

**(12) STANDARD PATENT**  
**(19) AUSTRALIAN PATENT OFFICE**

**(11) Application No. AU 2006210837 B2**

(54) Title  
**Anti-EphB2 antibodies and methods using same**

(51) International Patent Classification(s)  
**C07K 16/28** (2006.01)      **C12N 5/10** (2006.01)  
**A61K 39/395** (2006.01)      **C12N 15/13** (2006.01)  
**A61K 47/48** (2006.01)      **C12N 15/85** (2006.01)  
**A61P 35/00** (2006.01)      **G01N 33/574** (2006.01)  
**C07K 16/30** (2006.01)      **G01N 33/577** (2006.01)  
**C12N 1/21** (2006.01)

(21) Application No: **2006210837**      (22) Date of Filing: **2006.01.31**

(87) WIPO No: **WO06/083936**

(30) Priority Data

(31) Number	(32) Date	(33) Country
<b>60/756,844</b>	<b>2006.01.05</b>	<b>US</b>
<b>60/648,541</b>	<b>2005.01.31</b>	<b>US</b>

(43) Publication Date: **2006.08.10**

(44) Accepted Journal Date: **2012.07.12**

(71) Applicant(s)  
**Genentech, Inc.**

(72) Inventor(s)  
**Polakis, Paul;Mao, Weiguang**

(74) Agent / Attorney  
**Griffith Hack, GPO Box 1285, Melbourne, VIC, 3001**

(56) Related Art  
**Mao et al: Cancer Research, (2004) vol. 64(3), , p. 781-788.**  
**Handra-Luca et al: " The American Journal of Pathology, (2003) vol. 163(3), pages 957-967.**

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

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
10 August 2006 (10.08.2006)

PCT

(10) International Publication Number  
WO 2006/083936 A3

(51) International Patent Classification:

*C07K 16/28* (2006.01) *C12N 1/21* (2006.01)  
*C07K 16/30* (2006.01) *A61K 39/395* (2006.01)  
*C12N 15/13* (2006.01) *A61P 35/00* (2006.01)  
*C12N 15/85* (2006.01) *G01N 33/577* (2006.01)  
*A61K 47/48* (2006.01) *G01N 33/574* (2006.01)  
*C12N 5/10* (2006.01)

(72) Inventors; and

(75) Inventors/Applicants (for US only): **MAO, Weiguang** [US/US]; 1613 De Anza Boulevard, San Mateo, California 94403 (US). **POLAKIS, Paul** [US/US]; 1449 Cortez Avenue, Burlingame, California 94010 (US).

(74) Agents: **COBURN, Cara M.** et al.; c/o GENENTECH, INC., 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH,

*[Continued on next page]*

(21) International Application Number:

PCT/US2006/003502

(22) International Filing Date: 31 January 2006 (31.01.2006)

(25) Filing Language: English

(26) Publication Language: English

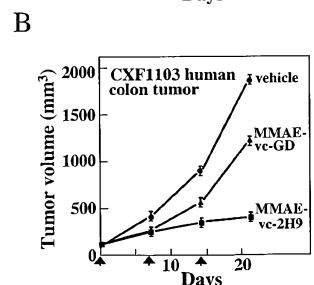
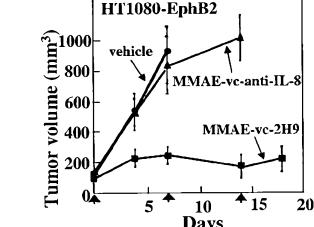
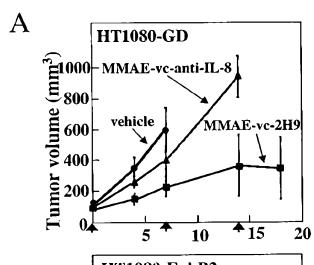
(30) Priority Data:

60/648,541 31 January 2005 (31.01.2005) US  
60/756,844 5 January 2006 (05.01.2006) US

(71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; 1 DNA Way, MS 49, South San Francisco, California 94080 (US).

(54) Title: ANTI-EPHB2 ANTIBODIES AND METHODS USING SAME

(57) Abstract: The application provides anti-EphB2 antibodies, immunoconjugates, and compositions comprising and methods of using these antibodies and immunoconjugates.



WO 2006/083936 A3



GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

**(88) Date of publication of the international search report:**

11 January 2007

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

5

**ANTI-EPHB2 ANTIBODIES AND METHODS USING SAME****CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority under 35 USC §119 to US Provisional Application 60/648,541, filed January 31, 2005, and US Provisional Application 60/756,844, filed January 5, 2006, the entire contents of which are hereby incorporated by reference.

**FIELD OF THE INVENTION**

The present invention relates generally to the fields of molecular biology. More specifically, the invention concerns anti-EphB2 antibodies, and uses of same.

15

**BACKGROUND OF THE INVENTION**

The EphB2 receptor (“EphB2” or “EphB2R”) is a member of the eph receptor family, which constitutes the largest family of tyrosine kinase receptors in the human genome (reviewed in Dodelet, *Oncogene*, 19: 5614-5619, 2000). The human eph receptor tyrosine kinases are categorized by sequence identity into an A class and a B class with corresponding A-type and B-type ligands referred to as ephrins. 20 Signaling can occur in a forward manner, in which the receptor tyrosine kinase is activated by the ligand, and in a reverse manner, in which the transmembrane ephrinB ligands are activated by interaction with receptors. Eph receptor ligand interactions have been implicated in a wide range of biological functions including axon guidance, tissue border formation, vasculogenesis, and cell motility (Kullander et al. *Nat. Rev. Mol. Cell. Biol.*, 3: 475-486, 2002; Cheng et al. *Cytokine Growth Factor Rev.*, 13: 75-85, 2002; Coulthard et al. *Int. J. Dev. Biol.*, 46: 375-384, 2002).

The EphB2 receptor has an extracellular region with a cysteine-rich motif extending over its amino-terminal half followed by two fibronectin type II motifs. There is an intracellular domain featuring a conserved kinase region and a transmembrane domain. EphB2 binds ligands such as ephrin-B1, ephrin-B2, and ephrin-B3. The cytoplasmic regions of the activated EphB2 receptor has been reported to interact with 30 myriad familiar signaling molecules such as Src, Grb2, and Abl (Holland et al., *EMBO J.*, 16: 3877-3888, 1997; Zisch et al., *Oncogene*, 16: 2657-2670, 1998; Yu et al., *Oncogene*, 20: 3995-4006, 2001). The EphB2 receptor tyrosine kinase down-regulates the ras/mitogen-activated protein (MAP) kinase signaling pathway and also inhibits the abl tyrosine kinase in endothelial and neuronal cells (Yu et al., *Oncogene*, 20: 3995-4006, 2001; Kim et al., *FASEB J.*, 16: 1126-1128, 2002; Elowe et al. *Mol. Cell. Biol.*, 21: 7429-7441, 2001).

35

Upregulation of both ephrin ligand and Eph receptor family members has been described in a range of human tumors and cell lines. For instance, EphB2 is reported to be over-expressed in small cell lung cancer (Tang et al., *Clin Cancer Res* 1999;5:455-60), neuroblastomas, (Tang et al., *Med Pediatr Oncol* 2001;36:80-2), melanoma (Vogt et al. *Clin Cancer Res* 1998;4:791-7), breast carcinoma (Wu et al., *Pathol Oncol Res* 2004;10:26-33), colorectal cancer (CRC) (Jubb et al., co-owned, co-pending U.S. patent application no. 40 60/642,164, filed Jan. 6, 2005, and Cairns et al., WO2003/000113) and hepatocellular carcinoma (Hafner et al., *Clin Chem* 2004;50:490-9).

27 Jun 2012

2006210837

Antibody-based therapy has proved very effective in the treatment of various disorders. For example, HERCEPTIN™ and RITUXAN™ (both from Genentech, S. San Francisco), have been used successfully to treat breast cancer and non-Hodgkin's lymphoma, respectively. HERCEPTIN™ is a recombinant DNA-derived humanized monoclonal antibody that selectively binds to the extracellular domain of the human epidermal growth factor receptor 2 (HER2) proto-oncogene. HER2 protein overexpression is observed in 25-30% of primary breast cancers. RITUXAN™ is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. Both these antibodies are produced in CHO cells.

The use of antibody-drug conjugates (ADC), i.e. immunoconjugates, for the local delivery of agents such as cytotoxic or cytostatic agents to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drug Del. Rev.* 26:151-172; US 4975278) allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al (1986) *Lancet* pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications*, A. Pinchera et al (eds), pp. 475-506).

It is clear that there continues to be a need for agents that have clinical attributes that are optimal for development as therapeutic agents. The invention described herein meets this need and provides other benefits.

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

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

## 25 SUMMARY OF THE INVENTION

A first aspect provides an anti-EphB2 antibody comprising hypervariable region (HVR) sequences:

- (i) (a) HVR-I.1 comprising sequence KSSQSI.I.NSGNQFNYI.A (SEQ ID NO:1);
- 30 (b) HVR-L2 comprising sequence GASTRES (SEQ ID NO:2);
- (c) HVR-L3 comprising sequence QNDHSYPFT (SEQ ID NO:3);
- (d) HVR-H1 comprising sequence SYWMH (SEQ ID NO:4);
- (e) HVR-H2 comprising sequence FINPSTGYTDYNQFKD (SEQ ID NO:5); and
- (f) HVR-H3 comprising sequence RLKLLRYAMDY (SEQ ID NO:6); or

(ii) HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 and HVR-H3 of the antibody produced by hybridoma cell line deposited as American Type Culture Collection (ATCC) No. PTA-6606, wherein the antibody specifically binds human EphB2.

A second aspect provides a polynucleotide encoding the antibody of the first aspect.

A third aspect provides a vector comprising the polynucleotide of the second aspect.

A fourth aspect provides a composition comprising the antibody of the first aspect.

27 Jun 2012

2006210837

A fifth aspect provides a composition comprising the polynucleotide of the second aspect or the vector of the third aspect.

A sixth aspect provides a host cell comprising the vector of third aspect.

5 A seventh aspect provides a method for making an anti-EphB2 antibody, said method comprising (a) expressing the vector of the third aspect in a suitable host cell, and (b) recovering the antibody.

An eighth aspect provides a method for making an anti-EphB2 immunoconjugate, said method comprising (a) expressing the vector of the third aspect in a suitable host cell, (b) recovering the antibody, and (c) conjugating the antibody to a drug.

10 A ninth aspect provides a method for treating or preventing a tumor, a cancer, or a cell proliferative disorder associated with increased expression or activity of EphB2, the method comprising administering an effective amount of the antibody of the first aspect or the composition of the fourth or fifth aspect to a subject in need of such treatment.

A tenth aspect provides a method for killing a cancer cell or tumor cell, the method comprising administering an effective amount of the antibody of the first aspect to a subject in need of such treatment.

15 An eleventh aspect provides a method for detecting EphB2, the method comprising detecting EphB2-anti-EphB2 antibody complex in a biological sample, wherein the anti-EphB2 antibody is the antibody of the first aspect.

20 A twelfth aspect provides a method for diagnosing a disorder associated with EphB2 expression, the method comprising detecting EphB2-anti-EphB2 antibody complex in a biological sample from a subject having or suspected of having the disorder, wherein the anti-EphB2 antibody is the antibody of the first aspect.

A thirteenth aspect provides use of the antibody of the first aspect in the manufacture of a medicament for treating or preventing a tumor, a cancer, or a cell proliferative disorder associated with increased expression or activity of EphB2.

25 A fourteenth aspect provides use of the antibody of the first aspect in the manufacture of a medicament for killing a cancer cell or tumor cell.

The invention is in part based on the identification of a variety of EphB2 binding agents (such as immunoconjugates, antibodies, and fragments thereof). EphB2 presents as an important and advantageous therapeutic target, and the invention provides compositions and methods based on binding EphB2. EphB2 binding agents of the invention, as described herein, provide important therapeutic and diagnostic agents for use 30 in targeting pathological conditions associated with expression and/or activity of the EphB2-EphB2 ligand pathways. Accordingly, the invention provides methods, compositions, kits and articles of manufacture related to EphB2 binding.

The present invention provides antibodies that bind to EphB2.

Disclosed herein is the antibody produced by hybridoma cell line 2H9.11.14 having American Type 35 Culture Collection (ATCC) No. PTA-6606, deposited on February 24, 2005.

Also disclosed is an isolated antibody comprising heavy and/or light chain variable domain(s) of the antibody produced by hybridoma cell line 2H9.11.14 having American Type Culture Collection (ATCC) No. PTA-6606, wherein said isolated antibody specifically binds human EphB2.

27 Jun 2012

2006210837

Also disclosed is an isolated antibody comprising at least one (at least 2, at least 3, at least 4, at least 5, and/or 6) hypervariable sequence(s) (HVR(s)) comprising a sequence selected from the group consisting of HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and/or HVR-H3 of the antibody produced by hybridoma cell line 2H9.11.14 having American Type Culture Collection (ATCC) No. PTA-6606, wherein said isolated antibody specifically binds human EphB2.

Also disclosed is an isolated antibody that binds to the same epitope on human EphB2 as the antibody produced by hybridoma cell line 2H9.11.14 having American Type Culture Collection (ATCC) No. PTA-6606.

Also disclosed is an isolated antibody that competes with the antibody produced by hybridoma cell line 2H9.11.14 having American Type Culture Collection (ATCC) No. PTA-6606 for binding to human EphB2.

Also disclosed is an isolated anti-EphB2 antibody, wherein a full length IgG form of the antibody specifically binds human EphB2 with a binding affinity of 70 pM or better. As is well-established in the art, binding affinity of a ligand to its receptor can be determined using any of a variety of assays, and expressed in terms of a variety of quantitative values. Accordingly, in one embodiment, the binding affinity is expressed as Kd values and reflects intrinsic binding affinity (e.g., with minimized avidity effects). Generally and preferably, binding affinity is measured in vitro, whether in a cell-free or cell-associated setting. Any of a number of assays known in the art, including those described herein, can be used to obtain binding affinity measurements, including, for example, Biacore, radioimmunoassay (RIA) and ELISA.

Also disclosed is an isolated antibody that binds a ligand binding region of EphB2. In some embodiments, the isolated antibody binds a polypeptide comprising, consisting of or consisting essentially of amino acids about 19 to about 208 of human EphB2 (Figure 12).

Also disclosed is an isolated anti-EphB2 antibody that competes with EphB2 ligand for binding of EphB2.

Also disclosed is an isolated anti-EphB2 antibody that inhibits, reduces, and/or blocks EphB2 activity. In some embodiments, EphB2 autophosphorylation is inhibited, reduced, and/or blocked.

Also disclosed is an anti-EphB2 antibody comprising: at least one, two, three, four, five, and/or six hypervariable region (HVR) sequences selected from the group consisting of: (a) HVR-L1 comprising sequence KSSQSLNSGNQENYLA (SEQ ID NO:1); (b) HVR-L2 comprising sequence GASTRES (SEQ ID NO:2); (c) HVR-L3 comprising sequence QNDHSYPFT (SEQ ID NO:3); (d) HVR-H1 comprising sequence SYWMH (SEQ ID NO:4); (e) HVR-H2 comprising sequence FINPSTGYTDYNQKFKD (SEQ ID NO:5); and (f) HVR-H3 comprising sequence RLKLLRYAMDY (SEQ ID NO:6).

In one embodiment, an antibody of the disclosure comprises a light chain comprising at least one, at least two or all three of HVR sequences selected from the group consisting of KSSQSLNSGNQENYLA (SEQ ID NO:1), GASTRES (SEQ ID NO:2), and QNDHSYPFT (SEQ ID NO:3). In one embodiment, the antibody comprises light chain HVR-L1 having amino acid sequence KSSQSLNSGNQENYLA (SEQ ID NO:1). In one embodiment, the antibody comprises light chain HVR-L2 having amino acid sequence GASTRES (SEQ ID NO:2). In one embodiment, the antibody comprises light chain HVR-L3 having amino acid sequence QNDHSYPFT (SEQ ID NO:3). In one embodiment, an antibody of the invention comprises a heavy chain comprising at least one, at least two or all three of HVR sequences selected from the group consisting of SYWMH (SEQ ID NO:4), FINPSTGYTDYNQKFKD (SEQ ID NO:5), and RLKLLRYAMDY (SEQ ID NO:6).

27 Jun 2012

2006210837

NO:6). In one embodiment, the antibody comprises heavy chain HVR-H1 having amino acid sequence SYWMH (SEQ ID NO:4). In one embodiment, the antibody comprises heavy chain HVR-H2 having amino acid sequence FINPSTGYTDYNQKFKD (SEQ ID NO:5). In one embodiment, the antibody comprises heavy chain HVR-H3 having amino acid sequence RLKLLRYAMDY (SEQ ID NO:6). In one embodiment, an antibody of the invention comprises a heavy chain comprising at least one, at least two or all three of HVR sequences selected from the group consisting of SYWMH (SEQ ID NO:4), FINPSTGYTDYNQKFKD (SEQ ID NO:5), and RLKLLRYAMDY (SEQ ID NO:6) and a light chain comprising at least one, at least two or all three of HVR sequences selected from the group consisting of KSSQSLNSGNQENYLA (SEQ ID NO:1), GASTRES (SEQ ID NO:2), and QNDHSYPFT (SEQ ID NO:3).

In one embodiment, an antibody of the disclosure comprises a light chain variable domain having the sequence:

DIVMTQSPSSLSVSAGEKVTMNCKSSQSLNSGNQENYLAWYQQKPGQPPKLLIYGASTRESGVPDRF TGSGSGTDFTLTISSVQAEDLAVYYCQNDHSYPFTFGAGTKVEIKR (SEQ ID NO:7).

In one embodiment, an antibody of the disclosure comprises a heavy chain variable domain having the sequence:

QVQLQQSGAELAKPGASVKMSCKASGYTFTSYWMHWVKQRPGQGLEWIGFINPSTGYTDYNQKFKD KATLTVKSSNTAYMQLSRLTSEDSA VYYCTRRLKLLRYAMDYWGQGTTLVSA (SEQ ID NO:8).

In one embodiment, an antibody of the disclosure comprises a light chain variable domain having the sequence:

DIVMTQSPSSLSVSAGEKVTMNCKSSQSLNSGNQENYLAWYQQKPGQPPKLLIYGASTRESGVPDRF TGSGSGTDFTLTISSVQAEDLAVYYCQNDHSYPFTFGAGTKVEIKR (SEQ ID NO:7); and comprises a heavy chain variable domain having the sequence:

QVQLQQSGAELAKPGASVKMSCKASGYTFTSYWMHWVKQRPGQGLEWIGFINPSTGYTDYNQKFKD KATLTVKSSNTAYMQLSRLTSEDSA VYYCTRRLKLLRYAMDYWGQGTTLVSA (SEQ ID NO:8).

Also disclosed is an antibody that competes with any of the above-mentioned antibodies for binding to EphB2. Also disclosed is an antibody that binds to the same epitope on EphB2 as any of the above-mentioned antibodies.

As is known in the art, and as described in greater detail hereinbelow, the amino acid position/boundary delineating a hypervariable region of an antibody can vary, depending on the context and the various definitions known in the art (as described below). Some positions within a variable domain may be viewed as hybrid hypervariable positions in that these positions can be deemed to be within a hypervariable region under one set of criteria while being deemed to be outside a hypervariable region under a different set of criteria. One or more of these positions can also be found in extended hypervariable regions (as further defined below).

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

27 Jun 2012

2006210837

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

Humanized antibodies include those that have amino acid substitutions in the FR and affinity maturation variants with changes in the grafted CDRs. The substituted amino acids in the CDR or FR are not limited to those present in the donor or recipient antibody. In other embodiments, the antibodies further comprise changes in amino acid residues in the Fc region that lead to improved effector function including enhanced CDC and/or ADCC function and B-cell killing. Other antibodies include those having specific changes that improve stability. Antibodies also include fucose deficient variants having improved ADCC function in vivo. In other embodiments, the antibodies comprise changes in amino acid residues in the Fc region that lead to decreased effector function, e.g. decreased CDC and/or ADCC function and/or decreased B-cell killing. In some embodiments, the antibodies are characterized by decreased binding (such as absence of binding) to human complement factor C1q and/or human Fc receptor on natural killer (NK) cells. In some embodiments, the antibodies are characterized by decreased binding (such as the absence of binding) to human Fc<sub>Y</sub>RI, Fc<sub>Y</sub>RIIA, and/or Fc<sub>Y</sub>RIIIA. In some embodiments, the antibodies are of the IgG class (e.g., IgG1 or IgG4) and comprises at least one mutation in E233, L234, L235, G236, D265, D270, N297, E318, K320, K322, A327, A330, P331 and/or P329 (numbering according to the EU index). In some embodiments, the antibodies comprise the mutation L234A/L235A or D265A/N297A.

Also disclosed is anti-EphB2 polypeptides comprising any of the antigen binding sequences provided herein, wherein the anti-EphB2 polypeptides specifically bind to EphB2.

Also disclosed is an immunoconjugate (interchangeably termed "antibody drug conjugate" or "ADC") comprising any of the anti-EphB2 antibodies disclosed herein conjugated to an agent, such as a drug. EphB2 overexpression has been observed in gastric cancer, small cell lung cancer, neuroblastomas, melanoma, breast carcinoma, and hepatocellular carcinoma and in all stages of colorectal tumorogenesis, indicating that EphB2 is a suitable target for immunoconjugate therapy. EphB2 expression has been observed in colon adenomas, indicating that EphB2 is a suitable target for disorders characterized by colon adenomas.

In some embodiments, the drug in the immunoconjugate is a chemotherapeutic agent, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate). In some embodiments, the drug is a maytansinoid, an auristatin, a dolastatin, or a calicheamicin. In some embodiments, the drug is DM1, DM3, DM4, MMAE, or MMAF.

In the immunoconjugates of the disclosure, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a linker (L). In some embodiments, the linker comprises linker components selected from one or more of 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-

27 Jun 2012

2006210837

aminobenzyloxycarbonyl (“PAB”), N-Succinimidyl 4-(2-pyridylthio) pentanoate (“SPP”), N-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate (“SMCC”), and/or N-Succinimidyl (4-iodo-acetyl) aminobenzoate (“SIAB”). In some embodiments, the linker comprises MC-val-cit-PAB.

In some embodiments, the immunoconjugate comprises SPP-DM1, SMCC-DM1, BMPEO-DM1, MC-vc-PAB-MMAF, MC-vc-PAB-MMAE, MC-MMAF, or MC-MMAE.

Also disclosed is an immunoconjugate comprising an anti-EphB2 antibody that kills tumor cells. In some embodiments, tumor cells are killed in vitro (in some embodiments, with an IC<sub>50</sub> of about 50 ng/ml, 40 ng/ml, 30 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 1 ng/ml, or less, such as 900 pg/ml, 800 pg/ml, 700 pg/ml, 600 pg/ml, 500 pg/ml, or less). In some embodiments, tumor cells are killed in vivo. In some embodiments, administration of the immunoconjugate reduces tumor growth (in some embodiments, having about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10%, or less reduction in tumor growth compared to a control tumor) or reduces time to tumor doubling.

The antibodies and immunoconjugates of the disclosure bind (such as specifically bind) EphB2, and in some embodiments, may modulate one or more aspects of EphB2-associated effects, including but not limited to EphB2 activation, EphB2 downstream molecular signaling, EphB2 ligand activation, EphB2 ligand downstream molecular signaling, disruption of ligand (e.g., ephrin-B1, ephrin-B2, and/or ephrin-B3) binding to EphB2, EphB2 phosphorylation and/or EphB2 multimerization, and/or EphB2 ligand phosphorylation, and/or disruption of any biologically relevant EphB2 and/or EphB2 ligand biological pathway, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with EphB2 expression and/or activity (such as increased EphB2 expression and/or activity). In some embodiments, the antibody or immunoconjugate specifically binds to EphB2. In some embodiments, the antibody or immunoconjugate specifically binds to the EphB2 extracellular domain (ECD). In some embodiments, the antibody or immunoconjugate specifically binds to a polypeptide consisting of or consisting essentially of amino acids about 19 to about 208 of human EphB2. In some embodiments, the antibody or immunoconjugate specifically binds EphB2 with a KD of 70 pM or stronger. In some embodiments, the antibody or immunoconjugate reduces, inhibits, and/or blocks EphB2 activity in vivo and/or in vitro. In some embodiments, the antibody or immunoconjugate reduces, inhibits and/or blocks EphB2 autophosphorylation. In some embodiments, the antibody or immunoconjugate competes for binding with EphB2-ligand (reduces and/or blocks EphB2 ligand binding to EphB2). In some embodiments, the antibody or immunoconjugate is internalized upon binding to EphB2 expressed on a mammalian cell.

Also disclosed is use of an antibody or immunoconjugate of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is characterized by colon adenomas.

Also disclosed are compositions comprising one or more antibodies or immunoconjugates of the disclosure and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

Also disclosed are nucleic acids encoding an anti-EphB2 antibody of the disclosure.

Also disclosed are vectors comprising a nucleic acid of the disclosure.

Also disclosed are compositions comprising one or more nucleic acid of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

27 Jun 2012

2006210837

Also disclosed are host cells comprising a nucleic acid or a vector of the disclosure. A vector can be of any type, for example a recombinant vector such as an expression vector. Any of a variety of host cells can be used. In one embodiment, a host cell is a prokaryotic cell, for example, *E. coli*. In one embodiment, a host cell is a eukaryotic cell, for example a mammalian cell such as Chinese Hamster Ovary (CHO) cell.

5 Also disclosed are methods of making an antibody or immunoconjugate of the disclosure. For example, the invention provides methods of making an anti-EphB2 antibody (which, as defined herein includes full length and fragments thereof) or immunoconjugate, said method comprising expressing in a suitable host cell a recombinant vector of the invention encoding said antibody (or fragment thereof), and recovering said antibody. Also disclosed are methods of making an anti-EphB2 immunoconjugate, said method comprising 10 expressing in a suitable host cell a recombinant vector of the disclosure encoding an anti-EphB2 antibody (or fragment thereof) or the disclosure, recovering said anti-EphB2 antibody, and conjugating the anti-EphB2 antibody to a drug.

15 Also disclosed is an article of manufacture comprising a container; and a composition contained within the container, wherein the composition comprises one or more anti-EphB2 antibodies or immunoconjugates of the disclosure. In one embodiment, the composition comprises a nucleic acid of the disclosure. In one embodiment, a composition comprising an antibody or immunoconjugate further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, an article of manufacture further comprises instructions for administering the composition (for e.g., the antibody) to a subject (such as 20 instructions for any of the methods described herein).

25 Also disclosed is a kit comprising a first container comprising a composition comprising one or more anti-EphB2 antibodies or immunoconjugates of the disclosure; and a second container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, a composition comprising an antibody or immunoconjugate further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, a kit further comprises instructions for administering the composition (for e.g., the antibody) to a subject.

Also disclosed is use of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody or immunoconjugate of the disclosure) in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

30 Also disclosed is use of a nucleic acid of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

Also disclosed is use of an expression vector of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

35 Also disclosed is use of a host cell of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

27 Jun 2012

2006210837

Also disclosed is use of an article of manufacture of the disclosure in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

Also disclosed is use of a kit of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder.

Also disclosed are methods and compositions useful for modulating disease states associated with expression and/or activity of EphB2, such as increased expression and/or activity or undesired expression and/or activity or decreased expression and/or activity.

Also disclosed are methods for treating or preventing a tumor, a cancer, and/or a cell proliferative disorder associated with increased expression and/or activity of EphB2, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment.

Also disclosed are methods for killing a cell (such as a cancer or tumor cell), the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment.

Also disclosed are methods for reducing, inhibiting, blocking, or preventing growth of a tumor or cancer, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment.

Also disclosed are methods for treating or preventing a neuropathy or neurodegenerative disease, or repairing a damaged nerve cell, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment.

Also disclosed are methods for promoting the development, proliferation, maintenance or regeneration of neurons, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment.

Also disclosed are methods for inhibiting angiogenesis, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody of the disclosure) to a subject in need of such treatment. In some embodiments, the site of angiogenesis is a tumor or cancer.

Methods of the disclosure can be used to affect any suitable pathological state. Exemplary disorders are described herein, and include a cancer selected from the group consisting of small cell lung cancer, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and hepatocellular carcinoma; and disorders characterized by colon adenomas.

In one embodiment, a cell that is targeted in a method of the invention is a cancer cell. For example, a cancer cell can be one selected from the group consisting of a breast cancer cell, a colorectal cancer cell, a lung cancer cell, a papillary carcinoma cell, a colon cancer cell, a pancreatic cancer cell, an ovarian cancer cell, a cervical cancer cell, a central nervous system cancer cell, an osteogenic sarcoma cell, a renal carcinoma cell, a

27 Jun 2012

2006210837

hepatocellular carcinoma cell, a bladder cancer cell, a gastric carcinoma cell, a head and neck squamous carcinoma cell, a melanoma cell, a leukemia cell, and a colon adenoma cell. In one embodiment, a cell that is targeted in a method of the invention is a hyperproliferative and/or hyperplastic cell. In one embodiment, a cell that is targeted in a method of the invention is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the invention is a metastatic cell. In some embodiments, the cell that is targeted is a colon adenoma cell. In some embodiments, the cell that is targeted expresses on the cell membrane at least about 20,000; 30,000; 40,000; 50,000; 60,000; 70,000; 80,000; 90,000; 100,000; 150,000; 200,000; 250,000; 300,000; 350,000; 400,000; 450,000; 500,000; 550,000; 600,000; 650,000; 700,000; 750,000; 800,000; 850,000, or more EphB2 molecules.

10 Methods of the disclosure can further comprise additional treatment steps. For example, in one embodiment, a method further comprises a step wherein a targeted cell and/or tissue (for e.g., a cancer cell) is exposed to radiation treatment or a chemotherapeutic agent.

15 Also disclosed are methods for detection of EphB2, the methods comprising detecting EphB2-anti-EphB2 antibody complex in the sample. The term "detection" as used herein includes qualitative and/or quantitative detection (e.g., measuring levels) with or without reference to a control.

20 Also disclosed are methods for diagnosing a disorder associated with EphB2 expression and/or activity, the methods comprising detecting EphB2-anti-EphB2 antibody complex in a biological sample from a patient having or suspected of having the disorder. In some embodiments, the EphB2 expression is increased expression or abnormal expression. In some embodiments, the disorder is a tumor, cancer, and/or a cell proliferative disorder.

25 Also disclosed are any of the anti-EphB2 antibodies described herein, wherein the anti-EphB2 antibody comprises a detectable label.

Also disclosed is a complex of any of the anti-EphB2 antibodies described herein and EphB2. In some embodiments, the complex is *in vivo* or *in vitro*. In some embodiments, the complex comprises a cancer cell.

25 In some embodiments, the anti-EphB2 antibody is detectably labeled.

Also disclosed is a polypeptide comprising, consisting of, or consisting essentially of amino acids 19-208 of EphB2 (Figure 12).

#### BRIEF DESCRIPTION OF THE FIGURES

30 FIGURE 1: depicts the amino acid sequences of monoclonal antibody 2H9 heavy chain variable region. Amino acids are numbered according to Kabat.

FIGURE 2: depicts the amino acid sequence of monoclonal antibody 2H9 light chain variable regions. Amino acids are numbered according to Kabat.

35 FIGURE 3: EphB2 mRNA is overexpressed in human cancer tissues versus normal human tissues. A, oligonucleotide microarray analysis was performed on RNA extracted from 38 human colorectal tumors and 7 specimens of normal human colonic mucosa. The average difference represents signal intensity (scaled to

1500). B, real-time PCR was performed on RNA extracted from 11 human colorectal cancer biopsy specimens and the corresponding patient-matched normal colon tissue specimens.

5 FIGURE 4: EphB2 expression in cancer and normal human colon tissues. A, *in situ* hybridization. EphB2 transcript is detected as deposited silver grains in the dark-field images, and the corresponding bright-field images were generated by staining the sections with H&E. Top and bottom panels are from colon tumor and normal colon tissues, respectively. B, EphB2 protein expression was detected using immunohistochemistry. Frozen serial tissue sections of a human adenocarcinoma were stained with anti-EphB2 monoclonal antibody 2H9 (right panel) or with control antibody (left panel) and counterstained with hematoxylin.

10 FIGURE 5: Monoclonal antibody (MAb) 2H9 antagonizes the ephrin-EphB2 interaction. A, the SVT2-EphB2 cell line was incubated with purified Fc-EphrinB2 fusion protein, and EphB2 protein was recovered from cell lysates by immunoprecipitation (IP) through the GD epitope tag fused to its NH2 terminus. Western blotting (WB) was performed with anti-phosphotyrosine (PTyr; top panel), to detect phosphotyrosine, and anti-GD (bottom panel), to detect total EphB2 protein. During ligand incubation, MAb 2H9 or control 15 antibody (EGVEGF MAb) was included in the culture medium. B, binding of purified human Fc-EphrinB1 fusion protein to HT1080 cells overexpressing EphB2 was assessed by flow cytometry using human anti-Fc conjugated to FITC. Relative fluorescence intensity was then measured in the presence of increasing concentrations of MAb 2H9. As controls, Fc-EphrinB1 was omitted in the presence or absence of Mab 2H9 or secondary antibody.

20 FIGURE 6: Internalization of anti-EphB2 monoclonal antibody (Mab) 2H9. A, MAb 2H9 was incubated on ice with HT1080 cells overexpressing EphB2. The cells either remained on ice or were shifted to 37°C for 1 h before fixing and staining with secondary antibody. B, Mab 2H9 was labeled with <sup>125</sup>I and incubated with HT1080-EphB2 cells on ice. Cells remained on ice (4°C) or were shifted to 37°C for 1 hour, and then the percentage of internalized <sup>125</sup>I was determined by scintillation counting.

25 FIGURE 7: Immunoconjugate 2H9-MC-valine-citrulline (vc)-PAB- monomethylauristatin E (MMAE) kills tumor cells in vitro. The HT1080 cell line stably overexpressing EphB2 (HT1080-EphB2) or a vector control HT1080 cell line (HT1080-GD) was treated with increasing concentrations of naked (i.e. not conjugated) monoclonal antibody 2H9, immunoconjugate 2H9-MC-vc-PAB-MMAE (abbreviated “MMAE-vc-2H9” in this figure), or immunoconjugate anti-interleukin-8-MC-vc-PAB-MMAE (abbreviated “MMAE-vc-anti-IL8” in this figure), and cell viability was measured after 2 days. The antibody concentration required 30 to attain half-maximal killing is indicated as IC50.

35 FIGURE 8: Characterization of HT1080 cell lines and immunoconjugate 2H9-MC-vc-PAB-MMAE. A, monoclonal antibody 2H9 binding assays were performed on the HT1080-EphB2 and HT1080-GD cell lines. The estimated number of total binding sites and the dissociation constants (Kd) were determined by Scatchard analysis. B, flow cytometry was performed with MAb 2H9 on the HT1080-EphB2 and HT1080-GD cell lines. Relative fluorescence intensity is presented in the histogram for MAb 2H9 and for secondary antibody only (control). C, saturation binding was performed on the HT1080-EphB2 and HT1080-GD cell lines with both naked Mab 2H9 (abbreviated “2H9” in this figure) and immunoconjugate 2H9-MC-vc-PAB-MMAE (abbreviated “2H9-vc-MMAE” in this figure). Relative binding was determined using a horseradish 40 peroxidase-conjugated secondary antibody, and absorbance was read at 450 nm.

FIGURE 9: Immunoconjugates 2H9-SPP-Dm1, 2H9-SMCC-DM1, 2H9-MC-vc-PAB-MMAE, and 2H9-MC-vc-PAB-MMAF kill tumor cells in vitro. A, the HT1080 cell line stably overexpressing EphB2 (HT1080-EphB2) was treated with increasing concentrations of 2H9-MC-vc-PAB-MMAE (abbreviated “vc-MMAE” in this figure), 2H9-SMCC-DM1 (abbreviated “SMCC-DM1” in this figure), 2H9-SPP-DM1 (abbreviated “Spp-DM1” in this figure), or anti-interleukin-8-MC-vc-PAB-MMAE (abbreviated “IL8MMAE” in this figure), and cell viability was measured after 2 days. B, the HT1080 cell line stably overexpressing EphB2 (HT1080-EphB2) was treated with increasing concentrations of 2H9-MC-vc-PAB-MMAE (abbreviated “vc-MMAE” in this figure), 2H9-MC-vc-PAB-MMAF (abbreviated “vc-MMAF” in this figure), 2H9-SMCC-DM1 (abbreviated “SMCC-DM1” in this figure), or 2H9-SPP-DM1 (abbreviated “Spp-DM1” in this figure), and cell viability was measured after 2 days.

FIGURE 10: 2H9-MC-vc-PAB-MMAE specifically inhibits growth of human tumors in vivo. A, nude mice were inoculated s.c. on one flank with HT1080-GD cells and on the other with HT1080-EphB2, and the resulting tumor xenografts were grown to an average size of 150 mm<sup>3</sup> each. Ten animals in each group received vehicle control (●) or 3 mg/kg body weight of either immunoconjugate 2H9 MC-vc-PAB-MMAE (■) (abbreviated “MMAE-vc-2H9” in this figure) or immunoconjugate anti-interleukin 8 MC-vc-PAB-MMAE (▲) (abbreviated “MMAE-vc-anti-IL-8” in this figure) once per week. B, the CXF1103 human colon tumor line was grown as a xenograft in nude mice that underwent a treatment protocol identical to that described for the HT1080 model. The control antibody was anti-GD conjugated to MC-vc-PAB-MMAE (abbreviated “MMAE-vc-GD” in this figure). Mean tumor volumes with SEs are presented.

FIGURE 11: depicts an amino acid sequence of the ligand binding domain of EphB2 (SEQ ID NO:9).

#### DETAILED DESCRIPTION OF THE INVENTION

The invention herein provides anti-EphB2 antibodies, or immunoconjugates comprising anti-EphB2 antibodies, that are useful for, e.g., treatment or prevention of disease states associated with expression and/or activity of EphB2, such as increased expression and/or activity or undesired expression and/or activity. In some embodiments, the antibodies or immunoconjugates of the invention are used to treat a tumor, a cancer, and/or a cell proliferative disorder.

In another aspect, the anti-EphB2 antibodies of the invention find utility as reagents for detection and/or isolation of EphB2, such as detection of EphB2 in various tissues and cell type.

The invention further provides methods of making anti-EphB2 antibodies, and polynucleotides encoding anti-EphB2 antibodies.

#### *General techniques*

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

27 Jun 2012

2006210837

**Definitions**

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

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

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat", and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The phrase "substantially similar," or "substantially the same", as used herein, denotes a sufficiently high degree of similarity between two numeric values (generally one associated with an antibody of the invention and the other associated with a reference/comparator antibody) such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is preferably less than about 50%, preferably less than about 40%, preferably less than

27 Jun 2012

2006210837

about 30%, preferably less than about 20%, preferably less than about 10% as a function of the value for the reference/comparator antibody.

“Binding affinity” generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects

12a

3389309\_1 (GHMatters) P72403.AU 27-Jun-12

a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and 5 tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative embodiments are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as 10 described by the following assay that measures solution binding affinity of Fabs for antigen by equilibrating Fab with a minimal concentration of (<sup>125</sup>I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (Chen, et al., (1999) J. Mol Biol 293:865-881). To establish conditions for the assay, microtiter plates (Dynex) are coated overnight with 5 ug/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently 15 blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [<sup>125</sup>I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of an anti-VEGF antibody, Fab-12, in Presta et al., (1997) Cancer Res. 57:4593-4599). The Fab of interest is then incubated overnight; however, the 20 incubation may continue for a longer period (e.g., 65 hours) to insure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates have dried, 150 ul/well of scintillant (MicroScint-20; Packard) is added, and the plates are counted on a Topcount 25 gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays. According to another embodiment the Kd or Kd value is measured by using surface plasmon resonance assays using a BIAcore<sup>TM</sup>-2000 or a BIAcore<sup>TM</sup>-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10mM sodium acetate, pH 4.8, into 5ug/ml (~0.2uM) before 30 injection at a flow rate of 5ul/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% Tween 20 (PBST) at 25°C at a flow rate of approximately 25ul/min. Association rates (k<sub>on</sub>) and dissociation rates (k<sub>off</sub>) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) 35 by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) is calculated as the ratio k<sub>off</sub>/k<sub>on</sub>. See, e.g., Chen, Y., et al., (1999) J. Mol Biol 293:865-881. If the on-rate exceeds 10<sup>6</sup> M<sup>-1</sup> S<sup>-1</sup> by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a 40

spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stir red cuvette.

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

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

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-

nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups 5 ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls 10 may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and a basic 15 nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR<sub>2</sub> ("amide"), P(O)R, 20 P(O)OR', CO or CH<sub>2</sub> ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

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

30 The term "EphB2" (interchangeably termed "EphB2R"), as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) EphB2 polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated or secreted forms (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term "wild type EphB2" generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring EphB2 protein. The term "wild type EphB2 sequence" 35 generally refers to an amino acid sequence found in a naturally occurring EphB2.

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

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or 5 hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a  $\beta$ -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, 10 contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

15 Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an  $F(ab')_2$  fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

20 "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can 25 associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

30 The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group.  $F(ab')_2$  antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

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

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, and IgA<sub>2</sub>. The heavy-chain constant domains that correspond to the different classes of

immunoglobulins are called  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an *in vivo* half life substantially similar to an intact antibody. For e.g., such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring *in vivo* stability to the fragment.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, *etc.*, and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (*e.g.*, Kohler *et al.*, *Nature*, 256:495 (1975); Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681, (Elsevier, N.Y., 1981)), recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567), phage display technologies (see, *e.g.*, Clackson *et al.*, *Nature*, 352:624-628 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991); Sidhu *et al.*, *J. Mol. Biol.* 338(2):299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, *e.g.*, WO

1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggemann *et al.*, *Year in Immuno.*, 7:33 (1993); U.S. Patent Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); 5,545,807; WO 1997/17852; U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks 5 *et al.*, *Bio/Technology*, 10: 779-783 (1992); Lonberg *et al.*, *Nature*, 368: 856-859 (1994); Morrison, *Nature*, 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnology*, 14: 845-851 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.*, 13: 65-93 (1995).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are 10 human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient 15 antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region 20 (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and 25 Gross, *Curr. Op. Biotech.* 5:428-433 (1994).

25 "Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. 30 Patent No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

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

35 The term "hypervariable region", "HVR", or "HV", when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3); and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most 40 commonly used (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service,

National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" hypervariable regions are based on an analysis of the available 5 complex crystal structures. The residues from the Kabat, Chothia and contact hypervariable regions are noted below.

	Loop	Kabat	Chothia	Contact
	---	---	-----	-----
	L1	L24-L34	L24-L34	L30-L36
10	L2	L50-L56	L50-L56	L46-L55
	L3	L89-L97	L89-L97	L89-L96
	H1	H31-H35B	H26-H32	H30-H35B
		(Kabat Numbering)		
	H2	H50-H65	H52-H56	H47-H58
15	H3	H95-H102	H95-H102	H93-H101

Hypervariable regions may comprise "extended hypervariable regions" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102 or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat et al., *supra* 20 for each of these definitions.

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

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further 25 comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which 30 fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an 35 antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof 40 which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even

picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Natl Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al. *J. Mol. Biol.* 226:889-896 (1992).

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

10 Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express Fc $\gamma$ RIII only, whereas monocytes express Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 20 5,500,362 or 5,821,337 or Presta U.S. Patent No. 6,737,056 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

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

20 "Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc $\gamma$ RI, Fc $\gamma$ RII, and Fc $\gamma$ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc $\gamma$ RII receptors include Fc $\gamma$ RIIA (an "activating receptor") and Fc $\gamma$ RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc $\gamma$ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc $\gamma$ RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer

*et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)) and regulates homeostasis of immunoglobulins.

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

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

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

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

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

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

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

30 The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, 35 pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplastics include but are not limited those described above. Non-

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

The terms "neurodegenerative disease" and "neurodegenerative disorder" are used in the broadest sense to include all disorders the pathology of which involves neuronal degeneration and/or dysfunction, including, without limitation, peripheral neuropathies; motorneuron disorders, such as amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy or paralysis; and other human neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease.

"Peripheral neuropathy" is a neurodegenerative disorder that affects the peripheral nerves, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic dysfunction. Peripheral neuropathies may, for example, be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent, such as a neurotoxic drug, e. g. antineoplastic agent, or industrial or environmental pollutant. "Peripheral sensory neuropathy" is characterized by the degeneration of peripheral sensory neurons, which may be idiopathic, may occur, for example, as a consequence of diabetes (diabetic neuropathy), cytostatic drug therapy in cancer (e.g. treatment with chemotherapeutic agents such as vincristine, cisplatin, methotrexate, 3'-azido-3'-deoxythymidine, or taxanes, e.g. paclitaxel [TAXOL®, Bristol- Myers Squibb Oncology, Princeton, N.J.] and doxetaxel [TAXOTERE®, Rhône- Poulenc Rorer, Antony, France]), alcoholism, acquired immunodeficiency syndrome (AIDS), or genetic predisposition. Genetically acquired peripheral neuropathies include, for example, Refsum's disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, Dejerine-Sottas syndrome, Abetalipoproteinemia, and Charcot-Marie-Tooth (CMT) Disease (also known as Proneal Muscular Atrophy or Hereditary Motor Sensory Neuropathy (HMSN)). Most types of peripheral neuropathy develop slowly, over the course of several months or years. In clinical practice such neuropathies are called chronic. Sometimes a peripheral neuropathy develops rapidly, over the course of a few days, and is referred to as acute. Peripheral neuropathy usually affects sensory and motor nerves together so as to cause a mixed sensory and motor neuropathy, but pure sensory and pure motor neuropathy are also known.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing 5 metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or disorder.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs and horses), 10 primates, mice and rats.

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

An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to 15 achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule of the invention, agonist or antagonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability 20 of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function 25 of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., At<sup>211</sup>, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Bi<sup>212</sup>, P<sup>32</sup> and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamycin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, 30 mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of 35 chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylololomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan 40 (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolactin, and 9-

aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as 5 chlorambucil, chlornaphazine, chlophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gammaI and calicheamicin omegaI (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); 10 dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, 15 esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as 20 fludarabine, 6-mercaptopurine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxuryidine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as folinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestramycin; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; el fornithine; elliptinium acetate; an epothilone; etoglucid; gallium 25 nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; niraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); 30 dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepe; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANETM Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum 35 analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovorin; vinorelbine (NAVELBINE®); novantrone; edatraxate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or 40 more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin,

vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATINTM) combined with 5-FU and leucovorin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVISTA® raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin acetate, buserelin acetate and triptorelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestane, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole. In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), DIDROCAL® etidronate, NE-58095, ZOMETA® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREDIA® pamidronate, SKELID® tiludronate, or ACTONEL® risedronate; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

25 A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell (such as a cell expressing EphB2) either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells (such as a cell expressing EphB2) in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

“Doxorubicin” is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxo-hexapyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

***Compositions of the invention and methods of making same***

5 This invention encompasses compositions, including pharmaceutical compositions, comprising an anti-EphB2 antibody; and polynucleotides comprising sequences encoding an anti-EphB2 antibody. As used herein, compositions comprise one or more antibodies that bind to EphB2, and/or one or more polynucleotides comprising sequences encoding one or more antibodies that bind to EphB2. These compositions may further comprise suitable carriers, such as pharmaceutically acceptable excipients including buffers, which are well known in the art.

10 The invention also encompasses isolated antibody and polynucleotide embodiments. The invention also encompasses substantially pure antibody and polynucleotide embodiments.

15 The anti- EphB2 antibodies of the invention are preferably monoclonal. Also encompassed within the scope of the invention are Fab, Fab', Fab'-SH and F(ab')<sub>2</sub> fragments of the anti- EphB2 antibodies provided herein. These antibody fragments can be created by traditional means, such as enzymatic digestion, or may be generated by recombinant techniques. Such antibody fragments may be chimeric or humanized. These fragments are useful for the diagnostic and therapeutic purposes set forth below.

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

The anti- EphB2 monoclonal antibodies of the invention can be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (e.g., U.S. Patent No. 4,816,567).

25 In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies to EphB2 generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of EphB2 and an adjuvant. EphB2 may be prepared using methods well-known in the art, some of which are further described herein. For example, recombinant production of EphB2 is described below. In one embodiment, animals are immunized with a derivative of EphB2 that contains the extracellular domain (ECD) of EphB2 fused to the Fc portion of an immunoglobulin heavy chain. In a preferred embodiment, animals are immunized with an EphB2-IgG1 fusion protein. Animals ordinarily are immunized against immunogenic conjugates or derivatives of EphB2 with monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT) and the solution is injected intradermally at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti- EphB2 titer. Animals are boosted until titer plateaus.

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

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

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California 10 USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, **133**:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

15 Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against EphB2. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

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

25 After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

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

30 The anti-EphB2 antibodies of the invention can be made by using combinatorial libraries to screen for synthetic antibody clones with the desired activity or activities. In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted 35 from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the anti-EphB2 antibodies of the invention can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length anti-EphB2 antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, 40 Bethesda MD (1991), vols. 1-3.

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

5 Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter *et al.*, *Ann. Rev. Immunol.*, **12**: 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths *et al.*, *EMBO J.*, **12**: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unarranged V-gene segments from stem 10 cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, *J. Mol. Biol.*, **227**: 381-388 (1992).

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

20 In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-EphB2 clones is desired, the subject is immunized with EphB2 to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In a preferred embodiment, a human antibody gene fragment library biased in favor of anti-human EphB2 clones is obtained by generating an anti- 25 human EphB2 antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that EphB2 immunization gives rise to B cells producing human antibodies against EphB2. The generation of human antibody-producing transgenic mice is described below.

30 Additional enrichment for anti- EphB2 reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing EphB2-specific membrane bound antibody, e.g., by cell separation with EphB2 affinity chromatography or adsorption of cells to fluorochrome-labeled EphB2 followed by flow-activated cell sorting (FACS).

35 Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which EphB2 is not antigenic. For 40

libraries incorporating in vitro antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

5 Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **86**: 3833-3837 (1989), thereby making diverse V 10 gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward primers based within the J- segment as described in Orlandi *et al.* (1989) and in Ward *et al.*, *Nature*, **341**: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones *et al.*, *Biotechnol.*, **9**: 88-89 (1991), and forward primers within the constant region as described in Sastry *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **86**: 5728-5732 (1989). To maximize complementarity, degeneracy can be 15 incorporated in the primers as described in Orlandi *et al.* (1989) or Sastry *et al.* (1989). Preferably, the library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g. as described in the method of Marks *et al.*, *J. Mol. Biol.*, **222**: 581-597 (1991) or as described in the method of Orum *et al.*, *Nucleic Acids 20 Res.*, **21**: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites 20 can be introduced within the PCR primer as a tag at one end as described in Orlandi *et al.* (1989), or by further PCR amplification with a tagged primer as described in Clackson *et al.*, *Nature*, **352**: 624-628 (1991).

Repertoires of synthetically rearranged V genes can be derived in vitro from V gene segments. Most 25 of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson *et al.*, *J. Mol. Biol.*, **227**: 776-798 (1992)), and mapped (reported in Matsuda *et al.*, *Nature Genet.*, **3**: 88-94 (1993); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH 30 gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, *J. Mol. Biol.*, **227**: 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**: 4457-4461 (1992). Human V $\kappa$  and V $\lambda$  segments have been cloned and sequenced (reported in 35 Williams and Winter, *Eur. J. Immunol.*, **23**: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged in vitro according to the methods of Hoogenboom and Winter, *J. Mol. Biol.*, **227**: 381-388 (1992).

Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires 40 together in several ways. Each repertoire can be created in different vectors, and the vectors recombined in vitro, e.g., as described in Hogrefe *et al.*, *Gene*, **128**: 119-126 (1993), or in vivo by combinatorial infection, e.g., the loxP system described in Waterhouse *et al.*, *Nucl. Acids Res.*, **21**: 2265-2266 (1993). The *in vivo* recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size

imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about  $10^{12}$  clones). Both vectors contain in vivo recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. 5 These huge libraries provide large numbers of diverse antibodies of good affinity (e.g.,  $K_d^{-1}$  of about  $10^{-8}$  M).

Alternatively, the repertoires may be cloned sequentially into the same vector, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**: 7978-7982 (1991), or assembled together by PCR and then cloned, e.g. as described in Clackson *et al.*, *Nature*, **352**: 624-628 (1991). PCR assembly can also be used to 10 join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton *et al.*, *Nucl. Acids Res.*, **20**: 3831-3837 (1992).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity 15 ( $K_d^{-1}$  of about  $10^6$  to  $10^7$  M $^{-1}$ ), but affinity maturation can also be mimicked *in vitro* by constructing and reselecting from secondary libraries as described in Winter *et al.* (1994), *supra*. For example, mutation can be introduced at random *in vitro* by using error-prone polymerase (reported in Leung *et al.*, *Technique*, **1**: 11-15 (1989)) in the method of Hawkins *et al.*, *J. Mol. Biol.*, **226**: 889-896 (1992) or in the method of Gram *et al.*, *Proc. Natl. Acad. Sci USA*, **89**: 3576-3580 (1992). Additionally, affinity maturation can be performed by 20 randomly mutating one or more CDRs, e.g. using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 (published 14 March 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V 25 domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks *et al.*, *Biotechnol.*, **10**: 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities in the  $10^{-9}$  M range.

Nucleic acid sequence encoding the EphB2 can be designed using the amino acid sequence of the desired region of EphB2, e.g. the extracellular domain spanning amino acids 19 to 542 of the amino acid 30 sequence shown in GenBank Accession Nos. NM\_017449, or NM\_004442, or Figure 101 of WO03/000113, and/or the polypeptide comprising amino acids about 19 to about 208 of the amino acid sequence shown in GenBank Accession Nos. NM\_017449, or NM\_004442, or Figure 101 of WO03/000113.

Alternatively, the cDNA sequence (or fragments thereof) of GenBank Accession Nos. NM\_017449, or NM\_004442, or shown in Figure 23 of WO03/000113 may be used. Additional EphB2 sequences are further 35 disclosed in, e.g., *Annu. Rev. Neurosci.* 21:309-345 (1998), *Int. Rev. Cytol.* 196:177-244 (2000); WO2003042661; WO200053216; WO2004065576; WO2004020583; WO2003004529 (Page 128-132); and WO200053216. DNAs encoding EphB2 can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, **28**: 716-734 (1989), such as the triester, phosphite, phosphoramidite and H- 40 phosphonate methods. In one embodiment, codons preferred by the expression host cell are used in the design

of the EphB2 encoding DNA. Alternatively, DNA encoding the EphB2 can be isolated from a genomic or cDNA library.

Following construction of the DNA molecule encoding the EphB2, the DNA molecule is operably linked to an expression control sequence in an expression vector, such as a plasmid, wherein the control sequence is recognized by a host cell transformed with the vector. In general, plasmid vectors contain replication and control sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. Suitable vectors for expression in prokaryotic and eukaryotic host cells are known in the art and some are further described herein. Eukaryotic organisms, such as yeasts, or cells derived from multicellular organisms, such as mammals, may be used.

Optionally, the DNA encoding the EphB2 is operably linked to a secretory leader sequence resulting in secretion of the expression product by the host cell into the culture medium. Examples of secretory leader sequences include stII, ecotin, lamB, herpes GD, lpp, alkaline phosphatase, invertase, and alpha factor. Also suitable for use herein is the 36 amino acid leader sequence of protein A (Abrahmsen *et al.*, *EMBO J.*, **4**: 3901 (1985)).

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

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

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

Prokaryotic host cells used to produce the EphB2 can be cultured as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the EphB2 can be cultured in a variety of media, which is well known in the art and some of which is described herein.

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

Purification of EphB2 may be accomplished using art-recognized methods, some of which are described herein.

The purified EphB2 can be attached to a suitable matrix such as agarose beads, acrylamide beads, glass beads, cellulose, various acrylic copolymers, hydroxyl methacrylate gels, polyacrylic and polymethacrylic copolymers, nylon, neutral and ionic carriers, and the like, for use in the affinity chromatographic separation of phage display clones. Attachment of the EphB2 protein to the matrix can be accomplished by the methods described in *Methods in Enzymology*, vol. 44 (1976). A commonly employed

technique for attaching protein ligands to polysaccharide matrices, e.g. agarose, dextran or cellulose, involves activation of the carrier with cyanogen halides and subsequent coupling of the peptide ligand's primary aliphatic or aromatic amines to the activated matrix.

5 Alternatively, EphB2 can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other art-known method for panning phage display libraries.

The phage library samples are contacted with immobilized EphB2 under conditions suitable for binding of at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to 10 the solid phase are washed and then eluted by acid, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**: 7978-7982 (1991), or by alkali, e.g. as described in Marks *et al.*, *J. Mol. Biol.*, **222**: 581-597 (1991), or by EphB2 antigen competition, e.g. in a procedure similar to the antigen competition method of Clackson *et al.*, *Nature*, **352**: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection.

Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

15 The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The 20 selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass *et al.*, *Proteins*, **8**: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks *et al.*, *Biotechnol.*, **10**: 779-783 (1992).

It is possible to select between phage antibodies of different affinities, even with affinities that differ 25 slightly, for EphB2. However, random mutation of a selected antibody (e.g. as performed in some of the affinity maturation techniques described above) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting EphB2, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess biotinylated EphB2, but with the biotinylated EphB2 at a concentration of lower molarity than the target molar affinity constant for EphB2. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such 30 "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

Anti-EphB2 clones may be activity selected. In one embodiment, the invention provides anti-EphB2 35 antibodies that block the binding between an EphB2 ligand (such as ephrin-B1, ephrin-B2 and/or ephrin-B3) and EphB2, but do not block the binding between an EphB2 ligand and a second protein (such as EphB1, EphB3, EphB4, EphB5 and/or EphB6). Fv clones corresponding to such anti- EphB2 antibodies can be selected by (1) isolating anti- EphB2 clones from a phage library as described in Section B(I)(2) above, and 40 optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting EphB2 and a second protein against which blocking and non-blocking activity,

respectively, is desired; (3) adsorbing the anti- EphB2 phage clones to immobilized EphB2; (4) using an excess of the second protein to elute any undesired clones that recognize EphB2-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can 5 be further enriched by repeating the selection procedures described herein one or more times.

DNA encoding the hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention is readily isolated and sequenced using conventional procedures (e.g. by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then 10 transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra *et al.*, *Curr. Opinion in Immunol.*, 5: 256 (1993) and Pluckthun, *Immunol. Revs*, 130: 151 (1992).

15 DNA encoding the Fv clones of the invention can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g. the appropriate DNA sequences can be obtained from Kabat *et al.*, *supra*) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. A 20 Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid", full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In a preferred embodiment, a Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for all human, full or partial length heavy and/or light chains.

25 DNA encoding anti- EphB2 antibody derived from a hybridoma of the invention can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (e.g. as in the method of Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81: 6851-6855 (1984)). DNA encoding a hybridoma or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding 30 sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the invention.

#### *Antibody Fragments*

The present invention encompasses antibody fragments. In certain circumstances there are 35 advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan *et al.*, *Science*, 229:81 (1985)). 40 However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody

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

15 ***Humanized Antibodies***

The present invention encompasses humanized antibodies. Various methods for humanizing non-human antibodies are known in the art. For example, a humanized antibody can have one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization 20 can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein 25 substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence 30 of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody (Sims *et al.* (1993) *J. Immunol.* 151:2296; Chothia *et al.* (1987) *J. Mol. Biol.* 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several 35 different humanized antibodies (Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; Presta *et al.* (1993) *J. Immunol.*, 151:2623.

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to one method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using 40 three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin

models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the 5 ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

#### ***Human antibodies***

10 Human anti-EphB2 antibodies of the invention can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequences(s) as described above. Alternatively, human monoclonal anti-EphB2 antibodies of the invention can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, **133**: 15 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, **147**: 86 (1991).

15 It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region 20 (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci USA*, **90**: 2551 (1993); Jakobovits *et al.*, *Nature*, **362**: 255 (1993); Brugermann *et al.*, *Year in Immunol.*, **7**: 33 (1993).

25 Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or 30 Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, *i.e.* the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional 35 humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

#### ***Bispecific Antibodies***

40 Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for EphB2 and the other is for any other antigen. Exemplary bispecific antibodies may bind to two different

5 epitopes of the EphB2 protein. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express EphB2. These antibodies possess an EphB2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon- $\alpha$ , vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')<sub>2</sub> bispecific antibodies).

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

15 According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH<sub>2</sub>, and CH<sub>3</sub> regions. It is preferred to have the first heavy-chain constant region (CH<sub>1</sub>), containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate 20 expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no 25 particular significance.

25 In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the 30 bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986).

35 According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C<sub>H</sub>3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains

with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

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

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

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

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, **148**(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, **152**:5368 (1994).

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

40 *Multivalent Antibodies*

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the 5 polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one 10 polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)<sub>n</sub>-VD2-(X2)<sub>n</sub>-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide 15 chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

#### *Antibody Variants*

20 In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino 25 acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

30 A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence 35 variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

40 Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal

methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

5 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglucosamine, galactose, or xylose to 10 a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

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

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

25 The preferred glycosylation variant herein comprises an Fc region, wherein a carbohydrate structure attached to the Fc region lacks fucose. Such variants have improved ADCC function. Optionally, the Fc region further comprises one or more amino acid substitutions therein which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Examples of publications related to "defucosylated" or "fucose-deficient" antibodies include: US 30 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO 2005/053742; Okazaki *et al.* *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki *et al.* *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka *et al.* *Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8*, knockout CHO cells (Yamane-Ohnuki *et al.* *Biotech. Bioeng.* 87: 614 (2004)).

35 Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for 40 substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated.

Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

5 Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

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

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

- (3) acidic: asp, glu;
- (4) basic: his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

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

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are 10 generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable 15 region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as 20 described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide- 25 mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of the immunoglobulin polypeptides of the invention, thereby generating a Fc region variant. The Fc region variant 30 may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody used in methods of the invention may comprise one or more alterations as compared to the wild type counterpart antibody, e.g. in the Fc region. These antibodies would nonetheless 35 retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For example, it is thought that certain alterations can be made in the Fc region that would result in altered (i.e., either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in WO99/51642. See also Duncan & Winter *Nature* 322:738-40 (1988); US Patent No. 5,648,260; US Patent No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants.

WO00/42072 (Presta) and WO 2004/056312 (Lowman) describe antibody variants with improved or diminished binding to FcRs. The content of these patent publications are specifically incorporated herein by reference. See, also, Shields *et al.* *J. Biol. Chem.* 9(2): 6591-6604 (2001). Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton *et al.*). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1, WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al.* *J. Immunol.* 164: 4178-4184 (2000).

#### *Antibody Derivatives*

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

#### *Screening for antibodies with desired properties*

The antibodies of the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art. In some embodiments, antibodies are characterized for any one or more of reduction or blocking of EphB2 activation, reduction or blocking of EphB2 downstream molecular signaling, reduction or blocking of EphB2 ligand activation, reduction or blocking of EphB2 ligand downstream molecular signaling, disruption or blocking of ligand (e.g., ephrin-B1, ephrin-B2, and/or ephrin-B3) binding to EphB2, EphB2 phosphorylation and/or EphB2 multimerization, and/or EphB2 ligand phosphorylation, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with EphB2 expression and/or activity (such as increased EphB2 expression and/or activity).

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

In certain embodiments of the invention, the antibodies produced herein are analyzed for their biological activity. In some embodiments, the antibodies of the present invention are tested for their antigen binding activity. The antigen binding assays that are known in the art and can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, 5 radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. Illustrative antigen binding assay are provided below in the Examples section.

10 In another embodiment, the invention provides the anti- EphB2 monoclonal antibody produced by hybridoma cell line 2H9.11.14 (ATCC Deposit No. PTA-6606) (interchangeably termed "2H9" or "Mab 2H9" herein).

15 In still another embodiment, the invention provides anti- EphB2 monoclonal antibodies that compete with 2H9 antibody for binding to EphB2. Such competitor antibodies include antibodies that recognize an EphB2 epitope that is the same as or overlaps with the EphB2 epitope recognized by antibody 2H9. Such competitor antibodies can be obtained by screening anti- EphB2 hybridoma supernatants for binding to immobilized EphB2 in competition with labeled 2H9 antibody. A hybridoma supernatant containing competitor antibody will reduce the amount of bound, labeled antibody detected in the subject competition binding mixture as compared to the amount of bound, labeled antibody detected in a control binding mixture containing irrelevant (or no) antibody. Any of the competition binding assays described herein are suitable for use in the foregoing procedure.

20 In another aspect, the invention provides an anti- EphB2 monoclonal antibody that comprises one or more (such as 2, 3, 4, 5, and/or 6) HVRs of the 2H9 antibody. An anti- EphB2 monoclonal antibody that comprises one or more HVR(s) of 2H9 can be constructed by grafting one or more HVR(s) of 2H9 onto a template antibody sequence, e.g. a human antibody sequence which is closest to the corresponding murine sequence of the parental antibody, or a consensus sequence of all human antibodies in the particular subgroup 25 of the parental antibody light or heavy chain, and expressing the resulting chimeric light and/or heavy chain variable region sequence(s), with or without accompanying constant region sequence(s), in recombinant host cells as described herein.

30 Anti-EphB2 antibodies of the invention possessing the unique properties described herein can be obtained by screening anti- EphB2 hybridoma clones for the desired properties by any convenient method. For example, if an anti- EphB2 monoclonal antibody that blocks or does not block the binding of EphB2 ligands to EphB2 is desired, the candidate antibody can be tested in a binding competition assay, such as a competitive binding ELISA, wherein plate wells are coated with EphB2, and a solution of antibody in an excess of the Eph ligand of interest is layered onto the coated plates, and bound antibody is detected enzymatically, e.g. contacting the bound antibody with HRP-conjugated anti-Ig antibody or biotinylated anti-Ig antibody and 35 developing the HRP color reaction., e.g. by developing plates with streptavidin-HRP and/or hydrogen peroxide and detecting the HRP color reaction by spectrophotometry at 490 nm with an ELISA plate reader.

If an anti-EphB2 antibody that inhibits EphB2 activation is desired, the candidate antibody can be tested in an EphB2 phosphorylation assay. Such assays are known in the art and one such assay is described and exemplified in the Examples section. If an antibody that interferes with antibody internalization is desired,

the candidate assay can be tested in a cell internalization assay. Such assays are known in the art and one such assay is described and exemplified in the Examples section.

If an anti-EphB2 antibody or immunoconjugate that kills cells or inhibits cell growth is desired, the candidate antibody or immunoconjugate can be tested in in vitro and/or in vivo assays that measure cell killing and/or inhibition of cell growth. Such assays are known in the art and are further described and exemplified herein.

In one embodiment, the present invention contemplates an altered antibody that possesses some but not all effector functions, which make it a desired candidate for many applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the produced immunoglobulin are measured to ensure that only the desired properties are maintained. In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in US Patent No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. PNAS (USA) 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed. FcRn binding and in vivo clearance/half life determinations can also be performed using methods known in the art, e.g. those described in the Examples section.

#### ***Vectors, Host Cells and Recombinant Methods***

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

35       a. Generating antibodies using prokaryotic host cells:

          i. *Vector Construction*

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

polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each 5 vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

10 In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy 15 means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237.

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

25 The expression vector of the invention may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g. the presence or absence of a nutrient or a change in temperature.

30 A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the 35 promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the  $\beta$ -galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled

worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) Cell 20: 269) using linkers or adaptors to supply any required restriction sites.

In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, 5 the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline 10 phosphatase, penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In one embodiment of the invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the immunoglobulins according to the invention can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within 15 each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the *E. coli* *trxB*- strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits (Proba and Pluckthun Gene, 159:203 (1995)).

Prokaryotic host cells suitable for expressing antibodies of the invention include Archaeabacteria and 20 Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (e.g., *E. coli*), *Bacilli* (e.g., *B. subtilis*), *Enterobacteria*, *Pseudomonas* species (e.g., *P. aeruginosa*), *Salmonella typhimurium*, *Serratia marcescens*, *Klebsiella*, *Proteus*, *Shigella*, *Rhizobia*, *Vitreoscilla*, or *Paracoccus*. In one embodiment, gram-negative cells are used. In one embodiment, *E. coli* cells are used as 25 hosts for the invention. Examples of *E. coli* strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* λ 1776 (ATCC 31,537) and *E. coli* RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing 30 derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, *Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell 35 should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

#### *ii. Antibody Production*

Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the 40 genes encoding the desired sequences.

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

10 Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include Luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin 15 resistant gene.

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

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

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

30 In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further 35 isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

35 In one aspect of the invention, antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 40 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially

glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

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

10 To improve the production yield and quality of the polypeptides of the invention, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al. (1999) J Bio Chem 274:19601-19605; Georgiou et al., U.S. Patent No. 6,083,715; Georgiou et al., U.S. Patent No. 6,027,888; Bothmann and Pluckthun (2000) J. Biol. Chem. 275:17100-17105; Ramm and Pluckthun (2000) J. Biol. Chem. 275:17106-17113; Arie et al. (2001) Mol. Microbiol. 39:199-210.

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

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

### *iii. Antibody Purification*

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

35 In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products of the invention. Protein A is a 41kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity to the Fc region of antibodies. Lindmark et al (1983) *J. Immunol. Meth.* 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific 40 adherence of contaminants.

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

5       b. Generating antibodies using eukaryotic host cells:

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

(i) *Signal sequence component*

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

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

(ii) *Origin of replication*

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

(iii) *Selection gene component*

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

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

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

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

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

*(iv) Promoter component*

5 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody polypeptide nucleic acid. Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

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

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

*(v) Enhancer element component*

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

*(vi) Transcription termination component*

25 Expression vectors used in eukaryotic host cells will typically also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding an antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

*(vii) Selection and transformation of host cells*

30 Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture)

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

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

10 (viii) *Culturing the host cells*

15 The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 20 20 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCINT<sup>TM</sup> drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

25 (ix) *Purification of antibody*

30 When using recombinant techniques, the antibody can be produced intracellularly, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon 35 ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

40 The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to

purify antibodies that are based on human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss et al., *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or

5 poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX<sup>TM</sup>resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE<sup>TM</sup> chromatography on an anion or cation exchange resin (such as a polyaspartic acid

10 column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

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

15 **Immunoconjugates**

The invention also provides immunoconjugates (interchangeably termed "antibody-drug conjugates" or "ADC"), comprising any of the anti-EphB2 antibodies described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

20 The use of antibody-drug conjugates for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drg Del. Rev.* 26:151-172; U.S. patent 4,975,278) allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells

25 as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) *Lancet* pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications*, A. Pinchera et al. (ed.s), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) *Cancer Immunol. Immunother.*, 21:183-87).

30 Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) *supra*). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) *Jour. of the Nat. Cancer Inst.* 92(19):1573-1581; Mandler et al (2000) *Bioorganic & Med. Chem. Letters* 10:1025-1028; Mandler et al (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), and calicheamicin (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

40 ZEVALIN<sup>®</sup> (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal

and malignant B lymphocytes and  $^{111}\text{In}$  or  $^{90}\text{Y}$  radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) Eur. Jour. Nucl. Med. 27(7):766-77; Wiseman et al (2002) Blood 99(12):4336-42; Witzig et al (2002) J. Clin. Oncol. 20(10):2453-63; Witzig et al (2002) J. Clin. Oncol. 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged 5 cytopenias in most patients. MYLOTARG<sup>TM</sup> (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (Drugs of the Future (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine 10 (Immunogen, Inc.), an antibody drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin 15 (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) Nature Biotechnology 21(7):778-784) and are under therapeutic development.

Chemotherapeutic agents useful in the generation of immunoconjugates are described herein (above). Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding 20 active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the trichothecenes. See, e.g., WO 93/21232 published October 28, 1993. A variety of radionuclides are available for the production of 25 radioconjugated antibodies. Examples include  $^{212}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{131}\text{In}$ ,  $^{90}\text{Y}$ , and  $^{186}\text{Re}$ . Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine 30 compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., *Science*, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

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

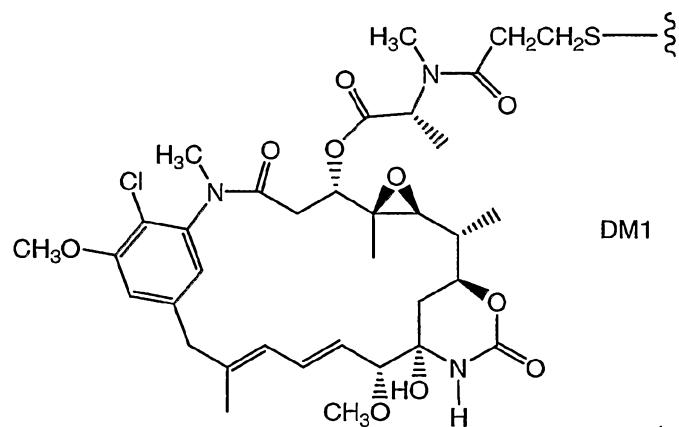
i. Maytansine and maytansinoids

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

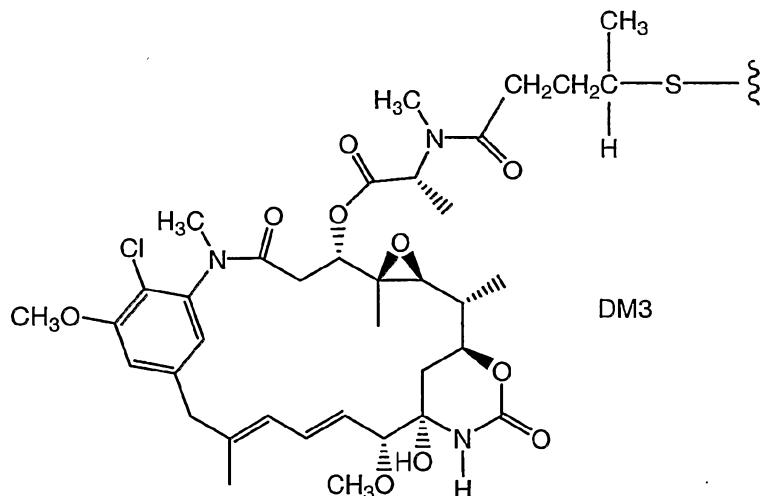
Maytansinoids are mitotic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata* (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, 5 for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

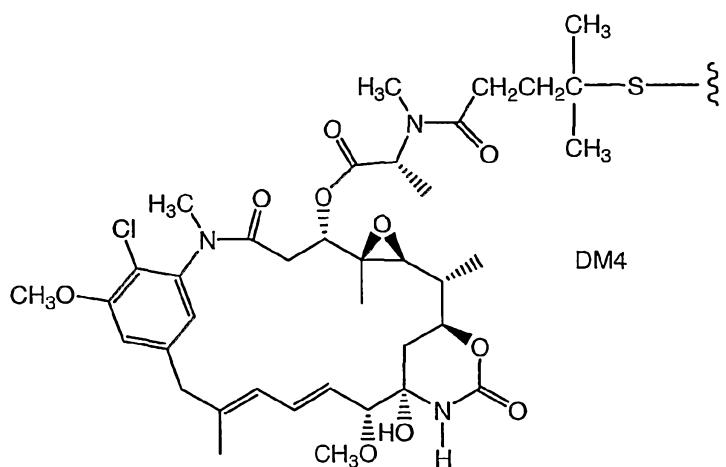
Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation 10 products, (ii) amenable to derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

Exemplary embodiments of maytansinoid drug moieties include: DM1; DM3; and DM4, having the structures:



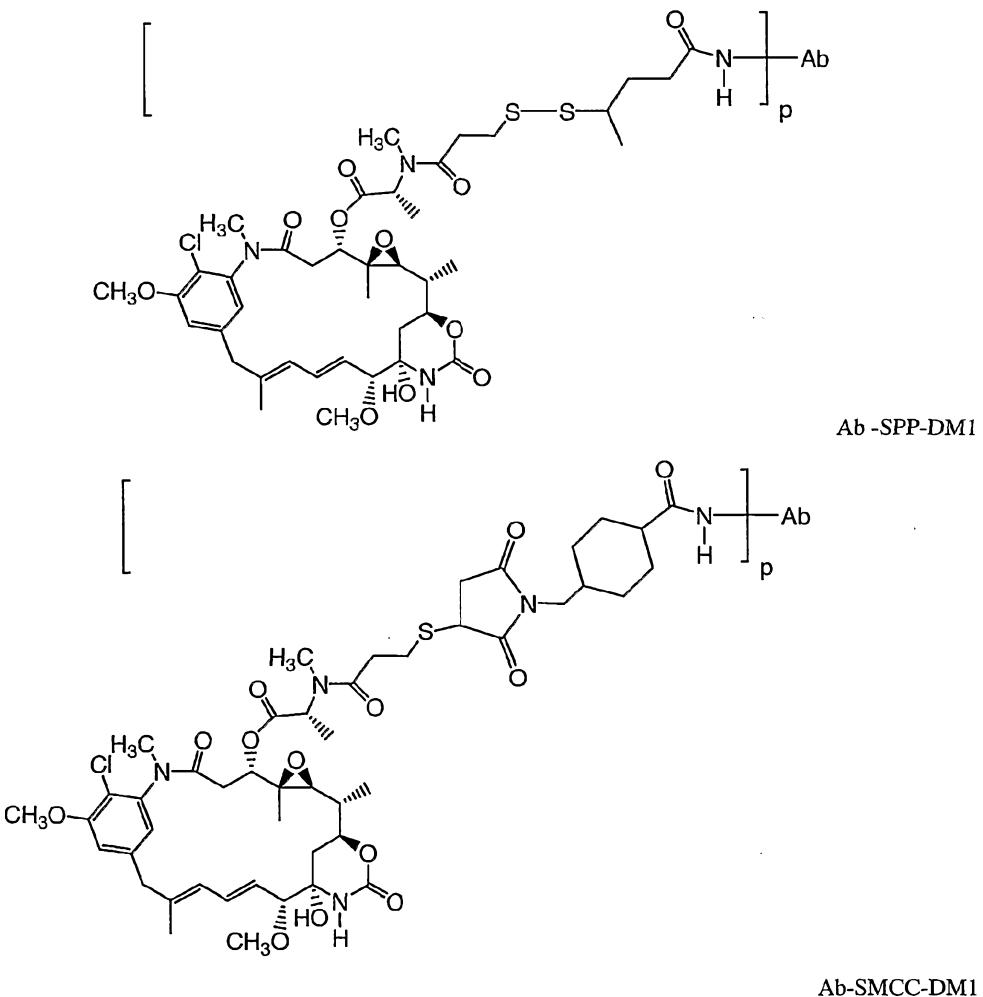
15



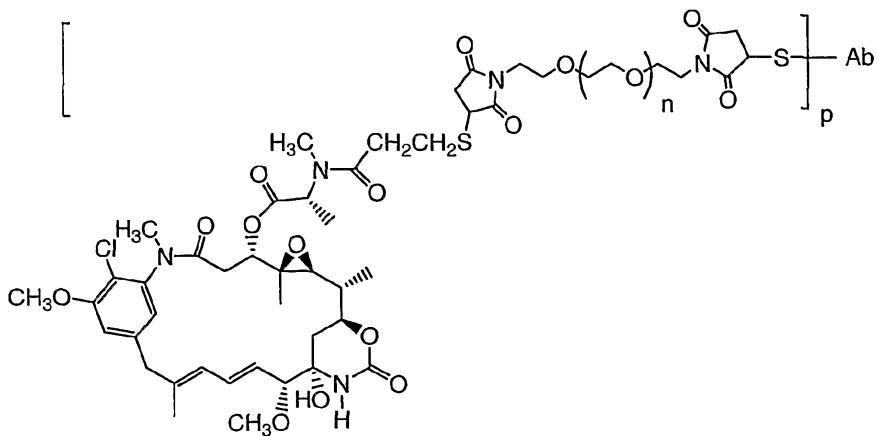


wherein the wavy line indicates the covalent attachment of the sulfur atom of the drug to a linker (L) of an antibody drug conjugate.

Other exemplary maytansinoid antibody drug conjugates have the following structures and 5 abbreviations, (wherein Ab is antibody and p is 1 to about 8):



Exemplary antibody drug conjugates where DM1 is linked through a BMPEO linker to a thiol group of the antibody have the structure and abbreviation:



5

Ab-BMPEO-DM1

wherein Ab is antibody; n is 0, 1, or 2; and p is 1, 2, 3, or 4.

Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al., Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The cytotoxicity of the TA.1-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses  $3 \times 10^5$  HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

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

and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, Chari et al.,

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

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., 20 Biochem. J. 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

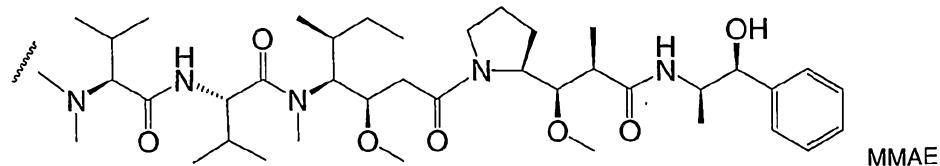
The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-25 14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

## ii. Auristatins and dolastatins

In some embodiments, the immunoconjugate comprises an antibody of the invention conjugated to dolastatins or dolostatin peptidic analogs and derivatives, the auristatins (US Patent Nos. 5635483; 5780588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke et al (2001) Antimicrob. Agents and Chemother. 45(12):3580-3584) and have anticancer (US 5663149) and antifungal activity (Pettit et al (1998) Antimicrob. Agents Chemother. 42:2961-2965). The dolastatin or auristatin drug moiety may be attached to the antibody through the N 35 (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (WO 02/088172).

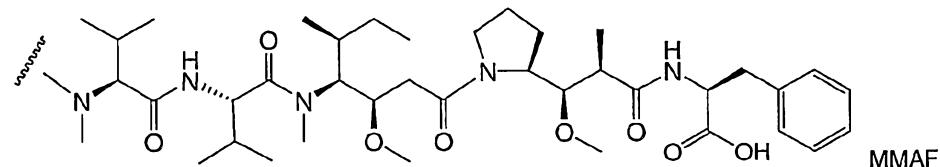
Exemplary auristatin embodiments include the N-terminus linked monomethylauristatin drug moieties DE and DF, disclosed in "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the disclosure of which is expressly incorporated by reference in its entirety.

An exemplary auristatin embodiment is MMAE (wherein the wavy line indicates the covalent 40 attachment to a linker (L) of an antibody drug conjugate).



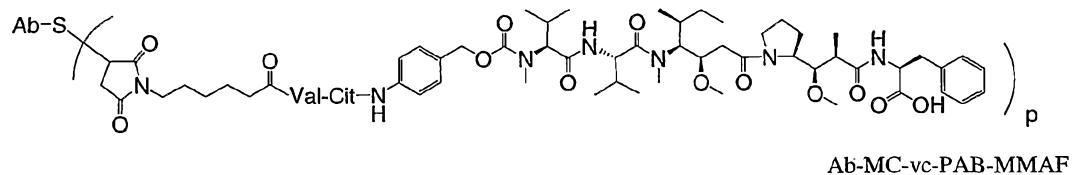
Another exemplary auristatin embodiment is MMAF (wherein the wavy line indicates the covalent attachment to a linker (L) of an antibody drug conjugate):

5 attachment to a linker (L) of an antibody drug conjugate):

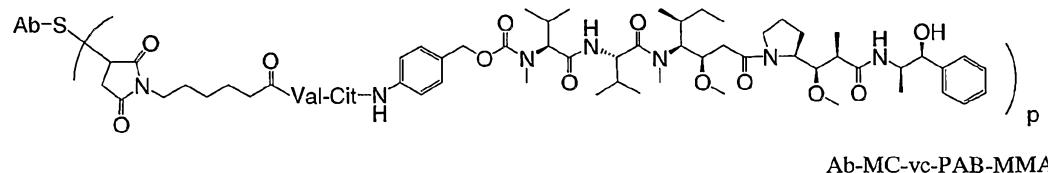


Additional exemplary embodiments comprising MMAE or MMAF and various linker components (described further herein) have the following structures and abbreviations (wherein Ab means antibody and p is 1 to about 8):

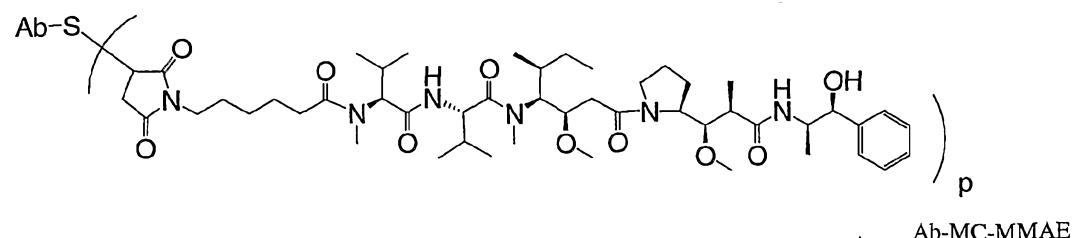
10

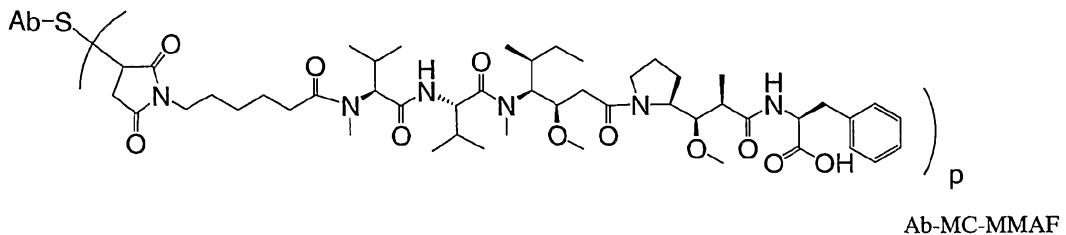


15



20





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

iii. Calicheamicin

In other embodiments, the immunoconjugate comprises an antibody of the invention conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to,  $\gamma_1^I$ ,  $\alpha_2^I$ ,  $\alpha_3^I$ , N-acetyl- $\gamma_1^I$ , PSAG and  $\theta^I_1$  (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

iv. Other cytotoxic agents

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

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

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

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At<sup>211</sup>, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Bi<sup>212</sup>, P<sup>32</sup>, Pb<sup>212</sup> and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc<sup>99m</sup> or I<sup>123</sup>, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

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

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

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

v. Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC) of the invention, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents

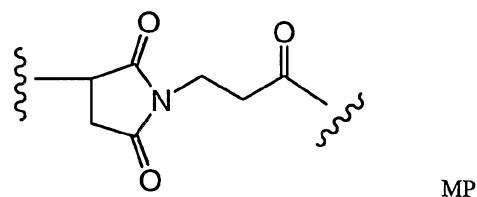
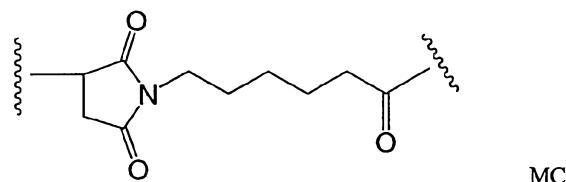
known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody. Additional methods for preparing ADC are 5 described herein.

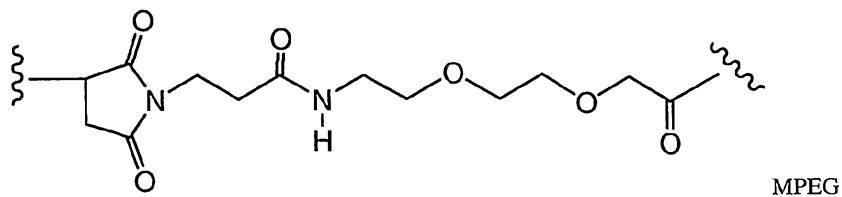


The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-aminobenzylloxycarbonyl ("PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"),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

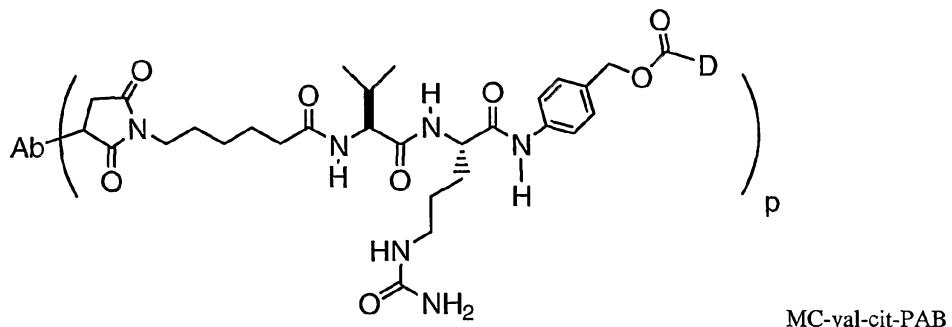
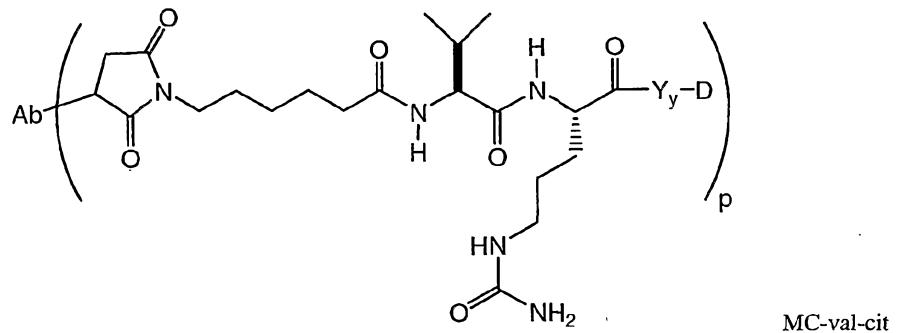
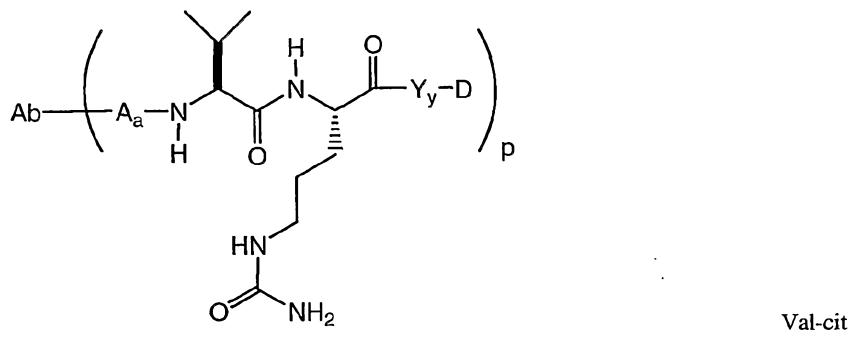
In some embodiments, the linker may comprise amino acid residues. Exemplary amino acid linker 15 components include a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide. Exemplary dipeptides include: valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe). Exemplary tripeptides include: glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). Amino acid residues which comprise 20 an amino acid linker component include those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline. Amino acid linker components can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzymes, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

Exemplary linker component structures are shown below (wherein the wavy line indicates sites of covalent attachment to other components of the ADC):





Additional exemplary linker components and abbreviations include (wherein the antibody (Ab) and linker are depicted, and p is 1 to about 8):



10

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents

including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each 5 cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into the antibody (or fragment thereof) by introducing one, two, three, four, or more cysteine residues (e.g., preparing mutant antibodies comprising one or more non-native cysteine amino acid residues).

10 Antibody drug conjugates of the invention may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents 15 to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glucose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan 20 & Stroh, (1992) Bioconjugate Chem. 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, 25 hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

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

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

35 Antibody (Ab)-MC-MMAE may be prepared by conjugation of any of the antibodies provided herein with MC-MMAE as follows. Antibody, dissolved in 500mM sodium borate and 500 mM sodium chloride at pH 8.0 is treated with an excess of 100mM dithiothreitol (DTT). After incubation at 37 °C for about 30 minutes, the buffer is exchanged by elution over Sephadex G25 resin and eluted with PBS with 1mM DTPA. The thiol/Ab value is checked by determining the reduced antibody concentration from the absorbance at 280 40 nm of the solution and the thiol concentration by reaction with DTNB (Aldrich, Milwaukee, WI) and

determination of the absorbance at 412 nm. The reduced antibody dissolved in PBS is chilled on ice. The drug linker reagent, maleimidocaproyl-monomethyl auristatin E (MMAE), i.e. MC-MMAE, dissolved in DMSO, is diluted in acetonitrile and water at known concentration, and added to the chilled reduced antibody 2H9 in PBS. After about one hour, an excess of maleimide is added to quench the reaction and cap any 5 unreacted antibody thiol groups. The reaction mixture is concentrated by centrifugal ultrafiltration and 2H9-MC-MMAE is purified and desalted by elution through G25 resin in PBS, filtered through 0.2  $\mu$ m filters under sterile conditions, and frozen for storage.

Antibody-MC-MMAF may be prepared by conjugation of any of the antibodies provided herein with MC-MMAF following the protocol provided for preparation of Ab-MC-MMAE.

10 Antibody-MC-val-cit-PAB-MMAE may be prepared by conjugation of any of the antibodies provided herein with MC-val-cit-PAB-MMAE following the protocol provided for preparation of Ab-MC-MMAE.

Antibody-MC-val-cit-PAB-MMAF may be prepared by conjugation of any of the antibodies provided herein with MC-val-cit-PAB-MMAF following the protocol provided for preparation of Ab-MC-MMAE.

15 Antibody-SMCC-DM1 may be prepared by conjugation of any of the antibodies provided herein with SMCC-DM1 as follows. Purified antibody is derivatized with (Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce Biotechnology, Inc) to introduce the SMCC linker. Specifically, antibody is treated at 20 mg/mL in 50mM potassium phosphate/ 50 mM sodium chloride/ 2 mM EDTA, pH 6.5 with 7.5 molar equivalents of SMCC (20 mM in DMSO, 6.7 mg/mL). After stirring for 2 hours under argon at ambient temperature, the reaction mixture is filtered through a Sephadex G25 column equilibrated 20 with 50mM potassium phosphate/ 50 mM sodium chloride/ 2 mM EDTA, pH 6.5. Antibody containing fractions are pooled and assayed.

25 Antibody-SMCC prepared thus is diluted with 50mM potassium phosphate/50 mM sodium chloride/2 mM EDTA, pH 6.5, to a final concentration of about 10 mg/ml, and reacted with a 10 mM solution of DM1 in dimethylacetamide. The reaction is stirred at ambient temperature under argon 16.5 hours. The conjugation reaction mixture is filtered through a Sephadex G25 gel filtration column (1.5 x 4.9 cm) with 1 x PBS at pH 6.5. The DM1 drug to antibody ratio (p) may be about 2 to 5, as measured by the absorbance at 252 nm and at 280 nm.

30 Ab-SPP-DM1 may be prepared by conjugation of any of the antibodies provided herein with SPP-DM1 as follows. Purified antibody is derivatized with N-succinimidyl-4-(2-pyridylthio)pentanoate to introduce dithiopyridyl groups. Antibody (376.0 mg, 8 mg/mL) in 44.7 mL of 50 mM potassium phosphate buffer (pH 6.5) containing NaCl (50 mM) and EDTA (1 mM) is treated with SPP (5.3 molar equivalents in 2.3 mL ethanol). After incubation for 90 minutes under argon at ambient temperature, the reaction mixture is gel filtered through a Sephadex G25 column equilibrated with 35 mM sodium citrate, 154 mM NaCl, 2 mM EDTA. Antibody containing fractions were pooled and assayed. The degree of modification of the antibody is 35 determined as described above.

35 Antibody-SPP-Py (about 10  $\mu$ moles of releasable 2-thiopyridine groups) is diluted with the above 35 mM sodium citrate buffer, pH 6.5, to a final concentration of about 2.5 mg/mL. DM1 (1.7 equivalents, 17  $\mu$ moles) in 3.0 mM dimethylacetamide (DMA, 3% v/v in the final reaction mixture) is then added to the antibody solution. The reaction proceeds at ambient temperature under argon for about 20 hours. The reaction 40 is loaded on a Sephadex S300 gel filtration column (5.0 cm x 90.0 cm, 1.77 L) equilibrated with 35 mM

sodium citrate, 154 mM NaCl, pH 6.5. The flow rate may be about 5.0 mL/min and 65 fractions (20.0 mL each) are collected. The number of DM1 drug molecules linked per antibody molecule (p') is determined by measuring the absorbance at 252 nm and 280 nm, and may be about 2 to 4 DM1 drug moieties per antibody.

Antibody-BMPEO-DM1 may be prepared by conjugation of any of the antibodies provided herein 5 with BMPEO-DM1 as follows. The antibody is modified by the bis-maleimido reagent BM(PEO)4 (Pierce Chemical), leaving an unreacted maleimido group on the surface of the antibody. This may be accomplished by dissolving BM(PEO)4 in a 50% ethanol/water mixture to a concentration of 10 mM and adding a tenfold molar excess to a solution containing antibody in phosphate buffered saline at a concentration of approximately 1.6 mg/ml (10 micromolar) and allowing it to react for 1 hour to form antibody-linker 10 intermediate, antibody-BMPEO. Excess BM(PEO)4 is removed by gel filtration (HiTrap column, Pharmacia) in 30 mM citrate, pH 6 with 150 mM NaCl buffer. An approximate 10 fold molar excess DM1 is dissolved in dimethyl acetamide (DMA) and added to the antibody-BMPEO intermediate. Dimethyl formamide (DMF) may also be employed to dissolve the drug moiety reagent. The reaction mixture is allowed to react overnight 15 before gel filtration or dialysis into PBS to remove unreacted DM1. Gel filtration on S200 columns in PBS is used to remove high molecular weight aggregates and furnish purified antibody-BMPEO-DM1.

#### ***Pharmaceutical Formulations***

Therapeutic formulations comprising an antibody of the invention are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or 20 stabilizers (*Remington: The Science and Practice of Pharmacy* 20th edition (2000)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecylmethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; 25 catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter- 30 ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN<sup>TM</sup>, PLURONICS<sup>TM</sup> or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose 35 intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions.

40 Such techniques are disclosed in *Remington: The Science and Practice of Pharmacy* 20th edition (2000).

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

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

#### *Uses*

An antibody of the present invention may be used in, for example, *in vitro*, *ex vivo* and *in vivo* therapeutic methods.

In one aspect, the invention provides methods for treating or preventing a tumor, a cancer, and/or a cell proliferative disorder associated with increased expression and/or activity of EphB2, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate to a subject in need of such treatment.

In one aspect, the invention provides methods for killing a tumor cell, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate (in some embodiments, an anti-EphB2 antibody or immunoconjugate of the invention) to a subject in need of such treatment.

In one aspect, the invention provides methods for reducing, inhibiting, or preventing growth of a tumor or cancer, the methods comprising administering an effective amount of an anti-EphB2 antibody or immunoconjugate to a subject in need of such treatment.

Moreover, at least some of the antibodies of the invention can bind antigen from other species. Accordingly, the antibodies of the invention can be used to bind specific antigen activity, *e.g.*, in a cell culture containing the antigen, in human subjects or in other mammalian subjects having the antigen with which an antibody of the invention cross-reacts (*e.g.* chimpanzee, baboon, marmoset, cynomolgus and rhesus, pig or mouse). In one embodiment, the antibody of the invention can be used for inhibiting antigen activities by contacting the antibody with the antigen such that antigen activity is inhibited. Preferably, the antigen is a human protein molecule.

In one embodiment, an antibody of the invention can be used in a method for binding an antigen in a subject suffering from a disorder associated with increased antigen expression and/or activity, comprising

administering to the subject an antibody of the invention such that the antigen in the subject is bound. Preferably, the antigen is a human protein molecule and the subject is a human subject. Alternatively, the subject can be a mammal expressing the antigen with which an antibody of the invention binds. Still further the subject can be a mammal into which the antigen has been introduced (e.g., by administration of the antigen 5 or by expression of an antigen transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes. Moreover, an antibody of the invention can be administered to a non-human mammal expressing an antigen with which the immunoglobulin cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time 10 courses of administration).

The antibodies of the invention can be used to treat, inhibit, delay progression of, prevent/delay recurrence of, ameliorate, or prevent diseases, disorders or conditions associated with expression and/or activity of one or more antigen molecules.

Exemplary disorders include carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid 15 malignancies. More particular examples of such cancers include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon 20 cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain, as well as head and neck cancer, and associated metastases. In some embodiments, the cancer is selected from the group consisting of small cell lung cancer, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and 25 hepatocellular carcinoma. In some embodiments, the cancer is colorectal cancer.

The antibodies of the invention are also useful in the treatment (including prevention) of disorders the pathology of which involves cellular degeneration or dysfunction, such as treatment of various (chronic) neurodegenerative disorders and acute nerve cell injuries. Such neurodegenerative disorders include, without limitation, peripheral neuropathies; motoneuron disorders, such as amyotrophic lateral sclerosis (ALS, Lou 30 Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy or paralysis; and other human neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease, and acute nerve cell injuries, for example due to trauma or spinal cord injury.

The antibodies of the invention are also useful for inhibiting angiogenesis. In some embodiments, the 35 site of angiogenesis is a tumor or cancer.

In some embodiments, the disorder is characterized by colon adenoma(s). The methods of the invention are particularly suitable for disorders characterized by a plurality of colon adenomas (such as more than 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, or more colon adenomas), including at least the following: autosomal dominant familial adenomatous polyposis (FAP) disorder caused by 40 mutation in the APC gene (Tomlinson et al., J Med Genet 1996;33:268-73); Peutz-Jegher's syndrome (PJS),

Juvenile Polyposis Syndrome (JPS), Attenuated FAP caused by mutations in the MYH gene (Sieber et al. N Eng J Med 348:791-9 (2003)), Hereditary Mixed Polyposis syndrome (HMPS), Cowden disease, and Bannayan-Ruvalcaba-Riley syndrome.

EphB2 has been implicated in motoaxon guidance and neural crest cell migration in the developing embryo. Accordingly, the antibodies of the invention are also useful for modulating axon guidance, neuronal development, and/or neural crest migration *in vitro* or *in vivo*.

In certain embodiments, an immunoconjugate comprising an antibody conjugated with one or more cytotoxic agent(s) is administered to the patient. In some embodiments, the immunoconjugate and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the target cell to which it binds. In one embodiment, the cytotoxic agent targets or interferes with nucleic acid in the target cell. In one embodiment, the cytotoxic agent targets or interferes with microtubule polymerization. Examples of such cytotoxic agents include any of the chemotherapeutic agents noted herein (such as a maytansinoid, auristatin, dolastatin, or a calicheamicin), a radioactive isotope, or a ribonuclease or a DNA endonuclease.

Antibodies and immunoconjugates of the invention can be used either alone or in combination with other compositions in a therapy. For instance, an antibody of the invention may be co-administered with another antibody, chemotherapeutic agent(s) (including cocktails of chemotherapeutic agents), other cytotoxic agent(s), anti-angiogenic agent(s), cytokines, and/or growth inhibitory agent(s). Where an antibody of the invention inhibits tumor growth, it may be particularly desirable to combine it with one or more other therapeutic agent(s) which also inhibits tumor growth. For instance, anti-VEGF antibodies blocking VEGF activities may be combined with anti-ErbB antibodies (e.g. HERCEPTIN® anti-HER2 antibody) in a treatment of metastatic breast cancer. Alternatively, or additionally, the patient may receive combined radiation therapy (e.g. external beam irradiation or therapy with a radioactive labeled agent, such as an antibody). Such combined therapies noted above include combined administration (where the two or more agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, and/or following, administration of the adjunct therapy or therapies.

The antibody of the invention (and adjunct therapeutic agent) is/are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

The antibody composition of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such

other agents depends on the amount of antibodies of the invention present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention  
5 (when used alone or in combination with other agents such as chemotherapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease,  
10 about 1  $\mu$ g/kg to 15 mg/kg (e.g. 0.1mg/kg-10mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1  $\mu$ g/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. One exemplary  
15 dosage of the antibody would be in the range from about 0.05mg/kg to about 10mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg or 10mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen  
20 comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The anti-EphB2 antibodies of the invention are useful in assays detecting EphB2 expression (such as diagnostic or prognostic assays) in specific cells or tissues wherein the antibodies are labeled as described  
25 below and/or are immobilized on an insoluble matrix.

In another aspect, the invention provides methods for detection of EphB2, the methods comprising detecting EphB2-anti-EphB2 antibody complex in the sample. The term "detection" as used herein includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control.

In another aspect, the invention provides methods for diagnosing a disorder associated with EphB2  
30 expression and/or activity, the methods comprising detecting EphB2-anti-EphB2 antibody complex in a biological sample from a patient having or suspected of having the disorder. In some embodiments, the EphB2 expression is increased expression or abnormal (undesired) expression. In some embodiments, the disorder is a tumor, cancer, and/or a cell proliferative disorder.

In another aspect, the invention provides methods for evaluation (prognostic evaluation) of a patient  
35 having or suspected of having cancer, the method comprising: (a) obtaining a biological sample from the patient; (b) detecting EphB2 expression in the biological sample; (c) comparing EphB2 expression in the biological sample with expression of EphB2 in a control sample (control reference value); and (d) predicting cancer prognosis of the patient based on the comparison in (a), wherein increased EphB2 expression in the patient biological sample relative to the control sample is prognostic for cancer in the patient. Increased  
40 EphB2 expression is prognostic for cancer. See Jubb et al, U.S. Patent Application No. \_\_, filed Jan. 6, 2005.

Thus, determining cancer prognosis can provide for convenient, efficient, and potentially cost-effective means to obtain data and information useful in assessing future course of the disorder, including selection of appropriate therapies for treating patients.

5 In another aspect, the invention provides any of the anti-EphB2 antibodies described herein, wherein the anti-EphB2 antibody comprises a detectable label.

In another aspect, the invention provides a complex of any of the anti-EphB2 antibodies described herein and EphB2. In some embodiments, the complex is *in vivo* or *in vitro*. In some embodiments, the complex comprises a cancer cell. In some embodiments, the anti-EphB2 antibody is detectably labeled.

10 Anti- EphB2 antibodies can be used for the detection of EphB2 in any one of a number of well known detection assay methods. For example, a biological sample may be assayed for EphB2 by obtaining the sample from a desired source, admixing the sample with anti- EphB2 antibody to allow the antibody to form antibody/ EphB2 complex with any EphB2 present in the mixture, and detecting any antibody/ EphB2 complex present in the mixture. The biological sample may be prepared for assay by methods known in the art which are suitable for the particular sample. The methods of admixing the sample with antibodies and the methods of 15 detecting antibody/ EphB2 complex are chosen according to the type of assay used. Such assays include immunohistochemistry, competitive and sandwich assays, and steric inhibition assays.

Analytical methods for EphB2 all use one or more of the following reagents: labeled EphB2 analogue, immobilized EphB2 analogue, labeled anti- EphB2 antibody, immobilized anti- EphB2 antibody and steric conjugates. The labeled reagents also are known as "tracers."

20 The label used is any detectable functionality that does not interfere with the binding of EphB2 and anti- EphB2 antibody. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include: The label used is any detectable functionality that does not interfere with the binding of EphB2 and anti- 25 EphB2 antibody. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and 30 bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydropthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, 35 and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter *et al.*, *Nature*, **144**: 945 (1962); David *et al.*, *Biochemistry*, **13**: 1014-1021 (1974); Pain *et*

al., *J. Immunol. Methods*, **40**: 219-230 (1981); and Nygren, *J. Histochem. and Cytochem.*, **30**: 407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase. The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan *et al.*, "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in Enzymology*, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166.

5 Immobilization of reagents is required for certain assay methods. Immobilization entails separating the anti- EphB2 antibody from any EphB2 that remains free in solution. This conventionally is accomplished by either insolubilizing the anti- EphB2 antibody or EphB2 analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*, U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the anti-EphB2 antibody or EphB2 analogue afterward, e.g., by immunoprecipitation.

10 The expression of proteins in a sample may be examined using immunohistochemistry and staining protocols. Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing or detecting presence of proteins in a sample. Immunohistochemistry ("IHC") techniques utilize an antibody to probe and visualize cellular antigens *in situ*, generally by chromogenic or fluorescent methods. For sample preparation, a tissue or cell sample from a mammal (typically a human patient) may be used. Examples of samples include, but are not limited to, cancer cells such as colon, breast, prostate, ovary, lung, stomach, pancreas, lymphoma, and leukemia cancer cells. The sample can be obtained by a variety of 15 procedures known in the art including, but not limited to surgical excision, aspiration or biopsy. The tissue may be fresh or frozen. In one embodiment, the sample is fixed and embedded in paraffin or the like. The tissue sample may be fixed (*i.e.* preserved) by conventional methodology. One of ordinary skill in the art will appreciate that the choice of a fixative is determined by the purpose for which the sample is to be histologically stained or otherwise analyzed. One of ordinary skill in the art will also appreciate that the length of fixation 20 depends upon the size of the tissue sample and the fixative used.

25 IHC may be performed in combination with additional techniques such as morphological staining and/or fluorescence *in-situ* hybridization. Two general methods of IHC are available; direct and indirect assays. According to the first assay, binding of antibody to the target antigen (e.g., EphB2) is determined directly. This direct assay uses a labeled reagent, such as a fluorescent tag or an enzyme-labeled primary antibody, which can be visualized without further antibody interaction. In a typical indirect assay, 30 unconjugated primary antibody binds to the antigen and then a labeled secondary antibody binds to the primary antibody. Where the secondary antibody is conjugated to an enzymatic label, a chromogenic or fluorogenic substrate is added to provide visualization of the antigen. Signal amplification occurs because several secondary antibodies may react with different epitopes on the primary antibody.

35 The primary and/or secondary antibody used for immunohistochemistry typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

Aside from the sample preparation procedures discussed above, further treatment of the tissue section prior to, during or following IHC may be desired, For example, epitope retrieval methods, such as heating the

tissue sample in citrate buffer may be carried out (see, e.g., Leong *et al. Appl. Immunohistochem.* 4(3):201 (1996)).

Following an optional blocking step, the tissue section is exposed to primary antibody for a sufficient period of time and under suitable conditions such that the primary antibody binds to the target protein antigen in the tissue sample. Appropriate conditions for achieving this can be determined by routine experimentation. The extent of binding of antibody to the sample is determined by using any one of the detectable labels discussed above. Preferably, the label is an enzymatic label (e.g. HRPO) which catalyzes a chemical alteration of the chromogenic substrate such as 3,3'-diaminobenzidine chromogen. Preferably the enzymatic label is conjugated to antibody which binds specifically to the primary antibody (e.g. the primary antibody is rabbit polyclonal antibody and secondary antibody is goat anti-rabbit antibody).

Specimens thus prepared may be mounted and coverslipped. Slide evaluation is then determined, e.g. using a microscope, and staining intensity criteria, routinely used in the art, may be employed. Staining intensity criteria may be evaluated as follows:

15

TABLE 2

Staining Pattern	Score
No staining is observed in cells.	0
Faint/barely perceptible staining is detected in more than 10% of the cells.	1+
Weak to moderate staining is observed in more than 10% of the cells.	2+
Moderate to strong staining is observed in more than 10% of the cells.	3+

Typically, a staining pattern score of about 2+ or higher in an IHC assay is diagnostic and/or prognostic. In some embodiments, a staining pattern score of about 1+ or higher is diagnostic and/or prognostic. In other embodiments, a staining pattern score of about 3 or higher is diagnostic and/or prognostic. It is understood that when cells and/or tissue from a tumor or colon adenoma are examined using IHC, staining is generally determined or assessed in tumor cell and/or tissue (as opposed to stromal or surrounding tissue that may be present in the sample).

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer EphB2 analogue to compete with the test sample EphB2 for a limited number of anti- EphB2 antibody antigen-binding sites. The anti- EphB2 antibody generally is insolubilized before or after the competition and then the tracer and EphB2 bound to the anti-EphB2 antibody are separated from the unbound tracer and EphB2. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample EphB2 is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of EphB2 are prepared and compared with the test results to quantitatively determine the amount of

EphB2 present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the EphB2 is prepared and used such that when anti- EphB2 antibody binds to the EphB2 the presence of the anti-EphB2 antibody modifies the enzyme activity. In this case, the EphB2 or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with anti- EphB2 antibody so that binding of the anti- EphB2 antibody inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

10 Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small EphB2 fragment so that antibody to hapten is substantially unable to bind the conjugate at the same time as anti- EphB2 antibody. Under this assay procedure the EphB2 present in the test sample will bind anti-EphB2 antibody, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in 15 fluorescence when the hapten is a fluorophore.

20 Sandwich assays particularly are useful for the determination of EphB2 or anti- EphB2 antibodies. In sequential sandwich assays an immobilized anti- EphB2 antibody is used to adsorb test sample EphB2, the test sample is removed as by washing, the bound EphB2 is used to adsorb a second, labeled anti- EphB2 antibody and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional 25 to test sample EphB2. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled anti- EphB2. A sequential sandwich assay using an anti- EphB2 monoclonal antibody as one antibody and a polyclonal anti- EphB2 antibody as the other is useful in testing samples for EphB2.

The foregoing are merely exemplary detection assays for EphB2. Other methods now or hereafter developed that use anti- EphB2 antibody for the determination of EphB2 are included within the scope hereof, 25 including the bioassays described herein.

#### *Articles of Manufacture*

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of 30 manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or when combined with another composition(s) effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper 35 pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice, such as cancer. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic 40 agent. The article of manufacture in this embodiment of the invention may further comprise a package insert

indicating that the first and second antibody compositions can be used to treat a particular condition, e.g. cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials 5 desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

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

10

## EXAMPLES

The following materials and methods were used in the Examples.

*Antibodies and Recombinant Proteins.*

Monoclonal anti-phosphotyrosine conjugated with horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-epidermal growth factor (EGF), EGF, phospho-p44/42 15 MAP kinase, and p44/42 MAP kinase were purchased from Cell Signaling Technology (Beverly, MA). Antibody to the GD epitope and the fusion proteins ephrin-B1-Fc and ephrin-B2-Fc were produced at Genentech (South San Francisco, CA).

*Cell Lines and Plasmids.*

The human colon adenocarcinoma cell lines SW480, SW620, and Colo 205 and the fibrosarcoma cell line 20 HT1080 were obtained from the American Type Culture Collection (Manassas, VA). The HT1080-EphB2 and HT1080-GD cell lines were generated by cotransfection with a SV40-driven vector encoding an NH2-terminal GD epitope-tagged form of EphB2 or empty vector, respectively, and with a cytomegalovirus promoter-driven puromycin vector. Cells were selected in 1  $\mu$ g/ml puromycin. The SVT2-EphB2 cell line was established in the same fashion, except that mouse 3T3 cells were cotransfected with a cytomegalovirus promoter driving Neo, and 25 cells were selected in Geneticin (Life Technologies, Inc.) at 400  $\mu$ g/ml. Cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin (100 units/ml).

EphB2 ligand binding domain construct (ss.EphB2LBD.gD.GPI) was prepared by cloning a polynucleotide 30 encoding amino acids 19 to 208 of human EphB2 into a vector encoding a COOH-terminal GPI linker and an NH2-terminal GD epitope tag (ss.his.gD.GPI). This vector was transiently transfected into 293 cells. Cells were grown in F12 DMEM Mix (50:50) media with 10% FBS. The transfected cells were subjected to further analysis 48 hours following transfection.

*RNA Expression Analysis.*

For the analysis of tumor and normal colon tissue specimens (Figure 3A), approximately 10  $\mu$ g of total RNA from each human tumor or normal colon tissue sample served as starting material for the 35 preparation of probes required for oligonucleotide array analysis on the Affymetrix GeneChip. Probes were prepared according to the manufacturer's recommendations. After hybridization, the arrays were washed and stained with streptavidin-phycoerythrin and then scanned with the Gene Array scanner (Agilent Technologies). Default parameters provided in the Affymetrix data analysis software package were applied in determining the signal intensities, referred to as average differences. Sample normalization was done using global scaling as

stated in the Affymetrix Expression Analysis Technical Manual, and a target intensity of 1500 was used to determine average difference expression values. For the analysis of EphB2 mRNA expression in multiple human tumor and normal biopsy samples, the Affymetrix data were obtained from Gene Logic, Inc.

(Gaithersburg, MD). In the analysis shown, there are a total of 4841 samples (1808 normal samples; 1545

5 cancer samples; and 1488 non-cancer diseased samples). Gene Logic data were also normalized using global scaling, but in this instance, the target intensity was 100. The Affymetrix data for EphB2 mRNA expression was generated from the U95 probe set ID 41678\_at. Real-time PCR (TaqMan; Perkin-Elmer, Applied Biosystems) for EphB2 mRNA was performed using gene-specific primers (5'-CGA-GCC-ACG-TTA-CAT-CA-3' (SEQ ID NO:10) and 5'-TCA-GTA-ACG-CCG-TTC-ACA-GC-3' (SEQ ID NO:11)) and probe (5'-  
10 CCC-ACA-CCC-AGT-ACA-CCT-TCG-AGA-TCC-3' (SEQ ID NO:12)). For *in situ* hybridization, a 458-bp <sup>33</sup>P-labeled antisense riboprobe was generated from an EphB2 PCR product using a primer with oligonucleotide sequence 5'-TCTGTCCATCTGTCCCCGTCCT-3' (SEQ ID NO:13) and a sense control riboprobe with the primer 5'-GCCCTCCTGGTGCTCTATCC-3' (SEQ ID NO:14).

*Monoclonal Anti-EphB2 Antibodies.*

15 BALB/c mice (Charles River Laboratories, Wilmington, DE) were immunized with baculovirus-derived His8-tagged EphB2 receptor diluted in Ribi adjuvant (Corixia, Hamilton, MT) twice a week, via footpad, five doses. B cells from lymph nodes were harvested from five mice demonstrating high serum titers and were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection). After 10–14 days, the supernatants were screened for antibody production by direct ELISA and by flow cytometry on  
20 HT1080-GD and HT1080-EphBR cells. Positives were subcloned twice to achieve monoclonality. For large-scale production of purified antibody, hybridoma cells were injected i.p. into pristine-primed BALB/c mice. The ascites fluids were pooled and purified by protein A affinity chromatography (Pharmacia Fast Protein Liquid Chromatography; Pharmacia, Uppsala, Sweden).

*Determination of 2H9 Fv sequences*

25 Total RNA was extracted from hybridoma cells producing the mouse anti human EphB2 monoclonal antibody 2H9, using RNeasy Mini Kit (Qiagen, Germany). The variable light (VL) and variable heavy (VH) domains were amplified using RT-PCR with the following degenerate primers: Light chain (LC) forward: 5'-GATCGATATCGTGATGACMCAGTCTCCATC-3' (SEQ ID NO:15)

Light chain reverse: 5'-TTTDAKYTCCAGCTTGGTACC-3' (SEQ ID NO:16)

30 Heavy chain (HC) forward: 5'-GATCCGTACGCTCAGGTYCARCTSCAGCAGTCTGG-3' (SEQ ID NO:17)

Heavy chain reverse: 5'-ACAGTGGGCCCTGGTGGAGGCTGMRGAGACDGTGASHRDRGT-3' (SEQ ID NO:17)

35 The forward primers were specific for the N-terminal amino acid sequence of the VL and VH region. Respectively, the LC and HC reverse primers were designed to anneal to a region in the constant light (CL) and constant heavy domain 1 (CH1), which is highly conserved across species. Amplified VL was cloned into a pRK mammalian cell expression vector (Shields et al. J Biol Chem (2000) 276:659-604). Amplified VH was inserted to a pRK mammalian cell expression vector. The polynucleotide sequence of the inserts was determined using routine sequencing methods.

40 *Analysis of EphB2 activation*

The analysis of EphB2 activation by soluble Ephrin-Fc ligand was performed by stimulating the SVT2-EphB2 cell line with purified Fc-EphrinB2 (5  $\mu$ g/ml, 15min) and detecting EphB2 autophosphorylation using the following protocol. Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% deoxycholate, 1% NP40, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 complete proteinase inhibitor mixture (Roche Molecular Biochemicals)). Ten  $\mu$ g of anti-GD MAb were added to the lysates, followed by protein G-agarose (Life Technologies, Inc.), and incubated overnight at 4°C. The immunoprecipitates were recovered, washed with lysis buffer, and subjected to SDS-PAGE and immunoblotting. Blots were incubated with 1  $\mu$ g/ml anti-phosphotyrosine mouse MAb conjugated with horseradish peroxidase (Santa Cruz Biotechnology) or anti-GD mouse MAb. Goat antimouse horseradish peroxidase was used as a secondary antibody, and the blots were washed and developed using the Enhanced Chemiluminescence system (Pierce). Analysis of MAP kinase activation was carried out using the HT1080-EphB2 cell line. Cells were serum starved for 12 h and either left untreated or stimulated with EGF 100 ng/ml in the absence or presence of 5  $\mu$ g human Fc-EphrinB2/ml. Cell lysates were equalized for protein concentration, subjected to SDS-PAGE, and immunoblotted with anti-phospho-EGF receptor or phospho-MAP kinase antibody.

*Flow cytometry*

For flow cytometry, cells were grown to 90% confluence and removed from plates using Cell Dissociation Buffer (Invitrogen). Cells were washed and resuspended in fluorescence-activated cell-sorting buffer (PBS with 1% BSA) and incubated for 45 min with anti-EphB2 MAb 2H9 or anti-GD antibody (Genentech) followed by 30-min incubation with antimouse secondary antibody conjugated to phycoerythrin. Analysis was performed on FACSScan.

*Binding affinity, ELISA, and isotype testing*

Binding affinity of Mab 2H9 was determined by surface plasmon resonance using Pharmacia BIAcore® 3000 (BIAcore AB, Uppsala, Sweden) at room temperature (see, e.g., Morton et al. (1998) Methods in Enzymology, 295, 268-294). Anti-EphB2 antibodies were immobilized to the sensor chip (CM5) through primary amine groups. The carboxymethylated sensor chip surface matrix was activated by injecting 20  $\mu$ l of a mixture of 0.025 M N-hydroxysuccinimide and 0.1 M N-ethyl-N'(dimethylaminopropyl) carbodiimide at 5  $\mu$ l/min. 5-10  $\mu$ l of 10  $\mu$ g/ml solution of anti-EphB2 antibodies in 10 mM sodium acetate, pH 4.5, were injected at 5  $\mu$ l/min. After coupling, unoccupied sites on the chip were blocked by injecting 20  $\mu$ l of 1M ethanalamine, pH 8.5. The running buffer was PBS containing 0.05% polysorbate 20. For kinetic measurements, two-fold serial dilutions of polyhis-tagged EphB2 in running buffer were injected over the flow cells for 3 minutes at a flow rate of 30  $\mu$ l/min and the bound polyhis tagged EphB2 was allowed to dissociate for 20 minutes. The binding surface was regenerated by injecting 20  $\mu$ l of 10 mM glycine-HCl (pH 1.5). Flow cell one, which was activated but did not have antibody immobilized, was used as a reference cell. There was no significant non-specific binding of polyhis tagged EphB2 to flow cell one. For calculate apparent binding affinity, data were analyzed using a 1:1 binding model using global fitting. The association and dissociation rate constants were fitted simultaneously (BIAevaluation software).

Mab 2H9 was determined to bind human and mouse EphB2 polypeptide using ELISA according to the following protocol. Microtiter plates (Nunc, Roskilde, Sweden), were coated with 100  $\mu$ l/well of baculovirus-derived His8-tagged EphB2 receptor at 1  $\mu$ g/ml in 0.05 M carbonate buffer, pH 9.6, overnight at

4°C. Plates were washed with PBS/0.05%T20 and blocked with PBS bovine serum albumin (BSA)/T20. Hybridoma supernatants were added (100 µl/well), incubated for 1 h at room temperature with agitation. Following the wash step, bound antibodies were detected with goat anti-mouse IgG (Fc specific) peroxidase conjugate (Sigma, St. Louis, MO; diluted 1:10 K in PBS/BSA/T20 buffer; incubated for 1 h at RT). The plates were developed with TMB substrate solution (KPL/Kirkegaard & Perry Laboratories, Gaithersburg, MD) and reaction was stopped with TMB 1-component stop solution (KPL). Plates were read at absorbance of 450nm, with reference at 630 nm using an automated plate reader.

Reactivity of Mab 2H9 with related Eph receptor EphB3 was tested as follows. 293 cells expressing EphB3 were generated by transient transfection with a vector containing EphB3 ECD sequences, an NH2-terminal GD tag and a COOH-terminal GPI linker. After 48 hours, Mab 2H9 was incubated with the cells, then, following washing, the cell were subjected to FACS analysis as described above. Mab 2H9 showed very low cross-binding with EphB3.

Mab 2H9 was determined to be isotype IgG1, as determined using standard methods.

*Antibody Binding and internalization, immunohistochemistry.*

Purified MAB 2H9 was iodinated using the lactoperoxidase method, and the radiolabeled antibody was purified from free <sup>125</sup>I-Na by gel filtration chromatography using a Pharmacia PD-10 column. Assessment of internalization was carried out essentially as described previously (Gladhaug I. P. et al., J. Biol. Chem., 263: 12199-12203, 1988). Cells were incubated with iodinated antibody on ice and then shifted to 37°C for 4 h, followed by an acid/salt/urea incubation at 4°C to dissociate surface-bound ligand. Total surface-bound and internalized antibody was determined by scintillation counting. Internalization of EphB2 was also assessed by immunofluorescence staining of cells.

Immunohistochemical staining of human colon tumor sections with anti-EphB2 antibody was performed on frozen tissue sections. Sections containing malignant epithelial cells of a colorectal adenocarcinoma were incubated with primary antibody 2H9 at a concentration of 5 µg/ml, followed a biotinylated horse antimouse IgG affinity-purified antiserum. As control, an adjacent section was incubated with an irrelevant primary antibody and counterstained with hematoxylin.

Slides containing HT1080-EphB2 cells were incubated with 1 µg/ml 2H9 antibody for 30 min on ice and then shifted to 37°C in a CO2 incubator for 1 h. The slides were washed in PBS, fixed in 3% paraformaldehyde, and incubated with rhodamine-conjugated antimouse IgG antibody (Jackson 30 Immunoresearch Laboratories) at a 1:200 dilution for 20 min at room temperature. The number of cell surface MAb 2H9 binding sites was estimated by incubating cells for 4 h on ice with a fixed concentration of <sup>125</sup>I-labeled MAb 2H9 combined with increasing concentrations of unlabeled MAb 2H9, essentially as described previously (Holmes W. et al., Science, 256: 1205-1210, 1992).

*Determination of EphB2 ligand binding domain and mapping of 2H9 binding determinant*

To determine EphB2's ligand binding domain, 48 hours following transient transfection, 1 µl EphB2 ligand (ephrinB1-IgG at 91ng/µl) was added to ss.EphB2LBD.gD.GPI-293 cells, and cells were kept on ice for 20 minutes. Following two washes, cells were incubated with FITC-conjugated Fc specific anti-human IgG antibody (Sigma catalog no. F9512) on ice for 30 minutes. Following two more washed, cells were subjected to FACS analysis as described above. The results demonstrated that EphB2 ligand EprinB1 bound EphB2

amino acids 19-208, thus defining EphB2 amino acids 19-208 as containing the EphB2 ligand binding domain. No binding was observed using cells that expressed a control vector.

To determine reactivity of Mab 2H9 with amino acid numbers 19-208 of EphB2, Mab 2H9 (1 $\mu$ g/ml) or anti-GD antibody (4 $\mu$ g/ml) were incubated with ss.EphB2LBD.gD.GPI-293 cells, then, following washing, 5 cells were subjected to FACS analysis as described above. The results demonstrated that GD protein and EphB2 ligand binding domain protein was detected by these antibodies. These results indicated that Mab 2H9 bound the EphB2 ligand binding domain. No 2H9 or anti-GD antibody binding was observed using cells that expressed a control vector.

*Preparation of Anti EphB2 Mab 2H9 immunoconjugates*

10 The conjugation of the anti-EphB2 antibody 2H9 and control anti-interleukin (IL)-8 antibody with MC-vc-PAB-MMAE and the conjugation of 2H9 with MC-vc-PAB-MMAF were performed as described elsewhere (Doronina S. O. et al. Nat. Biotechnol., 21: 778-784, 2003; US Ser. No. 10/983,340, filed Nov. 5, 2004). The conjugation of 2H9 with MC-MMAE and MC-MMAF was performed as described in US Ser. No. 10/983,340, filed Nov. 5, 2004, following the protocol provided in Doronina, supra. The conjugation of 2H9 15 with SMCC-DM1 and SPP-DM1 was performed as described in U.S. Ser. No. 10/960,602, filed Oct. 8, 2004.

*In Vitro Tumor Cell Killing Assays.*

The HT1080-EphB2 cell line or the vector control cell line were added to each well of 96-well microtiter plates at 1.5 x 10<sup>3</sup> cells/well, 100  $\mu$ l/well, and incubated overnight at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were exposed to various concentrations of MAb 2H9-MC-vc-PAB-MMAE or 20 Mab anti-IL-8-MC-vc-PAB-MMAE based on 1:3 serial dilutions. After incubation for 48 h, Cell Titer-Glo reagent (Promega, Madison, WI) was added to the wells at 100  $\mu$ l/well, and after a 10-min incubation at room temperature, the luminescent signal was recorded.

25 In a separate experiment, HT1080-EphB2 cells were exposed to various concentrations of 2H9-SPP-Dm1, 2H9-SMCC-DM1, 2H9-MC-vc-PAB-MMAE, 2H9-MC-vc-PAB-MMAF or anti-interleukin-8-MC-vc-PAB-MMAE, essentially as described above, and cell viability was measured after 2 days as described above.

*In Vivo Tumor Growth Assays.*

Female nude mice (Charles River Laboratories, Hollister, CA) were maintained in accordance with the guide for the care and use of laboratory animals. HT1080-EphB2 and HT1080-GD cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks (1 x 10<sup>6</sup> cells/flank), respectively, of 6-8-week-old mice. When tumors reached approximately 100 mm<sup>3</sup>, animals were dosed i.p. with 0.2 ml of native EphB2 MAb or 2H9-MC-vc-PAB-MMAE or Mab anti-IL-8-MC-vc-PAB-MMAE once a week i.v. at a final dose of 3 mg/kg body weight. The tumor volumes were determined by measuring the length (l) and width (w) and calculating the volume (V = lw<sup>2</sup>/2) as described previously.

30 Assays with the CXF1103 tumor line were performed by Oncotest GmbH (Feiburg, Germany). See U.S. Pat. No. 6,271,342. Affymetrix oligonucleotide array analysis was performed on tumors from the Oncotest collection, which demonstrated expression of EphB2 mRNA in CXF1103. This was confirmed by real-time PCR and immunohistochemistry. CXF1103 is a human colon tumor established by serial passage in nude mice. Groups of 10 nude mice of NMRI background received s.c. tumor implants to obtain 30 mice bearing tumors of similar sizes for randomization. Tumors were grown to an average size of 100-200 mg, 35 whereupon treatment with vehicle control, control antibody conjugate anti-GD-MC-vc-PAB-MMAE, or anti-40

EphB2 antibody conjugate 2H9-MC-vc-PAB-MMAE was initiated by i.v. injection. Antibody conjugates were administered at 3 mg/kg body weight at 7-day intervals for 3 weeks.

## RESULTS

### Example 1: analysis of EphB2 mRNA and protein expression in cancer and normal human tissues

5       EphB2 mRNA and protein are overexpressed in cancer tissues. Oligonucleotide-based microarray expression analysis was performed on 38 human colorectal tumors and 7 normal colon biopsy samples. Data mining revealed that EphB2 was overexpressed by 2-6-fold in the majority of the tumors relative to the average expression level of the normal samples (Figure 3A). To confirm the overexpression in colon cancers, real-time PCR (TaqMan) was performed on 11 additional human colorectal tumors, each referenced to a  
10 patient-matched normal colon sample. These data were highly consistent with the microarray data (Figure 3B). On mining of a larger database, containing microarray data from over 4800 human biopsy samples, it was noted that EphB2 mRNA was preferentially expressed in intestinal tissue, with increased expression in colorectal, soft tissue cancers (such as fibrosarcoma and other sarcomas), and gastric cancers relative to numerous other tissues.

15       EphB2 mRNA expression was also examined by *in situ* hybridization on tissue microarrays. By this method, the only normal human tissues in which EphB2 protein expression was detected were the colon and small intestine. *In situ* hybridization was also carried out on a gastrointestinal tissue microarray containing samples from a variety of cancers. Here, EphB2 mRNA expression was seen in 12 of 18 primary colonic adenocarcinomas, 6 of 8 metastatic adenocarcinomas to the liver, 2 of 9 primary gastric carcinomas, and 1 of 4  
20 esophageal carcinomas. No expression was observed in four of four pancreatic adenocarcinomas. Examples of expression in normal colonic mucosa and colorectal cancer by *in situ* hybridization are shown Figure 4A.

25       Mab 2H9 reacted strongly with tissue sections obtained from human colon adenocarcinomas (Figure 4B). EphB2 protein expression has been demonstrated at all stages of colorectal tumorigenesis, including all normal crypts, and overexpression in 77% of adenomas, 82% of primary cancers, and 64% of metastases. See co-pending co-owned U.S. Patent Application No. 60/642,164, filed Jan. 6, 2005.

### Example 2: Preparation and characterization of anti-EphB2 monoclonal antibody 2H9

30       Antibodies to the extracellular sequence of EphB2 were prepared as described above, and several hybridomas were cloned that expressed MAbs reactive with the purified EphB2 immunogen and with the full-length EphB2 by flow cytometry (fluorescence-activated cell sorting) of cells expressing EphB2. MAbs from positive hybridomas were purified and compared for reactivity against colorectal cancer cell lines that endogenously express EphB2 and against cell lines engineered to overexpress the receptor (HT-1080EphB2 and SVT2EphB2). A MAb designated 2H9 performed well in these assays.

35       Elisa analysis demonstrated that Mab 2H9 bound human and mouse EphB2. Isotype analysis revealed that Mab 2H9 possessed an IgG1 isotype. Mab 2H9 binding affinity was also determined by BiaCore analysis. Mab 2H9 bound human EphB2 with  $K_a$  (1/Ms) of 8.28E+04,  $K_d$  (1/s) of 1.03E-05, and  $K_d$  (nM) of 0.12. Mab 2H9 showed very low cross-reactivity with related Eph receptor, EphB3, in a FACS-based analysis.

### Example 3: Mab 2H9 inhibits EphB2 activation by competitively inhibiting EphB2 ligand binding

40       The tyrosine kinase activity of EphB2 can be activated on binding of the receptor with ephrinB ligands (Davis S. et al. *Science*, 266: 816-819, 1994). This was observed when the murine 3T3 cell line expressing human EphB2 was incubated with a purified Fc-ephrinB2 fusion protein (Figure 5A). MAb 2H9

was tested in this assay. The tyrosine autophosphorylation of EphB2 by Fc-ephrinB2 was inhibited when the cells were preincubated with MAb 2H9, whereas a control antibody had no effect (Figure 5A). The mechanism by which MAb 2H9 inhibits EphB2 activation was investigated and found to involve competitive inhibition of ligand binding. This was determined by performing flow cytometry on cells incubated with the 5 Fc-ephrinB1 ligand after their incubation with or without MAb 2H9. In this experiment, a positive fluorescence-activated cell-sorting signal results from the specific binding of FITC-conjugated antihuman Fc antibody to the bound Fc-ephrinB1 ligand. Increasing amounts of MAb 2H9 resulted in a corresponding decrease in Fc-ephrinB1 ligand binding (Figure 5B). The loss of binding was not due to antibody-mediated receptor uptake because all incubations were performed on ice, where receptor internalization is minimal.

10 Thus, MAb 2H9 inhibited the binding of ephrin ligands to the EphB2 receptor.

To determine whether MAb 2H9 was able to affect the mitogenic or tumorigenic potential of cancer cells, growth effects of Mab 2H9 on various cell lines in vitro was determined. However, MAb 2H9 did not exhibit any specific effect on the growth of various cell lines in vitro that express the receptor (cell lines HT-1080-EphB2 and PC3-EphB2). An inhibition of cell growth might not be expected, however, because Eph 15 receptors are not considered to be mitogenic and have even been reported to interfere with mitogenic signaling as assessed by the activity of the MAP kinase pathway (Kim et al., FASEB J., 16: 1126-1128, 2002; Elowe et al., Mol. Cell. Biol., 21: 7429-7441, 2001). To assess this in a tumorigenic cell line strongly responsive to EGF, stable clones of the fibrosarcoma cell line HT1080 overexpressing EphB2 (HT1080-EphB2) were generated. The HT1080 cell line produced ephrinB1 transcript. EphrinB2 did not inhibit activation of the 20 MAP kinase pathway by EGF in these cells but rather appeared to enhance it slightly.

Example 4: Mab 2H9 is internalized after binding EphB2

Internalization of MAb 2H9 after EphB2 binding was investigated. HT1080-EphB2 cells were incubated with MAb 2H9 on ice for 30 minutes and then shifted to 37°C for 1 hour before fixation and staining with secondary antibody. Compared with cells kept on ice for the course of the experiment, the cells shifted to 25 37°C contained significant amounts of internalized antibody (Figure 6A). MAb 2H9 uptake was examined by incubating cells with <sup>125</sup>I-radiolabeled antibody at 4°C. The amount of <sup>125</sup>I-labeled MAb 2H9 that was internalized after cells were shifted to 37°C for 1 hour was approximately double that of cells maintained at 4°C (Figure 6B). MAb 2H9 was readily internalized on binding to EphB2. Thus, Mab 2H9 is suitable for immunoconjugate therapy approaches wherein the uptake or internalization of the antigen-antibody complex 30 results in preferential release of drug inside of the cancer cells.

Example 5: Mab 2H9 binds to the EphB2 ligand binding domain

The EphB2 ligand binding domain was defined by incubating ss.EphB2LBD.gD.GPI-293 cells with EprinB1-IgG, then subjecting the cells to FACS analysis. The results demonstrated that an EphB2 ligand, EphrinB1, bound EphB2 amino acids 19-208, thus defining EphB2 amino acids 19-208 (Figure 11) as 35 containing the EphB2 ligand binding domain. No binding was observed using cells that expressed a control vector.

Mab 2H9 binding to the EphB2 ligand binding domain was investigated by incubating Mab 2H9 or anti-GD antibody with ss.EphB2LBD.gD.GPI-293 cells, then subjecting the cells to FACS analysis. The result demonstrated that GD protein and EphB2 ligand binding domain was detected by these antibodies, indicating

that Mab 2H9 bound the EphB2 ligand binding domain. No 2H9 or anti-GD antibody binding was observed using cells that expressed a control vector.

Example 6: Mab 2H9 immunoconjugate effectively kills tumor cells in vitro

MAb 2H9 was covalently coupled to the drug MMAE through the linker MC-vc-PAB that is  
5 susceptible to cleavage by cathepsin B (Weiner L. M. Semin. Oncol., 26: 43-51, 1999). On cleavage by cathepsin B, active MMAE is released, disrupting the dynamics of tubulin polymerization in the cell. The 2H9-MC-vc-PAB antibody drug conjugate (immunoconjugate) was tested in vitro by treating the HT1080 cancer cells with increasing concentrations of antibody. Both the vector control HT1080 cell line (HT1080-GD) and the clonal derivative overexpressing EphB2 (HT1080-EphB2) were killed by 2H9-MC-vc-PAB-  
10 MMAE at significantly lower concentrations relative to control antibody conjugate anti-IL-8-MC-vc-PAB (Figure 7). No effect was seen with underivatized MAb 2H9 up to a concentration of 10 mg/ml. The antibody concentration required for half-maximal cell killing (IC50) was 0.006  $\mu$ g/ml for cancer cells HT1080-EphB2.

To relate cell killing to receptor copy number, quantitative binding assays were performed using MAb 2H9 on the HT1080-GD and HT1080-EphB2 cell lines. By Scatchard analysis, approximately 71,000 and  
15 308,000 copies/cell for these two lines, respectively, were estimated (Figure 8A). For both cell lines, the apparent dissociation constant for MAb 2H9 was approximately 4 nM. The difference in EphB2 copy number was also evident from the relative fluorescence intensity observed when flow cytometry was performed with MAb 2H9 on the two cell lines (Figure 8B). Also, the conjugation of MAb 2H9 with MC-vc-PAB-MMAE did not appreciably affect its binding properties to the HT1080 cells. The relative difference in receptor copy  
20 number on the two HT1080 cell lines was again evident from the binding of 2H9-MC-vc-PAB-MMAE (Figure 8C). These results indicated that target copy number is an important factor in determining the efficacy of a drug-conjugated antibody. In the case of EphB2, a moderate difference in copy number resulted in a much greater difference in the IC50 values determined in vitro.

In a separate experiment, HT1080-EphB2 cells were exposed to various concentrations of 2H9-SPP-  
25 DM1, 2H9-SMCC-DM1, 2H9-MC-vc-PAB-MMAE, 2H9-MC-vc-PAB-MMAF or anti-interleukin-8-MC-vc-PAB-MMAE essentially as described above, and cell viability was measured after 2 days as described above. HT1080-EphB2 cells were killed by 2H9-MC-vc-PAB-MMAE, 2H9-SMCC-DM1 and 2H9-SPP-DM1 at significantly lower concentrations relative to control antibody conjugate anti-IL-8-MC-vc-PAB-MMAE (Figure 9A). HT1080-EphB2 cells were killed by 2H9-MC-vc-PAB, 2H9-MC-vc-PAB-MMAF, 2H9-SPP-  
30 DM1 and 2H9-SMCC-DM1 (Figure 9B).

Example 7: Mab 2H9 antibody drug conjugate effectively kills tumor cells in vivo

2H9-MC-vc-PAB-MMAE was tested for in vivo efficacy by administration to nude mice bearing human tumor xenografts. Tumors were established by inoculating mice on one flank with the HT1080-GD cells and on the opposite flank with HT1080-EphB2 cells. Tumors were grown to a size of 100-200 mm<sup>3</sup>  
35 before i.v. administration of 2H9-MC-vc-PAB-MMAE once per week at a dose of 3 mg/kg. Additional animals were treated with vehicle control or with control antibody anti-IL-8-MC-vc-PAB-MMAE at 3 mg/kg. In this experiment, the HT1080-EphB2 cell line grew more rapidly than the HT1080-GD vector control cell line under control conditions. It is not believed that this is due to overexpression of EphB2 because previous testing of HT1080-EphB2 clones did not reveal a reproducible effect on tumor growth rate. Nevertheless, both  
40 types of tumors responded well to treatment with 2H9-MC-vc-PAB-MMAE relative to vehicle control and

control antibody conjugate anti-IL-8-MC-vc-PAB-MMAE (Figure 10A). Control animal groups were terminated between day 7 and 14 due to the aggressive growth of their tumors. Animals treated with 2H9-MC-vc-PAB-MMAE were maintained out to 4 weeks, when treatment was discontinued. By contrast, naked Mab 2H9 antibody was tested in a by growing the HT1080-EphB2 cell line as a tumor xenograft in nude mice that 5 were then given MAb 2H9 at a dose of 10 mg/kg body weight twice per week. However, treatment with naked MAb 2H9 did not result in any significant effect on the rate of tumor growth in this assay.

As an additional test of in vivo efficacy, a human colon tumor established by serial passage in nude mice was implanted s.c., and growth was measured during a treatment course identical to that described for the HT1080 model above. Significant growth retardation was again observed with 2H9-MC-vc-PAB-MMAE, 10 relative to vehicle control or control antibody anti-GD-MC-vc-PAB-MMAE (Figure 10B). Overall, these results demonstrated specificity and efficacy of 2H9-MC-vc-PAB-MMAE in in vivo tumor growth models.

The following hybridoma has been deposited with the American Type Culture Collection, PO Box 1549, Manassas, VA, 20108, USA (ATCC):

15	<u>Cell Lines</u>	<u>ATCC Accession No.</u>	<u>Deposit Date</u>
	Hybridoma 2H9.11.14	PTA-6606	February 24, 2005

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations 20 thereunder (Budapest Treaty). This assures maintenance of a viable deposit for 30 years from the date of deposit. These cell lines will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the cell lines to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any 25 U.S. or foreign patent application, whichever comes first, and assures availability of the cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the deposited cell lines should be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a 30 specimen of the same cell line. Availability of the deposited cell lines is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Although the foregoing invention has been described in some detail by way of illustration and 35 example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention.

27 Jun 2012

2006210837

## WHAT IS CLAIMED IS:

1. An anti-EphB2 antibody comprising hypervariable region (HVR) sequences:

5 (i) (a) HVR-L1 comprising sequence KSSQSLLNSGNQENYLA (SEQ ID NO:1);  
(b) HVR-L2 comprising sequence GASTRES (SEQ ID NO:2);  
(c) HVR-L3 comprising sequence QNDHSYPFT (SEQ ID NO:3);  
(d) HVR-H1 comprising sequence SYWMH (SEQ ID NO:4);  
(e) HVR-H2 comprising sequence FINPSTGYTDYNQKFKD (SEQ ID NO:5); and  
(f) HVR-H3 comprising sequence RLKLLRYAMDY (SEQ ID NO:6); or

10 (ii) HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2 and HVR-H3 of the antibody produced by hybridoma cell line deposited as American Type Culture Collection (ATCC) No. PTA-6606,  
wherein the antibody specifically binds human EphB2.

15 2. The antibody of claim 1, wherein the antibody comprises a light chain variable domain comprising the sequence:

DIVMTQSPSSLSVSAGEKVTMNCKSSQSLNSGNQENYLAWYQQKPGQPPKLLIYGASTRESGVPDFR  
TGSGSGTDFTLTISSVQAEDLAVYYCQNDHSYPFTGAGTKVEIKR (SEQ ID NO:7).

20 3. The antibody of claim 1 or claim 2, wherein the antibody comprises a heavy chain variable domain comprising the sequence:

QVQLQQSGAELAKPGASVKMSCKASGYTFTSYWMHWVKQRPGQGLEWIGFINPSTGYTDYNQKFKD  
KATLTVKSSNTAYMQLSRLTSEDSAVYYCTRRLLRYAMDYWGQGTTLTVA (SEQ ID NO:8).

25 4. The antibody of claim 1 comprising the heavy and light chain variable domains of the antibody produced by hybridoma cell line deposited as ATCC No. PTA-6606.

30 5. The antibody of claim 1, wherein the antibody is encoded by antibody coding sequences of hybridoma cell line deposited as ATCC No. PTA-6606

35 6. The antibody of any one of claims 1 to 5, wherein the antibody is a monoclonal antibody.

7. The antibody of any one of claims 1 to 6, wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody, an affinity matured antibody, a human antibody, and a bispecific antibody.

35 8. The antibody of any one of claims 1 to 6, wherein the antibody is an antibody fragment.

9. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to a growth inhibitory agent.

27 Jun 2012

2006210837

10. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to a cytotoxic agent.

11. The antibody of claim 10, wherein the cytotoxic agent is selected from the group consisting of  
5 toxins, antibiotics, radioactive isotopes and nucleolytic enzymes.

12. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to an agent selected  
from the group consisting of a maytansinoid, an auristatin, a dolastatin, and a calicheamicin.

10 13. The antibody of claim 12, wherein the agent is selected from the group consisting of DM1, DM3,  
DM4, MMAE, and MMAF.

14. A polynucleotide encoding the antibody of any one of claims 1 to 8.

15 15. A vector comprising the polynucleotide of claim 14.

16. The vector of claim 15, wherein the vector is an expression vector.

17. A composition comprising the antibody of any one of claims 1 to 13.

20 18. A composition comprising the polynucleotide of claim 14 or the vector of claim 15 or claim 16.

19. The composition of claim 17 or claim 18, wherein the composition further comprises a carrier.

25 20. A host cell comprising the vector of claim 15 or claim 16.

21. The host cell of claim 20, wherein the host cell is prokaryotic.

22. The host cell of claim 20, wherein the host cell is eukaryotic.

30 23. The host cell of claim 22, wherein the host cell is mammalian.

24. A method for making an anti-EphB2 antibody, said method comprising (a) expressing the vector  
of claim 16 in a suitable host cell, and (b) recovering the antibody.

35 25. A method for making an anti-EphB2 immunoconjugate, said method comprising (a) expressing the  
vector of claim 16 in a suitable host cell, (b) recovering the antibody, and (c) conjugating the antibody to a drug.

26. The method of claim 24 or claim 25, wherein the host cell is prokaryotic.

27 Jun 2012

2006210837

27. The method of claim 24 or claim 25, wherein the host cell is eukaryotic.

28. A method for treating or preventing a tumor, a cancer, or a cell proliferative disorder associated  
5 with increased expression or activity of EphB2, the method comprising administering an effective amount of the  
antibody of any one of claims 1 to 13 or the composition of claim 17 or claim 19 to a subject in need of such  
treatment.

29. The method of claim 28, wherein the cancer is selected from the group consisting of small cell  
10 lung cancer, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer, and hepatocellular  
carcinoma.

30. The method of claim 29, wherein the cancer is colorectal cancer.

31. A method for killing a cancer cell or tumor cell, the method comprising administering an effective  
15 amount of the antibody of any one of claims 9 to 13 to a subject in need of such treatment.

32. The method of claim 31, wherein the cancer cell or tumor cell is a colon cancer cell or a gastric  
cancer cell.

33. A method for detecting EphB2, the method comprising detecting EphB2-anti-EphB2 antibody  
20 complex in a biological sample, wherein the anti-EphB2 antibody is the antibody of any one of claims 1 to 8.

34. A method for diagnosing a disorder associated with EphB2 expression, the method comprising  
25 detecting EphB2-anti-EphB2 antibody complex in a biological sample from a subject having or suspected of  
having the disorder, wherein the anti-EphB2 antibody is the antibody of any one of claims 1 to 8.

35. The method of claim 34, wherein the disorder is a tumor, cancer, and/or a cell proliferative  
disorder.

36. The method of any one of claims 33 to 35, wherein the anti-EphB2 antibody is detectably labeled.

37. Use of the antibody of any one of claims 1 to 13 in the manufacture of a medicament for treating  
or preventing a tumor, a cancer, or a cell proliferative disorder associated with increased expression or activity  
35 of EphB2.

38. Use of the antibody of any one of claims 9 to 13 in the manufacture of a medicament for killing a  
cancer cell or tumor cell.

27 Jun 2012

2006210837

39. The antibody of claim 1, the polynucleotide of claim 14, the vector of claim 15, the composition of claim 17 or claim 18, the host cell of claim 20, the method of any one of claims 24, 25, 28, 31, 33 or 34, or the use of claim 37 or claim 38, substantially as hereinbefore described with reference to the examples and figures.

5

86

3389309\_1 (GMMatters) P72403.AU 27-Jun-12

1/9

## FIGURE 1 (SEQ ID NO:8)

Kabat#	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40																	
	Kabat - CDR H1																	
2H9	Q V Q L Q Q S G A E L A K P G A S V K M S C K A S G Y T F T S Y W M H W V K Q R																	
	Hypothetical - CDR H1																	
	Contact - CDR H1																	
Kabat#	41 42 43 44 45 46 47 48 49 50 51 52 A B C 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78																	
	Kabat - CDR H2																	
2H9	P G Q G L E W I G F I N P S T G Y T D Y N Q K F K D K A T L T V - K S S N T A																	
	Hypothetical - CDR H2																	
	Contact - CDR H2																	
Kabat#	79 80 81 82 A B C 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 A B C 101 102 103 104 105 106 107 108 109 110 111 112 113																	
	Kabat - CDR H3																	
2H9	Y M Q L S R L T S E D S A V Y C T R R L K L R Y A M D Y W G Q G T T L T V S A																	
	Hypothetical - CDR H3																	
	Contact - CDR H3																	

## FIGURE 2 (SEQ ID NO:7)

Kabat#	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 A B C D E F 28 29 30 31 32 33 34 35 36 37	Kabat - CDR L1
2H9	D I V M T Q S P S L S V S A G E K V T M N C K S S Q S L N S G N Q E N Y L A W Y Q	Chothia - CDR L1
Kabat#	38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80	Kabat - CDR L2
2H9	Q K P G Q P P K L L I Y G A S T R E S G V P D R F T G S G S G T D F T L T I S S V Q A	Chothia - CDR L2
Kabat#	81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108	Kabat - CDR L3
2H9	E D L A V Y Y C Q N D H S Y P F T F G A G T K V E I K R	Chothia - CDR L3

3/9

FIGURE 3

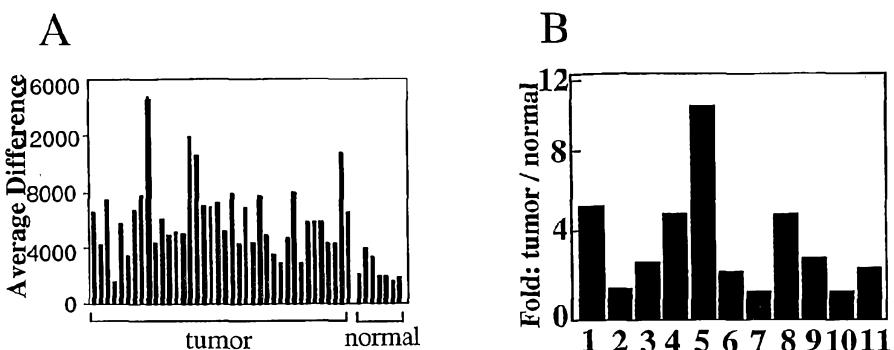
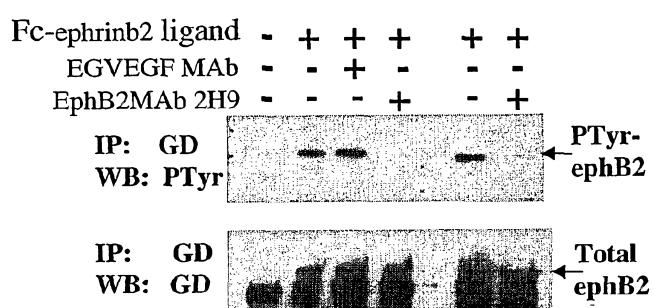
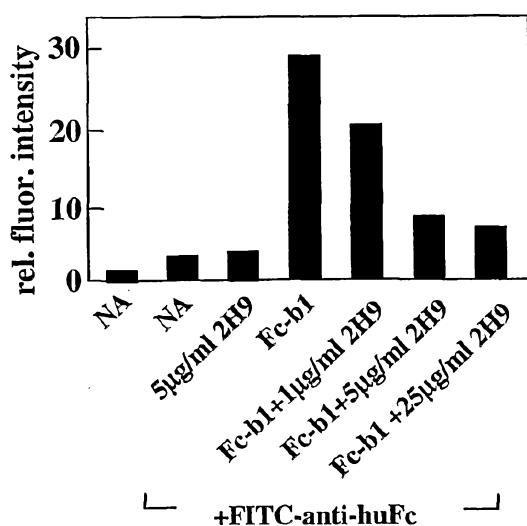
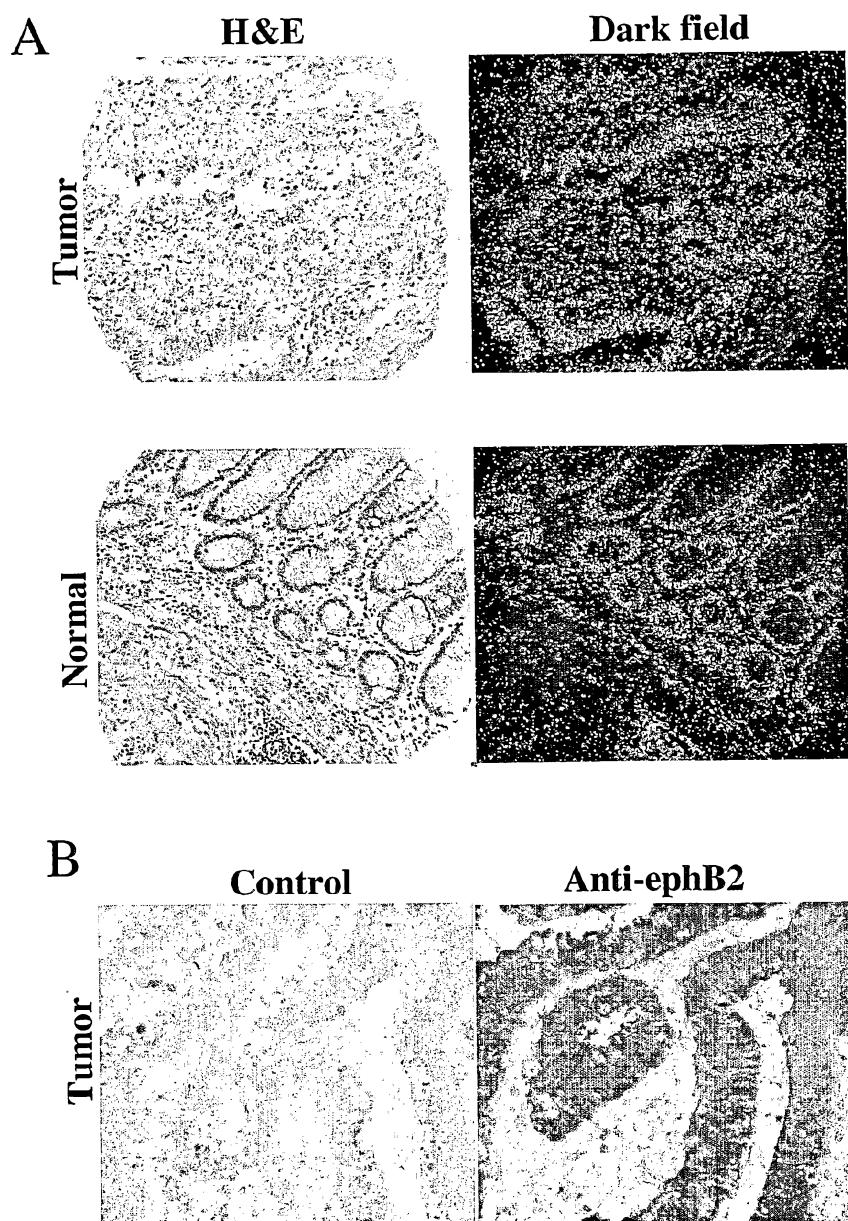
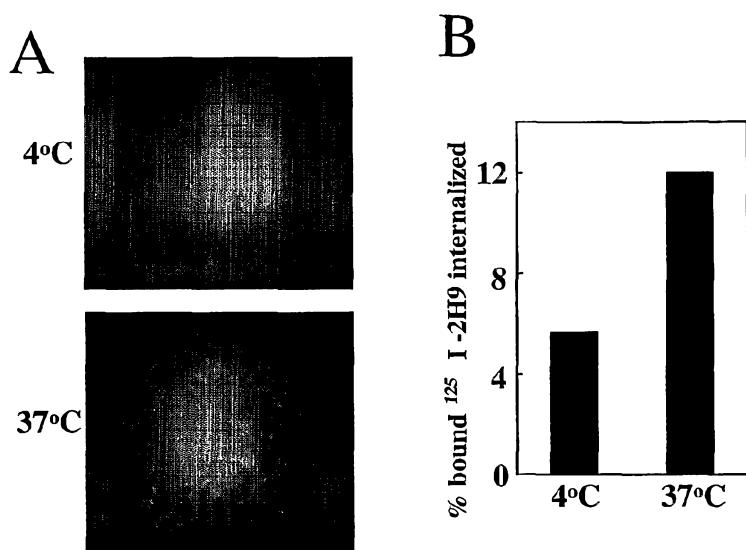


FIGURE 5

**A****B**

4/9

**FIGURE 4**

**FIGURE 6****FIGURE 11**  
EphB2 ligand binding domain (SEQ ID NO:9)

VEETLMDSTTATAELGWMVHPPSGWEEVSGYDENMNTIRT  
YQVCNVFESSQNNWLRTKFIRRRGAHRIHVEMKFSVRDCS  
SIPSPVPGSCKETFNLYYYEADFDSATKTFPNWMENPWVKV  
DTIAADESFSQVDLGGRVMKINTEVRSFGPVSRSGFYLAHQ  
DYGGCMSLIAVRVFYRKCPRIIQ

FIGURE 7

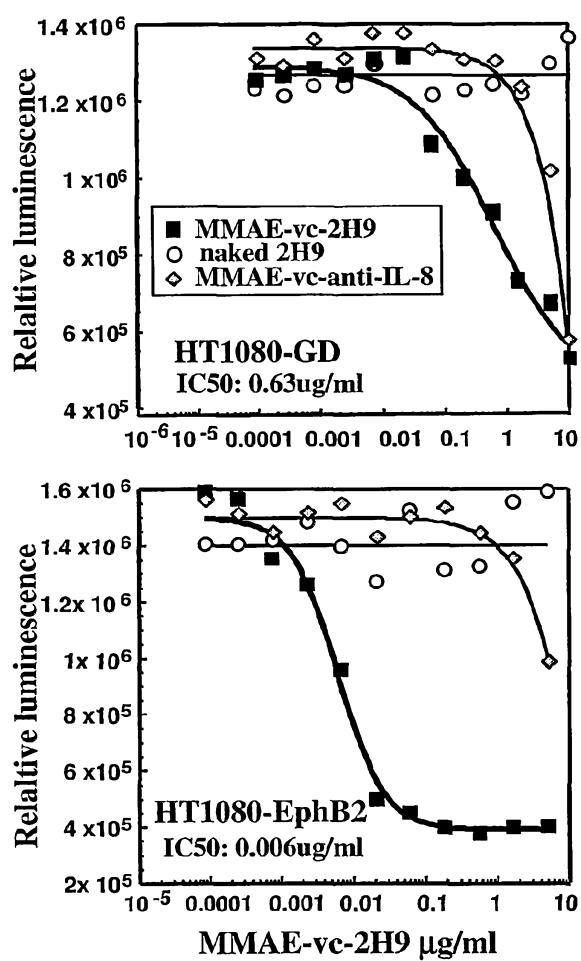
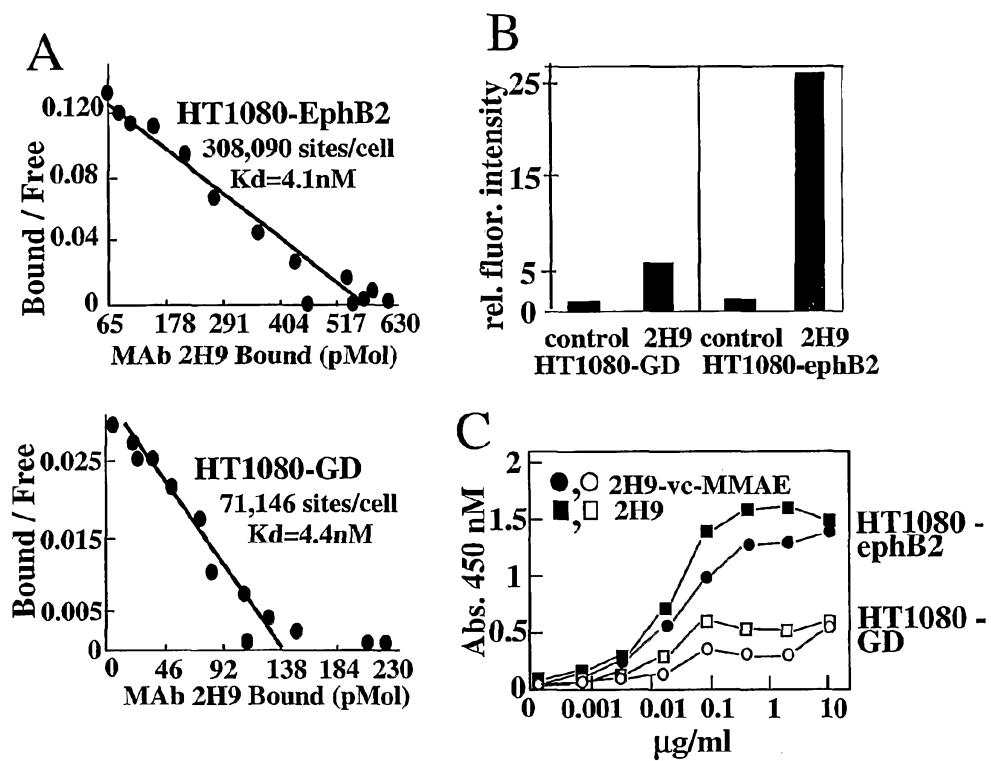
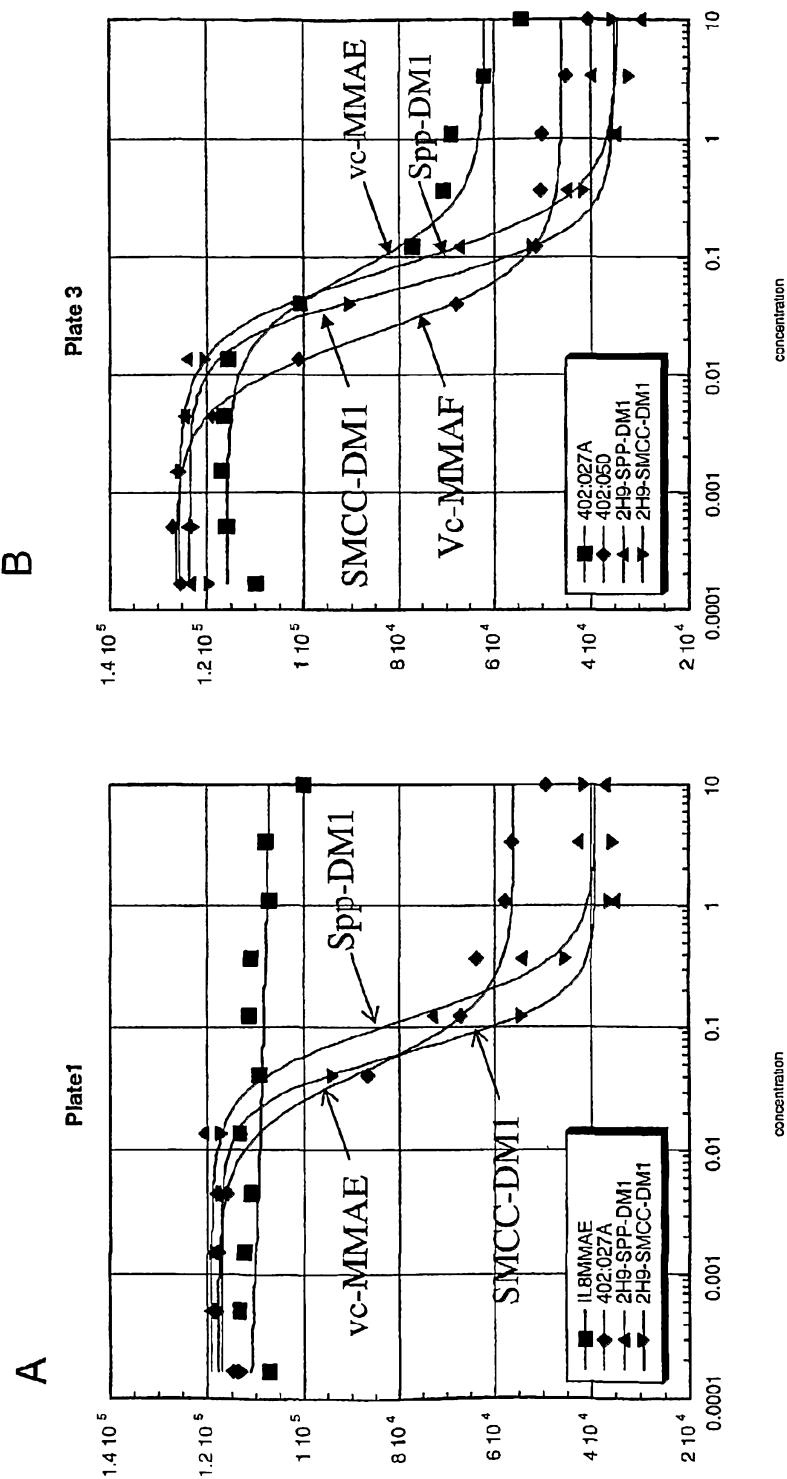


FIGURE 8



8/9

FIGURE 9

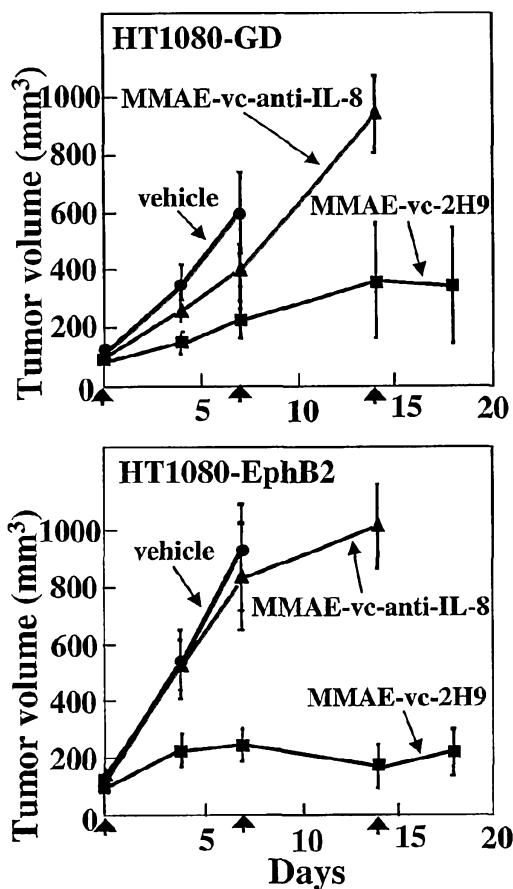


SUBSTITUTE SHEET (RULE 26)

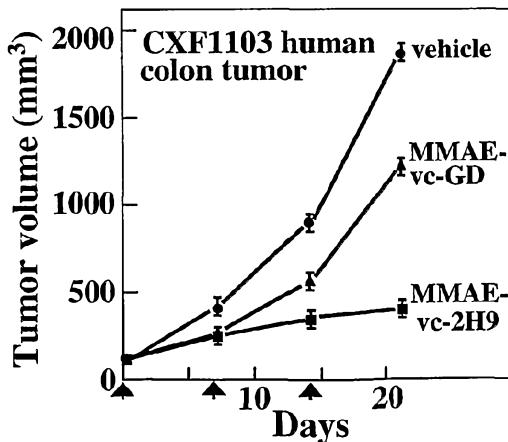
9/9

Figure 10

A



B



Sequence listing.txt  
Sequence Listing

<110> Genentech, Inc.  
MAO, Weiguang  
POLAKIS, Paul

<120> ANTI-EPHB2 ANTIBODIES AND METHODS USING SAME

<130> P2204R1

<150> US 60/648,541  
<151> 2004-01-31

<150> US 60/756,844  
<151> 2006-01-05

<160> 18

<210> 1  
<211> 17  
<212> PRT  
<213> Mus musculus

<400> 1  
Lys Ser Ser Gln Ser Leu Leu Asn Ser Gly Asn Gln Glu Asn Tyr  
1 5 10 15

Leu Ala

<210> 2  
<211> 7  
<212> PRT  
<213> Mus musculus

<400> 2  
Gly Ala Ser Thr Arg Glu Ser  
5

<210> 3  
<211> 9  
<212> PRT  
<213> Mus musculus

<400> 3  
Gln Asn Asp His Ser Tyr Pro Phe Thr  
5

<210> 4  
<211> 5  
<212> PRT  
<213> Mus musculus

<400> 4  
Ser Tyr Trp Met His  
5

<210> 5  
<211> 17  
<212> PRT  
<213> Mus musculus

<400> 5  
Phe Ile Asn Pro Ser Thr Gly Tyr Thr Asp Tyr Asn Gln Lys Phe  
1 5 10 15

Lys Asp

## Sequence listing.txt

<210> 6  
<211> 11  
<212> PRT  
<213> *Mus musculus*

<400> 6  
Arg Leu Lys Leu Leu Arg Tyr Ala Met Asp Tyr  
5 10

<210> 7  
<211> 114  
<212> PRT  
<213> *Mus musculus*

<400> 7  
Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Val Ser Ala  
1 5 10 15

Gly Glu Lys Val Thr Met Asn Cys Lys Ser Ser Gln Ser Leu Leu  
20 25 30

Asn Ser Gly Asn Gln Glu Asn Tyr Leu Ala Trp Tyr Gln Gln Lys  
35 40 45

Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Ala Ser Thr Arg  
50 55 60

Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr  
65 70 75

Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala  
80 85 90

Val Tyr Tyr Cys Gln Asn Asp His Ser Tyr Pro Phe Thr Phe Gly  
95 100 105

Ala Gly Thr Lys Val Glu Ile Lys Arg  
110

<210> 8  
<211> 119  
<212> PRT  
<213> *Mus musculus*

<400> 8  
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Ala Lys Pro Gly  
1 5 10 15

Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr  
20 25 30

Ser Tyr Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu  
35 40 45

Glu Trp Ile Gly Phe Ile Asn Pro Ser Thr Gly Tyr Thr Asp Tyr  
50 55 60

Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Lys Ser Ser  
65 70 75

Asn Thr Ala Tyr Met Gln Leu Ser Arg Leu Thr Ser Glu Asp Ser  
80 85 90

Ala Val Tyr Tyr Cys Thr Arg Arg Leu Lys Leu Leu Arg Tyr Ala  
95 100 105

## Sequence listing.txt

Met Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ala  
 110 115

<210> 9  
 <211> 184  
 <212> PRT  
 <213> Homo sapiens

<400> 9  
 Val Glu Glu Thr Leu Met Asp Ser Thr Thr Ala Thr Ala Glu Leu  
 1 5 10 15

Gly Trp Met Val His Pro Pro Ser Gly Trp Glu Glu Val Ser Gly  
 20 25 30

Tyr Asp Glu Asn Met Asn Thr Ile Arg Thr Tyr Gln Val Cys Asn  
 35 40 45

Val Phe Glu Ser Ser Gln Asn Asn Trp Leu Arg Thr Lys Phe Ile  
 50 55 60

Arg Arg Arg Gly Ala His Arg Ile His Val Glu Met Lys Phe Ser  
 65 70 75

Val Arg Asp Cys Ser Ser Ile Pro Ser Val Pro Gly Ser Cys Lys  
 80 85 90

Glu Thr Phe Asn Leu Tyr Tyr Tyr Glu Ala Asp Phe Asp Ser Ala  
 95 100 105

Thr Lys Thr Phe Pro Asn Trp Met Glu Asn Pro Trp Val Lys Val  
 110 115 120

Asp Thr Ile Ala Ala Asp Glu Ser Phe Ser Gln Val Asp Leu Gly  
 125 130 135

Gly Arg Val Met Lys Ile Asn Thr Glu Val Arg Ser Phe Gly Pro  
 140 145 150

Val Ser Arg Ser Gly Phe Tyr Leu Ala Phe Gln Asp Tyr Gly Gly  
 155 160 165

Cys Met Ser Leu Ile Ala Val Arg Val Phe Tyr Arg Lys Cys Pro  
 170 175 180

Arg Ile Ile Gln

<210> 10  
 <211> 17  
 <212> DNA  
 <213> Artificial sequence

<220>  
 <223> EphB2-specific primer 1

<400> 10  
 cgagccacgt tacatca 17

<210> 11  
 <211> 20  
 <212> DNA  
 <213> Artificial sequence

<220>  
 <223> EphB2-specific primer2

<400> 11

## Sequence listing.txt

tcagtaacgc cgttcacagc 20  
<210> 12  
<211> 27  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> EphB2-specific probe  
  
<400> 12  
cccacaccca gtacacccatc gagatcc 27  
  
<210> 13  
<211> 21  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> EphB2 riboprobe primer 1  
  
<400> 13  
tctgtccatc tgtcccgatc t 21  
  
<210> 14  
<211> 20  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> EphB2 riboprobe primer 1  
  
<400> 14  
gccctccctgg tgctctatcc 20  
  
<210> 15  
<211> 30  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> LC forward primer  
  
<400> 15  
gatcgatatc gtgtgacmc agtctccatc 30  
  
<210> 16  
<211> 21  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> LC reverse primer  
  
<400> 16  
tttdakytcc agcttggtagc c 21  
  
<210> 17  
<211> 35  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> HC forward primer  
  
<400> 17  
gatccgtacg ctcaggttca rctscagcag tctgg 35

## Sequence listing.txt

<210> 18  
<211> 43  
<212> DNA  
<213> Artificial sequence  
  
<220>  
<223> HC reverse primer  
  
<400> 18  
acagtgggcc cttggtgag gctgmrgaga cdgtgashrd rgt 43