors. The primary target genes of Notch activation include the HES (Hairy/Enhancer of Split) gene family and HES-related genes (Hey, CHF, HRT, HESR), which in turn regulate the downstream transcriptional effectors in a tissue and cell-type specific manner (Iso et al., 2003; Li and Harris, 2005).

DLL4 Internalization and Signaling

It is common that cell surface receptors present in the plasma membrane upon ligand stimulation are internalized (i.e. endocytosed) and either recycled to the cell membrane or degraded. The Notch ligands are also transmembrane proteins and it has been shown that

they are also internalized, reviewed in (LeBorgne R., et al., 2005, Development, 132, 1751-1762). The internalization of the Delta-like1 protein is required for the activation of the Notch receptor on the neighboring cell (Itoh M., et al., 2003, Dev Cell, 4, 67-82). It has also been proposed that Delta-like1 and Delta (the Drosophila orthologue of human DLL proteins) also have signaling properties on their own, independent Notch, as the intracellular part of DLL1 and Delta has been detected in the nucleus and been found to interact with transcription factors, (Bland et al., 2003, JBC, J. Biol. Chem., 278, 16, 13607-13610, Hiratochi M., et al., 2007, Nucleic Acids Res., 35(3):912-22). Thus, there is the potential that the DLL-Notch ligands and receptor has the potential for dual bi-directional signaling, i.e. both via the Notch receptor and the internalization of DLL ligands in separate cells.

Antibody-mediated Receptor Internalization and Antibody Valency

It has been suggested that targeting of antibodies against cell surface receptors leads to the internalization of the antibodies, while this is not the case for other targets not present in the plasma membrane (Lammert van Bueren et al., 2006, Cancer Research 66, 7630-7638, Baselga J, et al., J Clin Oncol 2000;18:904–14., Mould DR, et al., Clin Pharmacol Ther 1999;66:246–57., Rowinsky EK, et al., J Clin Oncol 2004;22:3003–15., Robert F, et al., J Clin Oncol 2001;19:3234–43., Bauer RJ, et al., J Pharmacokinet Biopharm 1999;27:397–420., Tokuda Y, et al., Br J Cancer 1999;81:1419–25., Kloft C, et al., Invest New Drugs 2004;22:39–52., Duconge J, et al., Eur J Drug Metab Pharmacokinet 2002;27:101–5., Lin YS, et al., J Pharmacol Exp Ther 1999;288:371–8., Benincosa LJ, et al., J Pharmacol Exp Ther 2000;292:810–6., Coffey GP, et al., J Pharmacol Exp Ther 2004;310:896–904., Shih LB, et al., Int J Cancer 1994;56:538–45., Shockley TR, et al., Cancer Res 1992;52:357–66. and Coffey GP, et al., Drug Metab Dispos 2005;33:623–9). It is known in the art that valency (valency of an antibody refers to the number of antigenic determinants that an individual antibody molecule can bind) of antibodies affect the degree of internalization of the antibodies, .e.g. bi-valent antibodies binding to and activating the HER2 receptor triggers internalization of the receptor while mono-valent versions of the antibodies have no capability of inducing receptor internalization (Yarden Y., 1990, PNAS, 87,2569-2573. and Srinivas U., et al., 1993, Cancer Immunology and Immunotherapy, 36,6 , 397-402).Therefore, it is possible to design antibodies that bind to proteins in the plasma membrane that that have different propensity of being internalized or not.

DLL4 Modulators

Modulators of DLL4 are molecules that modulate the activity of DLL4, e.g., agonists and antagonists. The term "DLL4 agonist" is used to refer to peptide and non-peptide analogs of DLL4 (such as the multimerized DLL4 described herein), and to other agents provided they have the ability to signal through a native Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4). The term "agonist" is defined in the context of the biological role of a Notch receptor. In certain embodiments, agonists possess the biological activities of a DLL4, as defined above, such as binding a Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, and activating a Notch receptor downstream molecular signaling. In some embodiments, DLL4 agonists inhibit endothelial cell proliferation, promote epithelial cell differentiation, and/or promote arterial development. In some embodiments, DLL4 agonists inhibit vascular development.

DLL4 modulators are known in the art, and some are described and exemplified herein. An exemplary and non-limiting list of DLL4 antagonists (such as an anti-DLL4 antibody) contemplated is provided herein under "Definitions."

The modulators useful in the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art. In some embodiments, DLL4 antagonists are characterized for any one or more of: binding to DLL4, binding to Notch receptor, reduction or blocking of Notch receptor activation, reduction or blocking of Notch receptor downstream molecular signaling, disruption or blocking of Notch receptor binding to DLL4, effect on DLL4 internalization, and/or promotion of endothelial cell proliferation, and/or inhibition of endothelial cell differentiation, and/or inhibition of arterial differentiation, and/or inhibition of tumor vascular perfusion, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with DLL4 expression and/or activity and/or treatment or prevention of a disorder associated with Notch receptor expression and/or activity. In some embodiments, DLL4 agonists are characterized for any one or more of: binding a Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, activating a Notch receptor downstream molecular signaling, inhibiting endothelial cell proliferation, promoting epithelial cell differentiation, and/or promoting arterial development. Methods for characterizing DLL4 antagonists and agonists are known in the art, and some are described and exemplified herein.

Antibodies

DLL4 antibodies are known in the art and some are described and exemplified herein. The anti-DLL4 antibodies are preferably monoclonal. Also encompassed within the scope of the invention are Fab, Fab', Fab'-SH and F(ab').sub.2 fragments of the anti-DLL4 antibodies provided herein. These antibody fragments can be created by traditional means, such as enzymatic digestion, or may be generated by recombinant techniques. Such antibody fragments may be chimeric or humanized. These fragments are useful for the diagnostic and therapeutic purposes set forth below.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

The anti-DLL4 monoclonal antibodies can be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies to DLL4 generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of DLL4 and an adjuvant. DLL4 may be prepared using methods well-known in the art, some of which are further described herein. For example, recombinant production of DLL4 is described below. In one embodiment, animals are immunized with a derivative of DLL4 that contains the extracellular domain (ECD) of DLL4 fused to the Fc portion of an immunoglobulin heavy chain. In a preferred embodiment, animals are immunized with an DLL4-IgG1 fusion protein. Animals ordinarily are immunized against immunogenic conjugates or derivatives of DLL4 with monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, Mont.) and the solution is injected intradermally at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti-DLL4 titer. Animals are boosted until titer plateaus.

Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC- 11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against DLL4. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., Anal. Biochem., 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by

standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The anti-DLL4 antibodies can be made by using combinatorial libraries to screen for synthetic antibody clones with the desired activity or activities. In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the anti-DLL4 antibodies can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length anti-DLL4 antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda Md. (1991), vols. 1-3.

The antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter et al., Ann. Rev. Immunol., 12: 433-455 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as "Fv phage clones" or "Fv clones".

Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter et al., Ann. Rev. Immunol., 12: 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., EMBO J, 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, J. Mol. Biol., 227: 381-388 (1992).

Filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, e.g. as described by Marks et al., J. Mol. Biol., 222: 581-597 (1991), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial host cell periplasm where assembly of a Fab-coat protein structure which becomes displayed on the phage surface by displacing some of the wild type coat proteins, e.g. as described in Hoogenboom et al., Nucl. Acids Res., 19: 4133-4137 (1991).

In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-DLL4 clones is desired, the subject is immunized with DLL4 to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In a preferred embodiment, a human antibody gene fragment library biased in favor of anti-DLL4 clones is obtained by generating an anti-DLL4 antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that DLL4 immunization gives rise to B cells producing human antibodies against DLL4. The generation of human antibody-producing transgenic mice is described below.

Additional enrichment for anti-DLL4 reactive cell populations can be obtained by using a

suitable screening procedure to isolate B cells expressing DLL4-specific membrane bound antibody, e.g., by cell separation with DLL4 affinity chromatography or adsorption of cells to fluorochrome-labeled DLL4 followed by flow-activated cell sorting (FACS).

Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which DLL4 is not antigenic. For libraries incorporating in vitro antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi et al., Proc. Natl. Acad. Sci. (USA), 86: 3833-3837 (1989), thereby making diverse V gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward primers based within the J-segment as described in Orlandi et al. (1989) and in Ward et al., Nature, 341: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones et al., Biotechnol., 9: 88-89 (1991), and forward primers within the constant region as described in Sastry et al., Proc. Natl. Acad. Sci. (USA), 86: 5728-5732 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi et al. (1989) or Sastry et al. (1989). Preferably, the library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g. as described in the method of Marks et al., J. Mol. Biol., 222: 581-597 (1991) or as described in the method of Orum et al., Nucleic Acids Res., 21: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi et al. (1989), or by further PCR amplification with a tagged primer as described in Clackson et al., Nature, 352: 624-628 (1991).

Repertoires of synthetically rearranged V genes can be derived in vitro from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson et al., J. Mol. Biol., 227: 776-798 (1992)), and mapped (reported in Matsuda et al., Nature Genet., 3: 88-94 (1993); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, J. Mol. Biol., 227: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas et al., Proc. Natl. Acad. Sci. USA, 89: 4457-4461 (1992). Human V.kappa. and V.lamda. segments have been cloned and sequenced (reported in Williams and Winter, Eur. J. Immunol., 23: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged in vitro according to the methods of Hoogenboom and Winter, J. Mol. Biol., 227: 381-388 (1992).

Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined in vitro, e.g., as described in Hogrefe et al., Gene, 128: 119-126 (1993), or in vivo by combinatorial infection, e.g., the loxP system described in Waterhouse et al., Nucl. Acids Res., 21: 2265-2266 (1993). The in vivo recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size imposed by E. coli transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10.sup.12 clones). Both vectors contain in vivo recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (Kd.sup.-1 of about 10.sup.-8 M).

Alternatively, the repertoires may be cloned sequentially into the same vector, e.g. as

described in Barbas et al., Proc. Natl. Acad. Sci. USA, 88: 7978-7982 (1991), or assembled together by PCR and then cloned, e.g. as described in Clackson et al., Nature, 352: 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton et al., Nucl. Acids Res., 20: 3831-3837 (1992).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (Kd-1 of about 106 to 107 M-1), but affinity maturation can also be mimicked in vitro by constructing and reselecting from secondary libraries as described in Winter et al. (1994), supra. For example, mutation can be introduced at random in vitro by using error-prone polymerase (reported in Leung et al., Technique, 1: 11-15 (1989)) in the method of Hawkins et al., J. Mol. Biol., 226: 889-896 (1992) or in the method of Gram et al., Proc. Natl. Acad. Sci USA, 89: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, e.g. using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 (published 14 Mar. 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks et al., Biotechnol., 10: 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities in the 10-9 M range.

DLL4 nucleic acid and amino acid sequences are known in the art and are further discussed herein. DNAs encoding DLL4 can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels et al., Agnew. Chem. Int. Ed. Engl., 28: 716-734 (1989), such as the triester, phosphite, phosphoramidite and H-phosphonate methods. In one embodiment, codons preferred by the expression host cell are used in the design of the DLL4 encoding DNA. Alternatively, DNA encoding the DLL4 can be isolated from a genomic or cDNA library.

Following construction of the DNA molecule encoding the DLL4, the DNA molecule is operably linked to an expression control sequence in an expression vector, such as a plasmid, wherein the control sequence is recognized by a host cell transformed with the vector. In general, plasmid vectors contain replication and control sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. Suitable vectors for expression in prokaryotic and eukaryotic host cells are known in the art and some are further described herein. Eukaryotic organisms, such as yeasts, or cells derived from multicellular organisms, such as mammals, may be used.

Optionally, the DNA encoding the DLL4 is operably linked to a secretory leader sequence resulting in secretion of the expression product by the host cell into the culture medium. Examples of secretory leader sequences include stII, ecotin, lamb, herpes GD, lpp, alkaline phosphatase, invertase, and alpha factor. Also suitable for use herein is the 36 amino acid leader sequence ofprotein A (Abrahmsen et al., EMBO J., 4: 3901 (1985)).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO.sub.4 precipitation and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell. Methods for transfection are well known in the art, and some are further described herein.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Methods for transformation are well known in the art, and some are further described herein.

Prokaryotic host cells used to produce the DLL4 can be cultured as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the DLL4 can be cultured in a variety of media, which is well known in the art and some of which is described herein.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

Purification of DLL4 may be accomplished using art-recognized methods, some of which are described herein.

The purified DLL4 can be attached to a suitable matrix such as agarose beads, acrylamide beads, glass beads, cellulose, various acrylic copolymers, hydroxyl methacrylate gels, polyacrylic and polymethacrylic copolymers, nylon, neutral and ionic carriers, and the like, for use in the affinity chromatographic separation of phage display clones. Attachment of the DLL4 protein to the matrix can be accomplished by the methods described in Methods in Enzymology, vol. 44 (1976). A commonly employed technique for attaching protein ligands to polysaccharide matrices, e.g. agarose, dextran or cellulose, involves activation of the carrier with cyanogen halides and subsequent coupling of the peptide ligand's primary aliphatic or aromatic amines to the activated matrix.

Alternatively, DLL4 can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other art-known method for panning phage display libraries.

The phage library samples are contacted with immobilized DLL4 under conditions suitable for binding of at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, e.g. as described in Barbas et al., Proc. Natl. Acad. Sci USA, 88: 7978-7982 (1991), or by alkali, e.g. as described in Marks et al., J. Mol. Biol., 222: 581-597 (1991), or by DLL4 antigen competition, e.g. in a procedure similar to the antigen competition method of

Clackson et al., Nature, 352: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass et al., Proteins, 8: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks et al., Biotechnol., 10: 779-783 (1992).

It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for DLL4. However, random mutation of a selected antibody (e.g. as performed in some of the affinity maturation techniques described above) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting DLL4, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess biotinylated DLL4, but with the biotinylated DLL4 at a concentration of lower molarity than the target molar affinity constant for DLL4. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

Anti-DLL4 clones may be activity selected. In one embodiment, the invention provides anti-DLL4 antibodies that block the binding between a Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and DLL4, but do not block the binding between a Notch receptor and a second protein. Fv clones corresponding to such anti-DLL4 antibodies can be selected by (1) isolating anti-DLL4 clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable

bacterial host; (2) selecting DLL4 and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-DLL4 phage clones to immobilized DLL4; (4) using an excess of the second protein to elute any undesired clones that recognize DLL4-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

DNA encoding the hybridoma-derived monoclonal antibodies or phage display Fv clones is readily isolated and sequenced using conventional procedures (e.g. by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra et al., Curr. Opinion in Immunol., 5: 256 (1993) and Pluckthun, Immunol. Revs, 130: 151 (1992).

DNA encoding the Fv clones can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g. the appropriate DNA sequences can be obtained from Kabat et al., supra) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. A Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid", full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In a preferred embodiment, a Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for all human, full or partial length heavy and/or light chains.

DNA encoding anti-DLL4 antibody derived from a hybridoma can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant

domains in place of homologous murine sequences derived from the hybridoma clone (e.g. as in the method of Morrison et al., Proc. Natl. Acad. Sci. USA, 81: 6851-6855 (1984)). DNA encoding a hybridoma or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies.

Antibody Fragments

The present invention encompasses antibody fragments. In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab').sub.2 fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, F(ab').sub.2 fragments can be isolated directly from recombinant host cell culture. Fab and F(ab').sub.2 fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat. No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and U.S. Pat. No. 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

Humanized Antibodies

The present invention encompasses humanized antibodies. Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody can have one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al. (1986) Nature 321:522-525; Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody (Sims et al. (1993) J. Immunol. 151:2296; Chothia et al. (1987) J. Mol. Biol. 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al. (1992) Proc. Natl. Acad. Sci. USA, 89:4285; Presta et al. (1993) J. Immunol., 151:2623.

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are

available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Human Antibodies

Human anti-DLL4 antibodies can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequences(s) as described above. Alternatively, human monoclonal anti-DLL4 antibodies can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor J. Immunol., 133: 3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., J. Immunol., 147: 86 (1991).

It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci USA, 90: 2551 (1993); Jakobovits et al., Nature, 362: 255 (1993); Bruggermann et al., Year in Immunol., 7: 33 (1993).

Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V

domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published Apr. 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for DLL4 and the other is for any other antigen. Exemplary bispecific antibodies may bind to two different epitopes of the DLL4 protein. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express DLL4. These antibodies possess an DLL4-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-.alpha., vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')2 bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305: 537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published May 13, 1993, and in Traunecker et al., EMBO J., 10: 3655 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain

constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1), containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one

of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')2 fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from E. coli, which can be chemically coupled to form bispecific antibodies. Shalaby et al., J. Exp. Med., 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')2 molecule. Each Fab' fragment was separately secreted from E. coli and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This

method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol. 147: 60 (1991).

Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fe region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n-VD2-(X2)n-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to

about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

Antibody Variants

In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) Science, 244:1081-1085. Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to

the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 (Presta, L.). See also US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO 2003/011878, Jean-Mairet et al. and U.S. Pat. No. 6,602,684, Umana et al. Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO 1997/30087, Patel et al. See, also, WO 1998/58964 (Raju, S.) and WO 1999/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof. See also US 2005/0123546 (Umana et al.) on antigen-binding molecules with modified glycosylation.

The preferred glycosylation variant herein comprises an Fc region, wherein a carbohydrate structure attached to the Fc region lacks fucose. Such variants have improved ADCC function. Optionally, the Fc region further comprises one or more amino acid substitutions

therein which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Examples of publications related to "defucosylated" or "fucose-deficient" antibodies include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; Okazaki et al. J. Mol. Biol. 336:1239-1249 (2004); Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004). Examples of cell lines producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. Arch. Biochem. Biophys. 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams et al., especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, FUT8, knockout CHO cells (Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004)).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 2 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 2, or as further described below in reference to amino acid classes, may be introduced and the products screened. TABLE-US-00003 TABLE 2 Original Exemplary Preferred Residue Substitutions Substitutions Ala (A) Val; Leu; Ile Val Arg (R) Lys; Gln; Asn Lys Asn (N) Gln; His; Asp, Lys; Arg Gln Asp (D) Glu; Asn Glu Cys (C) Ser; Ala Ser Gln (Q) Asn; Glu Asn Glu (E) Asp; Gln Asp Gly (G) Ala Ala His (H) Asn; Gln; Lys; Arg Arg Ile (I) Leu, Val, Met, Ala, Leu Phe; Norleucine Leu (L) Norleucine; Ile; Val; Ile Met; Ala; Phe Lys (K) Arg; Gln; Asn Arg Met (M) Leu; Phe; Ile Leu Phe (F) Trp; Leu; Val; Ile; Ala; Tyr Tyr Pro (P) Ala Ala Ser (S) Thr Thr Thr (T) Val; Ser Ser Trp (W) Tyr; Phe Tyr Tyr (Y) Trp; Phe; Thr; Ser Phe Val (V) Ile; Leu; Met; Phe; Leu Ala; Norleucine

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common

side-chain properties: [0215] (1) hydrophobic: norleucine, met, ala, val, leu, ile; [0216] (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; [0217] (3) acidic: asp, glu; [0218] (4) basic: his, lys, arg; [0219] (5) residues that influence chain orientation: gly, pro; and [0220] (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of the immunoglobulin polypeptides, thereby generating a Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG.sub.1, IgG.sub.2, IgG.sub.3 or IgG.sub.4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody used in methods may comprise one or more alterations as compared to the wild type counterpart antibody, e.g. in the Fc region. These antibodies would nonetheless retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For example, it is thought that certain alterations can be made in the Fc region that would result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in WO99/51642. See also Duncan & Winter Nature 322:738-40 (1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants. WO00/42072 (Presta) and WO 2004/056312 (Lowman) describe antibody variants with improved or diminished binding to FcRs. The content of these patent publications are specifically incorporated herein by reference. See, also, Shields et al. J. Biol. Chem. 9(2): 6591-6604 (2001). Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). These antibodies comprise an Fc reg on with one or more substitutions therein which improve binding of the Fc region to FcRn. Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C 1 q binding capability are described in U.S. Pat. No. 6,194,551B1, WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie et al. J. Immunol. 164: 4178-4184 (2000).

Antibody Derivatives

The antibodies can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. Preferably, the moieties suitable for derivatization of the antibody are water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl

pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymers are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

Screening for Antibodies with Desired Properties

The antibodies can be characterized for their physical/chemical properties and biological functions by various assays known in the art. In some embodiments, antibodies are characterized for any one or more of binding to DLL4, reduction or blocking of Notch receptor activation, reduction or blocking of Notch receptor downstream molecular signaling, disruption or blocking of Notch receptor binding to DLL4, triggering or hampering DLL4 internalization, and/or promotion of endothelial cell proliferation, and/or inhibition of endothelial cell differentiation, and/or inhibition of arterial differentiation, and/or inhibition of tumor vascular perfusion, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with DLL4 expression and/or activity and/or treatment or prevention of a disorder associated with Notch receptor expression and/or activity.

The purified antibodies can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion high pressure liquid chromatography (HPLC), mass spectrometry, ion exchange chromatography and papain digestion.

In certain embodiments of the invention, the antibodies produced herein are analyzed for their biological activity. In some embodiments, the antibodies of the present invention are tested for their antigen binding activity. The antigen binding assays that are known in the art

and can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. Illustrative antigen binding assay are provided below in the Examples section.

In certain embodiments of the invention, the antibodies produced herein are analyzed for their effect on DLL4 internalization.biological activity. In some embodiments, the antibodies of the present invention are tested for their effect on DLL4 internalization. Assays for internalization of cell surface expressed proteins are known in the art and can be used herein include without limitation any immunihistochemical localization techniques such as antibody based fluorescent localization techniques and cell surface biotinylation assays. Illustrative cell surface protein internalization assays are provided below in the Examples section.

Anti-DLL4 antibodies possessing the unique properties described herein can be obtained by screening anti-DLL4 hybridoma clones for the desired properties by any convenient method, some of which are described and exemplified herein. For example, if an anti-DLL4 monoclonal antibody that blocks or does not block the binding of Notch receptors to DLL4 is desired, the candidate antibody can be tested in a binding competition assay, such as a competitive binding ELISA, wherein plate wells are coated with DLL4, and a solution of antibody in an excess of the Notch receptor of interest is layered onto the coated plates, and bound antibody is detected enzymatically, e.g. contacting the bound antibody with HRP-conjugated anti-Ig antibody or biotinylated anti-Ig antibody and developing the HRP color reaction., e.g. by developing plates with streptavidin-HRP and/or hydrogen peroxide and detecting the HRP color reaction by spectrophotometry at 490 nm with an ELISA plate reader.

In one embodiment, the antibody is an altered antibody that possesses some but not all effector functions, which make it a desired candidate for many applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the produced immunoglobulin are measured to ensure that only the desired properties are maintained. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding

assays can be conducted to ensure that the antibody lacks Fc.gamma.R binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc.gamma.RIII only, whereas monocytes express Fc.gamma.RI, Fc.gamma.RII and Fc.gamma.RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in U.S. Pat. Nos. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed. FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art, e.g. those described in the Examples section.

Vectors, Host Cells and Recombinant Methods

For recombinant production of an antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species.

a. Generating Antibodies Using Prokaryotic Host Cells:

i. Vector Construction

Polynucleotide sequences encoding polypeptide components of the antibody can be obtained using standard recombinant techniques. Desired polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once

obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Pat. No. 5,648,237.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as .lamda.GEM.TM.-11 may be utilized in making a recombinant vector which can be used to transform susceptible host cells such as E. coli LE392.

The expression vector may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates

increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g. the presence or absence of a nutrient or a change in temperature.

A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the .beta.-galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) Cell 20: 269) using linkers or adaptors to supply any required restriction sites.

In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In one embodiment of the invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the immunoglobulins according to the invention can

occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the E. coli trxB-strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun Gene, 159:203 (1995).

[0246] Prokaryotic host cells suitable for expressing antibodies include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include Escherichia (e.g., E. coli), Bacilli (e.g., B. subtilis), Enterobacteria, Pseudomonas species (e.g., P. aeruginosa), Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla, or Paracoccus. In one embodiment, gram-negative cells are used. In one embodiment, E. coli cells are used as hosts for the invention. Examples of E. coli strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 AfhuA (AtonA) ptr3 lac Iq lacL8 .DELTA.ompT.DELTA.(nmpc-fepE) degP41 kanR (U.S. Pat. No.5,639,635). Other strains and derivatives thereof, such as E. coli 294 (ATCC 31,446), E. coli B, E. coli.lamda. 1776 (ATCC 31,537) and E. coli RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, E. coli, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

ii. Antibody Production

Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

Prokaryotic cells used to produce the polypeptides are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

The prokaryotic host cells are cultured at suitable temperatures. For E. coli growth, for example, the preferred temperature ranges from about 20.degree. C. to about 39.degree. C., more preferably from about 25.degree. C. to about 37.degree. C., even more preferably at about 30.degree. C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For E. coli, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

If an inducible promoter is used in the expression vector, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, e.g., Simmons et al., J. Immunol. Methods (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

In one aspect of the invention, antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD550 of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

To improve the production yield and quality of the polypeptides, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility

of heterologous proteins produced in bacterial host cells. Chen et al. (1999) J Bio Chem 274:19601-19605; Georgiou et al., U.S. Pat. No. 6,083,715; Georgiou et al., U.S. Pat. No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun (2000) J. Biol. Chem. 275:17106-17113; Arie et al. (2001) Mol. Microbiol. 39:199-210.

To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some E. coli protease-deficient strains are available and described in, for example, Joly et al. (1998), supra; Georgiou et al., U.S. Pat. No. 5,264,365; Georgiou et al., U.S. Pat. No. 5,508,192; Hara et al., Microbial Drug Resistance, 2:63-72 (1996).

In one embodiment, E. coli strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins are used as host cells in the expression system.

iii. Antibody Purification

Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products. Protein A is a 41 kD cell wall protein from Staphylococcus aureas which binds with a high affinity to the Fc region of antibodies. Lindmark et al (1983) J. Immunol. Meth. 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants.

As the first step of purification, the preparation derived from the cell culture as described above is applied onto the Protein A immobilized solid phase to allow specific binding of the antibody of interest to Protein A. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the antibody of interest is recovered from the solid phase by elution.

b. Generating Antibodies Using Eukaryotic Host Cells:

The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

A vector for use in a eukaryotic host cell may also contain a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide of interest. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of Replication

Generally, an origin of replication component is not needed for mammalian expression vectors. For example, the SV40 origin may typically be used only because it contains the early promoter.

(iii) Selection Gene Component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, where relevant, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (e.g., ATCC CRL-9096).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody polypeptide nucleic acid. Promoter sequences are known for eukaryotes. Virtually alleukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide (SEQ ID NO: 3). At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence (SEQ ID NO: 4). All of these sequences are suitably inserted into eukaryotic expression vectors.

Antibody polypeptide transcription from vectors in mammalian host cells is controlled, for

example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

(v) Enhancer Element Component

Transcription of DNA encoding the antibody polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, .alpha.-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody polypeptide-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells will typically also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding an antibody. One useful transcription termination component is the bovine growth hormone

polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

(viii) Culturing the Host Cells

The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem.102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES),

nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN.TM. drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of Antibody

When using recombinant techniques, the antibody can be produced intracellularly, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human .gamma.1, .gamma.2, or .gamma.4 heavy chains (Lindmark et al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human .gamma.3 (Guss et al., EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX.TM.resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE.TM. chromatography on an anion or

cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

Another type of covalent modification involves chemically or enzymatically coupling glycosides to a polypeptide of the invention. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 Sep. 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of any carbohydrate moieties present on a polypeptide of the invention may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al. Arch. Biochem. Biophys. 259:52 (1987) and by Edge et al. Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties, e.g., on antibodies, can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. Meth. Enzymol. 138:350 (1987).

Another type of covalent modification of a polypeptide of the invention comprises linking the polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Pharmaceutical Formulations

Therapeutic formulations comprising an antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington: The Science and Practice of Pharmacy 20th edition (2000)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN.TM., PLURONICS.TM. or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington: The Science and Practice of Pharmacy 20th edition (2000).

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and .gamma.ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT.TM. (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37.degree. C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

It is further contemplated that an agent useful in the invention can be introduced to a subject by gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In gene therapy applications, genes are introduced into cells in order to achieve in vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes in vivo. See, e.g., DLL4-SiRNA described in the Examples. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik et al., Proc. Natl. Acad. Sci. USA 83:4143-4146 (1986)). The oligonucleotides can

be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. For general reviews of the methods of gene therapy, see, for example, Goldspiel et al. Clinical Pharmacy 12:488-505 (1993); Wu and Wu Biotherapy 3:87-95 (1991); Tolstoshev Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan Science 260:926-932 (1993); Morgan and Anderson Ann. Rev. Biochem. 62:191-217 (1993); and May TIBTECH 11:155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. eds. (1993) Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler (1990) Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

Dosage and Administration

The molecules are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes, and/or subcutaneous administration.

In certain embodiments, the treatment of the invention involves the combined administration of a DLL4 antagonist and one or more anti-cancer agents, e.g., anti-angiogenesis agents. In one embodiment, additional anti-cancer agents are present, e.g., one or more different anti-angiogenesis agents, one or more chemotherapeutic agents, etc. The invention also contemplates administration of multiple inhibitors, e.g., multiple antibodies to the same antigen or multiple antibodies to different cancer active molecules. In one embodiment, a cocktail of different chemotherapeutic agents is administered with the DLL4 antagonist and/or one or more anti-angiogenesis agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and/or consecutive administration in either order. For example, a DLL4 antagonist may precede, follow, alternate with administration of the anti-cancer agents, or may be given simultaneously therewith. In one embodiment, there is a time period while both (or all) active agents simultaneously exert their biological activities.

For the prevention or treatment of disease, the appropriate dosage of DLL4 antagonist will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the inhibitor is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the inhibitor, and the discretion

of the attending physician. The inhibitor is suitably administered to the patient at one time or over a series of treatments. In a combination therapy regimen, the compositions of the invention are administered in a therapeutically effective amount or a therapeutically synergistic amount. As used herein, a therapeutically effective amount is such that administration of a composition of the invention and/or co-administration of DLL4 antagonist and one or more other therapeutic agents, results in reduction or inhibition of the targeting disease or condition. The effect of the administration of a combination of agents can be additive. In one embodiment, the result of the administration is a synergistic effect. A therapeutically synergistic amount is that amount of DLL4 antagonist and one or more other therapeutic agents, e.g., an angiogenesis inhibitor, necessary to synergistically or significantly reduce or eliminate conditions or symptoms associated with a particular disease.

Depending on the type and severity of the disease, about 1 .mu.g/kg to 50 mg/kg (e.g. 0.1-20 mg/kg) of DLL4 antagonist or angiogenesis inhibitor is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 .mu.g/kg to about 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. Typically, the clinician will administered a molecule(s) until a dosage(s) is reached that provides the required biological effect. The progress of the therapy of the invention is easily monitored by conventional techniques and assays.

For example, preparation and dosing schedules for angiogenesis inhibitors, e.g., anti-VEGF antibodies, such as AVASTIN.RTM. (Genentech), may be used according to manufacturers' instructions or determined empirically by the skilled practitioner. In another example, preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for chemotherapy are also described in Chemotherapy Service Ed., M. C. Perry, Williams & Wilkins, Baltimore, Md. (1992).

Efficacy of the Treatment

The efficacy of the treatment of the invention can be measured by various endpoints commonly used in evaluating neoplastic or non-neoplastic disorders. For example, cancer

treatments can be evaluated by, e.g., but not limited to, tumor regression, tumor weight or size shrinkage, time to progression, duration of survival, progression free survival, overall response rate, duration of response, and quality of life. Because the anti-angiogenic agents described herein target the tumor vasculature and not necessarily the neoplastic cells themselves, they represent a unique class of anticancer drugs, and therefore can require unique measures and definitions of clinical responses to drugs. For example, tumor shrinkage of greater than 50% in a 2-dimensional analysis is the standard cut-off for declaring a response. However, the inhibitors may cause inhibition of metastatic spread without shrinkage of the primary tumor, or may simply exert a tumouristatic effect. Accordingly, approaches to determining efficacy of the therapy can be employed, including for example, measurement of plasma or urinary markers of angiogenesis and measurement of response through radiological imaging.

The following Examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

The disclosures of all patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the Examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following Examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Va. 20108. References cited in the Examples are listed following the examples. All references cited herein are hereby incorporated by reference.

Example 1. Identification of DLL4-blocking antibodies.

Notch blocking ELISA. 96-well microtiter plates were coated with recombinant rat Notch1-Fc (rrNotch1-Fc, R&D Systems) at 0.5 .mu.g/ml. Conditioned medium containing DLL4-AP (amino acid 1-404 of DLL4 fused to human placenta alkaline phosphatase) was used in the assay. To prepare conditioned medium, 293 cells were transiently transfected with plasmid expressing DLL4-AP with Fugen6 reagent (Roche Molecular Biochemicals). Five days posttransfection, the conditioned medium was harvested, filtered and stored at 4.degree. C.

Purified antibodies titrated from 0.15 to 25 .mu.g/ml were preincubated for 1 hr at room temperature with DLL4-AP conditioned medium at a dilution that conferred 50% maximally achievable binding to coated rrNotch1-Fc. The antibody/DLL4-AP mixture was then added to rrNotch1-Fc coated plate for 1 hr at room temperature, after which plates were washed several times in PBS. The bound DLL4-AP was detected using 1-Step PNPP (Pierce) as substrate and OD 405 nm absorbance measurement. Identical assay was performed with DLL1-AP (human DLL1, amino acid 1-445). Similar assays were carried out with purified DLL4-His (C-terminal His-tagged human DLL4, amino acid 1-404) and Jag1-His (R& D system). The bound His-tagged ligands was detected with mouse anti-His mAb (1 .mu.g/ml, Roche Molecular Biochemicals), biotinylated goat-anti-mouse (Jackson ImmunoResearch) and Streptavidin-AP (Jackson ImmunoResearch).

Example 2. Visualization and quantification of cell surface expression and internalization of DLL4

Tagged (e.g. His or Myc tagged) or untagged full length or fragments of DLL4 were transfected into cells (e.g. COS7 cells). The localization of DLL4 was visualized using fluorescent antibodies recognizing the tagged or normal DLL4 protein. Membrane or nuclei stainings as well as or co-transfections of labeled proteins (e.g. Rab9- or RhoB-fused to e.g. Enhanced Green Fluorescent Protein) located to certain subcellular locations were used to determine the relative distribution of the DLL4 protein. The fluorescent proteins were detected using a confocal microscope.

The effects of DLL4-binding antibodies on the internalization of DLL4 were assessed by addition of specific antibodies to the cell culture medium and subsequent analysis of the cellular distribution of DLL4 protein.

The DLL4 expressing cells were also co-cultured with cells endogenously expressing Notch receptor(s) or cells transfected with Notch receptors to enhance the DLL4 internalization through ligand-receptor interaction/activation. Alternatively, the DLL4 expressing cells were cultured on plates coated with Notch-protein to enhance the internalization of DLL4.

Example 3. Quantification of the plasma membrane versus total amount of DLL4 protein.

Tagged (e.g. His or Myc tagged) or untagged full length or fragments of DLL4 were transfected into cells (e.g. COS7 cells), which were biotinylated prior to harvesting. DLL4 protein was immunoprecipitated using an anti-tag antibody or an antibody against DLL4 from

total lysates and blotted using streptavidin-HRP (Horseradish peroxidase) to detect the biotinylated DLL4. The total amount of DLL4 was detected by reprobing with an anti-tag antibody. The alteration of the relative distribution between cell surface and total amount of DLL4 was determined in the presence and absence of DLL4 binding antibodies.

The DLL4 expressing cells were also cultured together with cells endogenously expressing Notch receptor(s) or cells transfected with Notch receptors to enhance the DLL4 internalization through ligand-receptor activation. Alternatively, the DLL4 expressing cells were cultured on plates coated with Notch-protein.

Example 4. Internalization of DLL4-binding antibodies.

Living cells expressing DLL4 endogenously (e.g. human umbilical vein endothelial cells or human microvascular endothelial cells or human aorta endothelial cells) or cells transfected with DLL4 (e.g. COS7 cells) were incubated with DLL4-binding antibodies. The cellular localization of those antibodies was subsequently visualized using secondary labeled antibodies detecting the DLL4-binding antibodies.

Example 5. *in vivo* analysis of DLL4 internalization.

DLL4-binding antibodies were delivered to mouse pups where retinal angiogenesis was still ongoing (preferentially postnatal day 4 and 5). 3-24h after dosing of the DLL4 binding antibodies the pups were sacrificed and the eyes enucleated and fixed in 4% paraformaldehyde for 10-60min. The retinas were isolated through dissection under stereomicroscope. The retinas were blocked and permeabilized using 1% BSA (Bovine Serum Albumin) and 0.5% Triton X 100 (Sigma) in PBS (Phosphate Buffered Saline) for 1h to over night at room temperature or 4 degrees C. The DLL4 protein was visualized by incubation of an anti-DLL4 polyclonal goat serum from R&D systems (AF1389) diluted 1:100 in PBS with 0,5% BSA and 0.25% Triton X 100 over night at 4 degrees C. The retinas were washed 3 times 5 min in PBS. A secondary rabbit anti-goat antibody Alexa 555 (Invitrogen) was used to detect the primary antibody. The degree of endothelial cell surface versus internalized DLL4 staining from mouse pups treated with DLL4-binding and control antibodies was determined using images captured by a confocal microscope.

Some DLL4 positive cells exhibited intracellular accumulation of Dll4 staining suggesting prior engagement in Notch activation (**Figure 1**). Almost all tip cells (48 out of 50 analyzed) showed intracellular accumulation of Dll4 staining. Interestingly, many stalk cells immediately in contact with the tip-cells (33/58) also showed intracellular accumulations of Dll4 staining,

indicating active bi-directional Dll4 signaling between tip-cells and stalk cells at the sprouting front. Dll4 accumulation was similarly observed in arterioles, a known site of DLL4/Notch signaling but endothelial cells in the plexus behind the growing front or adjacent to arterioles showed no or minor intracellular accumulation of DLL4.

Example 6. Mouse tumor models

T241 fibrosarcoma, B16 melanoma and Lewis Lung Carcinoma were propagated in DMEM (Invitrogen) with 10% FCS (Fetal Calf Serum) (Invitrogen) and standard supplements. For tumor experiments, 0,5-1x10^6 tumor cells were suspended in 100 µL PBS and injected intradermally or subcutaneously on the back of C57Bl6 mice. The growth of the tumors were followed by measuring the length and width of the tumors and after 10-20 days, the tumors were 5 to 10 mm in diameter and the mice were sacrificed and the tumors removed, weighed, photographed and processed for histological and immunohistochemical analysis. The effects of DLL4-binding antibodies were compared to control antibodies.

Example 7. *in vitro* sprouting angiogenesis assay.

VEGF-A (25ng/ml, R&D Systems) driven sprouting of human umbilical vein endothelial cells (Promocell) in a three dimensional collagen (BD) matrix was quantified in the presence or absence of DLL4-binding antibodies essentially as described in (Korff T and Augustin HG., 1999, J Cell Sci. Oct;112 (Pt 19):3249-58). The 5 longest sprouts from 10 endothelial clusters were measured from each well and the effect of DLL4-binding antibodies were compared to that of VEGF-A.

Methods used in the examples

Cell Culture, Transfection, Immunoprecipitation, and Western Blot Analysis

COS7 cells were transiently transfected with 2–4 µg of plasmid DNA per 6 cm dish using either Fugene6 (Roche) or Gene Porter2 (Gene Therapy Systems). The total amount of plasmid DNA used for transfection was kept constant by adding an appropriate amount of the CS2+ vector plasmid. Two days after transfection, cells were harvested and lysed in TENT buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation and incubated with antibodies for 2 hr at 4°C, and then incubated with protein A or G Sepharose for 1 hr at 4°C. The Sepharose beads were washed with TENT buffer seven times. The beads were boiled in SDS gel loading buffer and eluted proteins were electrophoresed on an SDS-

polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Invitrogen). Blots were incubated with primary antibody (anti-FLAG M2, anti-Myc 9E10, and anti-HA 12C5, all at 1: 5000 dilution) for 2 hr. The signal was visualized using a secondary antibody (anti-mouse or -rat immunoglobulin-horseradish peroxidase, both at 1:10000 dilution) with a chemiluminescence detection system (Pierce).

Immunocytochemistry

Transfected COS7 cells were fixed, 24 hr posttransfection, in MeOH at −20°C for 5 min and air dried. Fixed cells were then incubated in blocking solution (10% normal goat serum in PBS) for 1 hr, followed by staining with appropriate primary antibodies (rabbit anti-Myc (A14) or FLAG polyclonal, biotinylated rat anti-HA, all at 1:1000 dilution) in blocking solution for 1 hr at room temperature. Subsequently, cells on coverslips were washed three times with PBS and incubated with goat anti-rabbit antibody conjugated with Alexa 488 or 594, or with Alexa 594- or 350-conjugated streptavidin, for 1 hr in the dark at room temperature. Coverslips were washed three times, mounted on glass slides, and analyzed on a Zeiss Axiophot fluorescent microscope. Images were collected on a Hamamatsu Orca camera and processed using Openlab and Photoshop software.

Internalization of DLL4 Antibodies

After 24 hr of transfection, DLL4-binding antibodies (10 µg/ml) and leupeptin (10 µg/ml) were added and the cells were further incubated for 9 hr. Following fixation and permeabilization, the DLL4-binding antibody was detected using a goat anti-mouse Alexa 594 secondary antibody.

Surface Biotinylation Assay

Transfected COS7 cells were washed three times with ice-cold PBS buffer. The cells were then incubated at 4°C for 30 min with biotinylation buffer (0.25 mg/ml EZ-link sulfo-NHS-LC-biotin [Pierce] in PBS). After removal of the biotinylation buffer, the cells were incubated for 20 min at 4°C in quenching buffer (100 mM glycine in PBS), washed once with ice-cold PBS, and lysed in 1 ml of TENT. Lysates were incubated with anti-HA-conjugated beads (Roche) for 1 hr and then the beads were washed five times with 1 ml of TENT and extracted in SDS sample buffer by boiling. The surface-biotinylated fraction was visualized using horseradish peroxidase-conjugated streptavidin (Vector Laboratory) by Western blot.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. However, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the

foregoing description and fall within the scope of the appended claims.

Claims

1. An antibody binding to DLL4 and blocking DLL4-mediated Notch signaling and hampering internalization of DLL4.
2. The antibody of claim 1 that is mono-valent.
3. The antibody of claim 1 that is bi-valent.
4. The antibody of claim 1 that is multi-valent.
5. A composition comprising an antibody according to any of claims 1-4.
6. The composition according to claim 5 comprising a further pharmaceutical for treatment of cancer.
7. A method for treatment of angiogenesis dependent diseases whereby an antibody hampering the signaling and internalization of DLL4 in a cell is administrated to an individual in need thereof.
8. The method according to claim 7, wherein the cell is an endothelial cell.
9. The method according to any of claims 7 and 8, wherein angiogenesis dependent diseases is any type of malign tumor such as cancer or a disease of endothelial cells.
10. The method according to any of claims 7 - 9, further comprising the treatment with another agent against angiogenesis dependent diseases.
11. A method for identifying a substance that affect DLL4 signalling wherein
    a) the cells are treated with a DLL4 binding substance
    b) DLL4 localization is determined as being present on the surface or inside the cell.
12. The method according to claim 11, wherein the cells are chosen from endothelial cells.
13. The method according to claim 11, wherein the tissue is chosen from retina.

Figure 1.

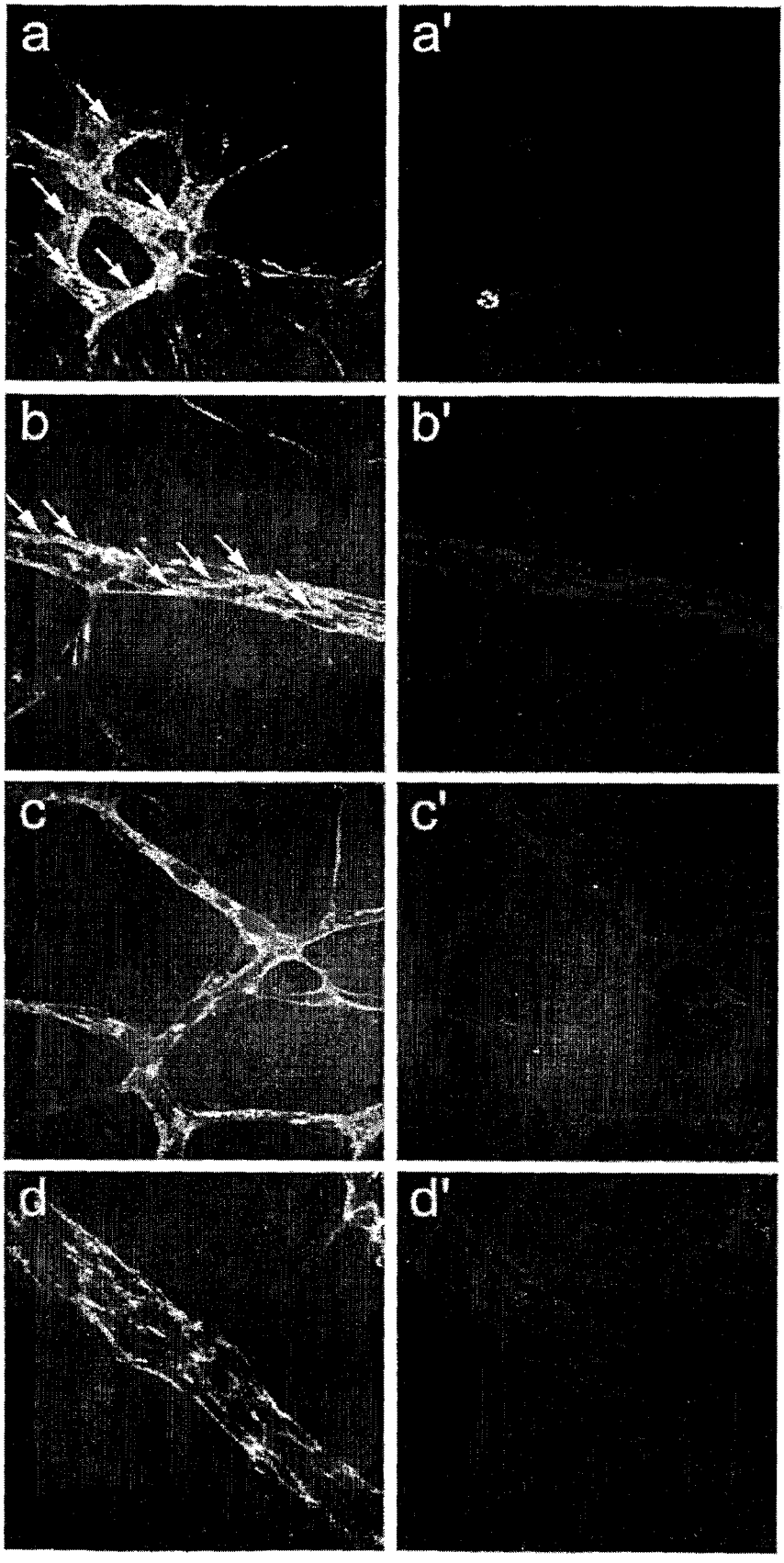

FIGURE 2.

MAAASRSASGWALLLLVALWQQRAAGSGVFQLQLQEFINERGVLASGRPCEPGCRTFFRVCL
KHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEAWHA
PGDDLRPEALPPDALISKIAIQGSLAVGQNWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSR
LCKKRNDHFGHYVCQPDGNLSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAECLCRPGWQGR
LCNECIPHNGCRHGTCSTPWQCTCDEGWGGLFCDQDLNYCTHHSPCKNGATCSNSGQRSYTC
TCRPGYTGVDCELELSECDSNPCRNGGSCKDQEDGYHCLCPPGYYGLHCEHSTLSCADSPCF
NGGSCRERNQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGPSRMCRCRPGFTG
TYCELHVSDCARNPCAHGGTCHDLENGLMCTCPAGFSGRRCEVRTSIDACASSPCFNRATCY
TDLSTDTFVCNCPYGFVGSRCEFPVGLPPSFPWVAVSLGVGLAVLLVLLGMVAVAVRQLRLR
RPDDGSREAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDCGLDKSNCGKQQNHTLDYNLAPG
PLGRGTMPGKFPHSDKSLGEKAPLRLHSEKPECRISAICSPRDSMYQSVCLISEERNECVIA
TEV

FIGURE 3.

ATGGCGGCAGCGTCCCGGAGCGCCTCTGGCTGGGCGCTACTGCTGCTGGTGGCACTTTGGCA
GCAGCGCGCGGCCGGCTCCGGCGTCTTCCAGCTGCAGCTGCAGGAGTTCATCAACGAGCGCG
GCGTACTGGCCAGTGGGCGGCCTTGCGAGCCCGGCTGCCGGACTTTCTTCCGCGTCTGCCTT
AAGCACTTCCAGGCGGTCGTCTCGCCCGGACCCTGCACCTTCGGGACCGTCTCCACGCCGGT
ATTGGGCACCAACTCCTTCGCTGTCCGGGACGACAGTAGCGGCGGGGGGCGCAACCCTCTCC
AACTGCCCTTCAATTTCACCTGGCCGGGTACCTTCTCGCTCATCATCGAAGCTTGGCACGCG
CCAGGAGACGACCTGCGGCCAGAGGCCTTGCCACCAGATGCACTCATCAGCAAGATCGCCAT
CCAGGGCTCCCTAGCTGTGGGTCAGAACTGGTTATTGGATGAGCAAACCAGCACCCTCACAA
GGCTGCGCTACTCTTACCGGGTCATCTGCAGTGACAACTACTATGGAGACAACTGCTCCCGC
CTGTGCAAGAAGCGCAATGACCACTTCGGCCACTATGTGTGCCAGCCAGATGGCAACTTGTC
CTGCCTGCCCGGTTGGACTGGGGAATATTGCCAACAGCCTATCTGTCTTTCGGGCTGTCATG
AACAGAATGGCTACTGCAGCAAGCCAGCAGAGTGCCTCTGCCGCCCAGGCTGGCAGGGCCGG
CTGTGTAACGAATGCATCCCCCACAATGGCTGTCGCCACGGCACCTGCAGCACTCCCTGGCA
ATGTACTTGTGATGAGGGCTGGGGAGGCCTGTTTTGTGACCAAGATCTCAACTACTGCACCC
ACCACTCCCCATGCAAGAATGGGGCAACGTGCTCCAACAGTGGGCAGCGAAGCTACACCTGC
ACCTGTCGCCCAGGCTACACTGGTGTGGACTGTGAGCTGGAGCTCAGCGAGTGTGACAGCAA
CCCCTGTCGCAATGGAGGCAGCTGTAAGGACCAGGAGGATGGCTACCACTGCCTGTGTCCTC
CGGGCTACTATGGCCTGCATTGTGAACACAGCACCTTGAGCTGCGCCGACTCCCCCTGCTTC
AATGGGGGCTCCTGCCGGGAGCGCAACCAGGGGGCCAACTATGCTTGTGAATGTCCCCCCAA
CTTCACCGGCTCCAACTGCGAGAAGAAAGTGGACAGGTGCACCAGCAACCCCTGTGCCAACG
GGGGACAGTGCCTGAACCGAGGTCCAAGCCGCATGTGCCGCTGCCGTCCTGGATTCACGGGC
ACCTACTGTGAACTCCACGTCAGCGACTGTGCCCGTAACCCTTGCGCCCACGGTGGCACTTG
CCATGACCTGGAGAATGGGCTCATGTGCACCTGCCCTGCCGGCTTCTCTGGCCGACGCTGTG
AGGTGCGGACATCCATCGATGCCTGTGCCTCGAGTCCCTGCTTCAACAGGGCCACCTGCTAC
ACCGACCTCTCCACAGACACCTTTGTGTGCAACTGCCCTTATGGCTTTGTGGGCAGCCGCTG
CGAGTTCCCCGTGGGCTTGCCGCCCAGCTTCCCCTGGGTGGCCGTCTCGCTGGGTGTGGGGC
TGGCAGTGCTGCTGGTACTGCTGGGCATGGTGGCAGTGGCTGTGCGGCAGCTGCGGCTTCGA
CGGCCGGACGACGGCAGCAGGGAAGCCATGAACAACTTGTCGGACTTCCAGAAGGACAACCT
GATTCCTGCCGCCCAGCTTAAAAACACAAACCAGAAGAAGGAGCTGGAAGTGGACTGTGGCC
TGGACAAGTCCAACTGTGGCAAACAGCAAAACCACACATTGGACTATAATCTGGCCCCAGGG
CCCCTGGGGCGGGGGACCATGCCAGGAAAGTTTCCCCACAGTGACAAGAGCTTAGGAGAGAA
GGCGCCACTGCGGTTACACAGTGAAAAGCCAGAGTGTCGGATATCAGCGATATGCTCCCCCA
GGGACTCCATGTACCAGTCTGTGTGTTTGATATCAGAGGAGAGGAATGAATGTGTCATTGCC
ACGGAGGTATAA

FIGURE 4.

MTPASRSACRWALLLLAVLWPQQRAAGSGIFQLRLQEFVNQRGMLANGQSCEPGCRTFFRIC
LKHFQATFSEGPCTFGNVSTPVLGTNSFVVRDKNSGSGRNPLQLPFNFTWPGTFSLNIQAWH
TPGDDLRPETSPGNSLISQIIIQGSLAVGKIWRTDEQNDTLTRLSYSYRVICSDNYYGESCS
RLCKKRDDHFGHYECQPDGSLSCLPGWTGKYCDQPICLSGCHEQNGYCSKPDECICRPGWQG
RLCNECIPHNGCRHGTCSIPWQCACDEGWGGLFCDQDLNYCTHHSPCKNGSTCSNSGPKGYT
CTCLPGYTGEHCELGLSKCASNPCRNGGSCKDQENSYHCLCPPGYYGQHCEHSTLTCADSPC
FNGGSCRERNQGSSYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGPSRTCRCRPGFT
GTHCELHISDCARSPCAHGGTCHDLENGPVCTCPAGFSGRRCEVRITHDACASGPCFNGATC
YTGLSPNNFVCNCPYGFVGSRCEFPVGLPPSFPWVAVSLGVGLVVLLVLLVMVVVAVRQLRL
RRPDDESREAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDCGLDKSNCGKLQNHTLDYNLAP
GLLGRGSMPGKYPHSDKSLGEKVPLRLHSEKPECRISAICSPRDSMYQSVCLISEERNECVI
ATEV

FIGURE 5.

CTCGCAGGCTAGGAACCCGAGGCCAAGAGCTGCAGCCAAAGTCACTTGGGTGCAGTGTACTC
CCTCACTAGCCCGCTCGAGACCCTAGGATTTGCTCCAGGACACGTACTTAGAGCAGCCACCG
CCCAGTCGCCCTCACCTGGATTACCTACCGAGGCATCGAGCAGCGGAGTTTTTGAGAAGGCG
ACAAGGGAGCAGCGTCCCGAGGGGAATCAGCTTTTCAGGAACTCGGCTGGCAGACGGGACTT
GCGGGAGAGCGACATCCCTAACAAGCAGATTCGGAGTCCCGGAGTGGAGAGGACACCCCAAG
GGATGACGCCTGCGTCCCGGAGCGCCTGTCGCTGGGCGCTACTGCTGCTGGCGGTACTGTGG
CCGCAGCAGCGCGCTGCGGGCTCCGGCATCTTCCAGCTGCGGCTGCAGGAGTTCGTCAACCA
GCGCGGTATGCTGGCCAATGGGCAGTCCTGCGAACCGGGCTGCCGGACTTTCTTCCGCATTT
GCCTTAAGCACTTCCAGGCAACCTTCTCCGAGGGACCCTGCACCTTTGGCAATGTCTCCACG
CCGGTATTGGGCACCAACTCCTTCGTCGTCAGGGACAAGAATAGCGGCAGTGGTCGCAACCC
TCTGCAGTTGCCCTTCAATTTCACCTGGCCGGGAACCTTCTCACTCAACATCCAAGCTTGGC
ACACACCGGGAGACGACCTGCGGCCAGAGACTTCGCCAGGAAACTCTCTCATCAGCCAAATC
ATCATCCAAGGCTCTCTTGCTGTGGGTAAGATTTGGCGAACAGACGAGCAAAATGACACCCT
CACCAGACTGAGCTACTCTTACCGGGTCATCTGCAGTGACAACTACTATGGAGAGAGCTGTT
CTCGCCTATGCAAGAAGCGCGATGACCACTTCGGACATTATGAGTGCCAGCCAGATGGCAGC
CTGTCCTGCCTGCCGGGCTGGACTGGGAAGTACTGTGACCAGCCTATATGTCTTTCTGGCTG
TCATGAGCAGAATGGTTACTGCAGCAAGCCAGATGAGTGCATCTGCCGTCCAGGTTGGCAGG
GTCGCCTGTGCAATGAATGTATCCCCCACAATGGCTGTCGTCATGGCACCTGCAGCATCCCC
TGGCAGTGTGCCTGCGATGAGGGATGGGGAGGTCTGTTTTGTGACCAAGATCTCAACTACTG
TACTCACCACTCTCCGTGCAAGAATGGATCAACGTGTTCCAACAGTGGGCCAAAGGGTTATA
CCTGCACCTGTCTCCCAGGCTACACTGGTGAGCACTGTGAGCTGGGACTCAGCAAGTGTGCC
AGCAACCCCTGTCGAAATGGTGGCAGCTGTAAGGACCAGGAGAATAGCTACCACTGCCTGTG
TCCCCCAGGCTACTATGGCCAGCACTGTGAGCATAGTACCTTGACCTGTGCGGACTCACCCT
GCTTCAATGGGGGCTCTTGCCGGGAGCGCAACCAGGGGTCCAGTTATGCCTGCGAATGCCCC
CCCAACTTTACCGGCTCTAACTGTGAGAAGAAAGTAGACAGGTGTACCAGCAACCCGTGTGC
CAATGGAGGCCAGTGCCTGAACAGAGGTCCAAGCCGAACCTGCCGCTGCCGGCCTGGATTCA
CAGGCACCCACTGTGAACTGCACATCAGCGATTGTGCCCGAAGTCCCTGTGCCCACGGGGGC
ACTTGCCACGATCTGGAGAATGGGCCTGTGTGCACCTGCCCCGCTGGCTTCTCTGGCAGGCG
CTGCGAGGTGCGGATAACCCACGATGCCTGTGCCTCCGGACCCTGCTTCAATGGGGCCACCT
GCTACACTGGCCTCTCCCCAAACAACTTCGTCTGCAACTGTCCTTATGGCTTTGTGGGCAGC
CGCTGCGAGTTTCCCGTGGGCTTGCCACCCAGCTTCCCCTGGGTAGCTGTCTCGCTGGGCGT
GGGGCTAGTGGTACTGCTGGTGCTGCTGGTCATGGTGGTAGTGGCTGTGCGGCAGCTGCGGC
TTCGGAGGCCCGATGACGAGAGCAGGGAAGCCATGAACAATCTGTCAGACTTCCAGAAGGAC
AACCTAATCCCTGCCGCCCAGCTCAAAAACACAAACCAGAAGAAGGAGCTGGAAGTGGACTG
TGGTCTGGACAAGTCCAATTGTGGCAAACTGCAGAACCACACATTGGACTACAATCTAGCCC

CGGGACTCCTAGGACGGGGCAGCATGCCTGGGAAGTATCCTCACAGTGACAAGAGCTTAGGA
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TCCCAGGGACTCTATGTACCAATCAGTGTGTTTGATATCAGAAGAGAGGAACGAGTGTGTGA
TTGCCACAGAGGTATAAGGCAGGAGCCTACTCAGACACCCAGCTCCGGCCCAGCAGCTGGGC
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GTTGAGGAGTTAGAGGAGCATCAGTTGAGCTGATATCTAAGGTGCCTCTCGAACTTGGACTT
GCTCTGCCAACAGTGGTCATCATGGAGCTCTTGACTGTTCTCCAGAGAGTGGCAGTGGCCCT
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CTCCCGCAGGCAGAACAGCCTTGGAGTTTGGCATTAAGCAGGAGCTACTCTGCAGGTGAGGA
AAGCCCGAGGAGGGGACACGTGTGACTCCTGCCTCCAACCCCAGCAGGTGGGGTGCCACCTG
CAGCCTCTAGGCAAGAGTTGGTCCTTCCCCTGGTCCTGGTGCCTCTGGGCTCATGTGAACAG
ATGGGCTTAGGGCACGCCCCTTTTGCCAGCCAGGGGTACAGGCCTCACTGGGGAGCTCAGGG
CCTTCATGCTAAACTCCCAATAAGGGAGATGGGGGGAAGGGGGCTGTGGCCTAGGCCCTTCC
CTCCCTCACACCCATTTTTGGGCCCTTGAGCCTGGGCTCCACCAGTGCCCACTGTTGCCCCG
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TTGGAATCTAGTATTTTGATAATTTAAGAATCAGAAGCACTGGCCTTTCTACATTTTATAAC
ATTATTTTGTATATAATGTGTATTTATAATATGAAACAGATGTGTACATAAAAAAAAAAAAA
AAAAAAAAAAAAAAA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/050099

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: A61K, C07K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 20060134121 A1 (THURSTON, G ET AL), 22 June 2006 (22.06.2006), claims 1-6,9-11, paragraphs [0003],[0007]-[0012],[0017]-[0018], [0023]-[0024],[0027]-[0029],[0031]-[0033],[0037], [0041],[0052],[0059],[0063]	1-10
Y	--	11-13
Y	Overstreet, E et al, "Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells", Published by The Company of Biologists 2004, p. 5355-5366, page 1, column 2, line 14 - page 2, column 2, line 30; page 3, column 2, line 35 - page 3, column 2, line 45; page 4, column 1, line 1 - line 15; figure 1; abstract	11-13
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2008	08 -05- 2008
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson/PR Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/050099

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Ridgway, J et al, "Inhibition of D114 signalling inhibits tumour growth by deregulating angiogenesis", Nature December 2006, Vol. 444, p. 1083-1087, entire document	1-10
A		11-13
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X	Noguera-Troise, I et aL, "Blockade of D114 inhibits tumour growth by promoting non-productive angiogenesis, Nature December 2006, Vol. 444, p. 1032-1037, page 1, column 1, line 1 - column 2, line 7; page 4, column 2, line 51 - page 5, column 2, line 11, figure 5, abstract	1-5,7-9
A		6,10-13
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X	US 20050137130 A1 (BODMER, M W ET AL), 23 June 2005 (23.06.2005), [0102],[0576],[0586], claims 1,4-7,26-27,59,62,65-68	1-5
A		6-13
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Y	De Renzis, S et al, "Dorsal-Ventral Pattern of Delta Trafficking Is Established by a Snail-Tom-Neuralized Pathway, Developmental Cell February 2006, Vol. 10, p. 257-264, page 1, column 1, line 1 - column 2, line 18; page 2, column 1, line 50 - column 2, line 13; page 7, column 1, line 26 - line 45, figure 1, abstract	1-13
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Y	Parks, A L et al, "Ligand endocytosis drives receptor dissociation and activation in the Notch pathway, Development 2000, Vol. 127, p. 1373-1385, page 1378, column 2, line 41 - line 55; page 1382, column 1, line 29 - column 2, line 10, figures 2,9, abstract	11-13
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/050099

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Sanz, L et al, "Antibody engineering: facing new challenges in cancer therapy, Acta Pharmacologica Sinica June 2005, Vol. 26, No. 6, p. 641-648, entire document	2-4
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PX	WO 2007143689 A2 (GENENTECH, INC.), 13 December 2007 (13.12.2007), page 3, line 29 - page 5, line 20; page 16, line 20 - line 25; page 18, line 8 - line 19, page 24, line 23 - line 25; page 59, line 8-line 30; page 93, line 9-page 94, line 16; claims 1,3-4, 6,9-25, example 13	1-10
PA		11-13
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PX	WO 2007070671 A2 (REGENERON PHARMACEUTICALS, INC.), 21 June 2007 (21.06.2007), claims 1-9, abstract, paragraphs [0003]-[0011],[0023]-[0027], [0035],[0039],[0043]-[0046]	1-10
PA		11-13
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PX	Gridley, T, "Vessel guidance", Nature February 2007, Vol. 445, p. 722-723, entire document	1-10
PA		11-13
	--	
E	WO 2008042236 A2 (ONCOMED PHARMACEUTICALS, INC.), 10 April 2008 (10.04.2008), abstract, claims 1, 10-17, 21-22,42,45,48-50,52,54-55,57	1-13
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/050099

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 7-10
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 7-10 relate to a method of treatment of the human or animal body by therapy /Rule 39.1(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

International patent classification (IPC)
A61K 39/395 (2006.01)
C07K 16/28 (2006.01)
A61P 35/00 (2006.01)

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Cited literature, if any, will be enclosed in paper form.

US	20060134121	A1	22/06/2006	NONE			
US	20050137130	A1	23/06/2005	CA	2465304	A	22/05/2003
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