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(54) Title: POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

(57) **Abrégé/Abstract:**

In certain embodiments, this present invention provides polypeptide compositions (e.g., antibodies and antigen binding portions thereof that bind to EphB4), and methods for inhibiting EphB4 activity. In other embodiments, the present invention provides methods and compositions for treating cancer or for treating angiogenesis-associated diseases.



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(54) Title: POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

(57) Abstract: In certain embodiments, this present invention provides polypeptide compositions (e.g., antibodies and antigen binding portions thereof that bind to EphB4), and methods for inhibiting EphB4 activity. In other embodiments, the present invention provides methods and compositions for treating cancer or for treating angiogenesis-associated diseases.



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**POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS
AND TUMOR GROWTH**

RELATED APPLICATIONS

5 This application claims the benefit of priority of U.S. Provisional Application number 60/612,908 filed September 23, 2004, U.S. Application number 10/949,720 filed September 23, 2004, and U.S. Application number 10/800,350 filed March 12, 2004. The entire teachings of the referenced Applications are incorporated herein by reference in their entirety.

10

BACKGROUND OF THE INVENTION

EphB4, sometimes referred to as Ephrin Receptor B4 or Hepatoma Transmembrane Kinase (HTK), belongs to a family of transmembrane receptor protein-tyrosine kinases. EphB4 has an extracellular domain composed of the
15 ligand-binding domain (also referred to as globular domain), a cysteine-rich domain, and a pair of fibronectin type III repeats (e.g., see Figure 5). The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α -motif (SAM) and a PDZ-domain binding motif. EphB4 interacts with the membrane-bound ligand Ephrin B2
20 (Sakano, S. et al Oncogene. 1996 Aug 15;13(4):813-22; Brambilla R. et al EMBO J. 1995 Jul 3;14(13):3116-26). EphB4, like other members of the Eph family, is activated by binding of clustered, membrane-attached ephrin ligands (Davis S et al, Science. 1994 Nov 4;266(5186):816-9), indicating that contact between cells expressing the receptor and cells expressing the ligand is required for the Eph
25 receptor activation. Upon ligand binding, an EphB4 receptor dimerizes and autophosphorylates the juxtamembrane tyrosine residues to acquire full activation. It has generally been thought that when an EphB4-expressing cell encounters an EphrinB2-expressing cell, the EphB4-EphrinB2 interaction and aggregation triggers signaling in both cells.

30 EphB4-EphrinB2 signaling has been implicated in angiogenesis (Wang et al. Cell. 1998 May 29;93(5):741-53; Gerety et al. Mol Cell. 1999 Sep;4(3):403-14). Angiogenesis, the development of new blood vessels from the endothelium of a

preexisting vasculature, is a critical process in the growth, progression, and metastasis of solid tumors within the host. During physiologically normal angiogenesis, the autocrine, paracrine, and amphicrine interactions of the vascular endothelium with its surrounding stromal components are tightly regulated both spatially and temporally. Additionally, the levels and activities of proangiogenic and angiostatic cytokines and growth factors are maintained in balance. In contrast, the pathological angiogenesis necessary for active tumor growth is sustained and persistent, representing a dysregulation of the normal angiogenic system. Solid and hematopoietic tumor types are particularly associated with a high level of abnormal angiogenesis.

It is generally thought that the development of a tumor consists of sequential, and interrelated steps that lead to the generation of an autonomous clone with aggressive growth potential. These steps include sustained growth and unlimited self-renewal. Cell populations in a tumor are generally characterized by growth signal self-sufficiency, decreased sensitivity to growth suppressive signals, and resistance to apoptosis. Genetic or cytogenetic events that initiate aberrant growth sustain cells in a prolonged "ready" state by preventing apoptosis.

It is a goal of the present disclosure to provide agents and therapeutic treatments for inhibiting angiogenesis and tumor growth.

20

SUMMARY OF THE INVENTION

In certain aspects, the disclosure provides polypeptide agents that inhibit EphB4 mediated functions, including antibodies and antigen binding portions thereof that bind to and affect EphB4 in particular ways. As demonstrated herein, EphB4 and EphrinB2 participate in various disease states, including cancers and diseases related to unwanted or excessive angiogenesis. Accordingly, certain polypeptide agents disclosed herein may be used to treat such diseases. In further aspects, the disclosure relates to the discovery that EphB4 and/or EphrinB2 are expressed, often at high levels, in a variety of tumors. Therefore, polypeptide agents that downregulate EphB4 or EphrinB2 function may affect tumors by a direct effect on the tumor cells as well as an indirect effect on the angiogenic processes recruited

30

by the tumor. In certain embodiments, the disclosure provides the identity of tumor types particularly suited to treatment with an agent that downregulates EphB4 or EphrinB2 function.

In certain aspects, the disclosure provides an isolated antibody or antigen
5 binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity. The isolated antibody or antigen binding portion thereof may binds to an epitope situated within amino acids 16-198 of the EphB4 sequence of Figure 1. For example, the epitope may be situated within the Globular Domain (GD) of EphB4 that binds to EphrinB2. The isolated antibody or
10 antigen binding portion thereof may inhibit the binding of EphB4 to the extracellular portion of EphrinB2. The isolated antibody or antigen binding portion thereof may bind to an epitope situated within amino acids 324-429 or 430-537 of the EphB4 sequence of Figure 1. For example, the isolated antibody or antigen binding portion thereof may bind to the first fibronectin-like domain (FND1) or the second
15 fibronectin-like domain (FND2) of EphB4. The isolated antibody or antigen binding portion thereof may inhibit EphB4 dimerization or multimerization and may optionally inhibit the EphrinB2-stimulated autophosphorylation of EphB4. The isolated antibody or antigen binding portion thereof may inhibit the formation of tubes by cultured endothelial cells, the vascularization of a tissue in vivo, the
20 vascularization of tissue implanted in the cornea of an animal, the vascularization of a Matrigel tissue plug implanted in an animal, and/or the growth of a human tumor xenograft in a mouse. Preferred antibodies that bind to an epitope situated within amino acids 16-198 of the EphB4 sequence of Figure 1 include antibodies denoted herein as No. 001, No. 023, No. 035, and No. 079. Preferred antibodies that bind to
25 an epitope situated within amino acids 428-537 of the EphB4 sequence of Figure 1 include antibodies denoted herein as No. 047, No. 057, No. 85H, No. 098, and No. 138.

In certain aspects, the disclosure provides an isolated antibody or antigen
binding portion thereof that binds to an epitope situated in the extracellular portion
30 of EphB4 and stimulates EphB4 kinase activity. For example, described herein are isolated antibodies or antigen binding portion thereof that bind to an epitope situated within amino acids 324-429 or 430-537 of the EphB4 sequence of Figure 1 and

stimulate EphB4 kinase activity. The isolated antibody or antigen binding portion thereof may bind to FND1 or FND2 of EphB4. The antibody may be selected from the group consisting of antibodies denoted herein as No. 85L, No. 091, No. 121, and No. 131.

5 The disclosure provides humanized versions of any of the antibodies disclosed herein, as well as antibodies and antigen binding portions thereof that comprise at least one CDR portion derived from an antibody disclosed herein, particularly the CDR3. In preferred embodiments, the antibody is a monoclonal antibody that is immunocompatible with the subject to which it is to be
10 administered, and preferably is clinically acceptable for administration to a human.

 In certain aspects, the disclosure provides a hybridoma that produces an antibody disclosed herein, and particularly a hybridoma that produces an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, No. 079, No. 047, No. 057, No. 85H, No. 098, No. 138, No. 085L, No.
15 091, and No. 131. Hybridomas producing antibody No. 023 (epitope within amino acids 16-198), antibody No. 091 (kinase activating antibody; epitope within amino acids 324-429), antibody No. 098 (epitope within amino acids 430-537), antibody No. 131 (epitope within amino acids 324-429), and antibody No. 138 (epitope within amino acids 430-537) were deposited in the American Type Culture
20 Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209. The ATCC Deposit Designation Nos. for antibody No. 023, No. 091, No. 098, No. 131, and No. 138 are PTA-6208, PTA-6209, PTA-6210, PTA-6214, and PTA-6211, respectively.

 Therefore, certain specific aspects of the disclosure provide a hybridoma cell
25 having an ATCC Deposit Designation No. selected from the group consisting of PTA-6208, PTA-6209, PTA-6210, PTA-6214, and PTA-6211.

 Surprisingly, antibodies that inhibit ligand binding, antibodies that inhibit EphB4 kinase activation and antibodies that activate EphB4 kinase activity all inhibit EphB4 mediated events in bioassays. Accordingly, the disclosure provides a
30 method of treating cancer, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion

thereof that binds to an epitope situated in the extracellular portion of EphB4 and either inhibits an EphB4 activity or activates EphB4 kinase activity. Optionally the patient has been diagnosed with a cancer selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia. The isolated antibody or antigen binding portion thereof may be administered systemically or locally. Additionally, the disclosure provides methods of inhibiting angiogenesis in a patient, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity or activates an EphB4 kinase activity. Optionally, the patient is diagnosed macular degeneration.

In certain aspects, the disclosure provides a pharmaceutical preparation comprising any of the isolated antibodies or antigen binding portions thereof disclosed herein, as well as the use of such antibodies or antigen binding portions thereof to make a pharmaceutical preparation for treating cancer. Optionally, the cancer is selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.

In certain aspects, the antibodies disclosed herein may be covalently linked (or otherwise stably associated with) an additional functional moiety, such as a label or a moiety that confers desirable pharmacokinetic properties. Exemplary labels include those that are suitable for detection by a method selected from the group consisting of fluorescence detection methods, positron emission tomography detection methods and nuclear magnetic resonance detection methods. Labels may, for example, be selected from the group consisting of a fluorescent label, a radioactive label, and a label having a distinctive nuclear magnetic resonance signature. Moieties such as a polyethylene glycol (PEG) moiety may be affixed to an antibody or antigen binding portion thereof to increase serum half-life.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an amino acid sequence of human EphB4 precursor protein (SEQ ID NO: 1).

Figure 2 shows a cDNA nucleotide sequence of human EphB4 protein.

Figure 3 shows amino acid sequence of the B4ECv3 protein (predicted
5 sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 4 shows amino acid sequence of the B4ECv3NT protein (predicted
sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 5 shows the monoclonal antibodies generated against EphB4 and
epitope mapping of these antibodies. The topology of the EphB4 extracellular
10 domain is shown, including a globular domain (G), a cystein-rich domain (C), and
two fibronectin type 3 domains (F1 and F2).

Figure 6 shows effects of Ephrin B2 polyclonal antibodies and EphB4
polyclonal antibodies tumor cell growth. A) H28 cell line; B) H2373 cell line; and
C) H2052 cell line.

15 Figure 7 shows results from affinity tests of EphB4 monoclonal antibodies.
The order of the affinity (from weakest to strongest) is shown.

Figure 8 shows mouse corneal micropocket assay with an exemplary EphB4
antibody (No. 138) in the presence or absence of bFGF.

Figure 9 shows that EphB4 antibodies inhibit the growth of SCC15 xenograft
20 tumors.

Figure 10 shows that EphB4 antibodies cause apoptosis, necrosis and
decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Figure 11 shows that systemic administration of EphB4 antibodies leads to
tumor regression.

25

DETAILED DESCRIPTION OF THE INVENTION

I. EphB4 Antibodies and Other Binding Polypeptides

The disclosure provides, in part, defined portions of the EphB4 molecule that
can be effectively targeted by polypeptide binding agents, such as antibodies,

antigen binding portions of antibodies, and non-immunoglobulin antigen binding scaffolds. The EphB4 polypeptide binding agents described herein may be used to treat a variety of disorders, particularly cancers and disorders related to unwanted angiogenesis. The disclosure provides antibodies and antigen binding portions thereof that inhibit one or more EphB4 mediated functions, such as EphrinB2 binding or EphB4 kinase activity. Such binding agents may be used to inhibit EphB4 function in vitro and in vivo, and preferably for treating cancer or disorders associated with unwanted angiogenesis. The disclosure also provides antibodies and antigen binding portions thereof that activate EphB4 kinase activity (typically assessed by evaluating EphB4 phosphorylation state). Surprisingly, such antibodies also inhibit EphB4 functions in cell based and in vivo assays. Accordingly, such binding agents may be used to inhibit EphB4 function in vitro and in vivo, and preferably for treating cancer or disorders associated with unwanted angiogenesis. While not wishing to be limited to any particular mechanism, it is expected that these antibodies stimulate not only EphB4 kinase activity, but also EphB4 removal from the membrane, thus decreasing overall EphB4 levels.

EphB4 belongs to a family of transmembrane receptor protein tyrosine kinases. The extracellular portion of EphB4 is composed of the ligand-binding domain (also referred to as globular domain), a cysteine-rich domain, and a pair of fibronectin type III repeats (e.g., see Figure 1). The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α -motif (SAM) and a PDZ-domain binding motif. EphB4 is specific for the membrane-bound ligand Ephrin B2 (Sakano, S. et al 1996; Brambilla R. et al 1995). EphB4 is activated by binding of clustered, membrane-attached ephrin ligands (Davis S et al, 1994), indicating that contact between cells expressing the receptor and cells expressing the ligand is required for the Eph receptor activation. Upon ligand binding, an EphB4 receptor dimerizes and autophosphorylates the juxtamembrane tyrosine residues to acquire full activation.

As used herein, the term "EphB4" refers to an EphB4 polypeptide from a mammal including humans. In one embodiment, the antibodies (immunoglobulins) are raised against an isolated and/or recombinant mammalian EphB4 or portion thereof (e.g., peptide) or against a host cell which expresses recombinant

mammalian EphB4. In certain aspects, antibodies of the invention specifically bind to an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide). For example, an EphB4 soluble polypeptide comprises a globular domain and is capable of binding to Ephrin B2. An example of EphB4 soluble polypeptides is provided in Figure 2. As used herein, the EphB4 soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide.

An "immunoglobulin" is a tetrameric molecule. In a naturally-occurring immunoglobulin, each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Human light chains are classified as kappa and lambda light chains. Heavy chains are classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)) (incorporated by reference in its entirety for all purposes). The variable regions of each light/heavy chain pair form the antibody binding site such that an intact immunoglobulin has two binding sites. Immunoglobulins may be organized into higher order structures. IgA is generally a dimer of two tetramers. IgM is generally a pentamer of five tetramers.

Immunoglobulin chains exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminus to C-terminus, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of

Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J. Mol. Biol. 196:901-917 (1987); Chothia et al. Nature 342:878-883 (1989).

An "antibody" refers to an intact immunoglobulin or to an antigen-binding
5 portion thereof that competes with the intact antibody for specific binding. Antigen-binding portions may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Antigen-binding portions include, inter alia, Fab, Fab', F(ab')₂, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric
10 antibodies, diabodies and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. The terms "anti-EphB4 antibody" and "EphB4 antibody" are used interchangeably herein.

An Fab fragment is a monovalent fragment consisting of the VL, VH, CL
15 and CH I domains; a F(ab')₂ fragment is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consists of the VH and CH1 domains; an Fv fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment (Ward et al., Nature 341:544-546, 1989) consists of a VH domain.

20 A single-chain antibody (scFv) is an antibody in which a VL and VH regions are paired to form a monovalent molecules via a synthetic linker that enables them to be made as a single protein chain (Bird et al., Science 242:423-426, 1988 and Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883, 1988). Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a
25 single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al., Proc. Natl. Acad. Sci. USA 90:6444-6448, 1993, and Poljak, R. J., et al., Structure 2:1121-1123, 1994). One or more CDRs may be
30 incorporated into a molecule either covalently or noncovalently.

An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally-occurring immunoglobulin has two identical binding sites, a single-chain antibody or Fab fragment has one binding site, while a "bispecific" or
5 "bifunctional" antibody has two different binding sites.

The term "human antibody" includes all antibodies that have one or more variable and constant regions derived from human immunoglobulin sequences. In a preferred embodiment, all of the variable and constant domains are derived from human immunoglobulin sequences (a fully human antibody). These antibodies may
10 be prepared in a variety of ways, as described below.

The term "chimeric antibody" refers to an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies. In a preferred embodiment, one or more of the CDRs are derived from a human anti-EphB4 antibody. In a more preferred embodiment, all of the CDRs are
15 derived from a human anti-EphB4 antibody. In another preferred embodiment, the CDRs from more than one human anti-EphB4 antibodies are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first human anti-EphB4 antibody may be combined with CDR2 and CDR3 from the light chain of a second human anti-EphB4 antibody, and the
20 CDRs from the heavy chain may be derived from a third anti-EphB4 antibody. Further, the framework regions may be derived from one of the same anti-EphB4 antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody.

A "neutralizing antibody" is an antibody that inhibits the binding of EphB4
25 to EphrinB2 when an excess of the anti-EphB4 antibody reduces the amount of EphB4 (soluble) bound to EphrinB2 by at least about 20% and preferably by at least 40%, more preferably 60%, even more preferably 80%, or even more preferably 85%. The binding reduction may be measured by any means known to one of ordinary skill in the art, for example, as measured in an in vitro competitive binding
30 assay. An example of measuring the reduction in binding is presented below in the Examples.

An "EphB4 kinase activating antibody" is an antibody that activates EphB4 kinase activity by at least about 20% when added to a cell, tissue or organism expressing EphB4. In a preferred embodiment, the antibody activates EphB4 kinase activity by at least 40%, more preferably 60%, even more preferably 80%, or even
5 more preferably 85%. Typically kinase activity is measured as the phosphorylation state of EphB4 itself (tyrosine autophosphorylation).

As used herein, the terms "label" or "labeled" refers to incorporation of another molecule in the antibody. In one embodiment, the label is a detectable marker, e.g., incorporation of a radiolabeled amino acid or attachment to a
10 polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). In another embodiment, the label or marker can be therapeutic, e.g., a drug conjugate or toxin. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used.
15 Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (e.g., ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups, predetermined
20 polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), magnetic agents, such as gadolinium chelates, toxins such as pertussis toxin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin,
25 dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

As shown in the Examples below, Applicants have generated a number of
30 monoclonal antibodies against EphB4 as well as hybridoma cell lines producing EphB4 monoclonal antibodies. These antibodies were further characterized in many ways, such as, their ability to inhibit interaction between EphB4 and its ligand (e.g.,

Ephrin B2 – neutralizing antibodies), their ability to inhibit dimerization or multimerization of EphB4 receptor, their ability to induce tyrosine phosphorylation of EphB4, their cross-reactivity with other Eph family members, their ability to inhibit angiogenesis, and their ability to inhibit tumor growth. Further, epitope mapping studies reveals that these EphB4 antibodies may specifically bind to one or more regions of EphB4 (e.g., a globular domain, a cysteine-rich domain, or a fibronectin type III domain). For example, an EphB4 antibody may bind to both fibronectin type III domains.

In certain aspects, antibodies of the invention specifically bind to an extracellular domain (ECD) of an EphB4 protein (also referred to herein as a soluble EphB4 polypeptide). A soluble EphB4 polypeptide may comprise a sequence encompassing the globular (G) domain (amino acids 29-197 of SEQ ID NO: 1), and optionally additional domains, such as the cysteine-rich domain (amino acids 239-321 of SEQ ID NO: 1), the first fibronectin type 3 domain (amino acids 324-429 of SEQ ID NO: 1) and the second fibronectin type 3 domain (amino acids 434-526 of SEQ ID NO: 1). Exemplary EphB4 soluble polypeptides are provided in Figures 3-4. As used herein, the EphB4 soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide.

In certain aspects, the present invention provides antibodies (anti-EphB4) having binding specificity for an EphB4 or a portion of EphB4. Examples of these antibodies include, but are not limited to, EphB4 antibody Nos. 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, and 138 as shown in Figure 5. Optionally, the immunoglobulins can bind to EphB4 with an affinity of at least about 1×10^{-6} , 1×10^{-7} , 1×10^{-8} , 1×10^{-9} M or less. Optionally, antibodies and portions thereof bind to EphrinB2 with an affinity that is roughly equivalent to that of a soluble extracellular EphB4 polypeptide comprising the globular ligand binding domain.

Antibodies disclosed herein will preferably be specific for EphB4, with minimal binding to other members of the Eph or Ephrin families. In another aspect of the invention, the anti-EphB4 antibody demonstrates both species and molecule selectivity. In one embodiment, the anti-EphB4 antibody binds to human, cynomolgous or rhesus EphB4. In a preferred embodiment, the anti-EphB4

antibody does not bind to mouse, rat, guinea pig, dog or rabbit EphB4. Optionally, the antibody does bind to multiple different EphB4s from different species, such as human and mouse. Following the teachings of the specification, one may determine the species selectivity for the anti-EphB4 antibody using methods well known in the art. For instance, one may determine species selectivity using Western blot, FACS, ELISA or RIA. In a preferred embodiment, one may determine the species selectivity using Western blot. In another embodiment, the anti-EphB4 antibody has a tendency to bind EphB4 that is at least 50 times greater than its tendency to bind other members of the EphB family from the same species, and preferably 100 or 200 times greater. One may determine selectivity using methods well known in the art following the teachings of the specification. For instance, one may determine the selectivity using Western blot, FACS, ELISA or RIA. In a preferred embodiment, one may determine the molecular selectivity using Western blot.

In certain embodiments, antibodies of the present invention bind to one or more specific domains of EphB4. For example, an antibody binds to one or more extracellular domains of EphB4 (such as the globular domain, the cysteine-rich domain, and the first fibronectin type 3 domain, and the second fibronectin type 3 domain). For example, EphB4 antibody Nos. 1, 23, 35, and 79 bind to an epitope in the region spanning amino acids 16-198 of the sequence in Figure 1, spanning the globular domain. EphB4 antibody Nos. 85L, 85H, 91, and 131 bind to an epitope in the region spanning amino acids 324-429, including the first fibronectin type 3 domain. EphB4 antibody Nos. 47, 57, 85H, 98, 121, and 138 bind to an epitope in the region spanning amino acids 430-537, including the second fibronectin type 3 domain. Optionally, the subject antibody (e.g., EphB4 antibody No. 85H) can bind to at least two domains of an EphB4 (Figure 5).

The anti-EphB4 antibody may be an IgG, an IgM, an IgE, an IgA or an IgD molecule. In a preferred embodiment, the antibody is an IgG and is an IgG1, IgG2, IgG3 or IgG4 subtype. In a more preferred embodiment, the anti-EphB4 antibody is subclass IgG2. The class and subclass of EphB4 antibodies may be determined by any method known in the art. In general, the class and subclass of an antibody may be determined using antibodies that are specific for a particular class and subclass of antibody. Such antibodies are available commercially. The class and subclass can

be determined by ELISA, Western Blot as well as other techniques. Alternatively, the class and subclass may be determined by sequencing all or a portion of the constant domains of the heavy and/or light chains of the antibodies, comparing their amino acid sequences to the known amino acid sequences of various class and subclasses of immunoglobulins, and determining the class and subclass of the antibodies. To illustrate, the classes and subclasses of the exemplary EphB4 antibodies are shown in Table 1 below.

In certain embodiments, single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single chain antibodies, comprising portions derived from different species, are also encompassed by the present invention as antigen binding portions of an antibody. The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly et al., U.S. Pat. No. 4,816,567; Cabilly et al., European Patent No. 0,125,023; Boss et al., U.S. Pat. No. 4,816,397; Boss et al., European Patent No. 0,120,694; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; and Winter, European Patent No. 0,239,400 B1. See also, Newman, R. et al., *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody. See, e.g., Ladner et al., U.S. Pat. No. 4,946,778; and Bird, R. E. et al., *Science*, 242: 423-426 (1988)), regarding single chain antibodies.

In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen binding function of a corresponding full-length antibody (e.g., specificity for an EphB4). Certain preferred functional fragments retain the ability to inhibit one or more functions characteristic of an EphB4, such as a binding activity, a signaling activity, and/or stimulation of a cellular response. For example, in one embodiment, a functional fragment of an

EphB4 antibody can inhibit the interaction of EphB4 with one or more of its ligands (e.g., Ephrin B2) and/or can inhibit one or more receptor-mediated functions, such as cell migration, cell proliferation, angiogenesis, and/or tumor growth.

For example, antibody fragments capable of binding to an EphB4 receptor or
5 portion thereof, including, but not limited to, Fv, Fab, Fab' and F(ab')₂ fragments are encompassed by the invention. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons
10 has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

A humanized antibody is an antibody that is derived from a non-human species, in which certain amino acids in the framework and constant domains of the
15 heavy and light chains have been mutated so as to avoid or abrogate an immune response in humans. Alternatively, a humanized antibody may be produced by fusing the constant domains from a human antibody to the variable domains of a non-human species. Examples of how to make humanized antibodies may be found in U.S. Pat. Nos. 6,054,297, 5,886,152 and 5,877,293. A humanized antibody may
20 comprise portions of immunoglobulins of different origin, wherein optionally at least one portion is of human origin. Accordingly, the present invention relates to a humanized immunoglobulin having binding specificity for an EphB4 (e.g., human EphB4), said immunoglobulin comprising an antigen binding region of nonhuman origin (e.g., rodent) and at least a portion of an immunoglobulin of human origin
25 (e.g., a human framework region, a human constant region or portion thereof). For example, the humanized antibody can comprise portions derived from an immunoglobulin of nonhuman origin with the requisite specificity, such as a mouse, and from immunoglobulin sequences of human origin (e.g., a chimeric immunoglobulin), joined together chemically by conventional techniques (e.g.,
30 synthetic) or prepared as a contiguous polypeptide using genetic engineering techniques (e.g., DNA encoding the protein portions of the chimeric antibody can be expressed to produce a contiguous polypeptide chain).

Another example of a humanized immunoglobulin of the present invention is an immunoglobulin containing one or more immunoglobulin chains comprising a CDR of nonhuman origin (e.g., one or more CDRs derived from an antibody of nonhuman origin) and a framework region derived from a light and/or heavy chain of human origin (e.g., CDR-grafted antibodies with or without framework changes).
5 In one embodiment, the humanized immunoglobulin can compete with murine monoclonal antibody for binding to an EphB4 polypeptide. Chimeric or CDR-grafted single chain antibodies are also encompassed by the term humanized immunoglobulin.

10 In certain embodiments, the present invention provides EphB4 antagonist antibodies. As described herein, the term "antagonist antibody" refers to an antibody that can inhibit one or more functions of an EphB4, such as a binding activity (e.g., ligand binding) and a signaling activity (e.g., clustering or phosphorylation of EphB4, stimulation of a cellular response, such as stimulation of
15 cell migration or cell proliferation). For example, an antagonist antibody can inhibit (reduce or prevent) the interaction of an EphB4 receptor with a natural ligand (e.g., Ephrin B2 or fragments thereof). Preferably, antagonist antibodies directed against EphB4 can inhibit functions mediated by EphB4, including endothelial cell migration, cell proliferation, angiogenesis, and/or tumor growth. Optionally, the
20 antagonist antibody binds to an extracellular domain of EphB4.

In other embodiments, the present invention provides EphB4 kinase activating antibodies. Such antibodies enhance EphB4 kinase activity, even independent of EphrinB2. In some instances, such antibodies may be used to stimulate EphB4. However, applicants note that in most cell-based and in vivo
25 assays, such antibodies surprisingly behaved like antagonist antibodies. Such antibodies appear to bind to at least one of the two fibronectin type III domains, particularly the region of amino acids 324-429 of Fig. 1.

In certain embodiments, anti-idiotypic antibodies are also provided. Anti-idiotypic antibodies recognize antigenic determinants associated with the antigen-
30 binding site of another antibody. Anti-idiotypic antibodies can be prepared against a second antibody by immunizing an animal of the same species, and preferably of the

same strain, as the animal used to produce the second antibody. See e.g., U.S. Pat. No. 4,699,880. In one embodiment, antibodies are raised against receptor or a portion thereof, and these antibodies are used in turn to produce an anti-idiotypic antibody. The anti-idiotypic antibodies produced thereby can bind compounds
5 which bind receptor, such as ligands of receptor function, and can be used in an immunoassay to detect or identify or quantitate such compounds. Such an anti-idiotypic antibody can also be an inhibitor of an EphB4 receptor function, although it does not bind receptor itself. Such an anti-idiotypic antibody can also be called an antagonist antibody.

10 In certain aspects, the present invention provides the hybridoma cell lines, as well as to the monoclonal antibodies produced by these hybridoma cell lines. The cell lines of the present invention have uses other than for the production of the monoclonal antibodies. For example, the cell lines of the present invention can be fused with other cells (such as suitably drug-marked human myeloma, mouse
15 myeloma, human-mouse heteromyeloma or human lymphoblastoid cells) to produce additional hybridomas, and thus provide for the transfer of the genes encoding the monoclonal antibodies. In addition, the cell lines can be used as a source of nucleic acids encoding the anti-EphB4 immunoglobulin chains, which can be isolated and expressed (e.g., upon transfer to other cells using any suitable technique (see e.g.,
20 Cabilly et al., U.S. Pat. No. 4,816,567; Winter, U.S. Pat. No. 5,225,539)). For instance, clones comprising a rearranged anti-EphB4 light or heavy chain can be isolated (e.g., by PCR) or cDNA libraries can be prepared from mRNA isolated from the cell lines, and cDNA clones encoding an anti-EphB4 immunoglobulin chain can be isolated. Thus, nucleic acids encoding the heavy and/or light chains of
25 the antibodies or portions thereof can be obtained and used in accordance with recombinant DNA techniques for the production of the specific immunoglobulin, immunoglobulin chain, or variants thereof (e.g., humanized immunoglobulins) in a variety of host cells or in an in vitro translation system. For example, the nucleic acids, including cDNAs, or derivatives thereof encoding variants such as a
30 humanized immunoglobulin or immunoglobulin chain, can be placed into suitable prokaryotic or eukaryotic vectors (e.g., expression vectors) and introduced into a suitable host cell by an appropriate method (e.g., transformation, transfection,

electroporation, infection), such that the nucleic acid is operably linked to one or more expression control elements (e.g., in the vector or integrated into the host cell genome). For production, host cells can be maintained under conditions suitable for expression (e.g., in the presence of inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc.), whereby the encoded polypeptide is produced. If desired, the encoded protein can be recovered and/or isolated (e.g., from the host cells or medium). It will be appreciated that the method of production encompasses expression in a host cell of a transgenic animal (see e.g., WO 92/03918, GenPharm International, published Mar. 19, 1992).

II. Methods of Antibody Production

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed as described herein, or using other suitable techniques. A variety of methods have been described. See e.g., Kohler et al., *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein et al., *Nature* 266: 550-552 (1977); Koprowski et al., U.S. Pat. No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F. M. et al., Eds., (John Wiley & Sons: New York, N.Y.), Chapter 11, (1991). Generally, a hybridoma can be produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cell, preferably those of the spleen or lymph nodes, are obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library, or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a full repertoire of human antibodies. See e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits et

al., Nature, 362: 255-258 (1993); Lonberg et al., U.S. Pat. No. 5,545,806; Surani et al., U.S. Pat. No. 5,545,807.

To illustrate, immunogens derived from an EphB4 polypeptide (e.g., an EphB4 polypeptide or an antigenic fragment thereof which is capable of eliciting an antibody response, or an EphB4 fusion protein) can be used to immunize a mammal, such as a mouse, a hamster or rabbit. See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an EphB4 polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In one embodiment, antibodies of the invention are specific for the extracellular portion of the EphB4 protein (e.g., SEQ ID NO: 2) or fragments thereof. In another embodiment, antibodies of the invention are specific for the intracellular portion or the transmembrane portion of the EphB4 protein.

Following immunization of an animal with an antigenic preparation of an EphB4 polypeptide, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an EphB4 polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

In certain embodiments, antibodies of the present invention can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab)₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab)₂ fragment can be treated to reduce disulfide bridges to produce Fab fragments.

In certain embodiments, antibodies of the present invention are further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for an EphB4 polypeptide conferred by at least one CDR region of the antibody. Techniques for the production of single chain antibodies (US Patent No. 4,946,778) can also be adapted to produce single chain antibodies. Also, transgenic mice or other organisms including other mammals, may be used to express humanized antibodies. Methods of generating these antibodies are known in the art. See, e.g., Cabilly et al., U.S. Pat. No. 4,816,567; Cabilly et al., European Patent No. 0,125,023; Queen et al., European Patent No. 0,451,216; Boss et al., U.S. Pat. No. 4,816,397; Boss et al., European Patent No. 0,120,694; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276; Winter, U.S. Pat. No. 5,225,539; winter, European Patent No. 0,239,400; Padlan, E. A. et al., European Patent Application No. 0,519,596 A1. See also, Ladner et al., U.S. Pat. No. 4,946,778; Huston, U.S. Pat. No. 5,476,786; and Bird, R. E. et al., Science, 242: 423-426 (1988)).

Such humanized immunoglobulins can be produced using synthetic and/or recombinant nucleic acids to prepare genes (e.g., cDNA) encoding the desired humanized chain. For example, nucleic acid (e.g., DNA) sequences coding for humanized variable regions can be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., et al., Nucl. Acids Res., 17: 5404 (1989)); Sato, K., et al., Cancer Research, 53: 851-856 (1993); Daugherty, B. L. et al., Nucleic Acids Res., 19(9): 2471-2476 (1991); and Lewis, A. P. and J. S. Crowe, Gene, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutagenized, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber et

al., U.S. Pat. No. 5,514,548; Hoogenboom et al., WO 93/06213, published Apr. 1, 1993)).

In certain embodiments, the antibodies are further attached to a label that is able to be detected (e.g., the label can be a radioisotope, fluorescent compound, enzyme or enzyme co-factor). The active moiety may be a radioactive agent, such as:
5 radioactive heavy metals such as iron chelates, radioactive chelates of gadolinium or manganese, positron emitters of oxygen, nitrogen, iron, carbon, or gallium, ^{43}K , ^{52}Fe , ^{57}Co , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{123}I , ^{125}I , ^{131}I , ^{132}I , or ^{99}Tc . A binding agent affixed to such a moiety may be used as an imaging agent and is administered in an amount
10 effective for diagnostic use in a mammal such as a human and the localization and accumulation of the imaging agent is then detected. The localization and accumulation of the imaging agent may be detected by radioscintigraphy, nuclear magnetic resonance imaging, computed tomography or positron emission tomography. Immunoscintigraphy using antibodies or other binding polypeptides
15 directed at EphB4 may be used to detect and/or diagnose cancers and vasculature. For example, monoclonal antibodies against the EphB4 marker labeled with ^{99}Tc Technetium, ^{111}In Indium, ^{125}I Iodine-may be effectively used for such imaging. As will be evident to the skilled artisan, the amount of radioisotope to be administered is dependent upon the radioisotope. Those having ordinary skill in the art can
20 readily formulate the amount of the imaging agent to be administered based upon the specific activity and energy of a given radionuclide used as the active moiety. Typically 0.1-100 millicuries per dose of imaging agent, preferably 1-10 millicuries, most often 2-5 millicuries are administered. Thus, compositions according to the present invention useful as imaging agents comprising a targeting moiety conjugated
25 to a radioactive moiety comprise 0.1-100 millicuries, in some embodiments preferably 1-10 millicuries, in some embodiments preferably 2-5 millicuries, in some embodiments more preferably 1-5 millicuries.

In certain preferred embodiments, an antibody of the invention is a monoclonal antibody, and in certain embodiments the invention makes available
30 methods for generating novel antibodies. For example, a method for generating a monoclonal antibody that binds specifically to an EphB4 polypeptide may comprise administering to a mouse an amount of an immunogenic composition comprising the

EphB4 polypeptide effective to stimulate a detectable immune response, obtaining antibody-producing cells (e.g., cells from the spleen) from the mouse and fusing the antibody-producing cells with myeloma cells to obtain antibody-producing hybridomas, and testing the antibody-producing hybridomas to identify a hybridoma
5 that produces a monoclonal antibody that binds specifically to the EphB4 polypeptide. Once obtained, a hybridoma can be propagated in a cell culture, optionally in culture conditions where the hybridoma-derived cells produce the monoclonal antibody that binds specifically to EphB4 polypeptide. The monoclonal antibody may be purified from the cell culture.

10 In addition, the techniques used to screen antibodies in order to identify a desirable antibody may influence the properties of the antibody obtained. For example, an antibody to be used for certain therapeutic purposes will preferably be able to target a particular cell type. Accordingly, to obtain antibodies of this type, it may be desirable to screen for antibodies that bind to cells that express the antigen of
15 interest (e.g., by fluorescence activated cell sorting). Likewise, if an antibody is to be used for binding an antigen in solution, it may be desirable to test solution binding. A variety of different techniques are available for testing antibody:antigen interactions to identify particularly desirable antibodies. Such techniques include ELISAs, surface plasmon resonance binding assays (e.g., the Biacore binding assay,
20 Bia-core AB, Uppsala, Sweden), sandwich assays (e.g., the paramagnetic bead system of IGEN International, Inc., Gaithersburg, Maryland), western blots, immunoprecipitation assays and immunohistochemistry.

The antibodies of the present invention are useful in a variety of applications, including research, diagnostic and therapeutic applications. For
25 instance, they can be used to isolate and/or purify receptor or portions thereof, and to study receptor structure (e.g., conformation) and function.

III. Diagnostic Applications

In certain aspects, the various antibodies of the present invention can be used to detect or measure the expression of EphB4 receptor, for example, on endothelial
30 cells (e.g., venous endothelial cells), or on cells transfected with an EphB4 receptor gene. Thus, they also have utility in applications such as cell sorting and imaging

(e.g., flow cytometry, and fluorescence activated cell sorting), for diagnostic or research purposes.

In certain embodiments, the antibodies or antigen binding fragments of the antibodies can be labeled or unlabeled for diagnostic purposes. Typically,
5 diagnostic assays entail detecting the formation of a complex resulting from the binding of an antibody to EphB4. The antibodies can be directly labeled. A variety of labels can be employed, including, but not limited to, radionuclides, fluorescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors and ligands (e.g., biotin, haptens). Numerous appropriate immunoassays are known to the skilled
10 artisan (see, for example, U.S. Pat. Nos. 3,817,827; 3,850,752; 3,901,654; and 4,098,876). When unlabeled, the antibodies can be used in assays, such as agglutination assays. Unlabeled antibodies can also be used in combination with another (one or more) suitable reagent which can be used to detect antibody, such as a labeled antibody (e.g., a second antibody) reactive with the first antibody (e.g.,
15 anti-idiotypic antibodies or other antibodies that are specific for the unlabeled immunoglobulin) or other suitable reagent (e.g., labeled protein A). An EphB4 antibody may also be derivatized with a chemical group such as polyethylene glycol (PEG), a methyl or ethyl group, or a carbohydrate group. These groups may be useful to improve the biological characteristics of the antibody, e.g., to increase
20 serum half-life or to increase tissue binding.

In one embodiment, the antibodies of the present invention can be utilized in enzyme immunoassays, wherein the subject antibodies, or second antibodies, are conjugated to an enzyme. When a biological sample comprising an EphB4 protein is combined with the subject antibodies, binding occurs between the antibodies and
25 EphB4 protein. In one embodiment, a sample containing cells expressing an EphB4 protein (e.g., endothelial cells) is combined with the subject antibodies, and binding occurs between the antibodies and cells bearing an EphB4 protein comprising an epitope recognized by the antibody. These bound cells can be separated from unbound reagents and the presence of the antibody-enzyme conjugate specifically
30 bound to the cells can be determined, for example, by contacting the sample with a substrate of the enzyme which produces a color or other detectable change when acted on by the enzyme. In another embodiment, the subject antibodies can be

unlabeled, and a second, labeled antibody can be added which recognizes the subject antibody.

In certain aspects, kits for use in detecting the presence of an EphB4 protein in a biological sample can also be prepared. Such kits will include an antibody
5 which binds to an EphB4 protein or portion of said receptor, as well as one or more ancillary reagents suitable for detecting the presence of a complex between the antibody and EphB4 or portion thereof. The antibody compositions of the present invention can be provided in lyophilized form, either alone or in combination with additional antibodies specific for other epitopes. The antibodies, which can be
10 labeled or unlabeled, can be included in the kits with adjunct ingredients (e.g., buffers, such as Tris, phosphate and carbonate, stabilizers, excipients, biocides and/or inert proteins, e.g., bovine serum albumin). For example, the antibodies can be provided as a lyophilized mixture with the adjunct ingredients, or the adjunct ingredients can be separately provided for combination by the user. Generally these
15 adjunct materials will be present in less than about 5% weight based on the amount of active antibody, and usually will be present in a total amount of at least about 0.001% weight based on antibody concentration. Where a second antibody capable of binding to the monoclonal antibody is employed, such antibody can be provided in the kit, for instance in a separate vial or container. The second antibody, if
20 present, is typically labeled, and can be formulated in an analogous manner with the antibody formulations described above.

Similarly, the present invention also relates to a method of detecting and/or quantitating expression of an EphB4 or portion of the receptor by a cell, wherein a composition comprising a cell or fraction thereof (e.g., membrane fraction) is
25 contacted with an antibody which binds to an EphB4 or portion of the receptor under conditions appropriate for binding of the antibody thereto, and antibody binding is monitored. Detection of the antibody, indicative of the formation of a complex between antibody and EphB4 or a portion thereof, indicates the presence of the receptor. Binding of antibody to the cell can be determined by standard methods,
30 such as those described in the working examples. The method can be used to detect expression of EphB4 on cells from an individual. Optionally, a quantitative expression of EphB4 on the surface of endothelial cells can be evaluated, for

instance, by flow cytometry, and the staining intensity can be correlated with disease susceptibility, progression or risk.

The present invention also relates to a method of detecting the susceptibility of a mammal to certain diseases. To illustrate, the method can be used to detect the susceptibility of a mammal to diseases which progress based on the amount of EphB4 present on cells and/or the number of EphB4-positive cells in a mammal. In one embodiment, the invention relates to a method of detecting susceptibility of a mammal to a tumor. In this embodiment, a sample to be tested is contacted with an antibody which binds to an EphB4 or portion thereof under conditions appropriate for binding of said antibody thereto, wherein the sample comprises cells which express EphB4 in normal individuals. The binding of antibody and/or amount of binding is detected, which indicates the susceptibility of the individual to a tumor, wherein higher levels of receptor correlate with increased susceptibility of the individual to a tumor. Applicants and other groups have found that expression of EphB4 has a correlation with tumor growth and progression. The antibodies of the present invention can also be used to further elucidate the correlation of EphB4 expression with progression of angiogenesis-associated diseases in an individual.

IV. Therapeutic Applications

In certain embodiments, the present invention provides compositions and methods for inhibiting angiogenesis and for treating angiogenesis-associated diseases (or disorders). In other embodiments, the present invention provides methods of inhibiting or reducing tumor growth and methods of treating an individual suffering from cancer. These methods involve administering to the individual a therapeutically effective amount of one or more EphB4 antibodies as described above. These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more particularly, humans.

As described herein, angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example, hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; inflammatory disorders such as immune and non-immune

inflammation; chronic articular rheumatism and psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque
5 neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation and wound healing; telangiectasia psoriasis scleroderma, pyogenic granuloma, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, hematopoiesis.

10 It is understood that methods and compositions of the invention are also useful for treating angiogenesis-independent cancers (tumors). As used herein, the term "angiogenesis-independent cancer" refers to a cancer (tumor) where there is no or little neovascularization in the tumor tissue.

In particular, antibodies of the present invention are useful for treating or
15 preventing a cancer (tumor), including, but not limited to, colon carcinoma, breast cancer, mesothelioma, prostate cancer, bladder cancer, squamous cell carcinoma of the head and neck (HNSCC), Kaposi sarcoma, and leukemia.

In certain embodiments of such methods, one or more EphB4 antibodies can be administered, together (simultaneously) or at different times (sequentially). In
20 addition, antibodies can be administered with another agent for treating cancer or for inhibiting angiogenesis. In a specific embodiment, the subject antibodies of the present invention can also be used with other antibody therapeutics (monoclonal or polyclonal).

In certain embodiments, the subject antibodies of the invention can be used
25 alone. Alternatively, the subject antibodies may be used in combination with other conventional anti-cancer therapeutic approaches directed to treatment or prevention of proliferative disorders (e.g., tumor). For example, such methods can be used in prophylactic cancer prevention, prevention of cancer recurrence and metastases after surgery, and as an adjuvant of other conventional cancer therapy. The present
30 invention recognizes that the effectiveness of conventional cancer therapies (e.g.,

chemotherapy, radiation therapy, phototherapy, immunotherapy, and surgery) can be enhanced through the use of one or more EphB4 antibodies of the invention.

A wide array of conventional compounds have been shown to have anti-neoplastic activities. These compounds have been used as pharmaceutical agents in
5 chemotherapy to shrink solid tumors, prevent metastases and further growth, or decrease the number of malignant cells in leukemic or bone marrow malignancies. Although chemotherapy has been effective in treating various types of malignancies, many anti-neoplastic compounds induce undesirable side effects. It has been shown that when two or more different treatments are combined, the treatments may work
10 synergistically and allow reduction of dosage of each of the treatments, thereby reducing the detrimental side effects exerted by each compound at higher dosages. In other instances, malignancies that are refractory to a treatment may respond to a combination therapy of two or more different treatments.

When a subject EphB4 antibody of the present invention is administered in
15 combination with another conventional anti-neoplastic agent, either concomitantly or sequentially, such antibody is shown to enhance the therapeutic effect of the anti-neoplastic agent or overcome cellular resistance to such anti-neoplastic agent. This allows decrease of dosage of an anti-neoplastic agent, thereby reducing the undesirable side effects, or restores the effectiveness of an anti-neoplastic agent in
20 resistant cells.

Pharmaceutical compounds that may be used for combinatory anti-tumor therapy include, merely to illustrate: aminoglutethimide, amsacrine, anastrozole, asparaginase, bcg, bicalutamide, bleomycin, buserelin, busulfan, campothecin, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate,
25 colchicine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, genistein, goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan,
30 ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine,

mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole, octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, suramin, tamoxifen, temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride, topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and
5 vinorelbine.

These chemotherapeutic anti-tumor compounds may be categorized by their mechanism of action into, for example, following groups: anti-metabolites/anti-cancer agents, such as pyrimidine analogs (5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine) and purine analogs, folate antagonists and related
10 inhibitors (mercaptopurine, thioguanine, pentostatin and 2-chlorodeoxyadenosine (cladribine)); antiproliferative/antimitotic agents including natural products such as vinca alkaloids (vinblastine, vincristine, and vinorelbine), microtubule disruptors such as taxane (paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and navelbine, epidipodophyllotoxins (etoposide, teniposide), DNA damaging
15 agents (actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchloroethamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol, taxotere, teniposide, triethylenethiophosphoramidate and etoposide (VP16)); antibiotics such as dactinomycin (actinomycin D),
20 daunorubicin, doxorubicin (adriamycin), idarubicin, anthracyclines, mitoxantrone, bleomycins, plicamycin (mithramycin) and mitomycin; enzymes (L-asparaginase which systemically metabolizes L-asparagine and deprives cells which do not have the capacity to synthesize their own asparagine); antiplatelet agents;
25 antiproliferative/antimitotic alkylating agents such as nitrogen mustards (mechlorethamine, cyclophosphamide and analogs, melphalan, chlorambucil), ethylenimines and methylmelamines (hexamethylmelamine and thiotepa), alkyl sulfonates-busulfan, nitrosoureas (carmustine (BCNU) and analogs, streptozocin),
30 trazenes - dacarbazine (DTIC); antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones,

hormone analogs (estrogen, tamoxifen, goserelin, bicalutamide, nilutamide) and aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen activator, streptokinase and urokinase), aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus (FK-506), sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF) inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisone, and prednisolone); growth factor signal transduction kinase inhibitors; mitochondrial dysfunction inducers and caspase activators; and chromatin disruptors.

In certain embodiments, pharmaceutical compounds that may be used for combinatory anti-angiogenesis therapy include: (1) inhibitors of release of “angiogenic molecules,” such as bFGF (basic fibroblast growth factor); (2) neutralizers of angiogenic molecules, such as an anti- β bFGF antibodies; and (3) inhibitors of endothelial cell response to angiogenic stimuli, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D₃ analogs, alpha-interferon, and the like. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-118 (1990), Moses et al., Science, 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and U.S. Pat. Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, and 6,573,256. In addition, there are a wide variety of compounds that can be used to inhibit angiogenesis, for example, peptides or agents that block the VEGF-mediated angiogenesis pathway, endostatin protein or derivatives, lysine binding fragments of angiostatin, melanin or melanin-promoting

compounds, plasminogen fragments (e.g., Kringles 1-3 of plasminogen), tropoin subunits, antagonists of vitronectin $\alpha_v\beta_3$, peptides derived from Saposin B, antibiotics or analogs (e.g., tetracycline, or neomycin), dienogest-containing compositions, compounds comprising a MetAP-2 inhibitory core coupled to a peptide, the compound EM-138, chalcone and its analogs, and naaladase inhibitors. See, for example, U.S. Pat. Nos. 6,395,718, 6,462,075, 6,465,431, 6,475,784, 6,482,802, 6,482,810, 6,500,431, 6,500,924, 6,518,298, 6,521,439, 6,525,019, 6,538,103, 6,544,758, 6,544,947, 6,548,477, 6,559,126, and 6,569,845.

Depending on the nature of the combinatory therapy, administration of the antibodies of the invention may be continued while the other therapy is being administered and/or thereafter. Administration of the antibodies may be made in a single dose, or in multiple doses. In some instances, administration of the antibodies is commenced at least several days prior to the conventional therapy, while in other instances, administration is begun either immediately before or at the time of the administration of the conventional therapy.

V. Pharmaceutical Compositions and Modes of Administration

In certain embodiments, the subject antibodies of the present invention are formulated with a pharmaceutically acceptable carrier. Such antibodies can be administered alone or as a component of a pharmaceutical formulation (composition). The compounds may be formulated for administration in any convenient way for use in human or veterinary medicine. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Formulations of the subject antibodies include those suitable for oral, dietary, topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), rectal, and/or intravaginal administration. Other suitable methods of administration can also include rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can

also be administered as part of a combinatorial therapy with other agents (either in the same formulation or in a separate formulation).

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of
5 active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

10 In certain embodiments, methods of preparing these formulations or compositions include combining another type of anti-tumor or anti-angiogenesis agent and a carrier and, optionally, one or more accessory ingredients. In general, the formulations can be prepared with a liquid carrier, or a finely divided solid carrier, or both, and then, if necessary, shaping the product.

15 Formulations for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or
20 sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of one or more subject antibodies as an active ingredient.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more antibodies of the present invention may be mixed with one or more pharmaceutically acceptable carriers, such as
25 sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch,
30 alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium

compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Methods of the invention can be administered topically, either to skin or to mucosal membranes such as those on the cervix and vagina. This offers the greatest opportunity for direct delivery to tumor with the lowest chance of inducing side effects. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl

alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples
5 are salicylic acid and sulfur.

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The subject antibodies may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or
10 propellants which may be required. The ointments, pastes, creams and gels may contain, in addition to an antibody, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an antibody, excipients such
15 as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Pharmaceutical compositions suitable for parenteral administration may
20 comprise one or more antibodies in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood
25 of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate.
30 Proper fluidity can be maintained, for example, by the use of coating materials, such

as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

Injectable depot forms are made by forming microencapsule matrices of one or more antibodies in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

Formulations for intravaginal or rectally administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

EXEMPLIFICATION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1. Effect of Ephrin B2 and EphB4 polyclonal antibodies on tumor cell growth

Two EphB4 polyclonal antibodies (H-200 and N-19) were purchased from Santa Cruz Biotech (Santa Cruz, CA). The H-200 antibody (also called sc-5536) has
5 an epitope region corresponding to amino acids 201-400 within an extracellular domain of human EphB4, while the N-19 antibody (also called sc-7285) has an epitope region within an N-terminal extracellular domain of human EphB4. In addition, an Ephrin B2 polyclonal was purchased from R&D Systems (Minneapolis, MN).

10 Three mesothelioma cell lines (H28, H2052, and H2373) were obtained from the ATCC (Manassas, VA) and used to test the anti-tumor activities of these EphB4 and Ephrin B2 polyclonal antibodies. These cells (about 5,000 cells/well) were plated in 48 well plates, and were treated the following day with different concentrations of each antibody. The cell viability assay (MTT) was done on day 4.
15 The effects of the Ephrin B2 and EphB4 polyclonal antibodies on tumor cell growth were shown in Figure 6.

Example 2. Effect of EphB4 monoclonal antibodies on angiogenesis and tumor growth

A. Generation And Functional Analysis of EphB4 Antibodies

20 Anti-EphB4 monoclonal antibodies were raised in mice against the extracellular domain (ECD) of EphB4. An EphB4ECD (see, e.g., Figure 5) was cloned into expression vectors (e.g., pGEX) to generate EphB4ECD fusion proteins (e.g., GST-ECD). EphB4ECD fusion protein expressed in BL21 *E. coli* was purified by affinity chromatography. In the case of GST fusion proteins, the GST
25 domain was cleaved by thrombin. Monoclonal antibody was purified from hybridoma supernatants by Protein A chromatography.

These monoclonal antibodies include EphB4 antibody Nos. 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, and 138 (Figure 5). Antibody mapping studies showed that the epitope domain for each of these antibodies (Figure 5). Binding
30 affinity of each EphB4 antibody was analyzed and shown in Figure 7.

Further experiments were carried out to analyze the functional activities of these antibodies, including their abilities to compete with their binding partner such as Ephrin B2, to activate EphB4 tyrosine phosphorylation, to inhibit in vitro tube formation in HUAEC, to inhibit in vivo angiogenesis by matrigel plug assay, to stimulate apoptosis or necrosis in SCC15 tumor cell, and to inhibit SCC15 xenotransplant growth. The results are summarized in Table 1 below.

Table 1. A summary of activities of EphB4 antibodies.

Ab. No.	Activation of EphB4 tyrosine phosphorylation	Inhibition of EphB4/Ephrin B2 interaction	Inhibition of HUAEC in vitro tube formation	Inhibition of in vivo angiogenesis (matrigel plug assay)	Stimulation of SCC15 tumor cell apoptosis or necrosis	Inhibition of SCC15 xeno-transplant growth	Ab. Subclass
1	--	+	+	Nd	N	Nd	IgG2b
23	--	+	+	+	A,N	--	IgG2b
35	--	+	+	Nd	A,N	--	IgG2b
47	--	--	+	--	Nd	+	IgG3
57	--	--	--	--	Nd	+	IgG3
79	--	+	--	Nd	A,N	--	IgG1
85L	+	--	--	--	Nd	--	IgG2b
85H	--	--	--	Nd	Nd	Nd	IgG2b
91	+	--	--	Nd	--	Nd	IgG2a
98	--	--	+	+	Nd	Nd	IgG2a
121	+	--	--	Nd	Nd	--	IgG1
131	+	--	+	Nd	Nd	+	IgG1
138	--	--	+	+	A,N	+	IgG2b

10 Nd = not determined (no data provided)

-- = no clear effect

+ = clear effect

A= apoptosis

N= necrosis

15 A,N = both apoptosis and necrosis

The effect of these antibodies on angiogenesis was further analyzed in mouse corneal micropocket assay. For example, EphB4 antibody No. 138 significantly inhibited angiogenesis as shown in Figure 8.

20 A representative experiment is shown in Figure 9 to illustrate the anti-tumor activities of EphB4 antibodies summarized in Table 1. BalbC nude mice were injected subcutaneously with 2.5×10^6 viable tumor cells (SCC15, a head and neck squamous cell carcinoma line). Tumors were initiated in nu/nu mice by injecting

2.5-5x10⁶ cells premixed with matrigel and Growth factors, and Ab's subcutaneously to initiate tumor xenografts. Mice were opened 14 days after injections. SCC15 is a head and neck squamous cell carcinoma line, B16 is a melanoma cell line, and MCF-7 is a breast carcinoma line. The responses of tumors to these treatments were compared to control treated mice, which receive PBS injections. Animals were observed daily for tumor growth and subcutaneous tumors were measured using a caliper every 2 days. Antibodies #1 and #23 showed significant regression of SCC15 tumor size compared to control, especially with no additional growth factor added, indicating that EphB4 antibodies inhibited the *in vivo* tumor growth of SCC15 cells.

Another representative experiment is shown in Figure 10 to illustrate the anti-tumor and anti-angiogenesis activities of EphB4 antibodies summarized in Table 1. Angiogenesis was assessed by CD-31 immunohistochemistry. Tumor tissue sections from treated and untreated mice were stained for CD31. Apoptosis was assessed by immunohistochemical TUNNEL, and proliferation by BrdU assay. Following surgical removal, tumors were immediately sliced into 2 mm serial sections and embedded in paraffin using standard procedures. Paraffin embedded tissue were sectioned at 5 μ m, the wax removed and the tissue rehydrated. The rehydrated tissues were microwave irradiated in antigen retrieval solution. Slides were rinsed in PBS, and TUNNEL reaction mixture (Terminal deoxynucleotidyl transferase and fluorescein labeled nucleotide solution), and BrdU were added in a humidity chamber completely shielded from light. The TUNNEL and BrdU reaction mixture were then removed, slides were rinsed and anti-flourescein antibody conjugated with horseradish peroxidase was added. After incubation and rinsing, 3, 3'-diaminobenzidine was added. Masson's Trichrome and Hematoxylin and Eosin were also used to stain the slides to visualize morphology. Masson's Trichrome allows to visualize necrosis and fibrosis. The tumor gets blood support from tumor/skin, muscle boundary. As tumor grows, inner regions get depleted of nutrients. This leads to necrosis (cell death), preferably at the tumor center. After cells die, (tumor) tissue gets replaced with fibroblastic tissue. Slides were visualized under 20-fold magnification with digital images acquired. A different morphology was obtained on SCC tumors with each antibody administered. Ab #1 showed an

increase in necrosis and fibrosis but not apoptosis. Ab #23 showed an increase in apoptosis, necrosis and fibrosis and a decrease in vessel infiltration. Ab #35 showed an increase in necrosis and fibrosis, and a small increase in apoptosis and a decrease in vessel infiltration. Ab #79 showed a large increase in apoptosis, and necrosis and fibrosis. Ab #91 showed no change in apoptosis but an increase in proliferation. And Ab #138 showed an increase in apoptosis, necrosis, fibrosis and a decrease in proliferation and vessel infiltration. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with EphB4 antibodies displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis. These results show that EphB4 antibodies caused apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

A further representative experiment is shown in Figure 11 to illustrate the anti-tumor activities of EphB4 antibodies summarized in Table 1. Alternate day treatment with EphB4 monoclonal antibody or an equal volume of PBS as control were initiated on day 4, after the tumors have established, and continued for 14 days. Systemic administration was administered either IP or SC with no significant difference. All the experiments were carried out in a double-blind manner to eliminate investigator bias. Mice were sacrificed at the conclusion of the two week treatment period. Tumors were harvested immediately postmortem and fixed and processed for immunohistochemistry. EphB4 antibodies 40 mg per kg body weight were administered. Treatment with EphB4 antibody significantly inhibited human SCC tumor growth compared with control-treated mice ($p < 0.05$). Treatment with EphB4 antibody significantly inhibited tumor weight compared with control-treated mice ($p < 0.05$). These results show that systemic administration of antibodies on xenografts led to tumor regression in SCC15 tumor xenografts.

Example 3. Materials and Methods

1) Immunohistochemistry

Formalin-fixed tissue sections were deparaffinized and incubated with 10% goat serum at -70°C for 10 minutes and incubated with the EphB4 monoclonal antibody 4°C overnight. Isotype-specific rabbit IgG was used as control. The

immunoreactivity for these receptors was revealed using an avidin-biotin kit from Vector Laboratories. Peroxidase activity was revealed by the diaminobenzidine (Sigma) cytochemical reaction. The slides were then counterstained with 0.12% methylene blue or H&E. For frozen sections, OCT-embedded tissues were
5 sectioned at 5 μ m and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed for 3 x 5 min in PBS and endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ in PBS for 10 min at room temperature. Sections were incubated with Eph4 (C-16) antibody (1:50) for 1 h at room temperature followed by three washes in PBS and incubation with donkey anti-goat secondary antibody
10 (Santa Cruz Biotech.) for 1 h at room temperature. After three washes in PBS, peroxidase activity was localized by incubation in DAB substrate solution (Vector Laboratories, Inc. Burlingame CA) for 10 min at room temperature. Sections were counterstained with Hematoxylin for 20 s, dehydrated and mounted. Negative control for staining was substitution of normal goat serum for primary antibody.

15 2) Western Blot

Whole cell lysates were prepared using Cell Lysis Buffer (GeneHunter, Basgavukke TN) supplemented with protease inhibitor cocktail (Pierce, Rockford IL), unless otherwise noted. Total protein was determined using the DC reagent system (Bio-Rad, Hercules CA). Typically, 20 μ g whole cell lysate was run on 4-20% Tris-
20 Glycine gradient gel. The samples were electro-transferred to PVDF membrane and the non-specific binding was blocked in TBST buffer (0.5 mM Tris-HCl, 45 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat milk. Membranes were first probed with primary antibody overnight, stripped with RestoreTM Western Blot stripping buffer (Pierce, Rockford IL) and reprobed with β -actin to confirm
25 equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

3) Tyrosine Kinase Phosphorylation Analysis

Cells growing in 60 mm dishes were either serum starved (1% FBS supplemented RPMI 1640, 24 hours) or cultured in normal conditions (10% FBS)
30 and then treated with or without 1 μ g/ml mouse ephrin B2/F_c for 10 min to activate EphB4 receptor. Cleared cell lysates were incubated with EphB4 monoclonal

antibody overnight at 4 °C. Antigen-antibody complex was immunoprecipitated by the addition of 100 µl of Protein G-Sepharose in 20 mM sodium phosphate, pH 7.0 with incubation overnight at 4 °C. Immunoprecipitates were analyzed by Western blot with phosphotyrosine (pTyr) specific antibody (Upstate, clone 4G10) at 1:1000
5 dilution followed by incubation with protein G-HRP (Bio-Rad) at 1:5000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed with EphB4 specific monoclonal antibody.

4) Cell Culture

Normal HUVECs were obtained from Cambrex (BioWhittaker) and
10 maintained in EBM2 medium supplemented with 0.1 mg/ml endothelial growth supplement (crude extract from bovine brain), penicillin (50 U/ml), streptomycin (50 U/ml), 2 mmol/l glutamine and 0.1 mg/ml sodium heparin. Aliquots of cells were preserved frozen between passages 1 and 3. For all experiments, HUVECs were used at passages 4 or below and collected from a confluent dish.

15 NCI H28 and NCI H2373 mesothelioma cell lines were obtained from the ATCC (Manassas, VA). Cells were maintained in RPMI 1640 media supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and antibiotics. Primary cells were obtained from pleural effusion of patients with mesothelioma.

20 5) Endothelial Cell Tube Formation Assay

Matrigel (60 µl of 10 mg/ml; Collaborative Lab, Cat. No. 35423) was placed in each well of an ice-cold 96-well plate. The plate was allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit Matrigel to polymerize. In the mean time, human umbilical vein endothelial cells
25 were prepared in EGM-2 (Clonetic, Cat. No. CC3162) at a concentration of 2×10^5 cells/ml. Cells (500 µl) and the test EphB4 antibody were mixed and 200 µl of this suspension were placed in duplicate on the polymerized Matrigel. After 24 h incubation, triplicate pictures were taken for each concentration using a Bioquant Image Analysis system. Protein addition effect (IC_{50}) was assessed compared to
30 untreated controls by measuring the length of cords formed and number of junctions.

6) Cell Migration Assay

Chemotaxis of HUVECs to VEGF was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24 well plates, 6.5 mm diam, 8 μ m pore size, 10 μ m thick matrigel coated, polycarbonate membranes (BD Biosciences). The cell suspensions of HUVECs (2×10^5 cells/ml) in 200 μ l of EBM were seeded in the upper chamber and the test EphB4 antibodies were added simultaneously with stimulant (VEGF or bFGF) to the lower compartment of the chamber and their migration across a polycarbonate filter in response to 10-20 ng/ml of VEGF with or without 100 nM-1 μ M test compound was investigated. After incubation for 4-24 h at 37 °C, the upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Ten random fields at 200 x mag were counted and the results expressed as mean # per field. Negative unstimulated control values were subtracted from stimulated control and protein treated sample values and the data was plotted as mean migrated cell $\pm$ S.D. IC₅₀ was calculated from the plotted data.

7) Growth Inhibition Assay

HUVEC (1.5×10^3 cells) were plated in a 96-well plate in 100 μ l of EBM-2 (Clonetic, Cat. No. CC3162). After 24 hours (day 0), the test EphB4 antibody is added to each well at the desired concentration in EBM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 °C. After 72 h, plates were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with 1:1 solution of ethanol: 0.1 M sodium citrate (including day 0 plate), and absorbance measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC₅₀ value was calculated from the plotted data.

8) Murine Matrigel Plug Angiogenesis Assay

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/ml, 0.5

ml) in liquid form at 4 °C was mixed with Endothelial Cell Growth Supplement (ECGS), test EphB4 antibodies plus ECGS or Matrigel plus vehicle alone (PBS containing 0.25% BSA). Matrigel (0.5 ml) was injected into the abdominal subcutaneous tissue of female nu/nu mice (6 wks old) along the peritoneal mid line.

5 There were 3 mice in each group. The animals were cared for in accordance with institutional and NIH guidelines. At day 6, mice were sacrificed and plugs were recovered and processed for histology. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin in PBS and embedded in paraffin. Sections of 3 μ m were cut and

10 stained with H&E or Masson's trichrome stain and examined under light microscope.

9) Mouse Corneal Micropocket Assay

Mouse corneal micropocket assay was performed according to that detailed by Kenyon et al., 1996. Briefly, hydron pellets (polyhydroxyethylmethacrylate [polyHEMA], Interferon Sciences, New Brunswick, NJ, U.S.A.) containing either

15 90 ng of bFGF (R&D) or 180 ng of VEGF (R&D Systems, Minneapolis, MN, U.S.A.) and 40 μ g of sucrose aluminium sulfate (Sigma) were prepared. Using an operating microscope, a stromal linear keratotomy was made with a surgical blade (Bard-Parker no. 15) parallel to the insertion of the lateral rectus muscle in an

20 anesthetized animal. An intrastromal micropocket was dissected using a modified von Graefe knife (2"30 mm). A single pellet was implanted and advanced toward the temporal corneal limbus (within $0\pm 7\pm 1\pm 0$ mm for bFGF pellets and 0 ± 5 mm for VEGF pellets). The difference in pellet location for each growth factor was determined to be necessary given the relatively weaker angiogenic stimulation of

25 VEGF in this model. Antibiotic ointment (erythromycin.) was then applied to the operated eye to prevent infection and to decrease surface irregularities. The subsequent vascular response was measured extending from the limbal vasculature toward the pellet and the contiguous circumferential zone of neovascularization. Data and clinical photos presented here were obtained on day 6 after pellet

30 implantation, which was found to be the day of maximal angiogenic response.

10) In vitro invasion assay

“Matrigel” matrix-coated 9-mm cell culture inserts (pore size, 8 μ m; Becton Dickinson, Franklin Lakes, NJ) were set in a 24-well plate. The HUVEC cells were seeded at a density of 5×10^3 cells per well into the upper layer of the culture insert and cultured with serum-free EBM in the presence of the test EphB4 antibodies for 24 h. The control group was cultured in the same media without EphB4 antibodies. Then 0.5 ml of the human SCC15 cell line, conditioned medium was filled into the lower layer of the culture insert as a chemo-attractant. The cells were incubated for 24 h, then the remaining cells in the upper layer were swabbed with cotton and penetrating cells in the lower layer were fixed with 5% glutaraldehyde and stained with Diff Quick. The total number of cells passing through the Matrigel matrix and each 8 μ m pore of the culture insert was counted using optical microscopy and designated as an invasion index (cell number/area).

11) SCC15 tumor growth in mice

Subcutaneously inject logarithmically growing SCC15, head and neck squamous cell carcinoma cell line, at 5×10^6 cell density; with or without the test EphB4 antibody in the presence or absence of human bFGF, into athymic Balb/c nude mice, along with Matrigel (BD Bioscience) synthetic basement membrane (1:1 v/v), and examine tumors within 2 weeks. Tumor volumes in the test EphB4 antibody group, in the presence and absence of growth factor after implantation were three-fold smaller than those in the vehicle groups. There was no difference in body weight between the groups. Immunohistochemical examination of cross-sections of resected tumors and TUNEL-positive apoptosis or necrosis, CD34 immunostaining, and BrdU proliferation rate will be performed, after deparaffinized, rehydrated, and quenched for endogenous peroxidase activity, and after 10 min permeabilization with proteinase K. Quantitative assessment of vascular densities will also be performed. Local intratumoral delivery or IV delivery of the test EphB4 antibody will also be performed twice a week.

30 athymic nude mice, BALB/c (nu/nu), were each injected with 1×10^6 B16 melanoma cells with 0.1 ml PBS mixed with 0.1 ml matrigel or 1.5×10^6 SCC15 cells resuspended in 200 μ l of DMEM serum-free medium and injected subcutaneously on day 0 on the right shoulder region of mice. Test EphB4

antibodies were injected intravenously or subcutaneously, around the tumor beginning on day 1 at a loading dose of 4 $\mu\text{g}/\text{mg}$, with weekly injections of 2 $\mu\text{g}/\text{mg}$ (10 $\mu\text{g}/\text{g}$, 50 $\mu\text{g}/\text{kg}/\text{day}$), and at 2 weeks post-inoculation. Mice are sacrificed on Day 14. Control mice received PBS 50 μl each day.

5 12) Tumor formation in nude mice

All animals were treated under protocols approved by the institutional animal care committees. Cancer cells (5×10^6) were subcutaneously inoculated into the dorsal skin of nude mice. When the tumor had grown to a size of about 100 mm^3 (usually it took 12 days), the test EphB4 antibody was either intraperitoneally or
10 subcutaneously injected once/day, and tumorigenesis was monitored for 2 weeks. Tumor volume was calculated according to the formula $a^2 \times b$, where a and b are the smallest and largest diameters, respectively. A Student's t test was used to compare tumor volumes, with $P < 0.05$ being considered significant.

13) Quantification of Microvessel Density

15 Tumors were fixed in 4% formaldehyde, embedded in paraffin, sectioned by 5 μm , and stained with hematoxylineosin. Vessel density was semi-quantitated using a computer-based image analyzer (five fields per section from three mice in each group).

20 INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

25 While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

Claims:

1. An isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity.
- 5 2. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof binds to an epitope situated within amino acids 16-198 of the EphB4 sequence of Figure 1.
3. The isolated antibody or antigen binding portion thereof of claim 2, wherein the antibody or antigen binding portion thereof inhibits the binding of EphB4
10 to the extracellular portion of EphrinB2.
4. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof binds to an epitope situated within amino acids 324-429 or 430-537 of the EphB4 sequence of Figure 1.
5. The isolated antibody or antigen binding portion thereof of claim 4, wherein
15 the antibody or antigen binding portion thereof inhibits the formation of EphB4 dimers or multimers.
6. The isolated antibody or antigen binding portion thereof of claim 3, wherein the antibody or antigen binding portion thereof binds to the first fibronectin-like domain (FND1) of EphB4.
- 20 7. The isolated antibody or antigen binding portion thereof of claim 3, wherein the antibody or antigen binding portion thereof binds to the second fibronectin-like domain (FND2) of EphB4.
8. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof inhibits the EphrinB2-
25 stimulated autophosphorylation of EphB4.
9. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof inhibits the formation of tubes by cultured endothelial cells.

10. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof inhibits the vascularization of a tissue in vivo.
11. The isolated antibody or antigen binding portion thereof of claim 10, wherein
5 the antibody or antigen binding portion thereof inhibits the vascularization of tissue implanted in the cornea of an animal.
12. The isolated antibody or antigen binding portion thereof of claim 10, wherein the antibody or antigen binding portion thereof inhibits the vascularization of a Matrigel tissue plug implanted in an animal.
- 10 13. The isolated antibody or antigen binding portion thereof of claim 1, wherein the antibody or antigen binding portion thereof decreases the growth of a human tumor xenograft in a mouse.
14. The isolated antibody of claim 2, wherein the antibody is selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035,
15 and No. 079.
15. The isolated antibody of claim 2, wherein the antibody is a humanized version of an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, and No. 079.
16. The isolated antibody or antigen binding portion thereof of claim 2, wherein
20 the antibody or antigen binding portion thereof comprises at least one CDR portion derived from an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, and No. 079.
17. The isolated antibody of claim 4, wherein the antibody is selected from the group consisting of antibodies denoted herein as No. 047, No. 057, No. 85H,
25 No. 098, and No. 138.
18. The isolated antibody of claim 4, wherein the antibody is a humanized version of an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, and No. 079.
19. The isolated antibody or antigen binding portion thereof of claim 4, wherein
30 the antibody or antigen binding portion thereof comprises at least one CDR

portion derived from an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, and No. 079.

20. The antibody of claim 1, wherein the antibody is a monoclonal antibody.
21. The antibody of claim 20, wherein the monoclonal antibody is clinically acceptable for administration to a human.
22. A hybridoma that produces an antibody of claim 1.
23. A hybridoma that produces an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, No. 079, No. 047, No. 057, No. 85H, No. 098, and No. 138.
24. A method of treating cancer, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity.
25. The method of claim 24, wherein the patient is diagnosed with a cancer selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.
26. The method of claim 24, wherein the isolated antibody or antigen binding portion thereof is administered systemically.
27. The method of claim 24, wherein the isolated antibody is administered locally.
28. A method of inhibiting angiogenesis in a patient, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity.
29. The method of claim 28, wherein the patient is diagnosed macular degeneration.
30. A pharmaceutical preparation comprising the isolated antibody or antigen binding portion thereof of claim 1.

31. Use of an isolated antibody or antigen binding portion thereof of claim 1 to make a pharmaceutical preparation for treating cancer.
32. The use of claim 31, wherein the cancer is selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous
5 cell carcinoma, Kaposi sarcoma, and leukemia..
33. An isolated antibody or antigen binding portion thereof that binds to an epitope situated within amino acids 324-429 or 430-537 of the EphB4 sequence of Figure 1 and stimulates EphB4 kinase activity.
34. The isolated antibody or antigen binding portion thereof of claim 33, wherein
10 the antibody binds to the first fibronectin-like domain (FND1) of EphB4.
35. The isolated antibody of claim 34, wherein the antibody binds to the second fibronectin-like domain (FND2) of EphB4.
36. The isolated antibody of claim 33, wherein the antibody is selected from the group consisting of antibodies denoted herein as No. 85L, No. 091, No. 121,
15 and No. 131.
37. The isolated antibody of claim 33, wherein the antibody is a humanized version of an antibody selected from the group consisting of antibodies denoted herein as No. 85L, No. 091, No. 121, and No. 131.
38. The isolated antibody or antigen binding portion thereof of claim 33, wherein
20 the antibody or antigen binding portion thereof comprises at least one CDR portion derived from an antibody selected from the group consisting of antibodies denoted herein as No. 85L, No. 091, No. 121, and No. 131.
39. The antibody of claim 33, wherein the monoclonal antibody is clinically acceptable for administration to a human.
- 25 40. A hybridoma that produces an antibody of claim 33.
41. A hybridoma that produces an antibody selected from the group consisting of antibodies denoted herein as No. 001, No. 023, No. 035, No. 079, No. 047, No. 057, No. 85H, No. 098, and No. 138.

42. A method of treating cancer, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and stimulates EphB4 kinase activity.
- 5 43. The method of claim 42, wherein the patient is diagnosed with a cancer selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.
- 10 44. The method of claim 42, wherein the isolated antibody or antigen binding portion thereof is administered systemically.
45. The method of claim 42, wherein the isolated antibody is administered locally.
- 15 46. A method of inhibiting angiogenesis in a patient, the method comprising administering to a patient in need thereof an effective amount of an isolated antibody or antigen binding portion thereof that binds to an epitope situated in the extracellular portion of EphB4 and stimulates EphB4 kinase activity.
47. The method of claim 46, wherein the patient is diagnosed macular degeneration.
- 20 48. A pharmaceutical preparation comprising the isolated antibody or antigen binding portion thereof of claim 33.
49. Use of an isolated antibody or antigen binding portion thereof of claim 33 to make a pharmaceutical preparation for treating cancer.
- 25 50. The use of claim 49, wherein the cancer is selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia..
51. An antibody that specifically binds to an epitope situated in the extracellular portion of EphB4, wherein the antibody is selected from the group consisting of Nos. 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, and 138.

52. An isolated antibody or antigen binding portion thereof of claim 1 or claim 33, wherein the isolated antibody or antigen binding portion thereof is covalently linked to an additional functional moiety.
53. The isolated antibody or antigen binding portion thereof of claim 52, wherein
5 the additional functional moiety is a label.
54. The isolated antibody or antigen binding portion thereof of claim 53, wherein the label is suitable for detection by a method selected from the group consisting of: fluorescence detection methods, positron emission tomography detection methods and nuclear magnetic resonance detection methods.
- 10 55. The isolated antibody or antigen binding portion thereof of claim 54, wherein the label is selected from the group consisting of: a fluorescent label, a radioactive label, and a label having a distinctive nuclear magnetic resonance signature.
56. The isolated antibody or antigen binding portion thereof of claim 52, wherein
15 the additional functional moiety confers increased serum half-life on the antibody or antigen binding portion thereof.
57. The isolated antibody or antigen binding portion thereof of claim 56, wherein the additional functional moiety comprises a polyethylene glycol (PEG) moiety.
- 20 58. An isolated antibody of claim 1 or 33, wherein the isolated antibody is expressed by a host cell having an ATCC Deposit Designation No. selected from PTA-6208, PTA-6209, PTA-6210, PTA-6211, and PTA-6214.
59. A host cell having an ATCC Deposit Designation No. selected from PTA-6208, PTA-6209, PTA-6210, PTA-6211, and PTA-6214.

25

Figure 1: human EphB4 receptor precursor sequence and structure.

1 melrvllcwa slaaaleetl lntkletadl kwvtfpqvdg qweelsglde eqhsvrtyev
 61 cdvqrapgga hwlrtgwwpr rgavhvyatl rftmleclsl pragscket ftvfyyesda
 121 dtataltpaw menpyikvdt vaaehltrkr pgaeatgkvn vktlrlgpls kagfylafqd
 181 qgacmallsl hlfykkcaql tvnltrfpet vprelvvpva gscvvdavpa pgpspslycr
 241 edgqwaegpv tgcscapgfe aaegntkcra caqgtfkpls gegscqpcpa nshsntigsa
 301 vcqcrvgyfr artdprgapc ttpsaprsv vsrlngsslh lewsaplesg gredltyalr
 361 crecrpggsc apcggdltfd pgprdlvepw vvvrglrpdf tytftevtaln gvsslatgpv
 421 pfepvnttd revppavsdv rvtrsspssl slawavprap sgavldyevk yhekgaegps
 481 svrflktsen raelrglkrg asylvqvrar seagygpfgg ehhsqqlde segwreqlal
 541 iagtavvgvv lvlvvivvav lclrkqsngr eaeysdkhgq ylighgtkvy idpftyedpn
 601 eavrefakei dvsvykleev igagefgeve rgrikapgkk escvaiktik ggyterqrre
 661 flseasingg fehpnirle gvvtnsmpvm iltefmenga ldsflrlndg qftviglvgm
 721 lrgiasgmry laemsvhrd laarnilvns nlvckvsdfg lsrfleenss dptytsslgg
 781 kipirwtape aiafrkftsa sdawsygvm wevmsfgerp ywdmsngdvi naieqdyrlp
 841 pppdcptslh qlmldewqkd rnarprfpqv vsalckmirn paslkivare nggashplld
 901 qrqphysafg svgewlraik mgryeesfaa agfgsfelvs qisaedllri gvtlaghqkk
 961 ilasvqhmks qakpgtpggt ggpapqy

Figure 2: EphB4 mRNA

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1  ctcgccccgg cggcgcgagc agagccactc cagggagggg gggagaccgc gagcgggccg
61  ctacagcccc gccacccggg gcgggacccc gagggccccg agggacccca actccagcca
121 cgtcttgctg cgcgcccggc cggcgcgggc actgccagca cgctccgggc ccgcccggc
181 cgcgcgcggc acagacgcgg ggccacactt ggcgcgcggc cccggtgccc cgcacgctcg
241 catgggcccg cgctgagggc cccgacgagg agtcccgcgc ggagtatcgg cgtccacccg
301 cccagggaga gtcagacctg ggggggagag ggccccccaa actcagttcg gatcctaccc
361 gagtgaggcg gcgccatgga gctccgggtg ctgctctgct gggcttcggt ggccgcagct
421 ttggaagaga ccctgctgaa caaaaattg gaaactgctg atctgaagtg ggtgacattc
481 cctcaggtgg acgggcagtg ggaggaactg agcggcctgg atgaggaaca gcacagcgtg
541 cgcacctacg aagtgtgtga cgtgcagcgt gccccggggc agggccactg gcttcgcaca
601 ggttgggtcc cacggcgggg cgcggtccac gtgtacgcca cgctgcgctt caccatgctc
661 gagtgcctgt ccctgcctcg ggctggggcg tccctgaagg agaccttcac cgtcttctac
721 tatgagagcg atgcggacac ggccacggcc ctacgcccag cctggatgga gaaccctac
781 atcaaggtgg acacggtggc cgcggagcat ctacccgga agcggcctgg ggccgaggcc
841 accgggaagg tgaatgtcaa gacgctgcgt ctgggaccgc tcagcaaggc tggcttctac
901 ctggccttcc aggaccaggg tgctgcatg gccctgctat ccctgcacct cttctacaaa
961 aagtgcgccc agctgactgt gaacctgact cgattcccgg agactgtgcc tcgggagctg
1021 gttgtgcccg tggccggtag ctgctggtg gatgccgtcc ccgcccctgg cccagcccc
1081 agcctctact gccgtgagga tggccagtgg gccgaacagc cggtcacggg ctgcagctgt
1141 gctccggggg tcgaggcagc tgagggggaa accaagtgcc gagcctgtgc ccagggcacc
1201 ttcaagcccc tgtcaggaga agggctctgc cagccatgcc cagccaatag ccactctaac
1261 accattggat cagccgtctg ccagtgccgc gtcgggtact tccgggcaag cacagacccc
1321 cggggtgcac cctgcaccac ccctccttcg gctccgcgga gcgtgggttc ccgcctgaac
1381 ggctcctccc tgcacctgga atggagtgcc ccctggagt ctggtggccg agaggacctc
1441 acctacgccc tccgctgccg ggagtgccga cccggaggct cctgtgcgcc ctgcggggga
1501 gacctgactt ttgaccccgg cccccgggac ctggtggagc cctgggtggt ggttcgaggg
1561 ctacgtcctg acttcacctt tacctttgag gtcactgcat tgaacggggg atcctcctta
1621 gccacggggc ccgtcccat ttagcctgtc aatgtcacca ctgaccgaga ggtacctcct
1681 gcagtgtctg acatccgggt gacgcggtcc tcaccagca gcttgagcct ggcctgggct
1741 gttccccggg caccagtggt ggctgtgctg gactacgagg tcaaatacca tgagaagggc
1801 gccgagggtc ccagcagcgt gcggttcctg aagacgtcag aaaaccgggc agagctgcgg
1861 gggctgaagc ggggagccag ctacctggtg caggtacggg cgcgctctga ggccggctac
1921 gggcccttcg gccaggaaca tcacagccag acccaactgg atgagagcga gggctggcgg
1981 gagcagctgg ccctgattgc gggcacggca gtcgtgggtg tggctcctgg cctggtggtc
2041 attgtggtcg cagttctctg cctcaggaag cagagcaatg ggagagaagc agaatactcg
2101 gacaaacacg gacagtatct catcggacat ggtactaagg tctacatcga ccccttact
2161 tatgaagacc ctaatgaggg tgtgagggaa tttgcaaaag agatcgatgt ctctacgtc
2221 aagattgaag aggtgattgg tgcaggtgag tttggcgagg tgtgccgggg gcggctcaag
2281 gccccaggga agaaggagag ctgtgtggca atcaagacct tgaagggtgg ctacacggag
2341 cggcagcggc gtgagtttct gagcgaggcc tccatcatgg gccagttcga gcacccaat
2401 atcatccgcc tggaggcggt ggtcaccaac agcatgcccg tcatgattct cacagagttc
2461 atggagaacg gcgccttgga ctcttcctg cggctaaacg acggacagtt cacagtcctc
2521 cagctcgtgg gcatgctgcg gggcatcgcc tccggcatgc ggtaccttgc cgagatgagc
2581 tacgtccacc gagacctggc tgctcgcaac atcctagtca acagcaacct cgtctgcaaa
2641 gtgtctgact ttggcctttc ccgattcctg gaggagaact cttccgatcc cacctacacg
2701 agctccctgg gaggaagat tcccatccga tggactgccc cggaggccat tgccttcggg
2761 aagttcactt ccgccagtga tgctggagt tacgggattg tgatgtggga ggtgatgtca
2821 tttggggaga ggccgtactg ggacatgagc aatcaggacg tgatcaatgc cattgaacag
2881 gactaccggc tgcccccgcc cccagactgt cccacctccc tccaccagct catgctggac
2941 tgttggcaga aagaccggaa tgcccggccc cgcttcccc cagggtgctcag cgccctggac
3001 aagatgatcc ggaacccccg cagcctcaaa atcgtggccc gggagaatgg cggggcctca
3061 caccctctcc tggaccagcg gcagcctcac tactcagctt ttggctctgt gggcgagtgg
3121 cttcgggcca tcaaaatggg aagatacgaa gaaagtctcg cagccgctgg ctttggctcc
3181 ttcgagctgg tcagccagat ctctgctgag gacctgctcc gaatcgaggt cactctggcg
3241 ggacaccaga agaaaatctt ggccagtgtc cagcacatga agtcccaggc caagccggga
3301 accccgggtg ggacaggagg accggccccg cagtactgac ctgcaggaaac tccccacccc

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3361 agggacaccg cctccccatt ttccggggca gaggggggac tcacagaggc cccagccct
3421 gtgccccgct ggattgcact ttgagcccggt ggggtgagga gttggcaatt tggagagaca
3481 ggatttgagg gttctgccat aataggagg gaaaatcacc cccagccac ctggggaac
3541 tccagaccaa gggtgagggc gcctttccct caggactggg tgtgaccaga ggaaaaggaa
3601 gtgcccaca tctcccagcc tccccagggt ccccccctac cttgatgggt gcgttccgc
3661 agaccaaaga gagtgtgact cccttgccag ctccagagtg ggggggctgt cccagggggc
3721 aagaaggggt gtcagggcc agtgacaaaa tcattgggggt ttgtagtccc aacttgctgc
3781 tgtcaccacc aaactcaatc atttttttcc cttgtaaatg cccctcccc agctgctgcc
3841 ttcataattga aggtttttga gttttgtttt tggctttaat tttctcccc gtccctttt
3901 tgtttcttcg ttttgttttt ctaccgtcct tgtcataact ttgtgttgga gggaacctgt
3961 ttcactatgg cctcctttgc ccaagttgaa acagggggccc atcatcatgt ctgtttccag
4021 aacagtgcct tggatcatccc acatccccgg acccgcctg ggaccccaa gctgtgtcct
4081 atgaaggggt gtgggggtgag gtagtgaaaa gggcggtagt tgggtgtgga accagaaac
4141 ggacgccggt gcttgagggt gttcttaaat tatatttaaa aaagtaactt tttgtataaa
4201 taaaagaaaa tgggacgtgt cccagctcca ggggt
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Fig. 2, cont.

Figure 3: Amino acid sequence of the B4ECv3 protein (EphB4 extracellular domain)

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWHEELSG
LDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVVYATLRFTM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNKTLRLGPLSKAGFYLAFAQDQGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVPVAGSCVVDVAVPAPGPSP
SLYCREDGQWAEQPVGTGCSCAPGFEEAEGNTKCRACAQGTFFKPLSGE
GSCQPCPANSHTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLES GGREDLTALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRAILQIEGKPIPNPLLGLDSTRTG
HHHHHH

Figure 4: Amino acid sequence of the B4ECv3NT protein (EphB4 extracellular domain)

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSG
LDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHUYATLRFTM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNKTLRLGPLSKAGFYLAQDQGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVPVAGSCVVDVAVPAPGPSP
SLYCREDGQWAEQPVGTGCSCAPGFEEAEGNTKCRACAQGTFFKPLSGE
GSCQPCPANSHTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLES GGREDLTALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRAILQISSTVAAARV

Figure 5: Summary of EphB4 Topology and Antibody Binding Sites

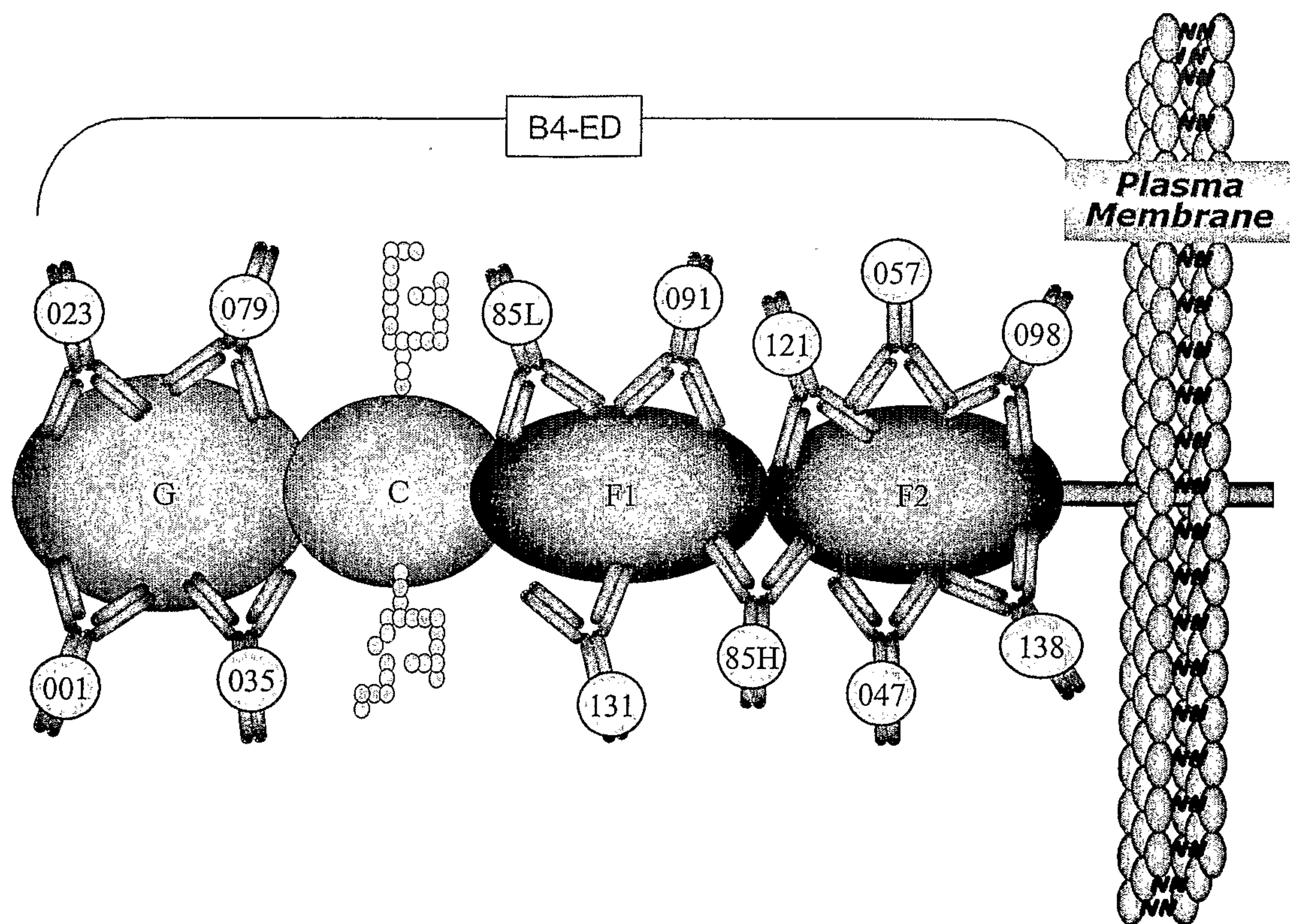


Figure 6A:

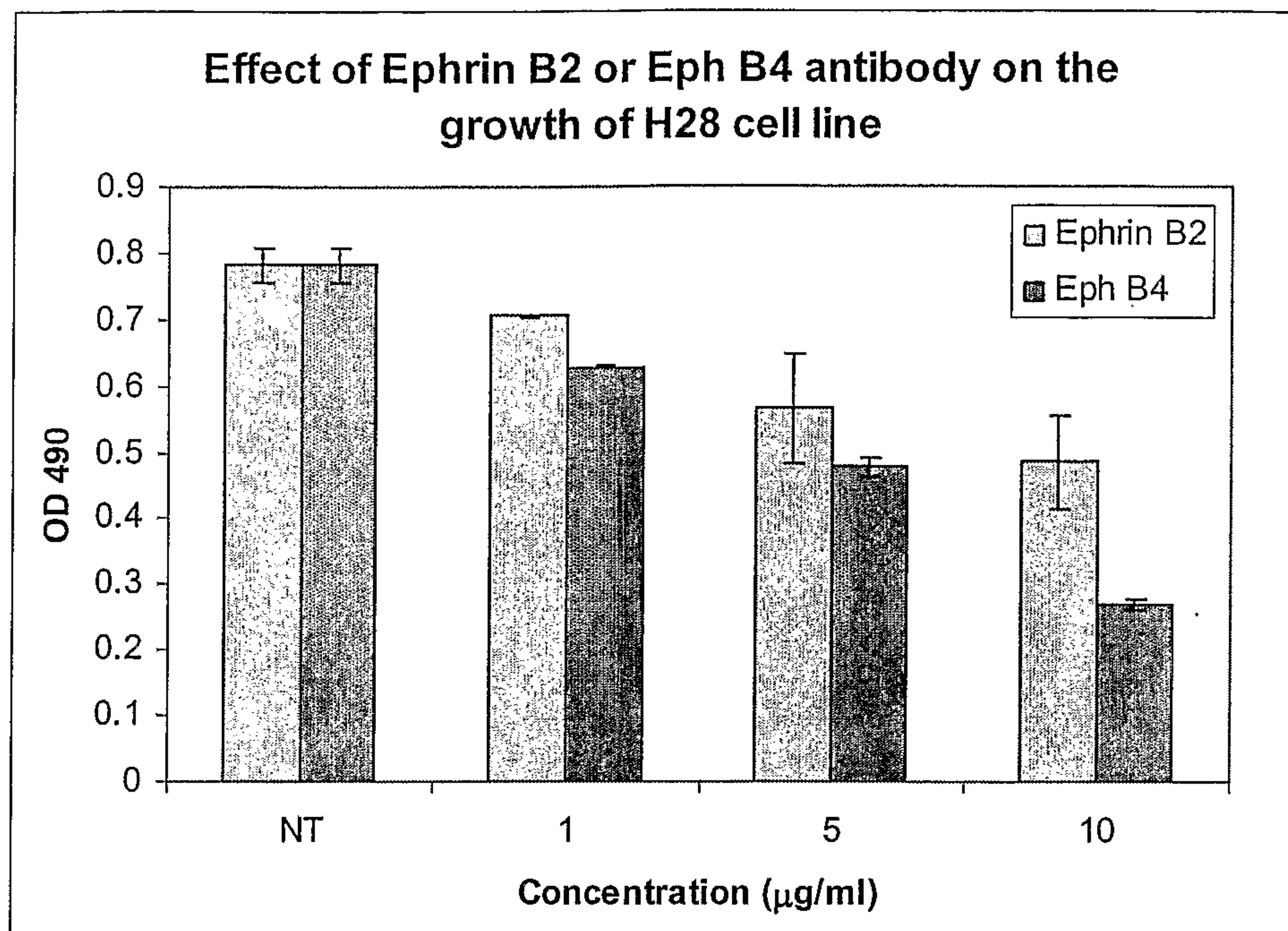


Figure 6B:

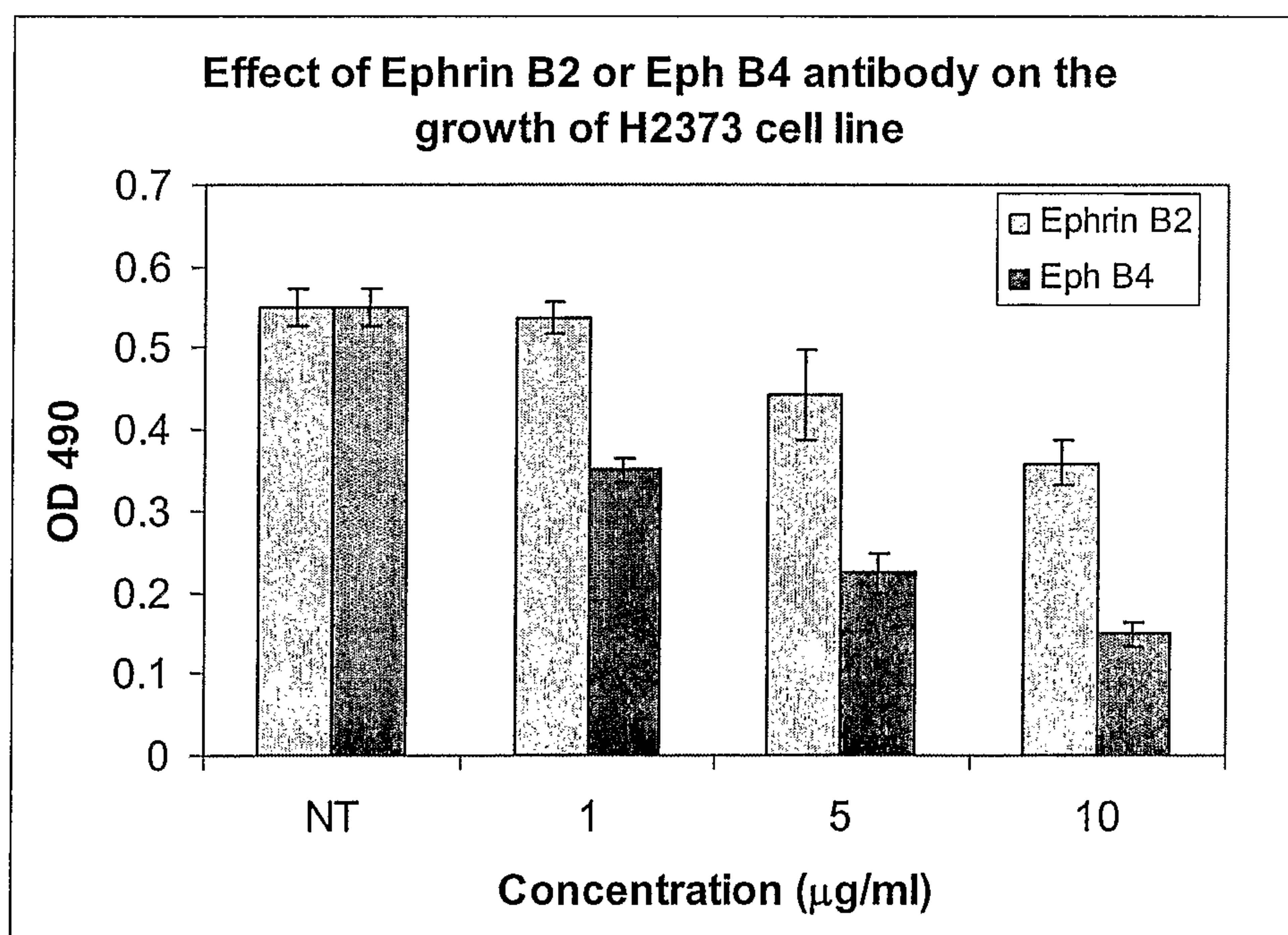
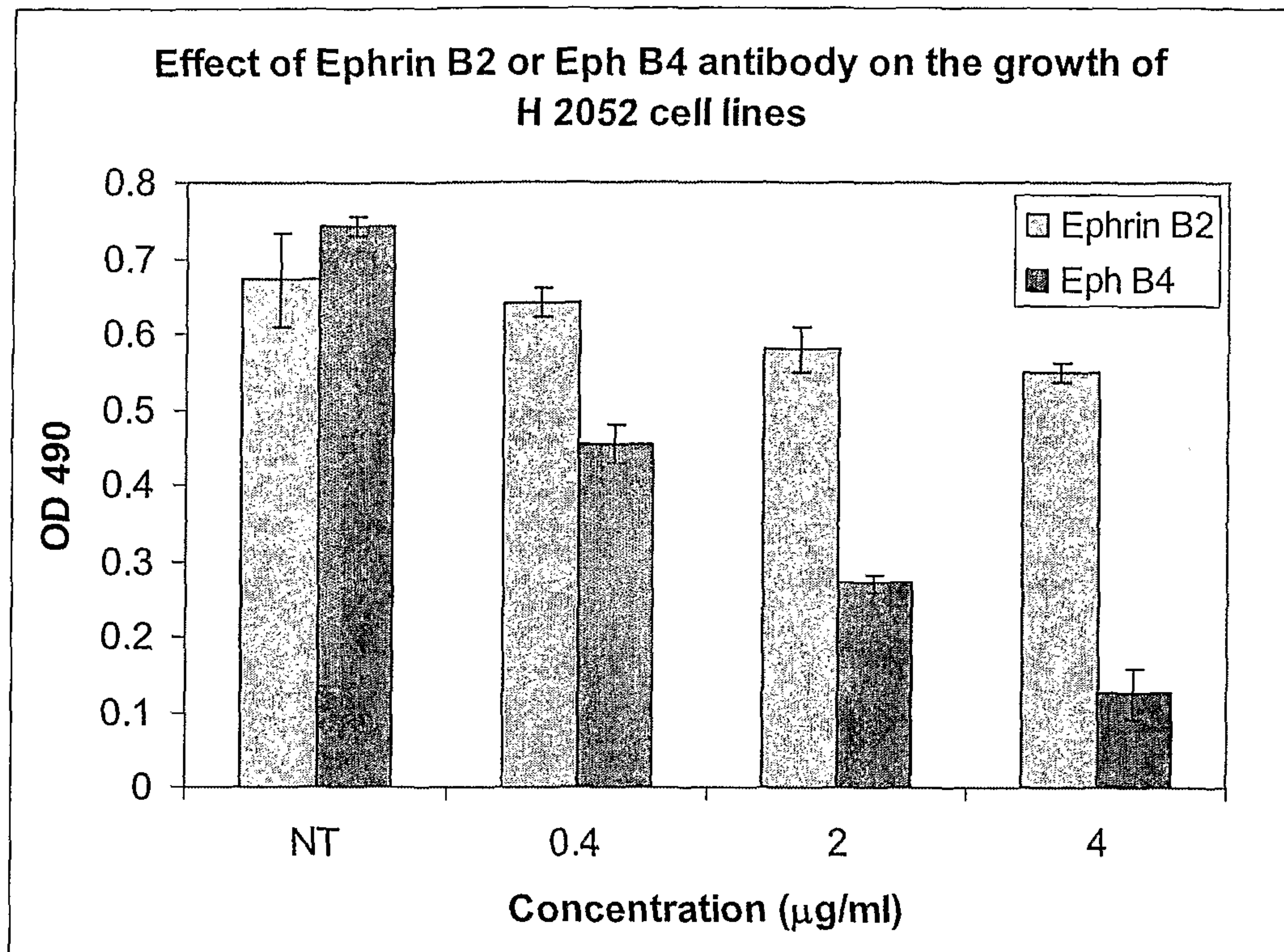


Figure 6C



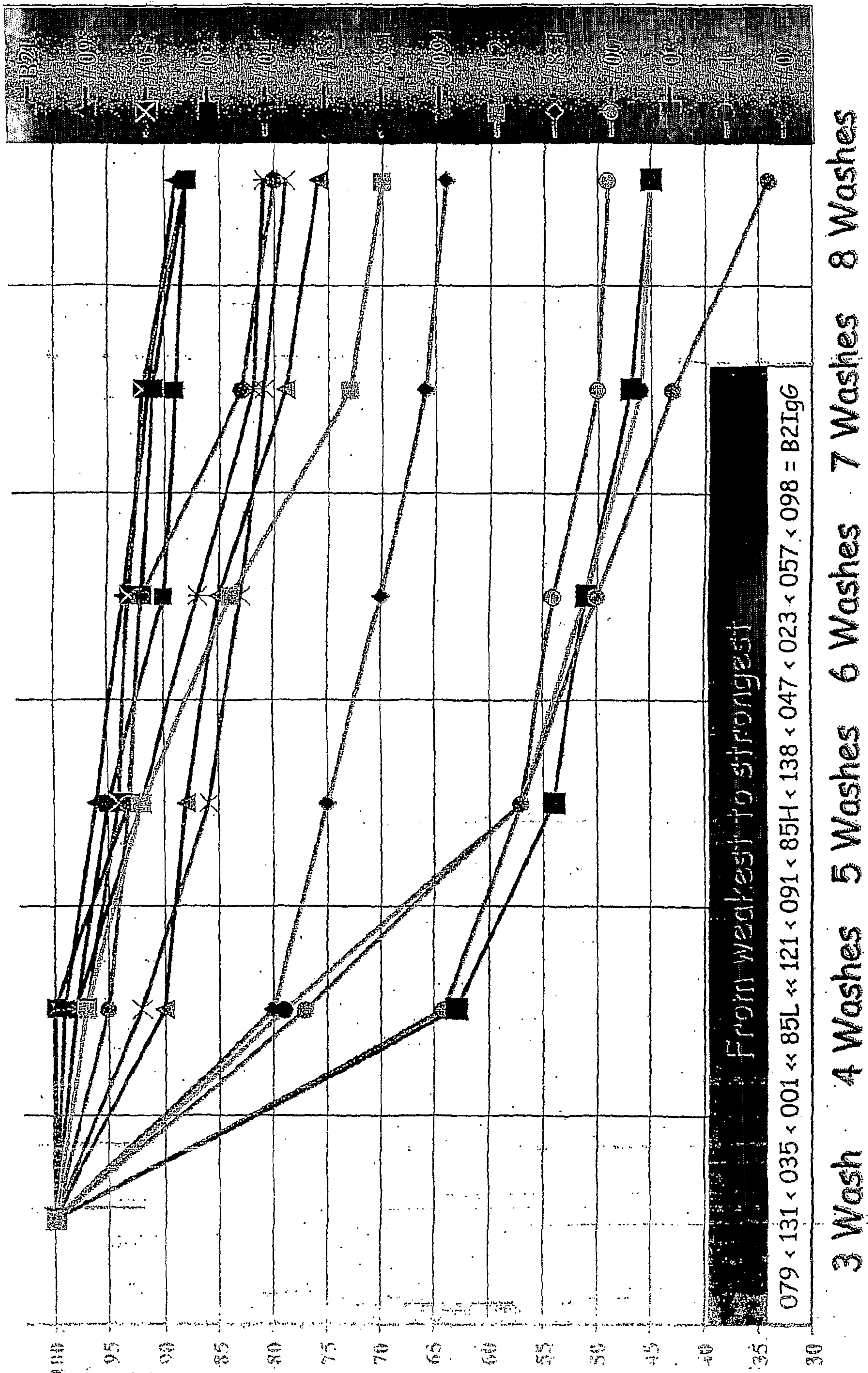


Fig 7

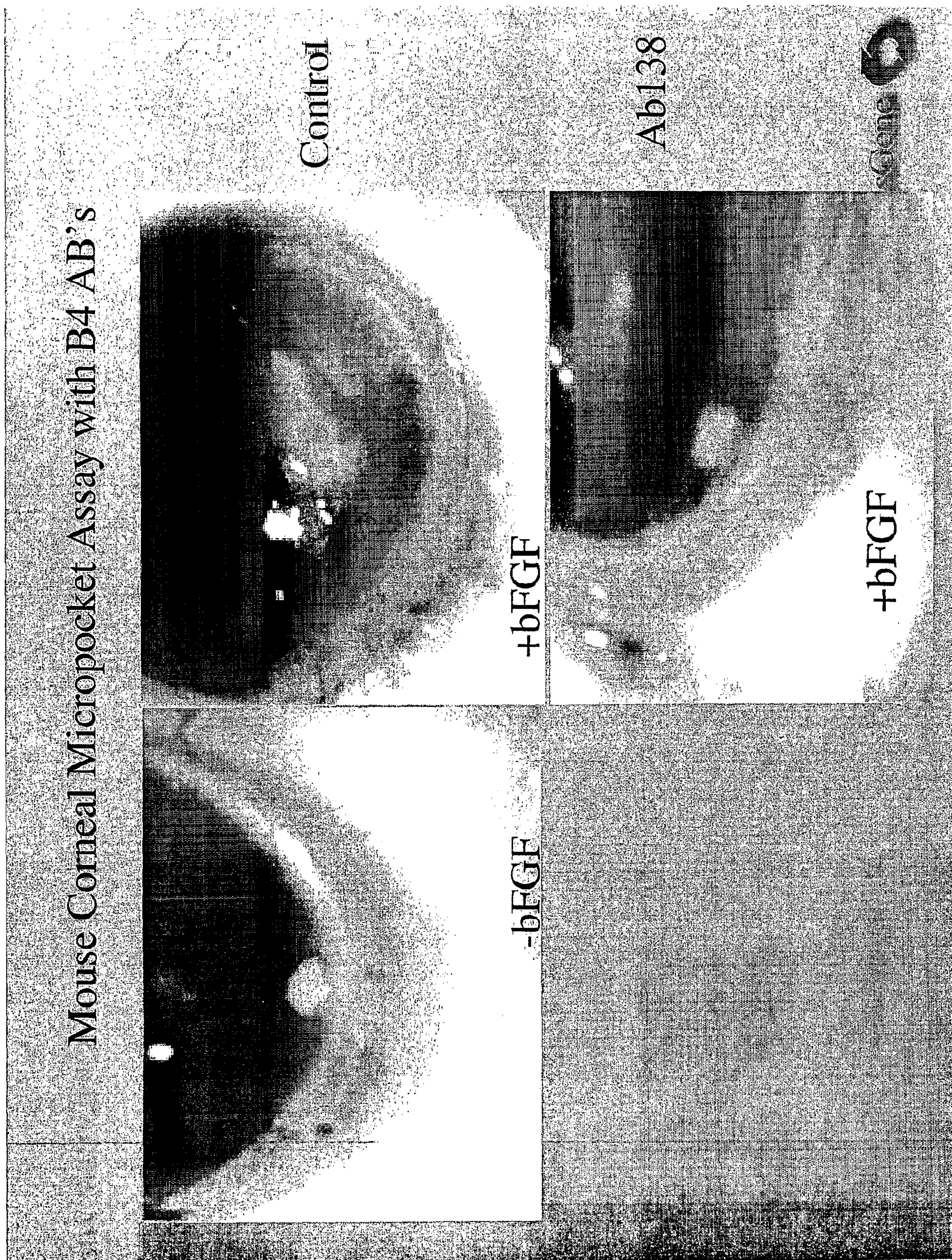


Figure 8

Figure 9: SCC15/MG xenograft tumor regression

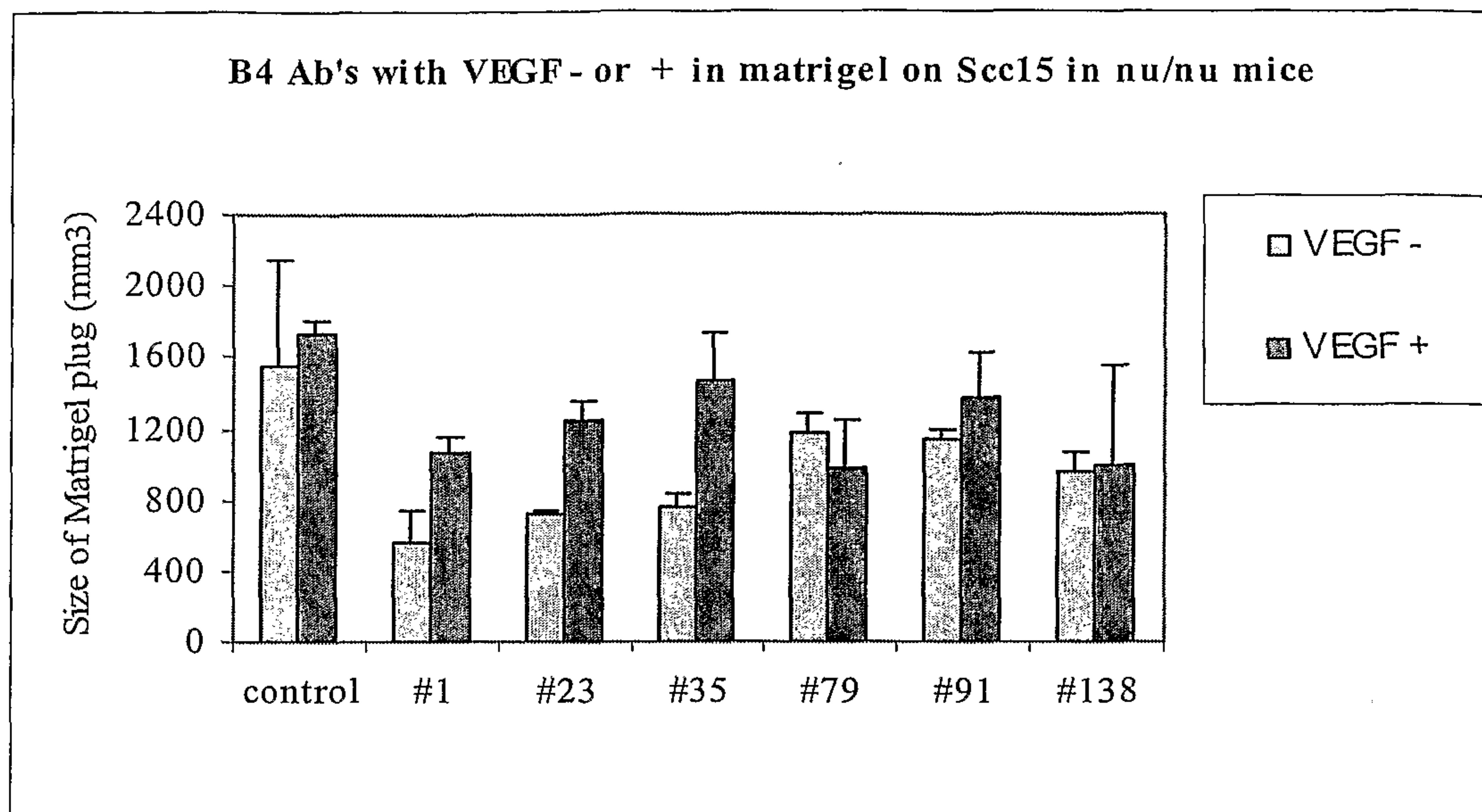


Figure 10: Effect of B4 antibodies on SCC15 Tumor histology

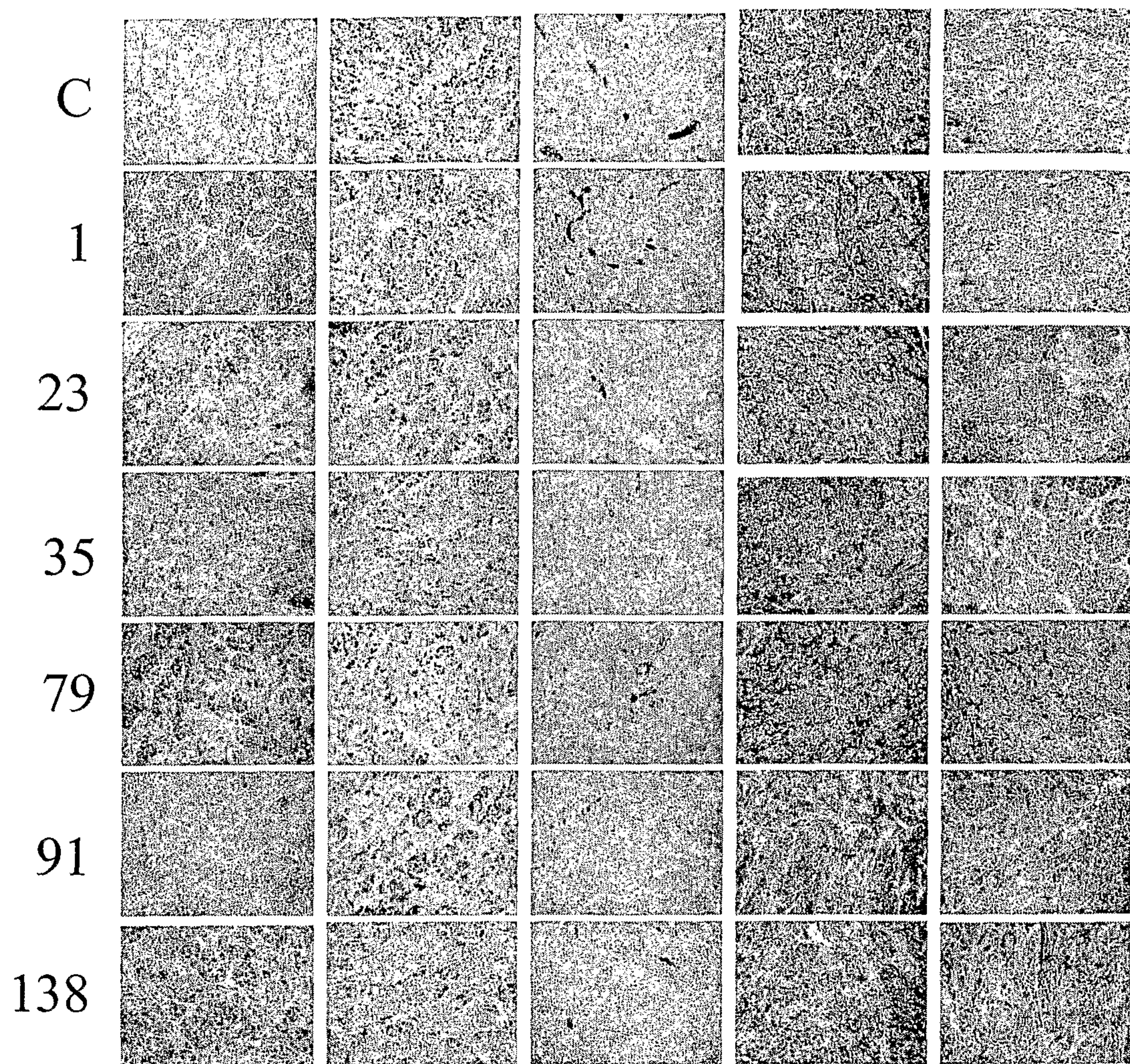


Figure 11: SCC15/IP,SC B4 Ab treated xenograft tumor regression

