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(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **COUTO, Suzana** [BR/US]; 37 Aztec Street, San Francisco, California 94110 (US). **HONGO, Jo-Anne S.** [US/US]; 31 Shaw Court, Redwood City, California 94061 (US). **KALLOP, Dara Y.** [US/US]; 2717 Sutter Street, San Francisco, California 94115 (US). **LI, Yongjun** [CN/US]; 144 Saint Marks Court, Daly City, California 94015 (US). **LIU, Jinfeng** [CN/US]; 3444 Edison Street, San Mateo, California 94403 (US). **LUDLAM, Mary** [GB/US]; 3534 24th Street, San Francisco, California 94110 (US). **PEI, Lin** [US/US]; 318 South Grant Street, #2C, San Mateo, California 94401 (US). **TAN, Christine** [US/US]; 3675 Edison Street, Apartment. F, San Mateo, California 94403 (US). **YE, Xiaofen** [CN/US]; 821 Rigel Lane, Foster City, California 94404 (US). **ZHA, Jiping** [US/US]; 371 Bramble Court, Foster City, California 94404 (US).

(74) Agents: **COBURN, Cara M.** et al.; Genentech, Inc., 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

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(54) Title: COMBINATION TREATMENTS

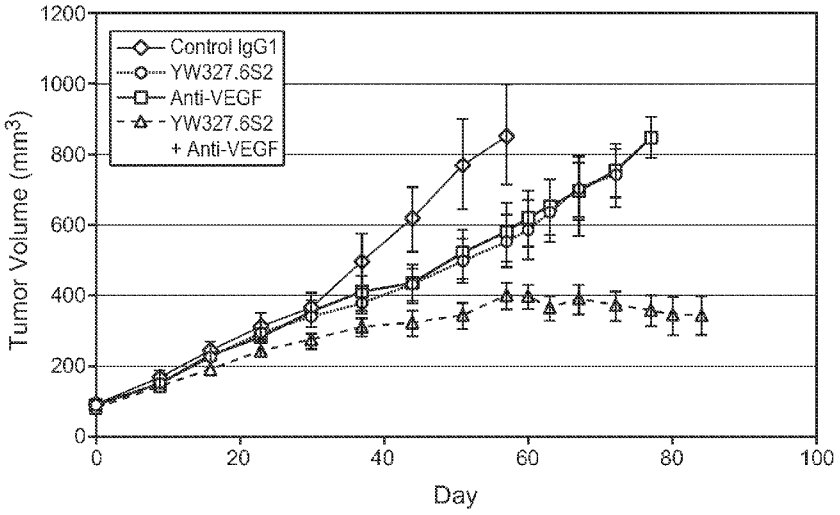

FIG. 15A

(57) Abstract: The invention provides combination treatments using Axl antagonists and VEGF antagonists.

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COMBINATION TREATMENTS COMPRISING AXL-ANTAGONISTS AND VEGF-ANTAGONISTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. patent application number 61/228,915, filed on July 27, 2009, and 61/356,498, filed June 18, 2010, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology. More specifically, the present invention relates to Axl antagonists and methods of using the same.

BACKGROUND

Axl belongs to the TAM subfamily of receptor tyrosine kinases (RTKs) that also includes Tyro3 and Mer (O'Bryan J.P. *et al.* (1991) *Mol. Cell Biol.* 11:5016-5031; Lai C. and G. Lemke (1991) *Neuron* 6:691-704). The TAM receptors are characterized by a combination of two immunoglobin–like domains and dual fibronectin type III repeats in the extracellular region and a cytoplasmic kinase domain. The ligands for TAM receptors are Gas6 (growth-arrest-specific 6) and protein S, two vitamin-K dependent proteins that show 43% amino acid sequence identity and share similar domain structures (Varnum B.C. *et al.* (1995) *Nature* 373:623-626; Stitt T.N. *et al.* (1995) *Cell* 80:661-670). Each protein has an N-terminal Gla domain containing 11 g-carboxyglutamic acid residues, followed by four epidermal growth factor (EGF)-like modules, and a C-terminal sex hormone-binding globlin (SHBG)-like structure consisting of two tandem laminin G domains. The SHBG domain is both necessary and sufficient for TAM receptor binding and activation, whereas the Gla domain binds the negatively charged membrane phospholipids and plays an important role in TAM-mediated phagocytosis of apoptotic cells (Sasaki T. *et al.* (2006) *EMBO J.* 25:80-87; Hasanbasic I. *et al.* (2005) *J. Thromb. Haemost.* 3:2790-2797).

TAM activation and signaling has been implicated in multiple cellular responses including cell survival, proliferation, migration and adhesion (Hafizi S. and B. Dahlbäck (2006) *FEBS J.* 273:5231-5244). TAM receptor signaling has been shown to regulate vascular smooth muscle homeostasis (Melaragno M.G. *et al.* (1999) *Trends Cardiovasc. Med.* 9:250-253; Korshunov V.A. *et al.* (2006) *Circ. Res.* 98:1446-1452; Korshunov V.A. *et al.* (2007) *Hypertension* 50:1057-1062), platelet function, thrombus stabilization

(Angelillo-Scherrer A. *et al.* (2001) *Nat. Med.* 7:215-221; Gould W.R. *et al.* (2005) *J. Thromb. Haemost.* 3:733-741) and erythropoiesis (Angelillo-Scherrer A. *et al.* (2008) *J. Clin. Invest.* 118:583-596). TAM receptors are also implicated in the control of oligodendrocyte cell survival (Shankar S.L. *et al.* (2006) *J. Neurosci.* 26:5638-5648) and in the regulation of osteoclast function (Katagiri M. *et al.* (2001) *J. Biol. Chem.* 276:7376-7382). Recent studies in knockout mice have revealed that TAM receptors play pivotal roles in innate immunity (Lemke G. and C.V., Rothlin (2008) *Nat. Rev. Immunol.* 8:327-336). TAM inhibits inflammation in macrophages and dendritic cells (Rothlin C.V. *et al.* (2007) *Cell* 131:1124-1136; Sharif M.N. *et al.* (2006) *J. Exp. Med.* 203:1891-1901), promotes the phagocytosis of apoptotic cells (Prasad D. *et al.* (2006) *Mol. Cell. Neurosci.* 33:96-108; Lu Q. *et al.* (1999) *Nature* 398:723-728) and stimulates the differentiation of natural killer cells (Caraux A. *et al.* (2006) *Nat. Immunol.* 7:747-754). In many of these instances, the primary downstream TAM signaling pathway appears to be PI3K/AKT pathway (Angelillo-Scherrer A. *et al.* (2001) *Nat. Med.* 7:215-221; Shankar S.L. *et al.* (2006) *J. Neurosci.* 26:5638-5648; Keating A.K. *et al.* (2006) *Oncogene* 25:6092-6100); however, the Janus kinase-STAT pathway is essential for TAM-mediated immune responses (Rothlin C.V. *et al.* (2007) *Cell* 131:1124-1136). In addition, cooperative interaction between TAM receptor and cytokine receptor signaling network is required for many TAM regulated biological functions (Rothlin C.V. *et al.* (2007) *Cell* 131:1124-1136; Budagian V. *et al.* (2005) *EMBO J.* 24:4260-4270).

Axl was originally cloned from patients with chronic myelogenous leukemia and when over-expressed exhibits transforming potential (O'Bryan J.P. *et al.* (1991) *Mol. Cell Biol.* 11:5016-5031; Janssen J.W. *et al.* (1991) *Oncogene* 6:2113-2120). Axl over-expression has been reported in a variety of human cancers (Berclaz G. *et al.* (2001) *Ann. Oncol.* 12:819-824; Craven R.J. *et al.* (1995) *Int. J. Cancer* 60:791-797; Shieh Y.S. *et al.* (2005) *Neoplasia* 7:1058-1064; Sun W. *et al.* (2004) *Oncology* 66:450-457; Ito T. *et al.* (1999) *Thyroid* 9:563-567), and is associated with invasiveness and metastasis in lung (Shieh Y.S. *et al.* (2005) *Neoplasia* 7:1058-1064), prostate (Sainaghi P.P. *et al.* (2005) *J. Cell. Physiol.* 204:36-44), breast (Meric F. *et al.* (2002) *Clin. Cancer Res.* 8:361-367), and gastric cancers (Wu C.W. *et al.* (2002) *Anticancer Res.* 22:1071-1078) as well as in renal cell carcinoma (Chung B.I. *et al.* (2003) *DNA Cell. Biol.* 22:533-540) and glioblastoma (Hutterer M. *et al.* (2008) *Clin. Cancer Res.* 14:130-138). A recent study showed that Axl over-expression via a "tyrosine kinase switch" leads to resistance to imatinib in

gastrointestinal stromal tumors (Mahadevan D. *et al.* (2007) *Oncogene* 26:3909-3919). Axl expression is induced by chemotherapy drugs and over-expression of Axl confers drug resistance in acute myeloid leukemia (Hong C.C. *et al.* (2008) *Cancer Lett.* 268:314-324). Axl has also been shown to regulate endothelial cell migration and tube formation (Holland S.J. *et al.* (2005) *Cancer Res.* 65:9294-9303). These findings suggest that Axl may be involved in the regulation of multiple aspects of tumorigenesis including tumor growth, invasion and angiogenesis. Other publications relating to Axl and anti-Axl antibodies include WO2004/039955, WO2009/063965; co-owned co-pending US patent application 61/356,508, filed June 18, 2010; co-owned, co-pending US patent application 61/356,514, filed June 18, 2010; WO2009/062690; WO2004/008147; and 5,468,634.

Development of a vascular system is a fundamental requirement for many physiological and pathological processes. Actively growing tissues such as embryos and tumors require adequate blood supply. They satisfy this need by producing pro-angiogenic factors, which promote new blood vessel formation via a process called angiogenesis. Vascular tube formation is a complex but orderly biological event involving all or many of the following steps: a) Endothelial cells (ECs) proliferate from existing ECs or differentiate from progenitor cells; b) ECs migrate and coalesce to form cord-like structures; c) vascular cords then undergo tubulogenesis to form vessels with a central lumen d) existing cords or vessels send out sprouts to form secondary vessels; e) primitive vascular plexus undergo further remodeling and reshaping; and f) peri-endothelial cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions to the vessels; such cells including pericytes for small capillaries, smooth muscle cells for larger vessels, and myocardial cells in the heart. Hanahan, D. Science 277:48-50 (1997); Hogan, B. L. & Kolodziej, P. A. Nature Reviews Genetics. 3:513-23 (2002); Lubarsky, B. & Krasnow, M. A. Cell. 112:19-28 (2003).

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 Garner A.,

"Vascular diseases", In: Pathobiology of Ocular Disease. A Dynamic Approach, Garner A., Klintworth GK, 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). 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 is 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 process of vascular development is tightly regulated. To date, a significant number of molecules, mostly secreted factors produced by surrounding cells, have been shown to regulate EC differentiation, proliferation, migration and coalescence into cord-like structures. For example, vascular endothelial growth factor (VEGF) has been identified as the key factor involved in stimulating angiogenesis and in inducing vascular permeability. Ferrara et al., Endocr. Rev. 18:4-25 (1997). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system. Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders. Ferrara et al., Endocr. Rev. supra. The VEGF mRNA is overexpressed by the majority of human tumors examined. Berkman et al., J. Clin. Invest. 91:153-159 (1993); Brown et al., Human Pathol. 26:86-91 (1995); Brown et al., Cancer Res. 53:4727-4735 (1993); Mattern et al., Brit. J. Cancer 73:931-934 (1996); Dvorak et al., Am. J. Pathol. 146:1029-1039 (1995).

Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim et al., Nature 362:841-844 (1993); Warren et al., J. Clin. Invest. 95:1789-1797 (1995); Borgström et al., Cancer Res. 56:4032-4039 (1996); Melnyk et al., Cancer Res. 56:921-924 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders. Adamis et al., Arch. Ophthalmol. 114:66-71 (1996). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of tumors and various intraocular neovascular disorders. Such antibodies are described, for example, in EP 817,648 published January 14, 1998; and in WO98/45331 and WO98/45332, both published October 15, 1998.

Cancer is one of the most deadly threats to human health. In the U.S. alone, cancer affects nearly 1.3 million new patients each year, and is the second leading cause of death after cardiovascular disease, accounting for approximately 1 in 4 deaths. Solid tumors are responsible for most of those deaths. Although there have been significant advances in the medical treatment of certain cancers, the overall 5-year survival rate for all cancers has improved only by about 10% in the past 20 years. Despite the significant advancement in the treatment of cancer, improved therapies are still being sought.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention provides combination therapies for treating a pathological condition, such as cancer, wherein an Axl antagonist is combined with a VEGF antagonist.

In one aspect, the invention provides methods of treating a cancer in an individual, comprising administering to the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

In another aspect, the invention provides methods of inhibiting metastasis of cancer in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

In another aspect, the invention provides methods of inhibiting angiogenesis in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

In another aspect, the invention provides methods of inhibiting cell proliferation in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

In certain embodiments, the VEGF antagonist is a compound that interferes with the binding of VEGF to a cellular receptor. Examples of such VEGF blocking antagonists include, but are not limited to, soluble VEGF receptors, apatmers or peptibodies that are specific to VEGF, and anti-VEGF antibodies. In one embodiment, the anti-VEGF antibody is bevacizumab.

In certain embodiments, when used in combination, bevacizumab is administered in the range from about 0.05 mg/kg to about 15 mg/kg. In one embodiment, one or more doses of about 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 3.0 mg/kg, 4.0 mg/kg, 5.0 mg/kg, 6.0 mg/kg, 7.0 mg/kg, 7.5 mg/kg, 8.0 mg/kg, 9.0 mg/kg, 10 mg/kg or 15 mg/kg (or any combination thereof) may be administered to the subject. Such doses may be administered intermittently, *e.g.* every day, every three days, every week or every two to three weeks.

Examples of Axl antagonists include, but are not limited to, soluble Axl receptors, soluble Axl ligand variants, apatmers or peptibodies that are specific to Axl or Axl ligands, Axl small molecules, anti-Axl antibodies and anti-Axl ligand antibodies. In some embodiment, the Axl antagonist is an anti-Axl antibody. In some embodiments, the Axl antagonist is not warfarin.

In some embodiments, the anti-Axl antibody comprises a heavy chain variable regions comprising sequence EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPYR GYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSSVG YAMDYWGQGTLV (SEQ ID NO:1) and a light chain variable region comprising sequence DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKR (SEQ ID NO:2).

In some embodiments, the Axl antagonist is R428 (Rigel; Holland et al, Cancer Research 70, 1544 (2010)) or PF02341066.

In certain embodiments, the method further comprises administering an EGFR antagonist. In some embodiments, the EGFR antagonist is erlotinib. In certain

embodiments, erlotinib may be administered at a dose of 150 mg, each day, or a dose of 100 mg, each day.

Methods of the invention can be used to affect any suitable pathological state. For example, methods of the invention can be used for treating different cancers, both solid and liquid tumors and soft-tissue tumors alike. Non-limiting examples of cancers amendable to the treatment of the invention include breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer (such as hepatocellular carcinoma), pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, gastric cancer, mesothelioma, and multiple myeloma. In certain aspects, the cancers are metastatic. In other aspects, the cancers are non-metastatic.

In some embodiment, an anti-Axl antibody and anti-VEGF antibody (such as bevacizumab) are used in combination therapies of cancers such as non-small cell lung carcinoma.

Depending on the specific indication to be treated, the combination therapy of the invention can be combined with additional therapeutic agents, such as chemotherapeutic agents, or additional therapies such as radiotherapy or surgery. Many known chemotherapeutic agents can be used in the combination therapy of the invention. In certain embodiments, the combination therapy of the invention can be combined with more than one chemotherapeutic agent. In certain embodiments, those chemotherapeutic agents that are standard for the treatment of the specific indications will be used. In another embodiment, dosage or frequency of each therapeutic agent to be used in the combination is the same as, or less than, the dosage or frequency of the corresponding agent when used without the other agent(s).

In certain embodiments, the cancer displays Axl expression (such as Axl overexpression), amplification, or activation.

In certain embodiments, the individual is diagnosed with an Axl-expressing cancer.

The invention also provides a method of inhibiting constitutive Axl activation comprising contacting a cell with an antibody (or antigen binding fragment thereof) that down-regulates Axl expression (decreases Axl expression, e.g., on a cell surface, such as a tumor cell surface and/or decreases total Axl expression in a cell, such as a tumor cell).

The invention also provides methods of treating cancer in an individual comprising administering to the individual an anti-Axl antibody that down-regulates Axl expression

(decreases Axl expression, e.g., on a cell surface, such as a tumor cell surface and/or decreases total Axl expression in a cell, such as a tumor cell).

The invention also provides a method of blocking ligand binding to Axl comprising contacting a cell that expresses Axl with an antibody or antigen-binding fragment of thereof that binds Axl and inhibits ligand binding to Axl wherein said antibody recognizes an epitope on human Axl but not mouse Axl. The monoclonal antibody may be a chimeric antibody, a humanized antibody, a single-chain antibody or a bispecific antibody, for example. In some embodiments the antibody binds to the same epitope as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8. In some embodiments, the antibody inhibits ligand binding to Axl. In some embodiments, the antibody down-regulates Axl expression. In some embodiments, the antibody recognizes a denatured epitope on Axl, as may be detected by Western blot. In some embodiments, the antibody has an IC_{50} of at least about 100 ng/ml. In some embodiments, the antibody inhibits Gas6-induced phosphorylation of Axl.

In one aspect, the invention provides the antibody produced by hybridoma cell line 3G9.19.7 having American Tissue Type Culture (ATCC) No. _____, deposited on _____.

In one aspect, the invention provides an isolated antibody comprising heavy and/or light chain variable domain(s) of the antibody produced by hybridoma cell line anti-Axl 3G9.19.7 having American Tissue Type Culture (ATCC) No. _____, wherein said isolated antibody specifically binds human Axl.

In one aspect, the invention provides an isolated antibody comprising at least one (at least 2, at least 3, at least 4, at least 5, and/or 6) hypervariable sequence(s) (HVR(s)) comprising a sequence selected from the group consisting of HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and/or HVR-H3 of the antibody produced by hybridoma cell line anti-Axl 3G9.19.7 having American Tissue Type Culture (ATCC) No. _____, wherein said isolated antibody specifically binds human Axl.

In one aspect, the invention provides an isolated antibody that binds to the same epitope on human Axl as the antibody produced by hybridoma cell line anti-Axl 3G9.19.7 having American Tissue Type Culture (ATCC) No. _____.

In one aspect, the invention provides an isolated antibody that competes with the antibody produced by hybridoma cell line anti-Axl 3G9.19.7 having American Tissue Type Culture (ATCC) No. _____ for binding to human Axl.

The invention also provides a method of down-regulating Axl expression in a cell comprising contacting said cell with an antibody that causes a reduction in Axl expression in said cell. The antibody may be a monoclonal antibody. The antibody may be a chimeric antibody, a humanized antibody, a single-chain antibody or a bispecific antibody, for example. In some embodiments the antibody binds to the same epitope as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8. In some embodiments, the antibody inhibits ligand binding to Axl. In some embodiments, the antibody recognizes a denatured epitope on Axl, as may be detected by Western blot. In some embodiments, the antibody has an IC_{50} of at least about 100 ng/ml. In some embodiments, the antibody inhibits Gas6-induced phosphorylation of Axl.

The invention also provides a method of detecting Axl in a sample comprising contacting said sample with an antibody that specifically binds to an epitope on Axl and does not cross react with other proteins in said sample. In some embodiments the antibody binds to the same epitope as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8. In some embodiments, the antibody recognizes a denatured epitope on Axl, as may be detected by Western blot. In some embodiments, the antibody has an IC_{50} of at least about 100 ng/ml.

The invention also provides a method of diagnosing a patient having a condition marked by expression (in some embodiments, over-expression) of Axl comprising obtaining a sample from said patient and assaying said sample for expression of Axl, wherein said assay comprises the use of an antibody that binds to the same epitope as an antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8, or an antigen-binding fragment thereof. In some embodiments the antibody is 3G9, 8B5, 12A11, 4F8, an antigen-binding fragment thereof, or a combination thereof. The antibody may be a monoclonal antibody, a chimeric antibody, a humanized antibody, a single chain antibody or an antigen-binding fragment thereof. In some embodiments, the antibody is conjugated to a detectable label, such as, but not limited to a radioactive isotope, a fluorescent compound or an enzymatic label.

The invention also provides a method of treating cancer in a patient comprising: (a) detecting over-expression of Axl in a sample from said patient using a first antibody; and (b) administering to said patient a composition comprising a second antibody and a pharmaceutically acceptable carrier, wherein said first antibody specifically binds to an epitope on human Axl, and said second antibody inhibits ligand binding to Axl,

phosphorylation of Axl, or down-regulates Axl expression. The cancer may be a cancer selected from the group consisting of myelogenous leukemia, lung cancer (e.g., non-small cell lung carcinoma (NSCLC)), gastric cancer, breast cancer, prostate cancer, renal cell cancer, pancreatic cancer and glioblastoma. In some embodiments, the first antibody binds to the same epitope of Axl as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8, or an antigen-binding fragment thereof. In some embodiments, the second antibody binds to the same epitope of Axl as a monoclonal antibody selected from the group consisting of 3G9, 8B5, and 12A11, or an antigen-binding fragment thereof. In some embodiments, the first antibody and the second antibodies are monoclonal antibodies, chimeric antibodies, humanized antibodies, single chain antibodies or antigen-binding fragments thereof. In some embodiments, the first antibody and/or the second antibody are conjugated to a cytotoxic agent, such as, but not limited to a chemotherapeutic agent, a toxin and a radioactive isotope. In some embodiments, the method further comprises administration of an anti-VEGF antibody, before, after or simultaneously with administration of the antibody that binds Axl.

The invention also provides a method of inhibiting angiogenesis in cells that over-express Axl comprising administering to said cells an antibody that specifically binds to an epitope on human Axl and inhibits ligand binding to Axl, phosphorylation of Axl, or down-regulates Axl expression. The cancer may be a cancer selected from the group consisting of myelogenous leukemia, lung cancer (e.g., non-small cell lung carcinoma (NSCLC)), gastric cancer, breast cancer, prostate cancer, renal cell cancer, pancreatic cancer and glioblastoma. In some embodiments, the antibody binds to the same epitope as an antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8, or an antigen-binding fragment thereof. In some embodiments, the antibody is a monoclonal antibody, a chimeric antibody, a humanized antibody, a single chain antibody or an antigen-binding fragment thereof. In some embodiments, the antibody is conjugated to a cytotoxic agent, such as, but not limited to a chemotherapeutic agent, a toxin and a radioactive isotope. In some embodiments, the method further comprises administration of an anti-VEGF antibody, before, after or simultaneously with administration of the antibody that binds Axl. The anti-Axl antibody and the anti-VEGF antibody may be in the form of a multi-specific antibody, such as, but not limited to a bispecific antibody.

In one aspect, the invention provides anti-Axl antibodies and methods of using the same.

In some embodiments, the invention provides a monoclonal antibody that specifically binds to an epitope on human Axl but not mouse Axl, or an antigen-binding fragment of such antibody. The monoclonal antibody may be a chimeric antibody, a humanized antibody, a single-chain antibody or a bispecific antibody, for example. In some embodiments the antibody binds to the same epitope as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8. In some embodiments, the antibody inhibits ligand binding to Axl. In some embodiments, the antibody down-regulates Axl expression. In some embodiments, the antibody recognizes a denatured epitope on Axl, as may be detected by Western blot. In some embodiments, the antibody has an IC_{50} of at least about 100 ng/ml. In some embodiments, the antibody inhibits Gas6-induced phosphorylation of Axl.

The invention also provides a pharmaceutical composition comprising a monoclonal antibody as described in the previous paragraph and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises an anti-VEGF antibody.

The invention further provides an article of manufacture comprising a container, a composition contained therein, and a package insert, wherein said composition comprises a monoclonal antibody that binds to the same epitope of Axl as a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8, or an antigen-binding fragment thereof, and said package insert comprises instructions for use of said composition. In some embodiments, the antibody is a monoclonal antibody selected from the group consisting of 3G9, 8B5, 12A11, and 4F8.

In one aspect, a polynucleotide encoding any of the above antibodies is provided. In one embodiment, a vector comprising the polynucleotide is provided. In one embodiment, a host cell comprising the vector is provided. In one embodiment, the host cell is eukaryotic. In one embodiment, the host cell is a CHO cell. In one embodiment, a method of making an anti-Axl antibody is provided, wherein the method comprises culturing the host cell under conditions suitable for expression of the polynucleotide encoding the antibody, and isolating the antibody.

In one, any of the above antibodies is a monoclonal antibody. In one embodiment, the antibody is an antibody fragment selected from a Fab, Fab'-SH, Fv, scFv, or $(Fab')_2$ fragment. In one embodiment, the antibody is humanized. In one embodiment, the antibody is human.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows Axl expression in cancer cell lines. Panel A: Axl mRNA levels in cancer cell lines: mRNA levels of Axl in cancer cell lines were compiled from several microarray experiments on Affymetrix GeneChip Human Genome U133 Plus 2.0 array, representing 244 samples (sample number for each tumor type is indicated). Probe set 202686_s_at was chosen to represent the expression of Axl. Panel B: Western blotting analysis of Axl and Gas6 in selected cancer cell lines. U87 & A172: glioma; DU145: prostate; Pan0327: pancreas; RKO: colon; MDA-MB-231: breast; Calu1, A549, H1299 & HCC366: NSCLC; SHSY5Y: neuroblastoma; Panel C: Phosphorylated Axl in cancer cell lines. Cells were starved overnight in serum free medium and treated with Gas6 (200 ng/ml) for 30 minutes. Phospho-Axl was measured by ELISA.

Figure 2 shows Axl knockdown in cancer cell lines reduces cell viability & attenuates migration. Panel A: Inducible Axl knockdown in cancer cell lines (Western blotting analysis). AxlshRNA expression was induced by treatment of cells with doxycycline for 72 hrs. Panel B: Axl knockdown abolished down-stream signaling. Cells were serum-starved for 24 hrs, pre-incubated with 100 ng/ml AxlFc for 2 hrs, and treated with Gas6 for 30 minutes. Western blotting analysis was performed for phospho-Akt and Akt; Panel C. Effect of Axl knockdown on cell viability. Cells were cultured either in 10 % serum (left), or in 1% serum (H1299, right) with or without Gas6. AxlshRNA was induced by treatment of cell with Dox for 72-hrs, and the cell viability was measured using CellTiter Glo assay (n=8). Panel D: Axl knockdown attenuates cell migration. The migration assay was performed as described. Error bars represent standard deviation (n=8).

Figure 3 shows that Axl knockdown reduces tumor growth in xenograft models. The graphs on the left show tumor growth curves and the Western blotting analysis of Axl expression in tumor lysates is shown on the right for each. Panel A: A549AxlshRNA. Panel B: H1299AxlshRNA. Panel C: MDA-MB-231AxlshRNA. The graphs are representative of three independent experiments. Error bars represent standard error of the mean (n=10 for each group in each experiment).

Figure 4 shows that Axl knockdown inhibits metastasis of MDA-MB-231 breast cancer cells to the lung. Panel A: Schematic presentation of the experimental design. Panel B: Bioluminescence imaging of the lung 5 weeks after removal of the primary tumors. Panel C: H&E staining of the lung 5 weeks after removal of the primary tumors. The circle

indicates the tumor mass. This figure is representative of three independent experiments (n=5 for each group in each experiment).

Figure 5 shows that Axl knockdown in HUVECs impairs endothelial tubule formation. Panel A: FACS analysis of Axl expression on HUVEC cell surface 96-hour post transfection with control (GL2) or Axl siRNA. B. Axl siRNAs reduce endothethial tube formation. Left panel: Tubes were viewed and photographed under ImageXpressMICRO imaging system. Right panel: quantification of total tube formation by measuring tube length using MetaXpress software.

Figure 6 shows the gene expression profile of HUVECs with Axl knockdown. Panel A: List of most up- and down-regulated genes following Axl knockdown. HUVECs were transfected with control or Axl siRNA and RNA was prepared 72-hr post transfection. Microarray and data analysis were described in Materials and Methods. Fold of change over control is shown. Definition for the genes in the list: MYCN: N-Myc; HLX: H2.0-like homeobox; GAS7: Growth Arrest Specific 7; HDAC9: histone deacetylase 9; E2F1: E2F transcription factor 1; CXCR4: chemokine (C-X-C motif) receptor 4; PMCH: promelanin-concentrating hormone; ANG-2: angiopoietin 2; IFI44L: interferon-induced protein 44-like; GJA4: gap junction protein, alpha 4; IFIT1: interferon-induced protein with tetratricopeptide repeats 1; SCG5: secretogranin V, CYTL1: cytokine-like 1; DPP4: dipeptidylpeptidase 4; DKK3: Dickkopf 3. Panel B: Quantitative RT–PCR for DKK3 and Ang-2 mRNA levels following Axl knockdown. Panel C: Effect of DKK3 or Ang-2 siRNA knockdown on endothelial tube formation. Left panel: Tubes were viewed and photographed under ImageXpressMICRO imaging system (Left). Quantification of total tube formation by measuring tube length using MetaXpress software (Right).

Figure 7 shows that Axl knockdown has an additive effect with Anti-VEGF. HUVEC cell were transfected with control, Axl or DKK3 siRNAs with or without anti-VEGF and cocultured with PASMCs, Tube formation were analyzed 3 days after co-culture by staining with FITC conjugated anti-CD31 antibodies. Upper panels: Tubes were viewed and photographed under ImageXpressMICRO imaging system. Lower panel: quantification of total tube formation by measuring tube length using MetaXpress software.

Figure 8 shows a characterization of Axl mAbs. Panel A: Axl mAbs inhibit Baf3Axl cell growth. Baf3Axl cells were grown in medium containing 200 ng/ml Gas6, and treated with Axl mAbs at indicated concentrations for 48 hrs. Cell viability was measure by CellTiter Glo assay. Panel B: mAbs 3G9 and 8B5 block Gas6 binding to Axl (B1:

ELISA & B2: FACS). Panel C: Down regulation of Axl expression in A549 (Western blotting analysis) & Gas6-induced phosphorylation of Axl in H1299 cells (ELISA) by mAbs. Panel D: Epitope mapping. Various portions of Axl ECD were *in vitro* transcribed and translated and were used as antigen in ELISA.

Figure 9 shows that Axl mAbs inhibit A549 xenograft tumor growth. Panel A: Tumor growth curve. Monoclonal antibodies were administrated IP at 30 mg/kg, twice a week, starting when the mean tumor size reached 100 mm3. The first (day 0) and last dose (day 40) of mAbs administration were indicated by arrows. The graph is representative of three independent experiments. Error bars represent standard error of the mean (n=10 for each group in each experiment). Panel B: Axl mAbs down-regulate Axl expression. Mice were treated with mAbs at 30 mg/kg and tumors excised at the indicated time points. Cell lysates from tumors were used in Western blot analysis for Axl expression. Panel C. Ki67 staining of tumor samples 72-hr after administration of Axl mAb12A11.

Figure 10 shows Axl knockdown by siRNA in HUVECs. HUVECs were transfected with Luciferase control (GL2) siRNA or with Axl-specific (D1-4, Axl2 & 4) siRNAs. 48-hr post transfection Axl expression on cell surface was analyzed by FACS. Panel A: FACS profiles of Axl expression. Bar graph represents quantification of FACS. Panel B: siRNA sequences (SEQ ID NO:3-16). Panel C: Effect of Axl knockdown on HUVEC growth. Cell viability was determined by CellTiter Glo Assay 72-hour post transfection.

Figure 11 shows that knockdown of DKK3 or Ang2 by RNAi does not affect Axl protein levels in HUVECs. HUVECs were transfected with control (GL2), Axl, Dkk3 or Ang-2 siRNAs. 48-hrs post transfection, protein levels of Axl were analyzed by FACS and protein levels of DKK3 and Ang-2 in the medium were determined by ELISA. Expressed as fold changes over control.

Figure 12 shows a cell-based assay for screening of Axl monoclonal antibodies (mAbs). Panel A: Stable expression of Axl in Baf3 cells (Western blotting). Panel B: Gas6-dependent growth of Baf3Axl cells. Panel C: Inhibition of Gas6-dependent growth of Baf3Axl cells by AxlFc. Panel D: Screen for Axl mAbs that block Gas6-dependent Baf3Axl growth. Baf3Axl cells were grown in the presence of 200 ng/ml Gas6 (C & D) with indicated dose of AxlFc (C), or hybridoma supernatants (D) for 72 hrs. Cell viability was determined by CellTiter Glo. Error bars represent standard deviation (n=8).

Figure 13 shows cross reactivity of Axl mAbs. Panel A: Axl mAbs do not cross react with murine Axl. Panel B: Axl mAbs do not cross react with Mer and Tyro-3. ELISA

performed using human AxlFc, murine AxlFc, and human MerFc and Tyro-3Fc as antigens for antibody binding.

Figure 14: Characterization of Axl mAb YW327.6S2. A. Affinity measurement of YW327.6S2 using BIAcore. Association rates (k_{on}) and dissociation rates (k_{off}) were calculated using the one-to-one Langmuir binding model. The equilibrium dissociation constant (Kd) was derived as the k_{on}/k_{off} ratio. B. Cross reactivity of YW327.6S2. YW327.6S2 cross reacts with murine and cynomolgus Axl but not with Tyro3 or Mer. Plates were coated with anti-human IgG Fc, and then incubated with human Axl, Mer, Tyro3 Fcs, mouse or cynomolgus AxlFcs. After washing, isotype control antibody or YW327.6S2 was added and followed by HRP conjugated anti-human IgG. C. YW327.6S2 blocks Gas6 binding to Axl. Upper panel: ELISA. Plates were coated with anti-human IgG Fc and incubated with human Axl-Fc. After washing, Gas6 was added with or without antibodies. Binding of Gas6 was detected by biotylated anti-Gas6 antibody and streptavidin-HRP conjugate. Lower panel: FACS. HUVECs were harvested and treated with YW327.6S2 or control antibody and then incubated with Gas6 for 30 minutes on ice. Binding of Gas6 to the cell surface was detected by biotylated anti-Gas6 antibody and streptavidin-PE conjugate. D. YW327.6S2 down-regulates Axl expression. A549 cells were incubated with 1 μg/ml YW327.6S2 for indicated time, and cell surface Axl expression was determined by FACS (upper panel), and total protein expression by Western Blotting analysis (lower panel). E. YW327.6S2 inhibits Gas6-induced Axl phosphorylation & signaling. H1299 cells were cultured in serum free medium over night, pre-incubated with YW327.6S2 for 4 hrs, and treated with Gas6 for 30 minutes. Phosphorylated Axl was measured by ELISA (upper panel), and phosphorylated Akt by Western Blotting analysis (lower panel). F. YW327.6S2 inhibits Baf3Axl cell growth. Baf3Axl cells were grown in medium containing 200 ng/ml Gas6, and treated with YW327.6S2 at indicated concentrations for 72 hrs. Cell viability was measured by CellTiter Glo assay.

Figure 15: YW327.6S2 attenuates A549 xenograft tumor growth and enhances the effect of anti-VEGF. A. Tumor growth curve. mAbs were administrated IP at 10 mg/kg (YW327.6S2 and isotype control antibody) or 1 mg/kg (anti-VEGF), twice a week, starting when the mean tumor size reached 100 mm^3 (day 0). Error bars represent standard error of the mean (n=10 for each group in each experiment). p=0.0003 (YW327.6S2 versus control), $p=10^{-11}$ (YW327.6S2 versus combination). B. Kaplan-Meier curve of various treatment groups. Mice were removed from the study when their tumor size reached 800 mm^3, and the

animals remaining in each group (% remaining) were plotted. C. 12A11 enhances the effect of anti-VEGF. 12A11 and anti-VEGF was administrated IP at 30 mg/kg and 1 mg/kg, respectively, twice a week, starting when the mean tumor size reached 100 mm^3 (day 0). Error bars represent standard error of the mean (n=10 for each group in each experiment). p=0.006 (12A11 vs control); p=0.0001 (12A11 vs combination). D. YW327.6S2 down-regulates Axl expression. Mice were treated with YW327.6S2 at 10 mg/kg and tumors excised at the indicated time points. Cell lysates from tumors were analyzed by Western blot for Axl expression. E. YW327.6S2 induces apoptosis. Tumors treated with control or YW327.6S2 for 2 weeks were excised and CC3 IHC was performed to measure apoptosis. F. YW327.6S2 enhances the effect of anti-VEGF in reducing intra-tumoral vascular density. Tumors from mice treated as above in D were excised at 0 and 72 hr post dosing and tumor vasculature was visualized by staining with MECA32 immunohistochemistry and quantified by image analysis (expressed as microns square). Student's t test was performed for each pair ($p<0.05$ for control vs combination).

Figure 16: YW327.6S2 enhances the anti-tumor effect of erlotinib and chemotherapy in A549 xenograft model. A. YW327.6S2 enhances the effect of erlotinib. Antibody administration was the same as in Fig. 2. Erlotinib was administered by oral gavage at 100 mg/kg/day. n=10 for each group. $p=1.7X10^{-9}$ (YW327.6S2 versus control), $p=2.3X10^{-10}$ (YW327.6S2 versus combination). B. YW327.6S2 enhances chemotherapy. Antibody administration was the same as in Fig.2. Paclitaxel and carboplatin were administrated subcutaneously at 6.25 mg/kg/day for 5 days, and 100 mg/kg for a single dose at the beginning of the treatment (day 0), respectively. n=10 for each group. $p=3X10^{-5}$ (YW327.6S2 versus control), $p=10^{-9}$ (chemotherapy versus control), $p=10^{-5}$ (combination versus chemotherapy alone).

Figure 17: YW327.6S2 attenuates MDA-MB-231 xenograft tumor growth by modulating tumor stromal functions. A & B. YW327.6S2 but not 12A11 reduces MDA-MB-231 tumor growth and enhances the effect of anti-VEGF. mAbs were administrated IP at 20 mg/kg (YW327.6S2 and isotype control antibody), 30 mg/kg (12A11) and 2 mg/kg (anti-VEGF), twice a week, starting when the mean tumor size reached 100 mm^3 (day 0). Error bars represent standard error of the mean (n=10 for each group in each experiment). $p=8.5X10^{-6}$ (YW327.6S2 versus control), $p=2.8X10^{-8}$ (YW327.6S2 versus combination), p=0.05 (12A11 vs control), p= 0.145 (anti-VEGF vs combination). C & D. YW327.6S2 down-regulates Axl expression. Mice bearing MDA-MB231 xenograft tumors (average size

500 mm^3) were treated with mAbs at 20 mg/kg and tumors excised at the indicated time points. Cell lysates from tumors were used in Western blot analysis for Axl expression. E. YW327.6S2 reduces the density of tumor-associated vasculature. Tumors from mice treated as above in C were excised at 0 hr and 1 week post dosing and tumor vasculature was visualized by staining with MECA32 immunohistochemistry and quantified by image analysis (expressed as microns square). Student's t test was performed for each pair ($p<0.05$ for YW327.6S2 vs control, anti-VEGF vs control, and anti-VEGF vs combination). F. Axl is highly expressed in infiltrating macrophages of primary human breast cancer. Immunohistochemistry was used to examine 79 primary tumors. 21% of these tumors express high levels of Axl in the infiltrating macrophages. Macrophages were identified by staining with anti-CD68, and expression of Axl on macrophages was determined by anti-Axl/CD68 dual IHC. Serial sections were used. G. YW327.6S2 inhibits inflammatory cytokine/chemokine secretion from tumor-associated macrophages. Mice bearing MDA-MB231 xenograft tumors (average size 500 mm^3) were treated with control antibody, YW327.6S2 or 12A11 at 20 mg/kg and tumors excised after I week treatment. Tumor associated macrophages were isolated by sorting for F4/80 positive cells and cultured overnight. Cytokines and chemokines secreted into the medium were measured using the Bio-Plex mouse cytokine assay kit.

Figure 18: YW327.6S2 reduces metastasis of MDA-MB-231 breast cancer cells to the bone. A. Bioluminescence imaging 4 weeks after tail vein injection of tumor cells. Mice were injected intraperitoneally (i.p.) with 200 μL of 25 mg/ml D-luciferin (Invitrogen) in PBS and were anesthetized during imaging using isoflurane via nose cone. Bioluminescence images were acquired on the Photon Imager (Biospace Lab, Paris, France) that has an intensified charge-coupled device camera. B. H & E staining of the tibia sections. Tissues were collected at the end of the experiments and fixed in 4% formaldehyde, sectioned and stained with H & E. The circle in the upper panel shows tumor cells invading the bone. The lower panel shows details of neoplastic cells in the bone marrow.

Figure 19: An exemplary human Axl sequence of RefSeq NM_001699 (SEQ ID NO:17).

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The invention provides methods using Axl antagonists and VEGF antagonists in combination therapy to treat a pathological condition, such as cancer.

The invention also provides isolated antibodies that bind to Axl and methods of using the same, *e.g.*, for the diagnosis or treatment of cancers such as, for example, myelogenous leukemia, lung cancer (e.g., non-small cell lung carcinoma (NSCLC)), gastric cancer, breast cancer, prostate cancer, renal cell cancer, pancreatic cancer and glioblastoma.

I. 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)); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R. I. Freshney), ed., 1987); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-8) J. Wiley and Sons; *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J. E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); *Immunobiology* (C. A. Janeway and P. Travers, 1997); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V. T. DeVita *et al.*, eds., J.B. Lippincott Company, 1993).

II. DEFINITIONS

For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice

versa. In the event that any definition set forth below conflicts with any document incorporated herein by reference, the definition set forth below shall control.

A polypeptide "variant" means a biologically active polypeptide having at least about 80% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the polypeptide. Ordinarily, a variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the native sequence polypeptide.

A "native sequence" polypeptide comprises a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally-occurring polypeptide from any mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring truncated or secreted forms of the polypeptide (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide.

An "anti-Axl antibody" is an antibody that binds to Axl with sufficient affinity and specificity. The antibody selected will normally have a sufficiently strong binding affinity for Axl, for example, the antibody may bind human Axl with a K_d value of between 100 nM-1 pM. In certain embodiments, an antibody that binds to Axl has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, or $\leq$ 0.1 nM. Antibody affinities may be determined by a surface plasmon resonance based assay (such as the BIAcore assay as described in PCT Application Publication No. WO2005/012359); enzyme-linked immunoabsorbent assay (ELISA); and competition assays (e.g. RIA's), for example. Preferably, the extent of binding of an anti-Axl antibody to an unrelated, non-Axl protein is less than about 10% of the binding of the antibody to Axl as measured, *e.g.*, by a radioimmunoassay (RIA). In certain embodiments, the anti-Axl antibody can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein Axl activity is involved. Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody.

An "Axl antagonist" (interchangeably termed "Axl inhibitor") is an agent that interferes with Axl activation or function. Examples of Axl inhibitors include Axl antibodies; Axl ligand antibodies; small molecule Axl antagonists; Axl tyrosine kinase inhibitors; antisense and inhibitory RNA (e.g., shRNA or siRNA) molecules (see, for example, WO2004/87207). Preferably, the Axl inhibitor is an antibody or small molecule which binds to Axl. In a particular embodiment, a Axl inhibitor has a binding affinity (dissociation constant) to Axl of about 1,000 nM or less. In another embodiment, a Axl inhibitor has a binding affinity to Axl of about 100 nM or less. In another embodiment, a Axl inhibitor has a binding affinity to Axl of about 50 nM or less. In a particular embodiment, a Axl inhibitor is covalently bound to Axl. In a particular embodiment, a Axl inhibitor inhibits Axl signaling with an IC50 of 1,000 nM or less. In another embodiment, a Axl inhibitor inhibits Axl signaling with an IC50 of 500 nM or less. In another embodiment, a Axl inhibitor inhibits Axl signaling with an IC50 of 50 nM or less.

"Axl activation" refers to activation, or phosphorylation, of the Axl receptor. Generally, Axl activation results in signal transduction (*e.g.* that caused by an intracellular kinase domain of a Axl receptor phosphorylating tyrosine residues in Axl or a substrate polypeptide). Axl activation may be mediated by Axl ligand (e.g., Gas6) binding to a Axl receptor of interest. Axl ligand binding to Axl may activate a kinase domain of Axl and thereby result in phosphorylation of tyrosine residues in the Axl and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s).

The term "ligand-independent" as used herein, as for example applied to receptor signaling activity, refers to signaling activity that is not dependent on the presence of a ligand. For example, EGFR signaling may result from dimerization with other members of the HER family such as HER2. A receptor having ligand-independent kinase activity will not necessarily preclude the binding of ligand to that receptor to produce additional activation of the kinase activity.

The term "constitutive" as used herein, as for example applied to receptor kinase activity, refers to continuous signaling activity of a receptor that is not dependent on the presence of a ligand or other activating molecules. For example, EGFR variant III (EGFRvIII) which is commonly found in glioblastoma multiforme has deleted much of its extracellular domain. Although ligands are unable to bind EGFRvIII it is nevertheless continuously active and is associated with abnormal proliferation and survival. Depending on the nature of the receptor, all of the activity may be constitutive or the activity of the

receptor may be further activated by the binding of other molecules (e. g. ligands). Cellular events that lead to activation of receptors are well known among those of ordinary skill in the art. For example, activation may include oligomerization, e.g., dimerization, trimerization, etc., into higher order receptor complexes. Complexes may comprise a single species of protein, i.e., a homomeric complex. Alternatively, complexes may comprise at least two different protein species, i.e., a heteromeric complex. Complex formation may be caused by, for example, overexpression of normal or mutant forms of receptor on the surface of a cell. Complex formation may also be caused by a specific mutation or mutations in a receptor.

The term "VEGF" or "VEGF-A" is used to refer to the 165-amino acid human vascular endothelial cell growth factor and related 121-, 189-, and 206- amino acid human vascular endothelial cell growth factors, as described by Leung et al. *Science*, 246:1306 (1989), and Houck et al. *Mol. Endocrin.*, 5:1806 (1991), together with the naturally occurring allelic and processed forms thereof. VEGF-A is part of a gene family including VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. VEGF-A primarily binds to two high affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), the latter being the major transmitter of vascular endothelial cell mitogenic signals of VEGF-A. Additionally, neuropilin-1 has been identified as a receptor for heparin-binding VEGF-A isoforms, and may play a role in vascular development. The term "VEGF" or "VEGF-A" also refers to VEGFs from non-human species such as mouse, rat, or primate. Sometimes the VEGF from a specific species is indicated by terms such as hVEGF for human VEGF or mVEGF for murine VEGF. The term "VEGF" is also used to refer to truncated forms or fragments of the polypeptide comprising amino acids 8 to 109 or 1 to 109 of the 165-amino acid human vascular endothelial cell growth factor. Reference to any such forms of VEGF may be identified in the present application, e.g., by "VEGF (8-109)," "VEGF (1-109)" or "$VEGF_{165}$." The amino acid positions for a "truncated" native VEGF are numbered as indicated in the native VEGF sequence. For example, amino acid position 17 (methionine) in truncated native VEGF is also position 17 (methionine) in native VEGF. The truncated native VEGF has binding affinity for the KDR and Flt-1 receptors comparable to native VEGF.

The term "VEGF variant" as used herein refers to a VEGF polypeptide which includes one or more amino acid mutations in the native VEGF sequence. Optionally, the one or more amino acid mutations include amino acid substitution(s). For purposes of

shorthand designation of VEGF variants described herein, it is noted that numbers refer to the amino acid residue position along the amino acid sequence of the putative native VEGF (provided in Leung et al., *supra* and Houck et al., *supra.*).

"VEGF biological activity" includes binding to any VEGF receptor or any VEGF signaling activity such as regulation of both normal and abnormal angiogenesis and vasculogenesis (Ferrara and Davis-Smyth (1997) *Endocrine Rev.* 18:4-25; Ferrara (1999) *J. Mol. Med.* 77:527-543); promoting embryonic vasculogenesis and angiogenesis (Carmeliet et al. (1996) *Nature* 380:435-439; Ferrara et al. (1996) *Nature* 380:439-442); and modulating the cyclical blood vessel proliferation in the female reproductive tract and for bone growth and cartilage formation (Ferrara et al. (1998) *Nature Med.* 4:336-340; Gerber et al. (1999) *Nature Med.* 5:623-628). In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx (Ferrara and Davis-Smyth (1997), *supra* and Cebe-Suarez et al. *Cell. Mol. Life Sci.* 63:601-615 (2006)). Moreover, recent studies have reported mitogenic effects of VEGF on a few non-endothelial cell types, such as retinal pigment epithelial cells, pancreatic duct cells, and Schwann cells. Guerrin et al. (1995) *J. Cell Physiol.* 164:385-394; Oberg-Welsh et al. (1997) *Mol. Cell. Endocrinol.* 126:125-132; Sondell et al. (1999) *J. Neurosci.* 19:5731-5740.

An "angiogenesis inhibitor" or "anti-angiogenesis agent" refers to a small molecular weight substance, a polynucleotide, 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. It should be understood that the anti-angiogenesis agent includes those agents that bind and block the angiogenic activity of the angiogenic factor or its receptor. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF-A or to the VEGF-A receptor (e.g., KDR receptor or Flt-1 receptor), anti-PDGFR inhibitors such as GLEEVEC® (Imatinib Mesylate). Anti-angiogenesis 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* 5:1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 2 listing known antiangiogenic

factors); and Sato. *Int. J. Clin. Oncol.*, 8:200-206 (2003) (e.g., Table 1 lists anti-angiogenic agents used in clinical trials.

A "VEGF antagonist" refers to a molecule (peptidyl or non-peptidyl) capable of neutralizing, blocking, inhibiting, abrogating, reducing, or interfering with VEGF activities including its binding to one or more VEGF receptors. In certain embodiments, the VEGF antagonist reduces or inhibits, by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more, the expression level or biological activity of VEGF. In one embodiment, the VEGF inhibited by the VEGF antagonist is VEGF (8-109), VEGF (1-109), or $VEGF_{165}$. VEGF antagonists useful in the methods of the invention include peptidyl or non-peptidyl compounds that specifically bind VEGF, such as anti-VEGF antibodies and antigen-binding fragments thereof, polypeptides, or fragments thereof that specifically bind to VEGF, and receptor molecules and derivatives that bind specifically to VEGF thereby sequestering its binding to one or more receptors (e.g., soluble VEGF receptor proteins, or VEGF binding fragments thereof, or chimeric VEGF receptor proteins); antisense nucleobase oligomers complementary to at least a fragment of a nucleic acid molecule encoding a VEGF polypeptide; small RNAs complementary to at least a fragment of a nucleic acid molecule encoding a VEGF polypeptide; ribozymes that target VEGF; peptibodies to VEGF; and VEGF aptamers.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. The antibody selected will normally have a sufficiently strong binding affinity for VEGF, for example, the antibody may bind hVEGF with a K_d value of between 100 nM-1 pM. Antibody affinities may be determined by a surface plasmon resonance based assay (such as the BIAcore assay as described in PCT Application Publication No. WO2005/012359); enzyme-linked immunoabsorbent assay (ELISA); and competition assays (e.g. RIA's), for example. In certain embodiments, the anti-VEGF antibody of the invention can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate its effectiveness as a therapeutic. Such assays are known in the art and depend on the target antigen and intended use for the antibody. Examples include the HUVEC inhibition assay (as described in the Examples below); tumor cell growth inhibition assays (as described in WO 89/06692, for example); antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) assays (US Patent 5,500,362); and agonistic activity or hematopoiesis assays (see

WO 95/27062). 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.

In certain embodiments, anti-VEGF antibodies include a monoclonal antibody that binds to the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709; a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. *Cancer Res.* 57:4593-4599 (1997). In one embodiment, the anti-VEGF antibody is "Bevacizumab (BV)", also known as "rhuMAb VEGF" or "AVASTIN®". 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 has been approved by the FDA for use in combination with chemotherapy regimens to treat metastatic colorectal cancer (CRC) and non-small cell lung cancer (NSCLC). Hurwitz et al., N. Engl. J. Med. 350:2335-42 (2004); Sandler et al., N. Engl. J. Med. 355:2542-50 (2006). Currently, bevacizumab is being investigated in many ongoing clinical trials for treating various cancer indications. Kerbel, *J. Clin. Oncol.* 19:45S-51S (2001); De Vore et al, *Proc. Am. Soc. Clin. Oncol.* 19:485a. (2000); Hurwitz et al., *Clin. Colorectal Cancer* 6:66-69 (2006); Johnson et al., *Proc. Am. Soc. Clin. Oncol.* 20:315a (2001); Kabbinavar et al. *J. Clin. Oncol.* 21:60-65 (2003); Miller et al., *Breast Can. Res. Treat.* 94:Suppl 1:S6 (2005).

Bevacizumab and other humanized anti-VEGF antibodies are further described in U.S. Pat. No. 6,884,879 issued Feb. 26, 2005. Additional antibodies include the G6 or B20 series antibodies (e.g., G6-31, B20-4.1), as described in PCT Publication No. WO2005/012359, PCT Publication No. WO2005/044853, and US Patent Application 60/991,302, the content of these patent applications are expressly incorporated herein by reference. For additional antibodies see U.S. Pat. Nos. 7,060,269, 6,582,959, 6,703,020; 6,054,297; WO98/45332; WO 96/30046; WO94/10202; EP 0666868B1; U.S. Patent Application Publication Nos. 2006009360, 20050186208, 20030206899, 20030190317, 20030203409, and 20050112126; and Popkov et al., Journal of Immunological Methods 288:149-164 (2004). Other antibodies include those that bind to a functional epitope on

human VEGF comprising of residues F17, M18, D19, Y21, Y25, Q89, I91, K101, E103, and C104 or, alternatively, comprising residues F17, Y21, Q22, Y25, D63, I83 and Q89.

A "G6 series antibody" according to this invention, is an anti-VEGF antibody that is derived from a sequence of a G6 antibody or G6-derived antibody according to any one of Figures 7, 24-26, and 34-35 of PCT Publication No. WO2005/012359, the entire disclosure of which is expressly incorporated herein by reference. See also PCT Publication No. WO2005/044853, the entire disclosure of which is expressly incorporated herein by reference. In one embodiment, the G6 series antibody binds to a functional epitope on human VEGF comprising residues F17, Y21, Q22, Y25, D63, I83 and Q89.

A "B20 series antibody" according to this invention is an anti-VEGF antibody that is derived from a sequence of the B20 antibody or a B20-derived antibody according to any one of Figures 27-29 of PCT Publication No. WO2005/012359, the entire disclosure of which is expressly incorporated herein by reference. See also PCT Publication No. WO2005/044853, and US Patent Application 60/991,302, the content of these patent applications are expressly incorporated herein by reference. In one embodiment, the B20 series antibody binds to a functional epitope on human VEGF comprising residues F17, M18, D19, Y21, Y25, Q89, I91, K101, E103, and C104.

A "functional epitope" according to this invention refers to amino acid residues of an antigen that contribute energetically to the binding of an antibody. Mutation of any one of the energetically contributing residues of the antigen (for example, mutation of wild-type VEGF by alanine or homolog mutation) will disrupt the binding of the antibody such that the relative affinity ratio (IC50mutant VEGF/IC50wild-type VEGF) of the antibody will be greater than 5 (see Example 2 of WO2005/012359). In one embodiment, the relative affinity ratio is determined by a solution binding phage displaying ELISA. Briefly, 96-well Maxisorp immunoplates (NUNC) are coated overnight at 4°C with an Fab form of the antibody to be tested at a concentration of 2ug/ml in PBS, and blocked with PBS, 0.5% BSA, and 0.05% Tween20 (PBT) for 2h at room temperature. Serial dilutions of phage displaying hVEGF alanine point mutants (residues 8-109 form) or wild type hVEGF (8-109) in PBT are first incubated on the Fab-coated plates for 15 min at room temperature, and the plates are washed with PBS, 0.05% Tween20 (PBST). The bound phage is detected with an anti-M13 monoclonal antibody horseradish peroxidase (Amersham Pharmacia) conjugate diluted 1:5000 in PBT, developed with 3,3', 5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry Labs, Gaithersburg, MD) substrate for approximately 5 min, quenched with 1.0 M

H3PO4, and read spectrophotometrically at 450 nm. The ratio of IC50 values (IC50,ala/IC50,wt) represents the fold of reduction in binding affinity (the relative binding affinity).

A "tyrosine kinase inhibitor" is a molecule which inhibits to some extent tyrosine kinase activity of a tyrosine kinase such as a Axl receptor.

A "chimeric VEGF receptor protein" is a VEGF receptor molecule having amino acid sequences derived from at least two different proteins, at least one of which is as VEGF receptor protein. In certain embodiments, the chimeric VEGF receptor protein is capable of binding to and inhibiting the biological activity of VEGF.

An antibody having a "biological characteristic" of a designated antibody is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule that contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, or more nucleotides or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200 amino acids or more.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already having a benign, pre-cancerous, or non-metastatic tumor as well as those in which the occurrence or recurrence of cancer is to be prevented.

The term "therapeutically effective amount" refers to an amount of a therapeutic agent to treat or prevent a disease or disorder in a mammal. In the case of cancers, the therapeutically effective amount of the therapeutic agent may reduce the number of cancer cells; reduce the primary tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, for example, be measured by assessing the duration of survival, time to disease progression (TTP), the response rates (RR), duration of response, and/or quality of life.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Included in this definition are benign and malignant cancers. By "early stage cancer" or "early stage tumor" is meant a cancer that is not invasive or metastatic or is classified as a Stage 0, I, or II cancer. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

By "metastasis" is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass.

By "reduce or inhibit" is meant the ability to cause an overall decrease of 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or greater. Reduce or inhibit can refer to the symptoms of the disorder being treated, the presence or size of metastases, the size of the primary tumor, or the size or number of the blood vessels in angiogenic disorders.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific

antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

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 research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater 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, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or 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.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

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 hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs 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 the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

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

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas *et al. Cellular and Mol. Immunology*, 4th ed. (W.B. Saunders, Co., 2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

A "naked antibody" for the purposes herein is an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

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-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) 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 HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs 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 contains the heavy- and light-chain variable domains and 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. $F(ab')_2$ 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.

"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, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315.

The term "diabodies" refers to 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 may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO 1993/01161; Hudson *et al.*, *Nat. Med.* 9:129-134 (2003); and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson *et al.*, *Nat. Med.* 9:129-134 (2003).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible mutations, *e.g.*, 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. In certain embodiments, such a 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 a 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, 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 and Milstein, *Nature*, 256:495-97 (1975); Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), 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. Patent 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 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. 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 Immunol.* 7:33 (1993); U.S. Patent 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 Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is 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 (see, *e.g.*,U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies include PRIMATIZED® antibodies wherein the antigen-binding region of the antibody is

derived from an antibody produced by, *e.g.*, immunizing macaque monkeys with the antigen of interest.

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, 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 may be made to further refine antibody performance. In general, a 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, *e.g.*, 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, *e.g.*, 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); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

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. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that

has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al., Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

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 HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Xu *et al.*, *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, *e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993); Sheriff *et al.*, *Nature Struct. Biol.* 3:733-736 (1996).

A number of HVR 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 of the structural loops (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

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

HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (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 HVR residues as herein defined.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al.*, *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.* residues 82a, 82b, and 82c, *etc.* according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g, Kabat *et al.*, *Sequences of Immunological Interest*. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The "EU numbering system"

or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain constant region (*e.g.*, the EU index reported in Kabat *et al., supra*). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody. Unless stated otherwise herein, references to residue numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system. Unless stated otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system (*e.g.*, see United States Provisional Application No. 60/640,323, Figures for EU numbering).

An "affinity matured" antibody is one with one or more alterations in one or more HVRs 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). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies may be produced using certain procedures known in the art. For example, Marks *et al. Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of HVR and/or framework residues is described by, for example, 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).

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

An "agonist antibody," as used herein, is an antibody which partially or fully mimics at least one of the functional activities of a polypeptide of interest.

"Growth inhibitory" antibodies are those that prevent or reduce proliferation of a cell expressing an antigen to which the antibody binds. For example, the antibody may prevent or reduce proliferation of cancer cells that express Axl *in vitro* and/or *in vivo*.

Antibodies that "induce apoptosis" are those that induce programmed cell death as determined by standard apoptosis assays, such as binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies).

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 (CDC); 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.

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.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; CDC; Fc receptor binding; 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 disclosed, for example, in definitions herein.

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-A and A 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.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, *e.g.*, Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, 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 "Fc receptor" or "FcR" 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 regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, *e.g.*, Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie *et al.*, *Nature Biotechnology*, 15(7):637-640 (1997); Hinton *et al.*, *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton *et al.*).

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 to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, *e.g.*, Shields *et al. J. Biol. Chem.* 9(2):6591-6604 (2001).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform

ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells, and neutrophils. The effector cells may be isolated from a native source, *e.g.*, from blood.

"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.* 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 primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII, and FcγRIII. 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 US Patent No. 5,500,362 or 5,821,337 or U.S. Patent No. 6,737,056 (Presta), may be performed. Useful effector cells for such assays include PBMC and NK cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"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), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, *e.g.*, in US Patent No. 6,194,551 B1 and WO 1999/51642. See also, *e.g.*, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

The term "Fc region-comprising antibody" refers to an antibody that 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 antibody or by recombinant engineering of the nucleic acid encoding the antibody. Accordingly, a composition comprising an antibody having an Fc region according to this invention can comprise an antibody with K447, with all K447 removed, or a mixture of antibodies with and without the K447 residue.

"Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, *e.g.*, Chen, *et al.*, *J. Mol. Biol.* 293:865-881(1999)). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (*e.g.*, consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta *et al.*, *Cancer Res.* 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (*e.g.*, about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (*e.g.*, for one hour). The solution is then removed and the plate washed eight times with 0.1% TWEEN-20™ in PBS. When the plates have dried, 150 μl/well of scintillant (MICROSCINT-20 ™; Packard) is added, and the plates are counted on a TOPCOUNT ™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that

give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the Kd or Kd value is measured by using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE ®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% TWEEN-20™ surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE ® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, *e.g.*, Chen *et al.*, *J. Mol. Biol.* 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

An "on-rate," "rate of association," "association rate," or "k_{on}" according to this invention can also be determined as described above using a BIACORE ®-2000 or a BIACORE ®-3000 system (BIAcore, Inc., Piscataway, NJ).

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical

significance within the context of the biological characteristic measured by said values (*e.g.*, Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein, denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (*e.g.*, Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

"Purified" means that a molecule is present in a sample at a concentration of at least 95% by weight, or at least 98% by weight of the sample in which it is contained.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is separated from at least one other nucleic acid molecule with which it is ordinarily associated, for example, in its natural environment. An isolated nucleic acid molecule further includes a nucleic acid molecule contained in cells that ordinarily express the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general,

expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may comprise modification(s) made after synthesis, such as conjugation to a label. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), those containing pendant moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, ply-L-lysine, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, *etc.*), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*), as well as unmodified forms of the polynucleotides(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl-, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs, and basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative

linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO, or CH2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single-stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

The term "Axl," as used herein, refers to any native Axl from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed Axl as well as any form of Axl that results from processing in the cell. The term also encompasses naturally occurring variants of Axl, e.g., splice variants or allelic variants.

The term "Axl ligand" or "Gas6"as used herein, refers to any native Axl ligand from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed Axl ligand as well as any form of Axl ligand that results from processing in the cell. The term also encompasses naturally occurring variants of Axl ligand, e.g., splice variants or allelic variants.

As used herein, "treatment" (and variations such as "treat" or "treating") 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 of the invention are used to delay development of a disease or disorder or to slow the progression of a disease or disorder.

An "individual," "subject," or "patient" is a vertebrate. In certain embodiments, the vertebrate is a mammal. 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. In certain embodiments, a mammal is a human.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations may be sterile.

A "sterile" formulation is aseptic or free from all living microorganisms and their spores.

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 of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, to elicit a desired response in the individual. A therapeutically effective amount encompasses an amount in which any toxic or detrimental effects of the substance/molecule 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 would be less than the therapeutically effective amount.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. The term is intended to include radioactive isotopes (*e.g.*, At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu), chemotherapeutic agents (*e.g.*, methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "toxin" is any substance capable of having a detrimental effect on the growth or proliferation of a cell.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, *e.g.*, Nicolaou *et al.*, *Angew. Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®), liposomal doxorubicin TLC D-99 (MYOCET®), peglylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin,

tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2'-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANE^{TM}), and docetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin (*e.g.*, ELOXATIN®), and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE®, FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as,

for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (sunitinib, SUTENT®, Pfizer); perifosine, COX-2 inhibitor (*e.g.* celecoxib or etoricoxib), proteosome inhibitor (*e.g.* PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE®); pixantrone; EGFR inhibitors (see definition below); tyrosine kinase inhibitors (see definition below); serine-threonine kinase inhibitors such as rapamycin (sirolimus, RAPAMUNE®); farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASAR^{TM}); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN^{TM}) combined with 5-FU and leucovorin.

Chemotherapeutic agents as defined herein include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to: anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), trioxifene, keoxifene, and selective estrogen receptor modulators (SERMs) such as SERM3; pure anti-estrogens without agonist properties, such as fulvestrant (FASLODEX®), and EM800 (such agents may block estrogen receptor (ER) dimerization, inhibit DNA binding, increase ER turnover, and/or suppress ER levels); aromatase inhibitors, including steroidal aromatase inhibitors such as formestane and exemestane (AROMASIN®), and nonsteroidal aromatase inhibitors such as anastrazole (ARIMIDEX®), letrozole (FEMARA®) and aminoglutethimide, and other aromatase inhibitors include vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, and 4(5)-imidazoles; lutenizing hormone-releaseing hormone agonists, including leuprolide (LUPRON® and ELIGARD®), goserelin, buserelin, and tripterelin; sex steroids, including progestines such as megestrol acetate and medroxyprogesterone acetate, estrogens such as diethylstilbestrol and premarin, and androgens/retinoids such as fluoxymesterone, all transretionic acid and fenretinide; onapristone; anti-progesterones; estrogen receptor

down-regulators (ERDs); anti-androgens such as flutamide, nilutamide and bicalutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell (such as a cell expressing Axl) either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells (such as a cell expressing Axl) in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in Mendelsohn and Israel, eds., *The Molecular Basis of Cancer*, Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (W.B. Saunders, Philadelphia, 1995), *e.g.*, p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

III. COMPOSITIONS AND METHODS

The present invention features the use of Axl antagonists and VEGF antagonists in combination therapy to treat a pathological condition, such as tumor. The present invention also provide anti-Axl antibodies and methods using same.

Axl antagonists

Axl antagonists useful in the methods of the invention include polypeptides that specifically bind to Axl, anti- Axl antibodies, Axl small molecules, receptor molecules and derivatives which bind specifically to Axl, and fusions proteins. Axl antagonists also include antagonistic variants of Axl polypeptides, siRNA, RNA aptamers and peptibodies against Axl and Axl ligands. Also included as Axl antagonists useful in the methods of the

invention are anti- Axl ligand antibodies, anti-Axl ligand polypeptides, Axl receptor molecules and derivatives which bind specifically to Axl ligand. Examples of each of these are described below. In some embodiments, the Axl antagonist is not warfarin.

Anti-Axl antibodies that are useful in the methods of the invention include any antibody that binds with sufficient affinity and specificity to Axl and can reduce or inhibit Axl activity.

Anti-Axl antibodies are well known in the art and are further described herein. See, e.g., WO2009/062690; WO2009/06396; co-owned co-pending US patent application 61/356,508, filed June 18, 2010; 5,468,634. In one embodiment, an anti-Axl antibody is a monoclonal antibody. In one embodiment, an anti-Axl antibody is an antibody fragment, *e.g.*, a Fab, Fab'-SH, Fv, scFv, or $(Fab')_2$ fragment. In one embodiment, an anti-Axl antibody is a chimeric, humanized, or human antibody. In one embodiment, an anti-Axl antibody is purified. In certain embodiments, a composition is a pharmaceutical formulation for the treatment of cancer.

Exemplary monoclonal antibodies produced by hybridomas are provided herein and described in Example 8. Those antibodies are designated 3G9, 8B5, 12A11, and 4F8. In one aspect, monoclonal antibodies that compete with 3G9, 8B5, 12A11, and 4F8 for binding to Axl are provided. Monoclonal antibodies that bind to the same epitope as 3G9, 8B5, 12A11, and 4F8 are also provided.

In some embodiments, the anti-Axl antibody comprises a heavy chain variable regions comprising sequence
EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPYR
GYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSSVG
YAMDYWGQGTLV (SEQ ID NO:) and a light chain variable region comprising sequence
DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG
VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKR (SEQ ID NO:).

Antibodies to Axl ligand Gas 6 may also be used in the methods of the invention.

Axl molecules or fragments thereof that specifically bind to Axl ligand (e.g. Gas6) can be used in the methods of the invention, e.g., to bind to and sequester the Axl ligand protein, thereby preventing it from signaling. Preferably, the Axl molecule, or Axl ligand binding fragment thereof, is a soluble form. In some embodiments, a soluble form of the

receptor exerts an inhibitory effect on the biological activity of the Axl protein by binding to Axl ligand, thereby preventing it from binding to its natural receptors present on the surface of target cells. Also included are Axl fusion proteins, examples of which are described below. See also Nagata, J Biol Chem 271:30022 (1996); 2005/0185471; McClosky et al., J Biol Chem 272: 23285 (1997); Fridell et al, J Biol Chem 273:7123 (1998).

A soluble Axl protein or chimeric Axl proteins includes Axl proteins which are not fixed to the surface of cells via a transmembrane domain. As such, soluble forms of Axl, including chimeric receptor proteins, while capable of binding to and inactivating Axl ligand, do not comprise a transmembrane domain and thus generally do not become associated with the cell membrane of cells in which the molecule is expressed.

Axl ligand molecules or fragments thereof that specifically bind to Axl and block or reduce activation of Axl, thereby preventing it from signaling, can be used in the methods of the invention.

Axl siRNA are described and exemplified herein and are also described in, e.g., WO2009/005813.

Aptamers are nucleic acid molecules that form tertiary structures that specifically bind to a target molecule, such as a Axl ligand polypeptide. The generation and therapeutic use of aptamers are well established in the art. See, e.g., U.S. Pat. No. 5,475,096. An Axl ligand aptamer is a pegylated modified oligonucleotide, which adopts a three-dimensional conformation that enables it to bind to extracellular Axl ligand. Additional information on aptamers can be found in U.S. Patent Application Publication No. 20060148748.

A peptibody is a peptide sequence linked to an amino acid sequence encoding a fragment or portion of an immunoglobulin molecule. Polypeptides may be derived from randomized sequences selected by any method for specific binding, including but not limited to, phage display technology. In a preferred embodiment, the selected polypeptide may be linked to an amino acid sequence encoding the Fc portion of an immunoglobulin. Peptibodies that specifically bind to and antagonize Axl ligand or Axl are also useful in the methods of the invention.

Axl antagonists include small molecules such as R 428 (Rigel; Holland et al, Cancer Research 70, 1544 (2010) and PF02341066. In some embodiments, the small molecule Axl antagonist is not warfarin.

VEGF antagonists

A VEGF antagonist refers to a molecule capable of binding to VEGF, reducing VEGF expression levels, or neutralizing, blocking, inhibiting, abrogating, reducing, or interfering with VEGF biological activities, including VEGF binding to one or more VEGF receptors and VEGF mediated angiogenesis and endothelial cell survival or proliferation. Included as VEGF- antagonists useful in the methods of the invention are polypeptides that specifically bind to VEGF, 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, fusions proteins (e.g., VEGF-Trap (Regeneron)), and $VEGF_{121}$-gelonin (Peregrine). VEGF antagonists also include antagonistic variants of VEGF polypeptides, RNA aptamers and peptibodies against VEGF. Examples of each of these are described below.

Anti-VEGF antibodies that are useful in the methods of the invention include any antibody, or antigen binding fragment thereof, that bind with sufficient affinity and specificity to VEGF and can reduce or inhibit the biological activity of VEGF. 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. Examples of such anti-VEGF antibodies include, but not limited to, those provided herein under "Definitions."

The two best characterized VEGF receptors are VEGFR1 (also known as Flt-1) and VEGFR2 (also known as KDR and FLK-1 for the murine homolog). The specificity of each receptor for each VEGF family member varies but VEGF-A binds to both Flt-1 and KDR. The full length Flt-1 receptor includes an extracellular domain that has seven Ig domains, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF and the intracellular domain is involved in signal transduction.

VEGF receptor molecules or fragments thereof that specifically bind to VEGF can be used in the methods of the invention to bind to and sequester the VEGF protein, thereby preventing it from signaling. In certain embodiments, the VEGF receptor molecule, or VEGF binding fragment thereof, is a soluble form, such as sFlt-1. A soluble form of the receptor exerts an inhibitory effect on the biological activity of the VEGF protein by binding to VEGF, thereby preventing it from binding to its natural receptors present on the surface

of target cells. Also included are VEGF receptor fusion proteins, examples of which are described below.

A chimeric VEGF receptor protein is a receptor molecule having amino acid sequences derived from at least two different proteins, at least one of which is a VEGF receptor protein (e.g., the flt-1 or KDR receptor), that is capable of binding to and inhibiting the biological activity of VEGF. In certain embodiments, the chimeric VEGF receptor proteins of the present invention consist of amino acid sequences derived from only two different VEGF receptor molecules; however, amino acid sequences comprising one, two, three, four, five, six, or all seven Ig-like domains from the extracellular ligand-binding region of the flt-1 and/or KDR receptor can be linked to amino acid sequences from other unrelated proteins, for example, immunoglobulin sequences. Other amino acid sequences to which Ig-like domains are combined will be readily apparent to those of ordinary skill in the art. Examples of chimeric VEGF receptor proteins include soluble Flt-1/Fc, KDR/Fc, or FLt-1/KDR/Fc (also known as VEGF Trap). (See for example PCT Application Publication No. WO97/44453).

A soluble VEGF receptor protein or chimeric VEGF receptor proteins of the present invention includes VEGF receptor proteins which are not fixed to the surface of cells via a transmembrane domain. As such, soluble forms of the VEGF receptor, including chimeric receptor proteins, while capable of binding to and inactivating VEGF, do not comprise a transmembrane domain and thus generally do not become associated with the cell membrane of cells in which the molecule is expressed.

Aptamers are nucleic acid molecules that form tertiary structures that specifically bind to a target molecule, such as a VEGF polypeptide. The generation and therapeutic use of aptamers are well established in the art. See, e.g., U.S. Pat. No. 5,475,096. A VEGF aptamer is a pegylated modified oligonucleotide, which adopts a three-dimensional conformation that enables it to bind to extracellular VEGF. One example of a therapeutically effective aptamer that targets VEGF for treating age-related macular degeneration is pegaptanib (Macugen™, OSI). Additional information on aptamers can be found in U.S. Patent Application Publication No. 20060148748.

A peptibody is a peptide sequence linked to an amino acid sequence encoding a fragment or portion of an immunoglobulin molecule. Polypeptides may be derived from randomized sequences selected by any method for specific binding, including but not limited to, phage display technology. In one embodiment, the selected polypeptide may be

linked to an amino acid sequence encoding the Fc portion of an immunoglobulin. Peptibodies that specifically bind to and antagonize VEGF are also useful in the methods of the invention.

Antibodies

1. Antibody Fragments

The present invention encompasses antibody fragments. Antibody fragments may be generated by traditional means, such as enzymatic digestion, or by recombinant techniques. 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. For a review of certain antibody fragments, see Hudson *et al.* (2003) *Nat. Med.* 9:129-134.

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 $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased *in vivo* half-life comprising 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 certain embodiments, an antibody is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. Fv and scFv are the only species with intact combining sites that are devoid of constant regions; thus, they may be suitable for reduced nonspecific binding during *in vivo* use. scFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an scFv. 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 antibodies may be monospecific or bispecific.

2. Humanized Antibodies

The 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. Patent 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 can be 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. See, *e.g.*, 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. See, *e.g.*, Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; Presta *et al.* (1993) *J. Immunol.*, 151:2623.

It is further generally desirable 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.

3. Human Antibodies

Human antibodies of the invention can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequence(s) as described above. Alternatively, human monoclonal antibodies of the invention 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 herein 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 April 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.

4. Bispecific Antibodies

Bispecific antibodies are monoclonal antibodies that have binding specificities for at least two different antigens. In certain embodiments, bispecific antibodies are human or humanized antibodies. In certain embodiments, one of the binding specificities is for Axl and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of Axl. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express Axl. These antibodies possess a Axl-binding arm and an arm which binds a cytotoxic agent, such as, *e.g.*, saporin, anti-interferon-α, 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 approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion, for example, is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. In

certain embodiments, the first heavy-chain constant region (CH1), containing the site necessary for light chain binding, is 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 one 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 interface comprises at least a part of the C_H3 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 (US Patent 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 method. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent 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).

5. 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. In certain embodiments, the 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 Fc region. In certain embodiments, a multivalent antibody comprises (or consists of) three to about eight antigen binding sites. In one such embodiment, a multivalent antibody comprises (or consists of) four antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (for example, 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 may further comprise at least two (for example, 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.

6. Single-Domain Antibodies

In some embodiments, an antibody of the invention is a single-domain antibody. A single-domain antibody is a single polyeptide chain comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see, e.g.*, U.S. Patent No. 6,248,516 B1). In one embodiment, a single-domain antibody consists of all or a portion of the heavy chain variable domain of an antibody.

7. 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 may be prepared by introducing appropriate changes into the nucleotide sequence encoding the antibody, 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 can be 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 (*e.g.*, 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. 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.

In certain embodiments, an antibody of the invention is altered to increase or decrease the extent to which the antibody is glycosylated. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of a 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 or deletion of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that one or more of the above-described tripeptide sequences (for N-linked glycosylation sites) is created or removed. The alteration may also be made by the addition, deletion, or substitution of 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. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, *e.g.*, Wright *et al.* (1997) *TIBTECH* 15:26-32. The oligosaccharide may include various carbohydrates, *e.g.*, mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

For example, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. Such variants may have

improved ADCC function. See, *e.g.*, US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants 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; WO2002/031140; Okazaki *et al. J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of 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 (see, *e.g.*, Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. *et al., Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, *e.g.*, in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, *e.g.*, in WO 2003/011878 (Jean-Mairet *et al.*); US Patent No. 6,602,684 (Umana *et al.*); and US 2005/0123546 (Umana *et al.*). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, *e.g.*, in WO 1997/30087 (Patel *et al.*); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Such substitutions may occur in combination with any of the variations described above.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable 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 antibody 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 FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, *e.g.* Hellstrom, I., *et al. Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I *et al.*, *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. *et al.*, *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). 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. Proc. Nat'l Acad. Sci. 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 assaymay be performed (see, for example, Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. *et al.*, *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, for example, Petkova, S.B. *et al.*, *Int'l. Immunol.* 18(12):1759-1769 (2006)).

Other antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions." More substantial changes, denominated "exemplary substitutions" are provided in Table 1, or as further described below in reference to amino acid classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened, *e.g.*, for a desired activity, such as improved antigen binding, decreased immunogenicity, improved ADCC or CDC, *etc.*

TABLE 1

Original Residue	Exemplary Substitutions	Preferred 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; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Axl(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; Ala; Norleucine	Leu

Modifications in the biological properties of an antibody may be accomplished by selecting substitutions that affect (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. Amino

acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Axl(P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, into the remaining (non-conserved) sites.

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 modified (*e.g.*, improved) biological properties relative to the parent antibody from which they are generated. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated using phage display-based affinity maturation techniques. 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 at least part of a phage coat protein (*e.g.*, 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). In order to identify candidate hypervariable region sites for modification, scanning mutagenesis (*e.g.*, alanine scanning) 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

techniques known in the art, including those elaborated herein. Once such variants are generated, the panel of variants is subjected to screening using techniques known in the art, including those described herein, and variants 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 antibodies of the invention, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 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 of the invention 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. Patent No. 5,648,260; U.S. Patent 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 region 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 C1q binding capability are described in US patent 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).

In another aspect, the invention provides antibodies comprising modifications in the interface of Fc polypeptides comprising the Fc region, wherein the modifications facilitate and/or promote heterodimerization. These modifications comprise introduction of a protuberance into a first Fc polypeptide and a cavity into a second Fc polypeptide, wherein the protuberance is positionable in the cavity so as to promote complexing of the first and second Fc polypeptides. Methods of generating antibodies with these modifications are known in the art, *e.g.*, as described in U.S. Pat. No. 5,731,168.

In yet another aspect, it may be desirable to create cysteine engineered antibodies, *e.g.*, "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region.

8. Antibody Derivatives

The antibodies of the present invention 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 polymer 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.*

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam *et al.*, *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

B. Certain Methods of Making Antibodies

1. Certain Hybridoma-Based Methods

Monoclonal antibodies of the invention can be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), and further described, *e.g.*, in Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), 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), and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) regarding human-human hybridomas. Additional methods include those described, for example, in U.S. Pat. No. 7,189,826 regarding production of monoclonal human natural IgM antibodies from hybridoma cell lines. Human hybridoma technology (Trioma technology) is described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).

For various other hybridoma techniques, see, *e.g.*, US 2006/258841; US 2006/183887 (fully human antibodies), US 2006/059575; US 2005/287149; US 2005/100546; US 2005/026229; and U.S. Pat. Nos. 7,078,492 and 7,153,507. An exemplary protocol for producing monoclonal antibodies using the hybridoma method is described as follows. In one embodiment, 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 are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of a

polypeptide comprising Axl or a fragment thereof, and an adjuvant, such as monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT). A polypeptide comprising Axl or a fragment thereof may be prepared using methods well known in the art, such as recombinant methods, some of which are further described herein. Serum from immunized animals is assayed for anti-Axl antibodies, and booster immunizations are optionally administered. Lymphocytes from animals producing anti-Axl antibodies are isolated. Alternatively, lymphocytes may be immunized *in vitro*.

Lymphocytes are then fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. See, *e.g.*, Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986). Myeloma cells may be used 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. Exemplary myeloma cells include, but are not limited to, 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, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland 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)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium, *e.g.*, a medium that 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. Preferably, serum-free hybridoma cell culture methods are used to reduce use of animal-derived serum such as fetal bovine serum, as described, for example, in Even *et al.*, *Trends in Biotechnology*, 24(3), 105-108 (2006).

Oligopeptides as tools for improving productivity of hybridoma cell cultures are described in Franek, *Trends in Monoclonal Antibody Research*, 111-122 (2005). Specifically, standard culture media are enriched with certain amino acids (alanine, serine,

asparagine, proline), or with protein hydrolyzate fractions, and apoptosis may be significantly suppressed by synthetic oligopeptides, constituted of three to six amino acid residues. The peptides are present at millimolar or higher concentrations.

Culture medium in which hybridoma cells are growing may be assayed for production of monoclonal antibodies that bind to Axl. The binding specificity of monoclonal antibodies produced by hybridoma cells may be 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 be determined, for example, by Scatchard analysis. See, *e.g.*, 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. See, *e.g.*, Goding, *supra*. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, hybridoma cells may be grown *in vivo* as ascites tumors in an animal. 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. One procedure for isolation of proteins from hybridoma cells is described in US 2005/176122 and U.S. Pat. No. 6,919,436. The method includes using minimal salts, such as lyotropic salts, in the binding process and preferably also using small amounts of organic solvents in the elution process.

2. Certain Library Screening Methods

Antibodies of the invention can be made by using combinatorial libraries to screen for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are described generally in Hoogenboom *et al.* in *Methods in Molecular Biology* 178:1-37 (O'Brien *et al.*, ed., Human Press, Totowa, NJ, 2001). For example, one method of generating antibodies of interest is through the use of a phage antibody library as described in Lee *et al.*, J. Mol. Biol. (2004), 340(5):1073-93.

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 antibodies of the invention 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 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.

In certain embodiments, 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 (HVRs) 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).

In certain embodiments, 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-Axl clones is desired, the subject is immunized with Axl 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- Axl clones is obtained by generating an anti- Axl antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that Axl immunization gives rise to B cells producing human antibodies against Axl. The generation of human antibody-producing transgenic mice is described below.

Additional enrichment for anti-Axl reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing Axl-specific membrane bound antibody, *e.g.*, by cell separation using Axl affinity chromatography or adsorption of cells to fluorochrome-labeled Axl 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 Axl 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). In certain embodiments, 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 Vκ and Vλ 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^{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 (K_d^{-1} of about 10^{-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 (K_d^{-1} of about 10^6 to 10^7 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 March 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 of about 10^{-9} M or less.

Screening of the libraries can be accomplished by various techniques known in the art. For example, Axl 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 method for panning phage display libraries.

The phage library samples are contacted with immobilized Axl under conditions suitable for binding 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 Axl 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 Axl. However, random mutation of a selected antibody

(*e.g.* as performed in some affinity maturation techniques) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting Axl, rare high affinity phage could be competed out. To retain all higher affinity mutants, phages can be incubated with excess biotinylated Axl, but with the biotinylated Axl at a concentration of lower molarity than the target molar affinity constant for Axl. 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-Axl clones may be selected based on activity. In certain embodiments, the invention provides anti-Axl antibodies that bind to living cells that naturally express Axl. In one embodiment, the invention provides anti-Axl antibodies that block the binding between a Axl ligand and Axl, but do not block the binding between a Axl ligand and a second protein. Fv clones corresponding to such anti-Axl antibodies can be selected by (1) isolating anti-Axl 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 Axl and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-Axl phage clones to immobilized Axl; (4) using an excess of the second protein to elute any undesired clones that recognize Axl-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 hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention 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 of the invention 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. An 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 certain embodiments, an Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for full- or partial-length human heavy and/or light chains.

DNA encoding anti-Axl antibody derived from a hybridoma of the invention 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 of the invention.

3. Vectors, Host Cells, and Recombinant Methods

Antibodies may also be produced using recombinant methods. For recombinant production of an anti-Axl antibody, nucleic acid encoding the antibody is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody may be 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 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.

a) Signal sequence component

An antibody of the invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is 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 a native antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.,* the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

b) Origin of replication

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

c) 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, or (c) supply critical nutrients not available from complex media, *e.g.,* the gene encoding D-alanine racemase for *Bacilli*.

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 antibody-encoding nucleic acid, such as DHFR, glutamine synthetase (GS), thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR gene are identified by culturing the transformants in a culture medium containing methotrexate (Mtx), a competitive antagonist of DHFR. Under these conditions, the DHFR gene is amplified along with any other co-transformed nucleic acid. A Chinese hamster ovary (CHO) cell line deficient in endogenous DHFR activity (*e.g.*, ATCC CRL-9096) may be used.

Alternatively, cells transformed with the GS gene are identified by culturing the transformants in a culture medium containing L-methionine sulfoximine (Msx), an inhibitor of GS. Under these conditions, the GS gene is amplified along with any other co-transformed nucleic acid. The GS selection/amplification system may be used in combination with the DHFR selection/amplification system described above.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding an antibody of interest, wild-type DHFR gene, 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. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg,

Bio/Technology, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

d) Promoter component

Expression and cloning vectors generally contain a promoter that is recognized by the host organism and is operably linked to nucleic acid encoding an antibody. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding an antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic 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. 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. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Antibody transcription from vectors in mammalian host cells can be 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, Simian Virus 40 (SV40), or 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. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

e) Enhancer element component

Transcription of a DNA encoding an antibody 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, α-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-encoding sequence, but is preferably located at a site 5' from the promoter.

f) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will 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 antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

g) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.*, *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*, *e.g.*, *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

Full length antibody, antibody fusion proteins, and antibody fragments can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (*e.g.*, a toxin) that by itself shows effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation. Production in *E. coli* is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, *e.g.*, U.S. 5,648,237 (Carter *et al.*), U.S. 5,789,199 (Joly *et al.*), U.S. 5,840,523 (Simmons *et al.*), which describes translation initiation region (TIR) and signal sequences for optimizing expression and secretion. *See also* Charlton, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*. After expression, the antibody may be isolated from the *E. coli* cell paste in a soluble fraction and can be purified through, *e.g.*, a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g,, in CHO cells.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP

402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g., Neurospora, Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*. For a review discussing the use of yeasts and filamentous fungi for the production of therapeutic proteins, see, *e.g.*, Gerngross, *Nat. Biotech.* 22:1409-1414 (2004).

Certain fungi and yeast strains may be selected in which glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. *See, e.g.*, Li *et al.*, *Nat. Biotech.* 24:210-215 (2006) (describing humanization of the glycosylation pathway in *Pichia pastoris*); and Gerngross *et al.*, *supra*.

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, duckweed (*Lemnaceae*), alfalfa (*M. truncatula*), and tobacco can also be utilized as hosts. *See, e.g.*, US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may be used as hosts, and 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); 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). Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); and myeloma cell lines such as NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, *e.g.*, Yazaki and Wu, *Methods in Molecular Biology, Vol. 248* (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 255-268.

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.

h) 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. Patent 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™ 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.

i) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, 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. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. 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, hydrophobic interaction chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being among one of the typically preferred purification steps. 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 γ1, γ2, or γ4 heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (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 C_H3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) 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™ 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).

In general, various methodologies for preparing antibodies for use in research, testing, and clinical are well-established in the art, consistent with the above-described methodologies and/or as deemed appropriate by one skilled in the art for a particular antibody of interest.

C. Immunoconjugates

The invention also provides immunoconjugates (interchangeably referred to as "antibody-drug conjugates," or "ADCs") comprising an antibody conjugated to one or more cytotoxic agents, such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (*e.g.*, a protein toxin, an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Immunoconjugates have been used for the local delivery of cytotoxic agents, *i.e.*, drugs that kill or inhibit the growth or proliferation of cells, in the treatment of cancer (Lambert, J. (2005) *Curr. Opinion in Pharmacology* 5:543-549; Wu et al (2005) *Nature Biotechnology* 23(9):1137-1146; Payne, G. (2003) i 3:207-212; Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drug Deliv. Rev.* 26:151-172; U.S. Pat. No. 4,975,278). Immunoconjugates allow for the targeted delivery of a drug moiety to a tumor, and intracellular accumulation therein, where systemic administration of unconjugated drugs may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin *et al.*, *Lancet* (Mar. 15, 1986) pp. 603-05; Thorpe (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications* (A. Pinchera *et al.*, eds) pp. 475-506. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland *et al.*, (1986) *Cancer Immunol. Immunother.* 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland *et al.*, (1986) *supra*). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) *J. Nat. Cancer Inst.* 92(19):1573-1581; Mandler et al (2000) *Bioorganic & Med. Chem. Letters* 10:1025-1028; Mandler et al (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), and calicheamicin (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). The toxins may exert their cytotoxic effects by mechanisms including tubulin binding, DNA

binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and 111In or 90Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) *Eur. Jour. Nucl. Med.* 27(7):766-77; Wiseman et al (2002) *Blood* 99(12):4336-42; Witzig et al (2002) *J. Clin. Oncol.* 20(10):2453-63; Witzig et al (2002) *J. Clin. Oncol.* 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody-drug conjugate composed of a huCD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (*Drugs of the Future* (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody-drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and other cancers. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody-drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) *Nature Biotechnol.* 21(7):778-784) and are under therapeutic development.

In certain embodiments, an immunoconjugate comprises an antibody and a chemotherapeutic agent or other toxin. Chemotherapeutic agents useful in the generation of immunoconjugates are described herein (*e.g.*, above). Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin,

enomycin, and the tricothecenes. See, *e.g.*, WO 93/21232 published October 28, 1993. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, dolastatins, aurostatins, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

1. Maytansine and maytansinoids

In some embodiments, the immunoconjugate comprises an antibody (full length or fragments) conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub Maytenus serrata (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to derivatization with

functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu *et al.*, Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an *in vivo* tumor growth assay. Chari *et al.*, Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansinoid conjugate was tested *in vitro* on the human breast cancer cell line SK-BR-3, which expresses 3 x 105 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. See, *e.g.*, U.S. Patent No. 5,208,020 (the disclosure of which is hereby expressly incorporated by reference). An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP

Patent 0 425 235 B1, Chari *et al.*, Cancer Research 52:127-131 (1992), and U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004, the disclosures of which are hereby expressly incorporated by reference. Antibody-maytansinoid conjugates comprising the linker component SMCC may be prepared as disclosed in U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004. The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred. Additional linking groups are described and exemplified herein.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson *et al.*, Biochem. J. 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

2. Auristatins and dolastatins

In some embodiments, the immunoconjugate comprises an antibody conjugated to dolastatins or dolostatin peptidic analogs and derivatives, the auristatins (US Patent Nos. 5635483; 5780588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke et al (2001) Antimicrob. Agents and Chemother. 45(12):3580-3584) and have anticancer (US 5663149) and antifungal activity (Pettit et al (1998) Antimicrob. Agents Chemother.

42:2961-2965). The dolastatin or auristatin drug moiety may be attached to the antibody through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (WO 02/088172).

Exemplary auristatin embodiments include the N-terminus linked monomethylauristatin drug moieties DE and DF, disclosed in "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the disclosure of which is expressly incorporated by reference in its entirety.

Typically, peptide-based drug moieties can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to the liquid phase synthesis method (see E. Schröder and K. Lübke, "The Peptides", volume 1, pp 76-136, 1965, Academic Press) that is well known in the field of peptide chemistry. The auristatin/dolastatin drug moieties may be prepared according to the methods of: US 5635483; US 5780588; Pettit et al (1989) J. Am. Chem. Soc. 111:5463-5465; Pettit et al (1998) Anti-Cancer Drug Design 13:243-277; Pettit, G.R., *et al.* Synthesis, 1996, 719-725; and Pettit et al (1996) J. Chem. Soc. Perkin Trans. 1 5:859-863. See also Doronina (2003) Nat Biotechnol 21(7):778-784; "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, hereby incorporated by reference in its entirety (disclosing, *e.g.*, linkers and methods of preparing monomethylvaline compounds such as MMAE and MMAF conjugated to linkers).

3. Calicheamicin

In other embodiments, the immunoconjugate comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ1I, α2I, α3I, N-acetyl-γ1I, PSAG and θI1 (Hinman *et al.*, Cancer Research 53:3336-3342 (1993), Lode *et al.*, Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore,

cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

4. Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. patents 5,053,394, 5,770,710, as well as esperamicins (U.S. patent 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (*e.g.*, a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as $tc^{99}m$ or I^{123}, Re^{186}, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57) can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari *et al.*, Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (*e.g.*, from Pierce Biotechnology, Inc., Rockford, IL., U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

5. Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC), an antibody (Ab) is conjugated to one or more drug moieties (D), *e.g.* about 1 to about 20 drug moieties per antibody, through a linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody. Additional methods for preparing ADC are described herein.

$$Ab–(L–D)_p \qquad I$$

The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-aminobenzyloxycarbonyl ("PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), N-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("SMCC'), and N-Succinimidyl (4-iodo-acetyl) aminobenzoate ("SIAB"). Additional linker components are known in the art and some are described herein. See also "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the contents of which are hereby incorporated by reference in its entirety.

In some embodiments, the linker may comprise amino acid residues. Exemplary amino acid linker components include a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide. Exemplary dipeptides include: valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe). Exemplary tripeptides include: glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). Amino acid residues which comprise an amino acid linker component include those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline. Amino acid linker components can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzymes, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, *e.g.* lysine, (iii) side chain thiol groups, *e.g.* cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, *i.e.* cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into the

antibody (or fragment thereof) by introducing one, two, three, four, or more cysteine residues (*e.g.*, preparing mutant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody drug conjugates may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, *e.g.* with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, *e.g.* by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, *e.g.*, by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionucleotide).

Anti-Axl antibodies of the invention may be characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

a) Activity assays

In one aspect, assays are provided for identifying anti-Axl antibodies thereof having biological activity. Biological activity may include, *e.g.*, inhibition of ligand binding to Axl, inhibition of phosphorylation of Axl, or down-regulation of Axl expression. Antibodies having such biological activity *in vivo* and/or *in vitro* are also provided.

In certain embodiments, an antibody of the invention is tested for its ability to inhibit ligand binding to Axl, inhibit phosphorylation of Axl, or down-regulate Axl expression.

b) Binding assays and other assays

In one aspect, an antibody of the invention is tested for its antigen binding activity, *e.g.*, by known methods such as ELISA, Western blot, *etc.* In another aspect, competition assays may be used to identify a monoclonal antibody that competes with 3G9, 8B5, 12A11, or 4F8 for binding to Axl. In certain embodiments, such a competing antibody binds to the same epitope (*e.g.*, a linear or a conformational epitope) that is bound by 3G9, 8B5, 12A11, or 4F8. Exemplary competition assays include, but are not limited to, routine assays such as those provided in Harlow and Lane (1988) *Antibodies: A Laboratory Manual* ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ). Two antibodies are said to bind to the same epitope if each blocks binding of the other by 50% or more.

In an exemplary competition assay, immobilized Axl is incubated in a solution comprising a first labeled antibody that binds to Axl (*e.g.*, 3G9, 8B5, 12A11, or 4F8) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to Axl. The second antibody may be present in a hybridoma supernatant. As a control, immobilized Axl is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to Axl, excess unbound antibody is removed, and the amount of label associated with immobilized Axl is measured. If the amount of label associated with immobilized Axl is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to Axl.

In one aspect, antibodies of the invention 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.

It is understood that any of the above assays may be carried out using an immunoconjugate of the invention in place of or in addition to an anti-Axl antibody.

Methods

Combination Therapies

The present invention features the combination use of an Axl antagonist and a VEGF antagonist. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. The present invention is particularly useful in treating cancers of various types at various stages.

The term cancer embraces a collection of proliferative disorders, including but not limited to pre-cancerous growths, benign tumors, and malignant tumors. Benign tumors remain localized at the site of origin and do not have the capacity to infiltrate, invade, or metastasize to distant sites. Malignant tumors will invade and damage other tissues around them. They can also gain the ability to break off from the original site and spread to other parts of the body (metastasize), usually through the bloodstream or through the lymphatic system where the lymph nodes are located. Primary tumors are classified by the type of tissue from which they arise; metastatic tumors are classified by the tissue type from which the cancer cells are derived. Over time, the cells of a malignant tumor become more abnormal and appear less like normal cells. This change in the appearance of cancer cells is called the tumor grade, and cancer cells are described as being well-differentiated (low grade), moderately-differentiated, poorly-differentiated, or undifferentiated (high grade). Well-differentiated cells are quite normal appearing and resemble the normal cells from which they originated. Undifferentiated cells are cells that have become so abnormal that it is no longer possible to determine the origin of the cells.

Cancer staging systems describe how far the cancer has spread anatomically and attempt to put patients with similar prognosis and treatment in the same staging group. Several tests may be performed to help stage cancer including biopsy and certain imaging tests such as a chest x-ray, mammogram, bone scan, CT scan, and MRI scan. Blood tests

and a clinical evaluation are also used to evaluate a patient's overall health and detect whether the cancer has spread to certain organs.

To stage cancer, the American Joint Committee on Cancer first places the cancer, particularly solid tumors, in a letter category using the TNM classification system. Cancers are designated the letter T (tumor size), N (palpable nodes), and/or M (metastases). T1, T2, T3, and T4 describe the increasing size of the primary lesion; N0, N1, N2, N3 indicates progressively advancing node involvement; and M0 and M1 reflect the absence or presence of distant metastases.

In the second staging method, also known as the Overall Stage Grouping or Roman Numeral Staging, cancers are divided into stages 0 to IV, incorporating the size of primary lesions as well as the presence of nodal spread and of distant metastases. In this system, cases are grouped into four stages denoted by Roman numerals I through IV, or are classified as "recurrent." For some cancers, stage 0 is referred to as "in situ" or "Tis," such as ductal carcinoma in situ or lobular carcinoma in situ for breast cancers. High grade adenomas can also be classified as stage 0. In general, stage I cancers are small localized cancers that are usually curable, while stage IV usually represents inoperable or metastatic cancer. Stage II and III cancers are usually locally advanced and/or exhibit involvement of local lymph nodes. In general, the higher stage numbers indicate more extensive disease, including greater tumor size and/or spread of the cancer to nearby lymph nodes and/or organs adjacent to the primary tumor. These stages are defined precisely, but the definition is different for each kind of cancer and is known to the skilled artisan.

Many cancer registries, such as the NCI's Surveillance, Epidemiology, and End Results Program (SEER), use summary staging. This system is used for all types of cancer. It groups cancer cases into five main categories:

In situ is early cancer that is present only in the layer of cells in which it began.

Localized is cancer that is limited to the organ in which it began, without evidence of spread.

Regional is cancer that has spread beyond the original (primary) site to nearby lymph nodes or organs and tissues.

Distant is cancer that has spread from the primary site to distant organs or distant lymph nodes.

Unknown is used to describe cases for which there is not enough information to indicate a stage.

In addition, it is common for cancer to return months or years after the primary tumor has been removed. Cancer that recurs after all visible tumor has been eradicated, is called recurrent disease. Disease that recurs in the area of the primary tumor is locally recurrent, and disease that recurs as metastases is referred to as a distant recurrence.

The tumor can be a solid tumor or a non-solid or soft tissue tumor. Examples of soft tissue tumors include leukemia (e.g., chronic myelogenous leukemia, acute myelogenous leukemia, adult acute lymphoblastic leukemia, acute myelogenous leukemia, mature B-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, polymphocytic leukemia, or hairy cell leukemia) or lymphoma (e.g., non-Hodgkin's lymphoma, cutaneous T-cell lymphoma, or Hodgkin's disease). A solid tumor includes any cancer of body tissues other than blood, bone marrow, or the lymphatic system. Solid tumors can be further divided into those of epithelial cell origin and those of non-epithelial cell origin. Examples of epithelial cell solid tumors include tumors of the gastrointestinal tract, colon, breast, prostate, lung, kidney, liver, pancreas, ovary, head and neck, oral cavity, stomach, duodenum, small intestine, large intestine, anus, gall bladder, labium, nasopharynx, skin, uterus, male genital organ, urinary organs, bladder, and skin. Solid tumors of non-epithelial origin include sarcomas, brain tumors, and bone tumors.

In addition to the hereindescribed therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

In some embodiments, the patient herein is subjected to a diagnostic test e.g., prior to and/or during and/or after therapy. Generally, if a diagnostic test is performed, a sample may be obtained from a patient in need of therapy. Where the subject has cancer, the sample may be a tumor sample, or other biological sample, such as a biological fluid, including, without limitation, blood, urine, saliva, ascites fluid, or derivatives such as blood serum and blood plasma, and the like.

In some embodiments, the subject's cancer expresses (in some embodiments, over-expresses) Axl and/or VEGF. Methods for determining Axl and VEGF expression are known in the art and certain methods are described herein.

In one aspect, the invention provides methods for treating cancer, inhibiting unwanted cellular proliferation, inhibiting metastasis of cancer and inducing apoptosis of tumor cells either *in vivo* or *in vitro*, the method comprising exposing a cell to an antibody of the invention under conditions permissive for binding of the antibody to Axl. In certain embodiments, the cell is a myelogenous leukemia cell, a lung cancer cell, a gastric cancer

cell, a breast cancer cell, a prostate cancer cell, a renal cell cancer cell, and a glioblastoma cell. In one embodiment, an antibody of the invention can be used for inhibiting an activity of Axl, the method comprising exposing Axl to an antibody of the invention such that the activity of Axl is inhibited.

In one aspect, the invention provides methods for treating cancer comprising administering to an individual an effective amount of an antibody of the invention. In certain embodiments, a method for treating cancer comprises administering to an individual an effective amount of a pharmaceutical formulation comprising an antibody of the invention and, optionally, at least one additional therapeutic agent, such as those provided below.

Antibodies of the invention can be used either alone or in combination with other compositions in a therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent and/or adjuvant. In certain embodiments, an additional therapeutic agent is an anti-VEGF antibody.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Antibodies of the invention can also be used in combination with radiation therapy.

In one embodiment, an antibody of the invention is used in a method for binding Axl in an individual suffering from a disorder associated with increased Axl expression and/or activity, the method comprising administering to the individual the antibody such that Axl in the individual is bound. In one embodiment, the Axl is human Axl, and the individual is a human individual.

Diagnostic methods and methods of detection

In one aspect, antibodies of the invention are useful for detecting the presence of Axl in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as tumor tissue.

In one aspect, the invention provides a method of detecting the presence of Axl in a biological sample. In certain embodiments, the method comprises contacting the biological

sample with an anti-Axl antibody under conditions permissive for binding of the anti-Axl antibody to Axl, and detecting whether a complex is formed between the anti-Axl antibody and Axl.

In one aspect, the invention provides a method of diagnosing a disorder associated with increased expression of Axl. In certain embodiments, the method comprises contacting a test cell with an anti-Axl antibody; determining the level of expression (either quantitatively or qualitatively) of Axl by the test cell by detecting binding of the anti-Axl antibody to Axl; and comparing the level of expression of Axl by the test cell with the level of expression of Axl by a control cell (*e.g.*, a normal cell of the same tissue origin as the test cell or a cell that expresses Axl at levels comparable to such a normal cell), wherein a higher level of expression of Axl by the test cell as compared to the control cell indicates the presence of a disorder associated with increased expression of Axl. In certain embodiments, the test cell is obtained from an individual suspected of having a disorder associated with increased expression of Axl. In certain embodiments, the disorder is a cell proliferative disorder, such as a cancer or a tumor.

Exemplary disorders that may be diagnosed using an antibody of the invention include myelogenous leukemia, lung cancer (e.g., non-small cell lung carconoma (NSCLC)), gastric cancer, breast cancer, prostate cancer, renal cell cancer, pancreatic cancer and glioblastoma.

Certain other methods can be used to detect binding of antibodies to Axl. Such methods include, but are not limited to, antigen-binding assays that are well known in the art, such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, protein A immunoassays, and immunohistochemistry (IHC).

In certain embodiments, antibodies are labeled. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, *e.g.*, through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, *e.g.*, firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase,

lysozyme, saccharide oxidases, *e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

In certain embodiments, antibodies are immobilized on an insoluble matrix. Immobilization may entail separating an anti-Axl antibody from any Axl that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-Axl antibody before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*., U.S. 3,720,760), or by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the anti-Axl antibody after formation of a complex between the anti-Axl antibody and Axl, *e.g.*, by immunoprecipitation.

It is understood that any of the above embodiments of diagnosis or detection may be carried out using an immunoconjugate of the invention in place of or in addition to an anti-Axl antibody.

The invention also provides methods of determining response to Axl antagonist treatment, comprising detection of expression (polypeptide, nucleic acid) expression of any of the genes shown in Figure 6. In some embodiments, one or more of MYCN, HLX, GAS7, HDAC9, E2F1, CXCR4, PMCH and ANG-2 expression is increased following treatment with an Axl antagonist (such as an anti-Axl antibody). In some embodiments, one or more of IFI44L, GJA4, Axl, IFIT1, SCG5, CYTL1, DPP4 and DKK3 expression is decreased following treatment with an Axl antagonist (such as an anti-Axl antibody). In some embodiments, increased and/or decreased expression is relative expression in an individual who has not been treated with an Axl antagonist (or an individual prior to treatment with an Axl antagonist).

Chemotherapeutic Agents

The combination therapy of the invention can further comprise one or more chemotherapeutic agent(s). The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

The chemotherapeutic agent, if administered, is usually administered at dosages known therefor, or optionally lowered due to combined action of the drugs or negative side effects attributable to administration of the antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner.

Various chemotherapeutic agents that can be combined are disclosed above. In some embodiments, chemotherapeutic agents to be combined are selected from the group consisting of a taxoid (including docetaxel and paclitaxel), vinca (such as vinorelbine or vinblastine), platinum compound (such as carboplatin or cisplatin), aromatase inhibitor (such as letrozole, anastrazole, or exemestane), anti-estrogen (e.g. fulvestrant or tamoxifen), etoposide, thiotepa, cyclophosphamide, methotrexate, liposomal doxorubicin, pegylated liposomal doxorubicin, capecitabine, gemcitabine, COX-2 inhibitor (for instance, celecoxib), or proteosome inhibitor (*e.g.* PS342). In some embodiments, the chemotherapeutic agent is a taxoid and a platinum compound (such as carboplatin).

Formulations, Dosages and Administrations

The therapeutic agents used in the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular subject being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, the drug-drug interaction of the agents to be combined, and other factors known to medical practitioners.

Therapeutic formulations are prepared using standard methods known in the art by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences (20^{th} edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, PA). Acceptable carriers, include saline, or buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; 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, asparagines, 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™, PLURONICS™, or PEG.

Optionally, but preferably, the formulation contains a pharmaceutically acceptable salt, preferably sodium chloride, and preferably at about physiological concentrations. Optionally, the formulations of the invention can contain a pharmaceutically acceptable preservative. In some embodiments the preservative concentration ranges from 0.1 to 2.0%, typically v/v. Suitable preservatives include those known in the pharmaceutical arts. Benzyl alcohol, phenol, m-cresol, methylparaben, and propylparaben are preferred preservatives. Optionally, the formulations of the invention can include a pharmaceutically acceptable surfactant at a concentration of 0.005 to 0.02%.

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's Pharmaceutical Sciences, *supra*.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, 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 γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (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 antibodies remain in the body for a long time, they may denature or aggregate

as a result of exposure to moisture at 37°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.

The therapeutic agents of the invention 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. In the case of VEGF antagonists, local administration is particularly desired if extensive side effects or toxicity is associated with VEGF antagonism. An *ex vivo* strategy can also be used for therapeutic applications. Ex vivo strategies involve transfecting or transducing cells obtained from the subject with a polynucleotide encoding an Axl and/or VEGF antagonist. The transfected or transduced cells are then returned to the subject. The cells can be any of a wide range of types including, without limitation, hemopoietic cells (e.g., bone marrow cells, macrophages, monocytes, dendritic cells, T cells, or B cells), fibroblasts, epithelial cells, endothelial cells, keratinocytes, or muscle cells.

For example, if the Axl and/or VEGF antagonist is an antibody, the antibody is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

In another example, the Axl and/or VEGF antagonist compound is administered locally, e.g., by direct injections, when the disorder or location of the tumor permits, and the injections can be repeated periodically. The Axl oand/or VEGF antagonist can also be delivered systemically to the subject or directly to the tumor cells, e.g., to a tumor or a tumor bed following surgical excision of the tumor, in order to prevent or reduce local recurrence or metastasis.

Where the inhibitor is an antibody, preferably the administered antibody is a naked antibody. However, the inhibitor administered may be conjugated with a cytotoxic agent. Preferably, the conjugated inhibitor and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the conjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

Suitable dosages of any of the therapeutic agents listed herein are those presently used and may be lowered or raised as deemed suitable by the treating physician.

Administration of the therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected). Combination therapy is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner.

The therapeutic agent can be administered by the same route or by different routes. For example, the VEGF and/or Axl antagonist in the combination may be administered by intravenous injection while the protein kinase inhibitor in the combination may be administered orally. Alternatively, for example, both of the therapeutic agents may be administered orally, or both therapeutic agents may be administered by intravenous injection, depending on the specific therapeutic agents. The sequence in which the therapeutic agents are administered also varies depending on the specific agents.

The present application contemplates administration of the VEGF and/or Axl antagonist by gene therapy. See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

It is understood that any of the above therapeutic methods may be carried out using an immunoconjugate of the invention in place of or in addition to an antibody.

D. Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, *etc.* The containers may be formed from a variety of materials such as glass or plastic. The container

holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody or immunoconjugate of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody or immunoconjugate of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

A. Materials and Methods

Antibodies. Antibodies were obtained from the following suppliers: Mouse monoclonal antibody (mAb) against human Axl (clone 6C8, Abnova, Taiwan), phospho-Akt (Ser 473) mouse mAb and Akt polyclonal antibody (Cell Signaling), mouse mAb against human Gas6 (R&D System). ELISA kit for phospho-Axl was purchased from R&D System.

Cell lines and culture. Human carcinoma cell lines were from ATCC. Primary human umbilical vein endothelial cells (HUVECs) and pulmonary artery smooth muscle cells (PASMCs) were from Cambrex (Walkersville, MD) and cultured following vendor's recommendations.

siRNA transfection. siRNA for Axl and luciferase control (GL2) were purchased from Dharmacon. The other Axl siRNAs (Axl2 and 4) based on previously published

sequence (Holland, 2005) were synthesized in house. For siRNA transfection, $3x10^5$ cells/well (6-well plate) HUVECs were transfected with 50 pmol siRNAs using lipofectamine RNAiMax (Invitrogen), and Axl knockdown was quantified 24 to 96 hours post transfection By FACS.

Generating inducible AxlshRNA cell lines. The shRNA vector (pHUSH-GW) comprises an shRNA expression shuttle plasmid and a retroviral vector backbone that contains a TetR-IRES-Puro cassette to enable tetracycline-regulated shRNA expression. 293GP2 cells were transfected using lipofectamine2000 reagent (Invitrogen). 48 hours post transfection, infectious supernatant was collected, filtered and was then incubated with targeting cancer cell lines for 24 hours. Cells resistant to puromycin treatment were pooled to generate stable cell lines that express AxlshRNA.

Co-culture. PASMCs (2.4×10^5 cells /well) were seeded into collagen-coated 12-well plates one day before adding HUVECs. Early-passage HUVECs ($p<5$) were transfected with 50pmol siRNA, and 24-hr post transfection cells were trypsinized and $4x10^4$ cells were placed onto the monolayer of PASMCs. Cells were co-cultured for 3 days and were characterized by staining with FITC conjugated anti-CD31 antibodies. Tubes were viewed and photographed under ImageXpress^{MICRO} imaging system. The total tube length was analyzed by MetaXpress software.

Microarray. HUVECs were transfected with Axl or control siRNA. 72-hour post transfection, RNA was extracted using RNeasy Plus mini kit (Qiagen). mRNA expression profiling of HUVECs was carried out on Affymetrix GeneChip Human Genome U133 Plus 2.0 Array per the manufacturer's protocol. The data analysis and probe sets were described herein.

Generation of anti-Axl monoclonal antibodies. The Axl extracellular domain was expressed in and purified from CHO cells. The purified AxlECD was used to immunize mice. Hybridoma supernatants were screened for their ability to inhibit Gas6-dependent proliferation of Baf3-Axl cells. The selected antibodies were subcloned and purified.

Xenograft experiments. All studies were conducted in accordance with the "Guidance for the Care and Use of Laboratory Animals" (NIH), and approved by Institutional Animal Care and Used Committee (IACUC). The detailed protocols are described herein.

Orthotopic metastasis model. Breast cancer cell line MDA-MB-231 was sequentially infected with AxlshRNA and then dsRedLuc to generate a stable cell line that

expresses both Dox-inducible AxlshRNA and the luciferase reporter gene. 10^5 cells were implanted into mammary fat pats of SCID mice. Mice were divided into Dox-treated and untreated groups (n=5 for each group) the day after implantation. When the primary tumor size reached 1000 mm^3, the tumors were removed. Lungs were removed for bioluminescence imaging 5 weeks after removal of the primary tumors.

Bioluminescence imaging. Mice were injected IP with 200 μL of 25 mg/ml D-luciferin (Invitrogen, Carlsbad, CA) in PBS and were anesthetized during imaging using isoflurane (Henry Schein, Sparks, NV) via nose cone. Bioluminescence images were acquired using a cooled intensified charge-coupled device camera fixed to a light-tight imaging chamber (Stanford Photonics, Palo Alto, CA). Image acquisition times were typically less than 5 minutes, and were processed by co-registering a reference image with the bioluminescence data image. For ex-vivo imaging, lungs were harvested, rinsed in PBS and bathed in 100 μl of 25 mg/ml D-luciferin sodium salt.

Example 1. Axl is frequently over-expressed in cancer cell lines.

We used microarray analysis to screen a large panel of cancer cell lines representing various tumor types for Axl mRNA expression. Axl exhibited a wide range of expression, from barely detectable to extremely high levels, even within the same tissue type (Fig. 1A). Axl was over-expressed in a variety of cancer cell lines, with highest frequency in glioblastoma multiform (GBM) and pancreatic cancer. We used immunoblotting to determine Axl protein levels in selected cancer cell lines. Axl was highly expressed in all the cancer cell lines tested, including GBM, prostate, pancreatic, colon, NSCLC and breast cancers (SH-SY5Y, a neuroblastoma cell line which is known not to express Axl was used as a negative control) (Fig. 1B). In addition, the ligand Gas6 was co-expressed in these cancer cell lines (Fig. 1B). To determine whether Axl is constitutively activated, we measured the level of phosphorylated Axl by ELISA. All cancer cell lines tested had detectable levels of phosphorylated Axl (Fig. 1C). Treatment of cells with exogenously added Gas6 could significantly induce further Axl phosphorylation in A172, Calu1, and H1299 cells, but not in other cell lines. These results suggest that Axl is constitutively activated to various levels in tumor cell lines, probably via autocrine action of endogenous Gas6.

Example 2. Axl Knockdown in cancer cell lines reduces cell viability and attenuates their migration.

To examine the functional significance of Axl expression in tumor growth, migration and metastasis, we established stable cell lines in which the expression of Axl could be knocked down by doxycycline-inducible AxlshRNA. We initially tested six Axl siRNA sequences for their ability to inhibit Axl expression in human umbilical vein endothelial cells (HUVECs). All six Axl-specific siRNAs reduced Axl protein levels by more than 90% as measured by FACS 48-hrs post transfection (Fig. 10A & 10B), whereas the control siRNA had no effect on Axl protein. We then used short hairpin RNA (shRNA) based on one of the Axl siRNAs (Axl-4) to generated stable, inducible Axl knockdown cell lines. These cell lines were generated as pools to
circumvent clonal variation.

Treatment of cancer cell lines stably expressing AxlshRNA with Dox for 72 hours resulted in almost complete inhibition of Axl protein expression (Fig. 2A). Axl knockdown was accompanied by inhibition of downstream signaling events. While Gas6 induced Akt phosphorylation in parental H1299 and H1299AxlshRNA cells without Dox treatment, Gas6-induced Akt phosphorylation was completely abolished in cells with Axl knockdown, similar to cells treated with soluble receptor AxlFc (Fig. 2B).

To test the effect of Axl knockdown on cancer cells *in vitro*, we performed cell proliferation and migration assays. Induction of Axl knockdown by Dox reduced cell viability in A549 (50%) and MDA-MB-231 (33%) (Fig. 2C). While Axl knockdown in H1299 cells did not affect cell viability in the absence of Gas6, it abolished Gas6-mediated increase in viability of cells cultured in low serum (Fig. 2C). Axl knockdown significantly attenuated the motility MDA-MB-231 (40%) and A549 (48%) cells (Fig. 2D). These results suggest that Gas6-Axl signaling promotes cancer cell growth and migration to various degrees depends on the cellular context.

Example 3. Axl knockdown reduces tumor growth in xenograft models.

To evaluate whether Axl expression is required for cancer cell growth *in vivo*, we tested the effect of Axl knockdown on the growth of NSCLC A549 and H1299, and breast cancer MDA-MB-231 xenografts in mice. Cells stably expressing AxlshRNA were implanted subcutaneously in nude (A549 and H1299) or SCID (MDA-MB-231) mice, respectively. For the A549 and H1299 models, mice were randomized into treatment and

control groups after implantation and administered with doxycycline (Dox) or sucrose respectively in drinking water. As compared with untreated mice, Dox-induced Axl silencing resulted in a markedly impaired ability of A549 tumors to grow (Fig. 3A). Dox-induced Axl knockdown in H1299 cells resulted in a moderate (25%) inhibition of tumor growth in nude mice (Fig. 3B). For the MDA-MB-231 model, xenograft tumors were allowed to grow to an average size of 200 mm3; mice were then randomized and divided into untreated or Dox treated groups. Axl knockdown significantly reduced growth of established MDA-MB-231 tumors (33% inhibition) (Fig. 3C). Similar results were obtained in A549 established tumor models (data not shown). The effective knockdown of Axl was verified by Western blotting analysis of tumor lysates at the end of the study (Fig. 3). These results suggest that Axl signaling contributes to the *in vivo* growth of A549, H1299 and MDA-MB-231 tumor xenografts.

Example 4. Axl knockdown inhibits metastasis of MDA-MB-231 breast cancer cells to the lung.

It has been demonstrated that Axl promotes motility and invasiveness of cancer cells *in vitro* (Fig. 2D, Zhang et al, 2008; Tai et al, 2008); however, the role of Axl in cancer metastasis remains to be determined. We approached this question by utilizing an orthotopic model and monitoring tumor metastasis by bioluminescence imaging. We generated a stable MDA-MB-231 cell line that expresses both a Dox-inducible AxlshRNA and a luciferase reporter gene. This cell line displayed high levels of bioluminescence as determined by an *in vitro* luciferase assay (data not shown).

Figure 4A outlines the experimental design for our study. At first we asked whether Axl expression affects dissemination of breast cancer cells to the distant organ sites. Doxycycline treatment was started the day after cell implantation. Primary tumors were well-established 2 weeks after implantation. The primary tumors were removed when they reached the size of 1000 mm3, and metastasis to the lung was monitored for 5 weeks after removal of the primary tumors. Five weeks after removal of the primary tumors, large metastatic foci were detected in the lungs of three out of five mice in the control group, whereas there was no sign of lung metastasis in the Dox-treated animals (n=5) (Fig. 4B, group 1). The presence of tumor foci in the lungs was confirmed by H & E staining (Fig. 4C), and by c-Met (a protein known to be expressed in MDA-MB-231 cells) IHC to definitively mark human tumor cells (not shown). These results suggest that Axl promotes

breast cancer cell metastasize to the lung. We then asked whether Axl expression is required for metastatic growth once cancer cells have colonized the lung. To address this question, we allowed primary tumor size to reach 1000 mm3, removed the primary tumors and then started treatment with Dox. Mice were monitored for 5 weeks after removal of primary tumors. Both the control and the Dox treated mice had metastatic foci (3 out of 5 in each group) (Fig. 4B, group 2). These results suggest that Axl is not critical for metastatic loci growth once cancer cells established themselves in the lung.

Example 5. Axl knockdown in HUVECs impairs endothelial tubule formation.

Previous study has shown that Axl is over-expressed not only in tumors but also in the surrounding vascular cells (Hutterer, et al). To evaluate further the potential role of Axl in the regulation of angiogenesis, we tested the effect of Axl knockdown on endothelial tube formation. FACS analysis demonstrated expression of Axl on the surface of HUVECs (Fig. 5A). 48-hrs post transfection, Axl protein level was down regulated more than 90% in HUVECs transfected with Axl specific siRNA, whereas the control luciferase siRNA (GL2) had no effect (Fig. 5A). Axl knockdown in HUVECs did not significantly affect cell growth (Fig. 10C).

We next used a primary HUVEC/PASMC (pulmonary artery smooth muscle cell) co-culture branching tube formation assay to assess the importance of Axl in angiogenesis. Tube formation as quantified by tube length was reduced ~40% in HUVECs transfected with Axl siRNA compared with cells transfected with the control siRNA (Fig. 5B). These results suggest that Axl is involved in the regulation of endothelial tube morphogenesis.

Example 6. Axl modulates expression of angiogenic factors Dickkopf-homologue 3 (DKK3) and angiopoietin-2 (Ang-2).

To gain insight into the mechanisms involved in the regulation of angiogenesis by Axl, we performed gene profiling in HUVECs transfected with Axl or control siRNAs. Fig. 6A lists the top eight genes that were up or down-regulated in HUVECs after Axl knockdown. Two of the genes, Ang-2 and DKK3, are known regulators of angiogenesis. We confirmed the microarray results by quantitative RT-PCR for DKK3 and Ang-2 (Fig. 6B). In addition, the amount of the DKK3 and Ang-2 proteins in HUVEC lysates and in the culture medium also changed following Axl knockdown as measured by ELISA (Fig. 11).

To understand the relationship between Axl and DKK3 or Ang-2, we tested the effect of DKK3 and Ang-2 siRNA knockdown on Axl expression. Knockdown of neither DKK3 nor Ang-2 affected Axl protein level (Fig. 11). In addition, knockdown of DKK3 had no effect on Ang-2, nor did Ang-2 knockdown affect DKK3 expression (Fig. 11). We next tested the effect of DKK3 and Ang-2 siRNAs on endothelial tube formation. Knockdown of DKK3 by RNAi reduced tube formation to a similar extent as did Axl knockdown, whereas Ang-2 siRNA alone had no significant effect on tubule formation (Fig. 6C). These results suggest that both DKK3 and Ang-2 are down-stream targets of Axl and that their expression may be regulated by different signaling pathways.

Example 7. Axl knockdown has additive effect with anti-VEGF.

Because VEGF is a major factor that regulates endothelial cell functions, we tested the effect of Axl knockdown together with anti-VEGF monoclonal antibody in tube formation assay. Treatment of cells with anti-VEGF alone resulted in dose-dependent reduction in tube formation (not shown), Axl knockdown together with anti-VEGF resulted in enhanced inhibition of tube formation (Fig. 7), similar results were seen with DKK3 knockdown (Fig. 7). These results suggest down-regulation of Axl expression has an additive effect with anti-VEGF to inhibit endothelial tube formation.

Example 8. Generation of monoclonal antibodies that block Axl function.

Our validation data using RNAi suggested that Axl is involved in tumor growth, metastasis as well as in the regulation of angiogenesis. We therefore set out to develop monoclonal antibodies against Axl. We developed a cell-based assay to directly test the ability of Axl mAbs to block Axl/Gas6 mediated cell growth. We generated a stable cell line in Baf3 pro-B cells that over-expresses Axl (Fig. 12A). Expression of Axl in Baf3 cells resulted in Gas6-dependent growth (Fig. 12B), which could be inhibited by AxlFc in a dose-dependent manner (Fig. 12C). We immunized mice with human AxlECD, and screened a panel of hybridoma supernatants using this assay. We identified four antibodies that blocked Gas6-induced Baf3Axl cell growth (Fig. 12D). Purified antibodies showed dose-dependent inhibition of Baf3Axl cell growth, with the strongest blocker, 12A11, exhibiting an IC50 of ~100 ng/ml (Fig. 8A).

To understand the mechanisms of Axl inhibition by these mAbs, we further characterized these antibodies. Axl mAbs 3G9 and 8B5 blocked ligand binding to the

receptor in solid phase ELISA, whereas 12A11 and 4F8 had no effect on ligand binding (Fig. 8B1). We confirmed this result by using FACS to determine the ability of Axl mAbs to block Gas6 binding to cell surface Axl on A549 cells (Fig. 8B2). Axl mAbs 12A11, 8B5 and 3G9 down-regulated receptor expression in A549 cells, whereas 4F8 had no effect (Fig. 8C). 4F8 was the only antibody that detected Axl in Western blotting, suggesting that it recognizes a linear epitope (data not shown). In addition, 12A11 and 3G9 inhibited Gas6-induced Axl phosphorylation in H1299 cells (Fig. 8C).

These monoclonal antibodies did not cross-react with murine Axl (Fig. 13A), nor did they cross-react with related receptors Tyro3 and Mer (Fig. 13B). The Axl mAbs did not compete with each other in crossblocking assays (data not shown), suggesting that they bind different epitopes. To localize the binding epitopes of mAbs, different portions of Axl ECD were *in vitro* transcribed and translated (Fig. 8D) and then used as antigens in ELISA. The epitopes for 3G9 and 8B5 appeared to be located within the first Ig-domain, consistent with their ability to block ligand binding to the receptor, while the epitopes for 12A11 and 4F8 were mapped to the first fibronectin domain (Fig. 8D).

Example 9. Axl mAbs inhibit A549 NSCLC xenograft tumor growth.

To evaluate whether mAb inhibition of Axl affects tumor cell growth *in vivo*, A549 cells were implanted subcutaneously in nude mice. When the tumor size reached 100 mm3 (Day 0 in Fig. 9A), animals were randomized and treated with either Axl mAbs or a control antibody at 30 mg/kg, twice weekly. Axl mAbs significantly attenuated A549 tumor growth compared with control, around 40% inhibition 40 days post dosing (Fig. 9A). To investigate the mechanism of tumor growth inhibition by Axl mAbs, we performed a pharmacodynamic study. Tumors were excised at 0, 24, 48, and 72 hours after administration of antibodies and tumor lysate was generated. Western blotting analysis showed that the Axl mAbs down-regulated Axl expression in tumors at 24 hours post dosing, which was sustained through 72 hours (Fig. 9B). Ki67 and Caspase 3 staining of tumor samples showed that treatment of tumors with Axl mAb 12A11 resulted in 20% decrease in Ki67 staining (Fig. 9C) and approximately 50% increase in caspase 3 staining (not shown) 72 hours post dosing. These results suggest that Axl mAbs attenuate A549 xenograft growth by downregulation of receptor expression, which in turn leads to increased apoptosis and deceased cell proliferation of tumor cells. These results are consistent with Axl knockdown in A549 cells,

confirming that Axl plays an important role in the regulation of tumor growth of this NSCLC cell line.

Discussion

In this study we have investigated various roles of Axl in tumorigenesis including tumor growth, metastasis as well as angiogenesis. We used inducible shRNA to inhibit Axl expression in human cancer cell lines and demonstrated that Axl expression promotes tumor growth in NSCLC and breast cancer models. Previous studies showed that Axl expression promotes tumor growth in xenograft models of glioblastoma (Vajkoczy et al, 2006) and breast cancer (Holland et al, 2005), using an Axl dominant negative mutant or shRNA, respectively. However, in both of these studies, tumor growth rate was compared between a control cell line and a cell line in which either Axl or its signaling was down regulated prior to implantation into mice. Although this approach is useful, it does not assess the direct impact of Axl inhibition on tumor growth. Our approach took the advantage of inducible expression of AxlshRNA and measured the growth of the same cell line in the presence or absence of Axl expression. Our results show that the impact of Axl expression on tumor growth depends on the cell model used. While Axl knockdown in A549 NSCLC almost completely inhibited tumor growth, the effect of Axl knockdown on H1299 NSCLC and MDA-MB-231 breast cancer was moderate (about ~30% growth inhibition). The higher sensitivity of A549 cells to Axl knockdown may be due to the presence of an increased gene copy number of Axl in these cells (unpublished result).

Metastasis accounts for approximately 90% of all cancer deaths; and of all processes involved in carcinogenesis, local invasion and formation of metastasis are clinically the most relevant. Recent experimental progress has identified several molecular pathways and cellular mechanisms that underlie the multistage process of metastasis. These include tumor invasion, tumor cell dissemination through the bloodstream or lymphatic system, colonization of distant organs and outgrowth of metastases (Christofori, 2006). Axl expression is associated with invasiveness and metastasis in various cancers including breast (Meris, et al, 2002; Zhang et al, 2008), lung (Shieh et al, 2005) and gastric (Sainaghi et al, 2005) cancers, as well as in glioblastoma (Hutterer et al, 2008). The involvement of Axl in promoting cancer cell migration and invasion has been demonstrated *in vitro* (Zhang et al, 2008; Tai et al, 2008; Vajkoczy et al., 2006). In breast cancer models, ectopic expression of Axl was sufficient to confer a highly invasive phenotype to weakly invasive

MCF7 cells. Conversely, inhibition of Axl signaling by shRNA knockdown or an anti-Axl antibody decreased mobility and invasiveness of highly invasive breast cancer cells (Zhang et al, 2008). Although these studies established a role for Axl in promoting cell migration and invasion *in vitro*, whether expression of Axl results in metastasis has not been elucidated. In this study we have used an orthotopic breast cancer model to investigate the functional significance of Axl in metastasis. Our results show that Axl expression is required for MDA-MB-231 cells to establish metastatic foci in the lung, since Axl knockdown by shRNA completely abolished the ability of cells emerging from the primary tumors to colonize the lungs. However, once metastatic foci had established in the lung, Axl did not seem to have a significant impact on the out growth of the metastases. These results implicate Axl in early stages of MDA-MB-231 cancer cell metastasis, and provided the first *in vivo* evidence that directly links Axl to metastasis.

Gas6/Axl signaling plays an important role in vascular biology by modulating survival and migration of vascular smooth muscle cells and endothelial cells (Melaragno et al, 1999). The expression of Gas6/Axl is up regulated in injured arteries and Gas6 induces class A scavenger receptor through activation of PI3K/Akt, leading to the formation of foam cells, an important step in atherosclerosis (Cao et al, 2001). Activation of Axl signaling mediates anti-apoptotic effects of laminar shear stress in endothelial cells possibly through its association with αvβ3 integrin complex (D'Arcangelo et al., 2006). Axl and Gas6 are co-expressed in tumor associated vascular cells in gliomas (Hutterer *et al.*, 2008). In primary human breast cancer samples, we found strong Axl staining in tumor stromal cells (unpublished observation). Therefore Axl may impact tumorigenesis not only in tumor cells but also by modulating tumor stromal function such as angiogenesis. In this study, we have performed experiments to understand the mechanisms involved in the regulation of endothelial cell functions by Axl. Our data shows that Axl expression is not required for endothelial cell proliferation, as Axl knockdown by RNAi had little impact on the growth of these cells. However, inhibition of Axl expression impaired endothelial tube formation.

To understand the mechanisms involved, we performed mRNA expression profiling to identify genes that may be modulated by Axl expression. Axl knockdown resulted in the down-regulation of DKK3 and up-regulation of Ang-2, two factors known to be involved in angiogenesis. DKK3 is among the Dickkopf family of secreted modulators of Wnt signaling (Krupnik et al, 1999), and is strongly expressed in the developing heart and blood vessels of mice and chickens (Monaghan et al, 1999). A recent study demonstrated DKK3 protein

expression in blood vessels of highly vascularized neoplasms, including GBM, high-grade non-Hodgkin's lymphoma, melanoma and colorectal carcinomas (Monaghan et al, 1999). Stable over-expression of murine DKK3 in B16F10 cells significantly increased microvessel density in the C57/BL6 melanoma model. In addition, an *in vitro* study in primary endothelial colony forming cells showed that siRNA knockdown of DKK3 did not significantly affect cell proliferation, but decreased tube formation in matrigel (Untergasser et al, 2008). These results are similar to our findings in HUVEC after Axl or DKK3 knockdown. Axl knockdown in HUVECs led to up regulation of Ang-2. The angiopoietin signaling system plays a key role in the regulation of angiogenesis, vascular homeostasis and vascular regression (Yancopoulos et al, 2000). This signaling system consists of angiopoietin 1 and 2 and their receptor Tie2 (Dumonut et al, 1994). Ang-1 is an agonist that supports EC survival and endothelium integrity (Suri et al, 1998) through the PI3K/Akt signaling pathway (Peters et al, 2004). On the other hand, Ang-2 (Maisonpierre et al, 1997) is a signaling antagonist and inhibits the action of Ang-1 to promote vessel destabilization. In the presence of VEGF, Ang-2 provides an important angiogenic stimulus (Yancopoulos et al, 2000). However, when VEGF signaling is inhibited or absent, the destabilizing action of Ang-2 on vessels leads to cell death and capillary regression (Yancopoulos et al, 2000). Our results demonstrate that Axl knockdown is additive with anti-VGEF to inhibit endothelial tube formation. This additive effect may be mediated through up- regulation of Ang-2 when Axl expression is inhibited. Taken together, our results suggest the following model for modulation of endothelial cell function by Axl. Expression of Axl in EC results in up-regulation of the pro-angiogenic factor DKK3 and down regulation of Ang-2, which in turn facilitates signaling of Ang-1/Tie2 and results in the increase in angiogenesis.

The multiple roles that Axl plays in tumorigenesis make it an attractive therapeutic target for cancer. Anti-Axl antibodies are specific reagents that can be used to investigate the role of Axl in tumorigenesis, and provide therapeutic potential to inhibit Axl-dependent signaling in patients. An antagonistic mAb can achieve its function by various mechanisms: (1) blocks ligand binding to the receptor, (2) promotes receptor degradation (3) blocks receptor dimerization, or (4) mediates ADCC. Since many tumors may exhibit ligand-independent activation of Axl, it is important to develop and compare mAbs that act through different modes of action, and select the lead mAb based on efficacy. In this study, we have characterized a panel of hybridoma antibodies against Axl. We developed a cell-based screen for monoclonal antibodies that block Axl biological function. The mAbs we

characterized here include two (3G9 & 8B5) that block ligand binding to the receptor, and one (12A11) that does not interfere with ligand binding, but inhibits receptor phosphorylation. All three mAbs induce down regulation of receptor expression. Most importantly, these mAbs attenuated A549 NSCLC tumor growth. These results not only confirm the role of Axl in promoting tumor growth as indicated by the shRNA studies, but also suggest that monoclonal antibodies may provide an effective strategy for treatment of cancers that over-express Axl. Since these mAbs do not cross-react with murine Axl, it was not possible to assess the importance of Axl for tumor angiogenesis *in vivo* and its impact on the tumor stroma. We are currently developing cross-reacting mAbs to investigate these potential functions of Axl *in vivo*.

In summary, we have validated a role for Axl in promoting tumor growth and provided the first evidence that Axl expression is required for metastasis of breast cancer cells to the lung. We demonstrated that Axl enhances endothelial tube formation by acting through the angiopoietin and DKK3 signaling system. Our data suggests that therapeutic antibodies against Axl could block Axl functions not only in the tumor itself but also possibly in the surrounding stroma. The additive effect of Axl inhibition with anti-VEGF suggests that blocking Axl function may enhance anti-angiogenic therapy.

Example 10

Materials and Methods

Antibodies and cell lines. Antibodies were obtained from the following suppliers: Mouse monoclonal antibody (mAb) against human Axl (Abnova, Taiwan), phospho-Akt mouse mAb and Akt polyclonal antibody (Cell Signaling). Mouse recombinant Gas6 and ELISA kit for phospho-Axl were purchased from R&D System. Human carcinoma cell lines were obtained from ATCC, and cultured in PRMI1640 medium supplemented with 10% FBS. Hybridoma anti-human Axl monoclonal antibodies 12A11 and 3G9 were provided by Genentech (see Li (2009)).

Generation of phage anti-Axl monoclonal antibodies. For antibody generation, human phage antibody libraries with synthetic diversities in the selected complementary determining regions (H1, H2, H3), mimicking the natural diversity of human IgG repertoire were used for panning. Fab fragments were displayed bivalently on the surface of M13 bacteriophage particles (Lee *et al,* 2004). The phage antibody libraries were panned against human and murine Axl ECD in alternative rounds. Phage antibodies that bound to human Axl ECD-His and murine Axl ECD-Fc fusion protein were identified by ELISA and DNA

sequencing, and antibody clones were reformatted to express full-length IgGs (Liang *et al,* 2007). Individual clones were transiently expressed in mammalian cells and purified with protein A columns (Carter *et al,* 1992).

Phage clones were screened for their ability to inhibit Gas6-dependent proliferation of Baf3-Axl cells. Two clones that exhibited highest potency in inhibition of Baf3Axl cell proliferation were chosen for affinity maturation.

For affinity maturation, phagemid displaying monovalent Fab on the surface of M13 bacteriophage (Liang *et al,* 2007) served as the library template for grafting light chain (V_L) and heavy chain (V_H) variable domains of the phage Ab. A soft randomization strategy was adopted for affinity maturation as described (Liang *et al,* 2007), and a high-throughput single-point competitive phage ELISA was used to rapidly screen for high-affinity clones as described (Sidhu *et al,* 2004).

Affinity Measurement of anti-Axl antibodies. For binding affinity determinations of anti-Axl antibodies, surface Plasmon Resonance (SRP) measurement with a BIAcore™-3000 instrument was used. To measure the affinity between anti-Axl antibodies and human Axl ECD-His protein, Anti-Axl human IgG was captured by CM5 biosensor chips coated with mouse anti-human IgG to achieve approximately 250 response units (RU). For kinetics measurements, two-fold serial dilutions of human Axl ECD-His (440nM – 28nM) were injected in PBT buffer (PBS with 0.05% Tween 20) at 25°C with a flow rate of 30μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) were calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2). The equilibrium dissociation constant (K_D) was calculated as the k_{off}/k_{on} ratio. To measure the affinity of anti-Axl antibodies to murine Axl ECD-Fc fusion protein, murine Axl ECD human IgG fusion protein was captured by CM5 biosensor chips coated mouse anti-human IgG to achieve approximately 150 response units (RU). For kinetics measurements, two-fold serial dilutions of the anti-Axl Fab fragment (200nM – 12nM) were injected in PBST buffer (PBS with 0.05% Tween 20) at 25°C with a flow rate of 30μl/min.

Cell proliferation assay. Cells were seeded at 5000 cells/well in 96-well plates and treated with Axl mAb at various concentrations for 72 hours. Cell proliferation was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer's instructions.

ELISA and Fluorescence-activated cell sorting (FACS). ELISA assays were performed as follows: goat anti-human IgG Fc coated plates were blocking with 0.5% BSA,

PBS, 0.05% Tween 20 (PBST). For cross reaction assay, coated plates were incubated with human Axl.Fc, mouse Axl.Fc or human Mer.Fc, Tyro-3.Fc for 1 hr at room temperature, washed four times in PBST, incubated with anti-Axl mAbs and HRP-conjugated anti-mouse Ig. For binding assay, coated plates were incubated with human Axl.Fc for 1 hr at room temperature, washed four times in PBST, incubated with rmGas 6 and anti-Axl mAbs for 1 hr at room temperature. Plates were washed four times in PBST, incubated with biotinylated anti-mGas6 and strepavidin-HRP. Secreted Ang-2 and DKK3 was measured using R&D ELISA kit, according to the manufacturer's instruction. Axl expression on cell surface was determined by FACS using standard techniques. Briefly, cells were harvested, stained with anti-Axl mAb (12A11, 10ug/ml) for 30 mins on ice, washed twice in PBS, and then staining with PE-conjugated second antibody. To determine antibodies blocking of Gas6 binding to Axl on cell surface, cells were harvested, stained with anti-Axl mAbs for 30-mins and incubated with rmGas6 for 30-mins on ice. They were washed twice in PBS, and stained with biotinylated anti-Gas6 and PE-conjugated strepavidin. Samples were analyzed on BD FACScalibur Flow Cytometer.

Xenograft experiments. All studies were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH), and approved by the Institutional Animal Care and Used Committee (IACUC). A total of $5X10^6$ (A549) or 10^7 cells in matrigel (MDA-MB-231) were implanted subcutaneously into right flank of nude (A549) or SCID mice (MDA-MB-231), respectively. When the average tumor sized reached 100 mm^3, mice were randomized and divided into different treatment groups (n=10 for each group). Anti-Axl or control IgG1 antibodies were at administrated at 10-30 mg/kg, anti-VEGF at 1-2 mg/kg, via intraperitoneal injection (IP), twice weekly. Erlotinib was administrated by oral gavage at 100 mg/kg/day. Paclitaxel and carboplatin were administrated subcutaneously at 6.25 mg/kg/day for 5 days, and 100 mg/kg for a single dose, respectively, at the beginning of the treatment. Statistic analyses were performed using Anova two-way for comparison of tumor growth in different treatment groups.

For pharmacodynamic (PD) studies, mice were treated with antibodies for 0, 24, 72 and 168 hours. At each time point, tumors were excised, processed for immunohistochemical staining and image analysis, and used to generate cell lysates for Western blot analysis.

For metastasis studies, $5X10^5$ MDA-MB-231 cells stably transfected with a luciferase reporter gene (Li *et al,* 2009) were implanted into SCID mice via tail vein

injection. Metastasis of tumor cells to various organs was monitor by bioluminescence detection as described (Li *et al,* 2009).

Immunohistochemistry. Xenograft tumor samples were fixed in 10% neutral buffered formalin, processed, embedded in paraffin, and sectioned at 4μm. Thin sections were then treated with primary antibodies for Ki67, cleaved caspase 3 and MECA32, followed by biotinylated secondary antibodies and the DAB chromagen.

Primary human breast cancer tissue microarray was obtained from Cureline Inc, including ductal and metastatic adenocarcinomas. Axl IHC was performed using anti-Axl monoclonal antibody described previously (Li et al, 2009), and macrophages were stained using CD68. For dual Axl/CD68 IHC, Axl staining was performed first at 2 μg/ml using Vector ABC Elite HRP reagents and DAB substrate. CD68 staining was run sequentially at 0.5 μg/ml, also using ABC Elite-HRP reagents but Vector SG chromogen (Blue/Grey) instead. A second target antigen retrieval step was performed in between the two complexes to elute off the first complex in order to avoid the cross reactivity of the two markers.

Vascular density measurement and data analysis. Tumor samples were stained with MECA32, a pan endothelial cell marker. Images were acquired by the Ariol SL-50 automated slide scanning platform (Genetix Ltd.; Hampshire, UK) at 100x final magnification. Tumor-specific areas were exported for analysis in the Metamorph software package (MDS Analytical Technologies; Ontario, Canada) as individual 8-bit images. The brown DAB-specific staining was isolated from the Hematoxylin counterstain using a blue-normalization algorithm as described (Brey *et al,* 2003). A segmentation algorithm identified vessels, and removed noise based on size and shape. Cells were identified as either tumor or non-tumor based on size, shape, and density of Hematoxylin staining. Non-tumor areas were identified by the density of non-tumor cells versus tumor cells. After analysis was complete, images were reviewed manually to remove artifacts identified incorrectly as vessels or tumor areas. Area measurements were recorded for individual vessels, as well as the tumor and non-tumor areas in each image. Raw values based on image analysis were analyzed using the JMP 8.0 software (SAS Institute, Inc. North Carolina, USA). A student's t test was performed to compare each pair of means, with $p < 0.05$.

Isolation of tumor-associated macrophages (TAM) and detection of secreted cytokines. Tumors were dissected, chopped into small pieces and incubated in RPMI1640 medium with 2.5% FBS, 0.2 units/ml Liberase Blendzymes II and 5 units/ml DNaseI,

(Roche). Tumor cells were disassociated using MACS Dissociator (Miltenyi Biotec) and maintained for 20 minutes at room temperature. EDTA (final concentration 0.002%) was added to stop the reaction. Single cell suspensions were prepared, and red blood cells were removed using RBC lysis buffer (eBioscience). Cells were resuspended at 10^7cells/ml in PBS containing 1% FBS and incubated with 20μg/ml FcRII, III and IV for 20 minutes. Anti-F4/80-PE (eBioscience) and anti-CD11c-APC (BD Pharmingen) (0.2μg/10^6 cells) were added and incubated for 30 minutes on ice. F4/80 and CD11c positive cells were sorted by FACSAria (BD Biosciences). A total of $2x10^5$ F4/80 and CD11c positive cells were seeded in 96-well plate and cultured overnight. Cultured media was collected and the levels of cytokines and chemokines were determined using Bio-Plex mouse cytokine assays (Bio-Rad) according to manufacturer's instructions.

Epitope Mapping

Axl-2 (PRK HuAxl(1-134Aa)/HuIgG1Fc), Axl-3 (PRK HuAxl(1-221Aa)/HuIgG1Fc), Axl-4 (PRK HuAxl(1-324Aa)/HuIgG1Fc), and Axl-5 (PRK HuAxl(1-435Aa)/HuIgG1Fc) plasmid constructs were made by standard molecular-biology techniques. All plasmids were confirmed by direct sequencing and/or restriction digestion. Plasmids encoding various portion of Axl extracellular domain (aa1-134, aa1-221, aa1-324, aa1-435) were in vitro transcribed and translated using Promega L2080 TNT SP6 Quick transcription/translation system kit and were used as antigens in ELISA. Axl portions used in these experiments included:

Amino acids 1-134 of human Axl (comprising Ig1 of Axl):

MAWRCPRMGRVPLAWCLALCGWACMAPRGTQAEESPFVGNPGNITGARG LTGTLRCQLQVQGEPPEVHWLRDGQILELADSTQTQVPLGEDEQDDWIVVSQLRITS LQLSDTGQYQCLVFLGHQTFVSQPGYVG (SEQ ID NO:18), and

amino acids 221-324 of human Axl (comprising Axl fibronectin domain):

ITVLPQQPRNLHLVSRQPTELEVAWTPGLSGIYPLTHCTLQAVLSDDGMGIQ AGEPDPPEEPLTSQASVPPHQLRLGSLHPHTPYHIRVACTSSQGPSSWTHWL (SEQ ID NO:19).

Antibody competition experiments.

To determine whether phage anti-Axl mabs YW327.6 or YW327.42 competed with hybridoma anti-Axl mAbs 12A11 and 3G9, A549 cells were co-stained with an anti-Axl phage antibody (each at 50ug/ml) and a hybridoma anti-Axl antibody (each at 10ug/ml) for 30 minutes. Cells were washed twice in PBS, and stained with anti-mouse Ig-PE. Samples

were analyzed on BD FACScalibur Flow Cytometer (BD Biosciences) as described above for FACS analysis.

Results

Generation of a phage derived monoclonal antibody that blocks Axl function.

To identify antibodies that cross-react with murine and human Axl, we employed phage-displayed antibody libraries with synthetic diversities in the selected complementary determining regions, mimicking the natural diversity of human IgG antibodies (Lee *et al,* 2004). Phage antibodies that bound to both human and murine Axl ECD were identified by ELISA and DNA sequencing, and antibody clones were reformatted to express full-length IgGs (Liang *et al,* 2007). A panel of full length IgGs was then screened for their ability to inhibit Gas6-dependent growth of Baf3Axl cells (Li *et al,* 2009), and one of the clones YW327.6 was affinity matured and purified.

Affinity matured Axl mAb YW327.6S2 binds to both human and murine Axl with high affinity, with a Kd of about 1 nM, and 545 pM, respectively (Fig. 14A). Specifically, Ka was 1.7 X 10^5, kd was 1.7 X 10^4, and KD was 9.9 X 10^{-10}. This antibody also binds to cynomolgus Axl, but it does not cross-react with related receptors Tyro3 and Mer (Fig. 14B). YW327.6S2 blocks binding of ligand Gas6 to Axl as demonstrated in both a cell free ELISA and on cell surface by FACS, in a dose-dependent manner (Fig. 14C).

Affinity matured Axl Mabs YW327.6S11, YW327.42S8 and YW327.42S31 were also characterized. YW327.6S11, YW327.42S8 and YW327.42S31 bind both human and murine Axl with high affinity. For example, biacore analysis of antibody binding to human Axl resulted in the following:

	Hu Axl		
	Ka	kd	KD
YW327.6S11	1.7 X 10^5	1.7 X 10^4	1.3 X 10^9
YW327.42S8	5.2.X 10^4	1.3X 10^4	2.5 X 10^9
YW327.42S31	6.3 X 10^4	1.5 X10^4	2.4 X 10^9

These antibodies do not cross-react with Tyro3 and Mer. YW327.6S2 (and parent antibody YW 327.6) blocks binding of ligand Gas6 to Axl, while YW327.42S8 and YW327.42S31 (and parent Mab YW327.42) do not block binding of ligand Gas6 to Axl.

YW327.6S2 comprises a heavy chain variable regions comprising sequence

EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPYR

GYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSSVG YAMDYWGQGTLV (SEQ ID NO:1) and a light chain variable region comprising sequence DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKR (SEQ ID NO:2).

Axl epitope analysis

Axl antibody binding to various portions of human Axl extracellular domain was analyzed using ELISA. The results were obtained.

	YW327.6	YW327.42
Axl2 (aa1-134)	+	-
Axl-3 (aa1-221)	+	-
Axl-4 (aa1-324)	+	+

YW327.6 bound to Axl-Fc fusions comprising amino acids 1-134 of human Axl. By contrast, YW327.42 bound an Axl-Fc fusion comprising amino acids 1-324 of human Axl, but did not bind Axl Fc fusions comprising amino acids 1-134, and 1-221. Thus, we concluded that YW327.6 binds a polypeptide consisting of amino acids 1-134 of human Axl, and that human Axl amino acids 222-234 was required for YW327.42 binding. Amino acids 1-134 contains Axl IgI domain, and amino acids 222-234 contain Axl fibronectin domain.

Competition experiments with anti-Axl hybridoma antibodies

We determined whether antibodies YW326.6 and YW327.42 could compete for human Axl binding with anti-Axl hybridoma antibodies 12A11 and 3G9 (Li, (2009)). In antibody competition binding experiments to human Axl, Antibody YW327.6 did not compete for human Axl binding with either of hybridoma antibodies 12A11 or 3G9, demonstrating that these antibodies do not recognize the same epitopes. Antibody YW327.42 competed for human Axl binding with hybridoma antibody 12A11, but did not compete for human Axl binding with hybridoma antibody 3G9.

YW327.6S2 down regulates Axl expression, inhibits its activation, signaling and Gas6-dependent Baf3Axl cell proliferation. To test whether YW327.6S2 affects Axl biological functions, we first evaluated its effect on Axl expression and signaling. Treatment of NSCLC cell line A549 with YW327.6S2 resulted in rapid down-regulation of Axl

expression on cell surface (Fig 14D, upper panel) and this down regulation is sustained for 24 hrs (Fig. 14D, lower panel). Gas6 treatment of H1299 NSCLC cells induces Axl phosphorylation that was inhibited when cells were pre-incubated with YW327.6S2 (Fig. 14E, upper panel). Consequently, pre-incubation of H1299 cells with YW327.6S2 blocks Gas6-induced phosphorylation of the down-stream signaling molecule Akt (Fig. 14E, lower panel). Down-regulation of Axl expression and inactivation of its signaling by YW327.6S2 potently inhibited Gas6-dependent growth of Baf3Axl cells, with an IC50 of 340 ng/ml (Fig. 14F).

YW327.6S2 reduces A549 xenograft growth and enhances the effect of Anti-VEGF. In a previous study, we showed that inhibition of Axl by either RNAi or treatment with anti-human Axl hybridoma monoclonal antibodies significantly attenuated A549 NSCLC tumor growth (Li *et al,* 2009). We therefore first tested the effect of YW327.6S2 on tumor growth in this model. YW327.6S2 alone at 10 mg/kg, twice a week dosing regimen significantly reduced A549 tumor growth (Fig. 15A), and this inhibitory effect is comparable to that of anti-human Axl hybridoma antibodies (Fig. 15C).

Axl is expressed on endothelial cells and enhances VEGF-induced endothelial tubule formation (Li *et al,* 2009; Holland *et al*, 2005); we tested whether anti-Axl mAb could enhance the anti-tumor growth property of anti-VEGF (Liang *et al*, 2006). Anti-VEGF antibody alone and YW327.6S2 alone had similar effects on A549 tumor growth (Fig. 15A). Combination of the two antibodies together resulted in enhanced tumor growth inhibition compared with either antibody alone, with 30% inhibition by single agent versus 60% inhibition by the combination treatment (Fig. 15A).

Animals in this study were dosed twice weekly for 60 days and followed to day 85 to examine the delay in tumor growth (animals were removed from the study when tumor sizes exceeded 800 mm^3; no animals were removed as a result of toxicity). YW327.6S2 in combination with anti-VEGF significantly delayed tumor growth as compared to a single agent (Fig. 15A). There was no tumor re-growth in the combination treatment group during the time elapsed from the last dose to the end of the experiment (day 85), which lead to the survival of all the animals in this group at the end of the experiment as shown in the Kaplan-Meier plot (Fig. 15B).

The anti-human Axl hybridoma monoclonal antibody 12A11, which does not cross-react with murine Axl, significantly attenuated A549 xenograft tumor growth (Li *et al,*

2009), it also enhances the effect of anti-VEGF as shown in Fig. 15C. This is expected, since 12A11 directly inhibits tumor cell growth and anti-VEGF affects tumor vasculature.

YW327.6S2 down-regulates receptor expression and induces apoptosis of A549 tumor cells. To begin to understand the mechanisms that mediate YW327.6S2 effect on reducing tumor growth, we performed a pharmacodynamic study. A549 tumor bearing mice was treated with YW327.6S2, and tumors were excised at 0, 24, 72, and 168 hrs post dosing. Western blotting analysis of tumor lysates showed that Axl expression was down-regulated 24hr after antibody administration and sustained over 168 hrs (Fig. 15D), suggesting that the anti-tumor growth effect of YW327.6S2 is mediated in part by down regulation of Axl expression.

To determine whether YW327.6S2 has direct effect on tumor cell proliferation and apoptosis, A549 xenograft tumors treated with control or YW327.6S2 for two weeks were excised and cleaved caspase 3 (CC3) and Ki67 IHC were performed. Tumors treated with YW327.6S2 exhibited increased CC3 compared with the control (Fig. 15E), suggesting that YW327.6S2 induces apoptosis of tumor cells. There was no significant difference in Ki67 positive nuclei between control and YW327.6S2 treated tumors, suggesting that YW327.6S2 does not directly affect tumor cell proliferation.

To investigate whether YW327.6S2 affects tumor-associated vasculature, we treated A549 tumor bearing mice with YW327.6S2 alone or in combination with anti-VEGF. Tumors were excised and stained with MECA32, a pan endothelial marker, to examine the intratumoral vascular density. YW327.6S2 alone did not significantly reduced vascular density compared with control but combination with anti-VEGF resulted in significant decrease of the tumor associated vascular density (Fig. 15F). In contrast, 12A11 has no significant effect on intratumoral vascular density by itself or in combination with anti-VEGF.

YW327.6S2 enhances the effect of erlotinib and chemotherapy. To test whether YW327.6S2 could enhance the therapeutic index of standard care for NSCLC, we performed combination treatment of YW327.6S2 with EGFR small molecule inhibitor (SMI) erlotinib and chemotherapy.

A549 contains wild type EGFR and is only moderately sensitive to erlotinib (Yauch *et al*, 2005); we therefore investigated whether anti-Axl mAb can sensitize these cells to EGFR SMI. YW327.6S2 and erlotinib when administrated as a single agent resulted in 30% reduction in tumor growth but in combination reduced the tumor growth rate by more than

50% (Fig. 16A), suggesting that anti-Axl mAb enhances the anti-tumor growth effect of erlotinib.

We then investigated whether anti-Axl mAb was able to enhance the therapeutic index of standard chemotherapy for NSCLC. Mice bearing A549 xenografts were treated with one cycle of chemotherapy consisting paclitaxel (6.25 mg/kg/day, 5 days) and carboplatin (100 mg/kg, one dose) administrated at the beginning of the treatment (day 0, Fig. 16B). Chemotherapy alone has similar effect on tumor growth as YW327.6S2 administrated alone, and combination of the two resulted in enhanced inhibition of tumor growth (Fig. 16B).

YW327.6S2 reduces vascular density and inhibits inflammatory cytokine secretion from tumor-associated macrophages in MDA-MB-231 breast cancer xenograft model. Since Axl knockdown by shRNA has only moderate effect on MDA-MB-231 xenograft tumor growth (Li *et al,* 2009), we asked whether YW327.6S2 is efficacious in this model. YW327.6S2 alone was able to reduce tumor growth (25%), and had similar effect to anti-VEGF used as a single agent in this model (Fig. 17A). The combination therapy leads to a 50% reduction in tumor growth, suggesting that YW327.6S2 potentiates the effect of anti-VEGF (Fig. 17A). In contrast, the anti-Axl hybridoma antibody 12A11 (Li *et al,* 2009) which does not cross-react with murine Axl, has no significant effect on tumor growth as a single agent in this model, nor does affects anti-VEGF (Fig. 17B). Western blot analysis showed that both YW327.6S2 and 12A11 down-regulate Axl expression in tumors (Fig. 17C & D). These results suggest that the anti-tumor growth effect of YW327.6S2 might be mediated by modulation of tumor stromal functions.

To investigate further how YW327.6S2 might modulate tumor stromal functions, we treated MDA-MB-231 tumor bearing mice with YW327.6S2 alone or in combination with anti-VEGF. At various time points after administration of the antibodies, tumors were excised and stained with MECA32 to examine the intra-tumoral vascular density. Both YW327.6S2 and anti-VEGF significantly reduced vascular density compared with control (Fig. 17E). And combination of the two antibodies resulted in further reduction of the tumor associated vascular density. These results suggest that YW327.6S2 reduces MDA-MB-231 tumor growth in part by altering vascular functions.

In primary human breast cancer specimens, we found that Axl protein is strongly expressed in infiltrating macrophages (Fig. 17F) and therefore asked whether Axl mAb YW327.6S2 might affect tumor associate macrophages (TAMs) functions. MDA-MB-231

xenograft tumors were treated for one week with YW327.6S2, 12A11 or control antibody, and TAMs were isolated by sorting for F4/80 positive cells. Cells were cultured in serum free media overnight, and the supernatant was collected and assayed for the presence of various cytokines and chemokines. TAMs from tumors treated with YW327.6S2 and 12A11 produced much lower levels of inflammatory cytokines and chemokines compared with TAMs treated with control antibody (Fig. 17G). Treatment with either Axl-mAb does not affect Axl expression levels on TAMs (data not shown) in contrast to down-regulation of Axl expression on tumor cells by theses antibodies (Fig 17C & D). These results suggest that Axl mAbs most likely modulate inflammatory cytokine/chemokine secretion from TAMs in an indirect manner, perhaps by blocking the crosstalk between tumor and stromal cells.

YW327.6S2 reduces MDA-MB-231 breast cancer cells metastasis to the bone. In a previous study we showed that Axl knockdown by shRNA inhibits metastasis of MDA-MB-231 breast cancer cells to the lung in an orthotopic model (Li *et al,* 2009), we therefore tested whether YW327.6S2 affects metastasis of these cells. MDA-MB-231 cells stably expressing the luciferase reporter gene were injected via tail vein into SCID mice. Four weeks after injection, strong luminescent signals were detected at craniofacial region, tibia and femur of all five animals in the control antibody treated group. The sites detected by bioluminescence in control groups are 5, 5, 4, 3 and 1 in each animal, with a total of 18 (Fig. 18A). In mice treated with YW327.6S2, the sites detected by bioluminescence were significantly reduced, with 0, 1, 2, 3, and 1 sites in each animal and a total of 7 for the entire group (Fig. 18A). The presence of metastatic foci in bone was verified by histological analysis (Fig. 18B). These results suggest that YW327.6S2 is able to reduce MDA-MB-231 breast cancer cells metastasis to distant organs.

Discussion

We have developed and characterized a human anti-Axl monoclonal antibody (YW327.6S2) that exhibits cross-species reactivity and blocks various functions of Axl in tumorigenesis. Besides being the first reported fully humanized blocking antibody for Axl, YW327.6S2 not only serves as a powerful tool to dissect out the impact of Axl activation/signaling in multiple aspects of cancer development and progression, but also represents a potential therapeutic for treatment of various cancers.

Our results show that YW327.6S2 blocks Axl functions by down-regulation of Axl expression as well as inhibition of ligand Gas6 binding to the receptor, leading to

inactivation of Axl and its down-stream signaling (Fig. 14). The ability of YW327.6S2 to down-regulate Axl expression in cancer cells represents an important mechanism for its inhibitory effects, since many cancers express constitutively activated Axl and are no longer responsive to exogenous Gas6 (Li *et al*, 2009).

In the A549 NSCLC model, YW327.6S2 significantly attenuated tumor growth when administrated as a single agent (Fig. 15A). This inhibitory effect is comparable to that of anti-Axl hybridoma antibodies in this model (Li et al (2009), Fig. 15C). YW327.6S2 rapidly down-regulates Axl expression in xenografts (Fig.15D), and induces apoptosis of tumor cells (Fig. 15E), which is likely one of the mechanisms that mediates its inhibitory effect on tumor growth. Our previous findings that Axl modulates endothelial cell functions by regulating the DKK3 and angiopoietin/Tie2 pathways (Li *et al*, 2009) raised the possibility that anti-Axl mAb could enhance the effect of anti-VEGF in reducing tumor growth. Our results (Fig. 15A & F) are consistent with this hypothesis, in that YW327.6S2 impacts tumor vasculature by enhancing the effect of anti-VEGF to reduce intra-tumoral vascular density. Indeed, co-administration of YW327.6S2 and anti-VEGF in A549 model resulted in tumor stasis that was maintained for at least 4 weeks after treatment cessation (Fig. 15B). The hybridoma antibody 12A11 also enhances the anti-tumor effect of anti-VEGF in this model (Fig. 15C). However, unlike YW327.6S2, 12A11 does not have direct effect on tumor vasculature; rather it directly inhibits tumor cell proliferation and induces apoptosis (Li et al. 2009). The effect of 12A11 on tumor growth and the anti-VEGF on tumor vasculature resulted in an increased effect when the two agents are used together.

EGFR small molecule inhibitors such as erlotinib are efficacious in treatment of NSCLC tumors that harbor EGFR mutations or amplification (Lynch *et al*, 2004; Paez *et al*, 2004; Eberhard *et al*, 2005; Giaccone *et al,* 2005; Tsao *et al*, 2005). It is also known that treatment of breast cancer cells with Her2/EGFR small molecules inhibitor Lapatinib or anti-Her2 antibody Herceptin leads to induction of Axl expression and consequently results in resistance of cancer cells to these therapies (Liu *et al,* 2009). We have found recently that Axl expression is induced in HCC827 NSCLC (a cell line that harbors both EGFR mutation and amplification) cells that have acquired resistance to erlotinib and Axl knockdown in resistant cells restores their sensitivity to erlotinib, suggesting that Axl may play a role in erlotinib resistance in NSCLC. Since A549 cells contains wild type EGFR and are only moderately sensitive to EGFR inhibition *in vitro* (Yauch *et al*, 2005), we asked whether anti-Axl mAb would sensitize these cells to erlotinib. Our results showed that YW327.6S2

potentiates the effect of erlotinib in reducing tumor growth (Fig. 16A), suggesting that anti-Axl mAb may enhance the efficacy of EGFR inhibitors in tumors that are refractory to EGFR inhibition alone, perhaps by directly reducing Axl expression in tumor cells.

Systemic chemotherapy plays the largest part in the treatment paradigms for NSCLC. For recurrent or advanced disease, patients treated with chemotherapy that consists of carboplatin/paclitaxel have a response rate of 15% and median survival of 10.3 months (Sandley *et al*, 2006). Our results in A549 NSCLC model showed that YW327.6S2 is able to enhance the anti-tumor efficacy of carboplatin/paclitaxel (Fig. 16B), suggesting blocking Axl functions might improve therapeutic index of chemotherapy in this disease. Our results are consistent with a recent report demonstrating an Axl small molecule inhibitor synergized with cisplatin to suppress liver micrometastasis in 4T1 breast cancer orthotopic model (Holland et al, 2010).

In the MDA-MB-231 breast cancer model, YW327.6S2 alone is able to significantly attenuate tumor growth (Fig. 17A). The anti-Axl hybridoma antibody, which does not cross react with murine Axl and therefore would only affect tumor cells, has no significant effect on tumor growth in this model (Fig. 17B). These results suggest that YW327.6S2 is likely to exert its anti-tumor effect through its actions on tumor stroma. Our results show that YW327.6S2 reduces intra-tumoral vascular density (Fig. 17E) and enhances the effect of anti-VEGF. Through its effect on tumor vasculature, YW327.6S2 may impact on tumor growth.

An association between the development of cancer and inflammation has long been appreciated (Balkwill & Mantovani, 2001; Coussens & Werb, 2002). The chronic inflammation associated with infection and irritation may lead to environments that foster genomic lesions and tumor initiation. There is increasing evidence that TAMs have causal roles in tumor progression including promotion of angiogenesis and matrix remodeling (Balkwill *et al*, 2005; Pollard, 2004). The signals responsible for this are thought to be inflammatory cytokines, including tumor necrosis factor- (TNF-), interleukin 6 (IL-6) and a plethora of chemokines. These cytokines and chemokines not only recruit immune cells to the specific sites that stimulate tumor progression, but it has been shown that their receptors are expressed on tumor cells where they can increase tumor growth and migration (Haghnegahdar et al, 2000). In primary human breast cancer, Axl protein is expressed at high levels on tumor associated macrophages (TAMs) (Fig. 17F). We provide evidence here that anti-Axl mAbs could modulate the functions of these cells through an indirect

mechanism. Our data showed that treatment of MDA-MB-231 xenografts with either YW327.6S2 or 12A11 (which does not cross react with murine Axl and therefore should not have direct effect on TAMs) inhibits secretion of inflammatory cytokines and chemokines from TAMs (Fig. 17G). Since Axl-mAbs do not seem to have significant effect on Axl expression on TAMs but down-regulate receptor expression on tumor cells, it is likely that anti-Axl-mAbs modulate cytokine/chemokine secretion from TAMs by blocking the crosstalk between tumor and stromal cells. These results are consistent with the recent reports that tumor cells could promote their growth by educating infiltrating leukocytes to induce expression of Gas6 (Loges et al, 2010); and a small molecule inhibitor of Axl reduced GM-CSF expression in 4T1 breast tumor cells (Holland et al, 2010).

Previous studies have established the role of Axl in promoting tumor cell migration, invasion and metastasis (Zhang *et al*, 2008; Li *et al*, 2009; Tai *et al*, 2008; Vajkoczy *et al*, 2006; Gjerdrum *et al*, 2010). Here we showed that YW327.6S2 is able to reduce metastasis of MDA-MB-231 breast cancer cells to the bone. These results are consistent with our previous data that Axl silencing by RNAi in breast cancer cells inhibits their metastasis to the lung in an orthotropic model (Li *et al*, 2009; Gjerdrum *et al*, 2010), suggesting that this anti-Axl antibody could have therapeutic potential not only in treatment of primary tumor but also in metastatic disease.

In conclusion, we have developed a human monoclonal antibody that blocks Axl functions. This anti-Axl mAb exerts its anti-tumor effect through multiple mechanisms including induction of tumor cell apoptosis, regulation of angiogenesis and modulation of tumor associated immune cells functions. Additionally, this anti-Axl mAb enhances the anti-tumor efficacy of anti-VEGF, EGFR SMI as well as chemotherapy, may therefore represent a novel therapeutic approach in clinical settings where these therapies are standard care.

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Hybridoma cell line 3G9.19.7 was deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, USA as described below:

Species	Material	Deposit No.	Deposit Date
Mouse	anti-Axl 3G9.19.7	_____	_____

The deposits herein were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposits for 30 years from the date of deposit. The deposits will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposits to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. 122 and the Commissioner's rules pursuant to thereto (including 37 C.F.R. 1.14 with particular reference to 886 OG 638).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

What is claimed is:

1. A method of treating a cancer in an individual, comprising administering to the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

2. A method of inhibiting metastasis of cancer in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

3. A method of inhibiting angiogenesis in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

4. A method of inhibiting cell proliferation in an individual, comprising administering the individual a therapeutically effective amount of an Axl antagonist and a VEGF antagonist.

5. The method of claim 1, 2, 3, or 4, wherein the cancer is breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer (such as hepatocellular carcinoma), pancreatic cancer, soft-tissue sarcoma, kaposi's sarcoma, carcinoid carcinoma, head and neck cancer, melanoma, ovarian cancer, gastric cancer, mesothelioma, and multiple myeloma.

6. The method of claim 1, 2, 3, or 4, wherein the Axl antagonist is an anti-Axl antibody.

7. The method of claim 6, wherein the anti-Axl antibody comprises a heavy chain variable regions comprising sequence EVQLVESGGGLVQPGGSLRLSCAASGFSLSGSWIHWVRQAPGKGLEWVGWINPYR GYAYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAREYSGWGGSSVG YAMDYWGQGTLV (SEQ ID NO:1) and a light chain variable region comprising sequence DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSG

VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIKR (SEQ ID NO:2).

8. The method of claim 6, wherein the anti-Axl antibody is antibody 3G9 or a humanized form of antibody 3G9.

9. The method of claim 1, 2, 3, or 4 wherein the VEGF antagonist is an anti-VEGF antibody.

10. The method of claim 9, wherein the anti-VEGF antibody is bevacizumab.

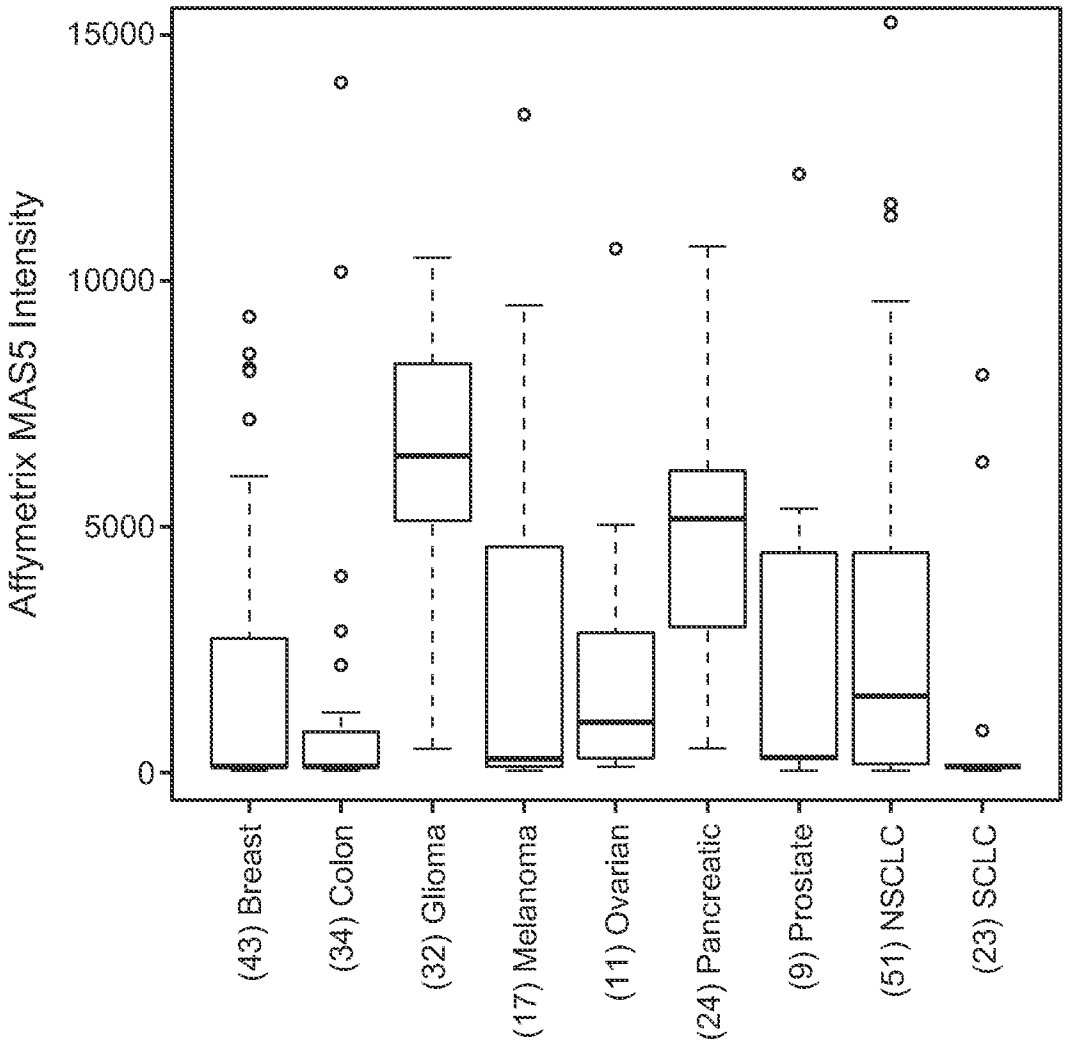

Affymetrix MAS5 Intensity
15000
10000
5000
0
(43) Breast
(34) Colon
(32) Glioma
(17) Melanoma
(11) Ovarian
(24) Pancreatic
(9) Prostate
(51) NSCLC
(23) SCLC

FIG. 1A

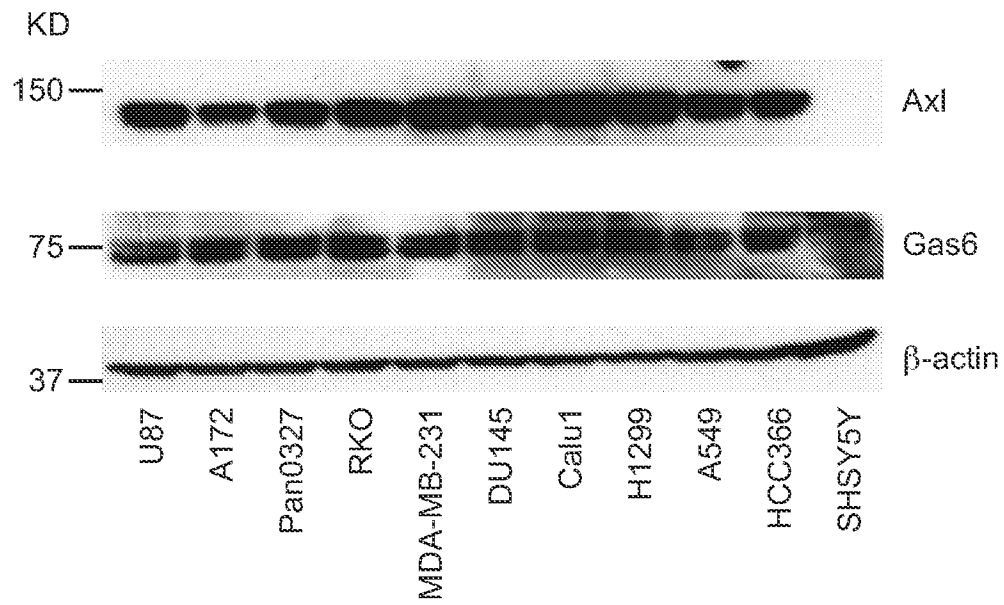

KD
150
75
37
Axl
Gas6
β-actin
U87
A172
Pan0327
RKO
MDA-MB-231
DU145
Calu1
H1299
A549
HCC366
SHSY5Y

FIG. 1B

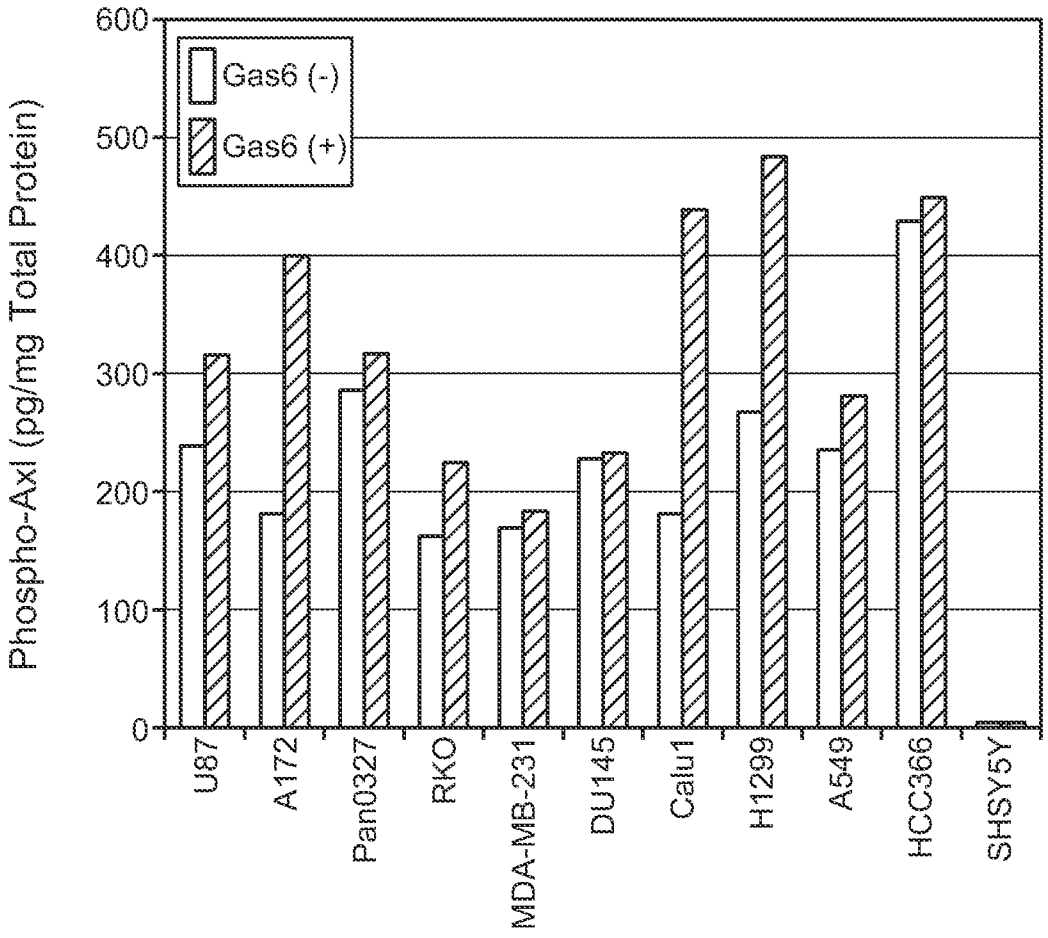

Phospho-Axl (pg/mg Total Protein)
600
500
400
300
200
100
0
Gas6 (-)
Gas6 (+)
U87
A172
Pan0327
RKO
MDA-MB-231
DU145
Calu1
H1299
A549
HCC366
SHSY5Y

FIG. 1C

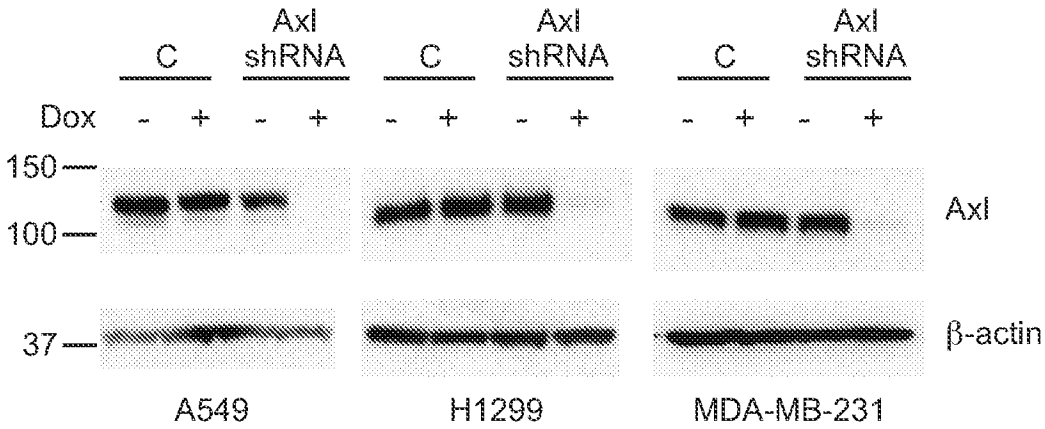

C
Axl shRNA
C
Axl shRNA
C
Axl shRNA
Dox
- + - + - + - + - + - +
150
100
37
Axl
β-actin
A549
H1299
MDA-MB-231

FIG. 2A

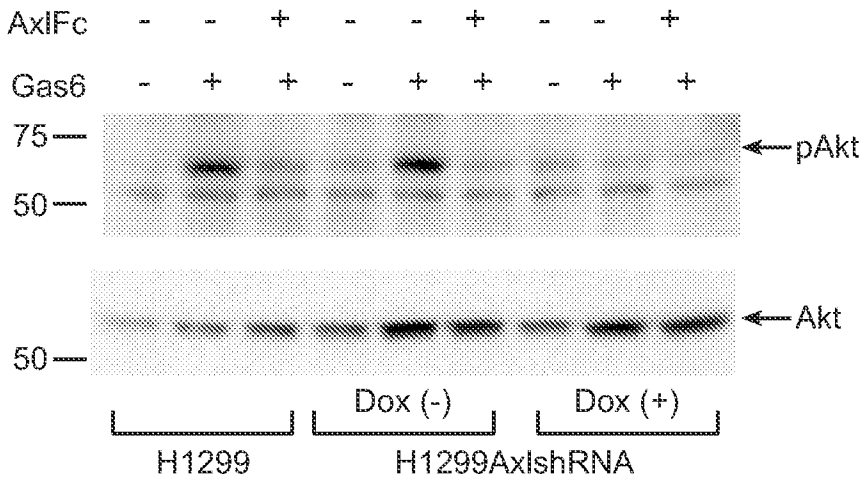

AxlFc
- - + - - + - - +
Gas6
- + + - + + - + +
75
50
pAkt
50
Akt
Dox (-)
Dox (+)
H1299
H1299AxlshRNA

FIG. 2B

FIG. 2C

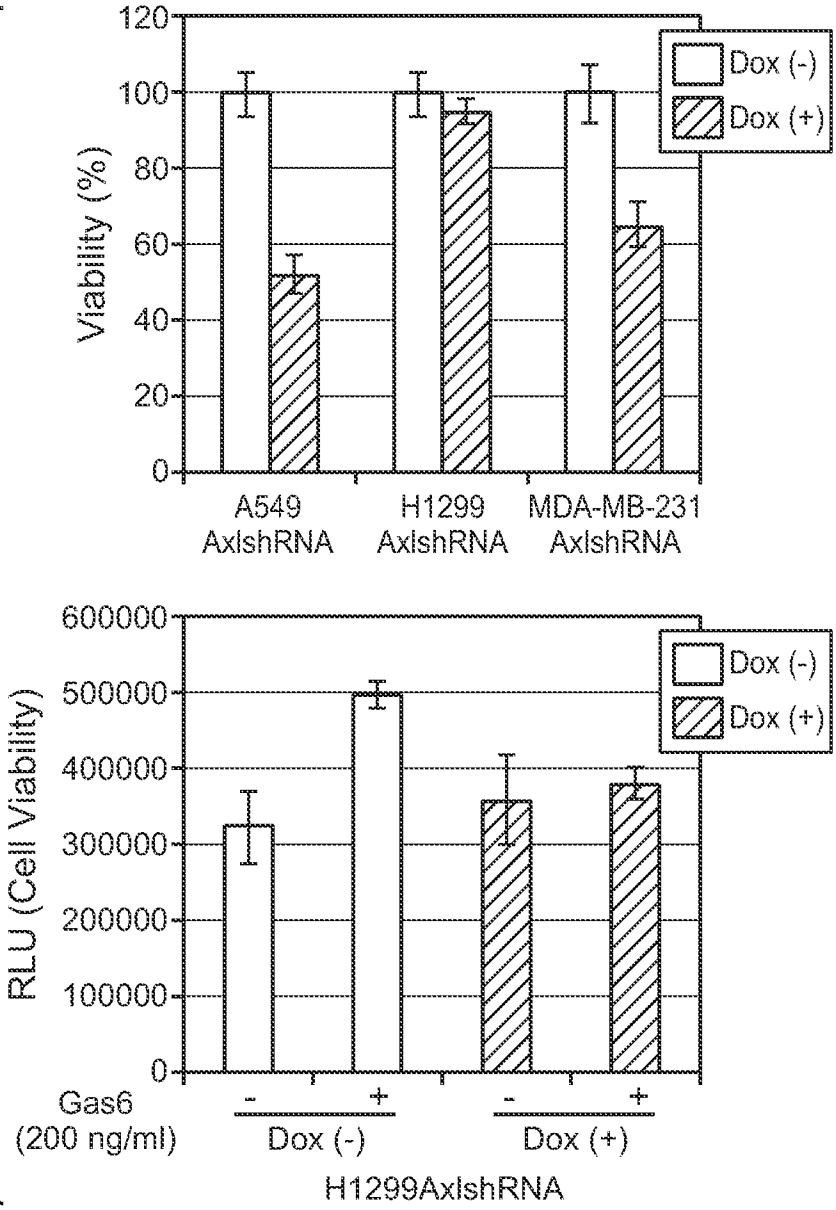
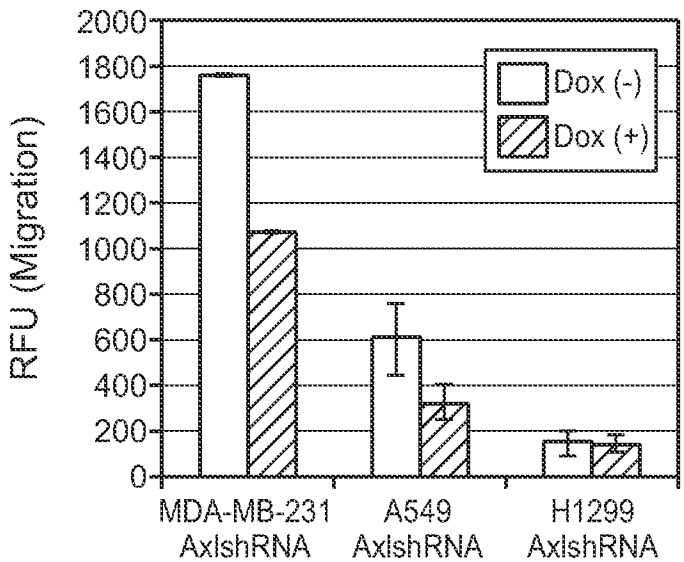

Viability (%)
120
100
80
60
40
20
0
Dox (-)
Dox (+)
A549 AxlshRNA
H1299 AxlshRNA
MDA-MB-231 AxlshRNA
RLU (Cell Viability)
600000
500000
400000
300000
200000
100000
0
Gas6 (200 ng/ml)
-
+
-
+
Dox (-)
Dox (+)
H1299AxlshRNA

FIG. 2D


RFU (Migration)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Dox (-)
Dox (+)
MDA-MB-231 AxlshRNA
A549 AxlshRNA
H1299 AxlshRNA

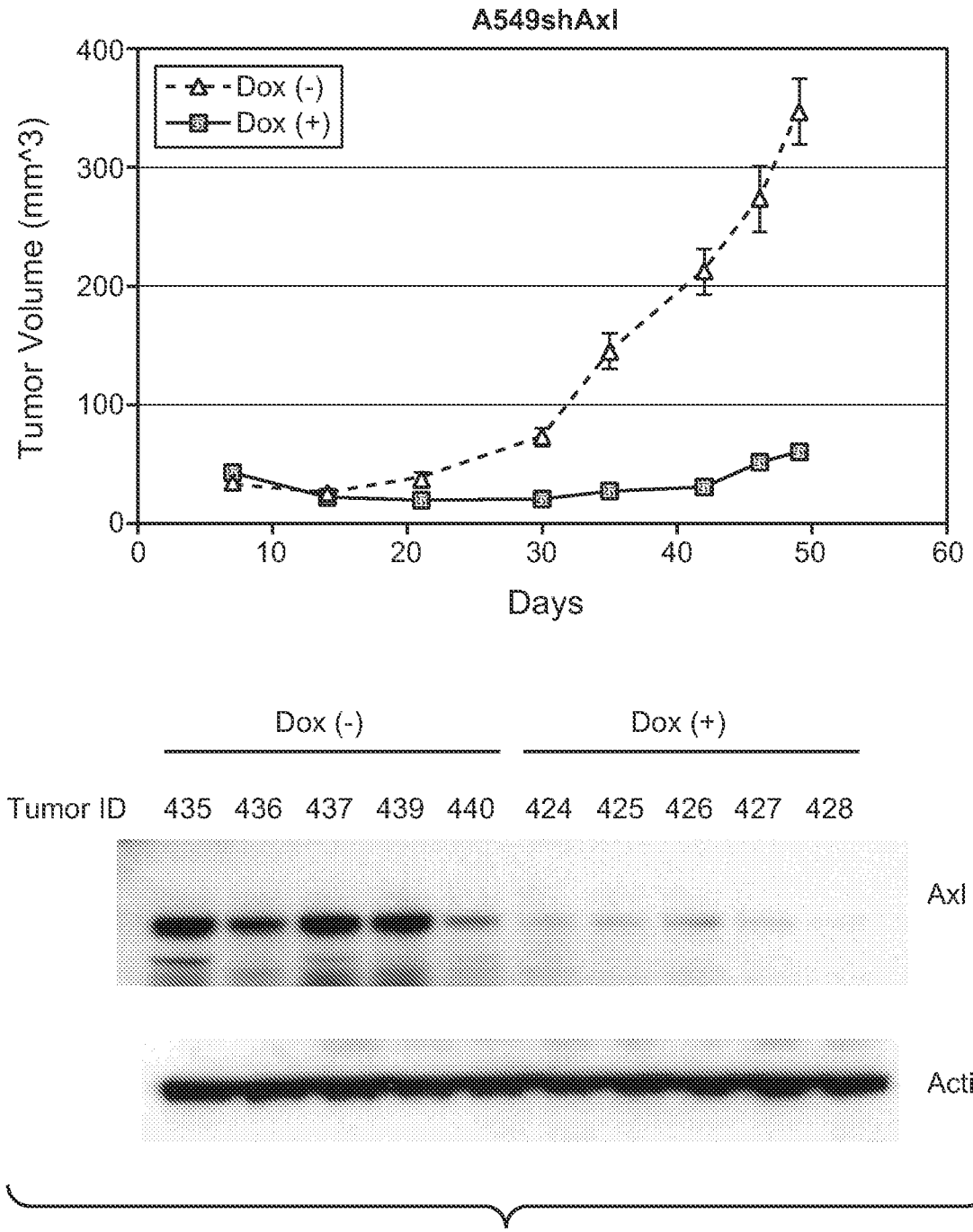

A549shAxl
Dox (-)
Dox (+)
Tumor Volume (mm^3)
400
300
200
100
0
0
10
20
30
40
50
60
Days
Dox (-)
Dox (+)
Tumor ID
435
436
437
439
440
424
425
426
427
428
Axl
Actin

FIG. 3A

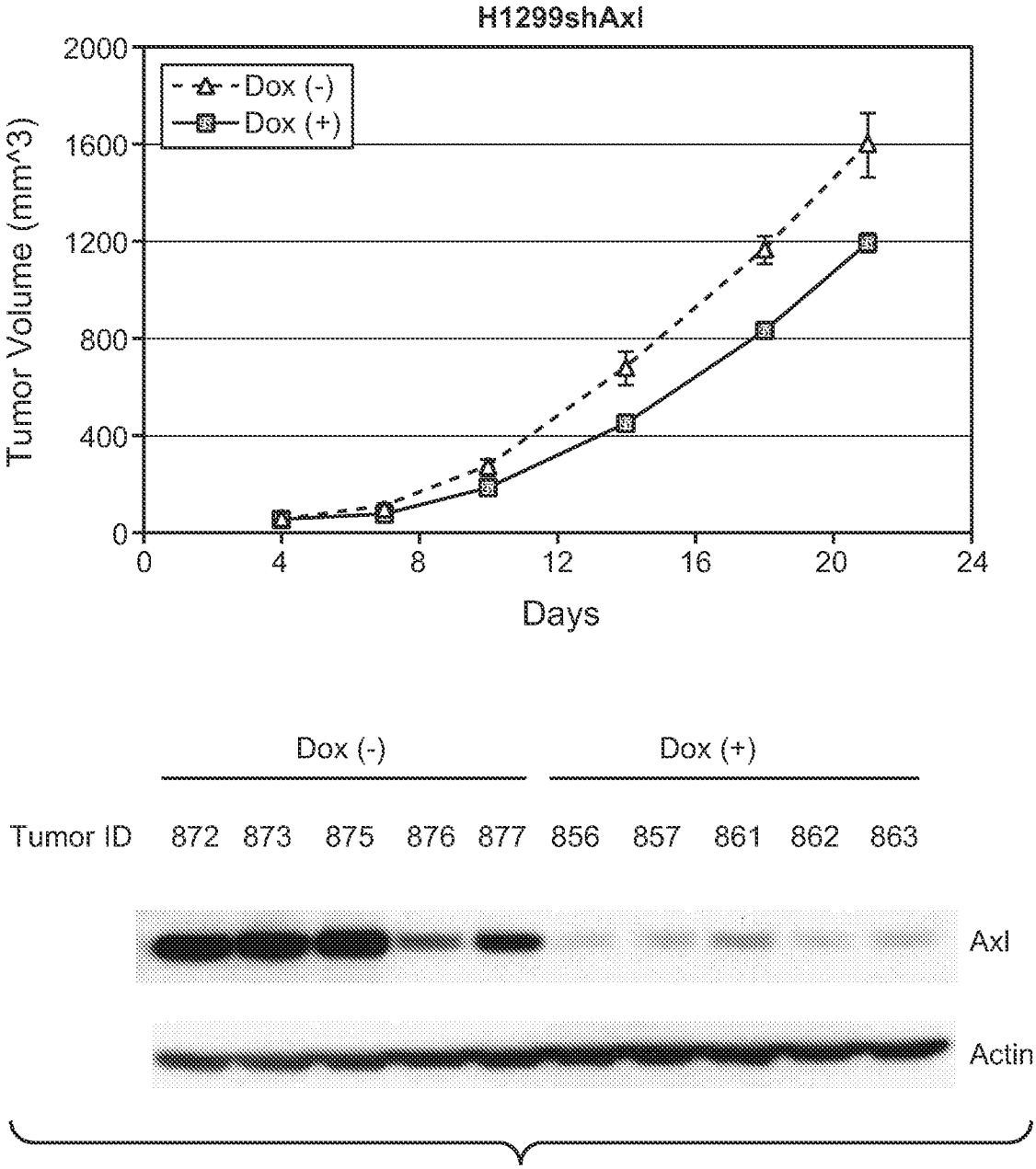

H1299shAxl
Tumor Volume (mm^3)
2000
1600
1200
800
400
0
Dox (-)
Dox (+)
0
4
8
12
16
20
24
Days
Dox (-)
Dox (+)
Tumor ID
872 873 875 876 877 856 857 861 862 863
Axl
Actin

FIG. 3B

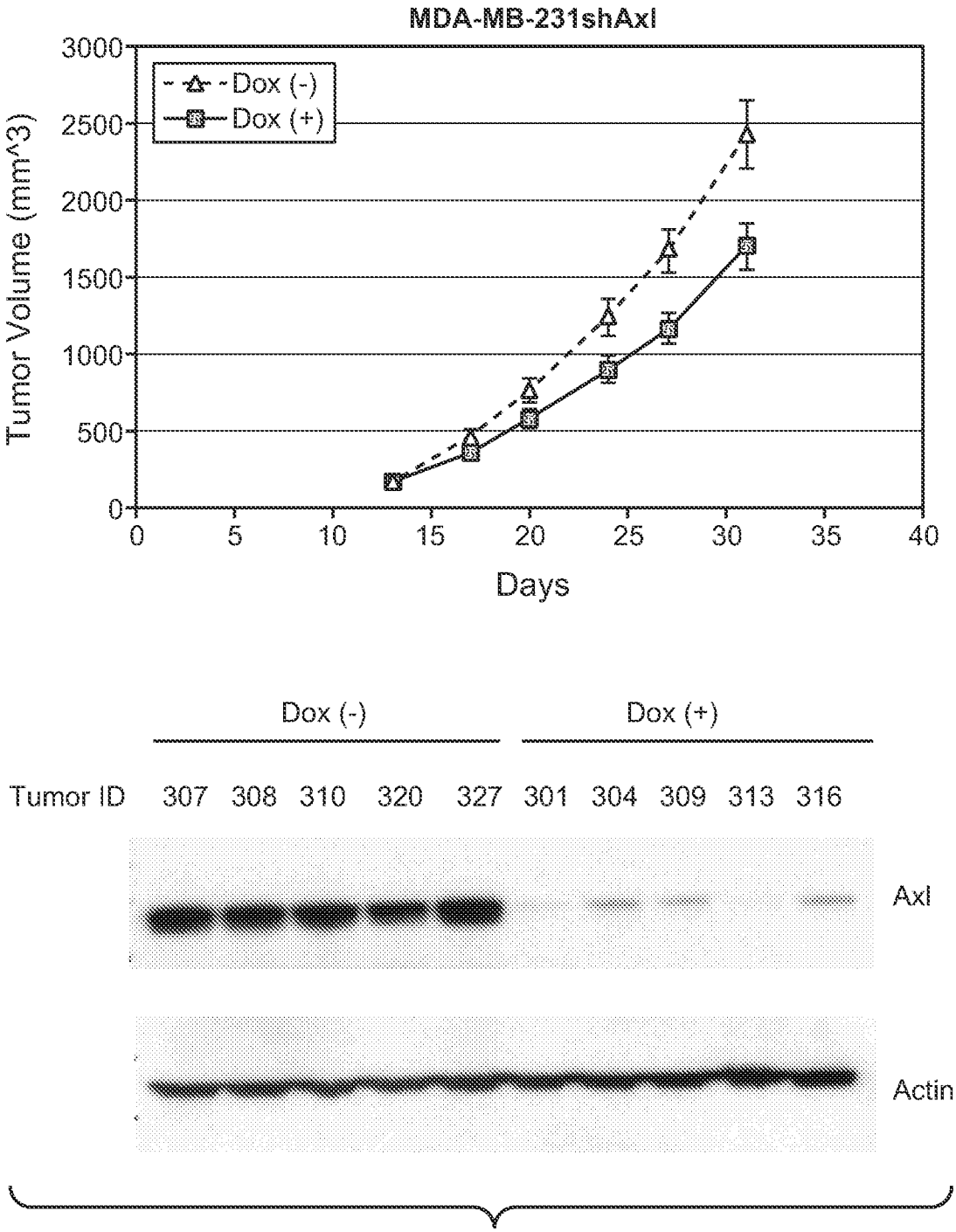

MDA-MB-231shAxl
Tumor Volume (mm^3)
3000
2500
2000
1500
1000
500
0
Dox (-)
Dox (+)
0
5
10
15
20
25
30
35
40
Days
Dox (-)
Dox (+)
Tumor ID
307
308
310
320
327
301
304
309
313
316
Axl
Actin

FIG. 3C

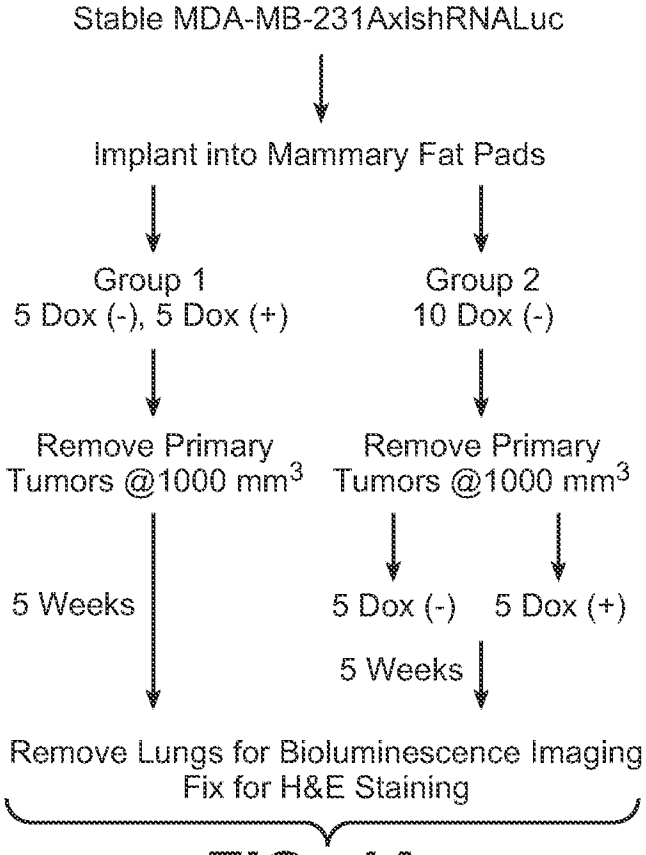


FIG. 4A

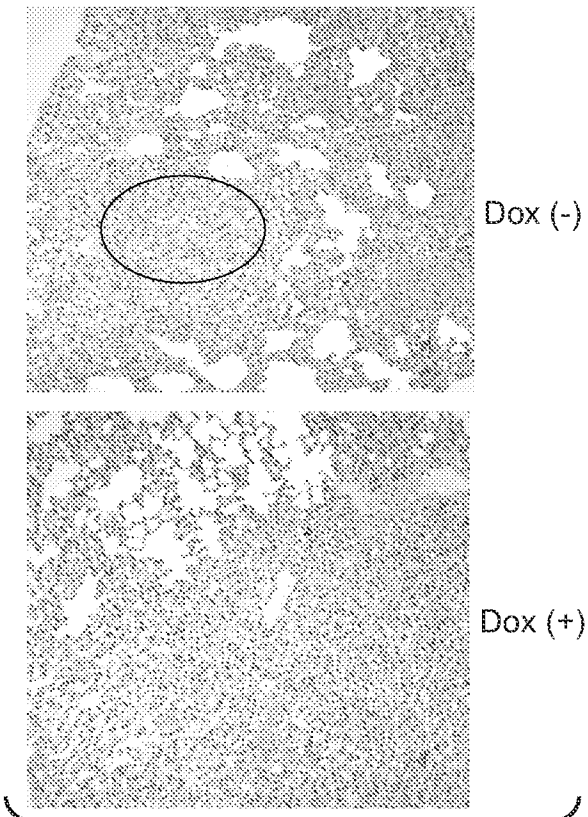


FIG. 4C

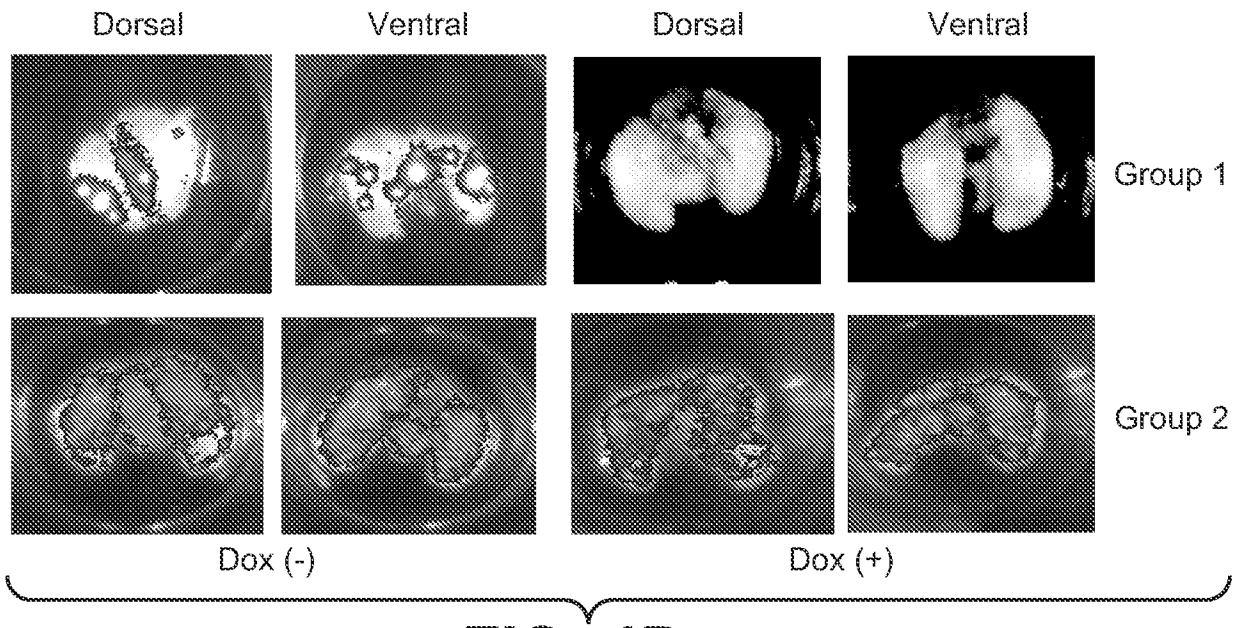


FIG. 4B

FIG. 5A

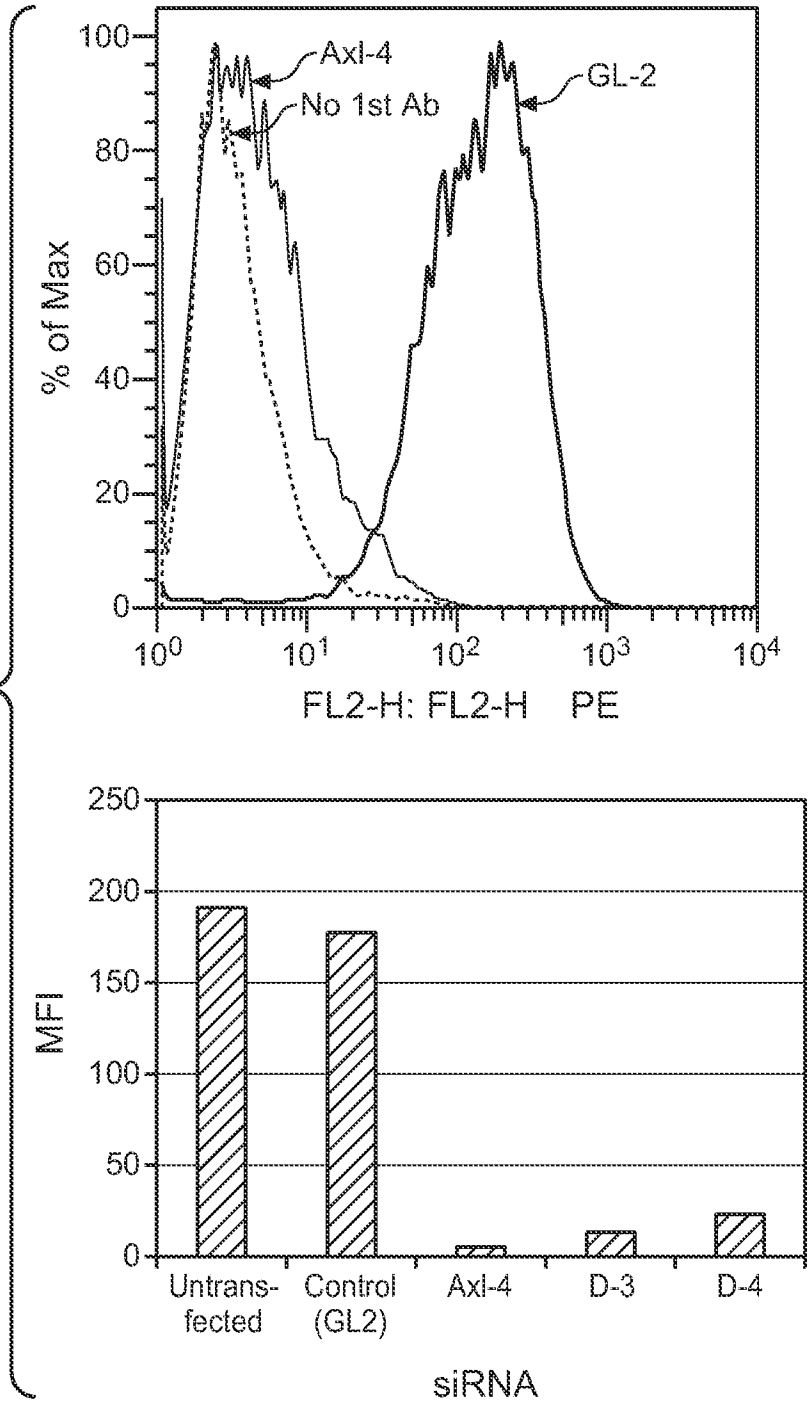
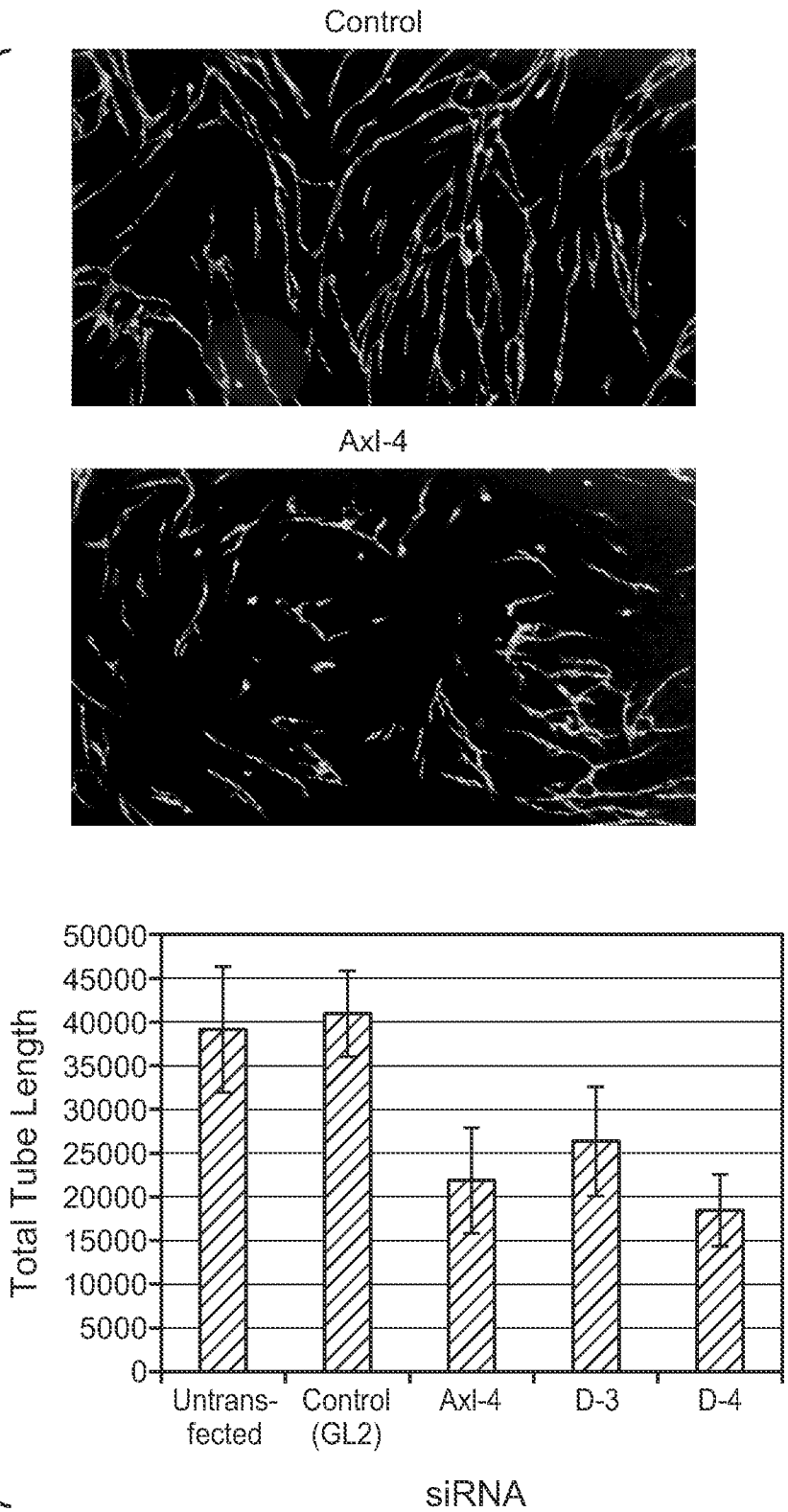

100
80
60
40
20
0
% of Max
Axl-4
No 1st Ab
GL-2
10^0
10^1
10^2
10^3
10^4
FL2-H: FL2-H PE
250
200
150
100
50
0
MFI
Untrans-fected
Control (GL2)
Axl-4
D-3
D-4
siRNA

FIG. 5B


Control
Axl-4
Total Tube Length
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Untrans-fected
Control (GL2)
Axl-4
D-3
D-4
siRNA

Up-regulated	Down-regulated
MYCN (9.1)	IFI44L (-98)
HLX (4.5)	GJA4 (-56)
GAS7 (4.5)	Axl (-20)
HDAC9 (4.1)	IFIT1 (-14)
E2F1 (3.9)	SCG5 (-8.2)
CXCR4 (3.6)	CYTL1 (-8.1)
PMCH (3.4)	DPP4 (-7.3)
ANG-2 (3.3)	DKK3 (-5.3)

FIG. 6A

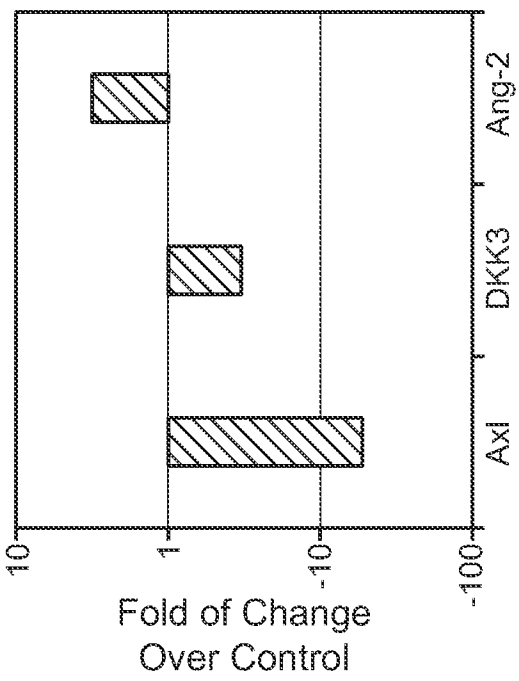
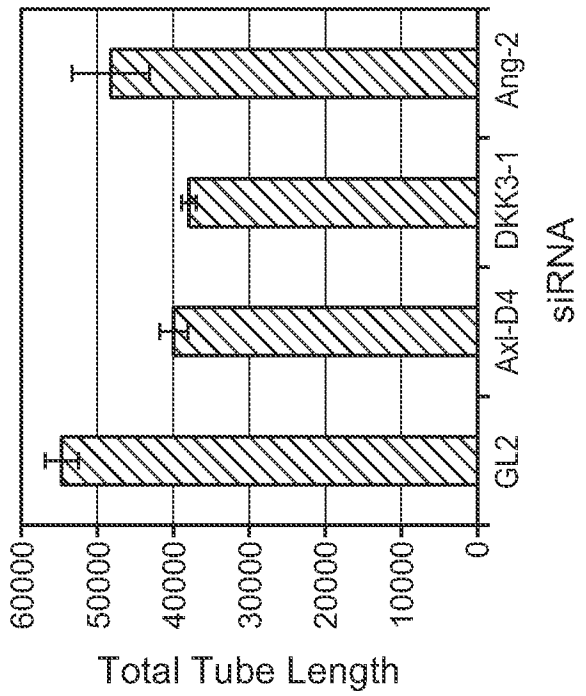


FIG. 6B



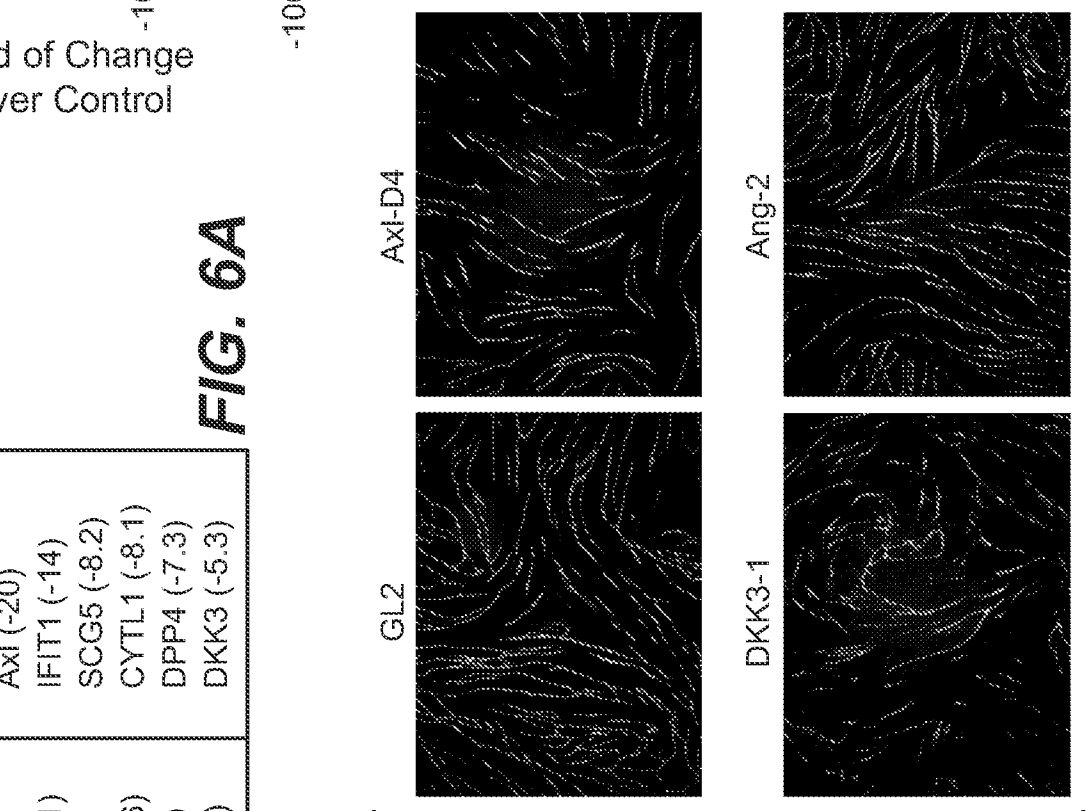
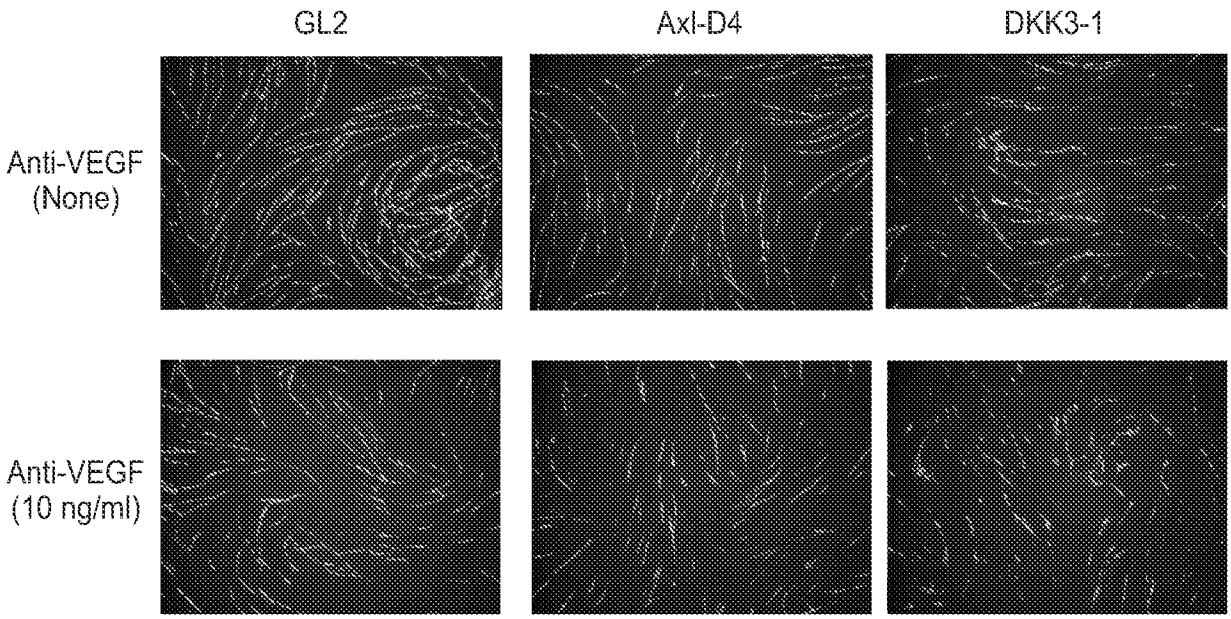


FIG. 6C


GL2
Axl-D4
DKK3-1
Anti-VEGF (None)
Anti-VEGF (10 ng/ml)

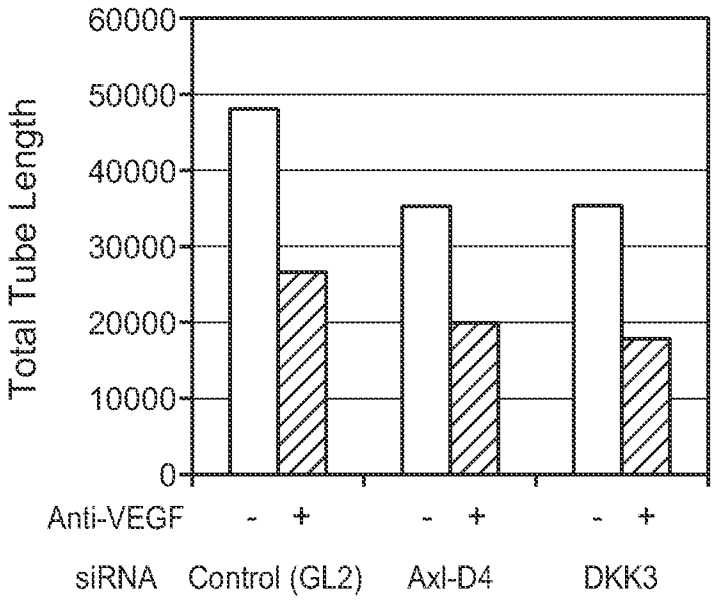

Total Tube Length
60000
50000
40000
30000
20000
10000
0
Anti-VEGF - + - + - +
siRNA Control (GL2) Axl-D4 DKK3

FIG. 7

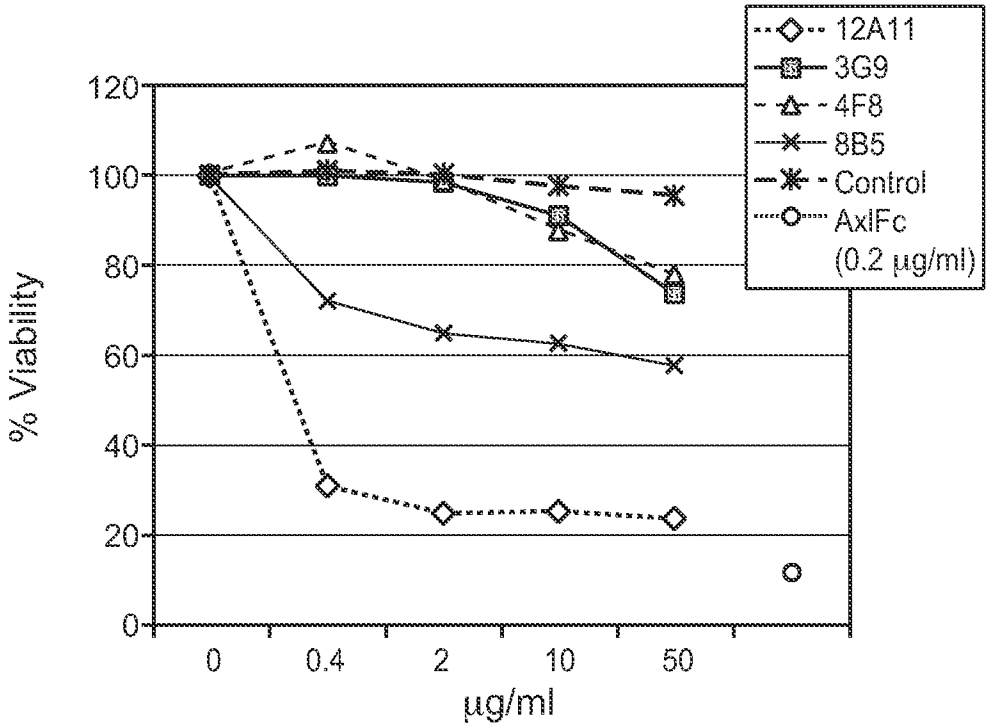


FIG. 8A

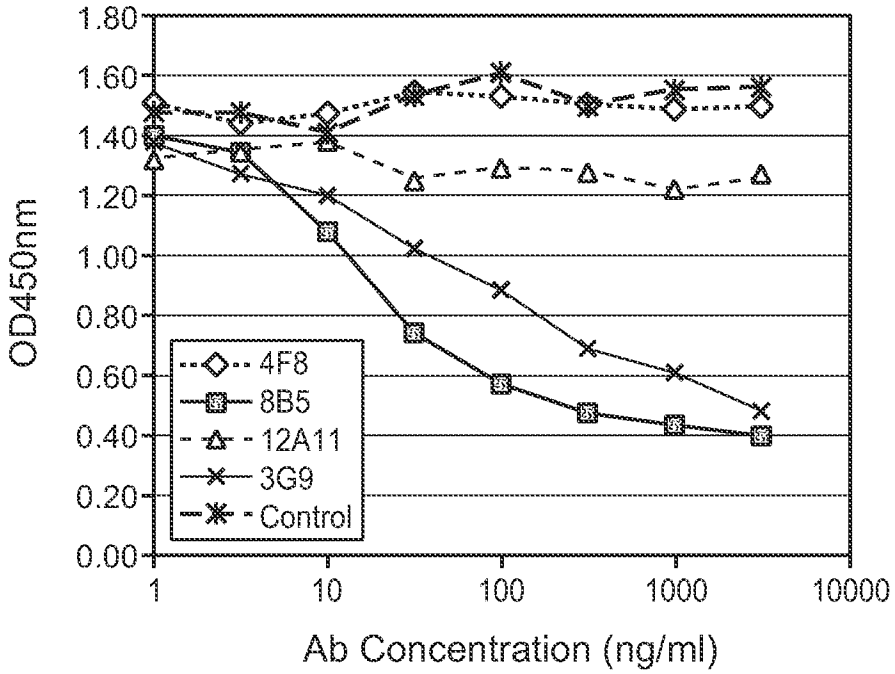


FIG. 8B-1

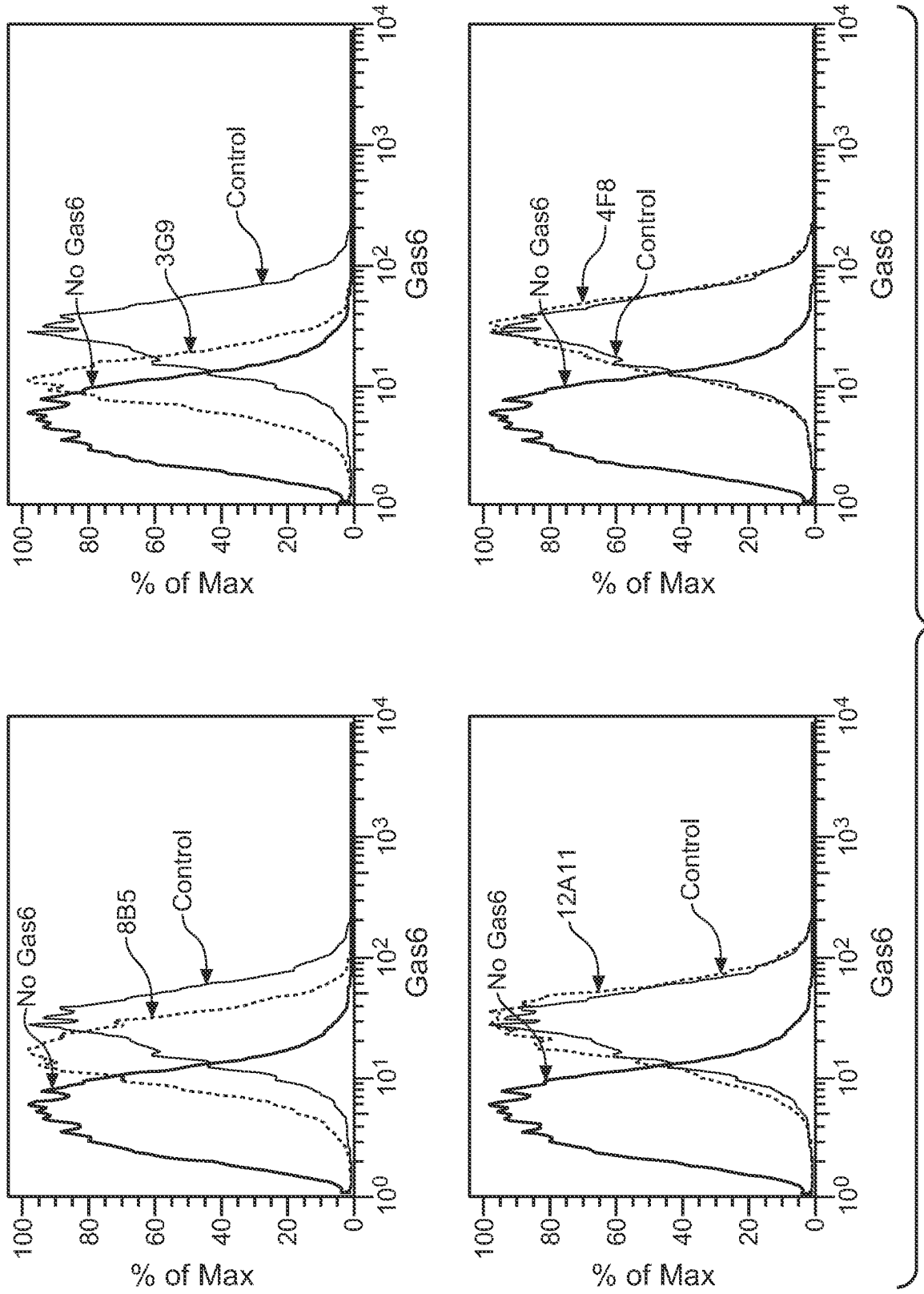
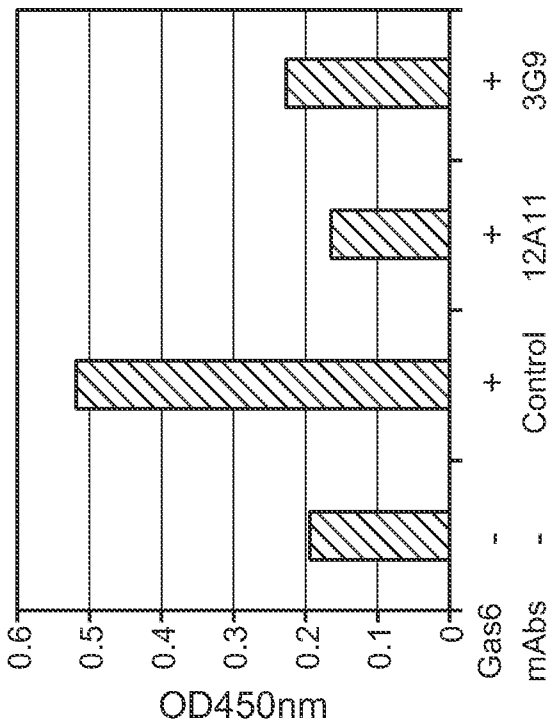
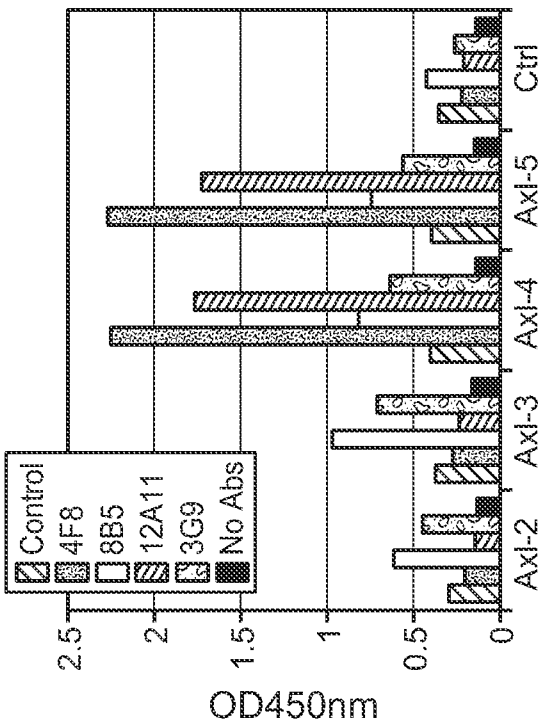

No Gas6
3G9
Control
% of Max
Gas6
No Gas6
4F8
Control
% of Max
Gas6
No Gas6
8B5
Control
% of Max
Gas6
No Gas6
12A11
Control
% of Max
Gas6

FIG. 8B-2


0.6
0.5
0.4
0.3
0.2
0.1
0
OD450nm
Gas6 - + + +
mAbs - Control 12A11 3G9


Control
4F8
8B5
12A11
3G9
No Abs
2.5
2
1.5
1
0.5
0
OD450nm
Axl-2
Axl-3
Axl-4
Axl-5
Ctrl

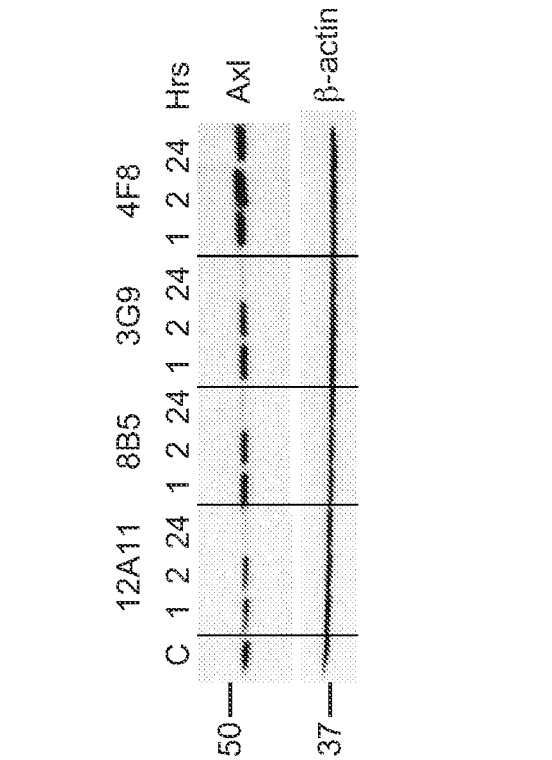

C 1 2 24 1 2 24 1 2 24 1 2 24 Hrs
12A11 8B5 3G9 4F8
Axl
β-actin
150
37

FIG. 8C

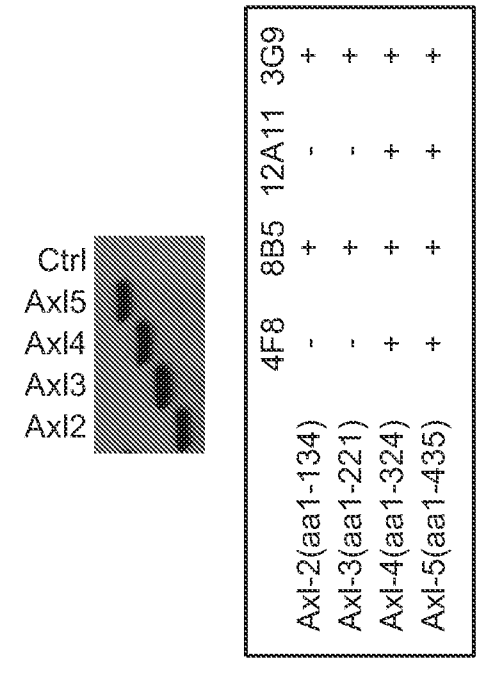

Ctrl
Axl5
Axl4
Axl3
Axl2
4F8 8B5 12A11 3G9
Axl-2(aa1-134) - + - +
Axl-3(aa1-221) - + - +
Axl-4(aa1-324) + + + +
Axl-5(aa1-435) + + + +

FIG. 8D

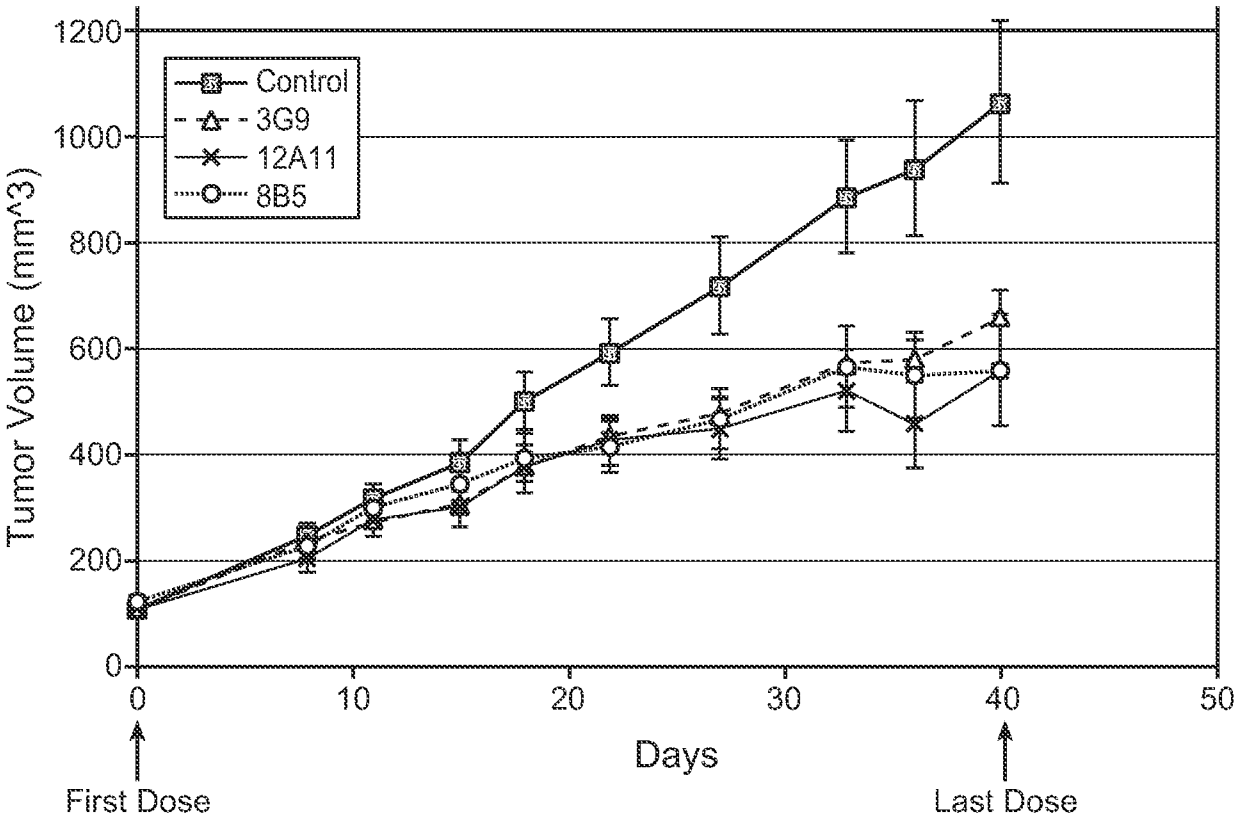

Tumor Volume (mm^3)
1200
1000
800
600
400
200
0
Control
3G9
12A11
8B5
0
10
20
30
40
50
Days
First Dose
Last Dose

FIG. 9A

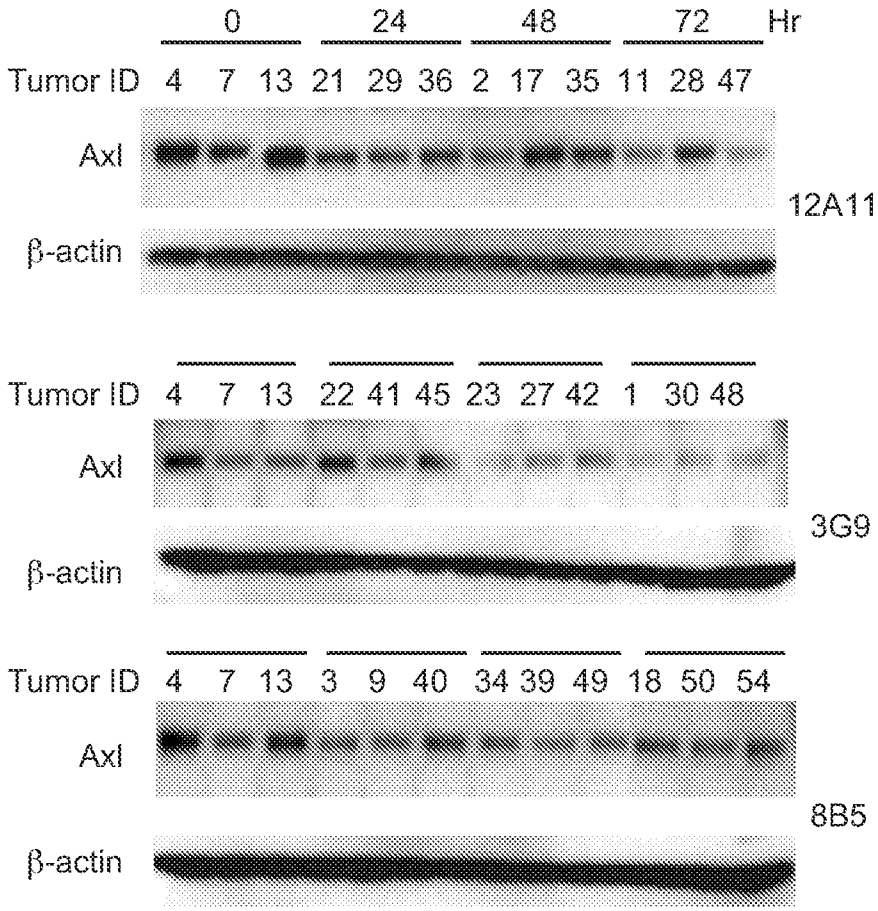

0
24
48
72
Hr
Tumor ID 4 7 13 21 29 36 2 17 35 11 28 47
Axl
12A11
β-actin
Tumor ID 4 7 13 22 41 45 23 27 42 1 30 48
Axl
3G9
β-actin
Tumor ID 4 7 13 3 9 40 34 39 49 18 50 54
Axl
8B5
β-actin

FIG. 9B

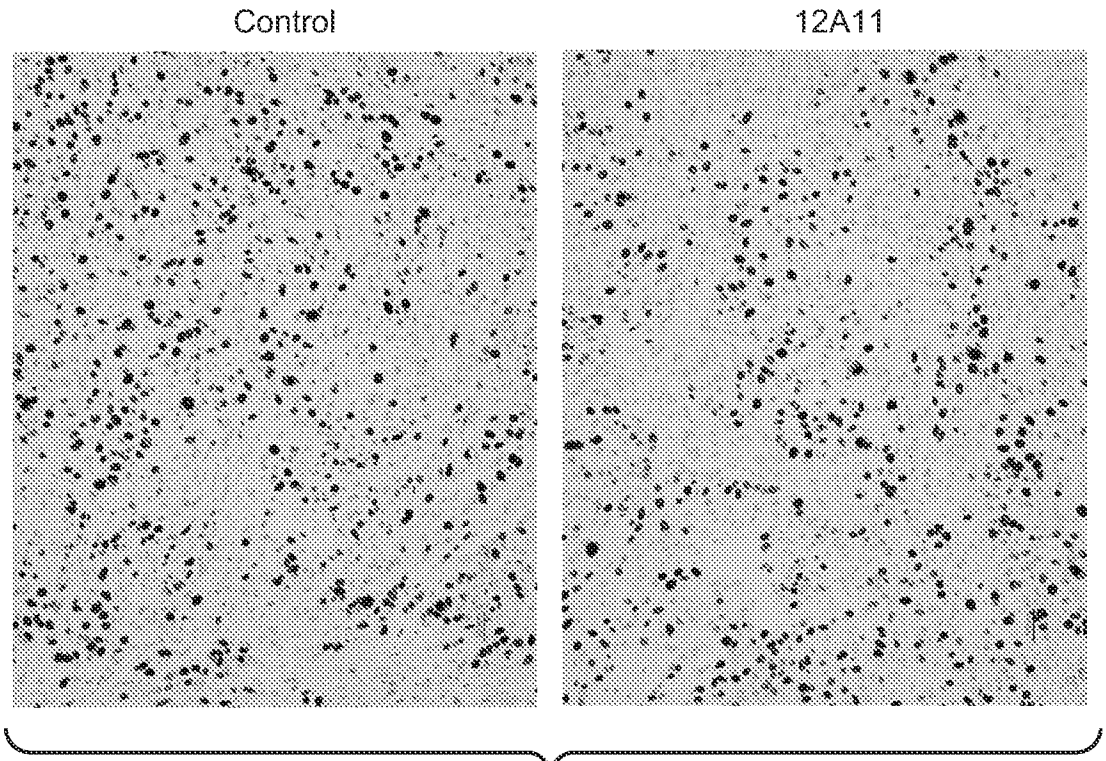


FIG. 9C

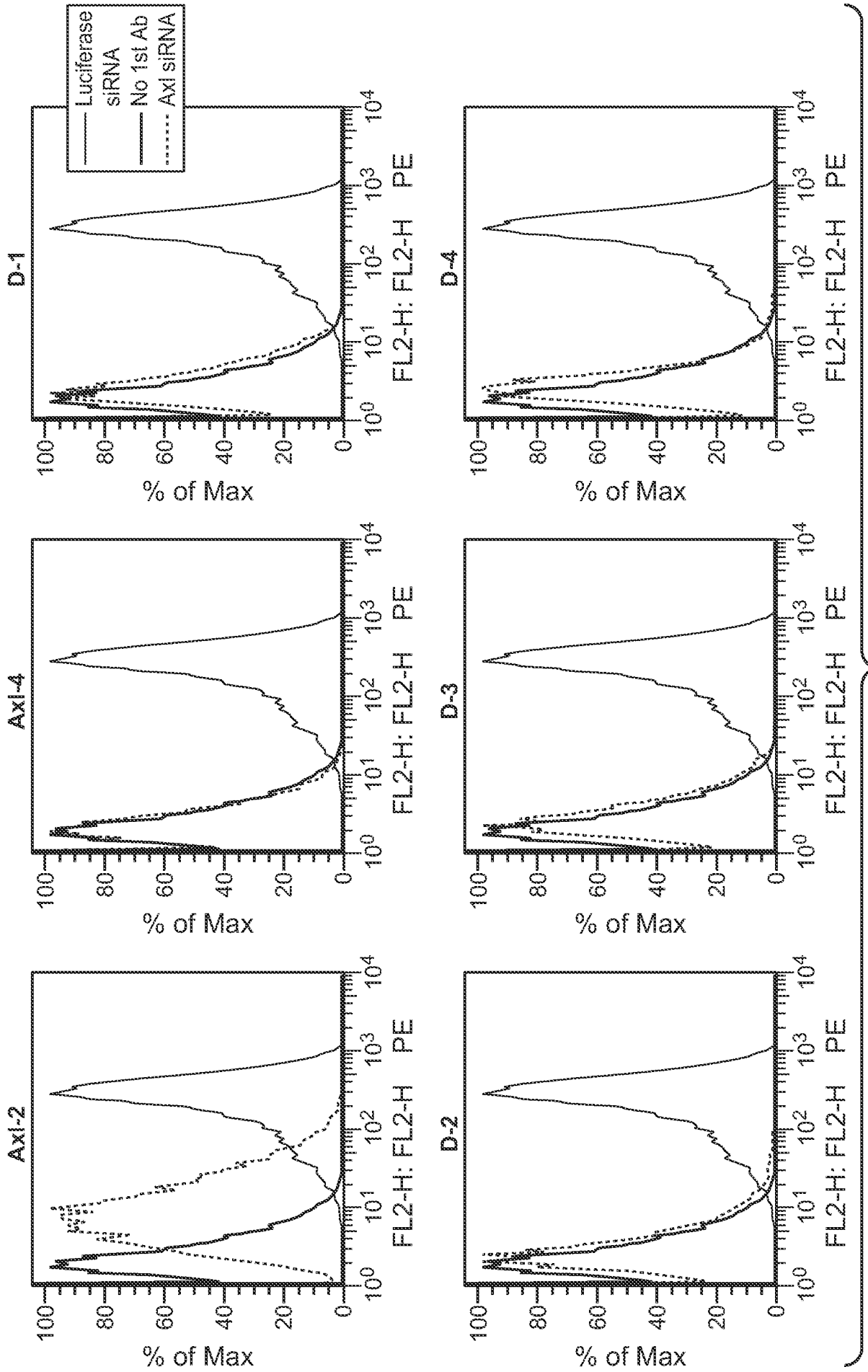

Luciferase siRNA
No 1st Ab
Axl siRNA
D-1
D-4
Axl-4
D-3
Axl-2
D-2
% of Max
FL2-H: FL2-H PE

FIG. 10A-1

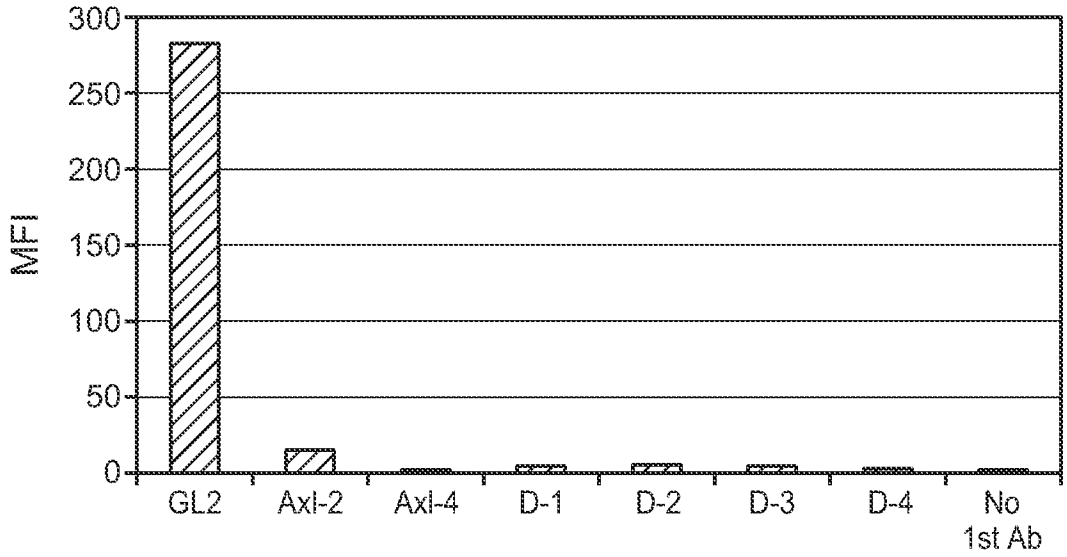


FIG. 10A-2

Luciferase GL2 siRNA
Forward: CGU ACG CGG AAU ACU UCG ATT
Reverse: UCG AAG UAU UCC GCG UAC GTT
Dharmacon-1
Forward: GAA AGA AGG AGA CCC GTT ATT
Reverse: TAA CGG GTC TCC TTC TTT CTT
Dharmacon-2
Forward: CCA AGA AGA TCT ACA ATG GTT
Reverse: CCA TTG TAG ATC TTC TTG GTT
Dharmacon-3
Forward: GGA ACT GCA TGC TGA ATG ATT
Reverse: TCA TTC AGC ATG CAG TTC CTT
Dharmacon-4
Forward: GAA GGA GAC CCG TTA TGG ATT
Reverse: TCC ATA ACG GGT CTC CTT CTT
Axl-2
Forward: GAC ATC CTC TTT CTC CTG CTT
Reverse: GCA GGA GAA AGA GGA TGT CTT
Axl-4
Forward: GAT TTG GAG AAC ACA CTG ATT
Reverse: TCA GTG TGT TCT CCA AAT CTT

FIG. 10B

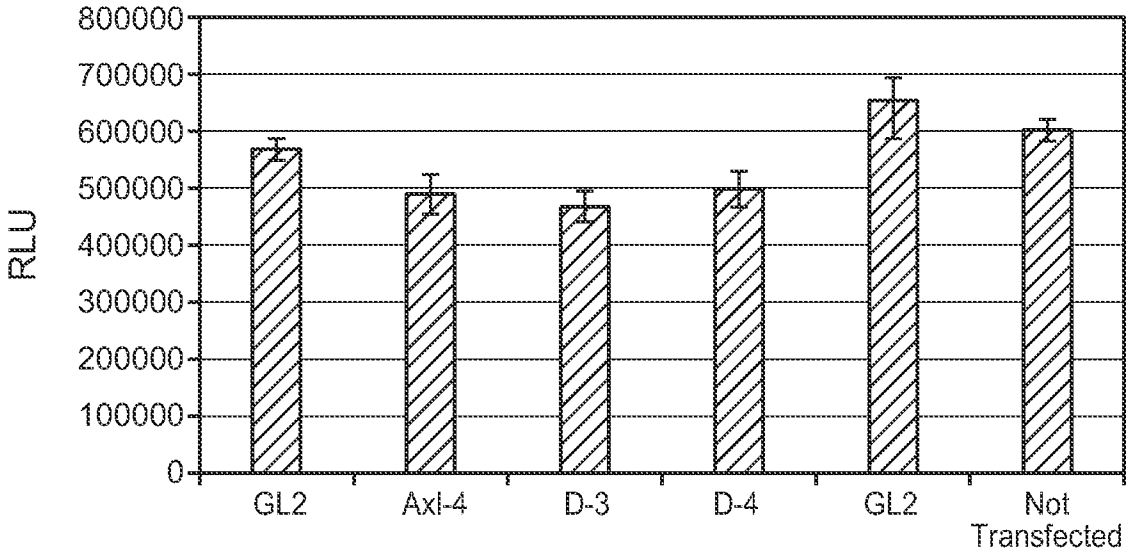


FIG. 10C

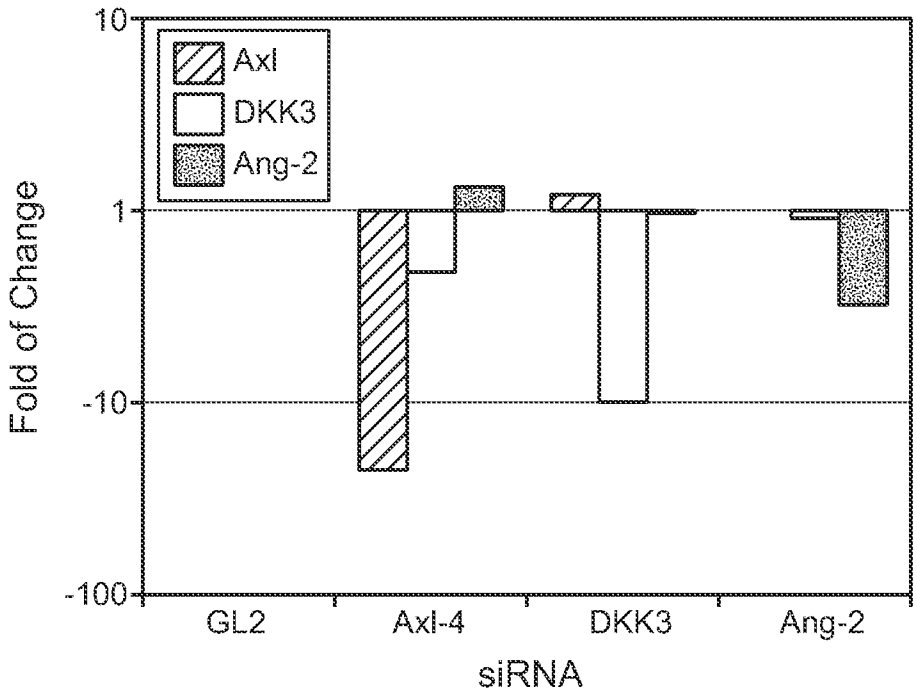


FIG. 11

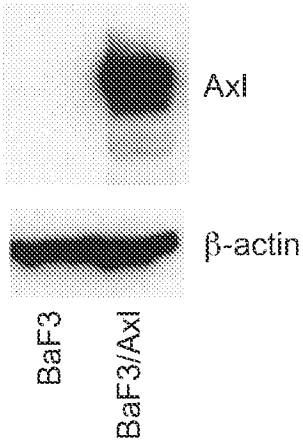


FIG. 12A

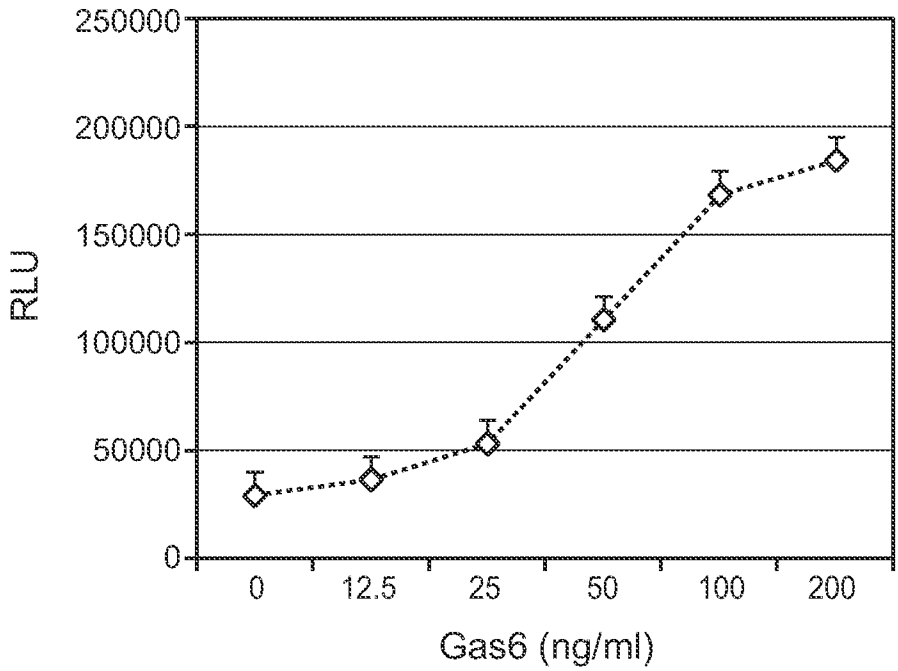


FIG. 12B

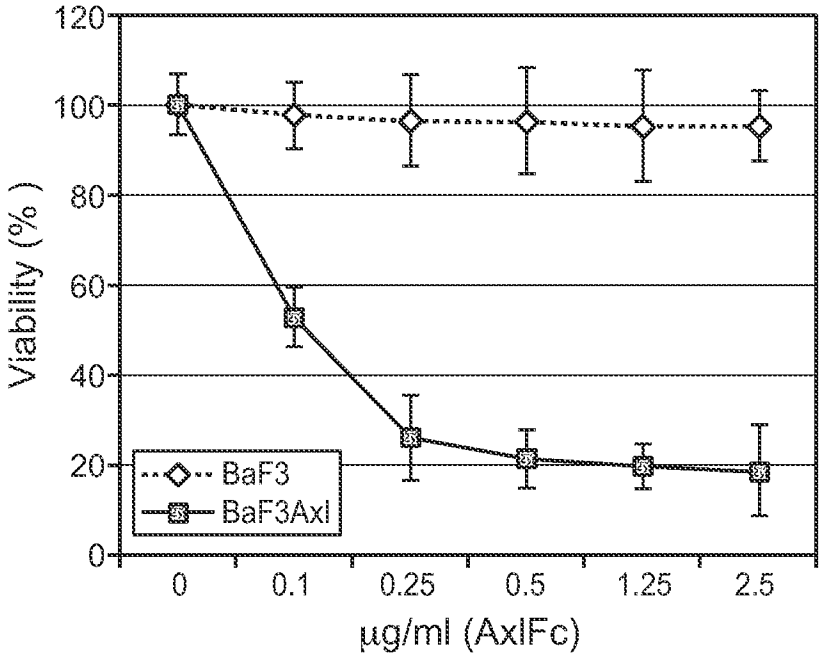


FIG. 12C

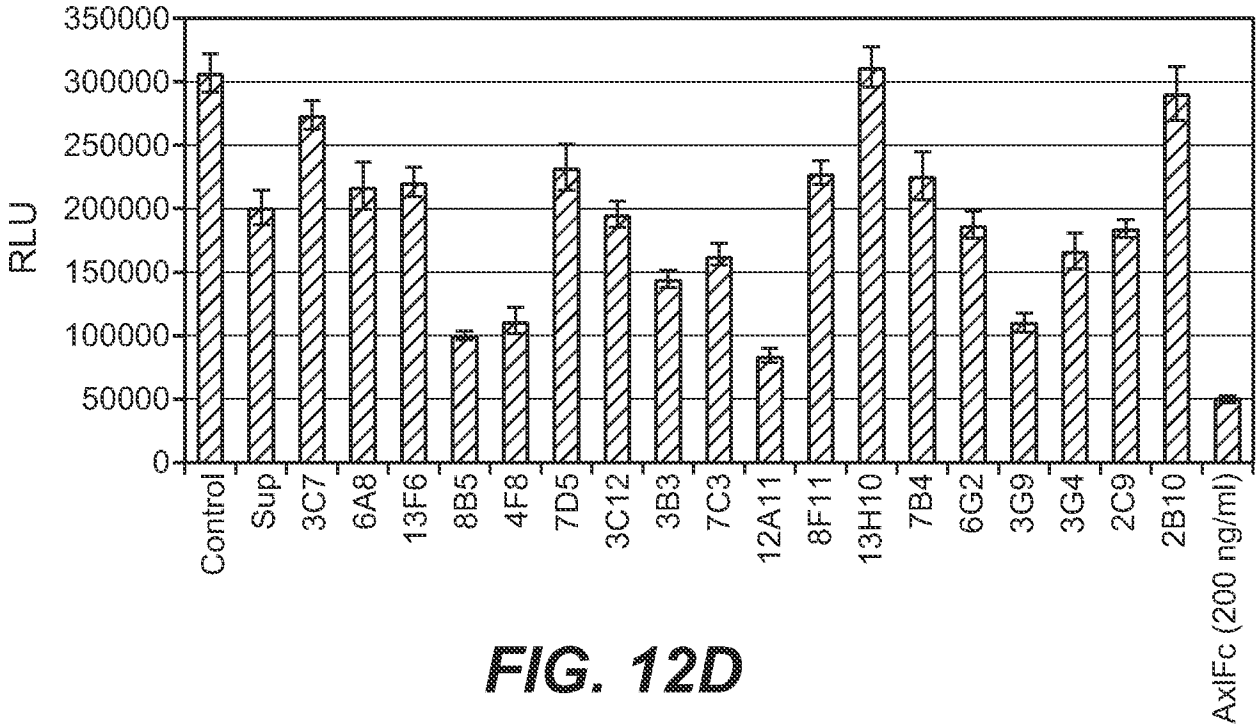
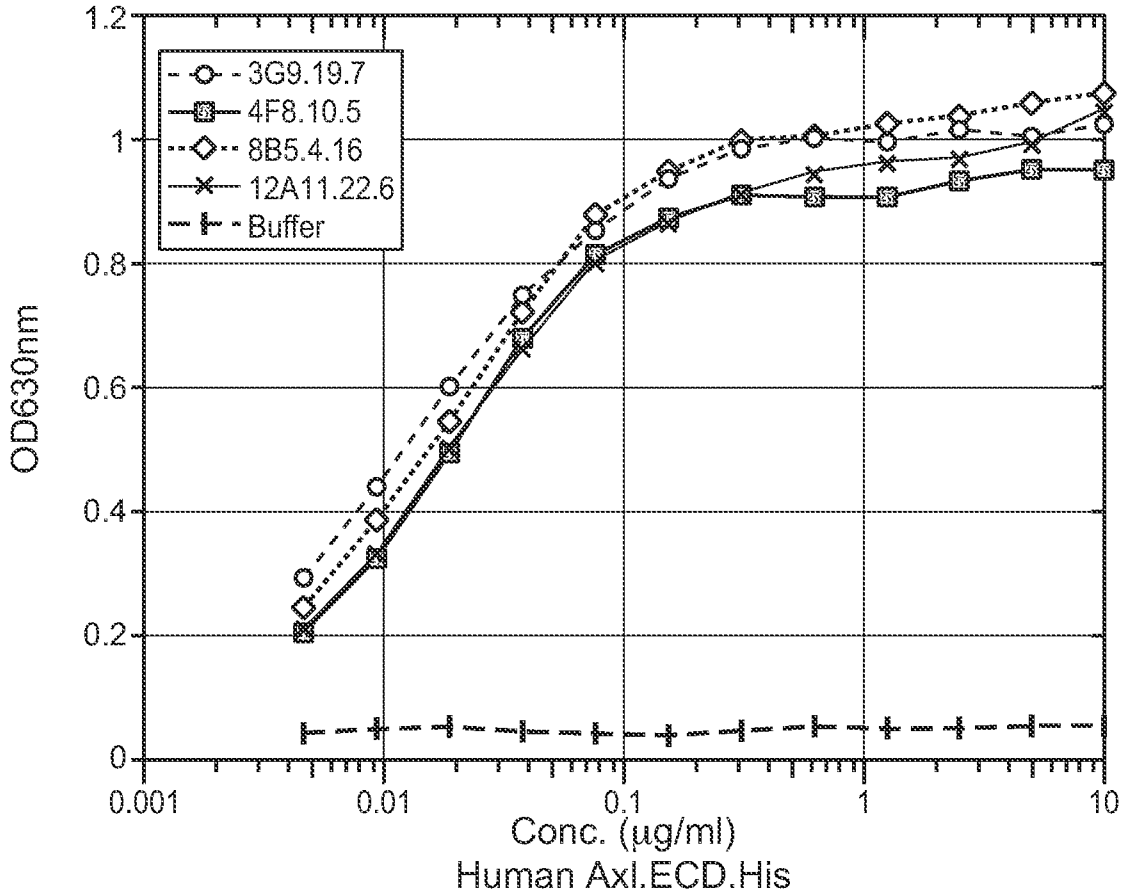


FIG. 12D


3G9.19.7
4F8.10.5
8B5.4.16
12A11.22.6
Buffer
OD630nm
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
Conc. (µg/ml)
Human Axl.ECD.His

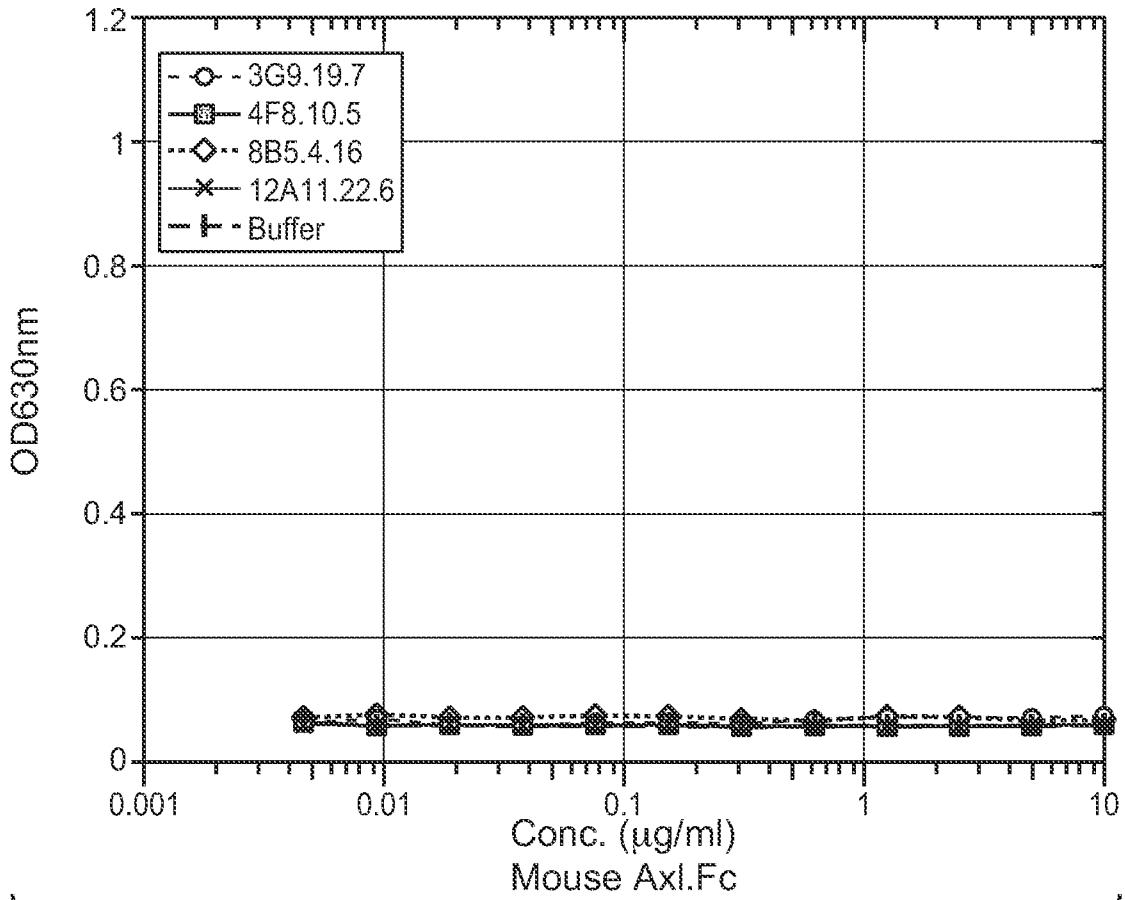

3G9.19.7
4F8.10.5
8B5.4.16
12A11.22.6
Buffer
OD630nm
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
Conc. (µg/ml)
Mouse Axl.Fc

FIG. 13A

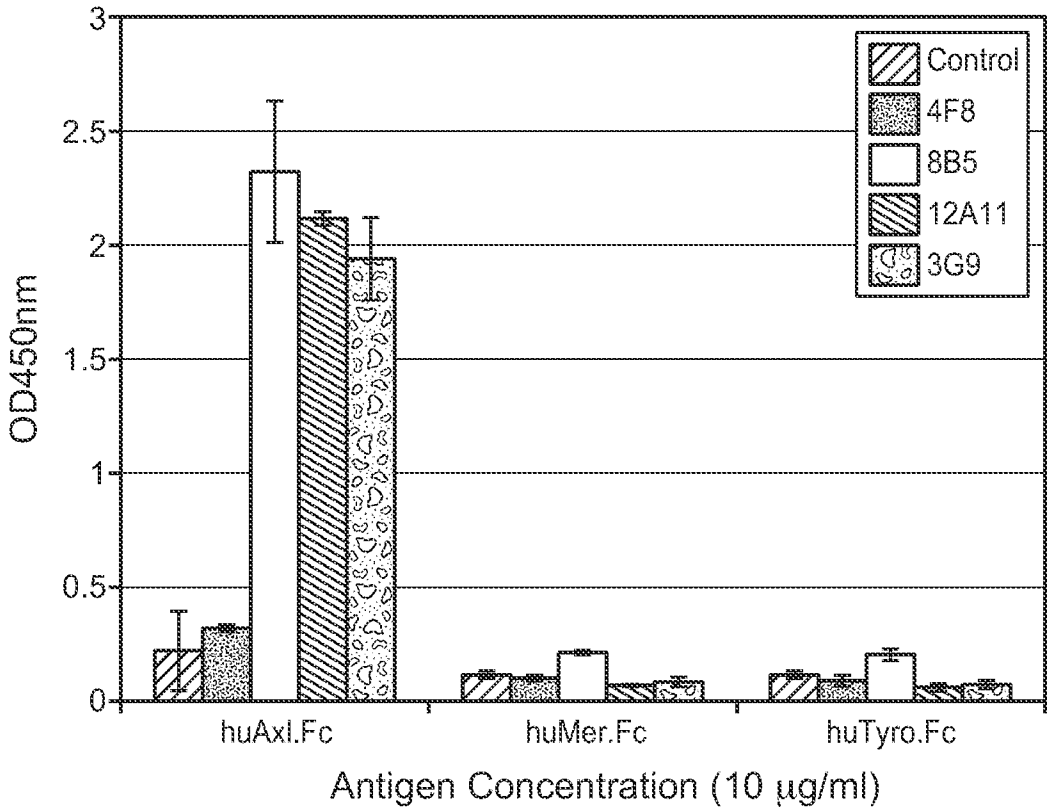

3
2.5
2
1.5
1
0.5
0
OD450nm
Control
4F8
8B5
12A11
3G9
huAxl.Fc
huMer.Fc
huTyro.Fc
Antigen Concentration (10 μg/ml)

FIG. 13B

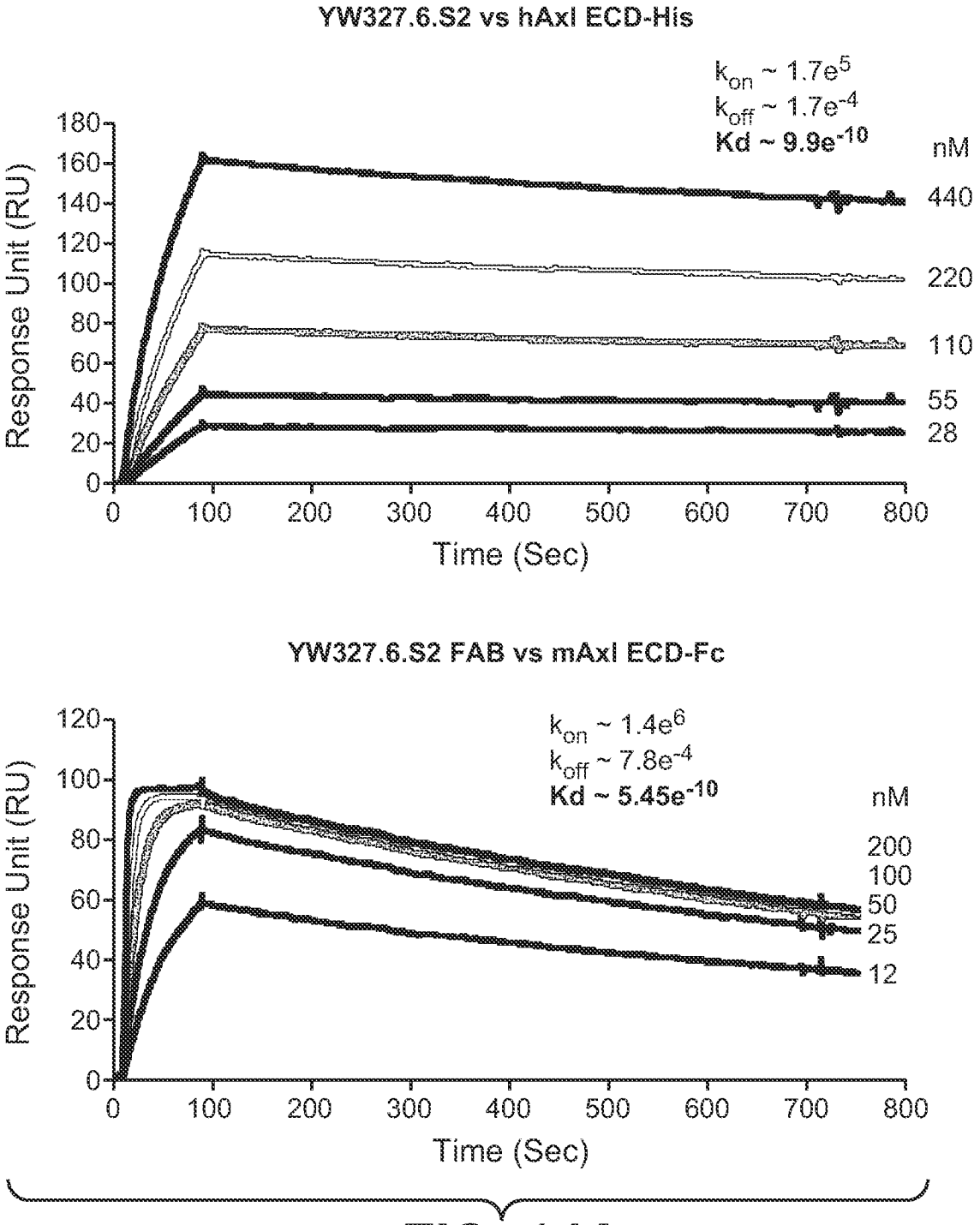

YW327.6.S2 vs hAxl ECD-His
k_{on} ~ 1.7e^{5}
k_{off} ~ 1.7e^{-4}
Kd ~ 9.9e^{-10}
nM
440
220
110
55
28
Response Unit (RU)
Time (Sec)
YW327.6.S2 FAB vs mAxl ECD-Fc
k_{on} ~ 1.4e^{6}
k_{off} ~ 7.8e^{-4}
Kd ~ 5.45e^{-10}
nM
200
100
50
25
12
Response Unit (RU)
Time (Sec)

FIG. 14A

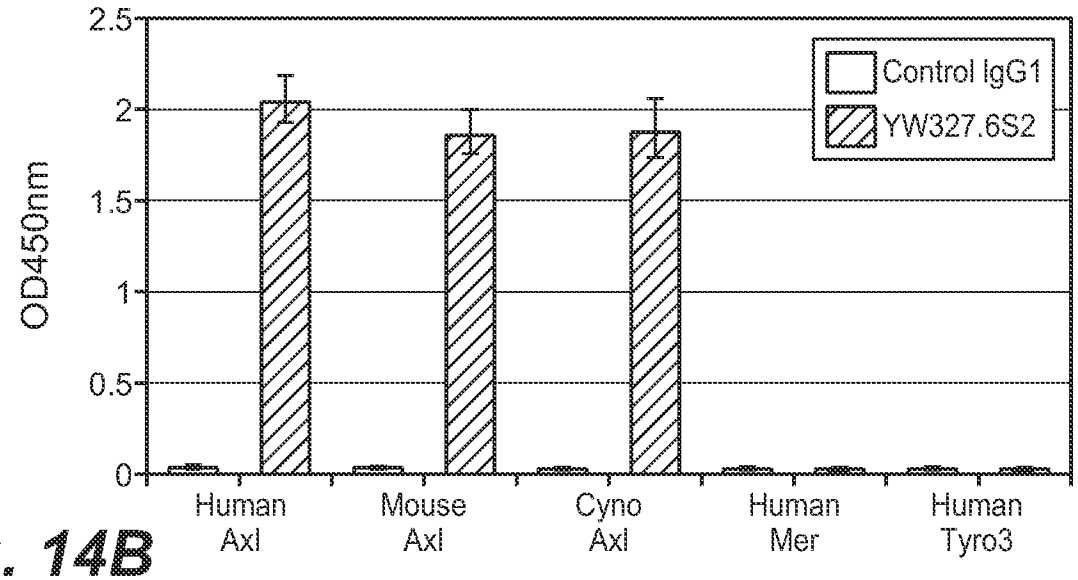


FIG. 14B

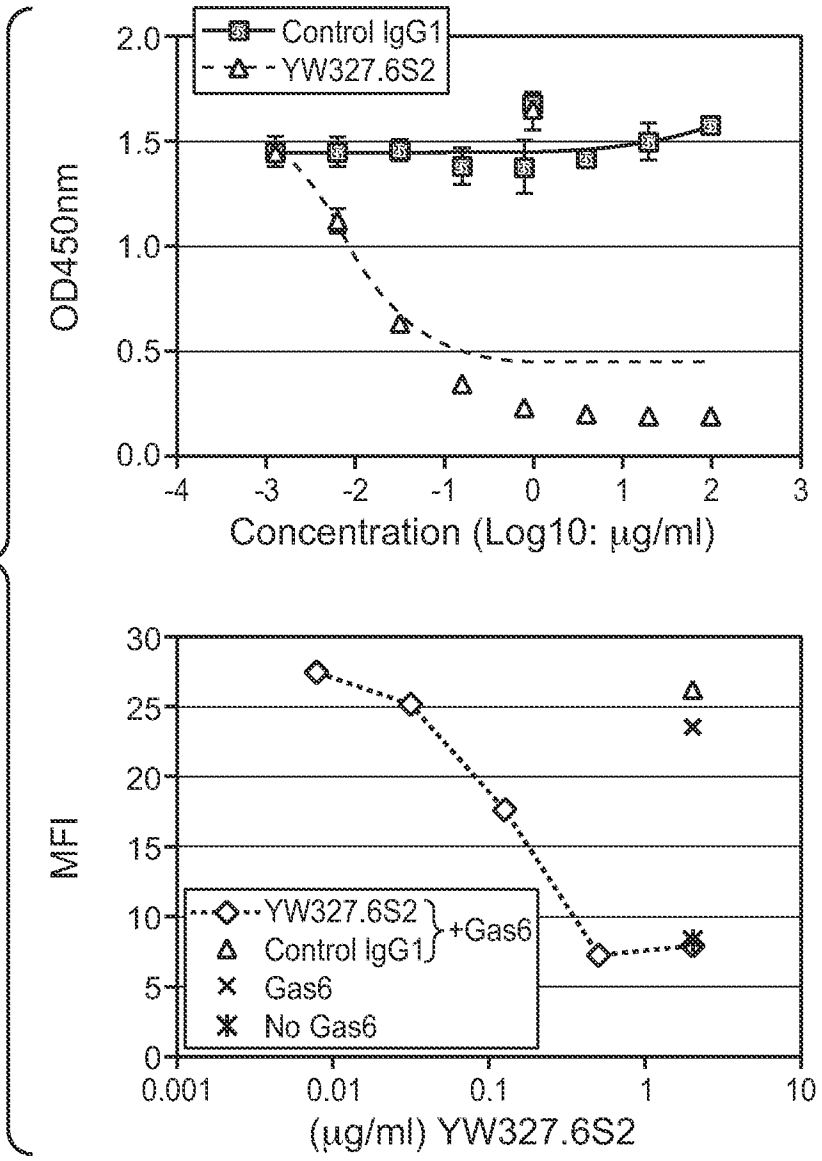
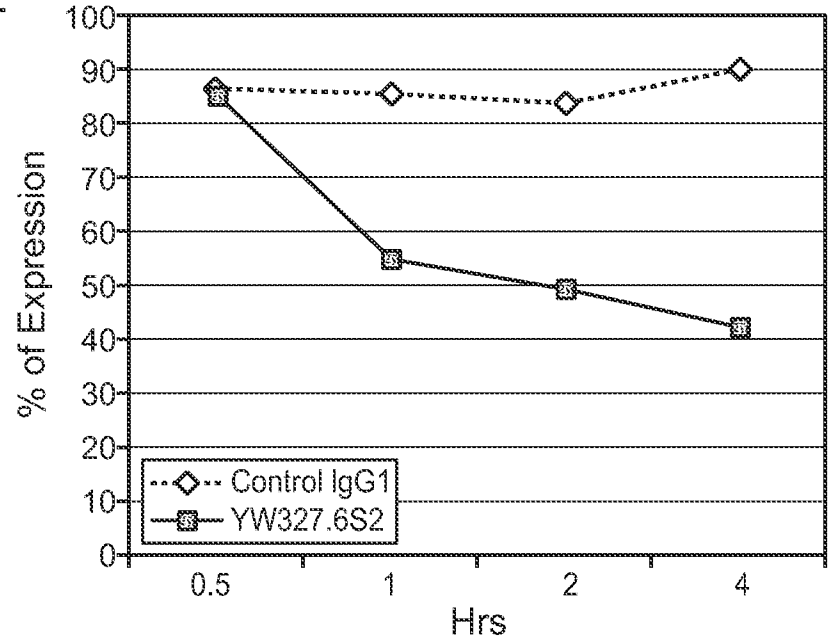


FIG. 14C

FIG. 14D


100
90
80
70
60
50
40
30
20
10
0
% of Expression
Control IgG1
YW327.6S2
0.5
1
2
4
Hrs

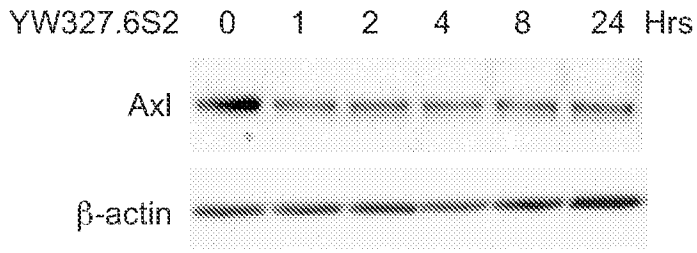

YW327.6S2 0 1 2 4 8 24 Hrs
Axl
β-actin

FIG. 14E

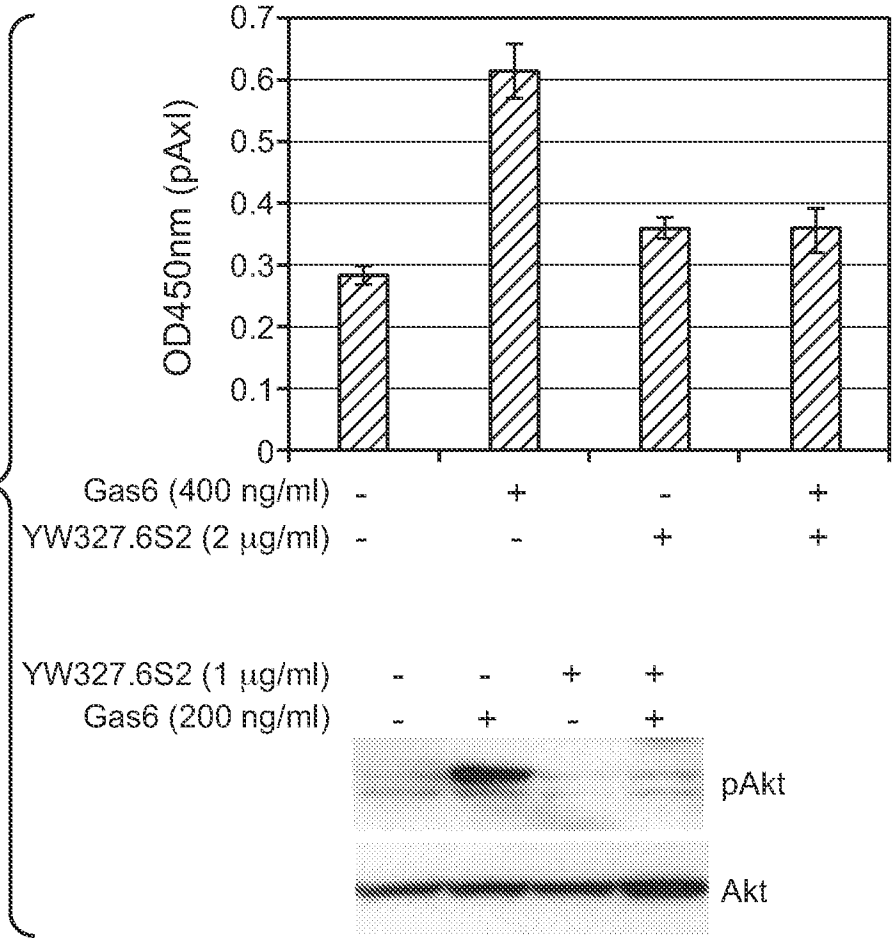
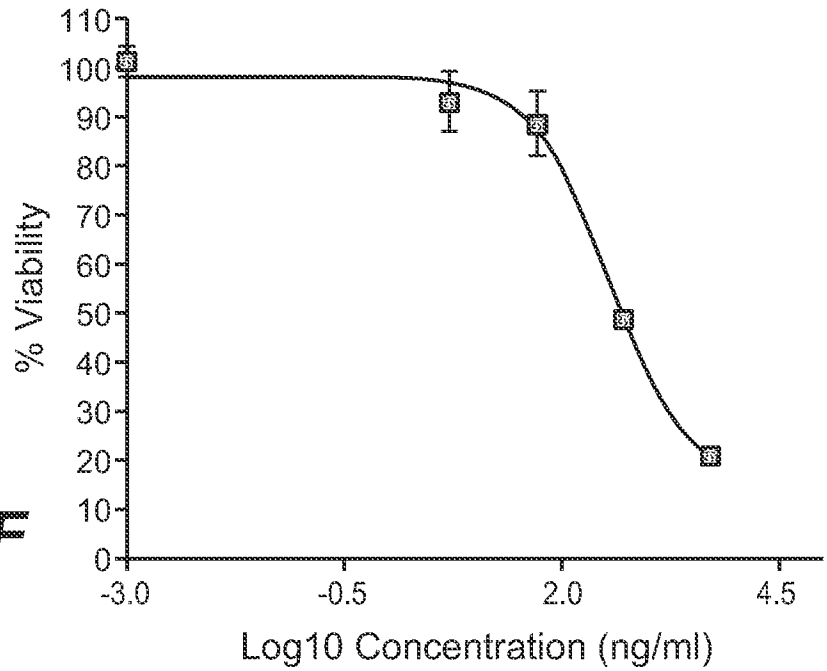


FIG. 14F



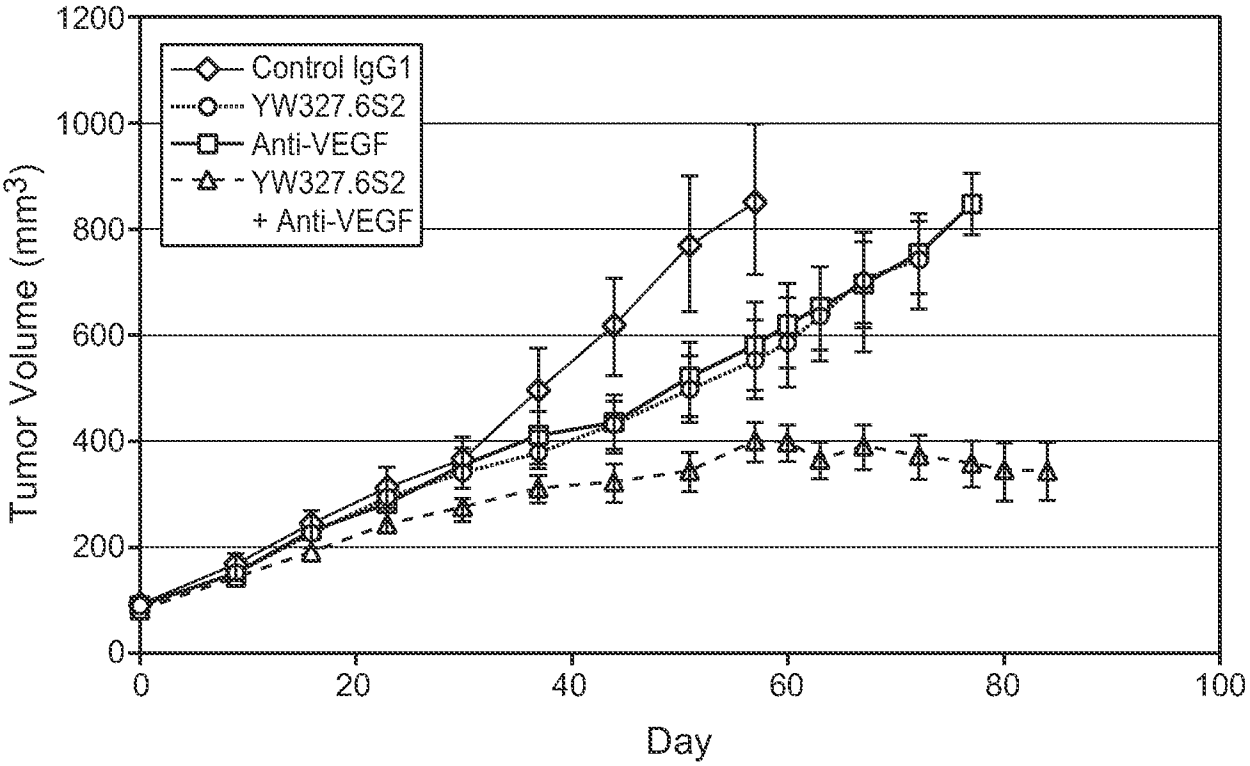


FIG. 15A

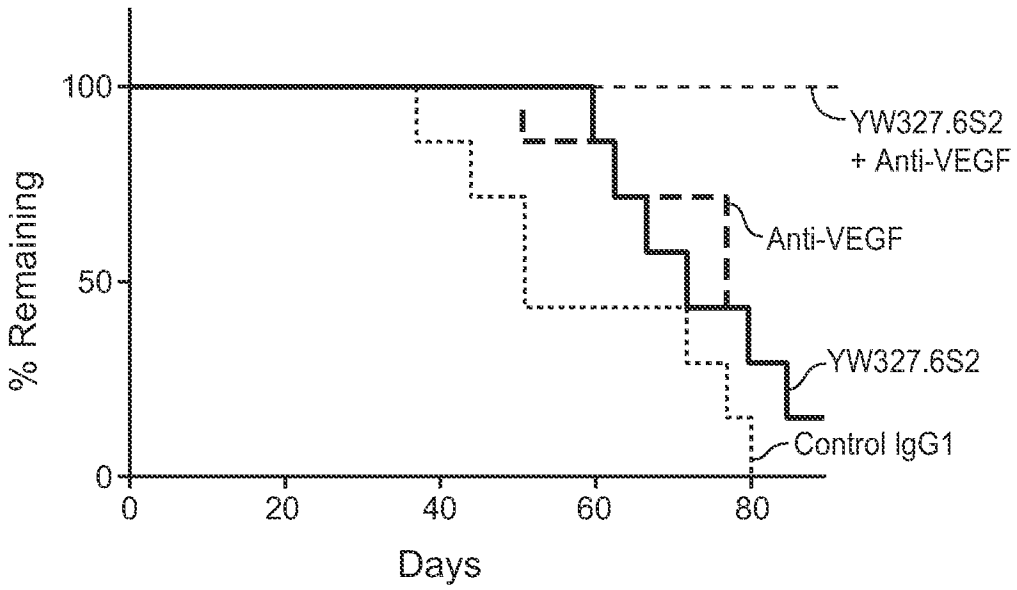


FIG. 15B

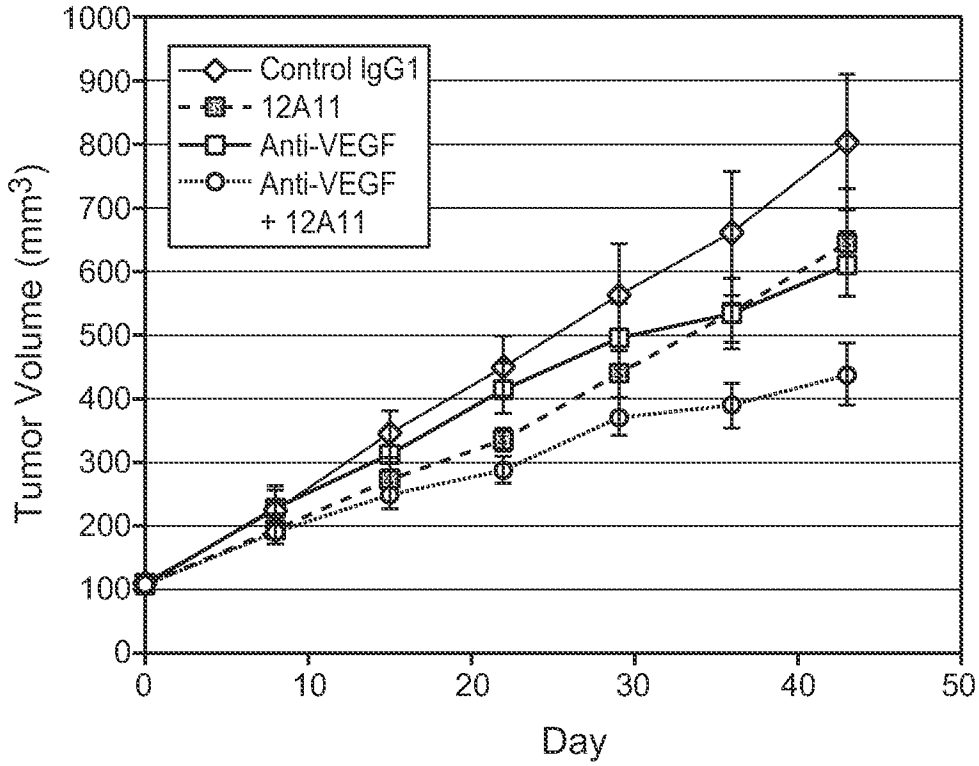


FIG. 15C

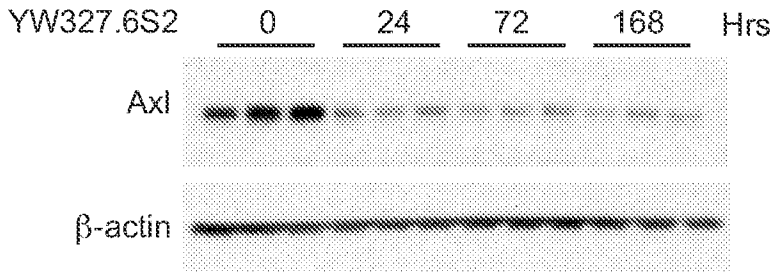


FIG. 15D

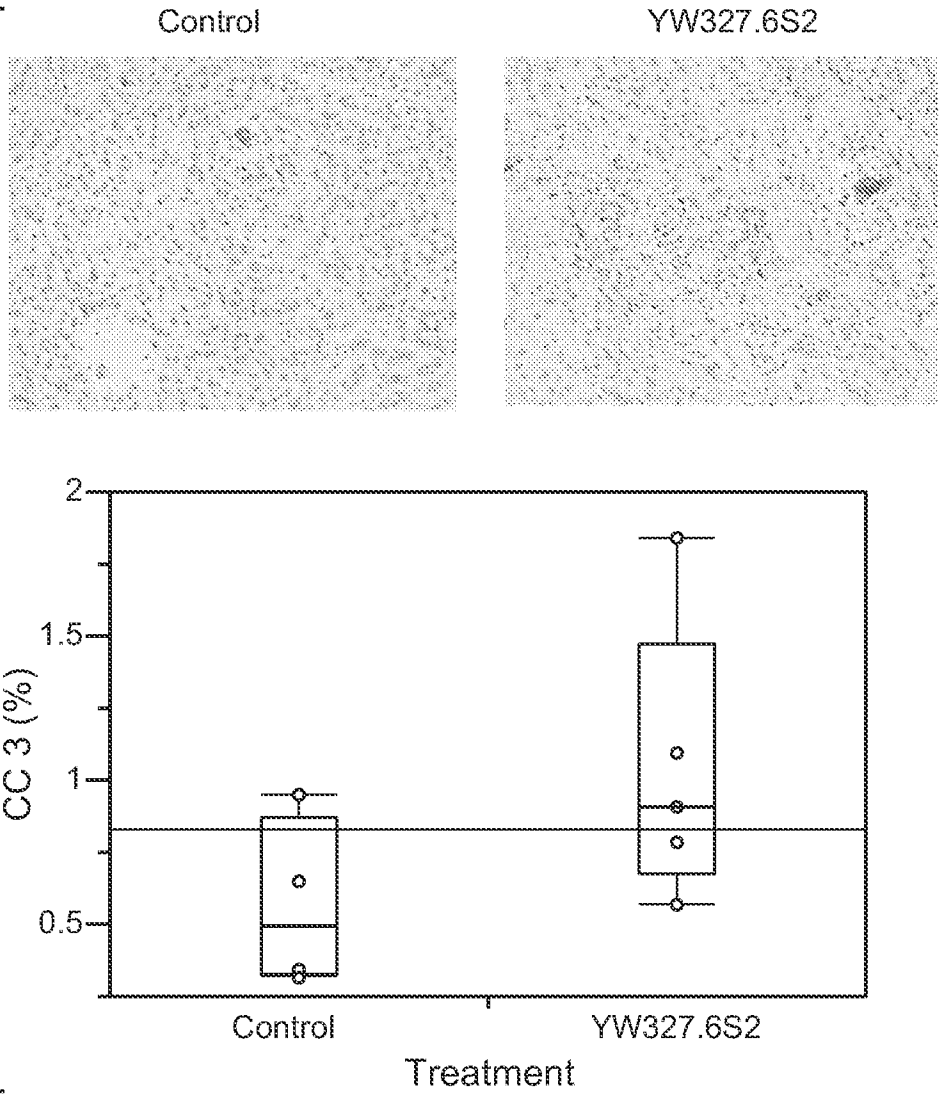


FIG. 15E

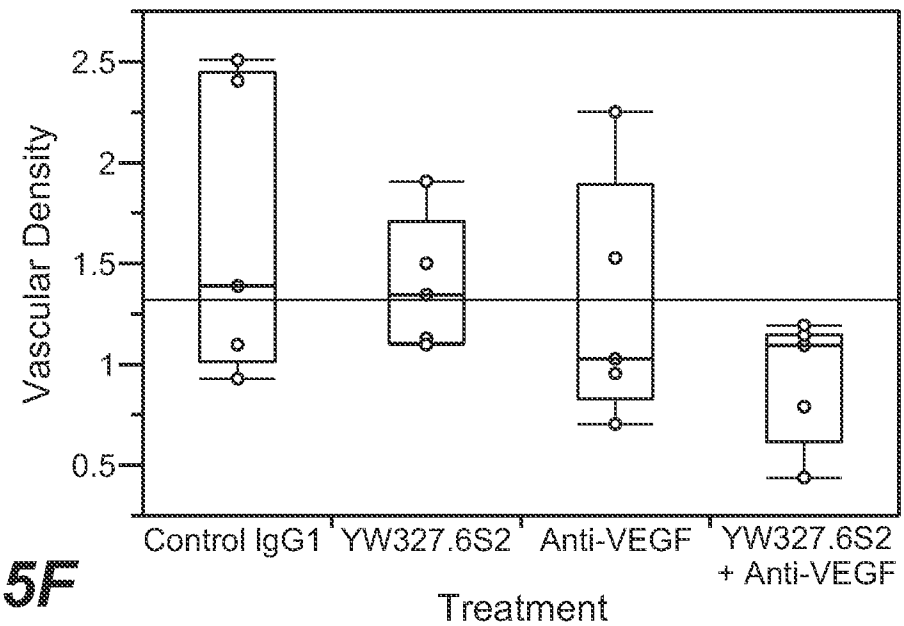


FIG. 15F

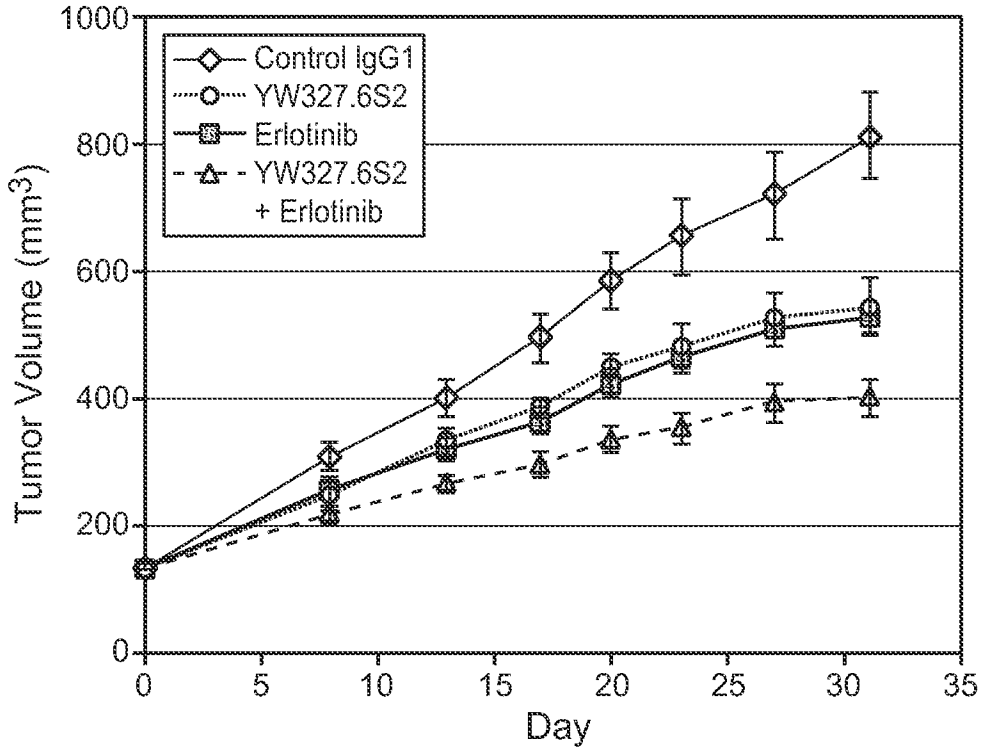


FIG. 16A

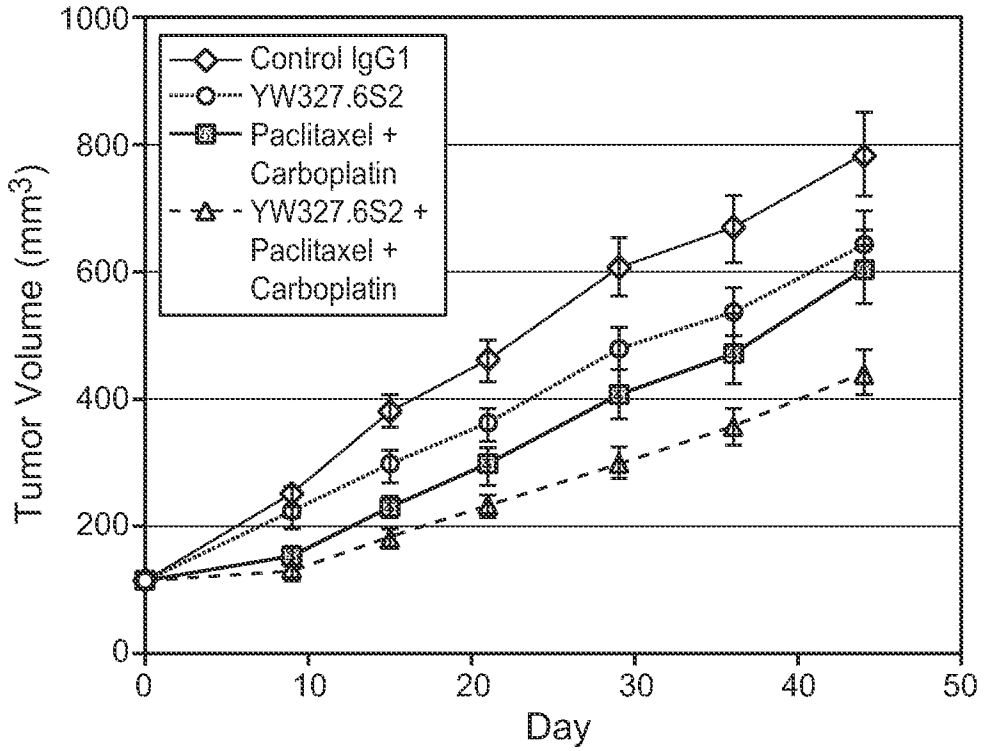


FIG. 16B

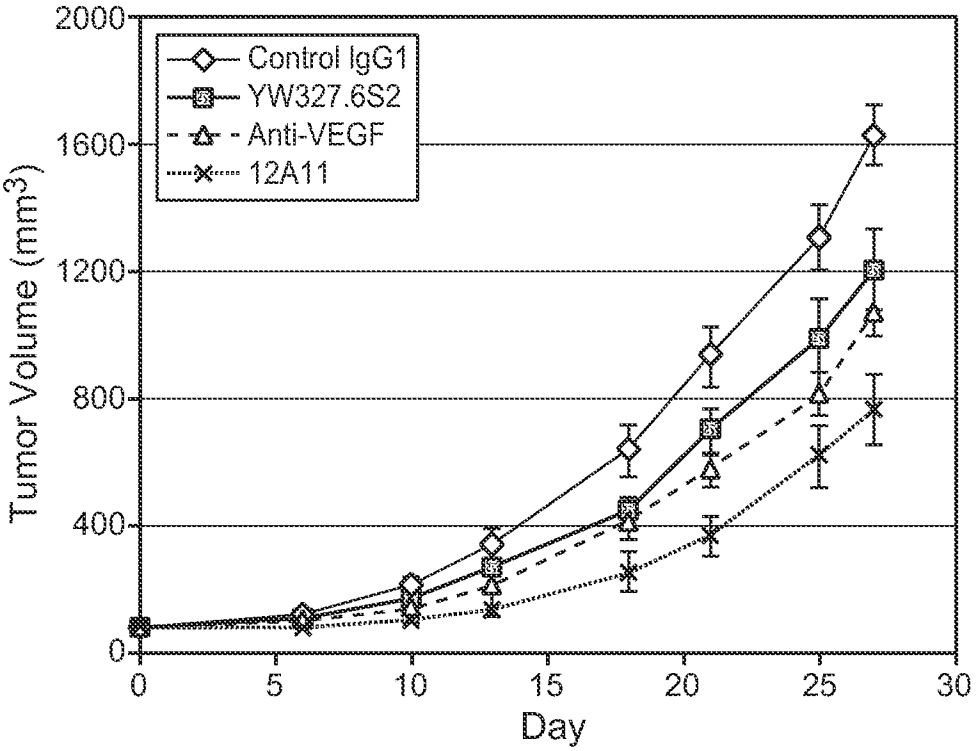


FIG. 17A

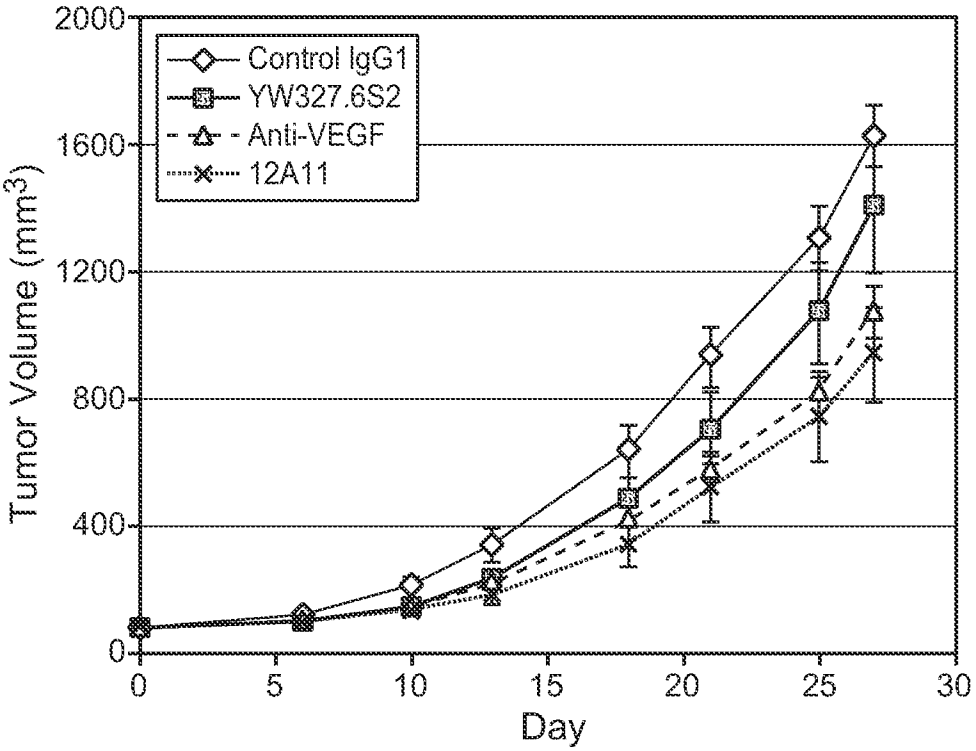


FIG. 17B

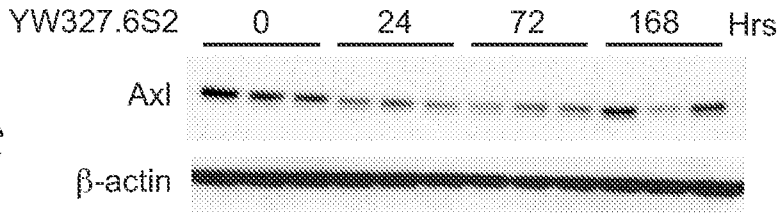


FIG. 17C

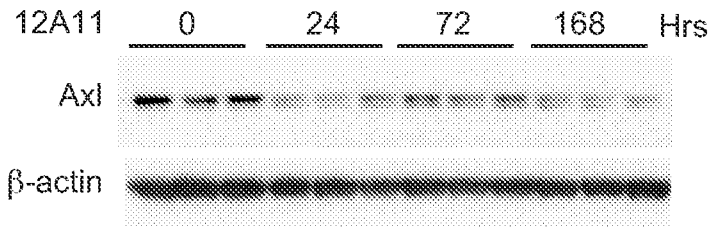
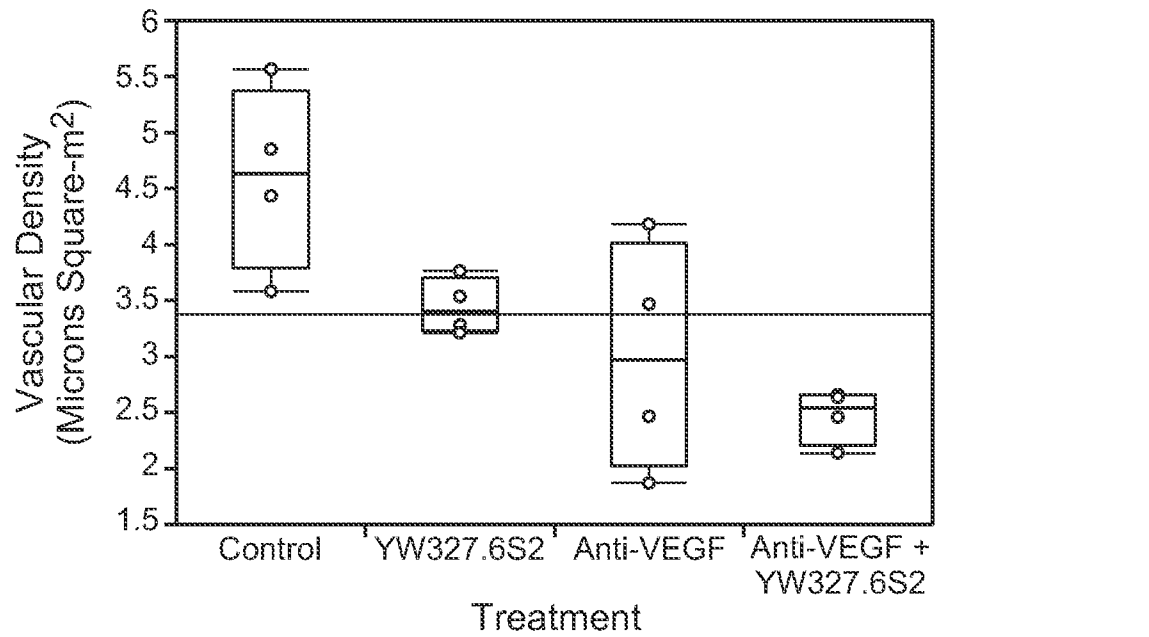


FIG. 17D



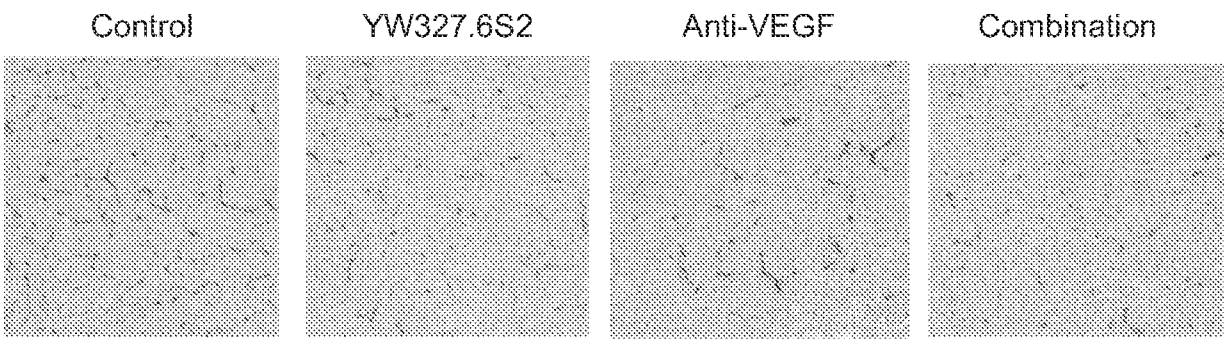


FIG. 17E

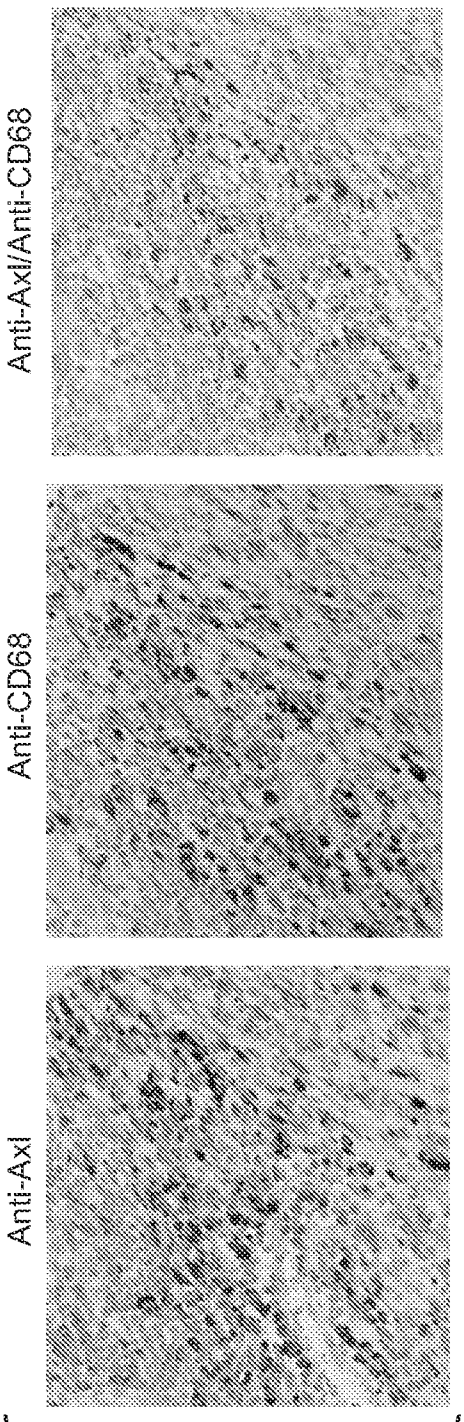


FIG. 17F

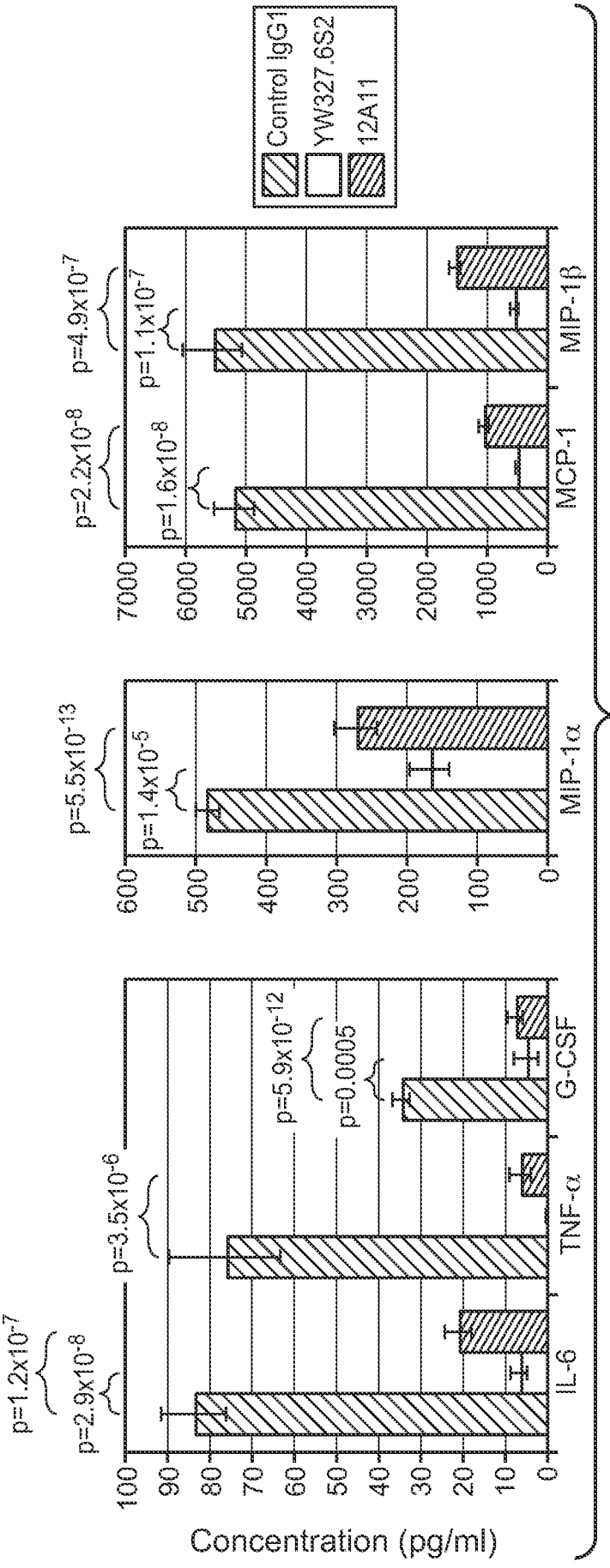


FIG. 17G

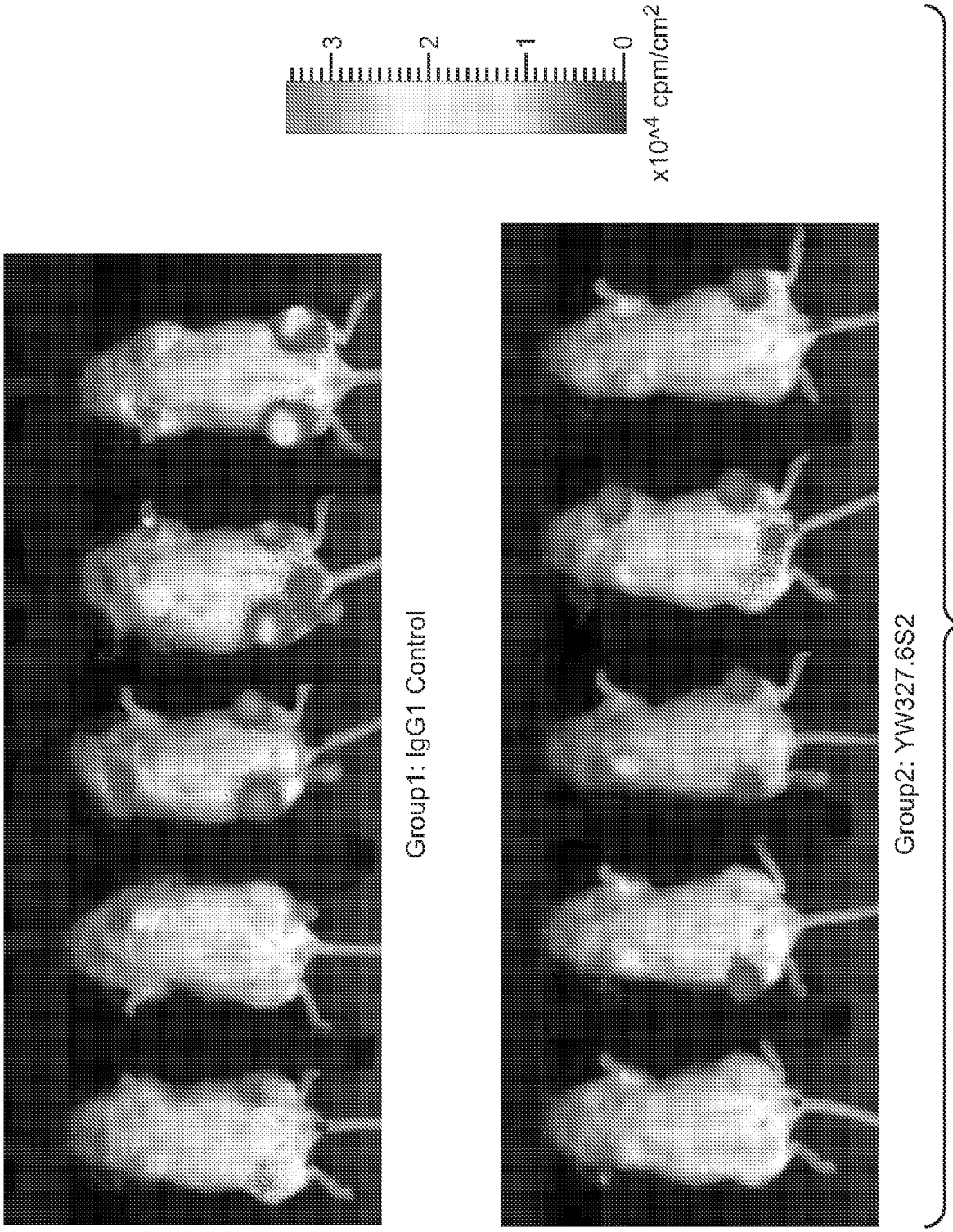


FIG. 18A

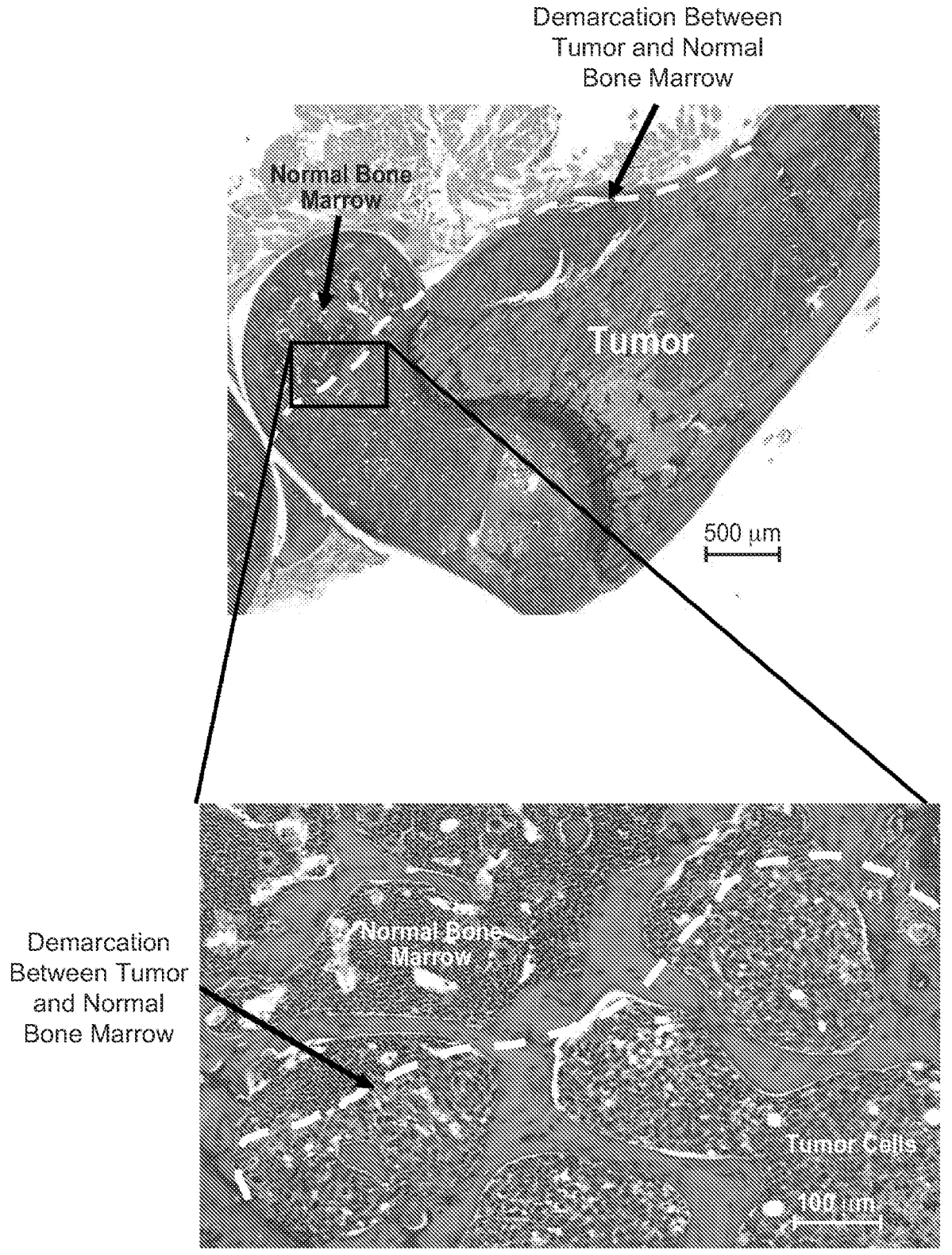

Demarcation Between Tumor and Normal Bone Marrow
Normal Bone Marrow
Tumor
500 µm
Demarcation Between Tumor and Normal Bone Marrow
Normal Bone Marrow
Tumor Cells
100 µm

FIG. 18B

MAWRCPRMGRVPLAWCLALCGWACMAPRGTQAEESPFVGNPGNI
TGARGLTGTLRCQLQVQGEPPEVHWLRDGQILELADSTQTQVPLGEDEQDDWIVVSQL
RITSLQLSDTGQYQCLVFLGHQTFVSQPGYVGLEGLPYFLEEPEDRTVAANTPFNLSC
QAQGPPEPVDLLWLQDAVPLATAPGHGPQRSLHVPGLNKTSSFSCEAHNAKGVTTSRT
ATITVLPQQPRNLHLVSRQPTELEVAWTPGLSGIYPLTHCTLQAVLSDDGMGIQAGEP
DPPEEPLTSQASVPPHQLRLGSLHPHTPYHIRVACTSSQGPSSWTHWLPVETPEGVPL
GPPENISATRNGSQAFVHWQEPRAPLQGTLLGYRLAYQGQDTPEVLMDIGLRQEVTLE
LQGDGSVSNLTVCVAAYTAAGDGPWSLPVPLEAWRPVKEPSTPAFSWPWWYVLLGAVV
AAACVLILALFLVHRRKKETRYGEVFEPTVERGELVVRYRVRKSYSRRTTEATLNSLG
ISEELKEKLRDVMVDRHKVALGKTLGEGEFGAVMEGQLNQDDSILKVAVKTMKIAICT
RSELEDFLSEAVCMKEFDHPNVMRLIGVCFQGSERESFPAPVVILPFMKHGDLHSFLL
YSRLGDQPVYLPTQMLVKFMADIASGMEYLSTKRFIHRDLAARNCMLNENMSVCVADF
GLSKKIYNGDYYRQGRIAKMPVKWIAIESLADRVYTSKSDVWSFGVTMWEIATRGQTP
YPGVENSEIYDYLRQGNRLKQPADCLDGLYALMSRCWELNPQDRPSFTELREDLENTL
KALPPAQEPDEILYVNMDEGGGYPEPPGAAGGADPPTQPDPKDSCSCLTAAEVHPAGR
YVLCPSTTPSPAQPADRGSPAAPGQEDGA (SEQ ID NO:17)

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/043248

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 C07K16/22 C07K16/28 A61K31/337 A61K31/517
A61K33/24 C07K14/705 G01N33/50 C12N15/113
ADD.
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)
A61K C07K G01N C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LORUSSO P ET AL: "Pharmacodynamics (pd) of XL880, a novel spectrum selective kinase inhibitor (SSKI), administered orally to patients (pts) with advanced solid tumors (AST)" EJC SUPPLEMENTS, vol. 4, no. 12, November 2006 (2006-11), page 124, XP025026779 & 18TH SYMPOSIUM ON MOLECULAR TARGETS AND CANCER THERAPEUTICS; PRAGUE, CZECH REPUBLIC; NOVEMBER 07 -10, 2006 ISSN: 1359-6349 * abstract	1-5

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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 document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"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
17 September 2010	01/10/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bumb, Peter

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	QIAN FAWN ET AL: "Inhibition of tumor cell growth, invasion, and metastasis by EXEL-2880 (XL880, GSK1363089), a novel inhibitor of HGF and VEGF receptor tyrosine kinases." CANCER RESEARCH 15 OCT 2009 LNKD-PUBMED:19808973, vol. 69, no. 20, 15 October 2009 (2009-10-15), pages 8009-8016, XP002601151 ISSN: 1538-7445 * abstract	1-5
X,P	LIU LI ET AL: "Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL." CANCER RESEARCH 1 SEP 2009 LNKD-PUBMED:19671800, vol. 69, no. 17, 1 September 2009 (2009-09-01), pages 6871-6878, XP002601152 ISSN: 1538-7445 page 6871, right-hand column	1-5
X	WO 2009/062690 A1 (U3 PHARMA GMBH [DE]; HETTMANN THORE [DE]; NIEWOEHNER JENS [DE]; RUHE J) 22 May 2009 (2009-05-22) examples 2-7,9-11,13-15,23-24 page 27, line 13 - line 14 figure 13	1-6,8-10
X	BELL R ET AL: "Bevacizumab: the first anti-angiogenic agent approved for the treatment of metastatic breast cancer" EUROPEAN JOURNAL OF CANCER. SUPPLEMENT, PERGAMON, OXFORD, GB LNKD-DOI:10.1016/S1359-6349(08)70286-6, vol. 6, no. 6, 1 March 2008 (2008-03-01), pages 1-6, XP022621253 ISSN: 1359-6349 [retrieved on 2008-03-01] the whole document	1-6,8-10
X,P	WO 2009/137429 A1 (SMITHKLINE BEECHAM CORP [US]; GILMER TONA M [US]; GREGER JAMES G [US];) 12 November 2009 (2009-11-12) page 28 - page 31	1-5

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMLET DAVID R ET AL: "Response to trastuzumab, erlotinib, and bevacizumab, alone and in combination, is correlated with the level of human epidermal growth factor receptor-2 expression in human breast cancer cell lines." MOLECULAR CANCER THERAPEUTICS OCT 2007 LNKD- PUBMED:17938260, vol. 6, no. 10, October 2007 (2007-10), pages 2664-2674, XP002601153 ISSN: 1535-7163 page 2665, left-hand column, line 42 - line 43	1-10
A	PYTEL DARIUSZ ET AL: "Tyrosine kinase blockers: new hope for successful cancer therapy." ANTI-CANCER AGENTS IN MEDICINAL CHEMISTRY JAN 2009 LNKD- PUBMED:19149483, vol. 9, no. 1, January 2009 (2009-01), pages 66-76, XP002601154 ISSN: 1875-5992 table 1	1-10
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Information on patent family members

International application No
PCT/US2010/043248

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		CA 2705164 A1	22-05-2009
		EP 2220121 A1	25-08-2010
WO 2009137429 A1	12-11-2009	AR 071631 A1	30-06-2010
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