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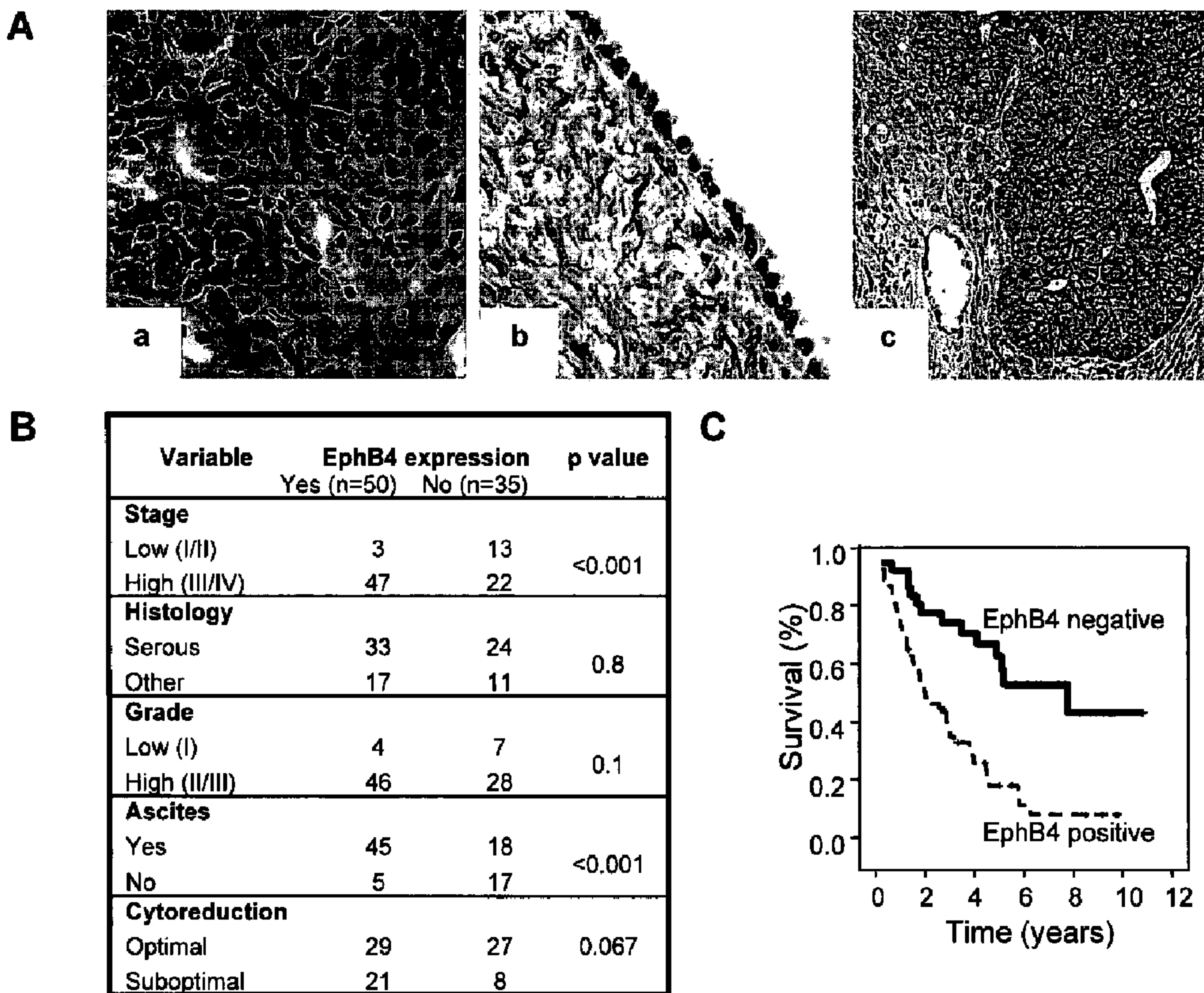
(54) Titre : UTILISATION DU RECEPTEUR EphB4 COMME MARQUEUR DIAGNOSTIQUE ET CIBLE THERAPEUTIQUE DANS LE CANCER DE L'OVAIRE

(54) Title: USE OF EphB4 AS A DIAGNOSTIC MARKER AND A THERAPEUTIC TARGET FOR OVARIAN CANCER



Variable	EphB4 expression Yes (n=50)	No (n=35)	p value
Stage			
Low (I/II)	3	13	<0.001
High (III/IV)	47	22	
Histology			
Serous	33	24	0.8
Other	17	11	
Grade			
Low (I)	4	7	0.1
High (II/III)	46	28	
Ascites			
Yes	45	18	<0.001
No	5	17	
Cytoreduction			
Optimal	29	27	0.067
Suboptimal	21	8	

(57) Abrégé/Abstract:
In certain embodiments, this present invention provides compositions, and methods for inhibiting EphB4 activity in ovarian cancer. In other embodiments, the present invention provides methods and compositions for treating ovarian cancer.

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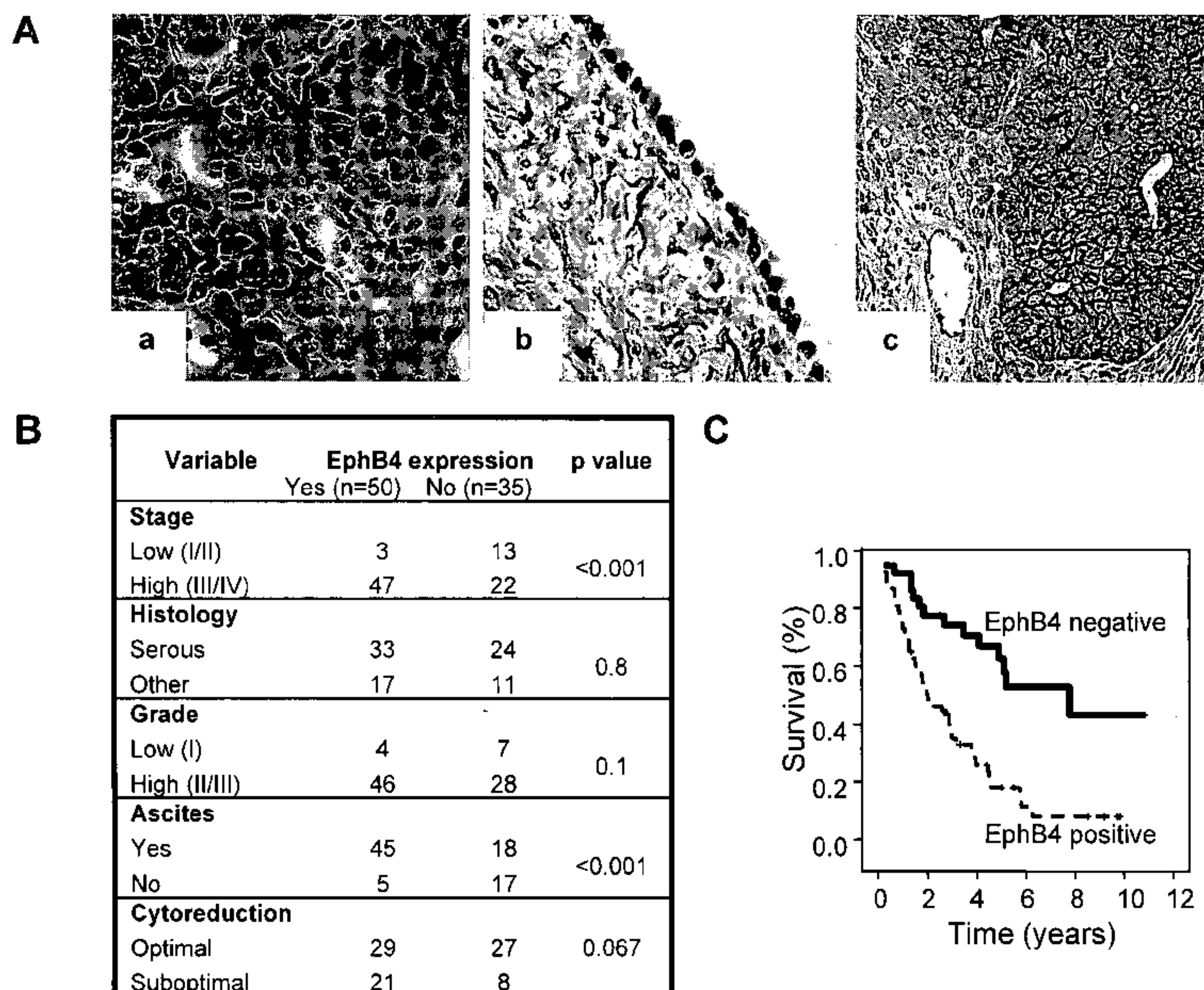
(54) Title: USE OF EPHB4 AS A DIAGNOSTIC MARKER AND A THERAPEUTIC TARGET FOR OVARIAN CANCER



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(57) Abstract: In certain embodiments, this present invention provides compositions, and methods for inhibiting EphB4 activity in ovarian cancer. In other embodiments, the present invention provides methods and compositions for treating ovarian cancer.

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Use of EphB4 as a Diagnostic Marker and a Therapeutic Target for Ovarian Cancer

RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional Application number 60/906,727 filed March 12, 2007, and US Provisional Application number 60/906,764 filed March 12, 2007. The entire teachings of the referenced Applications are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Ovarian cancer is the second most common gynecologic cancer in women, with an estimated 22,220 new cases in the United States in the year 2005 (American Cancer Society, 2005). Ovarian cancer causes more deaths than any other cancer of the female reproductive system. The vast majority of ovarian cancers is epithelial in origin. Ovarian cancer has a higher incidence in women who carry BRCA or mismatch repair gene mutations (Matais-Guiu, 1998). The role of female sex hormones on the progression of ovarian cancer has also been studied. It is believed that certain metabolites of estrogen may support tumor growth while progesterone may have a protective role (Seeger, 2006). No effective screening tool exists for ovarian cancer and in over 80% cases, the diagnosis is not made until the disease is advanced, making treatment particularly challenging. The current overall 5-year survival is 44%; however, the relative 5-year survival for women with distant disease is only 29% (American Cancer Society, 2005). There is hence a need to identify new molecular markers that play a role in the pathogenesis of ovarian cancer with a hope to offer novel, targeted, biological therapy.

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

SUMMARY OF THE INVENTION

In certain aspects, the disclosure provides a method of treating ovarian cancer, the method comprising administering to a patient in need thereof an effective amount of an EphB4 inhibitor. In certain embodiments, the inhibitor may be a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid, an RNAi construct, a nucleic acid analog, or a small molecule. In certain embodiments, said EphB4 inhibitor is an antibody or antigen binding fragment thereof.

In certain embodiments, said antibody or antigen binding fragment thereof is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully
5 human antibody or antibody fragment, a single chain antibody, an Fv, an Fd, an Fab, an Fab', and an F(ab')2. In certain embodiments, said antibody or antigen binding fragment thereof is a monoclonal antibody. In certain embodiments, said antibody or antigen binding fragment thereof binds the extracellular domain of EphB4.

In certain embodiments, the presense of EphB4 is a detectable level of EphB4. In certain
10 embodiments, a sample is selected from the group consisting of: a tissue sample, a blood sample, or a serum sample.

In certain embodiments, the antibody or antigen binding fragment thereof is covalently linked to an additional functional moiety. In certain embodiments, the additional functional moiety is a detectable label. In certain embodiments, the detectable label is selected from a
15 fluorescent or chromogenic label. In certain embodiments, said detectable label is selected from horseradish peroxidase or alkaline phosphatase. In certain embodiments, the additional functional moiety comprises a polyethylene glycol (PEG) moiety.

In certain embodiments, the antibody or antigen binding fragment thereof inhibits an EphB4 activity. In certain embodiments, the antibody or antigen binding fragment thereof
20 stimulates EphB4 kinase activity.

In certain embodiments, the antibody or antigen binding fragment thereof binds to an epitope situated in the extracellular portion of EphB4. In certain embodiments, the antibody or antigen binding fragment thereof binds to an epitope situated within amino acids 16-198 of human EphB4 sequence. In certain embodiments, the antibody or antigen binding fragment
25 thereof binds to an epitope situated within amino acids 327-427 or 428-537 of human EphB4.

In other embodiments, the antibody or antigen binding fragment thereof comprises a heavy chain variable region wherein the heavy chain variable region comprises one or more CDR regions having the amino acid sequence selected from the group consisting of SEQ ID NO: 261, SEQ ID NO: 262, and SEQ ID NO: 263. In another embodiment, the antibody or
30 antigen binding fragment thereof comprises a light chain variable region wherein the light chain variable region comprises one or more CDR regions having the amino acid sequence selected from the group consisting of SEQ ID NO: 264, SEQ ID NO: 265, and SEQ ID NO: 266.

In certain embodiments, said humanized antibody or antigen binding fragment thereof

comprises an immunoglobulin heavy chain and an immunoglobulin light chain, wherein the heavy chain comprises a CDR1 comprising SEQ ID NO:8, a CDR2 comprising SEQ ID NO:9, and a CDR3 comprising SEQ ID NO:10; and wherein the light chain comprises a CDR1 comprising SEQ ID NO:11, a CDR2 comprising SEQ ID NO:12, and a CDR3 comprising SEQ
5 ID NO:13. In certain embodiments, said humanized antibody or antigen binding fragment thereof comprises an immunoglobulin heavy chain and an immunoglobulin light chain, wherein the heavy chain comprises a CDR1 comprising SEQ ID NO:14, a CDR2 comprising SEQ ID NO:15, and a CDR3 comprising SEQ ID NO:16; and wherein the light chain comprises a CDR1 comprising SEQ ID NO:17, a CDR2 comprising SEQ ID NO:18, and a CDR3 comprising SEQ
10 ID NO:19.

In another embodiment, the EphB4 inhibitor is a soluble polypeptide selected from (i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; and (ii) a soluble polypeptide comprising an amino acid sequence of an
15 extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrinb B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide. In certain embodiments, the soluble polypeptide comprises a globular domain of an EphB4 protein. In other aspects, the soluble polypeptide comprises a sequence at least 90% identical to residues 1-522 of the EphB4 amino acid sequence. In still another aspect, the soluble polypeptide comprises a sequence at
20 least 90% identical to residues 1-412 of the EphB4 amino acid sequence. In further embodiments, the soluble polypeptide comprises a sequence at least 90% identical to residues 1-312 of the EphB4 amino acid sequence. Additionally, the soluble polypeptide comprises a sequence at least 90% identical to residues 1-225 of the EphB4 amino acid sequence. The soluble polypeptide may further be a fusion protein, or may comprise one or more modified
25 amino acid residues. In certain preferred embodiments, the soluble polypeptide inhibits the interaction between EphrinB2 and EphB4, or inhibits the clustering of Ephrin B2 and EphB4. In a further embodiment, the soluble polypeptide inhibits phosphorylation of Ephrin B2 or EphB4.

In other embodiments, said EphB4 inhibitor is selected from the group consisting of a nucleic acid compound and a nucleic acid analog. In certain embodiments, said nucleic acid
30 compound is about 15-100 nucleotides in length, hybridizes under physiological conditions to an EphB4 nucleic acid sequence set forth in SEQ ID NO:267 and reduces expression of said EphB4. In certain embodiments, said nucleic acid is selected from the group consisting of: an RNAi construct and an antisense oligonucleotide. In certain embodiments, said RNAi construct

is selected from the group consisting of: a dsRNA or an siRNA. In certain embodiment, said siRNA is around 19-30 nucleotides in length. In certain embodiment, said siRNA is around 21-23 nucleotides in length. In certain embodiment, said antisense oligonucleotide is around 19-30 nucleotides in length. In certain aspects, said siRNA sequence is selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2.

In certain aspects, the EphB4 inhibitor has an anti-cancer activity. In certain embodiments, said anti-cancer activity is selected from the group consisting of inhibiting tumor growth, inhibiting cancer cell proliferation, inhibiting cancer cell migration, inhibiting metastasis of cancer cells, inhibiting angiogenesis, and causing tumor cell death. In certain embodiments, the tumor cell death is due to apoptosis. In certain embodiments, said EphB4 inhibitor causes apoptosis via activation of caspase-8. In certain embodiments, the ovarian cancer comprises one or more cancer cells expressing EphB4. In certain embodiments, said EphB4 inhibitor is formulated with a pharmaceutically acceptable carrier.

In certain embodiments of the methods of the disclosure, the ovarian cancer cell expresses a higher level of EphB4 compared to a noncancerous cell from a comparable tissue. In certain embodiments, the ovarian cancer is metastatic. In further embodiments, the ovarian cancer is angiogenesis-dependent. In other embodiments, the ovarian cancer is angiogenesis-independent.

In certain aspects, the methods of the disclosure further include at least one additional anti-cancer chemotherapeutic agent that inhibits ovarian cancer cells in an additive or synergistic manner with the EphB4 inhibitor. In certain embodiments, said chemotherapeutic agent and said EphB4 inhibitor are administered serially. In certain embodiments, said chemotherapeutic agent and said EphB4 inhibitor are administered simultaneously.

In certain embodiments, the methods of the disclosure further include at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the EphB4 inhibitor. In certain embodiments, said anti-angiogenesis agent and said EphB4 inhibitor are administered serially. In certain embodiments, said anti-angiogenesis agent and said EphB4 inhibitor are administered simultaneously.

In certain aspects, the disclosure provides a method of reducing the growth rate of an ovarian cancer in a subject, comprising administering an amount of an EphB4 inhibitor to reduce the growth rate of the ovarian cancer. In other aspects, the disclosure provides a method for treating a patient suffering from ovarian cancer, comprising: (a) identifying in the patient a tumor having a plurality of cancer cells that express EphB4; and (b) administering to the patient

an EphB4 inhibitor. In certain aspects, the disclosure provides a method of reducing the growth rate of an ovarian cancer in a subject, comprising administering an amount of an EphB4 inhibitor sufficient to reduce the growth rate of the ovarian cancer.

In certain aspects, the disclosure provides the use of an EphB4 inhibitor in the
5 manufacture of a medicament for the treatment of ovarian cancer.

In still another aspect, the application provides a method of detecting whether a subject has ovarian cancer or is at risk for developing ovarian cancer comprising: (a) obtaining a sample from said subject; and (b) assessing the level of EphB4 protein and/or mRNA in the sample, wherein the increased level of EphB4 protein and/or mRNA is indicative that the subject has
10 ovarian cancer or is at risk of developing ovarian cancer. In some embodiments, the level of EphB4 protein or mRNA is at least two times the level in a normal ovarian tissue. In certain embodiments, the mammal is a human. In certain embodiments, said ovarian cancer is metastatic ovarian cancer. In some aspects, the the EphB4 protein expression level is assessed in an antibody-based assay. In certain embodiments, the methods can distinguish between a
15 subject who is at risk of developing ovarian cancer, a subject with early stage disease, and a subject with late stage disease.

In certain aspects, the disclosure provides a method of developing a prognosis for a patient suffering from ovarian cancer comprising: (a) obtaining a sample from said subject; (b) assessing expression level of EphB4 protein and/or mRNA in the sample, wherein an increased
20 a high level of EphB4 protein and/or mRNA is indicative of a poor prognosis. In certain embodiments, a high level of EphB4 protein and/or mRNA is two times the level of EphB4 in normal ovarian tissue. In certain embodiments, a high level of EphB4 is four times the level of EphB4 in normal ovarian tissue. In certain embodiments, a high level of EphB4 is ten times the level of EphB4 in normal ovarian tissue. In certain embodiments, a high level of EphB4 is a
25 detectable level of EphB4 in normal ovarian tissue. In certain embodiments, a sample is selected from the group consisting of: a tissue sample, a blood sample, or a serum sample.

In certain embodiments, the mammal is a human. In certain embodiments, said ovarian cancer is metastatic ovarian cancer.

In still another aspect, the application provides an EphB4 antibody or antigen binding
30 fragment comprising a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises a CDR1 comprising SEQ ID NO:261, a CDR2 comprising SEQ ID NO:262, and a CDR3 comprising SEQ ID NO:263; and wherein the light chain variable region comprises a CDR1 comprising SEQ ID NO:264, a CDR2 comprising SEQ

ID NO:265, and a CDR3 comprising SEQ ID NO:266. In certain embodiments, the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 42, and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 39. In certain embodiments, the antibody is humanized or antibody fragment thereof.

5 In yet another aspect, the application provides a kit for diagnosing ovarian cancer comprising an anti-EphB4 antibody or anti-EphB4-binding antibody fragment, a detectable label, and instructions for using the kit. In certain embodiments, the detectable label is fluorescent or chromogenic. In certain embodiments, the kit comprises horseradish peroxidase or alkaline phosphatase. In certain embodiments, the antibody or antibody fragment thereof 10 binds the C-terminal portion of EphB4.

The application contemplates combinations of any of the foregoing aspects and embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figures 1A-1C show that EphB4 is expressed in human ovarian tumor specimens and correlates with advanced stage, presence of ascites and poor survival. (A) Representative immunohistochemical peroxidase staining for EphB4 in a negative (deletion of primary antibody) control (a), normal ovarian epithelium (b) and invasive ovarian carcinoma (c). All pictures were taken at original magnification of X200. (B) Correlation of clinical and 20 pathological variables with EphB4 overexpression in ovarian carcinoma. (C) Kaplan-Meier survival of patients with invasive ovarian cancer based on EphB4 staining intensity, using the log-rank statistic.

Figures 2A-2D show that ovarian cancer cell lines express functional EphB4 that is downregulated by progesterone. (A) 20μg total cell lysate from each of the ovarian cancer cell 25 lines was run on 4-20% Tris-Glycine gel and transferred to PVDF membrane. The membrane was sequentially probed with anti-EphB4, anti-EphrinB2 and anti-β-actin antibody. (B) Hey cells were serum starved overnight and stimulated for the various time periods shown with 3μg/ml clustered EphrinB2/Fc or Fc alone. EphB4 was immunoprecipitated from 100μg whole cell lysates and phosphorylation status analyzed by anti-phosphotyrosine antibody 30 immunoblotting (top row). A duplicate membrane was probed for EphB4 to document immunoprecipitation efficiency (bottom row). (C) Hoc-7 (ovarian cancer) and MCV-50 (ovarian cystadenoma) cells were treated with varying doses of progesterone and estrogen for 36 hours and cell lysates were analyzed by immunoblotting for expression of EphB4, EphrinB2 and β-

actin. (D) 1×10^4 Hoc-7 cells were plated in each well of a 48-well plate and treated for 72 hours with varying doses of progesterone and estrogen. Cell viability was assessed by MTT assay and survival expressed as percentage of absorbance relative to untreated cells.

Figures 3A-3E show that EphB4 knockdown leads to tumor cell apoptosis. (A) Hev cells were transiently transfected with EphB4-specific siRNA (EphB4-siRNA)and 48 hours later, 20μg whole cell lysates were analyzed by immunoblotting for EphB4 and β-actin levels (top panel). 1×10^4 Hey cells were transfected with mutated EphB4 siRNAΔ or native EphB4-siRNA and plated in a 48-well plate. Cell viability was assessed by MTT assay at 48 hours and survival expressed as percentage of absorbance relative to untreated cells (bottom panel). (B) Hey cells were treated with varying doses of EphB4-specific ODN (AS-10). 72 hours later, 20μg whole cell lysates were analyzed by immunoblotting for EphB4 and β-actin levels (top panel). 1×10^4 Hey cells were treated with scrambled or AS-10 ODN. Cell viability was assessed by MTT assay at 72 hours and survival expressed as percentage of absorbance relative to untreated cells. (C) Hey cells were transiently transfected with EphB4-specific siRNA (EphB4-siRNA) or mutated siRNA (EphB4-siRNAΔ). Apoptosis was analyzed by ELISA for cytoplasmic nucleosomes as detailed in the Methods section using whole cell lysates. (D) Caspase-8 and caspase-9 activation was assayed colorimetrically in these cells and expressed as percent activity compared to lipofectamine-treated cells. (E) Cell cycle analysis of Hey cells treated wit lipofectamine alone (Control) or treated with 25nM EphB4-specific siRNA (EphB4 siRNA) or mutant siRNA (EphB4 siRNAΔ) for 36h. The % of cells in G0, G1, S, G2, and M phase are indicated. Similar results were obtained in three independent experiments.

Figures 4A-4B show that EphB4 favors tumor cell migration and invasion. (A) Confluent cultures of Hey cells were scraped with a plastic Pasteur pipette to produce 3mm wide cell-free zone in the monolayer. The ability of the cells to migrate and close the wound following transfection with 25nM EphB4-specific (EphB4 siRNA) or mutant siRNA (EphB4 siRNAΔ) was assessed over 9hr. (B) Invasion of Hey cells into Matrigel-coated inserts was studied as described in the methods. Cells invading the underside of the inserts in response to 10μg/ml EGF in the lower chamber were fixed and stained with Giemsa. Representative photomicrographs are shown.

Figures 5A-5B show that EphB4-specific antisense ODN inhibits tumor growth in a murine ovarian cancer xenograft model. (A) $2X10^6$ Hey cells were implanted in the flank of ten- to twelve-week old, female Balb/C athymic mice and tumor volume measured as detailed in the Methods section. Mice were administered vehicle alone (vehicle), or 10mg/kg scrambled ODN

(Scrambled) or EphB4-specific antisense ODN (AS-10) intraperitoneally daily starting day 4 after cell implantation. Animals were sacrificed five weeks later and tumors harvested. (B) 5μm sections of formalin-fixed paraffin embedded sections were stained with hematoxylin/eosin and analyzed by immunohistochemistry for Ki-67 and CD31 expression. Apoptosis was evaluated 5 by TUNEL with the in situ apoptosis staining kit. Number of cells staining positive was averaged over five random high-power fields by a blinded observer and indicated in each micrograph. *$p<0.05$ between AS-10 and control group. Bar in bottom left panel represents 200μm in H/E, 100μm in CD31 and 75μm in other photomicrographs.

Figure 6 shows an alignment of the MAB265 variable light chain sequence with 10 germline sequences.

Figure 7 shows an alignment of the MAB265 variable heavy chain sequence with germline sequences.

Figure 8 shows the monoclonal antibodies generated against EphB4 and epitope mapping of these antibodies. The topology of the EphB4 extracellular domain is shown, 15 including a globular domain (G), a cystein-rich domain (C), and two fibronectin type 3 domains (F1 and F2).

Figure 9 shows amino acid sequence of the B4ECv3 protein (sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 10 shows amino acid sequence of the B4ECv3NT protein (sequence of the 20 precursor including uncleaved Eph B4 leader peptide is shown).

Figure 11 shows amino acid sequence of the B4ECv3-FC protein (sequence of the precursor including uncleaved Eph B4 leader peptide is shown).

Figure 12 shows amino acid sequence of the B2EC protein (sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

25 Figure 13 shows amino acid sequence of the B2EC-FC protein (sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown).

Figure 14 shows an amino acid sequence of human Ephrin B2.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

The current invention is based in part on the discovery that signaling through the ephrin/ephrin receptor (ephrin/eph) pathway contributes to ovarian tumorigenesis. Applicants detected expression of EphB4 in ovarian tumor tissues and developed anti-tumor therapeutic agents for blocking signaling through the eph. Accordingly, in certain aspects, the disclosure provides numerous compounds (agents) that may be used to treat ovarian cancer.

Ovarian cancer is a disease produced by the rapid growth and division of cells within one or both ovaries reproductive glands in which the ova, or eggs, and the female sex hormones are made. The ovaries contain cells that, under normal circumstances, reproduce to maintain tissue health. When growth control is lost and cells divide too much and too fast, a cellular mass or tumor is formed. If the tumor is confined to a few cell layers, for example, surface cells, and it does not invade surrounding tissues or organs, it is considered benign. If the tumor spreads to surrounding tissues or organs, it is considered malignant, or cancerous. When cancerous cells break away from the original tumor, travel through the blood or lymphatic vessels, and grow within other parts of the body, the process is known as metastasis.

Ovarian tumors can be broadly classified into three categories, those derived from the surface epithelium, the germ cells and the specialized stroma. Tumors derived from the surface of the ovary account for the vast majority of ovarian tumors (approximately 80%) and are referred to as surface epithelial tumors. It is these tumors that constitute what is generally considered "ovarian cancer." Surface epithelial tumors are further subdivided into three categories, benign, borderline (low-malignant potential [LMP] or atypical proliferative) and invasive carcinoma. The behavior of the benign tumors and invasive carcinomas is reasonably well understood, but there is considerable controversy surrounding the diagnosis, prognosis and treatment of the intermediate (borderline) group. These tumors tend to occur in younger women and can often be treated conservatively. Conservative treatment allows women to preserve their fertility and retain ovarian hormone production which would be lost if both ovaries were removed as occurs with the treatment of invasive carcinoma. Conservative treatment, however, is dependent upon the correct, histologic (pathologic) diagnosis. It is in the category of "borderline" tumors where most of the errors in the diagnosis of ovarian tumors occur. Surface epithelial tumors are also subclassified based on the pattern of cellular differentiation and tumor grade. Finally, the stage and amount of residual tumor after surgery provide important

information that is used for predicting behavior and planning subsequent treatment (cross-reference).

Germ cell tumors are among the least common ovarian tumors, accounting for approximately 10-15% of ovarian tumors. They are derived from the oocytes (eggs). These tumors, like the surface epithelial tumors, can also be benign or malignant. There is, however, no intermediate group. The benign tumors are nearly always mature cystic teratomas or so-called "dermoids" and are successfully treated by the removal of the tumor with preservation of the uninvolved ovarian tissue. No further treatment is necessary. Malignant germ cell tumors require intensive multiagent chemotherapy after their removal. The treatment is completely different from the chemotherapy administered after surgical treatment of a surface epithelial tumor.

Finally, the least common type of ovarian tumor accounting for approximately 5-10% of ovarian tumors are those derived from the stromal component of the ovary. Since hormone production (female sex hormones such as estradiol and progesterone and male hormones such as testosterone, dehydroepiandrosterone [DHEA] and androstendione) occurs in the stroma, tumors derived from this part of the ovary can be associated with abnormal production of sex steroid hormones. This can lead to abnormal vaginal bleeding in reproductive age and postmenopausal women and precocious puberty in children. Ovarian tumors that produce male sex hormones can cause hirsutism (increased growth of hair on various parts of the body) and in extreme cases virilization characterized by an increase in body hair, deepening of the voice, balding, increase in muscle mass, and enlargement of the clitoris.

As used herein, the terms "Ephrin" and "Eph" are used to refer, respectively, to ligands and receptors. They can be from any of a variety of animals (e.g., mammals/non-mammals, vertebrates/non-vertebrates, including humans).

The work described herein, particularly in the examples, refers to Ephrin B2 and EphB4. However, the present invention contemplates any ephrin ligand and/or Eph receptor within their respective family, which is expressed in an ovarian tumor. The ephrins (ligands) are of two structural types, which can be further subdivided on the basis of sequence relationships and, functionally, on the basis of the preferential binding they exhibit for two corresponding receptor subgroups. Structurally, there are two types of ephrins: those which are membrane-anchored by a glycerophosphatidylinositol (GPI) linkage and those anchored through a transmembrane domain. Conventionally, the ligands are divided into the Ephrin-A subclass, which are GPI-

linked proteins which bind preferentially to EphA receptors, and the Ephrin-B subclass, which are transmembrane proteins which generally bind preferentially to EphB receptors.

The Eph family receptors are a family of receptor protein-tyrosine kinases which are related to Eph, a receptor named for its expression in an erythropoietin-producing human
5 hepatocellular carcinoma cell line. They are divided into two subgroups on the basis of the relatedness of their extracellular domain sequences and their ability to bind preferentially to Ephrin-A proteins or Ephrin-B proteins. Receptors which interact preferentially with Ephrin-A proteins are EphA receptors and those which interact preferentially with Ephrin-B proteins are EphB receptors (see published US patent application 20050164965 and 20050249736).

10 Eph receptors have an extracellular domain composed of the ligand-binding globular domain, a cysteine rich region followed by a pair of fibronectin type III repeats. The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α-motif (SAM) and a PDZ-domain binding motif. EphB4 is specific for the membrane-bound ligand Ephrin B2 (Sakano, S. et al 1996;
15 Brambilla R. et al 1995). Ephrin B2 belongs to the class of Eph ligands that have a transmembrane domain and cytoplasmic region with five conserved tyrosine residues and PDZ domain. Eph receptors are activated by binding of clustered, membrane attached ephrins (Davis S et al, 1994), indicating that contact between cells expressing the receptors and cells expressing the ligands is required for Eph activation.

20 Upon ligand binding, an Eph receptor dimerizes and autophosphorylate the juxtamembrane tyrosine residues to acquire full activation (Kalo MS et al, 1999, Binns KS, 2000). In addition to forward signaling through the Eph receptor, reverse signaling can occur through the ephrin Bs. Eph engagement of ephrins results in rapid phosphorylation of the conserved intracellular tyrosines (Bruckner K, 1997) and somewhat slower recruitment of PDZ
25 binding proteins (Palmer A 2002). Recently, several studies have shown that high expression of Eph/ephrins may be associated with increased potentials for tumor growth, tumorigenicity, and metastasis (Easty DJ, 1999; Kiyokawa E, 1994; Tang XX, 1999; Vogt T, 1998; Liu W, 2002; Stephenson SA, 2001; Steube KG 1999; Berclaz G, 1996).

30 *II. Nucleic Acid Therapeutic Agents*

This disclosure relates to methods for inhibiting or reducing gene expression of ephrin and/or ephrin receptor (Eph) in ovarian cancer. By “inhibit” or “reduce,” it is meant that the expression of the gene, or level of nucleic acids or equivalent nucleic acids encoding one or

more proteins or protein subunits, such as Ephrin B2 and/or EphB4, is reduced below that observed in the absence of the nucleic acid therapeutic agents of the disclosure. By "gene," it is meant a nucleic acid that encodes a RNA, for example, nucleic acid sequences including but not limited to structural genes encoding a polypeptide.

5 As used herein, the term "nucleic acid therapeutic agent" or "nucleic acid agent" or "nucleic acid compound" refers to any nucleic acid-based compound that contains nucleotides and has a desired effect on a target gene. The nucleic acid therapeutic agents can be single-, double-, or multiple-stranded, and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures, and combinations thereof. Examples of nucleic acid therapeutic
10 agents of the disclosure include, but are not limited to, antisense nucleic acids, dsRNA, siRNA, and enzymatic nucleic acid compounds.

In one embodiment, the disclosure features one or more nucleic acid therapeutic agents that independently or in combination modulate expression of the Ephrin B2 gene encoding an Ephrin B2 protein (e.g., Genbank Accession No.: NP_004084) or the EphB4 receptor gene
15 which encodes an EphB4 protein (e.g., Genbank Accession No.: NP_004435). Examples of nucleic acid constructs that may be used with the methods of this application are listed in published US patent application 20050164965 and 20050249736, such as those listed below, are herein incorporated by reference in their entirety.

20 Table 1. Inhibition of EphB4 Gene Expression by EphB4 antisense probes

SEQ ID NO:	Name	Sequence 5'→ 3'	position	Inhibition of EphB4 Expression	Percent reduction in viability
43	Eph B4 169	TCA GTA CTG CGG GGC CGG TCC	(2944-2963)	++	36
44	Eph B4 168	TCC TGT CCC ACC CGG GGT TC	(2924-2943)	++	51
45	Eph B4 167	CCG GCT TGG CCT GGG ACT TC	(2904-2923)	+++	66
46	Eph B4 166	ATG TGC TGG ACA CTG GCC AA	(2884-2903)	++++	70
47	Eph B4 165	GAT TTT CTT CTG GTG TCC CG	(2864-2883)	++++	75
48	Eph B4 164	CCA GAG TGA CTC CGA TTC GG	(2844-2863)	++	40
49	Eph B4 163	AGC AGG TCC TCA GCA GAG AT	(2824-2843)	++++	66
50	Eph B4 162	CTG GCT GAC CAG CTC GAA GG	(2804-2823)		25
51	Eph B4 161	AGC CAA AGC CAG CGG CTG CG	(2784-2803)	+	33
52	Eph B4 160	AAA CTT TCT TCG TAT CTT CC	(2763-2783)	+	25
53	Eph B4 159	CAT TTT GAT GGC CCG AAG CC	(2743-2762)	++	40
54	Eph B4 158	ACT CGC CCA CAG AGC CAA AA	(2723-2742)		30
55	Eph B4 157	GCT GAG TAG TGA GGC TGC CG	(2703-2722)	+	25
56	Eph B4 156	CTG GTC CAG GAG AGG GTG TG	(2683-2702)	++	30
57	Eph B4 155	AGG CCC CGC CAT TCT CCC GG	(2663-2682)		25
58	Eph B4 154	GCC ACG ATT TTG AGG CTG GC	(2643-2662)	++	40
59	Eph B4 153	GGG GTT CCG GAT CAT CTT GT	(2623-2642)	++	35

60	Eph B4 152	CCA GGG CGC TGA CCA CCT GG	(2603-2622)	+	30
61	Eph B4 151	GGG AAG CGG GGC CGG GCA TT	(2583-2602)	+	25
62	Eph B4 150	CCG GTC TTT CTG CCA ACA GT	(2563-2582)	++	25
63	Eph B4 149	CCA GCA TGA GCT GGT GGA GG	(2543-2562)	++	20
64	Eph B4 148	GAG GTG GGA CAG TCT GGG GG	(2523-2542)	+	30
65	Eph B4 147	CGG GGG CAG CCG GTA GTC CT	(2503-2522)	++	40
66	Eph B4 146	GTT CAA TGG CAT TGA TCA CG	(2483-2502)	++++	70
67	Eph B4 145	TCC TGA TTG CTC ATG TCC CA	(2463-2482)	++++	80
68	Eph B4 144	GTA CGG CCT CTC CCC AAA TG	(2443-2462)	+++	60
69	Eph B4 143	ACA TCA CCT CCC ACA TCA CA	(2423-2442)	++++	80
70	Eph B4 142	ATC CCG TAA CTC CAG GCA TC	(2403-2422)	++	40
71	Eph B4 141	ACT GGC GGA AGT GAA CTT CC	(2383-2402)	+++	50
72	Eph B4 140	GGA AGG CAA TGG CCT CCG GG	(2363-2382)	++	45
73	Eph B4 139	GCA GTC CAT CGG ATG GGA AT	(2343-2362)	++++	70
74	Eph B4 138	CTT TCC TCC CAG GGA GCT CG	(2323-2342)	++++	70
75	Eph B4 137	TGT AGG TGG GAT CGG AAG AG	(2303-2322)	++	40
76	Eph B4 136	TTC TCC TCC AGG AAT CGG GA	(2283-2302)	++	35
77	Eph B4 135	AAG GCC AAA GTC AGA CAC TT	(2263-2282)	++++	60
78	Eph B4 134	GCA GAC GAG GTT GCT GTT GA	(2243-2262)	++	50
79	Eph B4 133	CTA GGA TGT TGC GAG CAG CC	(2223-2242)	++	40
80	Eph B4 132	AGG TCT CGG TGG ACG TAG CT	(2203-2222)	++	40
81	Eph B4 131	CAT CTC GGC AAG GTA CCG CA	(2183-2202)	+++	50
82	Eph B4 130	TGC CCG AGG CGA TGC CCC GC	(2163-2182)	++	50
83	Eph B4 129	AGC ATG CCC ACG AGC TGG AT	(2143-2162)	++	50
84	Eph B4 128	GAC TGT GAA CTG TCC GTC GT	(2123-2142)	++	50
85	Eph B4 127	TTA GCC GCA GGA AGG AGT CC	(2103-2122)	+++	60
86	Eph B4 126	AGG GCG CCG TTC TCC ATG AA	(2083-2102)	++	50
87	Eph B4 125	CTC TGT GAG AAT CAT GAC GG	(2063-2082)	++++	80
88	Eph B4 124	GCA TGC TGT TGG TGA CCA CG	(2043-2062)	++++	70
89	Eph B4 123	CCC TCC AGG CGG ATG ATA TT	(2023-2042)	++	50
90	Eph B4 122	GGG GTG CTC GAA CTG GCC CA	(2003-2022)	++++	80
91	Eph B4 121	TGA TGG AGG CCT CGC TCA GA	(1983-2002)	++	50
92	Eph B4 120	AAC TCA CGC CGC TGC CGC TC	(1963-1982)	++	40
93	Eph B4 119	CGT GTA GCC ACC CTT CAG GG	(1943-1962)	++++	75
94	Eph B4 118	TCT TGA TTG CCA CAC AGC TC	(1923-1942)	++++	80
95	Eph B4 117	TCC TTC TTC CCT GGG GCC TT	(1903-1922)	++++	70
96	Eph B4 116	GAG CCG CCC CCG GCA CAC CT	(1883-1902)	++	50
97	Eph B4 115	CGC CAA ACT CAC CTG CAC CA	(1863-1882)	++++	60
98	Eph B4 114	ATC ACC TCT TCA ATC TTG AC	(1843-1862)	++++	65
99	Eph B4 113	GTA GGA GAC ATC GAT CTC TT	(1823-1842)	++++	90
100	Eph B4 112	TTG CAA ATT CCC TCA CAG CC	(1803-1822)	++++	70
101	Eph B4 111	TCA TTA GGG TCT TCA TAA GT	(1783-1802)	++++	70
102	Eph B4 110	GAA GGG GTC GAT GTA GAC CT	(1763-1782)	++++	80
103	Eph B4 109	TAG TAC CAT GTC CGA TGA GA	(1743-1762)	++	50
104	Eph B4 108	TAC TGT CCG TGT TTG TCC GA	(1723-1742)	++	45
105	Eph B4 107	ATA TTC TGC TTC TCT CCC AT	(1703-1722)	++++	70
106	Eph B4 106	TGC TCT GCT TCC TGA GGC AG	(1683-1702)	++++	70
107	Eph B4 105	AGA ACT GCG ACC ACA ATG AC	(1663-1682)	++	40
108	Eph B4 104	CAC CAG GAC CAG GAC CAC AC	(1643-1662)	++++	70
109	Eph B4 103	CCA CGA CTG CCG TGC CCG CA	(1623-1642)	++	40
110	Eph B4 102	ATC AGG GCC AGC TGC TCC CG	(1603-1622)	+++	50
111	Eph B4 101	CCA GCC CTC GCT CTC ATC CA	(1583-1602)	++++	80
112	Eph B4 100	GTT GGG TCT GGC TGT GAT GT	(1563-1582)	++++	80
113	Eph B4 99	TCC TGG CCG AAG GGC CCG TA	(1543-1562)	++	35
114	Eph B4 98	GCC GGC CTC AGA GCG CGC CC	(1523-1542)	++	50
115	Eph B4 97	GTA CCT GCA CCA GGT AGC TG	(1503-1522)	++++	80
116	Eph B4 96	GCT CCC CGC TTC AGC CCC CG	(1483-1502)	++	50
117	Eph B4 95	CAG CTC TGC CCG GTT TTC TG	(1463-1482)	++	50

118	Eph B4 94	ACG TCT TCA GGA ACC GCA CG	(1443-1462)	++++	80
119	Eph B4 93	CTG CTG GGA CCC TCG GCG CC	(1423-1442)	++	40
120	Eph B4 92	CTT CTC ATG GTA TTT GAC CT	(1403-1422)	++++	80
121	Eph B4 91	CGT AGT CCA GCA CAG CCC CA	(1383-1402)	++++	85
122	Eph B4 90	CTG GGT GCC CGG GGA ACA GC	(1363-1382)	+++	50
123	Eph B4 89	CCA GGC CAG GCT CAA GCT GC	(1343-1462)	++++	70
124	Eph B4 88	TGG GTG AGG ACC GCG TCA CC	(1323-1342)	++	40
125	Eph B4 87	CGG ATG TCA GAC ACT GCA GG	(1303-1322)	++++	60
126	Eph B4 86	AGG TAC CTC TCG GTC AGT GG	(1283-1302)	++	50
127	Eph B4 85	TGA CAT TGA CAG GCT CAA AT	(1263-1282)	++++	80
128	Eph B4 84	GGG ACG GGC CCC GTG GCT AA	(1243-1262)	++	50
129	Eph B4 83	GGA GGA TAC CCC GTT CAA TG	(1223-1242)	+++	60
130	Eph B4 82	CAG TGA CCT CAA AGG TAT AG	(1203-1222)	++++	70
131	Eph B4 81	GTG AAG TCA GGA CGT AGC CC	(1183-1202)	+++	60
132	Eph B4 80	TCG AAC CAC CAC CCA GGG CT	(1163-1182)	+++	50
133	Eph B4 79	CCA CCA GGT CCC GGG GGC CG	(1143-1162)	++	40
134	Eph B4 78	GGG TCA AAA GTC AGG TCT CC	(1123-1142)	++++	70
135	Eph B4 77	CCC GCA GGG CGC ACA GGA GC	(1103-1122)	+++	60
136	Eph B4 76	CTC CGG GTC GGC ACT CCC GG	(1083-1102)	+++	60
137	Eph B4 75	CAG CGG AGG GCG TAG GTG AG	(1063-1082)	++	40
138	Eph B4 74	GTC CTC TCG GCC ACC AGA CT	(1043-1062)	++	50
139	Eph B4 73	CCA GGG GGG CAC TCC ATT CC	(1023-1042)	++	50
140	Eph B4 72	AGG TGC AGG GAG GAG CCG TT	(1003-1022)	++++	70
141	Eph B4 71	CAG GCG GGA AAC CAC GCT CC	(983-1002)	++	40
142	Eph B4 70	GCG GAG CCG AAG GAG GGG TG	(963-982)	+++	50
143	Eph B4 69	GTG CAG GGT GCA CCC CGG GG	(943-962)	+++	50
144	Eph B4 68	GTC TGT GCG TGC CCG GAA GT	(923-942)	++	40
145	Eph B4 67	ACC CGA CGC GGC ACT GGC AG	(903-922)	++	40
146	Eph B4 66	ACG GCT GAT CCA ATG GTG TT	(883-902)	++	50
147	Eph B4 65	AGA GTG GCT ATT GGC TGG GC	(863-882(	++++	60
148	Eph B4 64	ATG GCT GGC AGG ACC CTT CT	(843-862)	++++	80
149	Eph B4 63	CCT GAC AGG GGC TTG AAG GT	(823-842)	++++	80
150	Eph B4 62	GCC CTG GGC ACA GGC TCG GC	(803-822)	+++	70
151	Eph B4 61	ACT TGG TGT TCC CCT CAG CT	(783-802)	++++	80
152	Eph B4 60	GCC TCG AAC CCC GGA GCA CA	(763-782)	+++	50
153	Eph B4 59	GCT GCA GCC CGT GAC CGG CT	(743-762)	+++	50
154	Eph B4 58	GTT CGG CCC ACT GGC CAT CC	(723-742)	++	45
155	Eph B4 57	TCA CGG CAG TAG AGG CTG GG	(703-722)	+++	70
156	Eph B4 56	GCT GGG GCC AGG GGC GGG GA	(683-702)	++	50
157	Eph B4 55	CGG CAT CCA CCA CGC AGC TA	(663-682)	++	50
158	Eph B4 54	CCG GCC ACG GGC ACA ACC AG	(643-662)	++	50
159	Eph B4 53	CTC CCG AGG CAC AGT CTC CG	(623-642)	+++	50
160	Eph B4 52	GGA ATC GAG TCA GGT TCA CA	(603-622)	++++	90
161	Eph B4 51	GTC AGC TGG GCG CAC TTT TT	(583-602)	+++	70
162	Eph B4 50	GTA GAA GAG GTG CAG GGA TA	(563-582)	++++	80
163	Eph B4 49	GCA GGG CCA TGC AGG CAC CC	(543-562)	++++	80
164	Eph B4 48	TGG TCC TGG AAG GCC AGG TA	(523-542)	++++	90
165	Eph B4 47	GAA GCC AGC CTT GCT GAG CG	(503-522)	++++	80
166	Eph B4 46	GTC CCA GAC GCA GCG TCT TG	(483-502)	++	40
167	Eph B4 45	ACA TTC ACC TTC CCG GTG GC	(463-482)	+++	50
168	Eph B4 44	CTC GGC CCC AGG GCG CTT CC	(443-462)	++	50
169	Eph B4 43	GGG TGA GAT GCT CCG CGG CC	(423-442)	+++	60
170	Eph B4 42	ACC GTG TCC ACC TTG ATG TA	(403-422)	++++	80
171	Eph B4 41	GGG GTT CTC CAT CCA GGC TG	(383-402)	++++	80
172	Eph B4 40	GCG TGA GGG CCG TGG CCG TG	(363-382)	++	50
173	Eph B4 39	TCC GCA TCG CTC TCA TAG TA	(343-362)	+++	60
174	Eph B4 38	GAA GAC GGT GAA GGT CTC CT	(323-342)	++++	80
175	Eph B4 37	TGC AGG AGC GCC CAG CCC GA	(303-322)	+++	50

176	Eph B4 36	GGC AGG GAC AGG CAC TCG AG	(283-302)	+++	45
177	Eph B4 35	CAT GGT GAA GCG CAG CGT GG	(263-282)	++	50
178	Eph B4 34	CGT ACA CGT GGA CGG CGC CC	(243-262)	++	40
179	Eph B4 33	CGC CGT GGG ACC CAA CCT GT	(223-242)	+++	60
180	Eph B4 32	GCG AAG CCA GTG GGC CTG GC	(203-222)	++++	70
181	Eph B4 31	CCG GGG CAC GCT GCA CGT CA	(183-202)	+++	60
182	Eph B4 30	CAC ACT TCG TAG GTG CGC AC	(163-182)	+++	70
183	Eph B4 29	GCT GTG CTG TTC CTC ATC CA	(143-162)	++++	80
184	Eph B4 28	GGC CGC TCA GTT CCT CCC AC	(123-142)	++	40
185	Eph B4 27	TGC CCG TCC ACC TGA GGG AA	(103-122)	++	50
186	Eph B4 26	TGT CAC CCA CTT CAG ATC AG	(83-102)	++++	70
187	Eph B4 25	CAG TTT CCA ATT TTG TGT TC	(63-82)	++++	70
188	Eph B4 24	AGC AGG GTC TCT TCC AAA GC	(43-62)	++++	80
189	Eph B4 23	TGC GGC CAA CGA AGC CCA GC	(23-42)	++	50
190	Eph B4 22	AGA GCA GCA CCC GGA GCT CC	(3-22)	+++	50
191	Eph B4 21	AGC AGC ACC CGG AGC TCC AT	(1-20)	+++	50
192	EphB4 AS-1	GTG CAG GGA TAG CAG GGC CAT	(552-572)		
193	EphB4 AS-2	AAG GAG GGG TGG TGC ACG GTG	(952-972)		
194	EphB4 AS-3	TTC CAG GTG CAG GGA GGA GCC	(1007-1027)		
195	EphB4 AS-4	GTG GTG ACA TTG ACA GGC TCA	(1263-1285)		
196	EphB4 AS-5	TCT GGC TGT GAT GTT CCT GGC	(1555-1575)		
197	EphB4 AS-6	GCC GCT CAG TTC CTC CCA	(123-140)		
198	EphB4 AS-7	TGA AGG TCT CCT TGC AGG	(316-333)		
199	EphB4 AS-8	CGC GGC CAC CGT GTC CAC CTT	(408-428)		
200	EphB4 AS-9	CTT CAG GGT CTT GAT TGC CAC	(1929-1949)		
201	EphB4 AS-10	ATG GAG GCC TCG CTC AGA AA	(1980-1999)		
202	Ephb4 AS-11	CAT GCC CAC GAG CTG GAT GAC	(2138-2158)		

Table 2. Inhibition of EphB4 Gene Expression by EphB4 RNAi probes

5

SEQ ID NO:	RNAi	EphB4 RNAi sequence			Inhibition of EphB4 Expression	Percent reduction in viability
203	1	446	aaattggaaactgctgatctg	466		
204	2	447	aattggaaactgctgatctga	467	+++	70
205	3	453	aaactgctgatctgaagtggg	473	++++	70
206	4	454	aactgctgatctgaagtgggt	474	+++	80
207	5	854	aatgtcaagacgctgcgtctg	874	+++	65
208	6	467	aagtgggtgacattccctcag	487	+	35
209	7	848	aaggtgaatgtcaagacgctg	868	++	50
210	8	698	aaggagaccttcaccgtcttc	718	+++	75
211	9	959	aaaaagtgcgcccagctgact	979	+	40
212	10	1247	aatagccactctaacaccatt	1267	++	50
213	11	1259	aacaccattggatcagccgtc	1279	++	50
214	12	1652	aatgtcaccactgaccgagag	1672	+	35
215	13	1784	aaataccatgagaagggcgcc	1804	+++	65
216	14	1832	aagacgtcagaaaaccgggca	1852	+	30
217	15	1938	aacatcacagccagacccaac	19	++	50
218	16	2069	aagcagagcaatgggagagaa	2089	++++	75
219	17	2078	aatgggagagaagcagaatat	2098	+++	65
220	18	2088	aagcagaatattcggacaaac	2108	+++	70

221	19	2094	aatattcggacaaacacggac	2114	++	40
222	20	2105	aaacacggacagtatctcatc	2125	++	50
223	21	2106	aacacggacagtatctcatcg	2126	+	35
224	22	2197	aaaagagatcgatgtctccta	2217	+++	65
225	23	2174	aatgaggctgtgagggaattt	2194	++	50
226	24	2166	aagaccctaatgaggctgtga	2186	++	50
227	25	2198	aaagagatcgatgtctcctac	2218	+++	55
228	26	2199	aagagatcgatgtctcctacg	2219	+++	70
229	27	2229	aagaggtgattggtgcaggtg	2249	+	33
230	28	2222	aagattgaagaggtgattggt	2242	+	30
231	29	2429	aacagcatgcccgtcatgatt	2449	++	40
232	30	2291	aagaaggagagctgtgtggca	2311	+++	50
233	31	2294	aaggagagctgtgtggcaatc	2314	+++	60
234	32	2311	aatcaagaccctgaagggtgg	2331	+++	70
235	33	2497	aaacgacggacagttcacagt	2517	+	35
236	34	2498	aacgacggacagttcacagtc	2518	+	40
237	35	2609	aacatcctagtcaacagcaac	2629	++	50
238	36	2621	aacagcaacctcgtctgcaaa	2641	+	35
239	37	2678	aactcttccgatcccacctac	2698	++	50
240	38	2640	aagtgtctgactttggccttt	2660	+++	70
241	39	2627	aacctcgtctgcaaagtgtct	2647	++	50
242	40	2639	aaagtgtctgactttggcctt	2659	+	25
243	41	2852	aatcaggacgtgatcaatgcc	2872	+++	75
244	42	2716	aaagattcccatccgatggac	2736	++	50
245	43	2717	aagattcccatccgatggact	2737	++	60
246	44	2762	aagttcacttccgccagtgat	2782	+++	70
247	45	3142	aagatacgaagaaagtttcgc	3162	++	50
248	46	3136	aatgggaagatacgaagaaag	3156	+++	66
249	47	2867	aatgccattgaacaggactac	2887		
250	48	3029	aaaatcgtggcccgggagaat	3049	+	33
251	49	3254	aaaatcttggccagtgtccag	3274	++	50
252	50	3255	aaatcttggccagtgtccagc	3275	+++	75
253	51	3150	aagaaagtttcgcagccgctg	3170	+++	80
254	52	3251	aagaaaatcttggccagtgtc	3271	++	50
255	53	3256	aatcttggccagtgtccagca	3276	++	50
256	Eph B4 50		gagacccugcugaacacaauu			
257	Eph B4 472		ggugaaugucaagacgcuguu			
258	Eph B4 1562		caucacagccagacccaacuu			
259	siRNA 2303		cucuuccgaucccaccuacuu			
260	Eph B4 2302		cucuuccgaucccaccuacuu			

A. Antisense nucleic acids

In certain embodiments, the disclosure relates to antisense nucleic acids. By "antisense nucleic acid," it is meant a non-enzymatic nucleic acid compound that binds to a target nucleic acid by means of RNA-RNA, RNA-DNA or RNA-PNA (protein nucleic acid) interactions and alters the activity of the target nucleic acid (for a review, see Stein and Cheng, 1993 Science 261, 1004 and Woolf et al., U.S. Pat. No. 5,849,902). Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can form a loop and binds to a substrate nucleic acid which forms a loop. Thus, an antisense molecule can be

complementary to two (or more) non-contiguous substrate sequences, or two (or more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence, or both. For a review of current antisense strategies, see Schmajuk et al., 1999, J. Biol. Chem., 274, 21783-21789, Delihas et al., 1997, Nature, 15, 751-753, Stein et al., 1997, Antisense N. A. Drug Dev., 7, 151, Crooke, 2000, Methods Enzymol., 313, 3-45; Crooke, 1998, Biotech. Genet. Eng. Rev., 15, 121-157, Crooke, 1997, Ad. Pharmacol., 40, 1-49.

In addition, antisense DNA can be used to target nucleic acid by means of DNA-RNA interactions, thereby activating RNase H, which digests the target nucleic acid in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region, which is capable of activating RNAse H to cleave a target nucleic acid. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof. By "RNase H activating region" is meant a region (generally greater than or equal to 4-25 nucleotides in length, preferably from 5-11 nucleotides in length) of a nucleic acid compound capable of binding to a target nucleic acid to form a non-covalent complex that is recognized by cellular RNase H enzyme (see for example Arrow et al., U.S. Pat. No. 5,849,902; Arrow et al., U.S. Pat. No. 5,989,912). The RNase H enzyme binds to a nucleic acid compound-target nucleic acid complex and cleaves the target nucleic acid sequence.

The RNase H activating region comprises, for example, phosphodiester, phosphorothioate, phosphorodithioate, 5'-thiophosphate, phosphoramidate or methylphosphonate backbone chemistry, or a combination thereof. In addition to one or more backbone chemistries described above, the RNase H activating region can also comprise a variety of sugar chemistries. For example, the RNase H activating region can comprise deoxyribose, arabino, fluoroarabino or a combination thereof, nucleotide sugar chemistry. Those skilled in the art will recognize that the foregoing are non-limiting examples and that any combination of phosphate, sugar and base chemistry of a nucleic acid that supports the activity of RNase H enzyme is within the scope of the definition of the RNase H activating region and the instant disclosure.

Thus, the antisense nucleic acids of the disclosure include natural-type oligonucleotides and modified oligonucleotides including phosphorothioate-type oligodeoxyribonucleotides, phosphorodithioate-type oligodeoxyribonucleotides, methylphosphonate-type oligodeoxyribonucleotides, phosphoramidate-type oligodeoxyribonucleotides, H-phosphonate-type oligodeoxyribonucleotides, triester-type oligodeoxyribonucleotides, alpha-anomer-type

oligodeoxyribonucleotides, peptide nucleic acids, other artificial nucleic acids, and nucleic acid-modified compounds.

Other modifications include those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesterol, cholesteryl, or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

Other examples of modifications to sugars include modifications to the 2' position of the ribose moiety which include but are not limited to 2'-O-substituted with an --O-- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an --O-aryl, or allyl group having 2-6 carbon atoms wherein such --O-alkyl, aryl or allyl group may be unsubstituted or may be substituted, (e.g., with halo, hydroxy, trifluoromethyl cyano, nitro acyl acyloxy, alkoxy, carboxy, carbalkoxyl, or amino groups), or with an amino, or halo group. Nonlimiting examples of particularly useful oligonucleotides of the disclosure have 2'-O-alkylated ribonucleotides at their 3', 5', or 31 and 5' termini, with at least four or five contiguous nucleotides being so modified. Examples of 2'-O-alkylated groups include, but are not limited to, 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, and 2'-O-butyls.

In certain cases, the synthesis of the natural-type and modified antisense nucleic acids can be carried out with, for example, a 381A DNA synthesizer or 394 DNA/RNA synthesizer manufactured by ABI (Applied Biosystems Inc.) in accordance with the phosphoramidite method (see instructions available from ABI, or F. Eckstein, Oligonucleotides and Analogues: A Practical Approach, IRL Press (1991)). In the phosphoramidite method, a nucleic acid-related molecule is synthesized by condensation between the 3'-terminus of a modified deoxyribonucleoside or modified ribonucleoside and the 5'-terminus of another modified deoxyribonucleoside, modified ribonucleoside, oligo-modified deoxyribonucleotide or oligo-modified-ribonucleotide by use of a reagent containing phosphoramidite protected with a group such as cyanoethyl group. The final cycle of this synthesis is finished to give a product with a protective group (e.g., dimethoxytrityl group) bound to a hydroxyl group at the 5'-terminus of

the sugar moiety. The oligomer thus synthesized at room temperature is cleaved off from the support, and its nucleotide and phosphate moieties are deprotected. In this manner, the natural-type oligonucleic acid compound is obtained in a crude form. The phosphorothioate-type nucleic acids can also be synthesized in a similar manner to the above natural type by the
5 phosphoramidite method with the synthesizer from ABI. The procedure after the final cycle of the synthesis is also the same as with the natural type.

The crude nucleic acids (natural type or modified) thus obtained can be purified in a usual manner e.g., ethanol precipitation, or reverse phase chromatography, ion-exchange chromatography and gel filtration chromatography in high performance liquid chromatography
10 (HPLC), supercritical fluid chromatography, and it may be further purified by electrophoresis. A cartridge for reverse phase chromatography, such as tC18-packed SepPak Plus (long body/ENV) (Waters), can also be used. The purity of the natural-type and modified (e.g., phosphorothioate-type) nucleic acids can be analyzed by HPLC.

In certain embodiments, the antisense nucleic acids of the disclosure can be delivered,
15 for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes an ephrin B2 or EphB4 polypeptide. Alternatively, the construct is an oligonucleotide which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences encoding an ephrin B2 or EphB4 polypeptide. Such
20 oligonucleotide probes are optionally modified oligonucleotide which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid compounds for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patent Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in
25 nucleic acid therapy have been reviewed, for example, by van der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al., (1988) Cancer Res 48:2659-2668.

B. dsRNA and RNAi Constructss

In certain embodiments, the disclosure relates to double stranded RNA (dsRNA) and
30 RNAi constructs. The term "dsRNA" as used herein refers to a double stranded RNA molecule capable of RNA interference (RNAi), including siRNA (see for example, Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., PCT Publication No. WO 00/44895; Zernicka-Goetz et al., PCT Publication No. WO 01/36646; Fire, PCT

Publication No. WO 99/32619; Plaetinck et al., PCT Publication No. WO 00/01846; Mello and Fire, PCT Publication No. WO 01/29058; Deschamps-Depaillette, PCT Publication No. WO 99/07409; and Li et al., PCT Publication No. WO 00/44914). In addition, RNAi is a term initially applied to a phenomenon observed in plants and worms where double-stranded RNA 5 (dsRNA) blocks gene expression in a specific and post-transcriptional manner. RNAi provides a useful method of inhibiting gene expression in vitro or in vivo.

The term "short interfering RNA," "siRNA," or "short interfering nucleic acid," as used herein, refers to any nucleic acid compound capable of mediating RNAi or gene silencing when processed appropriately be a cell. For example, the siRNA can be a double-stranded 10 polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises complementarity to a target nucleic acid compound (e.g., Ephrin B2 or EphB4). The siRNA can be a single-stranded hairpin polynucleotide having self-complementary sense and antisense regions, wherein the antisense region comprises complementarity to a target nucleic acid compound. The siRNA can be a circular single-15 stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises complementarity to a target nucleic acid compound, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siRNA capable of mediating RNAi. The siRNA can also comprise a single stranded polynucleotide having complementarity to a 20 target nucleic acid compound, wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez et al., 2002, Cell., 110, 563-574), or 5',3'-diphosphate.

Optionally, the siRNAs of the disclosure contain a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the 25 mRNA transcript for the gene to be inhibited (the "target" gene). The double-stranded RNA need only be sufficiently similar to natural RNA that it has the ability to mediate RNAi. Thus, the disclosure has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism or evolutionary divergence. The number of tolerated nucleotide mismatches between the target sequence and the siRNA sequence is no 30 more than 1 in 5 basepairs, or 1 in 10 basepairs, or 1 in 20 basepairs, or 1 in 50 basepairs. Mismatches in the center of the siRNA duplex are most critical and may essentially abolish cleavage of the target RNA. In contrast, nucleotides at the 3' end of the siRNA strand that is complementary to the target RNA do not significantly contribute to specificity of the target

recognition. Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the siRNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50 °C or 70 °C hybridization for 12-16 hours; followed by washing).

The double-stranded structure of dsRNA may be formed by a single self-complementary RNA strand, two complementary RNA strands, or a DNA strand and a complementary RNA strand. Optionally, RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition, while lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

As described herein, the subject siRNAs are around 19-30 nucleotides in length, and even more preferably 21-23 nucleotides in length. The siRNAs are understood to recruit nuclease complexes and guide the complexes to the target mRNA by pairing to the specific sequences. As a result, the target mRNA is degraded by the nucleases in the protein complex. In a particular embodiment, the 21-23 nucleotides siRNA molecules comprise a 3' hydroxyl group. In certain embodiments, the siRNA constructs can be generated by processing of longer double-stranded RNAs, for example, in the presence of the enzyme dicer. In one embodiment, the Drosophila *in vitro* system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. The siRNA molecules can be purified using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to purify siRNAs. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to purify the siRNA. In addition, chromatography (e.g., size

exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to purify siRNAs.

Production of the subject dsRNAs (e.g., siRNAs) can be carried out by chemical synthetic methods or by recombinant nucleic acid techniques. Endogenous RNA polymerase of the treated cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vitro*. As used herein, dsRNA or siRNA molecules of the disclosure need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. For example, the dsRNAs may include modifications to either the phosphate-sugar backbone or the nucleoside, e.g., to reduce susceptibility to cellular nucleases, improve bioavailability, improve formulation characteristics, and/or change other pharmacokinetic properties. To illustrate, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general response to dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNAs may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis. Methods of chemically modifying RNA molecules can be adapted for modifying dsRNAs (see, e.g., Heidenreich et al. (1997) Nucleic Acids Res, 25:776-780; Wilson et al. (1994) J Mol Recog 7:89-98; Chen et al. (1995) Nucleic Acids Res 23:2661-2668; Hirschbein et al. (1997) Antisense Nucleic Acid Drug Dev 7:55-61). Merely to illustrate, the backbone of an dsRNA can be modified with phosphorothioates, phosphoramidate, phosphodithioates, chimeric methylphosphonate-phosphodiesters, peptide nucleic acids, 5-propynyl-pyrimidine containing oligomers or sugar modifications (e.g., 2'-substituted ribonucleosides, a-configuration). In certain cases, the dsRNAs of the disclosure lack 2'-hydroxy (2'-OH) containing nucleotides.

In a specific embodiment, at least one strand of the siRNA molecules has a 3' overhang from about 1 to about 6 nucleotides in length, though may be from 2 to 4 nucleotides in length. More preferably, the 3' overhangs are 1-3 nucleotides in length. In certain embodiments, one strand having a 3' overhang and the other strand being blunt-ended or also having an overhang. The length of the overhangs may be the same or different for each strand. In order to further enhance the stability of the siRNA, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotide 3' overhangs by 2'-deoxythyinidine is tolerated

and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium and may be beneficial *in vivo.*

In another specific embodiment, the subject dsRNA can also be in the form of a long double-stranded RNA. For example, the dsRNA is at least 25, 50, 100, 200, 300 or 400 bases. In some cases, the dsRNA is 400-800 bases in length. Optionally, the dsRNAs are digested intracellularly, e.g., to produce siRNA sequences in the cell. However, use of long double-stranded RNAs *in vivo* is not always practical, presumably because of deleterious effects which may be caused by the sequence-independent dsRNA response. In such embodiments, the use of local delivery systems and/or agents which reduce the effects of interferon or PKR are preferred.

In a further specific embodiment, the dsRNA is in the form of a hairpin structure (named as hairpin RNA). The hairpin RNAs can be synthesized exogenously or can be formed by transcribing from RNA polymerase III promoters *in vivo.* Examples of making and using such hairpin RNAs for gene silencing in mammalian cells are described in, for example, Paddison et al., Genes Dev, 2002, 16:948-58; McCaffrey et al., Nature, 2002, 418:38-9; McManus et al., RNA, 2002, 8:842-50; Yu et al., Proc Natl Acad Sci U S A, 2002, 99:6047-52). Preferably, such hairpin RNAs are engineered in cells or in an animal to ensure continuous and stable suppression of a desired gene. It is known in the art that siRNAs can be produced by processing a hairpin RNA in the cell.

PCT application WO 01/77350 describes an exemplary vector for bi-directional transcription of a transgene to yield both sense and antisense RNA transcripts of the same transgene in a eukaryotic cell. Accordingly, in certain embodiments, the present disclosure provides a recombinant vector having the following unique characteristics: it comprises a viral replicon having two overlapping transcription units arranged in an opposing orientation and flanking a transgene for a dsRNA of interest, wherein the two overlapping transcription units yield both sense and antisense RNA transcripts from the same transgene fragment in a host cell.

C. Enzymatic Nucleic Acid Compounds

In certain embodiments, the disclosure relates to enzymatic nucleic acid compounds. By "enzymatic nucleic acid compound," it is meant a nucleic acid compound which has complementarity in a substrate binding region to a specified target gene, and also has an enzymatic activity which is active to specifically cleave a target nucleic acid. It is understood that the enzymatic nucleic acid compound is able to intermolecularly cleave a nucleic acid and thereby inactivate a target nucleic acid compound. These complementary regions allow

sufficient hybridization of the enzymatic nucleic acid compound to the target nucleic acid and thus permit cleavage. One hundred percent complementarity (identity) is preferred, but complementarity as low as 50-75% can also be useful in this disclosure (see for example Werner and Uhlenbeck, 1995, Nucleic Acids Research, 23, 2092-2096; Hammann et al., 1999, 5 Antisense and Nucleic Acid Drug Dev., 9, 25-31). The enzymatic nucleic acids can be modified at the base, sugar, and/or phosphate groups. As described herein, the term "enzymatic nucleic acid" is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, aptazyme or aptamer-binding ribozyme, regulatable ribozyme, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, 10 minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid compounds with enzymatic activity. The specific enzymatic nucleic acid compounds described in the instant application are not limiting in the disclosure and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid compound of this disclosure is that it has a specific substrate binding site which is complementary to one or more of the 15 target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving and/or ligation activity to the molecule (Cech et al., U.S. Pat. No. 4,987,071; Cech et al., 1988, 260 JAMA 3030).

Several varieties of naturally-occurring enzymatic nucleic acids are currently known. Each can catalyze the hydrolysis of nucleic acid phosphodiester bonds in trans (and thus can 20 cleave other nucleic acid compounds) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target nucleic acid. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target nucleic acid. Thus, the enzymatic nucleic acid first recognizes and then binds a target nucleic acid through complementary base-pairing, 25 and once bound to the correct site, acts enzymatically to cut the target nucleic acid. Strategic cleavage of such a target nucleic acid will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its nucleic acid target, it is released from that nucleic acid to search for another target and can repeatedly bind and cleave new targets.

30 In a specific embodiment, the subject enzymatic nucleic acid is a ribozyme designed to catalytically cleave an mRNA transcripts to prevent translation of mRNA (see, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225; and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA at site-

specific recognition sequences can be used to destroy particular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNAs have the following sequence of two bases: 5'-UG-3'. The
5 construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. The ribozymes of the present disclosure also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena thermophila (known as the IVS or L-19 IVS RNA) and which has been extensively described (see, e.g., Zaug, et al., 1984, Science, 224:574-578;
10 Zaug and Cech, 1986, Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216).

In another specific embodiment, the subject enzymatic nucleic acid is a DNA enzyme. DNA enzymes incorporate some of the mechanistic features of both antisense and ribozyme
15 technologies. DNA enzymes are designed so that they recognize a particular target nucleic acid sequence, much like an antisense oligonucleotide, however much like a ribozyme they are catalytic and specifically cleave the target nucleic acid. Briefly, to design an ideal DNA enzyme that specifically recognizes and cleaves a target nucleic acid, one of skill in the art must first identify the unique target sequence. Preferably, the unique or substantially sequence is a G/C
20 rich of approximately 18 to 22 nucleotides. High G/C content helps insure a stronger interaction between the DNA enzyme and the target sequence. When synthesizing the DNA enzyme, the specific antisense recognition sequence that will target the enzyme to the message is divided so that it comprises the two arms of the DNA enzyme, and the DNA enzyme loop is placed between the two specific arms. Methods of making and administering DNA enzymes can be
25 found, for example, in U.S. Patent No. 6,110,462.

In certain embodiments, the nucleic acid therapeutic agents of the disclosure can be between 12 and 200 nucleotides in length. In one embodiment, exemplary enzymatic nucleic acid compounds of the disclosure are between 15 and 50 nucleotides in length, including, for example, between 25 and 40 nucleotides in length (for example see Jarvis et al., 1996, J. Biol.
30 Chem., 271, 29107-29112). In another embodiment, exemplary antisense molecules of the disclosure are between 15 and 75 nucleotides in length, including, for example, between 20 and 35 nucleotides in length (see for example Woolf et al., 1992, PNAS., 89, 7305-7309; Milner et al., 1997, Nature Biotechnology, 15, 537-541). In another embodiment, exemplary siRNAs of

the disclosure are between 20 and 27 nucleotides in length, including, for example, between 21 and 23 nucleotides in length. Those skilled in the art will recognize that all that is required is that the subject nucleic acid therapeutic agent be of length and conformation sufficient and suitable for catalyzing a reaction contemplated herein. The length of the nucleic acid therapeutic
5 agents of the instant disclosure is not limiting within the general limits stated.

III. Nucleic Acid Target Sites

Targets for useful nucleic acid compounds of the disclosure (e.g., antisense nucleic acids, dsRNA, and enzymatic nucleic acid compounds) can be determined as disclosed in Draper et al.,
10 30 WO 93/23569; Sullivan et al., WO 93/23057; Thompson et al., WO 94/02595; Draper et al., WO 95/04818; McSwiggen et al., U.S. Pat. No. 5,525,468. Other examples include the following PCT applications inactivation of expression of disease-related genes: WO 95/23225, WO 95/13380, WO 94/02595. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art.
15 Enzymatic nucleic acid compounds, siRNA and antisense to such targets are designed as described in those applications and synthesized to be tested in vitro and in vivo, as also described. For examples, the sequences of human Ephrin B2 and/or EphB4 RNAs are screened for optimal nucleic acid target sites using a computer-folding algorithm. Potential nucleic acid binding/cleavage sites are identified. For example, for enzymatic nucleic acid compounds of the
20 disclosure, the nucleic acid compounds are individually analyzed by computer folding (Jaeger et al., 1989 Proc. Natl Acad. Sci. USA, 86, 7706) to assess whether the sequences fold into the appropriate secondary structure. Those nucleic acid compounds with unfavorable intramolecular interactions such as between the binding arms and the catalytic core can be eliminated from consideration.
25 The subject nucleic acid (e.g., antisense, RNAi, and/or enzymatic nucleic acid compound) binding/cleavage sites are identified and are designed to anneal to various sites in the nucleic acid target (e.g., Ephrin B2 and/or EphB4). The binding arms of enzymatic nucleic acid compounds of the disclosure are complementary to the target site sequences described above. Antisense and RNAi sequences are designed to have partial or complete
30 complementarity to the nucleic acid target. The nucleic acid compounds can be chemically synthesized. The method of synthesis used follows the procedure for normal DNA/RNA synthesis as described below and in Usman et al., 1987 J Am. Chem. Soc., 109, 7845; Scaringe

et al., 1990 Nucleic Acids Res., 18, 5433; and Wincott et al., 1995 Nucleic Acids Res. 23, 2677-2684; Caruthers et al., 1992, Methods in Enzymology 211,3-19.

Additionally, it is expected that nucleic acid therapeutic agents having a CpG motif are at an increased likelihood of causing a non-specific immune response. Generally, CpG motifs include a CG (Cytosine-Guanosine) sequence adjacent to one or more purines in the 5' direction and one or more pyrimidines in the 3' direction. Lists of known CpG motifs are available in the art. Preferred nucleic acid therapeutics will be selected so as to have a selective effect on the target gene (possibly affecting other closely related genes) without triggering a generalized immune response. By avoiding nucleic acid therapeutics having a CpG motif, it is possible to decrease the likelihood that a particular nucleic acid will trigger an immune response.

IV. Synthesis of Nucleic acid Therapeutic Agents

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this disclosure, small nucleic acid motifs (small refers to nucleic acid motifs less than about 100 nucleotides in length, preferably less than about 80 nucleotides in length, and more preferably less than about 50 nucleotides in length (e.g., antisense oligonucleotides, enzymatic nucleic acids, and RNAi constructs) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of RNA structure.

Exemplary molecules of the instant disclosure are chemically synthesized, and others can similarly be synthesized. To illustrate, oligonucleotides (e.g., DNA) are synthesized using protocols known in the art as described in Caruthers et al., 1992, Methods in Enzymology 211, 3-19, Thompson et al., International PCT Publication No. WO 99/54459, Wincott et al., 1995, Nucleic Acids Res. 23, 2677-2684, Wincott et al., 1997, Methods Mol. Bio., 74, 59, Brennan et al., 1998, Biotechnol Bioeng., 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides. Alternatively, syntheses can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle.

Optionally, the nucleic acid compounds of the present disclosure can be synthesized separately and joined together post-synthetically, for example by ligation (Moore et al., 1992,

Science 256, 9923; Draper et al., International PCT publication No. WO 93/23569; Shabarova et al., 1991, Nucleic Acids Research 19, 4247; Bellon et al., 1997, Nucleosides & Nucleotides, 16, 951; Bellon et al., 1997, Bioconjugate Chem. 8, 204).

Preferably, the nucleic acid compounds of the present disclosure are modified extensively
5 to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, TIBS 17, 34; Usman et al., 1994, Nucleic Acids Symp. Ser. 31, 163). Ribozymes are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Wincott et al., Supra, the totality of which is hereby incorporated herein by
10 reference) and are re-suspended in water

V. Optimizing Activity of the Nucleic acid compounds

Nucleic acid compounds with modifications (e.g., base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases and thereby increase their potency. There are
15 several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid compounds with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and
20 Cedergren, 1992, TIBS. 17, 34; Usman et al., 1994, Nucleic Acids Symp. Ser. 31, 163; Burgin et al., 1996, Biochemistry, 35, 14090). Sugar modification of nucleic acid compounds have been extensively described in the art (see Eckstein et al., PCT Publication No. WO 92/07065; Perrault et al. Nature, 1990, 344, 565-568; Pieken et al. Science, 1991, 253, 314-317; Usman and Cedergren, Trends in Biochem. Sci., 1992, 17, 334-339; Usman et al. PCT Publication No. WO
25 93/15187; Sproat, U.S. Pat. No. 5,334,711 and Beigelman et al., 1995, J. Biol. Chem., 270, 25702; Beigelman et al., PCT publication No. WO 97/26270; Beigelman et al., U.S. Pat. No. 5,716,824; Usman et al., U.S. Pat. No. 5,627,053; Woolf et al., PCT Publication No. WO 98/13526; Thompson et al., U.S. S No. 60/082,404 which was filed on Apr. 20, 1998; Karpeisky et al., 1998, Tetrahedron Lett., 39, 1131; Earnshaw and Gait, 1998, Biopolymers (Nucleic acid
30 Sciences), 48, 39-55; Verma and Eckstein, 1998, Annu. Rev. Biochem., 67, 99-134; and Burlina et al., 1997, Bioorg. Med. Chem., 5, 1999-2010). Similar modifications can be used to modify the nucleic acid compounds of the instant disclosure.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, an over-abundance of these modifications can cause toxicity. Therefore, the amount of these internucleotide linkages should be evaluated and appropriately minimized when designing the nucleic acid compounds. The reduction in the concentration of these linkages should lower toxicity resulting in increased efficacy and higher specificity of these molecules.

In one embodiment, nucleic acid compounds of the disclosure include one or more G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example, Lin and Matteucci, 1998, J. Am. Chem. Soc., 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid compounds of the disclosure results in both enhanced affinity and specificity to nucleic acid targets. In another embodiment, nucleic acid compounds of the disclosure include one or more LNA (locked nucleic acid) nucleotides such as a 2', 4'-C mythylene bicyclo nucleotide (see for example Wengel et al., PCT Publication Nos. WO 00/66604 and WO 99/14226).

In another embodiment, the disclosure features conjugates and/or complexes of nucleic acid compounds targeting Ephrin B2 and/or EphB4. Such conjugates and/or complexes can be used to facilitate delivery of nucleic acid compounds into a biological system, such as cells. The conjugates and complexes provided by the instant disclosure can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid compounds of the disclosure.

The present disclosure encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid compounds of the disclosure into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically

active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable nucleic acid linker molecule" as used herein, refers to a nucleic acid compound that is designed as a biodegradable linker to connect one molecule to another
5 molecule, for example, a biologically active molecule. The stability of the biodegradable nucleic acid linker molecule can be modulated by using various combinations of ribonucleotides, deoxyribonucleotides, and chemically modified nucleotides, for example, 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer,
10 tetramer or longer nucleic acid compound, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications. The term
15 "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

Therapeutic nucleic acid compounds, such as the molecules described herein, delivered exogenously are optimally stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies
20 between hours to days depending upon the disease state. These nucleic acid compounds should be resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid compounds described in the instant disclosure and in the art have expanded the ability to modify nucleic acid compounds by introducing nucleotide modifications to enhance their nuclease stability as described above.

25 In another aspect the nucleic acid compounds comprise a 5' and/or a 3'-cap structure. By "cap structure," it is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see for example Wincott et al., WO 97/26270). These terminal modifications protect the nucleic acid compound from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at
30 the 3'-terminus (3'-cap) or can be present on both terminus. In non-limiting examples, the 5'-cap includes inverted abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate

linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Wincott et al, PCT publication No. WO 97/26270). In other non-limiting examples, the 3'-cap includes, for example, 4',5'-methylene nucleotide; 1-(bela-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threopentofuranosy nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, Tetrahedron 49, 1925).

VI. Soluble Polypeptides

In certain aspects, the invention relates to a soluble polypeptide comprising an extracellular domain of an Ephrin B2 protein (referred to herein as an Ephrin B2 soluble polypeptide—see Figures 12 and 13) or comprising an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide—see Figures 9-11). Preferably, the subject soluble polypeptide is a monomer and is capable of binding with high affinity to Ephrin B2 or EphB4. In a specific embodiment, the EphB4 soluble polypeptide of the invention comprises a globular domain of an EphB4 protein. Specific examples EphB4 soluble polypeptides are provided in published US Patent Application No. 20050249736.

As used herein, the subject soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide or an Ephrin B2 soluble polypeptide. These fragments, functional variants, and modified forms of the subject soluble polypeptides antagonize function of EphB4, Ephrin B2 or both.

In certain embodiments, isolated fragments of the subject soluble polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of

the nucleic acid encoding an EphB4 or Ephrin B2 soluble polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments that can
5 function to inhibit function of EphB4 or Ephrin B2, for example, by testing the ability of the fragments to inhibit angiogenesis or tumor growth.

In certain embodiments, a functional variant of an EphB4 soluble polypeptide comprises an amino acid sequence that is at least 90%, 95%, 97%, 99% or 100% identical to residues 1-197, 29-197, 1-312, 29-132, 1-321, 29-321, 1-326, 29-326, 1-412, 29-412, 1-427, 29-427, 1-429,
10 29-429, 1-526, 29-526, 1-537 and 29-537 of the amino acid sequence. Such polypeptides may be used in a processed form, and accordingly, in certain embodiments, an EphB4 soluble polypeptide comprises an amino acid sequence that is at least 90%, 95%, 97%, 99% or 100% identical to residues 16-197, 16-312, 16-321, 16-326, 16-412, 16-427, 16-429, 16-526 and 16-537 of the amino acid sequence.

15 In other embodiments, a functional variant of an Ephrin B2 soluble polypeptide comprises a sequence at least 90%, 95%, 97%, 99% or 100% identical to residues 1-225 of the amino acid sequence or a processed form, such as one comprising a sequence at least 90%, 95%, 97%, 99% or 100% identical to residues 26-225 of the amino acid sequence.

In certain embodiments, the present invention contemplates making functional variants
20 by modifying the structure of the subject soluble polypeptide for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified soluble polypeptide are considered functional equivalents of the naturally-occurring EphB4 or Ephrin B2 soluble polypeptide. Modified soluble polypeptides can be produced, for instance, by amino acid substitution, deletion, or
25 addition. For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are
30 related in their side chains.

This invention further contemplates a method of generating sets of combinatorial mutants of the EphB4 or Ephrin B2 soluble polypeptides, as well as truncation mutants, and is especially useful for identifying functional variant sequences. The purpose of screening such

combinatorial libraries may be to generate, for example, soluble polypeptide variants which can act as antagonists of EphB4, EphB2, or both. Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring soluble polypeptide. Such variant proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding wild-type soluble polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the protein of interest (e.g., a soluble polypeptide). Such variants, and the genes which encode them, can be utilized to alter the subject soluble polypeptide levels by modulating their half-life. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant soluble polypeptide levels within the cell. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential soluble polypeptide sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, soluble polypeptide variants (e.g., the antagonist forms) can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993) Gene 137:109-118; Grodberg et al., (1993) Eur. J.

Biochem. 218:597-601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation
5 mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying
10 truncated (bioactive) forms of the subject soluble polypeptide.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial
15 mutagenesis of the subject soluble polypeptides. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of
20 the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In certain embodiments, the subject soluble polypeptides of the invention include a a small molecule such as a peptide and a peptidomimetic. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that
25 contain non-naturally occurring amino acids, peptoids, and the like. Peptidomimetics provide various advantages over a peptide, including enhanced stability when administered to a subject. Methods for identifying a peptidomimetic are well known in the art and include the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known
30 crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). Where no crystal structure of a target molecule is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems;

San Leandro Calif.), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of the EphB4 or Ephrin B2 soluble polypeptides.

To illustrate, by employing scanning mutagenesis to map the amino acid residues of a soluble polypeptide which are involved in binding to another protein, peptidomimetic compounds can be generated which mimic those residues involved in binding. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) J. Med. Chem. 29:295; and Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al., (1985) Tetrahedron Lett 26:647; and Sato et al., (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al., (1985) Biochem Biophys Res Commun 126:419; and Dann et al., (1986) Biochem Biophys Res Commun 134:71).

In certain embodiments, the soluble polypeptides of the invention may further comprise post-translational modifications. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified soluble polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a soluble polypeptide may be tested for its antagozing role in EphB4 or Ephrin B2 function, e.g, it inhibitory effect on angiogenesis or on tumor growth.

In certain aspects, functional variants or modified forms of the subject soluble polypeptides include fusion proteins having at least a portion of the soluble polypeptide and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, and an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), which are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt- conjugated resins are used. Another fusion domain well known in the art is green fluorescent protein (GFP). Fusion

domains also include "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain embodiments, the soluble polypeptides of the present invention contain one or more modifications that are capable of stabilizing the soluble polypeptides. For example, such modifications enhance the in vitro half life of the soluble polypeptides, enhance circulatory half life of the soluble polypeptides or reducing proteolytic degradation of the soluble polypeptides.

In certain embodiments, soluble polypeptides (unmodified or modified) of the invention can be produced by a variety of art-known techniques. For example, such soluble polypeptides can be synthesized using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In addition, automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Biosearch 9600). Alternatively, the soluble polypeptides, fragments or variants thereof may be recombinantly produced using various expression systems as is well known in the art (also see below).

VII. Nucleic acids encoding soluble polypeptides

In certain aspects, the invention relates to isolated and/or recombinant nucleic acids encoding an EphB4 or Ephrin B2 soluble polypeptide. The subject nucleic acids may be single-stranded or double-stranded, DNA or RNA molecules. These nucleic acids are useful as therapeutic agents. For example, these nucleic acids are useful in making recombinant soluble polypeptides which are administered to a cell or an individual as therapeutics. Alternative, these nucleic acids can be directly administered to a cell or an individual as therapeutics such as in gene therapy.

In certain embodiments, the invention provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to a region of the nucleotide sequence. One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to the subject nucleic acids, and variants of the subject nucleic acids

are also within the scope of this invention. In further embodiments, the nucleic acid sequences of the invention can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence or complement sequences thereof. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the subject nucleic acids due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In certain embodiments, the recombinant nucleic acids of the invention may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate for a host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known

in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

In certain aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an EphB4 or Ephrin B2 soluble polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the soluble polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a soluble polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

This invention also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject soluble polypeptide. The host cell may be any prokaryotic or eukaryotic cell. For example, a soluble polypeptide of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression
5 system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

VIII. Antibodies

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

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

EphB4 receptor dimerizes and autophosphorylates the juxtamembrane tyrosine residues to acquire full activation.

As used herein, the term "EphB4" refers to an EphB4 polypeptide from a mammal including humans. In one embodiment, the antibodies (immunoglobulins) are raised against an isolated and/or recombinant mammalian EphB4 or portion thereof (e.g., peptide) or against a host cell which expresses recombinant mammalian EphB4. In certain aspects, antibodies of the invention specifically bind to an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide). For example, an EphB4 soluble polypeptide comprises a globular domain and is capable of binding to Ephrin B2. As used herein, the EphB4 soluble polypeptides include fragments, functional variants, and modified forms of EphB4 soluble polypeptide.

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

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

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

An "antibody" refers to an intact immunoglobulin or to an antigen-binding portion
5 thereof that competes with the intact antibody for specific binding, and is intended to include monoclonal and polyclonal antibodies. An "isolated antibody" is simply an antibody that is substantially purified or produced so as to be free of other species of antibodies that bind to the same target. Monoclonal antibodies and most recombinant antibody forms are isolated, while an antibody species present in a polyclonal antibody mixture is not isolated. Antigen-binding
10 portions may be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. Antigen-binding portions include, inter alia, Fab, Fab', F(ab')2, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric antibodies, diabodies and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the
15 polypeptide. The terms "anti-EphB4 antibody" and "EphB4 antibody" are used interchangeably herein.

A Fab fragment is a monovalent fragment consisting of the VL, VH, CL and CH 1 domains; a F(ab').sub.2 fragment is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consists of the VH and CH1 domains; an Fv
20 fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment (Ward et al., Nature 341:544-546, 1989) consists of a VH domain.

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

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

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

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

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

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

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

Applicants have generated a number of monoclonal antibodies against EphB4 as well as hybridoma cell lines producing EphB4 monoclonal antibodies. These antibodies were further characterized in many ways, such as, their ability to inhibit interaction between EphB4 and its ligand (e.g., Ephrin B2), their ability to inhibit dimerization or multimerization of EphB4 receptor, their ability to induce tyrosine phosphorylation of EphB4, their cross-reactivity with other Eph family members, their ability to inhibit angiogenesis, and their ability to inhibit tumor growth. Further, epitope mapping studies reveals that these EphB4 antibodies may specifically bind to one or more regions of EphB4 (e.g., a globular domain, a cystein-rich domain, or a fibronectin type III domain). For example, an EphB4 antibody may bind to both fibronectin type 3 domains.

In certain aspects, antibodies of the invention specifically bind to an extracellular domain (ECD) of an EphB4 protein (also referred to herein as a soluble EphB4 polypeptide; also see Figure 9-11). A soluble EphB4 polypeptide may comprise a sequence encompassing the globular (G) domain (amino acids 29-197 of SEQ ID NO: 7), and optionally additional domains,

such as the cysteine-rich domain (amino acids 239-321 of SEQ ID NO: 7), the first fibronectin type 3 domain (amino acids 324-429 of SEQ ID NO: 7) and the second fibronectin type 3 domain (amino acids 434-526 of SEQ ID NO: 7) Exemplary EphB4 soluble polypeptides are provided in Figures 9-11. As used herein, the EphB4 soluble polypeptides include fragments,
5 functional variants, and modified forms of EphB4 soluble polypeptide.

In certain aspects, the present invention provides antibodies (anti-EphB4) having binding specificity for an EphB4 or a portion of EphB4. Examples of these antibodies include, but are not limited to, EphB4 Mab 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, and 138, as shown in Figure 8. Also included is Mab 265 (not shown in diagram). Optionally, the
10 immunoglobulins can bind to EphB4 with an affinity of at least about 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} M or less. Optionally, antibodies and portions thereof bind to EphrinB2 with an affinity that is roughly equivalent to that of a soluble extracellular EphB4 polypeptide comprising the globular ligand binding domain. Antibodies disclosed herein will preferably be specific for EphB4, with minimal binding to other members of the Eph or Ephrin families.

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

30 In certain embodiments, antibodies of the present invention bind to one or more specific domain of EphB4. For example, an antibody binds to one or more extracellular domains of EphB4 (such as the globular domain, the cystein-rich domain, and the first fibronectin type 3 domain, and the second fibronectin type 3 domain). For example, EphB4 antibody (Mab) Nos.

1, 23, 35, and 79 bind to an epitope in the region spanning amino acids 16-198 of the sequence in SEQ ID NO: 7, spanning the globular domain. EphB4 antibody Nos. 85L, 85H, 91, and 131 bind to an epitope in the region spanning amino acids 324-429, including the first fibronectin type 3 domain. EphB4 antibody Nos. 47, 57, 85H, 98, 121, and 138 bind to an epitope in the
5 region spanning amino acids 430-537, including the second fibronectin type 3 domain. Optionally, the subject antibody (e.g., EphB4 antibody No. 85H) can bind to at least two domains of an EphB4 (Figure 8).

The anti-EphB4 antibody may be an IgG, an IgM, an IgE, an IgA or an IgD molecule. In a preferred embodiment, the antibody is an IgG and is an IgG1, IgG2, IgG3 or IgG4 subtype. In
10 a more preferred embodiment, the anti-EphB4 antibody is subclass IgG2. The class and subclass of EphB4 antibodies may be determined by any method known in the art. In general, the class and subclass of an antibody may be determined using antibodies that are specific for a particular class and subclass of antibody. Such antibodies are available commercially. The class and subclass can be determined by ELISA, Western Blot as well as other techniques.
15 Alternatively, the class and subclass may be determined by sequencing all or a portion of the constant domains of the heavy and/or light chains of the antibodies, comparing their amino acid sequences to the known amino acid sequences of various class and subclasses of immunoglobulins, and determining the class and subclass of the antibodies. To illustrate, the classes and subclasses of the exemplary EphB4 antibodies are shown in Table 3 below.

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

In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen binding function of a corresponding full-length antibody (e.g., specificity for an EphB4). Certain preferred functional fragments retain the ability to inhibit one or more functions characteristic of an EphB4, such as a binding activity, a signaling activity, and/or stimulation of a cellular response. For example, in one embodiment, a functional fragment of an EphB4 antibody can inhibit the interaction of EphB4 with one or more of its ligands (e.g., Ephrin B2) and/or can inhibit one or more receptor-mediated functions, such as cell migration, cell proliferation, angiogenesis, and/or tumor growth.

For example, antibody fragments capable of binding to an EphB4 receptor or portion thereof, including, but not limited to, Fv, Fab, Fab' and $F(ab')_2$ fragments are encompassed by the invention. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can generate Fab or $F(ab')_2$ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a $F(ab')_2$ heavy chain portion can be designed to include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain.

The term "humanized immunoglobulin" as used herein refers to an immunoglobulin comprising portions of immunoglobulins of different origin, wherein at least one portion is of human origin. Accordingly, the present invention relates to a humanized immunoglobulin having binding specificity for an EphB4 (e.g., human EphB4), said immunoglobulin comprising an antigen binding region of nonhuman origin (e.g., rodent) and at least a portion of an immunoglobulin of human origin (e.g., a human framework region, a human constant region or portion thereof). For example, the humanized antibody can comprise portions derived from an immunoglobulin of nonhuman origin with the requisite specificity, such as a mouse, and from immunoglobulin sequences of human origin (e.g., a chimeric immunoglobulin), joined together chemically by conventional techniques (e.g., synthetic) or prepared as a contiguous polypeptide using genetic engineering techniques (e.g., DNA encoding the protein portions of the chimeric antibody can be expressed to produce a contiguous polypeptide chain).

Another example of a humanized immunoglobulin of the present invention is an immunoglobulin containing one or more immunoglobulin chains comprising a CDR of

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

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

In other embodiments, the present invention provides EphB4 kinase activating antibodies. Such antibodies enhance EphB4 kinase activity, even independent of EphrinB2. In some instances, such antibodies may be used to stimulate EphB4. However, applicants note that 20 in most cell-based and in vivo assays, such antibodies surprisingly behaved like antagonist antibodies. Such antibodies appear to bind to-the fibronectin type III domains, particularly the region of amino acids 327-427 of EphB4.

In certain embodiments, anti-idiotypic antibodies are also provided. Anti-idiotypic antibodies recognize antigenic determinants associated with the antigen-binding site of another 25 antibody. Anti-idiotypic antibodies can be prepared against a second antibody by immunizing an animal of the same species, and preferably of the same strain, as the animal used to produce the second antibody. See e.g., U.S. Pat. No. 4,699,880. In one embodiment, antibodies are raised against receptor or a portion thereof, and these antibodies are used in turn to produce an anti-idiotypic antibody. The anti-idiotypic antibodies produced thereby can bind compounds 30 which bind receptor, such as ligands of receptor function, and can be used in an immunoassay to detect or identify or quantitate such compounds. Such an anti-idotypic antibody can also be an inhibitor of an EphB4 receptor function, although it does not bind receptor itself. Such an anti-idotypic antibody can also be called an antagonist antibody.

In certain aspects, the present invention provides the hybridoma cell lines, as well as to the monoclonal antibodies produced by these hybridoma cell lines. The cell lines of the present invention have uses other than for the production of the monoclonal antibodies. For example, the cell lines of the present invention can be fused with other cells (such as suitably drug-marked
5 human myeloma, mouse myeloma, human-mouse heteromyeloma or human lymphoblastoid cells) to produce additional hybridomas, and thus provide for the transfer of the genes encoding the monoclonal antibodies. In addition, the cell lines can be used as a source of nucleic acids encoding the anti-EphB4 immunoglobulin chains, which can be isolated and expressed (e.g., upon transfer to other cells using any suitable technique (see e.g., Cabilly et al., U.S. Pat. No.
10 4,816,567; Winter, U.S. Pat. No. 5,225,539)). For instance, clones comprising a rearranged anti-EphB4 light or heavy chain can be isolated (e.g., by PCR) or cDNA libraries can be prepared from mRNA isolated from the cell lines, and cDNA clones encoding an anti-EphB4 immunoglobulin chain can be isolated. Thus, nucleic acids encoding the heavy and/or light chains of the antibodies or portions thereof can be obtained and used in accordance with
15 recombinant DNA techniques for the production of the specific immunoglobulin, immunoglobulin chain, or variants thereof (e.g., humanized immunoglobulins) in a variety of host cells or in an in vitro translation system. For example, the nucleic acids, including cDNAs, or derivatives thereof encoding variants such as a humanized immunoglobulin or immunoglobulin chain, can be placed into suitable prokaryotic or eukaryotic vectors (e.g.,
20 expression vectors) and introduced into a suitable host cell by an appropriate method (e.g., transformation, transfection, electroporation, infection), such that the nucleic acid is operably linked to one or more expression control elements (e.g., in the vector or integrated into the host cell genome). For production, host cells can be maintained under conditions suitable for expression (e.g., in the presence of inducer, suitable media supplemented with appropriate salts,
25 growth factors, antibiotic, nutritional supplements, etc.), whereby the encoded polypeptide is produced. If desired, the encoded protein can be recovered and/or isolated (e.g., from the host cells or medium). It will be appreciated that the method of production encompasses expression in a host cell of a transgenic animal (see e.g., WO 92/03918, GenPharm International, published Mar. 19, 1992).

30 Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed as described herein, or using other suitable techniques. A variety of methods have been described. See e.g., Kohler et al., Nature, 256: 495-497 (1975) and Eur. J. Immunol. 6: 511-519 (1976); Milstein et al., Nature 266: 550-552 (1977); Koprowski et al., U.S. Pat. No.

4,172,124; Harlow, E. and D. Lane, 1988, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.); Current Protocols In Molecular Biology, Vol. 2 (Supplement 27, Summer '94), Ausubel, F. M. et al., Eds., (John Wiley & Sons: New York, N.Y.), Chapter 11, (1991). Generally, a hybridoma can be produced by fusing a suitable
5 immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cell, preferably those of the spleen or lymph nodes, are obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

10 Other suitable methods of producing or isolating antibodies of the requisite specificity can used, including, for example, methods which select recombinant antibody from a library, or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a full repertoire of human antibodies. See e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90: 2551-2555 (1993); Jakobovits et al., Nature, 362: 255-258 (1993); Lonberg et al., U.S. Pat. No.
15 5,545,806; Surani et al., U.S. Pat. No. 5,545,807.

To illustrate, immunogens derived from an EphB4 polypeptide (e.g., an EphB4 polypeptide or an antigenic fragment thereof which is capable of eliciting an antibody response, or an EphB4 fusion protein) can be used to immunize a mammal, such as a mouse, a hamster or rabbit. See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold
20 Spring Harbor Press: 1988). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an EphB4 polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels
25 of antibodies. In one embodiment, antibodies of the invention are specific for the extracellular portion of the EphB4 protein or fragments thereof. In another embodiment, antibodies of the invention are specific for the intracellular portion or the transmembrane portion of the EphB4 protein.

Following immunization of an animal with an antigenic preparation of an EphB4
30 polypeptide, antisera can be obtained and, if desired, polyclonal antibodies can be isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well

known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an EphB4 polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

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

In certain embodiments, antibodies of the present invention are further intended to include bispecific, single-chain, and chimeric and humanized molecules having affinity for an EphB4 polypeptide conferred by at least one CDR region of the antibody. Techniques for the production of single chain antibodies (US Patent No. 4,946,778) can also be adapted to produce single chain antibodies. Also, transgenic mice or other organisms including other mammals, may be used to express humanized antibodies. Methods of generating these antibodies are known in the art. See, e.g., Cabilly et al., U.S. Pat. No. 4,816,567; Cabilly et al., European Patent No. 0,125,023 B1; Queen et al., European Patent No. 0,451,216 B1; Boss et al., U.S. Pat. No. 4,816,397; Boss et al., European Patent No. 0,120,694 E1; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; winter, European Patent No. 0,239,400 B1; Padlan, E. A. et al., European Patent Application No. 0,519,596 A1. See also, Ladner et al., U.S. Pat. No. 4,946,778; Huston, U.S. Pat. No. 5,476,786; and Bird, R. E. et al., Science, 242: 423-426 (1988)).

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

methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutagenized, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber et al., U.S. Pat. No. 5,514,548; Hoogenboom et al., WO 93/06213, published Apr. 1, 1993)).

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

In certain preferred embodiments, an antibody of the invention is a monoclonal antibody, and in certain embodiments the invention makes available methods for generating novel antibodies. For example, a method for generating a monoclonal antibody that binds
30 specifically to an EphB4 polypeptide may comprise administering to a mouse an amount of an immunogenic composition comprising the EphB4 polypeptide effective to stimulate a detectable immune response, obtaining antibody-producing cells (e.g., cells from the spleen) from the mouse and fusing the antibody–producing cells with myeloma cells to obtain antibody-

producing hybridomas, and testing the antibody-producing hybridomas to identify a hybridoma that produces a monocolonal antibody that binds specifically to the EphB4 polypeptide. Once obtained, a hybridoma can be propagated in a cell culture, optionally in culture conditions where the hybridoma-derived cells produce the monoclonal antibody that binds specifically to EphB4 polypeptide. The monoclonal antibody may be purified from the cell culture.

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

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

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

In certain embodiments, the antibodies or antigen binding fragments of the antibodies can be labeled or unlabeled for diagnostic purposes. Typically, diagnostic assays entail detecting the formation of a complex resulting from the binding of an antibody to EphB4. The antibodies can be directly labeled. A variety of labels can be employed, including, but not limited to, radionuclides, fluorescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors and ligands (e.g., biotin, haptens). Numerous appropriate immunoassays are known to the skilled artisan (see, for example, U.S. Pat. Nos. 3,817,827; 3,850,752; 3,901,654; and

4,098,876). When unlabeled, the antibodies can be used in assays, such as agglutination assays. Unlabeled antibodies can also be used in combination with another (one or more) suitable reagent which can be used to detect antibody, such as a labeled antibody (e.g., a second antibody) reactive with the first antibody (e.g., anti-idiotype antibodies or other antibodies that are specific for the unlabeled immunoglobulin) or other suitable reagent (e.g., labeled protein A).

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

In certain aspects, kits for use in detecting the presence of an EphB4 protein in a biological sample can also be prepared. Such kits will include an antibody which binds to an EphB4 protein or portion of said receptor, as well as one or more ancillary reagents suitable for detecting the presence of a complex between the antibody and EphB4 or portion thereof. Examples of these antibodies include, but are not limited to, EphB4 Mab 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, 138, and 265 (see published US Patent Application 20050249736). The antibody compositions of the present invention can be provided in lyophilized form, either alone or in combination with additional antibodies specific for other epitopes. The antibodies, which can be labeled or unlabeled, can be included in the kits with adjunct ingredients (e.g., buffers, such as Tris, phosphate and carbonate, stabilizers, excipients, biocides and/or inert proteins, e.g., bovine serum albumin). For example, the antibodies can be provided as a lyophilized mixture with the adjunct ingredients, or the adjunct ingredients can be separately provided for combination by the user. Generally these adjunct materials will be present in less than about 5% weight based on the amount of active antibody, and usually will be present in a total amount of at least about 0.001% weight based on antibody concentration. Where a second

antibody capable of binding to the monoclonal antibody is employed, such antibody can be provided in the kit, for instance in a separate vial or container. The second antibody, if present, is typically labeled, and can be formulated in an analogous manner with the antibody formulations described above.

5 Similarly, the present invention also relates to a method of detecting and/or quantifying expression of an EphB4 or portion of the receptor by a cell, wherein a composition comprising a cell or fraction thereof (e.g., membrane fraction) is contacted with an antibody which binds to an EphB4 or portion of the receptor under conditions appropriate for binding of the antibody thereto, and antibody binding is monitored. Detection of the antibody, indicative of the 10 formation of a complex between antibody and EphB4 or a portion thereof, indicates the presence of the receptor. Binding of antibody to the cell can be determined by standard methods, such as those described in the working examples. The method can be used to detect expression of EphB4 on cells from an individual. Optionally, a quantitative expression of EphB4 on the surface of endothelial cells can be evaluated, for instance, by flow cytometry, and the staining 15 intensity can be correlated with disease susceptibility, progression or risk.

The present invention also relates to a method of detecting the susceptibility of a mammal to certain diseases. To illustrate, the method can be used to detect the susceptibility of a mammal to diseases which progress based on the amount of EphB4 present on cells and/or the number of EphB4-positive cells in a mammal. In one embodiment, the invention relates to a 20 method of detecting susceptibility of a mammal to a tumor. In this embodiment, a sample to be tested is contacted with an antibody which binds to an EphB4 or portion thereof under conditions appropriate for binding of said antibody thereto, wherein the sample comprises cells which express EphB4 in normal individuals. The binding of antibody and/or amount of binding is detected, which indicates the susceptibility of the individual to a tumor, wherein higher levels 25 of receptor correlate with increased susceptibility of the individual to a tumor. Applicants and other groups have found that expression of EphB4 has a correlation with tumor growth and progression. The antibodies of the present invention can also be used to further elucidate the correlation of EphB4 expression with progression of angiogenesis-associated diseases in an individual.

30 Examples of EphB4 antibodies that may be used in the methods of the disclosure include mouse monoclonal antibodies 47 and 131, which have been described and characterized in US2005/0249736, which is hereby incorporated by reference in its entirety.

The CDR regions for the heavy chain of mouse monoclonal antibody (Mab) 47 are defined as SEQ ID NO:8 (CDR1), SEQ ID NO:9 (CDR2), and SEQ ID NO:10 (CDR3). The CDR regions for the light chain of mouse monoclonal antibody 47 are defined as SEQ ID NO:11 (CDR1), SEQ ID NO:12 (CDR2), and SEQ ID NO:13 (CDR3).

5 The CDR regions for the heavy chain of mouse monoclonal antibody 131 are defined as SEQ ID NO:14 (CDR1), SEQ ID NO:15 (CDR2), and SEQ ID NO:16 (CDR3). The CDR regions for the light chain of mouse monoclonal antibody 131 are defined as SEQ ID NO:17 (CDR1), SEQ ID NO:18 (CDR2), and SEQ ID NO:19 (CDR3).

The following are the sequences of the antibody variable domains and CDRs. The heavy
10 chain of mouse monoclonal antibody 47 (47VH), 47H1 (SEQ ID NO: 20), 47H2 (SEQ ID NO: 21), 47H3 (SEQ ID NO: 22), 47H4 (SEQ ID NO: 23), and 47H5(SEQ ID NO: 24). The light chain of #47 (47VL), 47K1 (SEQ ID NO: 25), 47K2 (SEQ ID NO: 26), 47K3 (SEQ ID NO: 27), and 47K4 (SEQ ID NO: 28). The heavy chain of mouse monoclonal antibody 131 (131VH), 131HV1 (SEQ ID NO:29), 131HV2 (SEQ ID NO:30), 131HV3 (SEQ ID NO:31), 131HV4
15 (SEQ ID NO:32), and 131HV5 (SEQ ID NO:33). The light chain of #131 (131VL), 131KV1 (SEQ ID NO:34), 131KV2 (SEQ ID NO:35), 131KV3 (SEQ ID NO:36), and 131KV4 (SEQ ID NO:37).

IX. Small Molecules

20 The inhibitors of the present application also include small molecules, which may inhibit the activity of proteins with enzymatic function, and/or the interactions of said proteins. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, less than 5,000, less than 1,000, or less than 500 daltons. This class of inhibitors includes chemically synthesized
25 molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the EphB4 protein or may be identified by screening compound libraries. Alternative appropriate inhibitors of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries
30 for EphB4-inhibiting activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science 151: 1964-1969(2000); Radmann J. and Gunther J., Science 151: 1947-1948 (2000)).

X. Diagnostic Methods

Antibodies and nucleic acid compositions disclosed herein are useful in diagnostic and prognostic evaluation of diseases and disorders, particularly cancers associated with EphB4 expression. At each stage of disease, compositions may be used to improve diagnostic accuracy and facilitate treatment decisions. Unlike standard diagnostic methods for tumors and cancer, such as computed topographic (CT) scans, which depend on a change in size or architecture of organs or lymph nodes, labeled antibodies can detect abnormal cells at an early stage, because of their expression of tumor antigens, such as EphB4. Once cancer is diagnosed, accurate staging is important in deciding on the most appropriate therapy. Later, during follow-up of surgery, rising serum levels of tumor antigens may indicate recurrence before it can be detected by conventional methods. These methods may be used with any of the compositions described herein.

Methods of diagnosis can be performed in vitro using a cellular sample (e.g., blood sample, lymph node biopsy or tissue) from a patient or can be performed by in vivo imaging.

In particular embodiments, the present application provides an antibody conjugate wherein the antibodies of the present application are conjugated to a diagnostic imaging agent. Compositions comprising the antibodies of the present application can be used to detect EphB4, for example, by radioimmunoassay, ELISA, FACS, etc. One or more detectable labels can be attached to the antibodies. Exemplary labeling moieties include radiopaque dyes, radiocontrast agents, fluorescent molecules, spin-labeled molecules, enzymes, or other labeling moieties of diagnostic value, particularly in radiologic or magnetic resonance imaging techniques.

A radiolabeled antibody in accordance with this disclosure can be used for in vitro diagnostic tests. The specific activity of an antibody, binding portion thereof, probe, or ligand, depends upon the half-life, the isotopic purity of the radioactive label, and how the label is incorporated into the biological agent. In immunoassay tests, the higher the specific activity, in general, the better the sensitivity. Radioisotopes useful as labels, e.g., for use in diagnostics, include iodine (^{131}I or ^{125}I), indium (^{111}In), technetium (^{99}Tc), phosphorus (^{32}P), carbon (^{14}C), and tritium (^{3}H), or one of the therapeutic isotopes listed above.

The radiolabeled antibody can be administered to a patient where it is localized to cancer cells bearing the antigen with which the antibody reacts, and is detected or "imaged" in vivo using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. See e.g., Bradwell et al., "Developments in Antibody Imaging", Monoclonal Antibodies for Cancer Detection and Therapy, Baldwin et al., (eds.), pp. 65-85 (Academic Press

1985), which is hereby incorporated by reference. Alternatively, a positron emission transaxial tomography scanner, such as designated Pet VI located at Brookhaven National Laboratory, can be used where the radiolabel emits positrons (e.g., ^{11}C, ^{18}F, ^{15}O, and ^{13}N).

Fluorophore and chromophore labeled biological agents can be prepared from standard moieties known in the art. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties may be selected to have substantial absorption at wavelengths above 310 nm, such as for example, above 400 nm. A variety of suitable fluorescers and chromophores are described by Stryer, Science, 162:526 (1968) and Brand et al., Annual Review of Biochemistry, 41:843-868 (1972), which are hereby incorporated by reference. The antibodies can be labeled with fluorescent chromophore groups by conventional procedures such as those disclosed in U.S. Patent Nos. 3,940,475, 4,289,747, and 4,376,110, which are hereby incorporated by reference.

The present application provides for a method of detecting a cancer comprising detecting the differential expression of mRNA or protein of EphB4 in said cancer cells in a subject in need of such detection. In one exemplary embodiment, the method of detecting cancer comprising: a) isolating a sample from a patient; b) contacting cells of said sample with the antibodies of the present application; c) contacting non-cancerous cells of the same type of said sample cells with the antibodies of the present application; and d) detecting and comparing the difference of expression of EphB4 in said sample cells with the non-cancerous cells.

In certain embodiments, antibody conjugates or nucleic acid compositions for diagnostic use in the present application are intended for use in vitro, where the composition is linked to a secondary binding ligand or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase and glucose oxidase. In certain embodiments, secondary binding ligands are biotin and avidin or streptavidin compounds.

In certain embodiments the diagnostic methods of the application may be used in combination with other ovarian cancer diagnostic tests.

The present application also provides for a diagnostic kit comprising anti- EphB4 antibodies or nucleic acid compositions that bind EphB4. Such a diagnostic kit may further comprise a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and co-factors required by the enzyme. In addition, other additives may be included such as stabilizers, buffers and the like. The relative amounts of the various reagents

may be varied widely to provide for concentrations in solution of the reagents that substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients that, on dissolution, will provide a reagent solution having the appropriate concentration.

5 In another aspect, the present application concerns immunoassays for binding, purifying, quantifying and otherwise generally detecting EphB4 protein components. As detailed below, immunoassays, in their most simple and direct sense, are binding assays. In certain embodiments, immunoassays are the various types of enzyme linked immunoadsorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection 10 using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and Western blotting, dot and slot blotting, FACS analyses, and the like may also be used.

The steps of various useful immunoassays have been described in the scientific literature, such as, e.g., Nakamura et al., in Enzyme Immunoassays: Heterogeneous and 15 Homogeneous Systems, Chapter 27 (1987), incorporated herein by reference.

In general, the immunobinding methods include obtaining a sample suspected of containing a protein or peptide, in this case, EphB4 and contacting the sample with a first antibody immunoreactive with EphB4 under conditions effective to allow the formation of immunocomplexes.

20 Immunobinding methods include methods for purifying EphB4 proteins, as may be employed in purifying protein from patients' samples or for purifying recombinantly expressed protein. They also include methods for detecting or quantifying the amount of EphB4 in a tissue sample, which requires the detection or quantification of any immune complexes formed during the binding process.

25 The biological sample analyzed may be any sample that is suspected of containing EphB4 such as a homogenized neoplastic ovarian tissue sample. Contacting the chosen biological sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of primary immune complexes) is generally a matter of adding the antibody composition to the sample and incubating the mixture for a period of time long enough 30 for the antibodies to form immune complexes with, i.e., to bind to, any EphB4 present. The sample-antibody composition is washed extensively to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are based upon the detection of radioactive, fluorescent, biological or enzymatic tags. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The anti- EphB4 antibody used in the detection may itself be conjugated to a detectable label, wherein one would then simply detect this label. The amount of the primary immune complexes in the composition would, thereby, be determined.

Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are washed extensively to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complex is detected.

An enzyme linked immunoadsorbent assay (ELISA) is a type of binding assay. In one type of ELISA, anti- EphB4 antibodies used in the diagnostic method of this application are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a suspected neoplastic tissue sample is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound EphB4 may be detected. Detection is generally achieved by the addition of another anti- EphB4 antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA." Detection may also be achieved by the addition of a second anti- EphB4 antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another type of ELISA, the neoplastic ovarian tissue samples are immobilized onto the well surface and then contacted with the anti- EphB4 antibodies used in this application. After binding and washing to remove non-specifically bound immune complexes, the bound anti-EphB4 antibodies are detected. Where the initial anti- EphB4 antibodies are linked to a detectable label, the immune complexes may be detected directly. Alternatively, the immune complexes may be detected using a second antibody that has binding affinity for the first anti-

EphB4 antibody, with the second antibody being linked to a detectable label.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes.

5 The radioimmunoassay (RIA) is an analytical technique which depends on the competition (affinity) of an antigen for antigen-binding sites on antibody molecules. Standard curves are constructed from data gathered from a series of samples each containing the same known concentration of labeled antigen, and various, but known, concentrations of unlabeled antigen. Antigens are labeled with a radioactive isotope tracer. The mixture is incubated in 10 contact with an antibody. Then the free antigen is separated from the antibody and the antigen bound thereto. Then, by use of a suitable detector, such as a gamma or beta radiation detector, the percent of either the bound or free labeled antigen or both is determined. This procedure is repeated for a number of samples containing various known concentrations of unlabeled antigens and the results are plotted as a standard graph. The percent of bound tracer antigens is 15 plotted as a function of the antigen concentration. Typically, as the total antigen concentration increases the relative amount of the tracer antigen bound to the antibody decreases. After the standard graph is prepared, it is thereafter used to determine the concentration of antigen in samples undergoing analysis.

In an analysis, the sample in which the concentration of antigen is to be determined is 20 mixed with a known amount of tracer antigen. Tracer antigen is the same antigen known to be in the sample but which has been labeled with a suitable radioactive isotope. The sample with tracer is then incubated in contact with the antibody. Then it can be counted in a suitable detector which counts the free antigen remaining in the sample. The antigen bound to the antibody or immunoadsorbent may also be similarly counted. Then, from the standard curve, the 25 concentration of antigen in the original sample is determined.

XI. Methods of Treatment

In certain embodiments, the present disclosure provides methods of inhibiting or reducing tumor growth and methods of treating an individual suffering from cancer, such as for 30 example ovarian cancer. These methods involve administering to the individual a therapeutically effective amount of one or more therapeutic agents as described above. These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more

particularly, humans. In certain embodiments,,the cancer (e.g., ovarian cancer) is angiogenesis-dependent, In other embodiments, the cancer (e.g., ovarian cancer) is angiogenesis-independent.

As described herein, the tumor includes a tumor inside an individual, a tumor xenograft, or a tumor cultured in vitro. In particular, therapeutic agents of the present disclosure are useful for treating or preventing an ovarian cancer of any type including epithelial, germ cell, and sex cord stromal.

In certain embodiments of such methods, one or more therapeutic agents can be administered, together (simultaneously) or at different times (sequentially). In addition, therapeutic agents can be administered with another type of compounds for treating cancer or for inhibiting angiogenesis.

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

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

When a therapeutic agent of the present disclosure is administered in combination with another conventional anti-neoplastic agent, either concomitantly or sequentially, such therapeutic agent is shown to enhance the therapeutic effect of the anti-neoplastic agent or overcome cellular resistance to such anti-neoplastic agent. This allows decrease of dosage of an

anti-neoplastic agent, thereby reducing the undesirable side effects, or restores the effectiveness of an anti-neoplastic agent in resistant cells.

Pharmaceutical compounds that may be used for combinatory anti-tumor therapy include, merely to illustrate: aminoglutethimide, amsacrine, anastrozole, asparaginase, bcg,
5 bicalutamide, bleomycin, buserelin, busulfan, campothecin, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, colchicine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, genistein,
10 goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan, ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole, octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, suramin,
15 tamoxifen, temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride, topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and vinorelbine.

These chemotherapeutic anti-tumor compounds may be categorized by their mechanism of action into, for example, following groups: anti-metabolites/anti-cancer agents, such as pyrimidine analogs (5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine) and
20 purine analogs, folate antagonists and related inhibitors (mercaptopurine, thioguanine, pentostatin and 2-chlorodeoxyadenosine (cladribine)); antiproliferative/antimitotic agents including natural products such as vinca alkaloids (vinblastine, vincristine, and vinorelbine), microtubule disruptors such as taxane (paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and navelbine, epidipodophyllotoxins (etoposide, teniposide), DNA damaging
25 agents (actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchlorehtamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol, taxotere, teniposide, triethylenethiophosphoramide and etoposide (VP16)); antibiotics such as
30 dactinomycin (actinomycin D), daunorubicin, doxorubicin (adriamycin), idarubicin, anthracyclines, mitoxantrone, bleomycins, plicamycin (mithramycin) and mitomycin; enzymes (L-asparaginase which systemically metabolizes L-asparagine and deprives cells which do not have the capacity to synthesize their own asparagine); antiplatelet agents;

antiproliferative/antimitotic alkylating agents such as nitrogen mustards (mechlorethamine, cyclophosphamide and analogs, melphalan, chlorambucil), ethylenimines and methylmelamines (hexamethylmelamine and thiotepa), alkyl sulfonates-busulfan, nitrosoureas (carmustine (BCNU) and analogs, streptozocin), trazenes - dacarbazinine (DTIC);
5 antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones, hormone analogs (estrogen, tamoxifen, goserelin, bicalutamide, nilutamide) and aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen
10 activator, streptokinase and urokinase), aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus (FK-506), sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF)
15 inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone,
20 methylpednisolone, prednisone, and prenisolone); growth factor signal transduction kinase inhibitors; mitochondrial dysfunction inducers and caspase activators; and chromatin disruptors.

In certain embodiments, pharmaceutical compounds that may be used for combinatory anti-angiogenesis therapy include: (1) inhibitors of release of "angiogenic molecules," such as bFGF (basic fibroblast growth factor); (2) neutralizers of angiogenic molecules, such as an anti-
25 βbFGF antibodies; and (3) inhibitors of endothelial cell response to angiogenic stimuli, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D_3 analogs, alpha-interferon, and the like. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-
30 118 (1990), Moses et al., Science, 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and U.S. Pat. Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, and 6573256. In addition, there are a wide variety of compounds that can be used to inhibit angiogenesis, for example, peptides or agents that block the VEGF-mediated angiogenesis pathway, endostatin

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

In certain embodiments, the methods of the application may be combined with known
10 treatments for ovarian cancer. In certain embodiments, the known treatment may include surgery, radiotherapy and/or chemotherapy. In certain embodiments, the chemotherapeutic compound used to treat ovarian cancer are a platinum chemotherapeutic agent, such as cisplatin or carboplatin. One of ordinary skill in the art will be able to determine the current known treatment protocols for ovarian cancer.

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

XII. Methods of Administration and Pharmaceutical Compositions

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

Formulations of the subject agents include those suitable for oral/ nasal, topical, parenteral, rectal, and/or intravaginal administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of

pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect.

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

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

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more therapeutic agents of the present disclosure may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceuticalal compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

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

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

15 In particular, methods of the disclosure can be administered topically, either to skin or to mucosal membranes such as those on the cervix and vagina. This offers the greatest opportunity for direct delivery to tumor with the lowest chance of inducing side effects. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-
20 pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and
25 sulfur.

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

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

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

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

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

30 Formulations for intravaginal or rectally administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the disclosure with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature,

but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Methods for delivering the subject nucleic acid compounds are known in the art (see, e.g., Akhtar et al., 1992, Trends Cell Bio., 2, 139; and Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995; Sullivan et al., PCT Publication No. WO 94/02595). These protocols can be utilized for the delivery of virtually any nucleic acid compound. Nucleic acid compounds can be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Other routes of delivery include, but are not limited to, oral (tablet or pill form) and/or intrathecal delivery (Gold, 1997, Neuroscience, 76, 1153-1158). Other approaches include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. For a comprehensive review on drug delivery strategies, see Ho et al., 1999, Curr. Opin. Mol. Ther., 1, 336-343 and Jain, Drug Delivery Systems: Technologies and Commercial Opportunities, Decision Resources, 1998 and Groothuis et al., 1997, J. NeuroVirol., 3, 387-400. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan et al., supra, Draper et al., PCT WO93/23569, Beigelman et al., PCT Publication No. WO99/05094, and Klimuk et al., PCT Publication No. WO99/04819.

In certain embodiments, the nucleic acids of the instant disclosure are formulated with a pharmaceutically acceptable agent that allows for the effective distribution of the nucleic acid compounds of the instant disclosure in the physical location most suitable for their desired activity. Non-limiting examples of such pharmaceutically acceptable agents include: PEG, phospholipids, phosphorothioates, P-glycoprotein inhibitors (such as Pluronic P85) which can enhance entry of drugs into various tissues, biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after implantation (Emerich, DF et al, 1999, Cell Transplant, 8, 47-58), and loaded nanoparticles such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (Prog Neuropsychopharmacol Biol Psychiatry, 23, 941-949, 1999).

In other embodiments, certain of the nucleic acid compounds of the instant disclosure can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985, Science, 229, 345; McGarry and Lindquist, 1986, Proc. Natl. Acad. Sci., USA 83, 399; Scanlon

et al., 1991, Proc. Natl. Acad. Sci. USA, 88, 10591-5; Kashani-Sabet et al., 1992, Antisense Res. Dev., 2, 3-15; Dropulic et al., 1992, J. Virol., 66, 1432-41; Weerasinghe et al., 1991, J. Virol., 65, 5531-4; Ojwang et al., 1992, Proc. Natl. Acad. Sci. USA, 89, 10802-6; Chen et al., 1992, Nucleic Acids Res., 20, 4581-9; Sarver et al., 1990 Science, 247, 1222-1225; Thompson et al.,
5 1995, Nucleic Acids Res., 23, 2259; Good et al., 1997, Gene Therapy, 4, 45). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by an enzymatic nucleic acid (Draper et al, PCT WO 93/23569, and Sullivan et al., PCT WO 94/02595; Ohkawa et al., 1992, Nucleic Acids Symp. Ser., 27, 15-6;
10 Taira et al., 1991, Nucleic Acids Res., 19, 5125-30; Ventura et al., 1993, Nucleic Acids Res., 21, 3249-55; Chowrira et al., 1994, J. Biol. Chem., 269, 25856; all of these references are hereby incorporated in their totalities by reference herein). Gene therapy approaches specific to the CNS are described by Blesch et al., 2000, Drug News Perspect., 13, 269-280; Peterson et al., 2000, Cent. Nerv. Syst. Dis., 485-508; Peel and Klein, 2000, J. Neurosci. Methods, 98, 95-104;
15 Hagihara et al., 2000, Gene Ther., 7, 759-763; and Herrlinger et al., 2000, Methods Mol. Med., 35, 287-312. AAV-mediated delivery of nucleic acid to cells of the nervous system is further described by Kaplitt et al., U.S. Pat. No. 6,180,613.

In another aspect of the disclosure, RNA molecules of the present disclosure are preferably expressed from transcription units (see for example Couture et al., 1996, TIG., 12,
20 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid compounds are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide
25 for transient expression of nucleic acid compounds. Such vectors can be repeatedly administered as necessary. Once expressed, the nucleic acid compound binds to the target mRNA. Delivery of nucleic acid compound expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow
30 for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

In one aspect, the disclosure contemplates an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid compounds of the instant disclosure. The nucleic acid sequence is operably linked in a manner which allows expression of the nucleic

acid compound of the disclosure. For example, the disclosure features an expression vector comprising: a) a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); c) a nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant disclosure;
5 and wherein said sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid compound. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the nucleic acid catalyst of the disclosure; and/or an intron (intervening sequences).

10

EXEMPLIFICATION

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

Example 1. EphB4 expression in human ovarian tumor samples

EphB4 expression in human ovarian samples was assessed by immunohistochemical staining of sections isolated from 7 normal ovaries and 85 invasive epithelial ovarian cancers (Figure 1A). All of the normal ovaries had little or absent EphB4 expression on the surface
20 epithelium (Figure 1Ab). Among the invasive ovarian cancers, 73 (86%) expressed EphB4 and moderate or strong expression was noted in 49 (58%) samples. We confirmed EphB4 expression by Western blot in a select group of 10 ovarian tumor samples for which frozen tissues were available. Seven of 10 tumor specimens expressed EphB4 on Western blotting (data not shown), confirming the staining data.

25

Example 2. EphB4 Expression correlates with clinicopathological features.

The demographic features of patients with invasive ovarian cancer are summarized in Figure 1B. Mean age at presentation was 59.4 (range 34-86) years. Sixty-nine of 85 patients (81%) had advanced stage (III or IV) disease, and 74 of 85 patients (87%) had high-grade (II or
30 III) disease. 56 of 85 patients (66%) underwent optimal surgical cytoreduction (< 1cm residual disease at completion of surgery). There was no association between expression of EphB4 and histological subtype, tumor grade or extent of cytoreduction.

EphB4 overexpression correlated with advanced disease stage. High expression of EphB4 was detected in 69% of high-stage ovarian cancers compared to 19% of low-stage ovarian cancer ($p < 0.001$). Ninety percent of patients with EphB4 overexpression had ascites compared to 49% of patients without EphB4 overexpression ($p < 0.001$). Survival rates of patients with invasive ovarian cancer and EphB4 expression were determined using the Kaplan-Meier method (Figure 1C). In univariate analysis, EphB4 overexpression was associated with significantly worse survival compared to low expression (median survival 7.75 years vs. 2.58 years; $p < 0.001$). On multiple regression analyses using a Cox proportional hazards model that included EphB4 expression, stage, grade, ascites, histology and level of cytoreduction, only advanced stage ($p = 0.04$) and EphB4 overexpression ($p = 0.003$) and suboptimal cytoreduction ($P=0.017$) were significant predictors of poor survival.

Example 3. EphB4 receptor is highly expressed in ovarian carcinoma cell lines

To study the biological role of EphB4, we wished to determine the expression level of EphB4 in ovarian carcinoma cell lines in comparison to benign ovarian tumor cell lines. Western blots revealed that EphB4 was indeed expressed highly in ovarian carcinoma cell lines Hey, CAOV-3, Hoc-7, and OVCAR-3, and least in the benign ovarian cell lines MCV-50 and ML-5 (Figure 2A). Hoc-7 expressed high levels of EphB4 compared to other ovarian cell lines. EphrinB2 is expressed at negligible to low levels in all ovarian cell lines, including the benign ovarian cell line (ML-5). Equal amount of protein loaded in each lane was confirmed by probing the same blots for β-actin (Figure 2A). EphB4 expressed on ovarian cancer cell lines is functional. Stimulation of Hey cells with 3μg/ml EphrinB2/Fc chimeric protein (in the absence of serum), but not Fc fragment alone, results in phosphorylation of EphB4 in ten minutes, which begins to diminish by 60 min (Figure 2B). Also, addition of fetal calf serum to serum-starved Hey cells induces a small degree of EphB4 phosphorylation above baseline.

Example 4. Progesterone reduces EphB4 expression and cell growth

Menstrual hormones are known to regulate ovarian tumor development and progression (Danforth *et al*, 2006). In particular, progesterone is growth inhibitory in several previous reports (Seeger, 2006). We were therefore interested in determining if progesterone and estrogen affect the expression of EphB4. Hoc-7 cells express functional progesterone and estrogen receptor. Treatment of Hoc-7 cells with progesterone led to a dose-dependent reduction in EphB4 expression, whereas estrogen had no effect on EphB4 levels (Figure 2C). Both hormones

had no effect on EphB4 expression in the benign MCV-50 cells (Figure 2C and data not shown). Progesterone, but not estrogen, similarly inhibited EphB4 expression in Hey cells as well (data not shown). Both Hoc-7 and Hey cells treated with progesterone showed a dose-dependent reduction in cell survival. 10μM progesterone resulted in nearly 50% reduction in cell survival
5 in Hoc-7 cells (Figure 2D)

Example 5. Inhibition of EphB4 results in reduced viability

In order to determine the biological function of EphB4 in ovarian cancer, we designed several siRNAs to knockdown EphB4 expression and selected the compound that best inhibited
10 EphB4 expression on Western blots (Figure 3A upper panel). EphB4 siRNA leads to a corresponding dose-dependent reduction in EphB4 expression levels in Hoc-7 cells as well (data not shown). Transfection with EphB4 siRNA mutated at three bases (EphB4 siRNAΔ) or non-specific GFP siRNA has no effect upon expression levels of EphB4 (data not shown), indicating that the reduction in EphB4 expression is a specific effect of EphB4 siRNA. Concomitant with
15 reduction in levels of EphB4 expression, EphB4 siRNA treatment, but not treatment with mutated EphB4 siRNAΔ, resulted in a dose-dependent reduction in Hey cell numbers. At a dose of 25nM siRNA, nearly 90% cell number was reduced (Figure 3A, lower panel). The dose of siRNA to achieve 50% cell number reduction was 4nM. Similarly, treatment of HOC-7 cells with 25nM EphB4 siRNA resulted in 75% reduction in cell numbers (data not shown).
20 Treatment of EphB4-negative tumor cell lines with EphB4 siRNA does not affect tumor cell numbers (Xia *et al*, 2005 and data not shown).

For use *in vivo*, we generated a panel of EphB4 antisense oligonulceotides and selected the molecule AS-10 that had maximal reduction in EphB4 levels for further studies (Figure 3B, upper panel). Similarly, treatment with 10μM AS-10, but not scrambled ODN, resulted in 90%
25 reduction in cell numbers, with an ED50 of 4μM (Figure 3B, lower panel).

Example 6. Knockdown of EphB4 leads to apoptosis and activation of the death receptor caspase pathway

We were interested in determining the cause for reduced cell numbers following EphB4
30 knockdown. Hey cells were transfected with 25nM EphB4 siRNA and 36 hours later, subjected to cell cycle analysis (Figure 3C). Whereas 90% of control cells and 88% of EphB4 siRNAΔ transfected cells entered the G0 phase and progressed along the cell cycle, only 48% of EphB4 siRNA treated cells progressed into the phases of the cell cycle.

Preventing cells from entering the G0 phase of the cell cycle is consistent with induction of apoptosis. To further corroborate this, we quantitated cytoplasmic nucleosomes by ELISA in Hey cells 48 hours following transfection with EphB4 siRNA (Figure 3D). Transfection with 25nM EphB4 siRNA resulted in a 3-fold increase in cytoplasmic nucelosomes, whereas
5 transfection with EphB4 siRNAΔ had no effect. Apoptosis can be induced by activation of the extrinsic, membrane-originating pathway that involves casapse-8, or the intrinsic mitochondrial pathway that does not involve casapse-8. Transfection of Hey cells with 25nM EphB4 siRNA resulted in 3.2-fold activation in caspase-8, whereas no such effect was observed with EphB4 siRNAΔ (Figure 3E). Neither the active nor the mutated siRNA resulted in appreciable casapse-
10 9 activation. EphB4 antisense oligonucleotides induced a similar cell cycle profile and caspase-8 activation (data not shown). Thus, EphB4 knockdown in Hey cells results in cell death due to induction of apoptosis predominantly via the extrinsic caspase-8 pathway.

Example 7. EphB4 regulates cell migration and invasion

15 The higher levels of expression of EphB4 in malignant compared to benign cell lines could be suggestive of higher EphB4 levels contributing to more malignant behavior by increasing cell migration and invasion of extracellular matrix proteins. We therefore determined if EphB4 participates in the migration of ovarian cell lines. These experiments were carried out over much shorter time-periods (9-12h) at which times no appreciable cell death was observed.
20 Confluent Hey cultures were wounded by a single scrape with a sterile plastic Pasteur pipette, which left a 3 mm cell-free zone with clearly defined borders. Migration of cells into the cleared zone in the presence of EphB4 siRNA and EphB4 AS-10 were evaluated and quantified over 9hr. Control cells and EphB4 siRNAΔ transfected cells rapidly migrate to cover the wound resulting in near complete wound healing by 9h, whereas, transfection of 25nM EphB4 siRNA
25 results in marked inhibition of cell migration and wound healing at 6h and 9h (Figure 4A). A similar reduction in cell migration was observed with AS-10 (data not shown).

Malignant cells are capable of degrading extracellular matrix and invading through tissues. To study this function, Hey cells were cultured on Matrigel coated on the inner chamber of a Boyden double chamber for 12h in the presence of 10μg EGF in the outer chamber. Cells
30 migrating to the under surface of the inner chamber were stained and visualized 12h later. Control cells readily migrated to the under surface of the membrane in 12h (Figure 4B). Whereas transfection with EphB4 siRNAΔ had no effect on cellular invasion, EphB4 siRNA nearly completely abolished cell migration across the basement membrane. A similar effect was

observed with AS-10 (data not shown). Thus, EphB4 confers to ovarian cancer cells the ability to migrate and invade basement membranes, known characteristics of an aggressive malignancy.

Example 8. EphB4 antisense ODN inhibits tumor growth in vivo

5 We then studied the effect of systemic antisense administration in mice bearing human ovarian cancer xenografts. $2X10^6$ Hey cells were injected in the flank of ten- to twelve-week old, female Balb/C athymic mice. On day 4 after cell implantation, mice were randomly divided into three groups (n=6 mice per group, experiment repeated twice) and treated by intraperitoneal injection with PBS (Vehicle), scrambled ODN or AS-10 at a dose of 10 mg/kg. Over five weeks, 10 AS-10 treatment resulted in greater than 85% smaller tumors compared to vehicle-treated tumors, while scrambled ODN treatment had no effect (Figure 4A). The mice appeared healthy during the entire time-period, fed well and were active. Serum levels of TNF-α, and IL-10 and spleen weights at sacrifice were similar between the three groups indicating that ODNs did not induce inflammatory cytokines (data not shown). Harvested tumors were evaluated by H/E 15 (Figure 4B, upper panel), which shows large areas of tumor necrosis in AS-10-treated tumors. Immunohistochemical evaluation with Ki-67 staining shows a 10-fold reduction in proliferative cells (Figure 4B, second panel) and a 12-fold increase in TUNEL positive cells (Figure 4B, third panel) consistent with induction of apoptosis as noted *in vitro*. Finally, AS-10 treatment resulted in 75% fewer tumor microvessels by CD31 immunostaining (Figure 4B, lower panel). Thus, 20 systemic administration of AS-10 inhibits growth of Hey tumors in mice, along with inhibition of tumor cell proliferation, induction of apoptosis and reduction of tumor microvasculature.

Example 9. Sequencing of MAB265

The variable heavy and light chains of MAB265 hybridoma clones by PCR using the 25 mouse Ig-primer set from Novagen. The variable heavy chain was 98.9% identical to germline J558.19. Alignments of germ line sequences and the variable light chain and an N-terminal sequence (SEQ ID NO: 39 and SEQ ID NO: 40) are shown in Figures 6. Alignments of germ line sequences and the variable heavy chain (SEQ ID NO: 42) are shown in Figures 7. CDRs were identified according to Kabat et al., 1991.

30 The CDR regions for the heavy chain of mouse monoclonal antibody 265 are defined as SEQ ID NO:261 (CDR1), SEQ ID NO:262 (CDR2), and SEQ ID NO:263 (CDR3). The CDR regions for the light chain of mouse monoclonal antibody 265 are defined as SEQ ID NO:264 (CDR1), SEQ ID NO:265 (CDR2), and SEQ ID NO:266 (CDR3).

Example 10. Effect of EphB4 monoclonal antibodies on angiogenesis and tumor growth

Anti-EphB4 monoclonal antibodies were raised in mice against the extracellular domain (ECD) of EphB4. An EphB4ECD (see, e.g., Figure 8) was cloned into expression vectors (e.g., pGEX) to generate EphB4ECD fusion proteins (e.g., GST-ECD). EphB4ECD fusion protein expressed in BL21 *E. coli* was purified by affinity chromatography. In the case of GST fusion proteins, the GST domain was cleaved by thrombin. Monoclonal antibody was purified from hybridoma supernatants by Protein A chromatography.

These monoclonal antibodies include EphB4 antibody Nos. 1, 23, 35, 47, 57, 79, 85L, 85H, 91, 98, 121, 131, and 138 (Figure 8). Antibody mapping studies showed the epitope domain for each of these antibodies (Figure 8).

Further experiments were carried out to analyze the functional activities of these antibodies, including their abilities to compete with their binding partner such as Ephrin B2, to activate EphB4 tyrosine phosphorylation, to inhibit in vitro tube formation in HUAEC, to inhibit in vivo angiogenesis by matrigel plug assay, to stimulate apoptosis or necrosis in SCC15 tumor cell, and to inhibit SCC15 xenotransplant growth. The results are summarized in Table 3 below.

Table 3. A summary of activities of EphB4 antibodies.

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

Nd = not determined (no data provided)
-- = no clear effect
+ = clear effect

A= apoptosis
N= necrosis
A,N = both apoptosis and necrosis

5 Example 11 MATERIALS AND METHODS

Reagents: EphrinB2 (P20) antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). The anti-phospho-tyrosine antibody 4G10 was from Upstate (Lake Placid, NY), monoclonal anti-actin antibody from Sigma Chemical Co. (St Louis, MO), anti-CD31 (M20) from Santa 10 Cruz Biotech (Santa Cruz, CA), monoclonal anti-Ki-67 from DAKO (Carpentaria, CA), and anti-human Fc from Jackson Labs (Bar Harbor, ME). EphrinB2/Fc chimeric protein was from R&D systems, Inc. (Minneapolis, MN). All antisense ODNs and siRNAs were synthesized from Qiagen (Valencia, CA).

15 Monoclonal antibodies to the extracellular domain of EphB4 were generated in house at Vasgene (three different clones designated MAb131,MAb 47 and MAb265). These antibodies have been extensively characterized in-house and are extremely sensitive and specific for the extra-cellular domain of EphB4 receptor. They do not cross react with other members of the Eph family of receptors (data not shown). MAb265 detects denatured human EphB4 only on Western 20 blot; MAb131 detects human EphB4 on tissue sections; while MAb47 detects human and murine EphB4 and is very effective in immunoprecipitation experiments.

Cell Culture: ML5 and ML10 cells were derived from human ovarian cystadenomas and transfected with SV40 large T antigen to increase their longevity *in vitro*. MCV 50 cells were 25 derived from a subclone of ML10 that became spontaneously immortalized in culture. HOC-7 ovarian carcinoma cells were obtained from Dr. R Buick, University of Toronto. OVCAR-3 cells were purchased from ATCC (ATCC#HTB161). ML5, ML10, and MCV50 were cultured in MEM medium supplemented with 10% FCS, 1mM glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). HOC-7 and OVCAR-3 cells were maintained in RPMI medium 30 supplemented with 10% FCS, 1mM glutamine, and 1% penicillin-streptomycin. Hey and CAOV-3 cells were cultured in DMEM medium containing 10% FCS, 1mM glutamine, 1mM MEM sodium pyruvate and 1% penicillin-streptomycin.

EphB4 siRNAs and Antisense oligodeoxynucleotides: EphB4-specific siRNA and phosphorothioate-modified antisense or scrambled oligodeoxynucleotide (ODN) were synthesized (Qiagen, Valencia, CA) and tested for specific inhibition of the EphB4 expression. EphB4-specific siRNA corresponded to the sequence 5'-GGU GAA UGU CAA GAC GCU
5 GUU-3' (SEQ ID NO:1) and 3'-UUC CAC UUA CAG UUC UGC GAC-5' (SEQ ID NO:2). The specific siRNA was mutated at three sites to yield siRNAΔ, 5'-AGU UAA UAU CAA GAC GCU GUU-3' (SEQ ID NO:3) and 3'-UUU CAA UUA UAG UUC UGC GAC-5' (SEQ ID NO:4), which had no effect on EphB4 levels and was used as control. SiRNA directed against GFP was used as an additional negative control. The AS-ODN used, AS-10, spanned
10 nucleotides 1980-1999 with a sequence 5'-ATG GAG GCC TCG CTC AGA AA-3' (SEQ ID NO:5). In order to negate non-specific cytokine-mediated effects from the CpG site, the cytosine in position 11 was methylated (AS-10M) without loss in EphB4 knockdown effect (data not shown). Scrambled ODN (sequence 5'-AAG GGC TAG GAT AGA CCC TC-3' (SEQ ID NO:6)) with the same nucleotides in a random sequence containing a CpG site as well was used
15 as control.

Immunohistochemistry: All of the human samples were collected in compliance with requirements of the Institutional Review Board for the Protection of Human Subjects. Formalin-fixed, paraffin-embedded samples were sectioned at 5μm. Sections were treated with antigen
20 retrieval buffer (modified citrate buffer with DIVA decloaker, BioCare Medical, Concord, CA) Specifically primary monoclonal EphB4 antibody MAb131 was applied at overnight at room temperature at a concentration of 30μg/ml in 1% BSA/TBST. Immunostaining was done using standard techniques (details available on request)

25 All of the samples were reviewed by a board-certified pathologist who was blinded to clinical outcome of the patients. EphB4 expression was determined by assessing the percentage of stained tumor cells and staining intensity. The percentage of positive cells was scored as follows: 0 points, 0-5%; 2 points, 6-50%; 3 points, > 50%. The staining intensity was scored as follows: 1 point, weak intensity; 2 points, moderate intensity; 3 points, strong intensity. Points
30 for expression and percentage of positive cells were added and an overall score between 0 and 6 was assigned. Tumors were categorized into four groups: negative (overall score = 0); weak expression (overall score = 1-2); moderate expression (overall score = 3-4),; and strong expression (overall score = 5-6). For xenograft staining, sections were incubated with primary

antibody (CD31 1:250 dilution, Ki-67 1:100) overnight at 4°C. Routine negative controls included deletion of primary and secondary antibody and substitution of normal IgG isotope for primary antibody. When using mouse-anti-human Ki-67 antibody, MOM kit (Vector Labs, Burlingame, CA) was used to block non-specific binding to mouse tissue. Number of cells
5 staining positive was counted by a blinded observer in five random high-power fields.

Western blot: Cell lysates were prepared as described (Masood *et al*, 2003). Typically, 10 µg proteins from whole cell lysate were fractionated on a 4-20% tris-glycine polyacrylamide gel, electro-transferred to PVDF membrane and probed with primary antibody overnight. Blot was
10 stripped with Restore™ Western Blot stripping buffer (Pierce, Rockford IL) and reprobed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), quantitated by X-ray densitometry using Fluro-S multi-Imager system (Bio-Rad).

15 Phosphorylation analysis of EphB4: Recombinant EphrinB2/Fc or Fc proteins were clustered using anti-Fc antibody for 1h at 4ºC. Hey and Hoc-7 cells were grown in 60 mm dishes until 100% confluence and were treated with clustered EphrinB2/Fc or Fc (Bar Harbor, ME) for various durations of time. Lysates were prepared with buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton-X100, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium vanadate,
20 and centrifuged at 50,000 x g for 60 min at 4°C. Clarified protein samples were incubated overnight with protein A/G coupled agarose beads pre-coated with anti-EphB4 monoclonal antibody (#47). The immunoprecipitated (IP) complexes were probed with anti-p-Tyr specific antibody 4G10. EphB4 precipitation efficiency was tested by probing with EphB4 specific monoclonal antibody (#265).
25

Wound healing migration assay: Hey cells were seeded onto 6-well plates and cultured until confluent. EphB4 antisense or scrambled ODN (1µM) or siRNA were introduced to the wells as described for the viability assay. Cell monolayer was wounded by scraping with a sterile pipette tip. Migration of the cells into scraped area over time was examined and recorded with a Nikon
30 Coolpix 5000 digital camera.

Cell migration Assay: Chemotaxis of ovarian cells was assessed using a modified Boyden chamber containing cell culture inserts with an 8µm pore size Matrigel-coated polycarbonate

membrane in 24 well plates (BD Biosciences). The cell suspensions of Hey ($2x10^5$cells/ml) in 200μl DMEM/1%FCS were seeded in the upper chamber following siRNA introduction or along with EphB4 AS-10. 500μl of DMEM/1%FCS containing chemotaxis agent EGF (20ng/ml) was added to the lower chamber. After incubation for 9h at 37°C, the upper surface of
5 the filter was scraped with swab and filters were fixed and stained with Diff Quick (vWR, West Chester, PA).

Cell viability assay: Hey and Hoc-7 cells were seeded on 48-well plates at a density of approximately $1x10^4$ cells/well in 200μl medium. Cells were treated with various concentrations
10 (1-10 μM) of EphB4 AS-10 or scrambled ODN on day 1 and day 3 after cell seeding. After three days, medium was changed and fresh ODNs added. Cell viability was assessed by MTT as described previously (Masood *et al*, 2003). EphB4 siRNAs (10-100 nM) were introduced into 2 x 10^4 cells/well of a 48-well plate using 2 μl of Lipofectamine™ 2000 according to the manufacturer's instructions. Four hr post- transfection the cells were returned to growth
15 medium. Viability was assayed after 48 hr as described (Masood *et al*, 2003).

Cell cycle analysis: 80% confluent cultures of Hey cells in 6-well plates were transfected with various siRNAs (100 nM) using Lipofectamine™ 2000 for variable amount of time, cells were trypsinized, washed in PBS and incubated for 1 h at 4 ^{0}C in 1 ml of hypotonic solution
20 containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100 and 20 μg/ml DNase-free RNaseA. Cells were analyzed in linear mode by flow cytometry. Results are expressed as percentages of elements detected in the different phases of the cell cycle, namely Sub G0 peak (apoptosis), G0/G1 (no DNA synthesis), S (active DNA synthesis), G2 (premitosis) and M (mitosis).
25

Apoptosis Assay: Apoptosis was studied using the Cell Death Detection ELISA plus Kit that detects cytoplasmic nucleosomes according to the manufacturer's instructions (Roche, Piscataway, NJ). Briefly, cell in 24-well plates cultured to 80% confluence were transfected using Lipofectamine™ 2000 with various concentrations (0-100 nM) of EphB4 siRNA(472) or
30 GFP siRNA. 16 hours later, cells were lysed and nuclei pelleted, incubated with anti-histone-biotin and anti-DNA POD in streptavidin-coated 96-well plate. Color was developed with ABST and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA). Caspase-8 and -9 activity was measured using the caspase-8 and caspase-9 colorimetric assay

kits that monitor cleavage of caspase-8 and -9 peptide substrates (R&D Systems, Inc., Minneapolis, MN). Apoptosis was detected in deparafinized sections of animal tumors by TUNEL assay using the *in situ* cell death detection kit (Roche, Piscataway, NJ) according to manufacturer's instructions.

5

Murine tumor xenograft model: Hey cells were propagated, collected by trypsin digestion and re-suspended in serum free medium. $2X10^6$ cells were injected in the flank of ten- to twelve-week old, female Balb/C athymic mice. Tumor growth was measured three times a week and volume estimated as $0.52 \times a \times b^2$, where a and b are the largest and smallest lengths of the
10 palpable tumor. On day 4 after cell implantation, tumor volumes were calculated to ensure uniformity in size and animals were randomly divided into three groups (n=6 mice per group). Each group was administered daily by intraperitoneal (i.p.) injection, AS-10 or scrambled ODN at a dose of 10 mg/kg or vehicle alone (sterile normal saline, pH 7.4). Animals were sacrificed and tumors and normal organs harvested after four weeks. A portion of the tumors was fixed in
15 formalin for paraffin-embedding and histologic analysis. The remaining tumor tissue and organs in each group were pooled and protein extracted. All procedures were approved by our Institutional Animal Care and Use Committee and performed in accordance with the Animal Welfare Act regulations.

20 Statistical Analysis. The X^2 test was used to determine differences among variables using SPSS (SPSS Inc., Chicago, IL). Survival curves were generated with the Kaplan-Meier method and compared with the log-rank statistic. The Cox proportional hazards model was used for multivariate analysis. Student's *t* test was used to compare tumor volumes. A p-value < 0.05 was considered statistically significant.

25

INCORPORATION BY REFERENCE

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

30 While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The

full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

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SEQUENCES

GGU GAA UGU CAA GAC GCU GUU-3’ (SEQ ID NO:1)

UUC CAC UUA CAG UUC UGC GAC-5' (SEQ ID NO:2)

AGU UAA UAU CAA GAC GCU GUU-3' (SEQ ID NO:3)

5 UUU CAA UUA UAG UUC UGC GAC-5' (SEQ ID NO:4)

ATG GAG GCC TCG CTC AGA AA-3' (SEQ ID NO:5)

AAG GGC TAG GAT AGA CCC TC-3' (SEQ ID NO:6)

10

Human EphB4 (Genbank ID No. NP_004435) (SEQ ID NO:7)

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHSVRT
YEVCDVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCKETFTVF
15 YYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKVNVKTLRLGPLSK
AGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVP
APGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGEGSCQP
CPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRLNGSSLHLEWSAPL
ESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLVEPWVVVRGLRPDFTYTFE
20 VTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAVL
DYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGPFGQEHH
SQTQLDESEGWREQLALIAGTAVVGVVLVLVVIVVAVLCLRKQSNGREAEYSDKHGQ
YLIGHGTKVYIDPFTYEDPNEAVREFAKEIDVSYVKIEEVIGAGEFGEVCRGRLKAPGKK
ESCVAIKTLKGGYTERQRREFLSEASIMGQFEHPNIIRLEGVVTNSMPVMILTEFMENGA
25 LDSFLRLNDGQFTVIQLVGMLRGIASGMRYLAEMSYVHRDLAARNILVNSNLVCKVSD
FGLSRFLEENSSDPTYTSSLGGKIPIRWTAPEAIAFRKFTSASDAWSYGIVMWEVMSFGE
RPYWDMSNQDVINAIEQDYRLPPPPDCPTSLHQLMLDCWQKDRNARPRFPQVVSALDK
MIRNPASLKIVARENGGASHPLLDQRQPHYSAFGSVGEWLRAIKMGRYEESFAAAGFG
SFELVSQISAEDLLRIGVTLAGHQKKILASVQHMKSQAKPGTPGGTGGPAPQY

30

SEQ ID NO:8:

DYYMN

SEQ ID NO:9:

DNNPNNGGTTYNQKF

5 SEQ ID NO:10:

GKYYGTSYGWYFDV

SEQ ID NO:11:

RISDNIDSYLA

10

SEQ ID NO:12:

DATVLAD

SEQ ID NO:13:

15 QVYYSIPWT

SEQ ID NO:14:

DYYIN

20 SEQ ID NO:15:

KIGPRIGTNYYNENFK

SEQ ID NO:16:

SEDYSGYVSYALDY

25

SEQ ID NO:17:

KASENVDTYVS

SEQ ID NO:18:

GASNRYT

5

SEQ ID NO:19:

GQTYRYPFT

SEQ ID NO:20:

10 EVQLVQSGAELKKPGASVKISCKASGYTFTDYYMNWVKQAHGKGLEWIGDNNPNNG
GTNYNQKFKGRATLTVDKSTSTAYMELRSLRSEDSAVYYCARGKYYGTSYGWYFDV
WGQGTTVTVSS

SEQ ID NO:21:

15 EVQLVQSGAELKKPGASVKISCKASGYTFTDYYMNWVKQAHGKGLEWIGDNNPNNG
GTNYNQKFKGRATLTVDKSTSTAYMELSSLRSEDSAVYYCARGKYYGTSYGWYFDV
WGQGTTVTVSS

SEQ ID NO:22:

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WGQGTTVTVSS

SEQ ID NO:23:

25 EVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYMNWVKQAPGKGLEWIGDNNPNNG
GTNYNQKFKGRVTLTVDKSTSTAYMELSSLRSEDTAVYYCARGKYYGTSYGWYFDV
WGQGTTVTVSS

SEQ ID NO:24:

EVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYMNWVRQAPGKGLEWIGDNNPNNG
GTNYNQKFKGRVTITVDKSTSTAYMELSSLRSEDTAVYYCARGKYYGTSYGWYFDVW
GQGTTVTVSS

5 SEQ ID NO:25:

DIQMTQSPSSLSASVGDRVTITCRISDNIDSYLAWFQQKQGKAPKLLVYDATVLADGVP
SRFSGSGSGTQYTLTINSLQSEDAARYYCQVYYSIPWTFGQGTKLEIK

SEQ ID NO:26:

10 DIQMTQSPSSLSASVGDRVTITCRISDNIDSYLAWFQQKPGKAPKLLVYDATVLADGVP
SRFSGSGSGTDYTLTINSLQAEDAARYYCQVYYSIPWTFGQGTKLEIK

SEQ ID NO:27:

DIQMTQSPSSLSASVGDRVTITCRISDNIDSYLAWFQQKPGKAPKLLVYDATVLADGVP
15 SRFSGSGSGTDYTLTINSLQAEDAATYYCQVYYSIPWTFGQGTKLEIK

SEQ ID NO:28:

DIQMTQSPSSLSASVGDRVTITCRISDNIDSYLAWYQQKPGKAPKLLVYDATVLADGVP
SRFSGSGSGTDYTLTINSLQAEDAATYYCQVYYSIPWTFGQGTKLEIK

20

SEQ ID NO:29:

QVQLVQSGAELKKPGASVKISCKASGYTFTDYYINWVKQAPGQGLEWIGKIGPRIGTNY
YNENFKGRATLTADISTNTAYMELSSLRSEDSAVYFCARSEDYSGYVSYALDYWGQGT
SVTVSS

25

SEQ ID NO:30:

QVQLVQSGAEVKKPGASVKISCKASGYTFTDYYINWVKQAPGQGLEWIGKIGPRIGTN
YYNENFKGRATLTADISTNTAYMELSSLRSEDTAVYFCARSEDYSGYVSYALDYWGQG
TLVTVSS

5 SEQ ID NO:31:

QVQLVQSGAEVKKPGASVKISCKASGYTFTDYYINWVKQAPGQGLEWIGKIGPRIGTN
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GTLVTVSS

10 SEQ ID NO:32:

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GTLVTVSS

15 SEQ ID NO:33:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYINWVRQAPGQGLEWIGKIGPRIGTN
YYNENFKGRVTLTADISTSTAYMELSSLRSEDTAVYYCARSEDYSGYVSYALDYWGQG
TLVTVSS

20 SEQ ID NO:34:

NIVMTQSPASLSLSPGERVTLSCKASENVDTYVSWYQQKPDQSPKLLIYGASNRYTGVP
DRFTGSGSATDFTLTISSLQAEDVADYHCGQTYRYPFTFGQGTKVEIK

SEQ ID NO:35:

25 NIVMTQSPATLSLSPGERVTLSCKASENVDTYVSWYQQKPDQSPKLLIYGASNRYTGVP
DRFTGSGSATDFTLTISSLQAEDVADYHCGQTYRYPFTFGQGTKVEIK

SEQ ID NO:36:

NIVMTQSPATLSLSPGERVTLSCKASENVDTYVSWYQQKPDQSPKLLIYGASNRYTGVP
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SEQ ID NO:37:

5 NIVMTQSPATLSLSPGERVTLSCKASENVDTYVSWYQQKPDQSPKLLIYGASNRYTGVP
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MAB265 VL3 DNA (SEQ ID NO:38)
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10 GAGTGGTATAATGTTGCTGACAGAAGTAATCTGCCAGGTCTTCAGCCTGCACACTGC
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TGCTGGTACCAGGCCAAATAGTTCTTTTGAGTGTTACTATTTAAAAGGCTCTGACTG
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15 ATAGCCAGGGAGGATGGAGACTGTGTCATCACAATGTCTGCACAGGCACCAGATAC
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CC

MAB265 VL3 AA (SEQ ID NO:39)
20 DIVMTQSPSSLAMSVGQKVTMNCKSSQSLLNSNTQKNYLAWYQQKPGHSPKLLVYLSS
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MAB265 VL N-terminal AA (SEQ ID NO:40)
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25 STRESGVPDRFIGSGSGTDFTLTISSVQAEDLADYFCQQHYTTPLTFGAGTKLELKR

MAB265 VH DNA (SEQ ID NO:41)
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30 TGGACAGGGTCTGGAATGGATTGGGGCTATTTATCCTGGAGATGGTGATACTAGGT
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GCCTACATGCAACTCAGCACCTTGGCATCTGAGGACTCTGCGGTCTATTACTGTGCA
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MAB265 VH AA (SEQ ID NO:42)
5 QQSGAELARPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGAIYPGDGDTRYT
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SEQ ID NO:261:
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10
SEQ ID NO:262:
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SEQ ID NO:263:
15 VGGWLLLAY

SEQ ID NO:264:
KSSQSLLNSNTQKNYLA

20 SEQ ID NO:265:
LSSTRES

SEQ ID NO:266:
QQHYTTPLT
25
Human EphB4 mRNA (Genbank No: NM_004444) SEQ ID NO:267:
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CACTCGGCCCGGCGGCGCGAGCAGAGCCACTCCAGGGAGGGGGGGAGACCGCGAG
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25 CAGAGCTGCGGGGGCTGAAGCGGGGAGCCAGCTACCTGGTGCAGGTACGGGCGCG
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45 CCGCTTCCCCCAGGTGGTCAGCGCCCTGGACAAGATGATCCGGAACCCCGCCAGCC
TCAAAATCGTGGCCCGGGAGAATGGCGGGGCCTCACACCCTCTCCTGGACCAGCGG
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50 AGAAAATCTTGGCCAGTGTCCAGCACATGAAGTCCCAGGCCAAGCCGGGAACCCCG

GGTGGGACAGGAGGACCGGCCCCGCAGTACTGACCTGCAGGAACTCCCCACCCCAG
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GAAACAGGGGCCCATCATCATGTCTGTTTCCAGAACAGTGCCTTGGTCATCCCACAT
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GGGACGTGTCCCAGCTCCAGGGGTAAAAAAAAAAAAAAAAA

WE CLAIM:

1. A method of treating ovarian cancer, the method comprising administering to a patient in need thereof an effective amount of an EphB4 inhibitor.

5

2. The method of claim 1, wherein said EphB4 inhibitor is selected from the group consisting of a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid, an RNAi construct, a nucleic acid analog, and a small molecule.

10 3. The method of claim 1, wherein said EphB4 inhibitor is an antibody or antigen binding fragment thereof.

4. The method of claim 3, wherein said antibody or antigen binding fragment thereof is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody
15 fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fd, an Fab, an Fab', and an F(ab')2.

20 5. The method of claim 4, wherein said antibody or antigen binding fragment thereof is a monoclonal antibody.

6. The method of claim 5, wherein said monoclonal antibody is a humanized antibody.

25 7. The method of claim 3, wherein said antibody or antigen binding fragment thereof binds to an epitope situated in the extracellular portion of EphB4 and inhibits an EphB4 activity.

8. The method of claim 3, wherein said antibody or antigen binding fragment thereof binds to an epitope situated within amino acids 16-198, 327-427 or 428-537 of the EphB4 sequence.

30

9. The method of claim 3, wherein said antibody or antigen binding fragment thereof comprises a heavy chain variable region wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO: 261, SEQ ID NO: 262, or SEQ ID NO: 263.

35

10. The method of claim 3, wherein said antibody or antigen binding fragment thereof comprises a light chain variable region, wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO: 264, SEQ ID NO: 265, or SEQ ID NO: 266.

40

11. The method of claim 3, wherein the antibody or antigen binding fragment thereof is covalently linked to an additional functional moiety.

12. The method of claim 11, wherein the additional functional moiety is a detectable label.

45

13. The method of claim 12, wherein the detectable label is selected from a fluorescent or chromogenic label.

50 14. The method of claim 12, wherein said detectable label is selected from horseradish

peroxidase or alkaline phosphatase.

15. The method of claim 11, wherein the additional functional moiety comprises a polyethylene glycol (PEG) moiety.

16. The method of claim 3, wherein the antibody or antigen binding fragment thereof inhibits an EphB4 activity.

17. The method of claim 3, wherein the antibody or antigen binding fragment thereof stimulates EphB4 kinase activity.

18. The method of claim 1, wherein said EphB4 inhibitor is a soluble polypeptide selected from:

(i) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein, wherein the EphB4 polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide; and

(ii) a soluble polypeptide comprising an amino acid sequence of an extracellular domain of an Ephrin B2 protein, wherein the soluble Ephrin B2 polypeptide is a monomer and binds with high affinity to an EphB4 polypeptide.

19. The method of claim 18, wherein the soluble polypeptide comprises a globular domain of an EphB4 protein.

20 The method of claim 18, wherein the soluble polypeptide comprises a sequence at least 90% identical to residues 1-522 of the EphB4 amino acid sequence.

21. The method of claim 18, wherein the soluble polypeptide comprises a sequence at least 90% identical to residues 1-412 of the EphB4 amino acid sequence.

22. The method of claim 18, wherein the soluble polypeptide comprises a sequence at least 90% identical to residues 1-312 of the EphB4 amino acid sequence.

23. The method of claim 18, wherein the soluble polypeptide comprises a sequence at least 90% identical to residues 1-225 of the Ephrin B2 amino acid sequence.

24. The method of claim 18, wherein the soluble polypeptide is a fusion protein.

25. The method of claim 18, wherein the soluble polypeptide comprises one or more modified amino acid residues.

26. The method of claim 18, wherein the soluble polypeptide inhibits the interaction between Ephrin B2 and EphB4.

27. The method of claim 18, wherein the soluble polypeptide inhibits clustering of Ephrin B2 or EphB4.

28. The method of claim 18, wherein the soluble polypeptide inhibits phosphorylation of Ephrin B2 or EphB4.

29. The method of claim 1, wherein said EphB4 inhibitor is a nucleic acid compound.

30. The method of claim 29, wherein said nucleic acid compound is about 15-100 nucleotides in length, hybridizes under physiological conditions to an EphB4 nucleic acid sequence and reduces expression of said EphB4.

5 31. The method of claim 29, wherein said nucleic acid is selected from the group consisting of: an RNAi construct and an antisense oligonucleotide.

32. The method of claim 31, wherein said RNAi construct is selected from the group consisting of: a dsRNA or an siRNA.

10

33. The method of claim 32, wherein said siRNA is around 19-30 nucleotides in length.

34. The method of claim 32, wherein said siRNA is around 21-23 nucleotides in length.

15 35. The method of claim 32, wherein said siRNA sequence is selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO:2.

36. The method of claim 29, wherein said antisense oligonucleotide is around 19-30 nucleotides in length.

20

37. The method of claim 36, wherein said antisense oligonucleotide sequence consists of SEQ ID NO: 5.

38. The method of claim 1, wherein said EphB4 inhibitor is administered systemically.

25

39. The method of claim 1, wherein said EphB4 inhibitor is administered locally.

40. The method of claim 1, wherein said EphB4 inhibitor has an anti-cancer activity.

30 41. The method of claim 40, wherein said anti-cancer activity is selected from the group consisting of promoting apoptosis, inhibiting tumor growth, inhibiting cancer cell proliferation, inhibiting cancer cell migration, inhibiting metastasis of cancer cells, inhibiting angiogenesis, and causing tumor cell death.

35 42. The method of claim 1, wherein said EphB4 inhibitor is formulated with a pharmaceutically acceptable carrier.

43. The method of claim 1, wherein the ovarian cancer comprises one or more cancer cells expressing EphB4.

40

44. The method of claim 1, wherein the ovarian cancer cell expresses a higher level of EphB4 compared to a noncancerous cell from a comparable tissue.

45. The method of claim 1, wherein the ovarian cancer is metastatic.

45

46. The method of claim 1, wherein the ovarian cancer is angiogenesis-dependent or angiogenesis-independent.

47. The method of claim 1, wherein said patient is a human.

50

48. The method of claim 1, further including at least one additional anti-cancer chemotherapeutic agent that inhibits ovarian cancer cells in an additive or synergistic manner with the EphB4 inhibitor.

5 49. The method of claim 1, further including at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the EphB4 inhibitor.

50. Use of an EphB4 inhibitor in the manufacture of a medicament for the treatment of ovarian cancer.

10

51. A method for treating a patient suffering from ovarian cancer, comprising: (a) identifying in the patient ovarian cancer having a plurality of cancer cells that express EphB4; and (b) administering to the patient an EphB4 inhibitor.

15 52. The method of claim 51, wherein said EphB4 inhibitor is selected from the group consisting of a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid, an RNAi construct, a nucleic acid analog, and a small molecule.

53. A method of reducing the growth rate of ovarian cancer in a subject, comprising
20 administering an amount of an EphB4 inhibitor sufficient to reduce the growth rate of the ovarian cancer.

54. The method of claim 53, wherein said EphB4 inhibitor is selected from the group consisting of a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid,
25 an RNAi construct, a nucleic acid analog, and a small molecule.

55. A method of inducing caspase-8 activation comprising administering an EphB4 inhibitor.

30 56. A method of detecting whether a subject has ovarian cancer comprising:

(a) obtaining a sample from said subject; and

(b) assessing expression level of EphB4 protein and/or mRNA in the sample;

wherein an increased level of EphB4 protein and/or mRNA relative to a control is indicative that the subject has ovarian cancer.

35

57. The method of claim 56, wherein said mammal is a human.

58. The method of claim 56, wherein said ovarian cancer is metastatic ovarian cancer.

40 59. The method of claim 56, wherein the EphB4 protein expression level is assessed in an antibody-based assay.

60. The method of claim 56, wherein the level of EphB4 protein or mRNA is at least two times the level in a normal ovarian tissue.

45

61. The method of claim 56, wherein a sample is selected from the group consisting of: a tissue sample, a blood sample, and a serum sample.

62. A method of developing a prognosis for a patient suffering from ovarian cancer

comprising:

(a) obtaining a sample from said subject;

(b) assessing expression level of EphB4 protein and/or mRNA in the sample;

wherein an increased level of EphB4 protein and/or mRNA relative to a control is indicative of a
5 poor prognosis.

63. The method of claim 62, wherein said mammal is a human.

64. The method of claim 62, wherein said ovarian cancer is metastatic ovarian cancer.
10

65. The method of claim 62, wherein the level of EphB4 protein or mRNA is at least two times the level in a normal ovarian tissue.

66. The method of claim 62, wherein a sample is selected from the group consisting of: a
15 tissue sample, a blood sample, and a serum sample.

67. A kit for diagnosing ovarian cancer comprising an anti-EphB4 antibody or antigen binding fragment thereof, a detectable label, and instructions for using the kit.

20 68. The kit of claim 67, wherein the detectable label is fluorescent or chromogenic.

69. The kit of claim 67, wherein said kit comprises horseradish peroxidase or alkaline phosphatase.

25 70. An EphB4 antibody or antigen binding fragment thereof comprising a heavy chain variable region, wherein the heavy chain variable region comprises a CDR1 comprising SEQ ID NO:261, a CDR2 comprising SEQ ID NO: 262, and a CDR3 comprising SEQ ID NO: 263.

71. The antibody of claim 70, wherein the heavy chain variable region comprises the amino
30 acid sequence of SEQ ID NO: 42.

72. The antibody of claim 70, further comprising a light chain variable region which comprises a CDR1 comprising SEQ ID NO: 264, a CDR2 comprising SEQ ID NO: 265, and a CDR3 comprising SEQ ID NO: 266.
35

73. The antibody of claim 72, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 39.

74. The antibody of claim 70, wherein the antibody is a humanized antibody or antibody
40 fragment thereof.

75. An EphB4 antibody or antigen binding fragment thereof comprising a light chain variable region, wherein the light chain variable region comprises a CDR1 comprising SEQ ID NO: 264, a CDR2 comprising SEQ ID NO: 265, and a CDR3 comprising SEQ ID NO: 266.
45

76. The antibody of claim 75, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 39.

77. The antibody of claim 75, further comprising a heavy chain variable region which

comprises a CDR1 comprising SEQ ID NO: 261, a CDR2 comprising SEQ ID NO: 262, and a CDR3 comprising SEQ ID NO: 263.

78. The antibody of claim 77, wherein the heavy chain variable region comprises the amino
5 acid sequence of SEQ ID NO: 42.

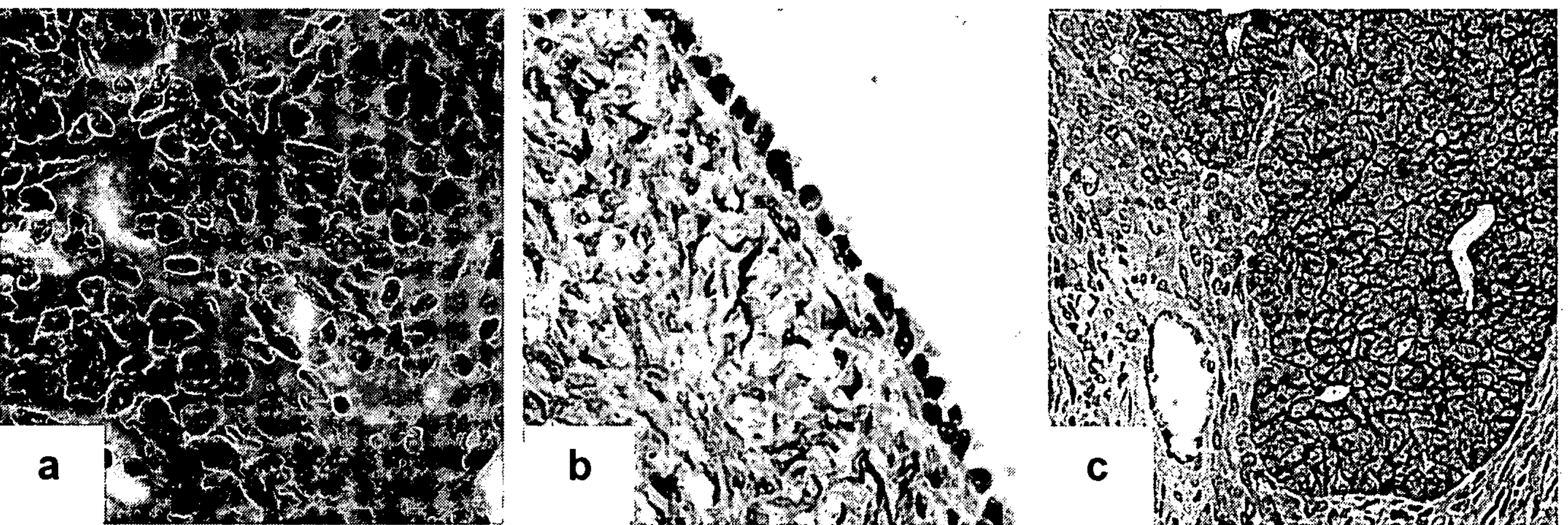
79. The antibody of claim 75, wherein the antibody is a humanized antibody or antibody fragment thereof.

10 80. An EphB4 antibody or antigen binding fragment thereof comprising a light chain variable region, wherein the light chain variable region comprises a CDR1 comprising SEQ ID NO: 11, a CDR2 comprising SEQ ID NO: 12, and a CDR3 comprising SEQ ID NO: 13.

15

Figure 1

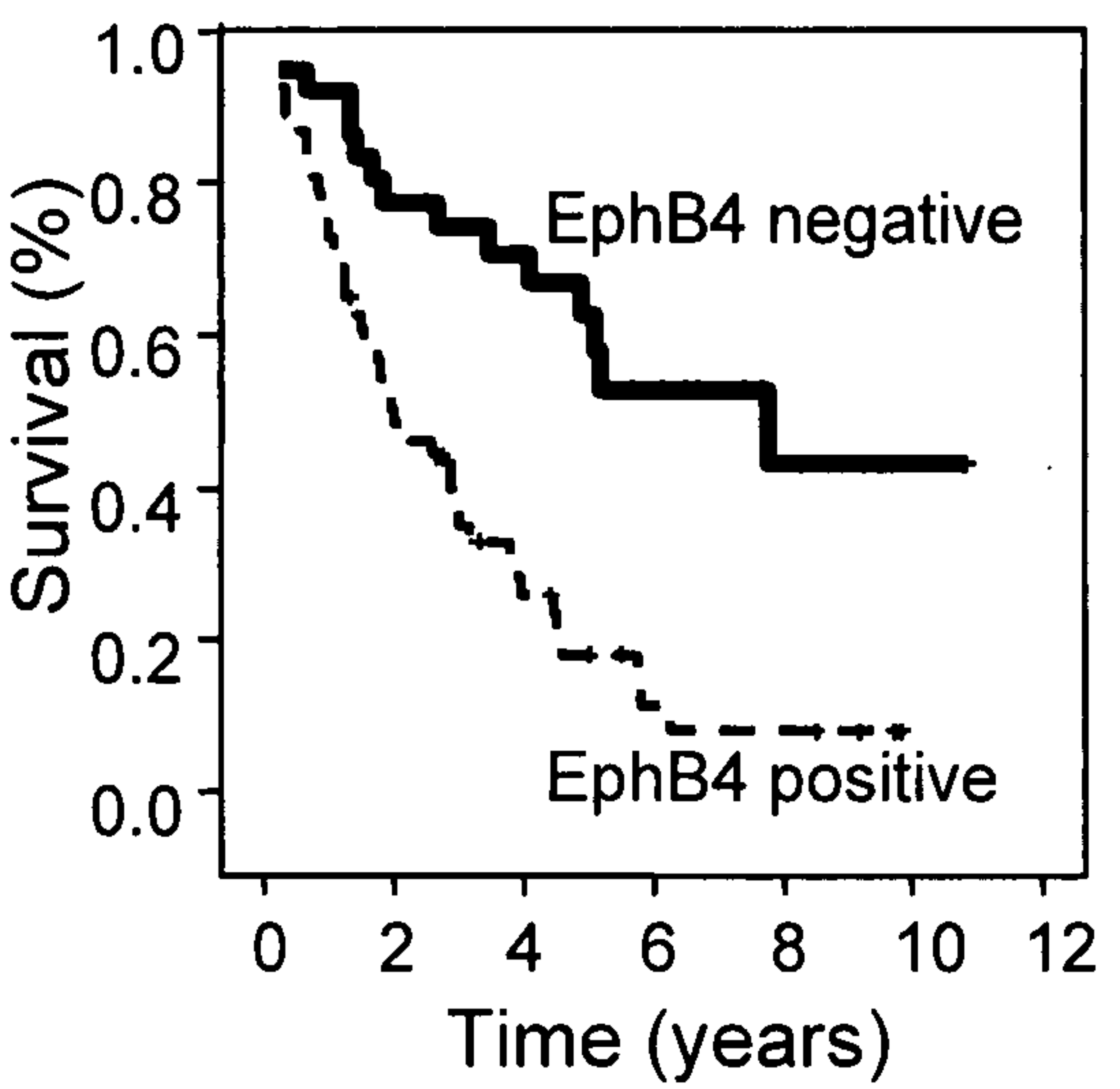
A



B

Variable	EphB4 expression Yes (n=50)	No (n=35)	p value
Stage			
Low (I/II)	3	13	<0.001
High (III/IV)	47	22	
Histology			
Serous	33	24	0.8
Other	17	11	
Grade			
Low (I)	4	7	0.1
High (II/III)	46	28	
Ascites			
Yes	45	18	<0.001
No	5	17	
Cytoreduction			
Optimal	29	27	0.067
Suboptimal	21	8	

C



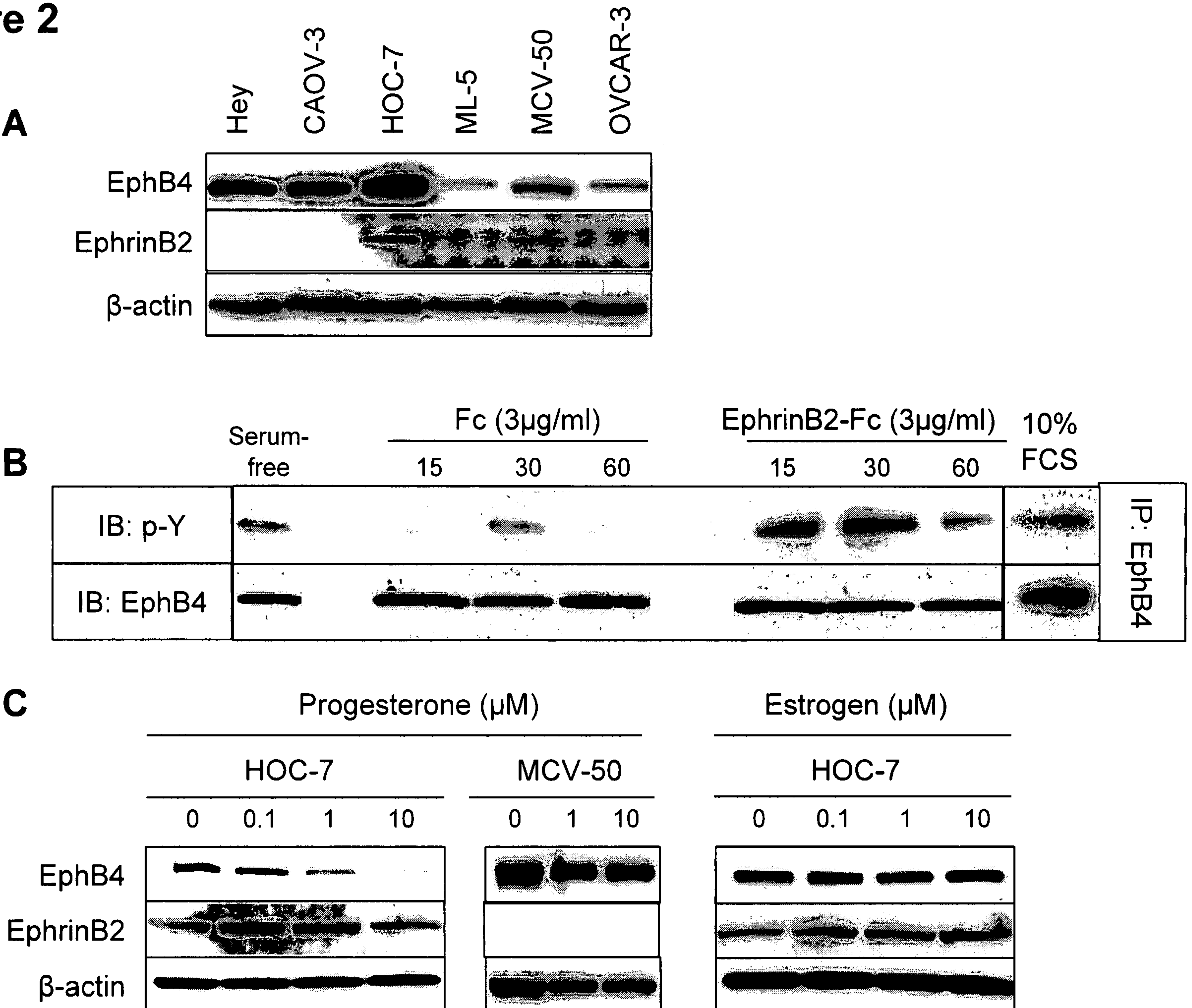
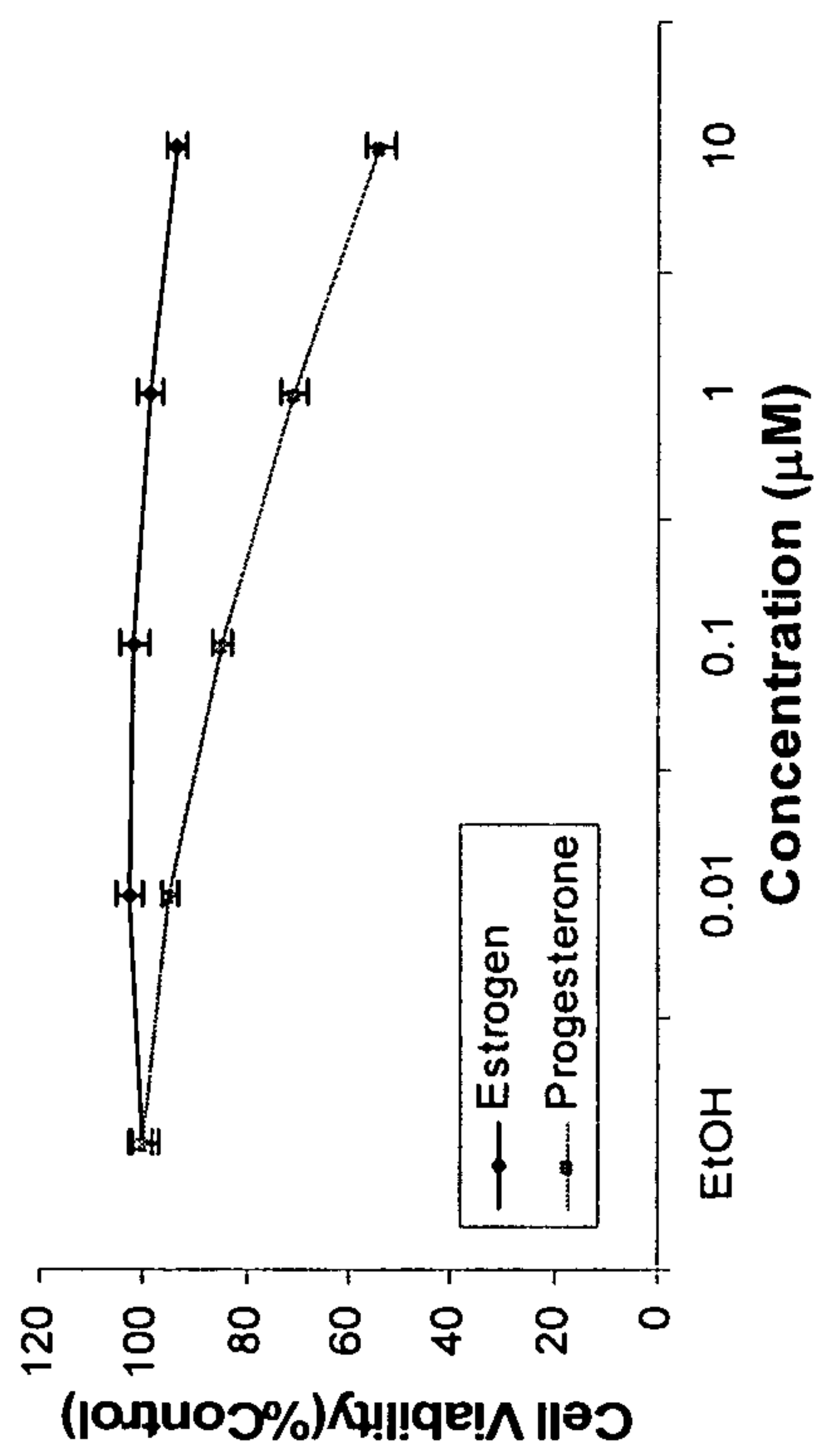


Figure 2
A
Hey
CAOV-3
HOC-7
ML-5
MCV-50
OVCAR-3
EphB4
EphrinB2
β-actin
B
Serum-free
Fc (3μg/ml)
15
30
60
EphrinB2-Fc (3μg/ml)
15
30
60
10% FCS
IB: p-Y
IB: EphB4
IP: EphB4
C
Progesterone (μM)
HOC-7
0
0.1
1
10
MCV-50
0
1
10
Estrogen (μM)
HOC-7
0
0.1
1
10
EphB4
EphrinB2
β-actin

Figure 2

D



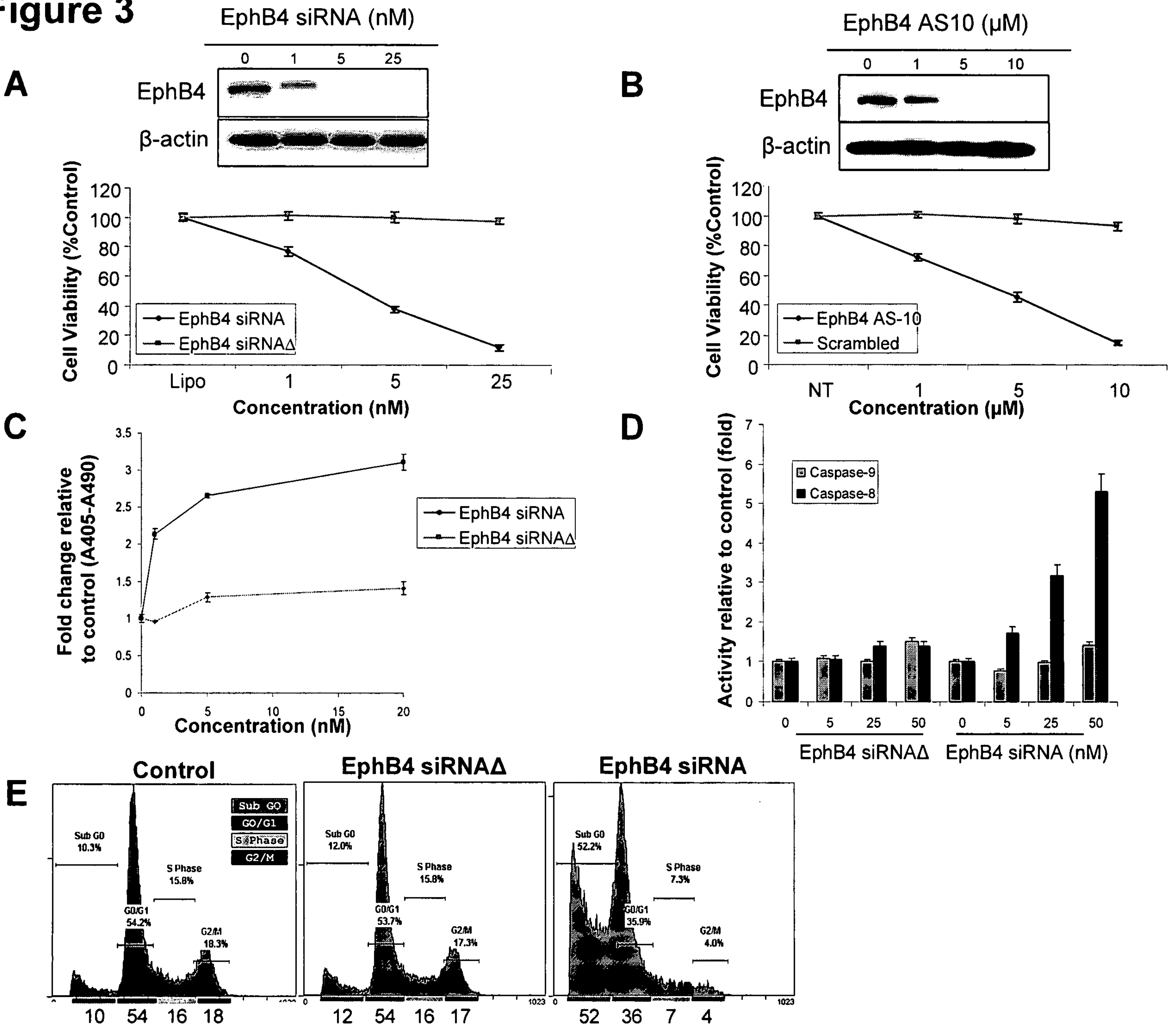
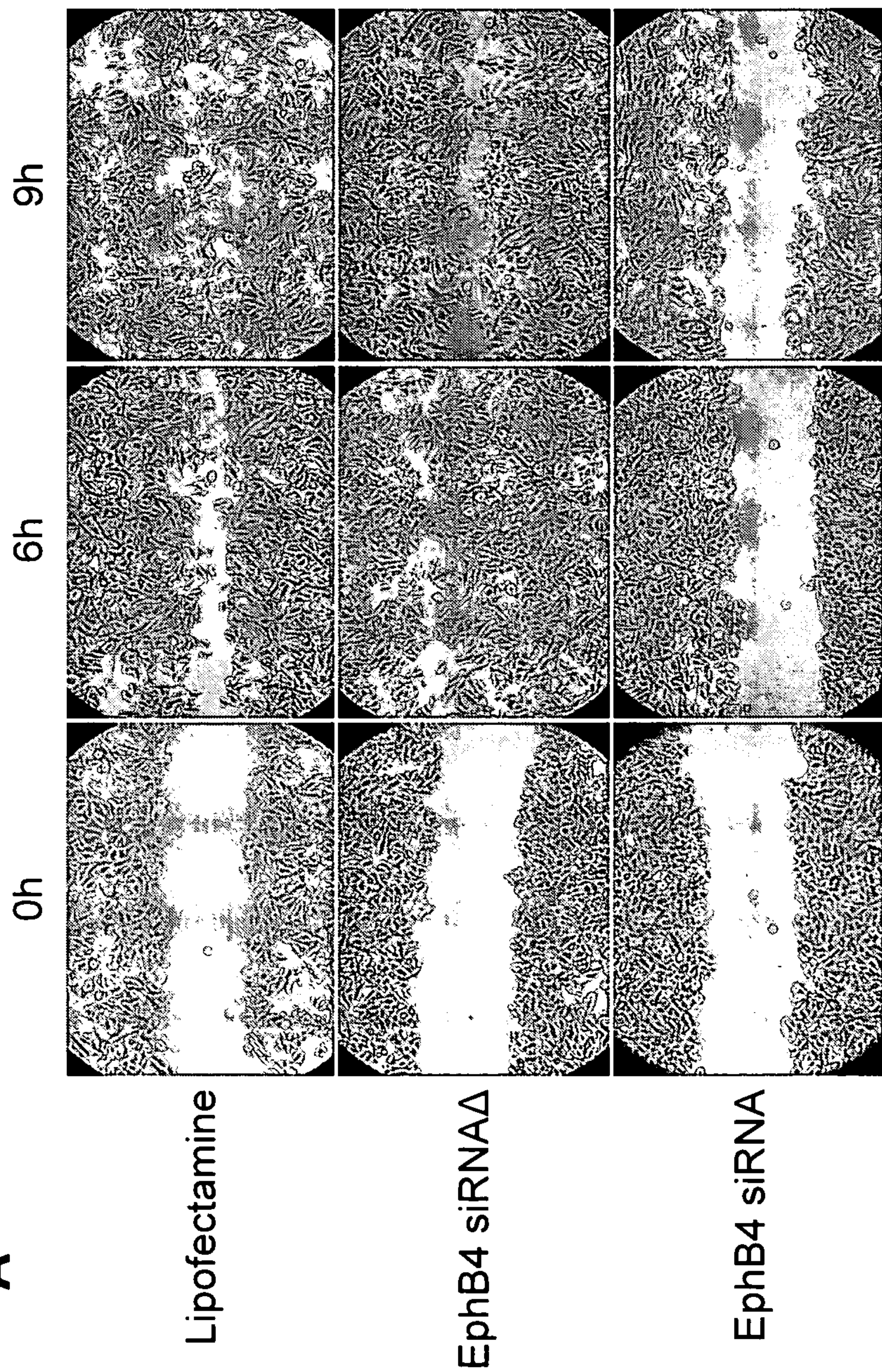


Figure 3
A
EphB4 siRNA (nM)
0 1 5 25
EphB4
β-actin
Cell Viability (%Control)
EphB4 siRNA
EphB4 siRNAΔ
Lipo 1 5 25
Concentration (nM)
B
EphB4 AS10 (μM)
0 1 5 10
EphB4
β-actin
Cell Viability (%Control)
EphB4 AS-10
Scrambled
NT 1 5 10
Concentration (μM)
C
Fold change relative to control (A405-A490)
EphB4 siRNA
EphB4 siRNAΔ
Concentration (nM)
D
Activity relative to control (fold)
Caspase-9
Caspase-8
0 5 25 50 0 5 25 50
EphB4 siRNAΔ
EphB4 siRNA (nM)
E
Control
EphB4 siRNAΔ
EphB4 siRNA
Sub G0
G0/G1
S Phase
G2/M
Sub G0 10.3%
G0/G1 54.2%
S Phase 15.8%
G2/M 18.3%
10 54 16 18
Sub G0 12.0%
G0/G1 53.7%
S Phase 15.8%
G2/M 17.3%
12 54 16 17
Sub G0 52.2%
G0/G1 35.9%
S Phase 7.3%
G2/M 4.0%
52 36 7 4

Figure 4



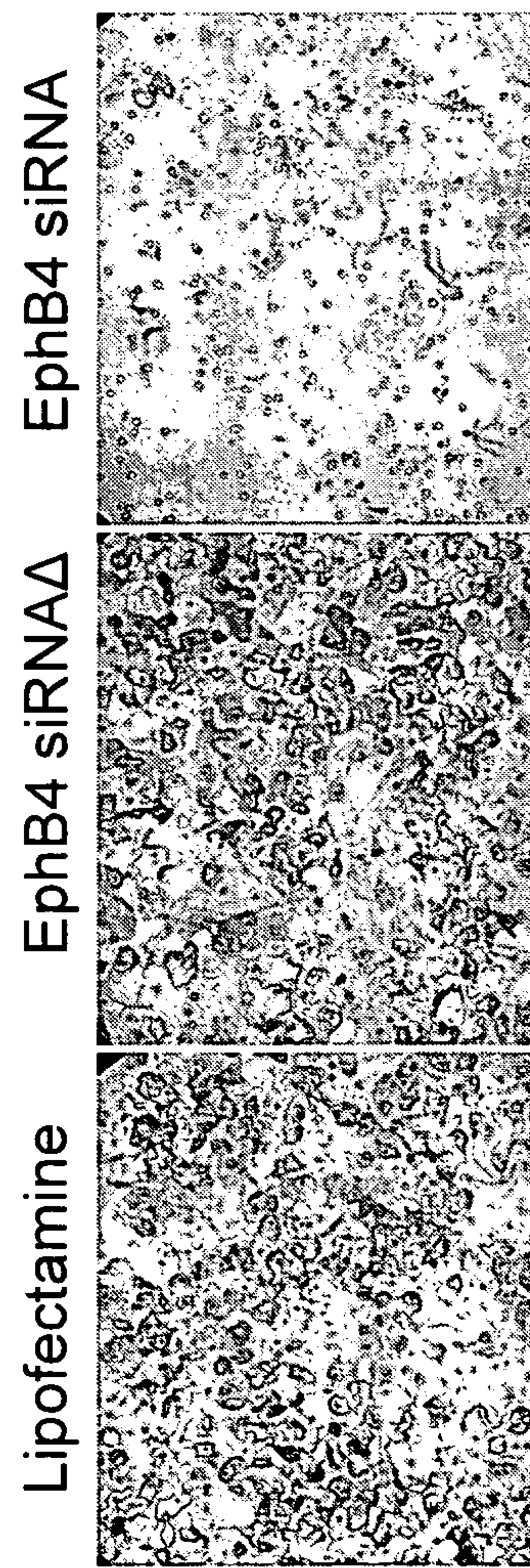
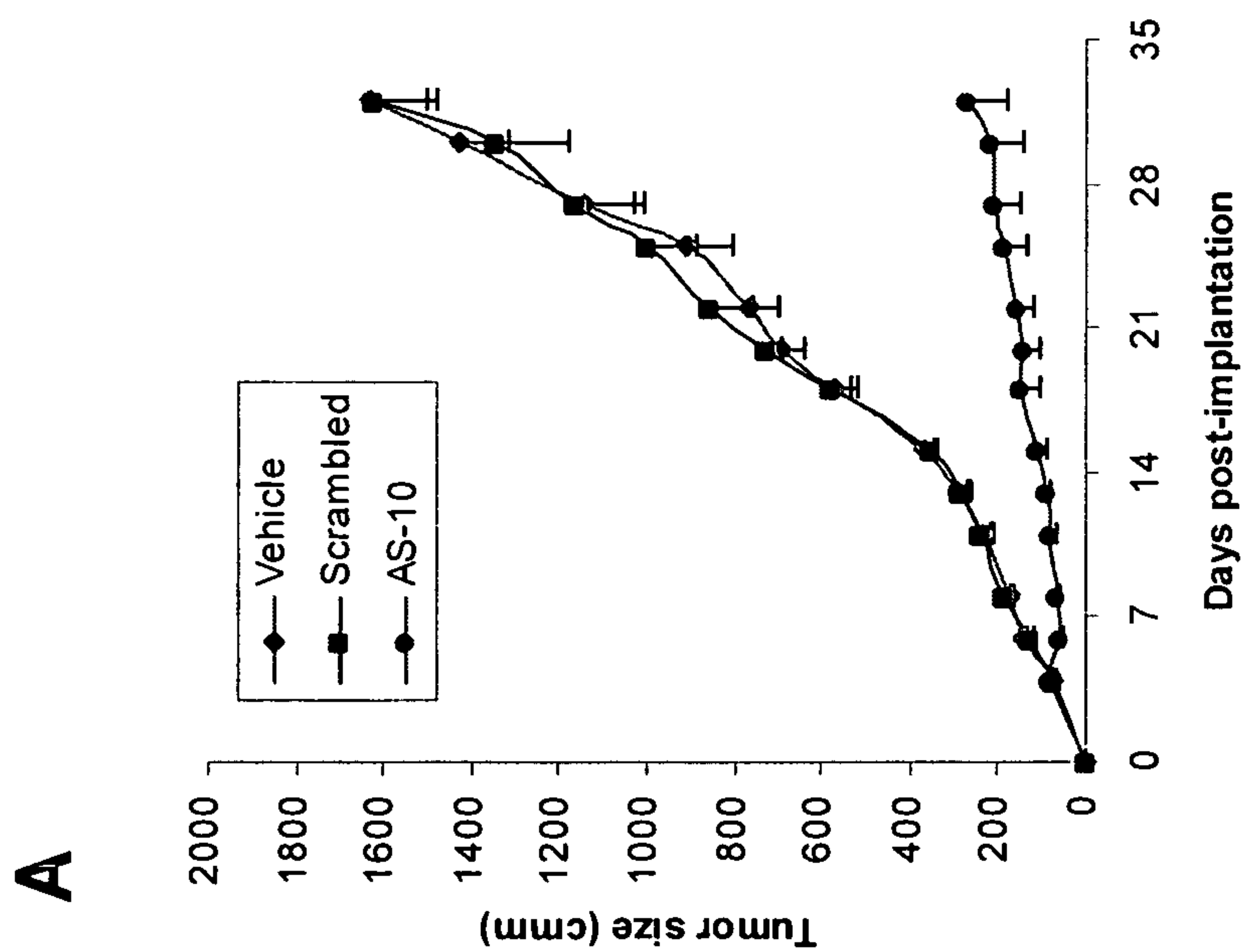


Figure 5

A



B

	Vehicle	Scrambled	AS-10
H/E			
Ki67	28	31	3*
CD31	46	42	11*
TUNEL	2	3	25*

Figure 6

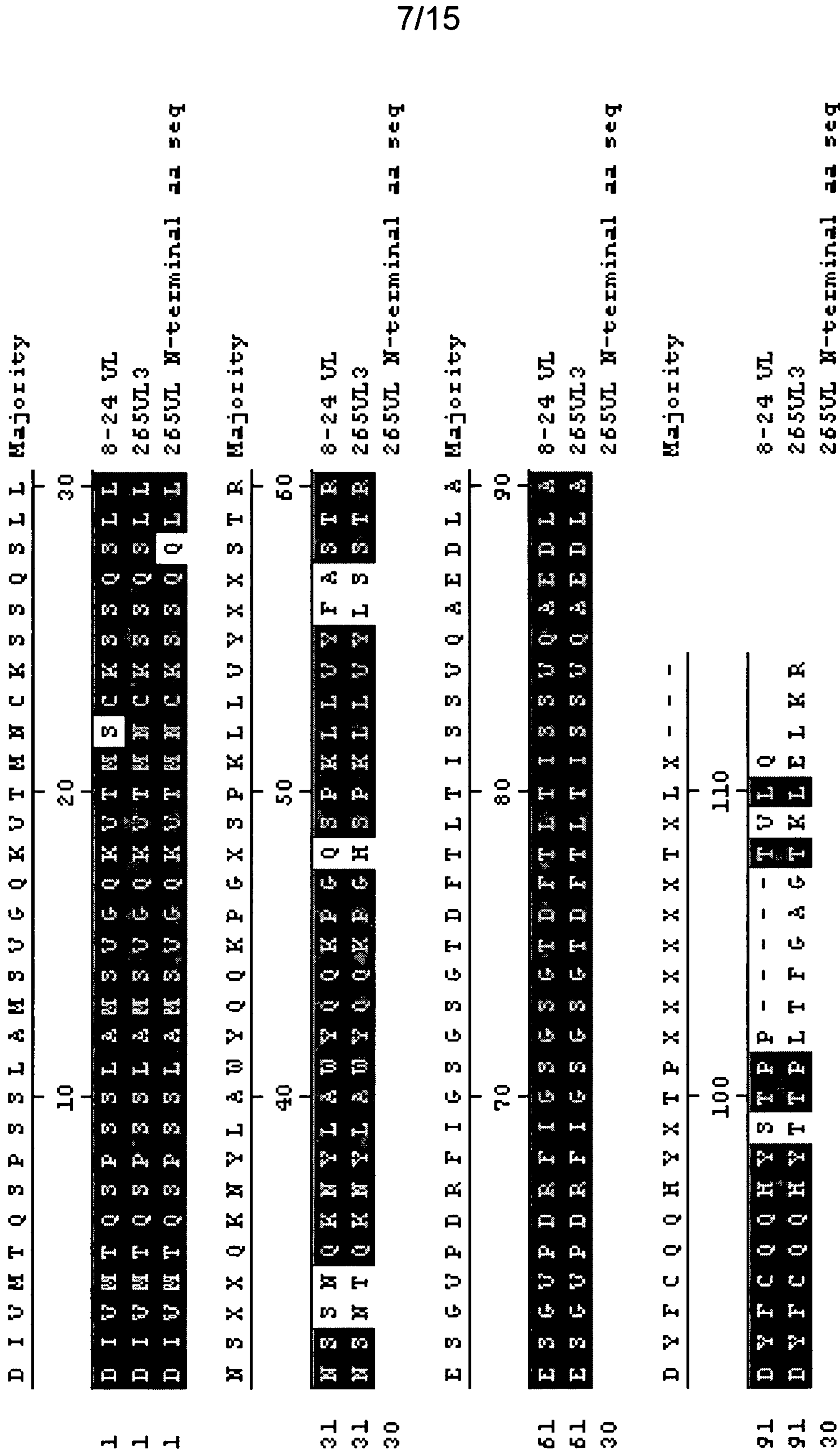


Figure 7

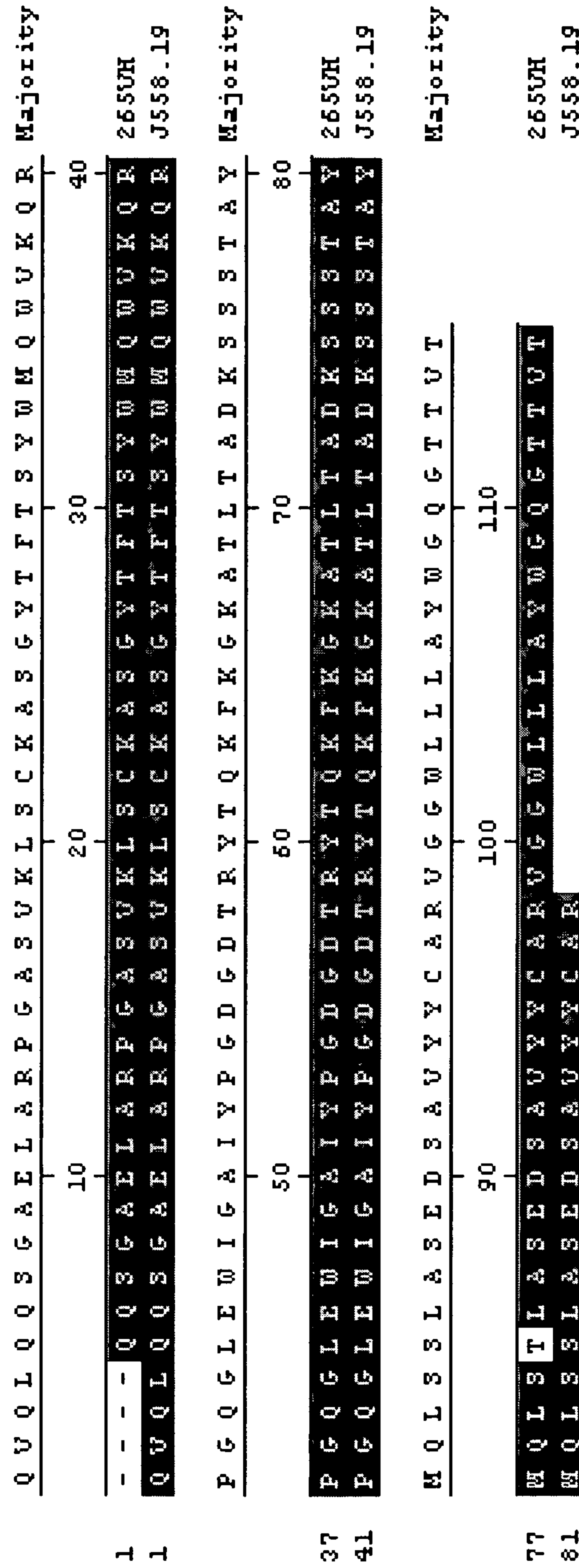


Figure 8

Summary of EphB4 Topology and Antibody Binding Sites

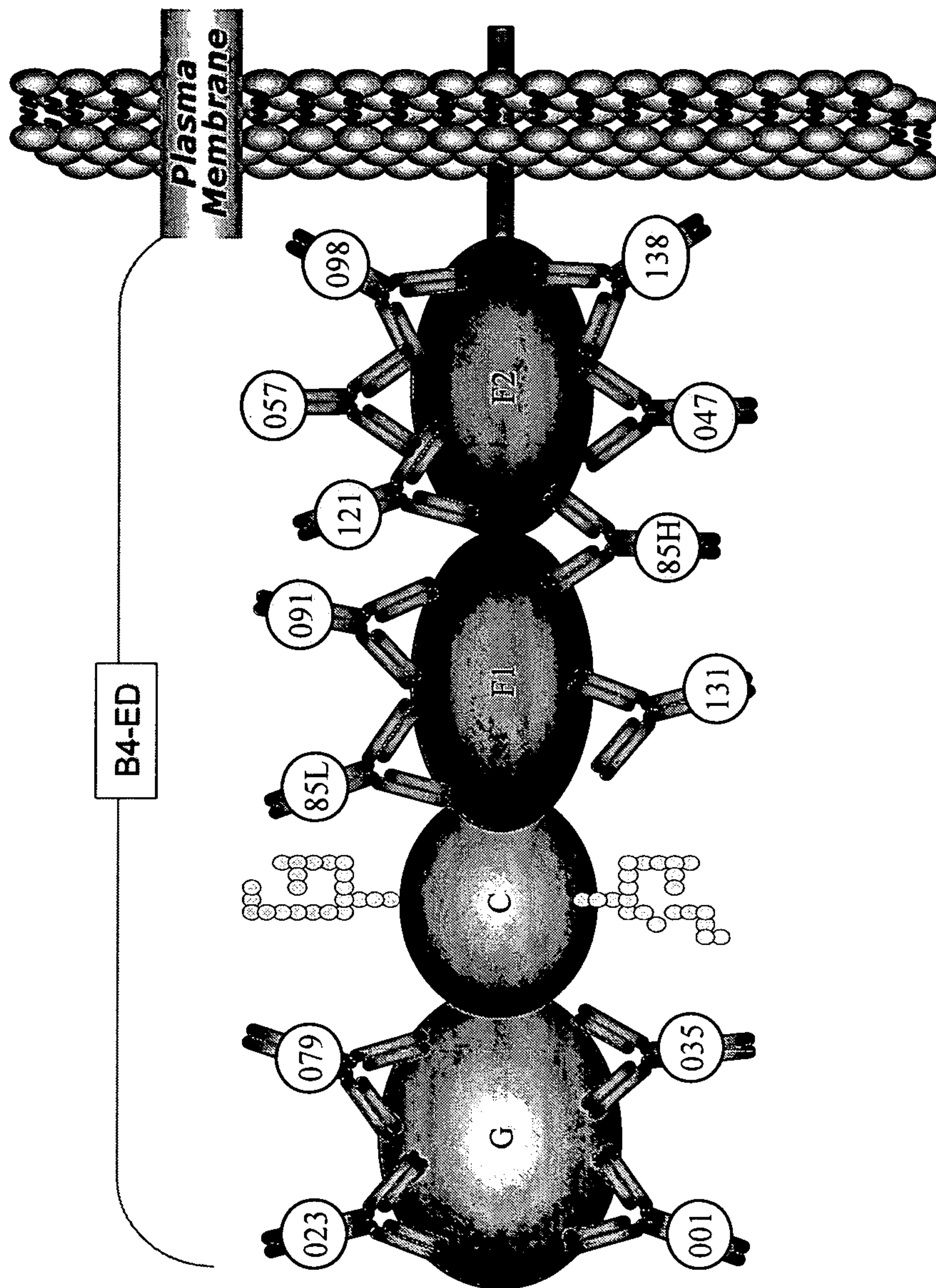


Figure 9

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

```
MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSG
LDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQDQGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVPAPGPSP
SLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGE
GSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRAILQIEGKPIPNPLLGLDSTRTG
HHHHHH
```

Figure 10

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

```
MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSG
LDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTM
LECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTV
AAEHLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQDQGACMALL
SLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVPAPGPSP
SLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGE
GSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRS
VVSRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGD
LTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFE
PVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVK
YHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGP
FGQEHHSQTQLDESEGWREQGSKRAILQISSTVAAARV
```

Figure 11

Amino acid sequence of the B4ECv3-FC protein (Eph B4 extracellular domain)

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHSVRTYEVCEVQRAPGQAH
WLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTVA
AEHLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPR
ELVVPVAGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGEGS
CQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRLNGSSLHLEWSAPLESGGREDL
TYALRCRECRPGGSCAPCGGDLTFDPGPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVN
VTTDREVPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGL
KRGASYLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQDPEPKSCDKTHTCPPCPAPELLGGPSVFLFP
PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Figure 12

Amino acid sequence of the B2EC protein (Ephrin B2 extracellular domain)

MAVRRDSVWKYCWGVLMVLCRTAISKSIVLEPIYWNSSNSKFLPGQGLVLYPQIGDKLDIICPKVDSKTVG
QYEYYKVYMVDKDQADRCTIKKENTPLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIISTSNGSL
EGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNKDPTRRPELEAGTNGRSSTTSPFVKPNPGSSTDGNSA
GHSGNNILGSEVGSHHHHHH

Figure 13

Amino acid sequence of the B2EC-FC protein (Ephrin B2 extracellular domain)

MAVRRDSVWKYCWGVLMVLCRTAISKSIVLEPIYWNSSNSKFLPGQGLVLYPQIGDKLDIICPKVDSKTVG
QYEYYKVYMVDKDQADRCTIKKENTPLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIISTSNGSL
EGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNKDPTRRPELEAGTNGRSSTTSPFVKPNPGSSTDGNSA
GHSGNNILGSEVDPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Figure 14

Ephrin B2

```
mavrrdsvwk ycwgvlmvlc rtaisksivl epiywnssns kflpgqglvl ypqigdkldi
icpkvdsktv gqyeyykvym vdkdqadrct ikkentplln cakpdqdikf tikfqefspn
lwglefqknk dyyiistsng slegldnqeg gvcqtramki lmkvgqdass agstrnkdpt
rrpeleagtn grssttspfv kpnpgsstdg nsaghsgnni lgsevalfag iasgciifiv
iiitlvvlll kyrrrhrkhs pqhtttlsls tlatpkrsgn nngsepsdii iplrtadsvf
cphyekvsgd yghpvyivqe mppqspaniy ykv
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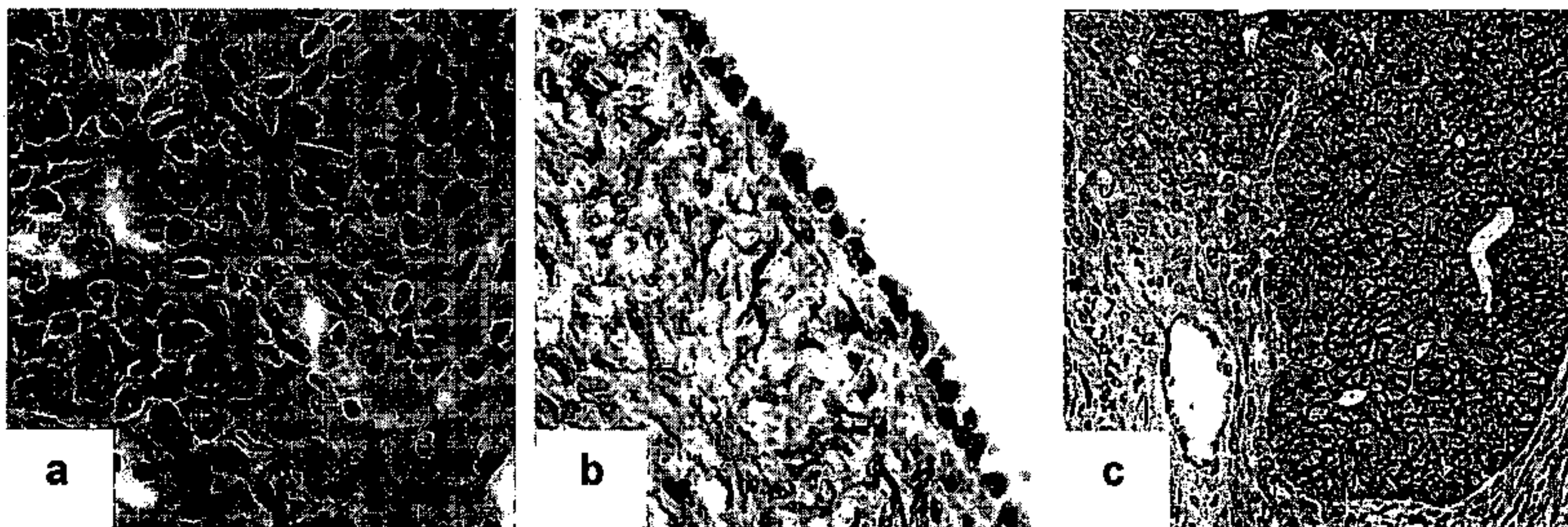
Figure 1

A



B

Variable	EphB4 expression Yes (n=50)	No (n=35)	p value
Stage			
Low (I/II)	3	13	<0.001
High (III/IV)	47	22	
Histology			
Serous	33	24	0.8
Other	17	11	
Grade			
Low (I)	4	7	0.1
High (II/III)	46	28	
Ascites			
Yes	45	18	<0.001
No	5	17	
Cytoreduction			
Optimal	29	27	0.067
Suboptimal	21	8	

C