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

Amino acid sequence of the B4ECv3 protein

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

(57) Abstract: In certain embodiments, this present invention provides polypeptide compositions,including compositions a modified polypeptide, and methods for inhibiting Ephrin B2 or EphB4 activity. In other embodiments, the present invention provides methods and compositions for treating cancer or for treating angiogenesis-associated diseases.

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POLYPEPTIDE COMPOUNDS FOR INHIBITING ANGIOGENESIS AND TUMOR GROWTH

RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Provisional Application No. 60/612,488, filed September 23, 2004, the specification of which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

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

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

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides an isolated soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4

protein fused to an albumin protein or a fragment thereof, wherein the polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide, and has an *in vivo* serum half-life at least 50% greater than that of the extracellular domain of the EphB4 alone, and wherein the polypeptide inhibits signaling that results from the interaction between EphB4 and EphrinB2.

According to a second aspect, the present invention provides a pharmaceutical composition comprising a polypeptide of the first aspect, and a pharmaceutically acceptable carrier.

According to a third aspect, the present invention provides use of a polypeptide of the first aspect for preparing a medicament for inhibiting signaling through Ephrin B2/EphB4 pathway.

According to a fourth aspect, the present invention provides use of a polypeptide of the first aspect for preparing a medicament for reducing the growth rate of the tumor.

According to a fifth aspect, the present invention provides use of a polypeptide of the first aspect for preparing a medicament for treating cancer in a patient.

According to a sixth aspect, the present invention provides use of a polypeptide of the first aspect for preparing a medicament for inhibiting angiogenesis in a patient.

According to a seventh aspect, the present invention provides use of a polypeptide of the first aspect for preparing a medicament for treating a patient suffering from an angiogenesis-associated disease.

According to an eighth aspect, the present invention provides a cosmetic composition comprising the polypeptide of the first aspect, and a pharmaceutically acceptable carrier

According to a ninth aspect, the present invention provides a method of inhibiting signaling through Ephrin B2/EphB4 pathway comprising administering a polypeptide of the first aspect.

According to a tenth aspect, the present invention provides a method of reducing the growth rate of a tumor comprising administering a polypeptide of the first aspect

According to an eleventh aspect, the present invention provides a method of treating cancer in a patient comprising administering a polypeptide the first aspect.

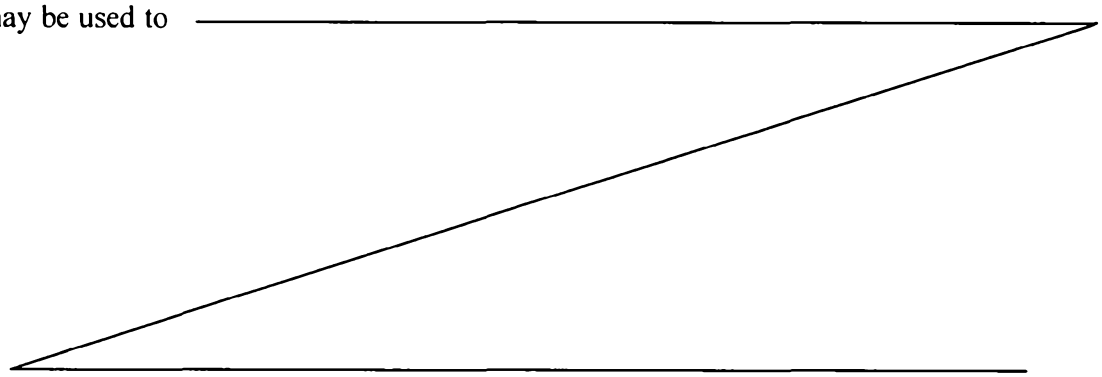
According to a twelfth aspect, the present invention provides a method of inhibiting angiogenesis in a patient comprising administering a polypeptide of the first aspect.

According to a twelfth aspect, the present invention provides a method of inhibiting angiogenesis in a patient comprising administering a polypeptide of the first aspect.

According to a thirteenth aspect, the present invention provides a method of treating a patient suffering from an angiogenesis-associated disease comprising administering a polypeptide of the first aspect.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

In certain aspects, the disclosure provides polypeptide agents that inhibit EphB4 or EphrinB2 mediated functions, including monomeric ligand binding portions of the EphB4 and EphrinB2 proteins. As demonstrated herein, EphB4 and EphrinB2 participate in various disease states, including cancers and diseases related to unwanted or excessive angiogenesis. Accordingly, certain polypeptide agents disclosed herein may be used to



treat such diseases. In further aspects, the disclosure relates to the discovery that EphB4 and/or EphrinB2 are expressed, often at high levels, in a variety of tumors. Therefore, polypeptide agents that down-regulate EphB4 or EphrinB2 function may affect tumors by a direct effect on the tumor cells as well as an indirect effect on the angiogenic processes recruited by the tumor. In certain embodiments, the disclosure provides the identity of tumor types particularly suited to treatment with an agent that downregulates EphB4 or EphrinB2 function. In preferred embodiments, polypeptides disclosed herein are modified so as to have increased serum half-life in vivo.

In certain aspects, the disclosure provides soluble EphB4 polypeptides comprising an amino acid sequence of an extracellular domain of an EphB4 protein. The soluble EphB4 polypeptides bind specifically to an EphrinB2 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphB4 for binding to ligand such as EphrinB2 and inhibit the signaling that results from EphB4 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. In certain embodiments the soluble EphB4 polypeptide comprises a globular domain of an EphB4 protein. A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-522 of the amino acid sequence defined by Figure 65 (SEQ ID NO:10). A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-412 of the amino acid sequence defined by Figure 65 (SEQ ID NO:10). A soluble EphB4 polypeptide may comprise a sequence at least 90% identical to residues 1-312 of the amino acid sequence defined by Figure 65 (SEQ ID NO:10). A soluble EphB4 polypeptide may comprise a sequence encompassing the globular (G) domain (amino acids 29-197 of Figure 65, SEQ ID NO:10), and optionally additional domains, such as the cysteine-rich domain (amino acids 239-321 of Figure 65, SEQ ID NO:10), the first fibronectin type 3 domain (amino acids 324-429 of Figure 65, SEQ ID NO:10) and the second fibronectin type 3 domain (amino acids 434-526 of Figure 65, SEQ ID NO:10). Preferred polypeptides described herein and demonstrated as having ligand binding activity include polypeptides corresponding to 1-537, 1-427 and 1-326, respectively, of the amino acid sequence shown in Figure 65 (SEQ ID NO:10). A soluble EphB4 polypeptide may comprise a sequence as set forth in Figure 1

or 2 (SEQ ID Nos. 1 or 2). As is well known in the art, expression of such EphB4 polypeptides in a suitable cell, such as HEK293T cell line, will result in cleavage of a leader peptide. Although such cleavage is not always complete or perfectly consistent at a single site, it is known that EphB4 tends to be cleaved so as to remove the first 15 amino acids of the sequence shown in Figure 65 (SEQ ID NO:10). Accordingly, as specific examples, the disclosure provides unprocessed soluble EphB4 polypeptides that bind to EphrinB2 and comprise an amino acid sequence selected from the following group (numbering is with respect to the sequence of Figure 65, SEQ ID NO:10): 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, 29-429, 1-526, 29-526, 1-537 and 29-537. Additionally, heterologous leader peptides may be substituted for the endogeneous leader sequences. Polypeptides may be used in a processed form, such forms having a predicted amino acid sequence selected from the following group (numbering is with respect to the sequence of Figure 65, SEQ ID NO:10): 16-197, 16-312, 16-321, 16-326, 16-412, 16-427, 16-429, 16-526 and 16-537. Additionally, a soluble EphB4 polypeptide may be one that comprises an amino acid sequence at least 90%, and optionally 95% or 99% identical to any of the preceding amino acid sequences while retaining EphrinB2 binding activity. Preferably, any variations in the amino acid sequence from the sequence shown in Figure 65 (SEQ ID NO:10) are conservative changes or deletions of no more than 1, 2, 3, 4 or 5 amino acids, particularly in a surface loop region. In certain embodiments, the soluble EphB4 polypeptide may inhibit the interaction between Ephrin B2 and EphB4. The soluble EphB4 polypeptide may inhibit clustering of or phosphorylation of Ephrin B2 or EphB4. Phosphorylation of EphrinB2 or EphB4 is generally considered to be one of the initial events in triggering intracellular signaling pathways regulated by these proteins. As noted above, the soluble EphB4 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

The present disclosure provides soluble EphB4 polypeptides having an additional component that confers increased serum half-life while still retaining EphrinB2 binding activity. In certain embodiments soluble EphB4 polypeptides are monomeric and are covalently linked to one or more polyoxyaklylene groups (e.g., polyethylene, polypropylene), and preferably polyethylene glycol (PEG) groups. Accordingly, one aspect of the invention provides modified EphB4 polypeptides, wherein the modification comprises a single polyethylene glycol group covalently bonded to the polypeptide. Other

aspects provide modified EphB4 polypeptides covalently bonded to one, two, three, or more polyethylene glycol groups.

The one or more PEG may have a molecular weight ranging from about 1 kDa to about 100 kDa, and will preferably have a molecular weight ranging from about 10 to about 60 kDa or about 10 to about 40 kDa. The PEG group may be a linear PEG or a branched PEG. In a preferred embodiment, the soluble, monomeric EphB4 conjugate comprises an EphB4 polypeptide covalently linked to one PEG group of from about 10 to about 40 kDa (monoPEGylated EphB4), or from about 15 to 30 kDa, preferably via an ϵ-amino group of EphB4 lysine or the N-terminal amino group. Most preferably, EphB4 is randomly PEGylated at one amino group out of the group consisting of the ϵ-amino groups of EphB4 lysine and the N-terminal amino group.

In one embodiment, the pegylated polypeptides provided by the invention have a serum half-life *in vivo* at least 50%, 75%, 100%, 150% or 200% greater than that of an unmodified EphB4 polypeptide. In another embodiment, the pegylated EphB4 polypeptides provided by the invention inhibit EphrinB2 activity. In a specific embodiment, they inhibit EphrinB2 receptor clustering, EphrinB2 phosphorylation, and/or EphrinB2 kinase activity.

Surprisingly, it has been found that monoPEGylated EphB4 according to the invention has superior properties in regard to the therapeutic applicability of unmodified soluble EphB4 polypeptides and poly-PEGylated EphB4. Nonetheless, the disclosure also provides poly-PEGylated EphB4 having PEG at more than one position. Such polyPEGylated forms provide improved serum-half life relative to the unmodified form.

In certain embodiments, a soluble EphB4 polypeptide is stably associated with a second stabilizing polypeptide that confers improved half-life without substantially diminishing EphrinB2 binding. A stabilizing polypeptide will preferably be immunocompatible with human patients (or animal patients, where veterinary uses are contemplated) and have little or no significant biological activity.

In a preferred embodiment, the stabilizing polypeptide is a human serum albumin, or a portion thereof. A human serum albumin may be stably associated with the EphB4 polypeptide covalently or non-covalently. Covalent attachment may be achieved by expression of the EphB4 polypeptide as a co-translational fusion with human serum albumin. The albumin sequence may be fused at the N-terminus, the C-terminus or at a non-disruptive internal position in the soluble EphB4 polypeptide. Exposed loops of the

EphB4 would be appropriate positions for insertion of an albumin sequence. Albumin may also be post-translationally attached to the EphB4 polypeptide by, for example, chemical cross-linking. An EphB4 polypeptide may also be stably associated with more than one albumin polypeptide. In some embodiments, the albumin is selected from the group consisting of a human serum albumin (HSA) and bovine serum albumin (BSA). In other embodiments, the albumin is a naturally occurring variant. In one preferred embodiment, the EphB4-HSA fusion inhibits the interaction between Ephrin B2 and EphB4, the clustering of Ephrin B2 or EphB4, the phosphorylation of Ephrin B2 or EphB4, or combinations thereof. In other embodiments, the EphB4-HSA fusion has enhanced in vivo stability relative to the unmodified wildtype polypeptide.

In certain aspects, the disclosure provides soluble EphrinB2 polypeptides comprising an amino acid sequence of an extracellular domain of an EphrinB2 protein. The soluble EphrinB2 polypeptides bind specifically to an EphB4 polypeptide. The term "soluble" is used merely to indicate that these polypeptides do not contain a transmembrane domain or a portion of a transmembrane domain sufficient to compromise the solubility of the polypeptide in a physiological salt solution. Soluble polypeptides are preferably prepared as monomers that compete with EphrinB2 for binding to ligand such as EphB4 and inhibit the signaling that results from EphrinB2 activation. Optionally, a soluble polypeptide may be prepared in a multimeric form, by, for example, expressing as an Fc fusion protein or fusion with another multimerization domain. Such multimeric forms may have complex activities, having agonistic or antagonistic effects depending on the context. A soluble EphrinB2 polypeptide may comprise residues 1-225 of the amino acid sequence defined by Figure 66 (SEQ ID NO:11). A soluble EphrinB2 polypeptide may comprise a sequence defined by Figure 3. As is well known in the art, expression of such EphrinB2 polypeptides in a suitable cell, such as HEK293T cell line, will result in cleavage of a leader peptide. Although such cleavage is not always complete or perfectly consistent at a single site, it is known that EphrinB2 tends to be cleaved so as to remove the first 26 amino acids of the sequence shown in Figure 66 (SEQ ID NO:11). Accordingly, as specific examples, the disclosure provides unprocessed soluble EphrinB2 polypeptides that bind to EphB4 and comprise an amino acid sequence corresponding to amino acids 1-225 of Figure 66 (SEQ ID NO:11). Such polypeptides may be used in a processed form, such forms having a predicted amino acid sequence selected from the following group (numbering is with respect to the sequence of Figure 66, SEQ ID NO:11): 26-225. In certain embodiments, the soluble EphrinB2 polypeptide may inhibit the interaction between Ephrin

B2 and EphB4. The soluble EphrinB2 polypeptide may inhibit clustering of or phosphorylation of EphrinB2 or EphB4. As noted above, the soluble EphrinB2 polypeptide may be prepared as a monomeric or multimeric fusion protein. The soluble polypeptide may include one or more modified amino acids. Such amino acids may contribute to desirable properties, such as increased resistance to protease digestion.

In certain aspects, the disclosure provides pharmaceutical formulations comprising a polypeptide reagent and a pharmaceutically acceptable carrier. The polypeptide reagent may be any disclosed herein, including, for example, soluble EphB4 or EphrinB2 polypeptides. Additional formulations include cosmetic compositions and diagnostic kits.

In certain aspects the disclosure provides methods of inhibiting signaling through Ephrin B2/EphB4 pathway in a cell. A method may comprise contacting the cell with an effective amount of a polypeptide agent, such as (a) 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; (b) 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.

In certain aspects the disclosure provides methods for reducing the growth rate of a tumor, comprising administering an amount of a polypeptide agent sufficient to reduce the growth rate of the tumor. The polypeptide agent may be selected from the group consisting of: (a) 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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (b) 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 Optionally, the tumor comprises cells expressing a higher level of EphB4 and/or EphrinB2 than noncancerous cells of a comparable tissue.

In certain aspects, the disclosure provides methods for treating a patient suffering from a cancer. A method may comprise administering to the patient a polypeptide agent. The polypeptide agent may be selected from the group consisting of: (a) 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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (b) 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. Optionally, the cancer comprises cancer cells expressing EphrinB2 and/or EphB4 at a higher level than noncancerous cells of a comparable tissue. The cancer may be a metastatic cancer. The cancer may be selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia. Optionally, the cancer is an angiogenesis-dependent cancer or an angiogenesis independent cancer. The polypeptide agent employed may inhibit clustering or phosphorylation of Ephrin B2 or EphB4. A polypeptide agent may be co-administered with one or more additional anti-cancer chemotherapeutic agents that inhibit cancer cells in an additive or synergistic manner with the polypeptide agent.

In certain aspects, the disclosure provides methods of inhibiting angiogenesis. A method may comprise contacting a cell with an amount of a polypeptide agent sufficient to inhibit angiogenesis. The polypeptide agent may be selected from the group consisting of: (a) 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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (b) 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.

In certain aspects, the disclosure provides methods for treating a patient suffering from an angiogenesis-associated disease, comprising administering to the patient a polypeptide agent. The polypeptide agent may be selected from the group consisting of: (a) 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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (b) 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. The soluble polypeptide may be formulated with a pharmaceutically acceptable carrier. An angiogenesis related disease or unwanted angiogenesis related

process may be selected from the group consisting of angiogenesis-dependent cancer, benign tumors, inflammatory disorders, chronic articular rheumatism and psoriasis, ocular angiogenic diseases, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, telangiectasia psoriasis scleroderma, pyogenic granuloma, rubeosis, arthritis, diabetic neovascularization, vasculogenesis. A polypeptide agent may be co-administered with at least one additional anti-angiogenesis agent that inhibits angiogenesis in an additive or synergistic manner with the soluble polypeptide.

In certain aspects, the disclosure provides for the use of a polypeptide agent in the manufacture of medicament for the treatment of cancer or an angiogenesis related disorder. The polypeptide agent may be selected from the group consisting of: (a) 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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (b) 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.

In certain aspects, the disclosure provides methods for treating a patient suffering from a cancer, comprising: (a) identifying in the patient a tumor having a plurality of cancer cells that express EphB4 and/or EphrinB2; and (b) administering to the patient a polypeptide agent. The polypeptide agent may be selected from the group consisting of: (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 optionally comprises an additional modification to increase serum half-life, such as a PEGylation or serum albumin or both; (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.

In certain aspects, the disclosure provides methods for identifying a tumor that is suitable for treatment with an EphrinB2 or EphB4 antagonist. A method may comprise detecting in the tumor cell one or more of the following characteristics: (a) expression of EphB4 protein and/or mRNA; (b) expression of EphrinB2 protein and/or mRNA; (c) gene

amplification (e.g., increased gene copy number) of the EphB4 gene; or (d) gene amplification of the EphrinB2 gene. A tumor cell having one or more of characteristics (a)-(d) may be suitable for treatment with an EphrinB2 or EphB4 antagonist, such as a polypeptide agent described herein.

Surprisingly, applicants have found that an EphB4 polypeptide lacking the globular domain can in fact inhibit tumor growth in a xenograft model, inhibit angiogenic tube formation of vascular endothelial cells and inhibit EphrinB2-activated autokinase activity of EphB4. While not wishing to be bound to any mechanism of action, it is expected that the polypeptide either prevents EphB4 aggregation or stimulates the elimination (e.g. by endocytosis) of EphB4 from the plasma membrane. Accordingly, the disclosure provides isolated soluble polypeptides comprising an amino acid sequence of a fibronectin type 3 domain of an EphB4 protein. Such polypeptides will preferably have a biological effect, such as inhibiting an activity (e.g. aggregation or kinase activity) of an EphB4 or EphrinB2 protein, and particularly the inhibition of tumor growth in a human or in a mouse xenograft model of cancer. Such polypeptides may also inhibit angiogenesis in vivo or in an cell-based assay system. Such polypeptides may not bind to EphrinB2 and may specifically exclude all of or the functional (e.g., EphrinB2 binding-) portions of the globular domain of an EphB4 protein. Such a polypeptide will preferably comprise amino acids corresponding to amino acids 324-429 and/or 434-526 of the sequence of Figure 65 (SEQ ID NO:10), or sequences at least 90%, 95%, 98%, 99% identical thereto. An example of such a polypeptide is shown in SEQ ID NO: 15. Such a polypeptide may be modified in any of the ways described herein, and may be produced as a monomer or as a dimer or multimer comprising two or more such polypeptides, such as an Fc fusion construct. Dimers or multimers may be desirable to enhance the effectiveness of such polypeptides. All of the methods for producing and using such polypeptides are similar to those described herein with respect to other EphB4 polypeptides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows amino acid sequence of the B4ECv3 protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown; SEQ ID NO:1).

Figure 2 shows amino acid sequence of the B4ECv3NT protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown; SEQ ID NO:2).

Figure 3 shows amino acid sequence of the B2EC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown; SEQ ID NO:3).

Figure 4 shows amino acid sequence of the B4ECv3-FC protein (predicted sequence of the precursor including uncleaved Eph B4 leader peptide is shown; SEQ ID NO:4).

Figure 5 shows amino acid sequence of the B2EC-FC protein (predicted sequence of the precursor including uncleaved Ephrin B2 leader peptide is shown; SEQ ID NO:5).

Figure 6 shows B4EC-FC binding assay (Protein A-agarose based).

Figure 7 shows B4EC-FC inhibition assay (Inhibition in solution).

Figure 8 shows B2EC-FC binding assay (Protein-A-agarose based assay).

Figure 9 shows chemotaxis of HUAEC in response to B4Ecv3.

Figure 10 shows chemotaxis of HHEC in response to B2EC-FC.

Figure 11 shows chemotaxis of HHAEC in response to B2EC.

Figure 12 shows effect of B4Ecv3 on HUAEC tubule formation.

Figure 13 shows effect of B2EC-FC on HUAEC tubule formation.

Figure 14 is a schematic representation of human Ephrin B2 constructs.

Figure 15 is a schematic representation of human EphB4 constructs.

Figure 16 shows the domain structure of the recombinant soluble EphB4EC proteins. Designation of the domains are as follows: L - leader peptide, G – globular (ligand-binding domain), C – Cys-rich domain, F1, F2 – fibronectin type III repeats, H – 6 x His-tag.

Figure 17 shows purification and ligand binding properties of the EphB4EC proteins. **A.** SDS-PAAG gel electrophoresis of purified EphB4-derived recombinant soluble proteins (Coomassie-stained). **B.** Binding of Ephrin B2-AP fusion to EphB4-derived recombinant proteins immobilized on Ni-NTA-agarose beads. Results of three independent experiments are shown for each protein. Vertical axis – optical density at 420 nm.

Figure 18 shows that EphB4v3 inhibits chemotaxis.

Figure 19 shows that EphB4v3 inhibits tubule formation on Matrigel. **A** displays the strong inhibition of tubule formation by B4v3 in a representative experiment. **B** shows a quantitation of the reduction of tube-length obtained with B4v3 at increasing concentrations as well as a reduction in the number of junctions, in comparison to cells with no protein. Results are displayed as mean values ± S.D. obtained from three independent experiments performed with duplicate wells.

Figure 20 shows that soluble EphB4 has no detectable cytotoxic effect as assessed by MTS assay.

Figure 21 shows that B4v3 inhibits invasion and tubule formation by endothelial cells in the Matrigel assay. (*A*) to detect total invading cells, photographed at 20X magnification or with Masson's Trichrome Top left of A B displays section of a Matrigel plug with no GF , *top right* of *A* displays section with B4IgG containing GF and *lower left* section contains GF, and lower right shows GF in the presence of B4v3. Significant invasion of endothelial cells is only seen in GF containing Matrigel. *Top right* displays an area with a high number of invaded cells induced by B4IgG, which signifies the dimeric form of B4v3. The *left upper parts* of the pictures correspond to the cell layers formed around the Matrigel plug from which cells invade toward the center of the plug located in the direction of the *right lower corner*. Total cells in sections of the Matrigel plugs were quantitated with Scion Image software. Results obtained from two experiments with duplicate plugs are displayed as mean values ± S.D.

Figure 22 shows tyrosine phosphorylation of EphB4 receptor in PC3 cells in response to stimulation with EphrinB2-Fc fusion in presence or absence of EphB4-derived recombinant soluble proteins.

Figure 23 shows effects of soluble EphB4ECD on viability and cell cycle. A) 3-day cell viability assay of two HNSCC cell lines. B) FACS analysis of cell cycle in HNSCC-15 cells treated as in A. Treatment of these cells resulted in accumulation in subG0/G1 and S/G2 phases as indicated by the arrows.

Figure 24 shows that B4v3 inhibits endovascular response in a murine corneal hydron micropocket assay.

Figure 25 shows that that SCC15, B16, and MCF-7 co-injected with sB4v3 in the presence of matrigel and growth factors, inhibits the in vivo tumor growth of these cells.

Figure 26 shows that soluble EphB4 causes apoptosis, necrosis and decreased angiogenesis in three tumor types, B16 (melanoma), SCC15 (head and neck carcinoma), and MCF-7 (breast carcinoma). Tumors were injected premixed with Matrigel plus growth factors and soluble EphB4 subcutaneously. After 10 to 14 days, the mice were injected intravenously with FITC-lectin (green) to assess blood vessel perfusion. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with sEphB4 displayed a decrease in tumor cell density and a marked

inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 27 shows expression of EphB4 in prostate cell lines. A) Western blot of total cell lysates of various prostate cancer cell lines, normal prostate gland derived cell line (MLC) and acute myeloblastic lymphoma cells (AML) probed with EphB4 monoclonal antibody. B) Phosphorylation of EphB4 in PC-3 cells determined by Western blot.

Figure 28 shows expression of EphB4 in prostate cancer tissue. Representative prostate cancer frozen section stained with EphB4 monoclonal antibody (top left) or isotype specific control (bottom left). Adjacent BPH tissue stained with EphB4 monoclonal antibody (top right). Positive signal is brown color in the tumor cells. Stroma and the normal epithelia are negative. Note membrane localization of stain in the tumor tissue, consistent with trans-membrane localization of EphB4. Representative QRT-PCR of RNA extracted from cancer specimens and adjacent BPH tissues (lower right).

Figure 29 shows downregulation of EphB4 in prostate cancer cells by tumor suppressors and RXR expression. A) PC3 cells were co-transfected with truncated CD4 and p53 or PTEN or vector only. 24 h later CD4-sorted cells were collected, lysed and analyzed sequentially by Western blot for the expression of EphB4 and β-actin, as a normalizer protein. B) Western blot as in (A) of various stable cell lines. LNCaP-FGF is a stable transfection clone of FGF-8, while CWR22R-RXR stably expresses the RXR receptor. BPH-1 was established from benign hypertrophic prostatic epithelium.

Figure 30 shows regulation of EphB4 in prostate cancer cells by EGFR and IGFR-1. A) Western blot of PC3 cells treated with or without EGFR specific inhibitor AG1478 (1 nM) for 36 hours. Decreased EphB4 signal is observed after AG 1478 treatment. The membrane was stripped and reprobed with β-actin, which was unaffected. B) Western Blot of triplicate samples of PC3 cells treated with or without IGFR-1 specific neutralizing antibody MAB391 (2 μg/ml; overnight). The membrane was sequentially probed with EphB4, IGFR-1 and β-actin antibodies. IGFR-1 signal shows the expected repression of signal with MAB391 treatment.

Figure 31 shows effect of specific EphB4 AS-ODNs and siRNA on expression and prostate cell functions. A) 293 cells stably expressing full-length construct of EphB4 was used to evaluate the ability of siRNA 472 to inhibit EphB4 expression. Cells were transfected with 50 nM RNAi using Lipofectamine 2000. Western blot of cell lysates 40 h post transfection with control siRNA (green fluorescence protein; GFP siRNA) or EphB4

siRNA 472, probed with EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody. B) Effect of EphB4 AS-10 on expression in 293 transiently expressing full-length EphB4. Cells were exposed to AS-10 or sense ODN for 6 hours and analyzed by Western blot as in (A). C) 48 h viability assay of PC3 cells treated with siRNA as described in the Methods section. Shown is mean ± s.e.m. of triplicate samples. D) 5-day viability assay of PC3 cells treated with ODNs as described in the Methods. Shown is mean ± s.e.m. of triplicate samples. E) Scrape assay of migration of PC3 cells in the presence of 50 nM siRNAs transfected as in (A). Shown are photomicrographs of representative 20x fields taken immediately after the scrape was made in the monolayer (0 h) and after 20h continued culture. A large number of cells have filled in the scrape after 20 h with control siRNA, but not with EphB4 siRNA 472. F) Shown is a similar assay for cells treated with AS-10 or sense ODN (both 10 μM). G) Matrigel invasion assay of PC3 cells transfected with siRNA or control siRNA as described in the methods. Cells migrating to the underside of the Matrigel coated insert in response to 5 mg/ml fibronectin in the lower chamber were fixed and stained with Giemsa. Shown are representative photomicrographs of control siRNA and siRNA 472 treated cells. Cell numbers were counted in 5 individual high-powered fields and the average ± s.e.m. is shown in the graph (bottom right).

Figure 32 shows effect of EphB4 siRNA 472 on cell cycle and apoptosis. A) PC3 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. 7.9% of the cell population is apoptotic (in the Sub G0 peak) when treated with siRNA 472 compared to 1% with control siRNA. B) Apoptosis of PC3 cells detected by Cell Death Detection ELISA^{plus} kit as described in the Methods. Absorbance at 405 nm increases in proportion to the amount of histone and DNA-POD in the nuclei-free cell fraction. Shown is the mean ± s.e.m. of triplicate samples at the indicated concentrations of siRNA 472 and GFP siRNA (control).

Figure 33 shows that EphB4 and EphrinB2 are expressed in mesothelioma cell lines as shown by RT-PCR (A) and Western Blot (B).

Figure 34 shows expression of ephrin B2 and EphB4 by in situ hybridization in mesothelioma cells. NCI H28 mesothelioma cell lines cultured in chamber slides hybridized with antisense probe to ephrin B2 or EphB4 (top row). Control for each hybridization was sense (bottom row). Positive reaction is dark blue cytoplasmic stain.

Figure 35 shows cellular expression of EphB4 and ephrin B2 in mesothelioma cultures. Immunofluorescence staining of primary cell isolate derived from pleural effusion of a patient with malignant mesothelioma and cell lines NCI H28, NCI H2373, and NCI H2052 for ephrin B2 and EphB4. Green color is positive signal for FITC labeled secondary antibody. Specificity of immunofluorescence staining was demonstrated by lack of signal with no primary antibody (first row). Cell nuclei were counterstained with DAPI (blue color) to reveal location of all cells. Shown are merged images of DAPI and FITC fluorescence. Original magnification 200X.

Figure 36 shows expression of ephrin B2 and EphB4 in mesothelioma tumor. Immunohistochemistry of malignant mesothelioma biopsy. H&E stained section reveals tumor architecture; bottom left panel is background control with no primary antibody. EphB4 and ephrin B2 specific staining is brown color. Original magnification 200X.

Figure 37 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on the growth of H28 cells.

Figure 38 shows effects of EPHB4 antisense probes (A) and EPHB4 siRNAs (B) on cell migration.

Figure 39 shows that EphB4 is expressed in HNSCC primary tissues and metastases. A) Top: Immunohistochemistry of a representative archival section stained with EphB4 monoclonal antibody as described in the methods and visualized with DAB (brown color) localized to tumor cells. Bottom: Hematoxylin and Eosin (H&E) stain of an adjacent section. Dense purple staining indicates the presence of tumor cells. The right hand column are frozen sections of lymph node metastasis stained with EphB4 polyclonal antibody (top right) and visualized with DAB. Control (middle) was incubation with goat serum and H&E (bottom) reveals the location of the metastatic foci surrounded by stroma which does not stain. B) In situ hybridization of serial frozen sections of a HNSCC case probed with EphB4 (left column) and ephrin B2 (right column) DIG labeled antisense or sense probes generated by run-off transcription. Hybridization signal (dark blue) was detected using alkaline-phosphatase-conjugated anti-DIG antibodies and sections were counterstained with Nuclear Fast Red. A serial section stained with H&E is shown (bottom left) to illustrate tumor architecture. C) Western blot of protein extract of patient samples consisting of tumor (T), uninvolved normal tissue (N) and lymph node biopsies (LN). Samples were fractionated by polyacrylamide gel electrophoresis in 4-20% Tris-glycine gels and subsequently electroblotted onto nylon membranes. Membranes were sequentially probed

with EphB4 monoclonal antibody and β-actin MoAb. Chemiluminescent signal was detected on autoradiography film. Shown is the EphB4 specific band which migrated at 120 kD and β-actin which migrated at 40 kD. The β-actin signal was used to control for loading and transfer of each sample.

Figure 40 shows that EphB4 is expressed in HNSCC cell lines and is regulated by EGF: A) Survey of EphB4 expression in SCC cell lines. Western blot of total cell lysates sequentially probed with EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody as described for Fig. 39C. B) Effect of the specific EGFR inhibitor AG1478 on EphB4 expression: Western blot of crude cell lysates of SCC15 treated with 0-1000 nM AG 1478 for 24 h in media supplemented with 10% FCS (left) or with 1 mM AG 1478 for 4, 8, 12 or 24 h (right). Shown are membranes sequentially probed for EphB4 and β-actin. C) Effect of inhibition of EGFR signaling on EphB4 expression in SCC cell lines: Cells maintained in growth media containing 10% FCS were treated for 24 hr with 1 μM AG 1478, after which crude cell lysates were analyzed by Western blots of cell lysates sequentially probed with for EGFR, EphB4, ephrin B2 and β-actin antibodies. Specific signal for EGFR was detected at 170 kD and ephrin B2 at 37 kD in addition to EphB4 and β-actin as described in Fig. 1C. β-actin serves as loading and transfer control.

Figure 41 shows mechanism of regulation of EphB4 by EGF: A) Schematic of the EGFR signaling pathways, showing in red the sites of action and names of specific kinase inhibitors used. B) SCC15 cells were serum-starved for 24 h prior to an additional 24 incubation as indicated with or without EGF (10 ng/ml), 3 μM U73122, or 5 μM SH-5, 5 μM SP600125, 25 nM LY294002, -- μM PD098095 or 5 μM SB203580. N/A indicates cultures that received equal volume of diluent (DMSO) only. Cell lysates were subjected to Western Blot with EphB4 monoclonal antibody. β-actin signal serves as control of protein loading and transfer.

Figure 42 shows that specific EphB4 siRNAs inhibit EphB4 expression, cell viability and cause cell cycle arrest. A) 293 cells stably expressing full length EphB4 were transfected with 50 nM RNAi using LipofectamineTM2000. 40 h post-transfection cells were harvested, lysed and processed for Western blot. Membranes were probed with EphB4 monoclonal antibody, stripped and reprobed with β-actin monoclonal antibody as control for protein loading and transfer. Negative reagent control was RNAi to scrambled green fluorescence protein (GFP) sequence and control is transfection with LipofectamineTM2000 alone. B) MTT cell viability assays of SCC cell lines treated with

siRNAs for 48 h as described in the Methods section. Shown is mean + s.e.m. of triplicate samples. C) SCC15 cells transfected with siRNAs as indicated were analyzed 24 h post transfection for cell cycle status by flow cytometry as described in the Methods. Shown are the plots of cell number vs. propidium iodide fluorescence intensity. Top and middle row show plots for cells 16 h after siRNA transfection, bottom row shows plots for cells 36 h post transfection. Specific siRNA and concentration are indicated for each plot. Lipo = LipofectamineTM200 mock transfection.

Figure 43 shows in vitro effects of specific EphB4 AS-ODNs on SCC cells. A) 293 cells transiently transfected with EphB4 full-length expression plasmid were treated 6 h post transfection with antisense ODNs as indicated. Cell lysates were collected 24 h after AS-ODN treatment and subjected to Western Blot. B) SCC25 cells were seeded on 48 well plates at equal densities and treated with EphB4 AS-ODNs at 1, 5, and 10 μM on days 2 and 4. Cell viability was measured by MTT assay on day 5. Shown is the mean + s.e.m. of triplicate samples. Note that AS-ODNs that were active in inhibiting EphB4 protein levels were also effective inhibitors of SCC15 cell viability. C) Cell cycle analysis of SCC15 cells treated for 36 h with AS-10 (bottom) compared to cells that were not treated (top). D) Confluent cultures of SCC15 cells scraped with a plastic Pasteur pipette to produce 3 mm wide breaks in the monolayer. The ability of the cells to migrate and close the wound in the presence of inhibiting EphB4 AS-ODN (AS-10) and non-inhibiting AS-ODN (AS-1) was assessed after 48 h. Scrambled ODN is included as a negative control ODN. Culture labeled no treatment was not exposed to ODN. At initiation of the experiment, all cultures showed scrapes of equal width and similar to that seen in 1 μM EphB4 AS-10 after 48 h. The red brackets indicate the width of the original scrape. E) Migration of SCC15 cells in response to 20 mg/ml EGF in two-chamber assay as described in the Methods. Shown are representative photomicrographs of non-treated (NT), AS-6 and AS-10 treated cells and 10 ng/ml Taxol as positive control of migration inhibition. F) Cell numbers were counted in 5 individual high-powered fields and the average + s.e.m. is shown in the graph.

Figure 44 shows that EphB4 AS-ODN inhibits tumor growth in vivo. Growth curves for SCC15 subcutaneous tumor xenografts in Balb/C nude mice treated with EphB4 AS-10 or scrambled ODN at 20 mg/kg/day starting the day following implantation of 5 x 106 cells. Control mice received and equal volume of diluent (PBS). Shown are the mean + s.e.m. of 6 mice/group. * P = 0.0001 by Student's t-test compared to scrambled ODN treated group.

Figure 45 shows that Ephrin B2, but not EphB4 is expressed in KS biopsy tissue. (A) In situ hybridization with antisense probes for ephrin B2 and EphB4 with corresponding H&E stained section to show tumor architecture. Dark blue color in the ISH indicates positive reaction for ephrin B2. No signal for EphB4 was detected in the Kaposi's sarcoma biopsy. For contrast, ISH signal for EphB4 is strong in squamous cell carcinoma tumor cells. Ephrin B2 was also detected in KS using EphB4-AP fusion protein (bottom left). (B) Detection of ephrin B2 with EphB4/Fc fusion protein. Adjacent sections were stained with H&E (left) to show tumor architecture, black rectangle indicates the area shown in the EphB4/Fc treated section (middle) detected with FITC-labeled anti-human Fc antibody as described in the methods section. As a control an adjacent section was treated with human Fc fragment (right). Specific signal arising from EphB4/Fc binding to the section is seen only in areas of tumor cells. (C) Co-expression of ephrin B2 and the HHV8 latency protein LANA1. Double-label confocal immunofluorescence microscopy with antibodies to ephrin B2 (red) LANA1 (green), or EphB4 (red) of frozen KS biopsy material directly demonstrates co-expression of LANA1 and ephrin B2 in KS biopsy. Coexpression is seen as yellow color. Double label confocal image of biopsy with antibodies to PECAM-1 (green) in cells with nuclear propidium iodide stain (red), demonstrating the vascular nature of the tumor.

Figure 46 shows that HHV-8 induces arterial marker expression in venous endothelial cells. (A) Immunofluorescence of cultures of HUVEC and HUVEC/BC-1 for artery/vein markers and viral proteins. Cultures were grown on chamber slides and processed for immunofluorescence detection of ephrin B2 (a, e, i), EphB4 (m, q, u), CD148 (j, v), and the HHV-8 proteins LANA1 (b, f, m) or ORF59 (r) as described in the Materials and Methods. Yellow color in the merged images of the same field demonstrate co-expression of ephrin B2 and LANA or ephrin B2 and CD148. The positions of viable cells were revealed by nuclear staining with DAPI (blue) in the third column (c, g, k, o, s, w). Photomicrographs are of representative fields. (B) RT-PCR of HUVEC and two HHV-8 infected cultures (HUVEC/BC-1 and HUVEC/BC-3) for ephrin B2 and EphB4. Ephrin B2 product (200 bp) is seen in HUVEC/BC-1, HUVEC/BC-3 and EphB4 product (400 bp) is seen in HUVEC. Shown also is β-actin RT-PCR as a control for amount and integrity of input RNA.

Figure 47 shows that HHV-8 induces arterial marker expression in Kaposi's sarcoma cells. (A) Western blot for ephrin B2 on various cell lysates. SLK-vGPCR is a stable clone of SLK expressing the HHV-8 vGPCR, and SLK-pCEFL is control stable

clone transfected with empty expression vector. SLK cells transfected with LANA or LANAΔ440 are SLK-LANA and SLK-Δ440 respectively. Quantity of protein loading and transfer was determined by reprobing the membranes with β-actin monoclonal antibody. (B) Transient transfection of KS-SLK cells with expression vector pvGPCR-CEFL resulted in the expression of ephrin B2 as shown by immunofluorescence staining with FITC (green), whereas the control vector pCEFL had no effect. KS-SLK cells (0.8 x 105/well) were transfected with 0.8 μg DNA using Lipofectamine 2000. 24 hr later cells were fixed and stained with ephrin B2 polyclonal antibody and FITC conjugated secondary antibody as described in the methods. (C) Transient transfection of HUVEC with vGPCR induces transcription from ephrin B2 luciferase constructs. 8 x 103 HUVEC in 24 well plates were transfected using Superfect with 0.8 μg/well ephrin B2 promoter constructs containing sequences from -2941 to -11 with respect to the translation start site, or two 5'-deletions as indicated, together with 80 ng/well pCEFL or pvGPCR-CEFL. Luciferase was determined 48 h post transfection and induction ratios are shown to the right of the graph. pGL3Basic is promoterless luciferase control vector. Luciferase was normalized to protein since GPCR induced expression of the cotransfected β-galactosidase. Graphed is mean + SEM of 6 replicates. Shown is one of three similar experiments.

Figure 48 shows that VEGF and VEGF-C regulate ephrin B2 expression. A) Inhibition of ephrin B2 by neutralizing antibodies. Cells were cultured in full growth medium and exposed to antibody (100 ng/ml) for 36 hr before collection and lysis for Western blot. B) For induction of ephrin B2 expression cells were cultured in EBM growth medium containing 5% serum lacking growth factors. Individual growth factors were added as indicated and the cells harvested after 36 h. Quantity of protein loading and transfer was determined by reprobing the membranes β-actin monoclonal antibody.

Figure 49 shows that Ephrin B2 knock-down with specific siRNA inhibits viability in KS cells and HUVEC grown in the presence of VEGF but not IGF, EGF or bFGF. A) KS-SLK cells were transfected with various siRNA to ephrin B2 and controls. After 48 hr the cells were harvested and crude cell lysates fractionated on 4-20% SDS-PAGE. Western blot was performed with monoclonal antibody to ephrin B2 generated in-house. The membrane was stripped and reprobed with β-actin monoclonal antibody (Sigma) to illustrate equivalent loading and transfer. B) 3 day cell viability assay of KS-SLK cultures in the presence of ephrin B2 and EphB4 siRNAs. 1 x 10^5 cells/well in 24-well plates were treated with 0, 10 and 100 ng/ml siRNAs as indicated on the graph. Viability of cultures was determined by MTT assay as described in the methods section. Shown are the mean +

standard deviation of duplicate samples. C) HUVE cells were seeded on eight wells chamber slides coated with fibronectin. The HUVE cells were grown overnight in EGM-2 media, which contains all growth supplements. On the following day, the media was replaced with media containing VEGF (10ng/ml) or EGF, FGF and IGF as indicated. After 2 hrs of incubation at 37 °C, the cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium containing 10 nM of siRNA to ephrin B2, Eph B4 or green fluorescence protein (GFP) as control. The cells were incubated for 2 hr and then the fresh media containing growth factors or VEGF alone was added to their respective wells. After 48 hrs, the cells were stained with crystal violet and the pictures were taken immediately by digital camera at 10X magnification.

Figure 50 shows that soluble EphB4 inhibits KS and EC cord formation and in vivo angiogenesis. Cord formation assay of HUVEC in MatrigelTM (upper row). Cells in exponential growth phase were treated overnight with the indicated concentrations of EphB4 extracellular domain (ECD) prior to plating on MatrigelTM. Cells were trypsinized and plated (1 x 10^5 cells/well) in a 24-well plate containing 0.5 ml MatrigelTM. Shown are representative 20X phase contrast fields of cord formation after 8 hr plating on MatrigelTM in the continued presence of the test compounds as shown. Original magnification 200 X. KS-SLK cells treated in a similar manner (middle row) in a cord formation assay on MatrigelTM. Bottom row shows in vivo MatrigelTM assay: MatrigelTM plugs containing growth factors and EphB4 ECD or PBS were implanted subcutaneously in the mid-ventral region of mice. After 7 days the plugs were removed, sectioned and stained with H&E to visualize cells migrating into the matrix. Intact vessels with large lumens are observed in the control, whereas EphB4 ECD almost completely inhibited migration of cells into the Matrigel.

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μM) (bottom panels). Upper panels show control cells.

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

Figure 57 shows comparison of EphB4 monoclonal antibodies by G250 and in pull-down assay.

Figure 58 shows that EphB4 antibodies inhibit the growth of SCC15 xenograft tumors.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Figure 60 shows that systemic administration of EphB4 antibodies leads to tumor regression.

Figure 61 shows a genomic nucleotide sequence of human EphB4 (SEQ ID NO:6).

Figure 62 shows a cDNA nucleotide sequence of human EphB4 (SEQ ID NO:7).

Figure 63 shows a genomic nucleotide sequence of human Ephrin B2 (SEQ ID NO:8).

Figure 64 shows a cDNA nucleotide sequence of human Ephrin B2 (SEQ ID NO:9).

Figure 65 shows an amino acid sequence of human EphB4 (SEQ ID NO:10).

Figure 66 shows an amino acid sequence of human Ephrin B2 (SEQ ID NO:11).

Figure 67 shows a comparison of the EphrinB2 binding properties of the HSA-EphB4 *fusion protein* and other EphB4 polypeptides.

Figure 68 shows a comparison between the in vivo stability of an EphB4-HSA fusion protein and an EphB4 polypeptide in mice.

Figure 69 shows the EphrinB2 binding activity of soluble EphB4 polypeptides pegylated under specific pH conditions.

Figure 70 shows the chromatographic separation of PEG derivatives of EphB4 protein on SP-Sepharose columns. Purity of the PEG-modified EphB4 protein was analyzed by PAGE. The EphrinB2 binding of the pegylation reaction products is also shown.

Figure 71 shows the purity, as determined by SDS-PAGE, of chromatography-separated unpegylated, monopegylated and poly-pegylated EphB4 fractions.

Figure 72 shows the EphrinB2-binding activity of the chromatography fractions from the EphB4 pegylation reaction.

Figure 73 shows the retention of EphrinB2-binding activity of the chromatography fractions from the EphB4 pegylation reaction after incubation in mouse serum at 37°C for three days.

Figure 74 shows the in vivo stability of unpegylated, monopegylated and polypegylated EphB4 in mice over time.

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 tumorigenesis. Applicants detected expression of ephrin B2 and EphB4 in tumor tissues and developed anti-tumor therapeutic agents for blocking signaling through the ephrin/eph. In addition, the disclosure provides polypeptide therapeutic agents and methods for polypeptide-based inhibition of the function of EphB4 and/or Ephrin B2. Accordingly, in certain aspects, the disclosure provides numerous polypeptide compounds (agents) that may be used to treat cancer as well as angiogenesis related disorders and unwanted angiogenesis related processes. Applicants have generated modified forms of EphrinB2 and EphB4 polypeptides and have demonstrated that such modified forms have markedly improved pharmacokinetic properties. Accordingly, in certain aspects, the disclosure provides numerous polypeptide compounds (agents) that may be used to treat cancer as well as angiogenesis related disorders and unwanted angiogenesis related processes.

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 nomenclature in this area has changed rapidly and the terminology used herein is that proposed as a result of work by the Eph Nomenclature Committee, which can be accessed, along with previously-used names at web site http://www.eph-nomenclature.com.

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 a 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 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.

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 (e.g., see Figure 16). The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues; a protein tyrosine kinase domain; a sterile α-motif (SAM) and a PDZ-domain binding motif. EphB4 is specific for the membrane-bound ligand Ephrin B2 (Sakano, S. et al 1996; Brambilla R. et al 1995). 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.

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 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).

In certain embodiments, the present invention provides polypeptide therapeutic agents that inhibit activity of Ephrin B2, EphB4, or both. As used herein, the term "polypeptide therapeutic agent" or "polypeptide agent" is a generic term which includes any polypeptide that blocks signaling through the Ephrin B2/EphB4 pathway. A preferred polypeptide therapeutic agent of the invention is a soluble polypeptide of Ephrin B2 or EphB4. Another preferred polypeptide therapeutic agent of the invention is an antagonist antibody that binds to Ephrin B2 or EphB4. For example, such polypeptide therapeutic agent can inhibit function of Ephrin B2 or EphB4, inhibit the interaction between Ephrin B2 and EphB4, inhibit the phosphorylation of Ephrin B2 or EphB4, or inhibit any of the downstream signaling events upon binding of Ephrin B2 to EphB4. Such polypeptides may include EphB4 or EphrinB2 that are modified so as to improve serum half-life, such as by PEGylation or stable association with a serum albumin protein.

II. 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) or comprising an extracellular domain of an EphB4 protein (referred to herein as an EphB4 soluble polypeptide). 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 Figures 1, 2, and 15. Specific examples of Ephrin B2 soluble polypeptides are provided in Figures 3 and 14.

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 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, 29-429, 1-526, 29-526, 1-537 and 29-537 of the amino acid sequence defined by Figure 65 (SEQ ID NO: 10). 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 defined by Figure 65 (SEQ ID NO:10).

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 defined by Figure 66 (SEQ ID NO: 11) 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 defined by Figure 66 (SEQ ID NO: 11).

In certain embodiments, the present invention contemplates making functional variants 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 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 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 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 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 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 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 small molecule such as a peptide and a peptidomimetic. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that 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 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.

In certain embodiments, the soluble polypeptides of the invention may further comprise post-translational modifications. Exemplary post-translational protein modification include phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination, glycosylation, carbonylation, sumoylation, biotinylation or addition of a polypeptide side chain or of a hydrophobic group. As a result, the modified soluble polypeptides may contain non-amino acid elements, such as 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 antagonizing role in EphB4 or Ephrin B2 function, e.g, it inhibitory effect on angiogenesis or on tumor growth.

In one specific embodiment of the present invention, modified forms of the subject soluble polypeptides comprise linking the subject soluble polypeptides to nonproteinaceous polymers. In one specific embodiment, the polymer is polyethylene glycol ("PEG"), polypropylene glycol, or polyoxyalkylenes, in the manner as set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. Examples of the modified polypeptide of the invention include PEGylated soluble Ephrin B2 and PEGylated soluble EphB4.

PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods well known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, Vol. 3, pages 138-161). The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented by the formula:

$X—O(CH_2CH_2O)_{n-1}CH_2CH_2OH$ (1), where n is 20 to 2300 and X is H or a terminal modification, *e.g.*, a C_{1-4} alkyl. In one embodiment, the PEG of the invention terminates on one end with hydroxy or methoxy, *i.e.*, X is H or CH_3 ("methoxy PEG"). A PEG can contain further chemical groups which are necessary for binding reactions; which results from the chemical synthesis of the molecule; or which is a spacer for optimal distance of parts of the molecule. In addition, such a PEG can consist of one or more PEG side-chains which are linked together. PEGs with more than one PEG chain are called multiarmed or branched PEGs. Branched PEGs can be prepared, for example, by the addition of

polyethylene oxide to various polyols, including glycerol, pentaerythriol, and sorbitol. For example, a four-armed branched PEG can be prepared from pentaerythriol and ethylene oxide. Branched PEG are described in, for example, EP-A 0 473 084 and U.S. Pat. No. 5,932,462. One form of PEGs includes two PEG side-chains (PEG2) linked via the primary amino groups of a lysine (Monfardini, C., et al., Bioconjugate Chem. 6 (1995) 62-69).

PEG conjugation to peptides or proteins generally involves the activation of PEG and coupling of the activated PEG-intermediates directly to target proteins/peptides or to a linker, which is subsequently activated and coupled to target proteins/peptides (see Abuchowski, A. et al, *J. Biol. Chem.*, 252, 3571 (1977) and *J. Biol. Chem.*, 252, 3582 (1977), Zalipsky, et al., and Harris et. al., in: Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications; (J. M. Harris ed.) Plenum Press: New York, 1992; Chap.21 and 22). It is noted that an EphB4containing a PEG molecule is also known as a conjugated protein, whereas the protein lacking an attached PEG molecule can be referred to as unconjugated.

Any molecular mass for a PEG can be used as practically desired, *e.g.*, from about 1,000 Daltons (Da) to 100,000 Da (n is 20 to 2300), for conjugating to Eph4 or EphrinB2 soluble peptides. The number of repeating units "n" in the PEG is approximated for the molecular mass described in Daltons. It is preferred that the combined molecular mass of PEG on an activated linker is suitable for pharmaceutical use. Thus, in one embodiment, the molecular mass of the PEG molecules does not exceed 100,000 Da. For example, if three PEG molecules are attached to a linker, where each PEG molecule has the same molecular mass of 12,000 Da (each n is about 270), then the total molecular mass of PEG on the linker is about 36,000 Da (total n is about 820). The molecular masses of the PEG attached to the linker can also be different, e.g., of three molecules on a linker two PEG molecules can be 5,000 Da each (each n is about 110) and one PEG molecule can be 12,000 Da (n is about 270).

In a specific embodiment of the invention, an EphB4 polypeptide is covalently linked to one poly(ethylene glycol) group of the formula: —CO— $(CH_2)_x$— $(OCH_2CH_2)_m$—OR , with the —CO (i.e. carbonyl) of the poly(ethylene glycol) group forming an amide bond with one of the amino groups of EphB4; R being lower alkyl; x being 2 or 3; m being from about 450 to about 950; and n and m being chosen so that the molecular weight of the conjugate minus the EphB4 protein is from about 10 to 40 kDa. In one embodiment, an EphB4 ϵ-amino group of a lysine is the available (free) amino group.

The above conjugates may be more specifically presented by formula (II): P—NHCO— $(CH_2)_x$— $(OCH_2CH_2)_m$—OR (II) , wherein P is the group of an EphB4 protein as described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in formula (II); and wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 950 and is chosen so that the molecular weight of the conjugate minus the EphB4 protein is from about 10 to about 40 kDa. As used herein, the given ranges of "m" have an orientational meaning. The ranges of "m" are determined in any case, and exactly, by the molecular weight of the PEG group.

One skilled in the art can select a suitable molecular mass for PEG, *e.g.*, based on how the pegylated EphB4 will be used therapeutically, the desired dosage, circulation time, resistance to proteolysis, immunogenicity, and other considerations. For a discussion of PEG and its use to enhance the properties of proteins, see N. V. Katre, Advanced Drug Delivery Reviews 10: 91-114 (1993).

In one embodiment of the invention, PEG molecules may be activated to react with amino groups on EphB4, such as with lysines (Bencham C. O. et al., Anal. Biochem., 131, 25 (1983); Veronese, F. M. et al., Appl. Biochem., 11, 141 (1985).; Zalipsky, S. et al., Polymeric Drugs and Drug Delivery Systems, adrs 9-110 ACS Symposium Series 469 (1999); Zalipsky, S. et al., Europ. Polym. J., 19, 1177-1183 (1983); Delgado, C. et al., Biotechnology and Applied Biochemistry, 12, 119-128 (1990)).

In one specific embodiment, carbonate esters of PEG are used to form the PEG-EphB4 conjugates. N,N'-disuccinimidylcarbonate (DSC) may be used in the reaction with PEG to form active mixed PEG-succinimidyl carbonate that may be subsequently reacted with a nucleophilic group of a linker or an amino group of EphB4 (see U.S. Pat. No. 5,281,698 and U.S. Pat. No. 5,932,462). In a similar type of reaction, 1,1'-(dibenzotriazolyl)carbonate and di-(2-pyridyl)carbonate may be reacted with PEG to form PEG-benzotriazolyl and PEG-pyridyl mixed carbonate (U.S. Pat. No. 5,382,657), respectively.

In one embodiment, additional sites for PEGylation are introduced by site-directed mutagenesis by introducing one or more lysine residues. For instance, one or more arginine residues may be mutated to a lysine residue. In another embodiment, additional PEGylation sites are chemically introduced by modifying amino acids on EphB4. In one specific embodiment, carboxyl groups in EphB4 are conjugated with diaminobutane,

resulting in carboxyl amidation (see Li et al., Anal Biochem. 2004;330(2):264-71). This reaction may be catalyzed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, a water-soluble carbodiimide. The resulting amides can then conjugated to PEG.

PEGylation of EphB4 can be performed according to the methods of the state of the art, for example by reaction of EphB4 with electrophilically active PEGs (supplier: Shearwater Corp., USA, www.shearwatercorp.com). Preferred PEG reagents of the present invention are, *e.g.*, N-hydroxysuccinimidyl propionates (PEG-SPA), butanoates (PEG-SBA), PEG-succinimidyl propionate or branched N-hydroxysuccinimides such as mPEG2-NHS (Monfardini, C., et al., Bioconjugate Chem. 6 (1995) 62-69). Such methods may used to PEGylated at an ϵ-amino group of an EphB4 lysine or the N-terminal amino group of EphB4.

In another embodiment, PEG molecules may be coupled to sulfhydryl groups on EphB4 (Sartore, L., et al., Appl. Biochem. Biotechnol., 27, 45 (1991); Morpurgo et al., Biocon. Chem., 7, 363-368 (1996); Goodson et al., Bio/Technology (1990) 8, 343; U.S. Patent No. 5,766,897). U.S. Patent Nos. 6,610,281 and 5,766,897 describes exemplary reactive PEG species that may be coupled to sulfhydryl groups.

In some embodiments where PEG molecules are conjugated to cysteine residues on EphB4, the cysteine residues are native to Eph4, whereas in other embodiments, one or more cysteine residues are engineered into EphB4. Mutations may be introduced into an EphB4 coding sequence to generate cysteine residues. This might be achieved, for example, by mutating one or more amino acid residues to cysteine. Preferred amino acids for mutating to a cysteine residue include serine, threonine, alanine and other hydrophilic residues. Preferably, the residue to be mutated to cysteine is a surface-exposed residue. Algorithms are well-known in the art for predicting surface accessibility of residues based on primary sequence or a protein. Alternatively, surface residues may be predicted by comparing the amino acid sequences of EphB4 an EphB2, given that the crystal structure of EphB2 has been solved (see Himanen et al., Nature. (2001) 20-27;414(6866):933-8) and thus the surface-exposed residues identified. In one embodiment, cysteine residues are introduced into EphB4 at or near the N- and/or C-terminus, or within loop regions. Loop regions may be identified by comparing the EphB4 sequence to that of EphB2.

In some embodiments, the pegylated EphB4 comprises a PEG molecule covalently attached to the alpha amino group of the N-terminal amino acid. Site specific N-terminal reductive amination is described in Pepinsky et al., (2001) JPET, 297,1059, and U.S. Pat.

No. 5,824,784. The use of a PEG-aldehyde for the reductive amination of a protein utilizing other available nucleophilic amino groups is described in U.S. Pat. No. 4,002,531, in Wieder et al., (1979) J. Biol. Chem. 254,12579, and in Chamow et al., (1994) Bioconjugate Chem. 5, 133.

In another embodiment, pegylated EphB4 comprises one or more PEG molecules covalently attached to a linker, which in turn is attached to the alpha amino group of the amino acid residue at the N-terminus of EphB4. Such an approach is disclosed in U.S. Patent Publication No. 2002/0044921 and in WO94/01451.

In one embodiment, EphB4 is pegylated at the C-terminus. In a specific embodiment, a protein is pegylated at the C-terminus by the introduction of C-terminal azido-methionine and the subsequent conjugation of a methyl-PEG-triarylphosphine compound via the Staudinger reaction. This C-terminal conjugation method is described in Cazalis et al., C-Terminal Site-Specific PEGylation of a Truncated Thrombomodulin Mutant with Retention of Full Bioactivity, *Bioconjug Chem.* 2004;15(5):1005-1009.

Monopegylation of EphB4 can also be produced according to the general methods described in WO 94/01451. WO 94/01451 describes a method for preparing a recombinant polypeptide with a modified terminal amino acid alpha-carbon reactive group. The steps of the method involve forming the recombinant polypeptide and protecting it with one or more biologically added protecting groups at the N-terminal alpha-amine and C-terminal alpha-carboxyl. The polypeptide can then be reacted with chemical protecting agents to selectively protect reactive side chain groups and thereby prevent side chain groups from being modified. The polypeptide is then cleaved with a cleavage reagent specific for the biological protecting group to form an unprotected terminal amino acid alpha-carbon reactive group. The unprotected terminal amino acid alpha-carbon reactive group is modified with a chemical modifying agent. The side chain protected terminally modified single copy polypeptide is then deprotected at the side chain groups to form a terminally modified recombinant single copy polypeptide. The number and sequence of steps in the method can be varied to achieve selective modification at the N- and/or C-terminal amino acid of the polypeptide.

The ratio of EphB4 (or EphrinB2) to activated PEG in the conjugation reaction can be from about 1:0.5 to 1:50, between from about 1:1 to 1:30, or from about 1:5 to 1:15. Various aqueous buffers can be used in the present method to catalyze the covalent addition of PEG to EphB4. In one embodiment, the pH of a buffer used is from about 7.0 to 9.0. In

another embodiment, the pH is in a slightly basic range, *e.g.*, from about 7.5 to 8.5. Buffers having a pKa close to neutral pH range may be used, e.g., phosphate buffer.

In one embodiment, the temperature range for preparing a mono-PEG-EphB4 is from about 4°C. to 40°C, or from about 18°C. to 25°C. In another embodiment, the temperature is room temperature.

The pegylation reaction can proceed from 3 to 48 hours, or from 10 to 24 hours. The reaction can be monitored using SE-HPLC to distinguish EphB4, mono-PEG-EphB4 and poly-PEG-EphB4. It is noted that mono-PEG-EphB4 forms before di-PEG-EphB4. When the mono-PEG-EphB4 concentration reaches a plateau, the reaction can be terminated by adding a quenching agent to react with unreacted PEG. In some embodiments, the quenching agent is a free amino acid, such as glycine, cysteine or lysine.

Conventional separation and purification techniques known in the art can be used to purify pegylated EphB4 or EphrinB2 products, such as size exclusion (e.g. gel filtration) and ion exchange chromatography. Products may also be separated using SDS-PAGE. Products that may be separated include mono-, di-, tri- poly- and un- pegylated EphB4, as well as free PEG. The percentage of mono-PEG conjugates can be controlled by pooling broader fractions around the elution peak to increase the percentage of mono-PEG in the composition. About ninety percent mono-PEG conjugates represents a good balance of yield and activity. Compositions in which, for example, at least ninety-two percent or at least ninety-six percent of the conjugates are mono-PEG species may be desired. In an embodiment of this invention the percentage of mono-PEG conjugates is from ninety percent to ninety-six percent.

In one embodiment, pegylated EphB4 proteins of the invention contain one, two or more PEG moieties. In one embodiment, the PEG moiety(ies) are bound to an amino acid residue which is on the surface of the protein and/or away from the surface that contacts EphrinB2. In one embodiment, the combined or total molecular mass of PEG in PEG-EphB4 is from about 3,000 Da to 60,000 Da, optionally from about 10,000 Da to 36,000 Da. In a one embodiment, the PEG in pegylated EphB4 is a substantially linear, straight-chain PEG.

In one embodiment of the invention, the PEG in pegylated EphB4 or EphrinB2 is not hydrolyzed from the pegylated amino acid residue using a hydroxylamine assay, *e.g.*, 450 mM hydroxylamine (pH 6.5) over 8 to 16 hours at room temperature, and is thus stable. In one embodiment, greater than 80% of the composition is stable mono-PEG-EphB4, more

preferably at least 90%, and most preferably at least 95%.

In another embodiment, the pegylated EphB4 proteins of the invention will preferably retain at least 25%, 50%, 60%, 70%least 80%, 85%, 90%, 95% or 100% of the biological activity associated with the unmodified protein. In one embodiment, biological activity refers to its ability to bind to EphrinB2. In one specific embodiment, the pegylated EphB4 protein shows an increase in binding to EphrinB2 relative to unpegylated EphB4.

In a preferred embodiment, the PEG-EphB4 has a half-life ($t_{1/2}$) which is enhanced relative to the half-life of the unmodified protein. Preferably, the half-life of PEG-EphB4 is enhanced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 400% or 500%, or even by 1000% relative to the half-life of the unmodified EphB4 protein. In some embodiments, the protein half-life is determined in vitro, such as in a buffered saline solution or in serum. In other embodiments, the protein half-life is an *in vivo* half life, such as the half-life of the protein in the serum or other bodily fluid of an animal.

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 a further embodiment, a soluble polypeptide of the present invention is fused to a cytotoxic agent. In this method, the fusion acts to target the cytotoxic agent to a specific tissue or cell (e.g., a tumor tissue or cell), resulting in a reduction in the number of afflicted cells. Such an approach can thereby reduce symptoms associated with cancer and angiogenesis-associated disorders. Cytotoxic agents include, but are not limited to, diphtheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin and the like, as well as radiochemicals.

In certain embodiments, the soluble polypeptides of the present invention may be fused to other therapeutic proteins or to other proteins such as Fc or serum albumin for pharmacokinetic purposes. See for example U.S. Pat. Nos. 5,766,883 and 5,876,969, both of which are incorporated by reference. In some embodiments, soluble peptides of the present invention are fused to Fc variants. In a specific embodiment, the soluble polypeptide is fused to an Fc variant which does not homodimerize, such as one lacking the cysteine residues which form cysteine bonds with other Fc chains.

In some embodiments, the modified proteins of the invention comprise fusion proteins with an Fc region of an immunoglobulin. As is known, each immunoglobulin heavy chain constant region comprises four or five domains. The domains are named sequentially as follows: CH1-hinge-CH2-CH3(-CH4). The DNA sequences of the heavy chain domains have cross-homology among the immunoglobulin classes, e.g., the CH2 domain of IgG is homologous to the CH2 domain of IgA and IgD, and to the CH3 domain of IgM and IgE. As used herein, the term, "immunoglobulin Fc region" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, preferably an immunoglobulin heavy chain constant region, or a portion thereof. For example, an immunoglobulin Fc region may comprise 1) a CH1 domain, a CH2 domain, and a CH3 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of two or more domains and an immunoglobulin hinge region. In a preferred embodiment the immunoglobulin Fc region comprises at least an immunoglobulin hinge region a CH2 domain and a CH3 domain, and preferably lacks the CH1 domain.

In one embodiment, the class of immunoglobulin from which the heavy chain constant region is derived is IgG (Igγ) (γ subclasses 1, 2, 3, or 4). The nucleotide and amino acid sequences of human Fc .gamma.-1 are set forth in SEQ ID NOS: 5 and 6. The nucleotide and amino acid sequences of murine Fcγ-2a are set forth in SEQ ID NOS: 7 and 8. Other classes of immunoglobulin, IgA (Igα), IgD (Igδ), IgE (Igε) and IgM (Igμ), may be used. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Pat. Nos. 5,541,087, and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. The portion of the DNA construct encoding the immunoglobulin Fc region preferably comprises at least a portion of a hinge domain, and preferably at least a portion of a CH_3 domain of Fc γ or the homologous domains in any of IgA, IgD, IgE, or IgM.

Furthermore, it is contemplated that substitution or deletion of amino acids within the immunoglobulin heavy chain constant regions may be useful in the practice of the invention. One example would be to introduce amino acid substitutions in the upper CH2 region to create a Fc variant with reduced affinity for Fc receptors (Cole et al. (1997) J. IMMUNOL. 159:3613). One of ordinary skill in the art can prepare such constructs using well known molecular biology techniques.

In a specific embodiment of the present invention, the modified forms of the subject soluble polypeptides are fusion proteins having at least a portion of the soluble polypeptide (e.g., an ectodomain of Ephrin B2 or EphB4) and a stabilizing domain such as albumin. As used herein, "albumin" refers collectively to albumin protein or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof (see EP 201 239, EP 322 094 WO 97/24445, WO95/23857) especially the mature form of human albumin, or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

The present invention describes that such fusion proteins are more stable relative to the corresponding wildtype soluble protein. For example, the subject soluble polypeptide (e.g., an ectodomain of Ephrin B2 or EphB4) can be fused with human serum albumin (HSA), bovine serum albumin (BSA), or any fragment of an albumin protein which has stabilization activity. Such stabilizing domains include human serum albumin (HSA) and bovine serum albumin (BSA).

In particular, the albumin fusion proteins of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin (See WO95/23857), for example those fragments disclosed in EP 322 094 (namely HA (Pn), where n is 369 to 419). The albumin may be derived from any vertebrate, especially any mammal, for example human, cow, sheep, or pig. Non-mammalian albumins include, but are not limited to, hen and salmon. The albumin portion of the albumin fusion protein may be from a different animal than the EphB4.

In some embodiments, the albumin protein portion of an albumin fusion protein corresponds to a fragment of serum albumin. Fragments of serum albumin polypeptides include polypeptides having one or more residues deleted from the amino terminus or from the C-terminus. Generally speaking, an HA fragment or variant will be at least 100 amino acids long, preferably at least 150 amino acids long. The HA variant may consist of or alternatively comprise at least one whole domain of HA. Domains, with reference to SEQ ID NO:18 in U.S. Patent Publication No. 2004/0171123, are as follows: domains 1 (amino acids 1-194), 2 (amino acids 195-387), 3 (amino acids 388-585), 1+2 (1-387), 2+3 (195-585) or 1+3 (amino acids 1-194 +amino acids 388-585). Each domain is itself made up of two homologous subdomains namely 1-105, 120-194, 195-291, 316-387, 388-491 and 512-585, with flexible inter-subdomain linker regions comprising residues Lys106 to Glu119, Glu292 to Val315 and Glu492 to Ala511.

In one embodiment, the EphB4-HSA fusion has one EphB4 soluble polypeptide linked to one HSA molecule, but other conformations are within the invention. For example, EphB4-HSA fusion proteins can have any of the following formula: R_1-L-R_2; R_2-L-R_1; R_1-L-R_2-L-R_1; or R_2-L-R1-L-R_2; R_1-R_2; R_2-R_1; R_1-R_2-R_1; or R_2-R_1-R_2; wherein R_1 is a soluble EphB4 sequence, R_2 is HSA, and L is a peptide linker sequence.

In a specific embodiment, the EphB4 and HSA domains are linked to each other, preferably via a linker sequence, which separates the EphB4 and HSA domains by a distance sufficient to ensure that each domain properly folds into its secondary and tertiary structures. Preferred linker sequences (1) should adopt a flexible extended conformation, (2) should not exhibit a propensity for developing an ordered secondary structure which could interact with the functional EphB4 and HSA domains, and (3) should have minimal hydrophobic or charged character, which could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Permutations of amino acid sequences containing Gly, Asn and Ser would be

expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, can also be used in the linker sequence.

In a specific embodiment, a linker sequence length of about 20 amino acids can be used to provide a suitable separation of functional protein domains, although longer or shorter linker sequences may also be used. The length of the linker sequence separating EphB4 and HSA can be from 5 to 500 amino acids in length, or more preferably from 5 to 100 amino acids in length. Preferably, the linker sequence is from about 5-30 amino acids in length. In preferred embodiments, the linker sequence is from about 5 to about 20 amino acids, and is advantageously from about 10 to about 20 amino acids. Amino acid sequences useful as linkers of EphB4 and HSA include, but are not limited to, $(SerGly_4)y$ wherein y is greater than or equal to 8, or $Gly_4SerGly_5Ser$. A preferred linker sequence has the formula $(SerGly_4)_4$. Another preferred linker has the sequence ((Ser-Ser-Ser-Ser-Gly)3-Ser-Pro).

In one embodiment, the polypeptides of the present invention and HSA proteins are directly fused without a linker sequence. In preferred embodiments, the C-terminus of a soluble EphB4 polypeptide can be directly fused to the N-terminus of HSA or the C-terminus of HSA can be directly fused to the N-terminus of soluble EphB4.

In some embodiments, the immunogenicity of the fusion junction between HSA and EphB4 may be reduced the by identifying a candidate T-cell epitope within a junction region spanning a fusion protein and changing an amino acid within the junction region as described in U.S. Patent Publication No. 2003/0166877.

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).

III. 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 depicted in SEQ ID Nos. 6-9. 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 depicted in SEQ ID Nos. 6-9, 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 system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject soluble polypeptides. For example, a host cell transfected with an expression vector encoding an EphB4 soluble polypeptide can be cultured under appropriate conditions to allow expression of the EphB4 soluble polypeptide to occur. The EphB4 soluble polypeptide may be secreted and isolated from a mixture of cells and medium containing the soluble polypeptides. Alternatively, the soluble polypeptides may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The soluble polypeptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the soluble polypeptides. In a preferred embodiment, the soluble polypeptide is a fusion protein containing a domain which facilitates its purification.

A recombinant nucleic acid of the invention can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant soluble polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli.*

The preferred mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant SLC5A8 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by

conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

IV. Drug Screening Assays

There are numerous approaches to screening for polypeptide therapeutic agents as antagonists of EphB4, Ephrin B2 or both. For example, high-throughput screening of compounds or molecules can be carried out to identify agents or drugs which inhibit angiogenesis or inhibit tumor growth. Test agents can be any chemical (element, molecule, compound, drug), made synthetically, made by recombinant techniques or isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and compiled into libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Test agents can also be natural or genetically engineered products isolated from lysates or growth media of cells -- bacterial, animal or plant -- or can be the cell lysates or growth media themselves. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps.

For example, an assay can be carried out to screen for compounds that specifically inhibit binding of Ephrin B2 (ligand) to EphB4 (receptor), or vice-versa, e.g., by inhibition of binding of labeled ligand- or receptor-Fc fusion proteins to immortalized cells. Compounds identified through this screening can then be tested in animals to assess their anti-angiogenesis or anti-tumor activity in vivo.

In one embodiment of an assay to identify a substance that interferes with interaction of two cell surface molecules (e.g., Ephrin B2 and EphB4), samples of cells expressing one type of cell surface molecule (e.g., EphB4) are contacted with either labeled ligand (e.g., Ephrin B2, or a soluble portion thereof, or a fusion protein such as a fusion of the extracellular domain and the Fc domain of IgG) or labeled ligand plus a test compound (or group of test compounds). The amount of labeled ligand which has bound to the cells is determined. A lesser amount of label (where the label can be, for example, a radioactive

isotope, a fluorescent or colorimetric label) in the sample contacted with the test compound(s) is an indication that the test compound(s) interferes with binding. The reciprocal assay using cells expressing a ligand (e.g., an Ephrin B2 ligand or a soluble form thereof) can be used to test for a substance that interferes with the binding of an Eph receptor or soluble portion thereof.

An assay to identify a substance which interferes with interaction between an Eph receptor and an ephrin can be performed with the component (e.g., cells, purified protein, including fusion proteins and portions having binding activity) which is not to be in competition with a test compound, linked to a solid support. The solid support can be any suitable solid phase or matrix, such as a bead, the wall of a plate or other suitable surface (e.g., a well of a microtiter plate), column pore glass (CPG) or a pin that can be submerged into a solution, such as in a well. Linkage of cells or purified protein to the solid support can be either direct or through one or more linker molecules.

In one embodiment, an isolated or purified protein (e.g., an Eph receptor or an ephrin) can be immobilized on a suitable affinity matrix by standard techniques, such as chemical cross-linking, or via an antibody raised against the isolated or purified protein, and bound to a solid support. The matrix can be packed in a column or other suitable container and is contacted with one or more compounds (e.g., a mixture) to be tested under conditions suitable for binding of the compound to the protein. For example, a solution containing compounds can be made to flow through the matrix. The matrix can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds. Compounds which remain bound can be released by a suitable elution buffer. For example, a change in the ionic strength or pH of the elution buffer can lead to a release of compounds. Alternatively, the elution buffer can comprise a release component or components designed to disrupt binding of compounds (e.g., one or more ligands or receptors, as appropriate, or analogs thereof which can disrupt binding or competitively inhibit binding of test compound to the protein).

Fusion proteins comprising all, or a portion of, a protein (e.g., an Eph receptor or an ephrin) linked to a second moiety not occurring in that protein as found in nature can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in which the second moiety comprises an affinity ligand (e.g., an enzyme, antigen, epitope). The fusion proteins can be produced by inserting the protein (e.g., an Eph receptor or an ephrin) or a portion thereof into a suitable expression vector

which encodes an affinity ligand. The expression vector can be introduced into a suitable host cell for expression. Host cells are disrupted and the cell material, containing fusion protein, can be bound to a suitable affinity matrix by contacting the cell material with an affinity matrix under conditions sufficient for binding of the affinity ligand portion of the fusion protein to the affinity matrix.

In one aspect of this embodiment, a fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand portion of the fusion protein to the matrix, and is contacted with one or more compounds (e.g., a mixture) to be tested, under conditions suitable for binding of compounds to the receptor or ligand protein portion of the bound fusion protein. Next, the affinity matrix with bound fusion protein can be washed with a suitable wash buffer to remove unbound compounds and non-specifically bound compounds without significantly disrupting binding of specifically bound compounds. Compounds which remain bound can be released by contacting the affinity matrix having fusion protein bound thereto with a suitable elution buffer (a compound elution buffer). In this aspect, compound elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the compound(s) tested to the receptor or ligand protein portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of compounds, or the elution buffer can comprise a release component or components designed to disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein (e.g., one or more ligands or receptors or analogs thereof which can disrupt binding of compounds to the receptor or ligand protein portion of the fusion protein). Immobilization can be performed prior to, simultaneous with, or after contacting the fusion protein with compound, as appropriate. Various permutations of the method are possible, depending upon factors such as the compounds tested, the affinity matrix selected, and elution buffer formulation. For example, after the wash step, fusion protein with compound bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with compound bound thereto. Bound compound can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

V. Methods of Treatment

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

As described herein, angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; inflammatory disorders such as immune and non-immune inflammation; chronic articular rheumatism and psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; telangiectasia psoriasis scleroderma, pyogenic granuloma, rubeosis, arthritis, diabetic neovascularization, vasculogenesis, hematopoiesis.

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

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

In certain embodiments of such methods, one or more polypeptide therapeutic agents can be administered, together (simultaneously) or at different times (sequentially). In addition, polypeptide 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 invention 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 invention recognizes that the effectiveness of conventional cancer therapies (e.g., chemotherapy, radiation therapy, photothérapy, immunotherapy, and surgery) can be enhanced through the use of a subject polypeptide therapeutic agent.

A wide array of conventional compounds have been shown to have anti-neoplastic activities. These compounds have been used as pharmaceutical 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 polypeptide therapeutic agent of the present invention 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, 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, 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, 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 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 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 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); antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones, hormone analogs (estrogen, tamoxifen, goserelin, bicalutamide, nilutamide) and aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen activator, streptokinase and urokinase), aspirin, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus (FK-506),

sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF) inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone, 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-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., 1032:89-118 (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 MetAP-2 inhibitory core coupled to a peptide, the compound EM-138, chalcone and its analogs, and naaladase inhibitors. See, for example, U.S. Pat. Nos. 6,395,718, 6,462,075, 6,465,431, 6,475,784, 6,482,802, 6,482,810, 6,500,431, 6,500,924, 6,518,298, 6,521,439, 6,525,019, 6,538,103, 6,544,758, 6,544,947, 6,548,477, 6,559,126, and 6,569,845.

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

VI. Methods of Administration and Pharmaceutical Compositions

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

Formulations of the subject polypeptide therapeutic 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 polypeptide 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 polypeptide therapeutic agents of the present invention 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 pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

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

Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters,

microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

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

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The subject polypeptide therapeutic 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 gels may contain, in addition to a subject polypeptide agent, 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 polypeptide 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.

Pharmaceutical compositions suitable for parenteral administration may comprise one or more polypeptide 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 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 pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

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

Injectable depot forms are made by forming microencapsule matrices of one or more polypeptide 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.

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

In other embodiments, the polypeptide therapeutic agents of the instant invention can be expressed within cells from eukaryotic promoters. For example, a soluble polypeptide of EphB4 or Ephrin B2 can be expressed in eukaryotic cells from an appropriate vector. The vectors are preferably DNA plasmids or viral vectors. Viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the vectors stably introduced in and persist in target

cells. Alternatively, viral vectors can be used that provide for transient expression. Such vectors can be repeatedly administered as necessary. Delivery of vectors encoding the subject polypeptide therapeutic agent 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 for introduction into the desired target cell (for a review see Couture et al., 1996, TIG., 12, 510).

EXEMPLIFICATION

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

Example 1. Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins

Soluble derivatives of the extracellular domains of human Ephrin B2 and EphB4 proteins represent either truncated full-length predicted extracellular domains of Ephrin B2 (B4ECv3, B2EC) or translational fusions of the domains with constant region of human immunoglobulins (IgG1 Fc fragment), such as B2EC-FC, B4ECv2-FC and B4ECv3-FC. Representative human Ephrin B2 constructs and human EphB4 constructs are shown Figures 14 and 15.

The cDNA fragments encoding these recombinant proteins were subcloned into mammalian expression vectors, expressed in transiently or stably transfected mammalian cell lines and purified to homogeneity as described in detail in Materials and Methods section (see below). Predicted amino acid sequences of the proteins are shown in Figures 1-5. High purity of the isolated proteins and their recognition by the corresponding anti-Ephrin B2 and anti-EphB4 monoclonal or polyclonal antibodies were confirmed. The recombinant proteins exhibit the expected high-affinity binding, binding competition and specificity properties with their corresponding binding partners as corroborated by the biochemical assays (see e.g., Figures 6-8).

Such soluble derivative proteins human Ephrin B2 and EphB4 exhibit potent biological activity in several cell-based assays and *in vivo* assays which measure angiogenesis or anti-cancer activities, and are therefore perspective drug candidates for

anti-angiogenic and anti-cancer therapy. B4ECv3 as well as B2EC and B2EC-FC proteins blocked chemotaxis of human endothelial cells (as tested with umbilical cord and hepatic AECs or VECs), with a decrease in degradation of the extracellular matrix, Matrigel, and a decrease in migration in response to growth factor stimuli (Figures 9-11). B4ECv3 and B2EC-FC proteins have potent anti-angiogenic effect as demonstrated by their inhibition of endothelial cell tube formation (Figures 12-13).

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

The sequence of the Globular domain + Cys-rich domain (B4EC-GC), precursor protein is (SEQ ID NO:12):

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDE
EQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAG
RSCKETFTVFYYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKV
NVKTLRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRE
LVVPVAGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCR
ACAQGTFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPS
AHHHHHH

For many uses, including therapeutic use, the leader sequence (first 15 amino acids, so that the processed form begins Leu-Glu-Glu...) and the c-terminal hexahistidine tag may be removed or omitted.

Sequence of the GCF precursor protein (SEQ ID NO:13):

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDE
EQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAG
RSCKETFTVFYYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKV
NVKTLRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRE
LVVPVAGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFAEGNTKCRAC
AQGTFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPR
SVVSRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPR
DLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVHHHHHH

For many uses, including therapeutic use, the leader sequence (first 15 amino acids, so that the processed form begins Leu-Glu-Glu...) and the c-terminal hexahistidine tag may be removed or omitted.

Amino acid sequence of encoded FL-hB4EC precursor (His-tagged) (SEQ ID NO:14):

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDE
EQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAG
RSCKETFTVFYYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKV
NVKTLRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRE
LVVPVAGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCR
ACAQGTFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPS
APRSVVSRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDP
GPRDLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAV
SDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAE
LRGLKRGASYLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQGSKRAILQIEG
KPIPNPLLGLDSTRTGHHHHHH

For many uses, including therapeutic use, the leader sequence (first 15 amino acids, so that the processed form begins Leu-Glu-Glu...) and the c-terminal hexahistidine tag may be removed or omitted.

EphB4 CF2 protein, precursor (SEQ ID NO:15):

MELRVLLCWASLAAALEETLLNTKLETQLTVNLTRFPETVPRELVVPVAGS
CVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFK
PLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRL
NGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLVEPW
VVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSP
SSLSLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKRGAS
YLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQGGRSSLEGPRFEGKPIPNPLL
GLDSTRTGHHHHHH

The precursor sequence of the preferred GCF2 protein (also referred to herein as GCF2F) is (SEQ ID NO:16):

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHS
VRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCK
ETFTVFYYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKVNVKT
LRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPV
AGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQG
TFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVV
SRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLV
EPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVT
RSSPSSLSLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKR
GASYLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQ

The processed sequence is (SEQ ID NO:17):

LEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHSVRTYEVCEVQR
APGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCKETFTVFYYESD
ADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKVNVKTLRLGPLSKAGF
YLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVP
APGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGEGS
CQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRLNGSSLHL
EWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLVEPWVVVRGL
RPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSPSSLSLA
WAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQV
RARSEAGYGPFGQEHHSQTQLDESEGWREQ

Biochemical Assays

A. Binding assay

10 µl of Ni-NTA-Agarose were incubated in microcentrifuge tubes with 50 µl of indicated amount of B4ECv3 diluted in binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin pH 8) After incubation for 30 min on shaking platform, Ni-NTA beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2-AP in the final concentration of 50 nM. Binding was performed for 30 min on shaking

platform, and then tubes were centrifuged and washed one time with 1.4 ml of BB. Amount of precipitated AP was measured colorimetrically after application of PNPP.

B. Inhibition assay

Inhibition in solution. Different amounts of B4ECv3 diluted in 50 µl of BB were pre-incubated with 50 µl of 5 nM B2EC-AP reagent (protein fusion of Ephrin B2 ectodomain with placental alkaline phosphatase). After incubation for 1 h, unbound B2EC-AP was precipitated with 5,000 HEK293 cells expressing membrane-associated full-length EphB4 for 20 min. Binding reaction was stopped by dilution with 1.2 ml of BB, followed by centrifugation for 10 min. Supernatants were discarded and alkaline phosphatase activities associated with collected cells were measured by adding para-nitrophenyl phosphate (PNPP) substrate.

Cell based inhibition. B4ECv3 was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8 and mixed with 5,000 HEK293 cells expressing membrane-associated full-length Ephrin B2. After incubation for 1 h, 50 µl of 5 nM B4EC-AP reagent (protein fusion of EphB4 ectodomain with placental alkaline phosphatase were added into each tube for 30 min to detect unoccupied Ephrin B2 binding sites. Binding reactions were stopped by dilution with 1.2 ml of BB and centrifugation. Colorimetric reaction of cell-precipitated AP was developed with PNPP substrate.

C. B4EC-FC binding assay

Protein A-agarose based assay. 10 µl of Protein A-agarose were incubated in Eppendorf tubes with 50 µl of indicated amount of B4EC-FC diluted in binding buffer BB (20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA pH 8). After incubation for 30 min on shaking platform, Protein AAagarose beads were washed twice with 1.4 ml of BB, followed by application of 50 µl of B2ECAP reagent at the final concentration of 50 nM. Binding was performed for 30 min on shaking platform, and then tubes were centrifuged and washed once with 1.4 ml of BB. Colorimetric reaction of precipitated AP was measured after application of PNPP (Fig. 6).

Nitrocellulose based assay. B4EC-FC was serially diluted in 20 mM Tris-HCl, 0.15 M NaCl, 50 µg/ml BSA, pH 8. 2 µl of each fraction were applied onto nitrocellulose strip and spots were dried out for 3 min. Nitrocellulose strip was blocked with 5% non-fat milk for 30 min, followed by incubation with 5 nM B2EC-AP reagent. After 45 min incubation

for binding, nitrocellulose was washed twice with 20 mM Tris-HCl, 0.15 M NaCl, 50 μg/ml BSA, pH 8 and color was developed by application of alkaline phosphatase substrate Sigma Fast (Sigma).

D. B4EC-FC inhibition assay

Inhibition in solution. See above, for B4ECv3. The results were shown in Figure 7.

Cell based inhibition. See above, for B4ECv3.

E. B2EC-FC binding assay

Protein-A-agarose based assay. See above, for B4EC-FC. The results were shown in Figure 8.

Nitrocellulose based assay. See above, for B4EC-FC.

6) Cell-Based Assays

A. Growth Inhibition Assay

Human umbilical cord vein endothelial cells (HUVEC) (1.5x103) are plated in a 96-well plate in 100 μl of EBM-2 (Clonetic # CC3162). After 24 hours (day 0), the test recombinant protein (100 μl) is added to each well at 2X the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate is stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates are incubated for 72 h at 37 °C. After 72 h, plates are stained with 0.5% crystal violet in 20% methanol, rinsed with water and airdried. The stain is eluted with 1:1 solution of ethanol: 0.1 M sodium citrate (including day 0 plate), and absorbance is measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance is subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC50 (drug concentration causing 50% inhibition) is calculated from the plotted data.

B. Cord Formation Assay (Endothelial Cell Tube Formation Assay)

Matrigel (60 μl of 10 mg/ml; Collaborative Lab # 35423) is placed in each well of an ice-cold 96-well plate. The plate is allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit the matrigel to polymerize. In the mean

time, HUVECs are prepared in EGM-2 (Clonetic # CC3162) at a concentration of $2X10^5$ cells/ml. The test compound is prepared at 2X the desired concentration (5 concentration levels) in the same medium. Cells (500 µl) and 2X drug (500 µl) is mixed and 200 µl of this suspension are placed in duplicate on the polymerized matrigel. After 24 h incubation, triplicate pictures are taken for each concentration using a Bioquant Image Analysis system. Drug effect (IC50) is assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

C. Cell Migration Assay

Migration is assessed using the 48-well Boyden chamber and 8 µm pore size collagen-coated (10 µg/ml rat tail collagen; Collaborative Laboratories) polycarbonate filters (Osmonics, Inc.). The bottom chamber wells receive 27-29 µl of DMEM medium alone (baseline) or medium containing chemo-attractant (bFGF, VEGF or Swiss 3T3 cell conditioned medium). The top chambers receive 45 µl of HUVEC cell suspension ($1X10^6$ cells/ml) prepared in DMEM+1% BSA with or without test compound. After 5 h incubation at 37 °C, the membrane is rinsed in PBS, fixed and stained in Diff-Quick solutions. The filter is placed on a glass slide with the migrated cells facing down and cells on top are removed using a Kimwipe. The testing is performed in 4-6 replicates and five fields are counted from each well. Negative unstimulated control values are subtracted from stimulated control and drug treated values and data is plotted as mean migrated cell ± S.D. IC50 is calculated from the plotted data.

Example 2. Extracellular domain fragments of EphB4 receptor inhibit angiogenesis and tumor growth.

A. Globular domain of EphB4 is required for EphrinB2 binding and for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

To identify subdomain(s) of the ectopic part of EphB4 necessary and sufficient for the anti-angiogenic activity of the soluble recombinant derivatives of the receptor, four recombinant deletion variants of EphB4EC were produced and tested (Fig. 16). Extracellular part of EphB4, similarly to the other members of EphB and EphA receptor family, contains N-terminal ligand-binding globular domain followed by cysteine-rich domain and two fibronectin type III repeats (FNIII). In addition to the recombinant B4-GCF2 protein containing the complete ectopic part of EphB4, we constructed three deletion variants of EphB4EC containing globular domain and Cys-rich domain (B4-GC); globular,

Cys-rich and the first FNIII domain (GCF1) as well as the ECD version with deleted globular domain (CF2). Our attempts to produce several versions of truncated EphB4EC protein containing the globular domain alone were not successful due to the lack of secretion of proteins expressed from all these constructs and absence of ligand binding by the intracellularly expressed recombinant proteins. In addition, a non-tagged version of B4-GCF2, called GCF2-F, containing complete extracellular domain of EphB4 with no additional fused amino acids was expressed, purified and used in some of the experiments described here.

All four C-terminally 6xHis tagged recombinant proteins were preparatively expressed in transiently transfected cultured mammalian cells and affinity purified to homogeneity from the conditioned growth media using chromatography on Ni^{2+}-chelate resin (Fig. 17). Apparently due to their glycosylation, the proteins migrate on SDS-PAAG somewhat higher than suggested by their predicted molecular weights of 34.7 kDa (GC), 41.5 (CF2), 45.6 kDa (GCF1) and 57.8 kDa (GCF2). Sequence of the extracellular domain of human EphB4 contains three predicted N-glycosylation sites (NXS/T) which are located in the Cys-rich domain, within the first fibronectin type III repeat and between the first and the second fibronectin repeats.

To confirm ability of the purified recombinant proteins to bind Ephrin B2, they were tested in an *in vitro* binding assay. As expected, GC, GCF1 and GCF2, but not CF2 are binding the cognate ligand Ephrin B2 as confirmed by interaction between Ephrin B2 – alkaline phosphatase (Ephrin B2-AP) fusion protein with the B4 proteins immobilized on Ni^{2+}-resin or on nitrocellulose membrane (Fig. 17).

All four proteins were also tested for their ability to block ligand-dependent dimerization and activation of Eph B4 receptor kinase in PC3 cells. The PC3 human prostate cancer cell line is known to express elevated levels of human Eph B4. Stimulation of PC3 cells with Ephrin B2 IgG Fc fusion protein leads to a rapid induction of tyrosine phosphorylation of the receptor. However, preincubation of the ligand with GCF2, GCF1 or GC, but not CF2 proteins suppresses subsequent EphB4 autophosphorylation. Addition of the proteins alone to the PC3 cells or preincubation of the cells with the proteins followed by changing media and adding the ligand does not affect EphB4 phosphorylation status.

Further, we found that globular domain of EphB4 is required for the activity of EphB4-derived soluble proteins in endothelial tube formation assay.

B. Effects of soluble EphB4 on HUV/AEC *in vitro*.

Initial experiments were performed to determine whether soluble EphB4 affected the three main stages in the angiogenesis pathway. These were carried out by establishing the effects of soluble EphB4 on migration / invasion, proliferation and tubule formation by HUV/AEC *in vitro*. Exposure to soluble EphB4 significantly inhibited both bFGF and VEGF-induced migration in the Boyden chamber assay in a dose-dependent manner, achieving significance at nM (Fig. 18). Tubule formation by HUV/AECS on wells coated with Matrigel was significantly inhibited by soluble EphB4 in a dose-dependent manner in both the absence and presence of bFGF and VEGF (Fig. 19). We also assessed *in vitro,* whether nM of soluble EphB4 was cytotoxic for HUVECS. Soluble EphB4 was found to have no detectable cytotoxic effect at these doses, as assessed by MTS assay (Fig. 20).

C. Soluble EphB4 receptor Inhibits Vascularization of Matrigel Plugs, in vivo

To demonstrate that soluble EphB4 can directly inhibit angiogenesis *in vivo*, we performed a murine matrigel plug experiment. Matrigel supplemented with bFGF and VEGF with and without soluble EphB4 was injected s.c. into Balb/C nu/nu mice, forming semi-solid plugs, for six days. Plugs without growth factors had virtually no vascularization or vessel structures after 6 days (Fig. 21). In contrast, plugs supplemented with bFGF and VEGF had extensive vascularization and vessels throughout the plug. Plugs taken from mice treated with μg of soluble EphB4 had markedly reduced vascularization of plugs, comparable to plugs without growth factor (Fig. 21). Furthermore, histological examination of plugs showed decreased vessel staining (Fig. 21). Treatment at 0 μg/dose significantly inhibited the amount of infiltration in Matrigel plugs compared to control (Fig. 21).

We examined EphB4 receptor phosphorylation in HUVECs by performing Western blot analyses with lysates from soluble EphB4-treated cells and antibodies against phosphor-tyrosine. We found that soluble EphB4 treatment of serum-starved HUVECs stimulated a rapid and transient decrease in the level of phosphorylated EphB4, in the presence of EphrinB2Fc, EphB4 ligand dimer. Ephrin B2Fc without the soluble EphB4 protein induced phosphorylation of EphB4 receptor (Fig. 22).

D. Effects of soluble EphB4 on tumor growth, *in vitro.*

We found that soluble EphB4 inhibits the growth of SCC15 tumors grown in Balb/C Nu/Nu mice (Fig. 23).

E. Soluble EphB4 inhibited corneal neovascularization

To further investigate the antiangiogenic activity of soluble EphB4 in vivo, we studied the inhibitory effect of administration of soluble EphB4 on neovascularization in the mouse cornea induced by bFGF. Hydron Pellets implanted into corneal micropocket could induce angiogenesis, in the presence of growth factors, in a typically avascular area. The angiogenesis response in mice cornea was moderate, the appearance of vascular buds was delayed and the new capillaries were sparse and grew slowly. Compared with the control group, on day 7 of implantation, the neovascularization induced by bFGF in mice cornea was markedly inhibited in soluble EphB4-treated group (Fig. 24).

F. Effects of soluble EphB4 on tumor growth, *in vivo.*

The same model was used to determine the effects of soluble EphB4 *in vivo*. SCC15 tumors implanted subcutaneously, pre-incubated with matrigel and with or w/o growth factors, as well as implanted sc alone, and mice treated sc or ip daily with 1-5ug of soluble EphB4 were carried out.

Tumors in the control group continued to grow steadily over the treatment period, reaching a final tumor volume of mm3. However, animals injected with soluble EphB4 exhibited a significantly ($p<0.0/$) reduced growth rate, reaching a final tumor volume of only mm3 (Fig. 25). Similar results were obtained in two further cohorts of such tumor-bearing mice. Soluble EphB4 administration appeared to be well tolerated *in vivo*, with no significant effect on body weight or the general well-being of the animals (as determined by the absence of lethargy, intermittent hunching, tremors or disturbed breathing patterns).

G. Effects of soluble EphB4 on tumor histology.

Histological analysis revealed the presence of a central area of necrosis in all SCC15 tumors, which was usually surrounded by a viable rim of tumor cells um in width. The central necrotic areas were frequently large and confluent and showed loss of cellular detail. Necrosis, assessed as a percentage of tumor section area, was significantly ($p<0.02$) more extensive in the soluble EphB4-treated group (% necrosis in treated vs. control). To determine whether the reduced volume of soluble EphB4 treated tumors was due to an effect of this protein on the tumor vascular supply, endothelial cells in blood vessels were identified in tumor sections using immunostaining with an anti-platelet cell adhesion molecule (PECAM-1; CD31) antibody (Fig. 26) and the density of microvessels was assessed. Microvessel density was similar in the outer viable rim of tumor cells (the

uniform layer of cells adjacent to the tumor periphery with well defined nuclei) in control and soluble EphB4-treated tumors. Microvessel density was significantly in the inner, less viable region of tumor cells abutting the necrotic central areas in soluble EphB4-treated than control tumors. Fibrin deposition, as identified by Masson's Trichrome staining, was increased in and around blood vessels in the inner viable rim and the central necrotic core of soluble EphB4 treated than control tumors. In the outer viable rim of soluble EphB4 treated tumors, although the vessel lumen remained patent and contained red blood cells, fibrin deposition was evident around many vessels. Soluble EphB4 was found to have no such effects on the endothelium in the normal tissues examined (lungs, liver and kidneys).

H. Materials and Methods

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

Cell-based EphB4 tyrosine kinase assay

The human prostate carcinoma cell line PC3 cells were maintained in RPMI medium with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics mix. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Typically, cells were grown in 60 mm dishes until confluency and were either treated with mouse Ephrin B2-Fc fusion at 1 μg/ml in RPMI for 10 min to activate EphB4 receptor or plain medium as a control. To study the effect of different derivatives of soluble EphB4 ECD proteins on EphB4 receptor activation, three sets of cells were used. In the first set, cells were treated with various proteins (5 proteins; GC, GCF1, GCF2, GCF2-F, CF2) at 5 μg/ml for 20 min. In the second set of cells, prior to application, proteins were premixed with ephrinB2-Fc at 1:5 (EphB4 protein: B2-Fc) molar ratio, incubated for 20 min and applied on cells for 10 min. In the third set of cells, cells were first treated with the proteins for 20 min at 5 μg/ml, media was replaced with fresh media containing 1 μg/ml of EphrinB2-Fc and incubated for another 10 min.

After the stimulation, cells were immediately harvested with protein extraction buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X100, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium vanadate. Protein extracts were clarified by centrifugation at 14,000 rpm for 20 min at 4 °C. Clarified protein samples were incubated overnight with protein A/G coupled agarose beads pre-coated with anti-EphB4 monoclonal antibodies. The IP complexes were washed twice with the same extraction buffer containing

0.1% Triton X100. The immunoprecipitated proteins were solubilized in 1X SDS-PAGE sample loading buffer and separated on 10% SDS-PAGE. For EphB4 receptor activation studies, electroblotted membrane was probed with anti-pTyr specific antibody 4G10 at 1:1000 dilution followed by Protein G-HRP conjugate at 1:5000 dilutions.

Endothelial Cell Tube Formation Assay

Matrigel (60 µl of 10mg/ml; Collaborative Lab, Cat. No. 35423) was placed in each well of an ice-cold 96-well plate. The plate was allowed to sit at room temperature for 15 minutes then incubated at 37 °C for 30 minutes to permit Matrigel to polymerize. In the mean time, human umbilical vein endothelial cells were prepared in EGM-2 (Clonetic, Cat. No. CC3162) at a concentration of $2x10^5$ cells/ml. The test protein was prepared at 2x the desired concentration (5 concentration levels) in the same medium. Cells (500 µl) and 2x protein (500 µl) were mixed and 200 µl of this suspension were placed in duplicate on the polymerized Matrigel. After 24 h incubation, triplicate pictures were taken for each concentration using a Bioquant Image Analysis system. Protein addition effect (IC_{50}) was assessed compared to untreated controls by measuring the length of cords formed and number of junctions.

Cell Migration Assay

Chemotaxis of HUVECs to VEGF was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24 well plates, 6.5 mm diam, 8 µm pore size, 10 µm thick matrigel coated, polycarbonate membranes (BD Biosciences). The cell suspensions of HUVECs ($2x\ 10^5$ cells/ml) in 200 µl of EBM were seeded in the upper chamber and the soluble EphB4 protein were added simultaneously with stimulant (VEGF or bFGF) to the lower compartment of the chamber and their migration across a polycarbonate filter in response to10- 20 ng/ml of VEGF with or without 100 nM-1 µM test compound was investigated. After incubation for 4-24 h at 37 °C, the upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Ten random fields at 200x mag were counted and the results expressed as mean # per field. Negative unstimulated control values were subtracted from stimulated control and protein treated sample values and the data was plotted as mean migrated cell ± S.D. IC_{50} was calculated from the plotted data.

Growth Inhibition Assay

HUVEC ($1.5x10^3$ cells) were plated in a 96-well plate in 100 µl of EBM-2 (Clonetic, Cat. No. CC3162). After 24 hours (day 0), the test recombinant protein (100 µl)

is added to each well at 2x the desired concentration (5-7 concentration levels) in EBM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 °C. After 72 h, plates were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with 1:1 solution of ethanol: 0.1M sodium citrate (including day 0 plate), and absorbance measured at 540 nm with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the 72 h plates and data is plotted as percentage of control proliferation (vehicle treated cells). IC_{50} value was calculated from the plotted data.

Murine Matrigel Plug Angiogenesis Assay

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/ml, 0.5 ml) in liquid form at 4 °C was mixed with Endothelial Cell Growth Supplement (ECGS), test proteins plus ECGS or Matrigel plus vehicle alone (PBS containing 0.25% BSA). Matrigel (0.5ml) was injected into the abdominal subcutaneous tissue of female nu/nu mice (6 wks old) along the peritoneal mid line. There were 3 mice in each group. The animals were cared for in accordance with institutional and NIH guidelines. At day 6, mice were sacrificed and plugs were recovered and processed for histology. Typically the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin in PBS and embedded in paraffin. Sections of 3 μm were cut and stained with H&E or Masson's trichrome stain and examined under light microscope

Mouse Corneal Micropocket assay

Mouse corneal micropocket assay was performed according to that detailed by Kenyon et al., 1996. Briefly, hydron pellets (polyhydroxyethylmethacrylate [polyHEMA], Interferon Sciences, New Brunswick, NJ, U.S.A.) containing either 90 ng of bFGF (R&D) or 180 ng of VEGF (R&D Systems, Minneapolis, MN, U.S.A.) and 40 µg of sucrose aluminium sulfate (Sigma) were prepared. Using an operating microscope, a stromal linear keratotomy was made with a surgical blade (Bard-Parker no. 15) parallel to the insertion of the lateral rectus muscle in an anesthetized animal. An intrastromal micropocket was dissected using a modified von Graefe knife (2"30 mm). A single pellet was implanted and advanced toward the temporal corneal limbus (within 0±7±1±0 mm for bFGF pellets and

0±5 mm for VEGF pellets). The difference in pellet location for each growth factor was determined to be necessary given the relatively weaker angiogenic stimulation of VEGF in this model. Antibiotic ointment (erythromycin.) was then applied to the operated eye to prevent infection and to decrease surface irregularities. The subsequent vascular response was measured extending from the limbal vasculature toward the pellet and the contiguous circumferential zone of neovascularization Data and clinical photos presented here were obtained on day 6 after pellet implantation, which was found to be the day of maximal angiogenic response.

In vitro invasion assay

"Matrigel" matrix-coated 9-mm cell culture inserts (pore size, 8 μm; Becton Dickinson, Franklin Lakes, NJ) were set in a 24-well plate. The HUVEC cells were seeded at a density of $5x10^3$ cells per well into the upper layer of the culture insert and cultured with serum-free EBM in the presence of EphB4 ECD for 24 h. The control group was cultured in the same media without EphB4. Then *0.5 ml of the human* SCC15 cell line, conditioned medium was filled into the lower layer of the culture insert as a chemo-attractant. The cells were incubated for 24 h, then the remaining cells in the upper layer were swabbed with cotton and penetrating cells in the lower layer were fixed with 5% glutaraldehyde and stained with Diff Quick. The total number of cells passing through the Matrigel matrix and each 8 μm pore of the culture insert wascounted using optical microscopy and designated as an invasion index (cell number/area).

SCC15 tumor growth in mice

Subcutaneously inject logarithmically growing SCC15, head and neck squamous cell carcinoma cell line, at $5X10^6$ cell density; with or without EphB4 ECD in the presence or absence of human bFGF, into athymic Balb/c nude mice, along with Matrigel (BD Bioscience) synthetic basement membrane (1:1 v/v), and examine tumors within 2 weeks. Tumor volumes in the EphB4 ECD group, in the presence and absence of growth factor after implantation were three-fold smaller than those in the vehicle groups. There was no difference in body weight between the groups. Immunohistochemical examination of cross-sections of resected tumors and TUNEL-positive apoptosis or necrosis, CD34 immunostaining, and BrdU proliferation rate will be performed, after deparaffinized, rehydrated, and quenched for endogenous peroxidase activity, and after 10 min permeabilization with proteinase K. Quantitative assessment of vascular densities will also

be performed. Local intratumoral delivery or IV delivery of EphB4 ECD will also be performed twice a week.

30 athymic nude mice, BALB/c (nu/nu), were each injected with 1 x 10^6 B16 melanoma cells with 0.1 ml PBS mixed with 0.1 ml matrigel or 1.5 x 10^6 SCC15 cells resuspended in 200 µl of DMEM serum-free medium and injected subcutaneously on day 0 on the right shoulder region of mice. Proteins were injected intravenously or subcutaneously, around the tumor beginning on day 1 at a loading dose of 4 µg/mg, with weekly injections of 2ug/mg. (10 µg/g, 50 µg/kg/day), and at 2 weeks post-inoculation. Mice are sacrificed on Day 14. Control mice received PBS 50 µl each day.

Tumor formation in nude mice

All animals were treated under protocols approved by the institutional animal care committees. Cancer cells ($5x10^6$) were subcutaneously inoculated into the dorsal skin of nude mice. When the tumor had grown to a size of about 100 mm^3 (usually it took ~12 days), sEphB4 was either intraperitoneally or subcutaneously injected once/day, and tumorigenesis was monitored for 2 weeks. Tumor volume was calculated according to the formula a^2xb, where a and b are the smallest and largest diameters, respectively. A Student's t test was used to compare tumor volumes, with $P<.05$ being considered significant.

Quantification of microvessel density

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

Example 3. EphB4 Is Upregulated and Imparts Growth Advantage in Prostate Cancer

A. Expression of EphB4 in prostate cancer cell lines

We first examined the expression of EphB4 protein in a variety of prostate cancer cell lines by Western blot. We found that prostate cancer cell lines show marked variation in the abundance of the 120 kD EphB4. The levels were relatively high in PC3 and even higher in PC3M, a metastatic clone of PC3, while normal prostate gland derived cell lines (MLC) showed low or no expression of EphB4 (Fig. 27A). We next checked the activation status of EphB4 in PC3 cells by phosphorylation study. We found that even under normal culture conditions, EphB4 is phosphorylated though it can be further induced by its ligand, ephrin B2 (Fig. 27B).

B. Expression of EphB4 in clinical prostate cancer samples

To determine whether EphB4 is expressed in clinical prostate samples, tumor tissues and adjacent normal tissue from prostate cancer surgical specimens were examined. The histological distribution of EphB4 in the prostate specimens was determined by immunohistochemistry. Clearly, EphB4 expression is confined to the neoplastic epithelium (Fig. 28, top left), and is absent in stromal and normal prostate epithelium (Fig. 28, top right). In prostate tissue array, 24 of the 32 prostate cancers examined were positive. We found EphB4 mRNA is expressed both in the normal and tumor tissues of clinical samples by quantitative RT-PCR. However, tumor EphB4 mRNA levels were at least 3 times higher than in the normal in this case (Fig. 28, lower right).

C. p53 and PTEN inhibited the expression of EphB4 in PC3 cells

PC3 cells are known to lack PTEN expression (Davis, et al., 1994, Science. 266:816-819) and wild-type p53 function (Gale, et al., 1997, Cell Tissue Res. 290:227-241). We investigated whether the relatively high expression of EphB4 is related to p53 and/or PTEN by re-introducing wild-type p53 and/or PTEN into PC3 cells. To compensate for the transfection efficiency and the dilution effect, transfected cells were sorted for the cotransfected truncated CD4 marker. We found that the expression of EphB4 in PC3 cells was reduced by the re-introduction of either wild-type p53 or PTEN. The co-transfection of p53 and PTEN did not further inhibit the expression of EphB4 (Fig. 29A).

D. Retinoid X receptor (RXR α) regulates the expression of EphB4

We previously found that RXRα was down-regulated in prostate cancer cell lines (Zhong, et al., 2003, Cancer Biol Ther. 2:179-184) and here we found EphB4 expression has the reverse expression pattern when we looked at "normal" prostate (MLC), prostate cancer (PC3), and metastatic prostate cancer (PC3M) (Fig. 27A), we considered whether RXRα regulates the expression of EphB4. To confirm the relationship, the expression of EphB4 was compared between CWR22R and CWR22R-RXRα, which constitutively expresses RXRα. We found a modest decrease in EphB4 expression in the RXRα overexpressing cell line, while FGF8 has no effect on EphB4 expression. Consistent with initial results, EphB4 was not found in "normal" benign prostate hypertrophic cell line BPH-1 (Fig. 29B).

E. Growth factor signaling pathway of EGFR and IGF-1R regulates EphB4 expression

EGFR and IGF-1R have both been shown to have autocrine and paracrine action on PC3 cell growth. Because we found that EphB4 expression is higher in the more aggressive cell lines, we postulated that EphB4 expression might correlate with these pro-survival growth factors. We tested the relationship by independently blocking EGFR and IGF-1R signaling. EphB4 was down-regulated after blocking the EGFR signaling using EGFR kinase inhibitor AG 1478 (Fig. 30A) or upon blockade of the IGF-1R signaling pathway using IGF-1R neutralizing antibody (Fig. 30B).

F. EphB4 siRNA and antisense ODNs inhibit PC3 cell viability

To define the significance of this EphB4 overexpression in our prostate cancer model, we concentrated our study on PC3 cells, which have a relatively high expression of EphB4. The two approaches to decreasing EphB4 expression were siRNA and AS-ODNs. A number of different phosphorothioate-modified AS-ODNs complementary to different segments of the EphB4 coding region were tested for specificity and efficacy of EphB4 inhibition. Using 293 cells transiently transfected with full-length EphB4 expression vector AS-10 was found to be the most effective (Fig. 31B). A Similar approach was applied to the selection of specific siRNA. EphB4 siRNA 472 effectively knocks down EphB4 protein expression (Fig. 31A). Both siRNA 472 and antisense AS-10 ODN reduced the viability of PC3 cells in a dose dependent manner (Fig. 31C, D). Unrelated siRNA or sense oligonucleotide had no effect on viability.

G. EphB4 siRNA and antisense ODNs inhibit the mobility of PC3 Cells

PC3 cells can grow aggressively locally and can form lymph node metastases when injected orthotopically into mice. In an effort to study the role of EphB4 on migration of PC3 cells *in vitro*, we performed a wound-healing assay. When a wound was introduced into a monolayer of PC3 cells, over the course of the next 20 hours cells progressively migrated into the cleared area. However, when cells were transfected with siRNA 472 and the wound was introduced, this migration was significantly inhibited (Fig. 31E). Pretreatment of PC3 cells with 10 μM EphB4 AS-10 for 12 hours generated the same effect (Fig. 31F). In addition, knock-down of EphB4 expression in PC3 cells with siRNA 472 severely reduced the ability of these cells to invade Matrigel as assessed by a double-chamber invasion assay (Fig. 31G), compared to the control siRNA.

H. EphB4 siRNA induces cell cycle arrest and apoptosis in PC3 cells

Since knock-down of EphB4 resulted in decreased cell viability (Fig. 31C) we sought to determine whether this was due to effects on the cell cycle. In comparison to

control siRNA transfected cells, siRNA 472 resulted in an accumulation of cells in the sub G0 and S phase fractions compared to cells treated with control siRNA. The sub G0 fraction increased from 1 % to 7.9%, and the S phase fraction from 14.9 % to 20.8 % in siRNA 472 treated cells compared to control siRNA treated cells (Fig. 32A). Cell cycle arrest at sub G0 and G2 is indicative of apoptosis. Apoptosis as a result of EphB4 knock-down was confirmed by ELISA assay. A dose-dependent increase in apoptosis was observed when PC3 cells were transfected with siRNA 472, but not with control siRNA (Fig. 32B). At 100 nM there was 15 times more apoptosis in siRNA 472 transfected than control siRNA transfected PC3 cells.

I. Materials and Methods

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

Example 4. Expression of EPHB4 in Mesothelioma: a candidate target for therapy

Malignant mesothelioma (MM) is a rare neoplasm that most often arises from the pleural and peritoneal cavity serous surface. The pleural cavity is by far the most frequent site affected (> 90%), followed by the peritoneum (6-10%) (Carbone et al., 2002, Semin Oncol. 29:2-17). There is a strong association with asbestos exposure, about 80% of malignant mesothelioma cases occur in individuals who have ingested or inhaled asbestos. This tumor is particularly resistant to the current therapies and, up to now, the prognosis of these patients is dramatically poor (Lee et al., 2000, Curr Opin Pulm Med. 6:267-74).

Several clinical problems regarding the diagnosis and treatment of malignant mesothelioma remain unsolved. Making a diagnosis of mesothelioma from pleural or abdominal fluid is notoriously difficult and often requires a thoracoscopic or laproscopic or open biopsy and Immunohistochemical staining for certain markers such as meosthelin expressed preferentially in this tumor. Until now, no intervention has proven to be curative, despite aggressive chemotherapeutic regimens and prolonged radiotherapy. The median survival in most cases is only 12–18 months after diagnosis.

In order to identify new diagnostic markers and targets to be used for novel diagnostic and therapeutic approaches, we assessed the expression of EPHB4 and its ligand EphrinB2 in mesothelioma cell lines and clinical samples.

A. EPHB4 and EphrinB2 is expressed in mesothelioma cell lines

The expression of Ephrin B2 and EphB4 in malignant mesothelioma cell lines was determined at the RNA and protein level by a variety of methods. RT-PCR showed that all of the four cell lines express EphrinB2 and EPHB4 (fig. 33A). Protein expression was determined by Western blot in these cell lines. Specific bands for EphB4 were seen at 120 kD. In addition, Ephrin B2 was detected in all cell lines tested as a 37 kD band on Western blot (fig. 33B). No specific band for Ephrin B2 was observed in 293 human embryonic kidney cells, which were included as a negative control.

To confirm the presence of EphB4 transcription in mesothelioma cells, *in situ* hybridization was carried out on NCI H28 cell lines cultured on chamber slides. Specific signal for EphB4 was detected using antisense probe Ephrin B2 transcripts were also detected in the same cell line. Sense probes for both EphB4 and Ephrin B2 served as negative controls and did not hybridize to the cells (figure 34). Expression of EphB4 and Ephrin B2 proteins was confirmed in the cell lines by immunofluorescence analysis (fig. 35). Three cell lines showed strong expression of EphB4, whereas expression of Ephrin B2 was present in H28 and H2052, and weakly detectable in H2373.

B. Evidence of Expression of EPHB4 and EphrinB2 in clinical samples

Tumor cells cultured from the pleural effusion of a patient diagnosed with pleural malignant mesothelioma were isolated and showed positive staining for both EphB4 and Ephrin B2 at passage 1 (figure 35, bottom row). These results confirm co-expression of EphB4 and Ephrin B2 in mesothelioma cell lines. To determine whether these results seen in tumor cell lines were a real reflection of expression in the disease state, tumor biopsy samples were subjected to immunohistochemical staining for EphB4 and Ephrin B2. Antibodies to both proteins revealed positive stain in the tumor cells. Representative data is shown in figure 36.

C. EPHB4 is involved in the cell growth and migration of mesothelioma

The role of EphB4 in cell proliferation was tested using EPHB4 specific antisepses oligonucleotides and siRNA. The treatment of cultured H28 with EPHB4 antisense reduced cell viability. One of the most active inhibitor of EphB4 expression is EPHB4AS-10 (fig. 37A). Transfection of EPHB4 siRNA 472 generated the same effect (fig. 37B).

MM is a locally advancing disease with frequent extension and growth into adjacent vital structures such as the chest wall, heart, and esophagus. In an effort to study this

process in vitro, we perform wound healing assay using previously described techniques (3:36). When a wound was introduced into sub confluent H28 cells, over the course of the next 28 hours cells would progressively migrate into the area of the wound. However, when cells were pretreated with EPHB4AS-10 for 24 hours, and the wound was introduced, this migration was virtually completely prevented (fig. 38A). The migration study with Boyden Chamber assay with EPHB4 siRNA showed that cell migration was greatly inhibited with the inhibition of EPHB4 expression (Fig. 38B).

D. Materials and Methods

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

Example 5. EphB4 Is Expressed in Squamous Cell Carcinoma of The Head and Neck: Regulation by Epidermal Growth Factor Signaling Pathway and Growth Advantage.

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide, with estimated 900,000 cases diagnosed each year. It comprises almost 50% of all malignancies in some developing nations. In the United States, 50,000 new cases and 8,000 deaths are reported each year. Tobacco carcinogens are believed to be the primary etiologic agents of the disease, with alcohol consumption, age, gender, and ethnic background as contributing factors.

The differences between normal epithelium of the upper aerodigestive tract and cancer cells arising from that tissue are the result of mutations in specific genes and alteration of their expression. These genes control DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis. For head and neck cancer, alterations of three signaling pathways occur with sufficient frequency and produce such dramatic phenotypic changes as to be considered the critical transforming events of the disease. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC.

Although high expression of EphB4 has been reported in hematologic malignancies, breast carcinoma, endometrial carcinoma, and colon carcinoma, there is limited data on the protein levels of EphB4, and complete lack of data on the biological significance of this protein in tumor biology such as HNSCC.

A. HNSCC tumors express EphB4

We studied the expression of EphB4 in human tumor tissues by immunohistochemistry, in situ hybridization, and Western blot. Twenty prospectively collected tumor tissues following IRB approval have been evaluated with specific EphB4 monoclonal antibody that does not react with other members of the EphB and EphA family. EphB4 expression is observed in all cases, with varying intensity of staining. Figure 39A (top left) illustrates a representative case, showing that EphB4 is expressed in the tumor regions only, as revealed by the H&E tumor architecture (Fig. 39A bottom left). Note the absence of staining for EphB4 in the stroma. Secondly, a metastatic tumor site in the lymph node shows positive staining while the remainder of the lymph node is negative (Fig. 39A, top right).

In situ hybridization was carried out to determine the presence and location of EphB4 transcripts in the tumor tissue. Strong signal for EphB4 specific antisense probe was detected indicating the presence of transcripts (Figure 39 B, top left). Comparison with the H&E stain (Fig. 39B, bottom left) to illustrate tumor architecture reveals that the signal was localized to the tumor cells, and was absent from the stromal areas. Ephrin B2 transcripts were also detected in tumor sample, and as with EphB4, the signal was localized to the tumor cells (Fig. 39B, top right). Neither EphB4 nor ephrin B2 sense probes hybridized to the sections, proving specificity of the signals.

B. High expression of EphB4 in primary and metastatic sites of HNSCC

Western blots of tissue from primary tumor, lymph node metastases and uninvolved tissue were carried out to determine the relative levels of EphB4 expression in these sites. Tumor and normal adjacent tissues were collected on 20 cases, while lymph nodes positive for tumor were harvested in 9 of these 20 cases. Representative cases are shown in figure 39C. EphB4 expression is observed in each of the tumor samples. Similarly, all tumor positive lymph nodes show EphB4 expression that was equal to or greater than the primary tumor. No or minimal expression is observed in the normal adjacent tissue.

C. EphB4 expression and regulation by EGFR activity in HNSCC cell lines

Having demonstrated the expression of EphB4 limited to tumor cells, we next sought to determine whether there was an in vitro model of EphB4 expression in HNSCC. Six HN SCC cell lines were surveyed for EphB4 protein expression by Western Blot (Fig. 40A). A majority of these showed strong EphB4 expression and thus established the basis for subsequent studies. Since EGFR is strongly implicated in HNSCC we asked whether

EphB4 expression is associated with the activation of EGFR. Pilot experiments in SCC-15, which is an EGFR positive cell line, established an optimal time of 24 h and concentration of 1 mM of the specific EGFR kinase inhibitor AG 1478 (Figure 40B) to inhibit expression of EphB4. When all the cell lines were studied, we noted robust EGFR expression in all but SCC-4, where it is detectable but not strong (Fig. 40C, top row). In response to EGFR inhibitor AG1478 marked loss in the total amount of EphB4 was observed in certain cell lines (SCC-15, and SCC-25) while no effect was observed in others (SCC-9, -12, -13 and -71). Thus SCC-15 and -25 serve as models for EphB4 being regulated by EGFR activity, while SCC-9, -12, -13 and -71 are models for regulation of EphB4 in HNSCC independent of EGFR activity, where there may be input from other factors such as p53, PTEN, IL-6 etc. We also noted expression of the ligand of EphB4, namely ephrin B2, in all of the cell lines tested. As with EphB4 in some lines ephrin B2 expression appears regulated by EGFR activity, while it is independent in other cell lines.

Clearly, inhibition of constitutive EGFR signaling repressed EphB4 levels in SCC15 cells. We next studied whether EGF could induce EphB4. We found that EphB4 levels were induced in SCC15 cells that had been serum starved for 24 h prior to 24 h treatment with 10 ng/ml EGF as shown in figure 41B (lanes 1 and 2). The downstream signaling pathways known for EGFR activation shown in figure 41A, (for review see Yarden & Slikowski 2001) were then investigated for their input into EGF mediated induction of EphB4. Blocking PLCg, AKT and JNK phosphorylation with the specific kinase inhibitors U73122, SH-5 and SP600125 respectively reduced basal levels and blocked EGF stimulated induction of EphB4 (Fig. 41B, lanes 3-8). In contrast, inhibition of ERK1/2 with PD098095 and PI3-K with LY294002 or Wortmannin had no discernible effect on EGF induction of EphB4 levels. However, basal levels of EphB4 were reduced when ERK1/2 phosphorylation was inhibited. Interestingly, inhibition of p38 MAPK activation with SB203580 increased basal, but not EGF induced EphB4 levels. Similar results were seen in the SCC25 cell line (data not shown).

D. Inhibition of EphB4 in high expressing cell lines results in reduced viability and causes cell-cycle arrest

We next turned to the role of EphB4 expression in HNSCC by investigating the effect of ablating expression using siRNA or AS-ODN methods. Several siRNAs to EphB4 sequence were developed (Table 1) which knocked-down EphB4 expression to varying degrees as seen in figure 42A. Viability was reduced in SCC-15, -25 and -71 cell lines

transfected with siRNAs 50 and 472, which were most effective in blocking EphB4 expression (Figure 42B). Little effect on viability was seen with EphB4 siRNA 1562 and 2302 or ephrin B2 siRNA 254. Note that in SCC-4, which does not express EphB4 (see Fig. 40A) there was no reduction in cell viability. The decreased cell viability seen with siRNA 50 and 472 treatment was attributable to accumulation of cells in sub G0, indicative of apoptosis. This effect was both time and dose-dependant (Figure 42C and Table 2). In contrast, siRNA2302 that was not effective in reducing EphB4 levels and had only minor effects on viability did not produce any changes in the cell cycle when compared with the mock Lipofectamine™2000 transfection.

A detailed description of the siRNA constructs for this example may be found in U.S. Patent Publication No. 20050084873.

Table: Effect of different EphB4 siRNA on Cell Cycle

Treatment	Sub G0	G1	S	G2
36hr				
Lipo alone	1.9	39.7	21.3	31.8
100 nM 2302	2.0	39.3	21.2	31.2
100 nM 50	18.1	31.7	19.7	24.4
100 nM 472	80.2	10.9	5.2	2.1
16hr				
Lipo alone	7.8	55.7	15.2	18.5
100 nM 2302	8.4	57.3	14.3	17.3
10 nM 50	10.4	53.2	15.7	17.7
100 nM 50	27.7	31.3	18.1	19.6
10 nM 472	13.3	50.2	15.8	17.5
100 nM 472	30.7	31.9	16.4	18.0

In addition, over 50 phosphorothioate AS-ODNs complementary to the human EphB4 coding sequences were synthesized and tested for their ability to inhibit EphB4 expression in 293 cells transiently transfected with full length EphB4 expression plasmid.

Figure 43A shows a representative sample of the effect of some of these AS-ODNs on EphB4 expression. Note that expression is totally abrogated with AS-10, while AS-11 has only a minor effect. The effect on cell viability in SCC15 cells was most marked with AS-ODNs that are most effective in inhibiting EphB4 expression as shown in figure 43B. The IC_{50} for AS-10 was approximately 1 μM, while even 10 μM AS-11 was not sufficient to attain 50 % reduction of viability. When the effect that AS-10 had on the cell cycle was investigated, it was found that the sub G0 fraction increased from 1.9 % to 10.5 % compared to non-treated cells, indicative of apoptosis (Fig. 43C).

E. EphB4 regulates Cell migration

We next wished to determine if EphB4 participates in the migration of HNSCC. Involvement in migration may have implications for growth and metastasis. Migration was assessed using the wound-healing/scrape assay. Confluent SCC15 and SCC25 cultures were wounded by a single scrape with a sterile plastic Pasteur pipette, which left a 3 mm band with clearly defined borders. Migration of cells into the cleared area in the presence of test compounds was evaluated and quantitated after 24, 48 and 72 hr. Cell migration was markedly diminished in response to AS-10 that block EphB4 expression while the inactive compounds, AS-1 and scrambled ODN had little to no effect as shown in figure 43D. Inhibition of migration with AS-10 was also shown using the Boyden double chamber assay (Fig. 43E).

F. EphB4 AS-10 in vivo anti-tumor activity

The effect of EphB4 AS-10, which reduces cell viability and motility, was determined in SCC15 tumor xenografts in Balb/C nude mice. Daily treatment of mice with 20 mg/kg AS-10, sense ODN or equal volume of PBS by I.P. injection was started the day following tumor cell implantation. Growth of tumors in mice receiving AS-10 was significantly retarded compared to mice receiving either sense ODN or PBS diluent alone (Figure 44). Non-specific effects attributable to ODN were not observed, as there was no difference between the sense ODN treated and PBS treated groups.

G. Materials and Methods

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

Example 6. Ephrin B2 Expression in Kaposi's Sarcoma Is Induced by Human Herpesvirus Type 8: Phenotype Switch from Venous to Arterial Endothelium

Kaposi's Sarcoma (KS) manifests as a multifocal angioproliferative disease, most commonly of the skin and mucus membranes, with subsequent spread to visceral organs (1) Hallmarks of the disease are angiogenesis, edema, infiltration of lymphomononuclear cells and growth of spindle-shaped tumor cells. Pathologically, established lesions exhibit an extensive vascular network of slit-like spaces. The KS vascular network is distinct from normal vessels in the lack of basement membranes and the abnormal spindle shaped endothelial cell (tumor cell) lining these vessels. Defective vasculature results in an accumulation of the blood components including albumin, red and mononuclear cells in the lesions (1). The KS tumor is endothelial in origin; the tumor cells express many endothelial markers, including lectin binding sites for *Ulex europeaus* agglutinin-1 (UEA-1), CD34, EN-4, PAL-E (2) and the endothelial cell specific tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), VEGFR-3 (Flt-4), Tie-1 and Tie-2 (3, RM & PSG unpublished data). KS cells co-express lymphatic endothelial cell related proteins including LYVE and podoplanin (4).

The herpesvirus HHV-8 is considered the etiologic agent for the disease. In 1994 sequences of this new herpes virus were identified in KS tumor tissue (5), and subsequent molecular-epidemiology studies have shown that nearly all KS tumors contain viral genome. Sero-epidemiology studies show that HIV infected patients with KS have the highest prevalence of HHV-8 and secondly that those with HIV infection but no KS have increased risk of developement of KS over the ensuing years if they are also seropositive for HHV-8 (6). Direct evidence for the role of HHV-8 in KS is the transformation of bone marrow endothelial cells after infection with HHV-8 (7). A number of HHV-8 encoded genes could contribute to cellular transformation (reviewed in 8). However, the most evidence has accumulated for the G-protein coupled receptor (vGPCR) in this role (9).

We investigated whether KS tumor cells are derived from arterial or venous endothelium. In addition, we investigated whether HHV-8 has an effect on expression of arterial or venous markers in a model of KS. KS tumor cells were found to express the ephrin B2 arterial marker. Further, ephrin B2 expression was induced by HHV-8 vGPCR in KS and endothelial cell lines. Ephrin B2 is a potential target for treatment of KS because inhibition of ephrin B2 expression or signaling was detrimental to KS cell viability and function.

A. KS tumors express Ephrin B2, but not EphB4

The highly vascular nature of KS lesions and the probable endothelial cell origin of the tumor cells prompted investigation of expression of EphB4 and ephrin B2 which are markers for venous and arterial endothelial cells, respectively. Ephrin B2, but not EphB4 transcripts were detected in tumor cells of KS biopsies by in situ hybridization (figure 45A). Comparison of the positive signal with ephrin B2 antisense probe and tumor cells as shown by H&E staining shows that ephrin B2 expression is limited to the areas of the biopsy that contain tumor cells. The lack of signal in KS with EphB4 antisense probe is not due to a defect in the probe, as it detected transcripts in squamous cell carcinoma, which we have shown expresses this protein (18). Additional evidence for the expression of ephrin B2 in KS tumor tissue is afforded by the localization of EphB4/Fc signal to tumor cells, detected by FITC conjugated anti human Fc antibody. Because ephrin B2 is the only ligand for EphB4 this reagent is specific for the expression of ephrin B2 (figure 45B, left). An adjacent section treated only with the secondary reagent shows no specific signal. Two-color confocal microscopy demonstrated the presence of the HHV-8 latency protein, LANA1 in the ephrin B2 positive cells (Fig. 45C, left), indicating that it is the tumor cells, not tumor vessels, which are expressing this arterial marker. Staining of tumor biopsy with PECAM-1 antibody revealed the highly vascular nature of this tumor (Fig. 45C, right). A pilot study of the prevalence of this pattern of ephrin B2 and EphB4 expression on KS biopsies was conducted by RT-PCR analysis. All six samples were positive for ephrin B2, while only 2 were weakly positive for EphB4 (data not shown).

B. Infection of venous endothelial cells with HHV-8 causes a phenotype switch to arterial markers

We next asked whether HHV-8, the presumed etiologic agent for KS, could itself induce expression of ephrin B2 and repress EphB4 expression in endothelial cells. Co-culture of HUVEC and BC-1 lymphoma cells, which are productively infected with HHV-8, results in effective infection of the endothelial cells (16). The attached monolayers of endothelial cells remaining after extensive washing were examined for ephrin B2 and EphB4 by RT-PCR and immunofluorescence. HUVEC express EphB4 venous marker strongly at the RNA level, but not ephrin B2 (figure 46B). In contrast, HHV-8 infected cultures (HUVEC/BC-1 and HUVEC/BC-3) express ephrin B2, while EphB4 transcripts are almost absent.

Immunofluorescence analysis of cultures of HUVEC and HUVEC/HHV-8 for artery/vein markers and viral proteins was undertaken to determine whether changes in protein expression mirrored that seen in the RNA. In addition, cellular localization of the proteins could be determined. Consistent with the RT-PCR data HUVEC are ephrin B2 negative and EphB4 positive (Fig. 46A(a & m)). As expected they do not express any HHV-8 latency associated nuclear antigen (LANA1) (Fig. 46A(b, n)). Co-culture of BC-1 cells, which are productively infected with HHV-8, resulted in infection of HUVEC as shown by presence of viral proteins LANA1 and ORF59 (Fig. 46A(f, r)). HHV-8 infected HUVEC now express ephrin B2 but not EphB4 (Fig. 46A(e, q, u), respectively). Expression of ephrin B2 and LANA1 co-cluster as shown by yellow signal in the merged image (Fig. 46A(h)). HHV-8 infected HUVEC positive for ephrin B2 and negative for Eph B4 also express the arterial marker CD148 (19) (Fig. 46A (j, v)). Expression of ephrin B2 and CD148 co-cluster as shown by yellow signal in the merged image (Fig. 46A(l)). Uninfected HUVEC expressing Eph B4 were negative for CD148 (not shown).

C. HHV-8 vGPCR induces ephrin B2 expression

To test whether individual viral proteins could induce the expression of ephrin B2 seen with the whole virus KS-SLK cells were stably transfected with HHV-8 LANA, or LANAΔ440 or vGPCR. Western Blot of stable clones revealed a five-fold induction of ephrin B2 in KS-SLK transfected with vGPCR compared to SLK-LANA or SLK-LANAΔ440 (Fig. 47A). SLK transfected with vector alone (pCEFL) was used as a control. SLK-vGPCR and SLK-pCEFL cells were also examined for ephrin B2 and Eph B4 expression by immunofluorescence in transiently transfected KS-SLK cells. Figure 47B shows higher expression of ephrin B2 in the SLK-vGPCR cells compared to SLK-pCEFL. No changes in Eph B4 were observed in SLK-vGPCR compared to SLK-pCEFL. This clearly demonstrates that SLK-vGPCR cells expressed high levels of ephrin B2 compared to SLK-pCEFL cells. This suggests that vGPCR of HHV-8 is directly involved in the induction of Ephrin B2 and the arterial phenotype switch in KS. Since we had shown that HHV-8 induced expression of ephrin B2 in HUVEC, we next asked if this could be mediated by a transcriptional effect. Ephrin B2 5'-flanking DNA-luciferase reporter plasmids were constructed as described in the Materials and Methods and transiently transfected into HUVECs. Ephrin B2 5'-flanking DNA sequences -2491/-11 have minimal activity in HUVEC cells (figure 47C). This is consistent with ephrin B2 being an arterial, not venous marker. However, we have noted that HUVEC in culture do express some

ephrin B2 at the RNA level. Cotransfection of HHV-8 vGPCR induces ephrin B2 transcription approximately 10-fold compared to the control expression vector pCEFL. Roughly equal induction was seen with ephrin B2 sequences -2491/-11, -1242/-11, or -577/-11, which indicates that elements between -577 and -11 are sufficient to mediate the response to vGPCR, although maximal activity is seen with the -1242/-11 luciferase construct.

D. Expression of Ephrin B2 is regulated by VEGF and VEGF-C

We next asked whether known KS growth factors could be involved in the vGPCR-mediated induction of ephrin B2 expression. SLK-vGPCR cells were treated with neutralizing antibodies to oncostatin-M, IL-6, IL-8, VEGF or VEGF-C for 36 hr. Figure 48A shows that neutralization of VEGF completely blocked expression of ephrin B2 in SLK-vGPCR cells. A lesser, but significant decrease in ephrin B2 was seen neutralization of VEGF-C and IL-8. No appreciable effect was seen with neutralization of oncostatin-M or IL-6. To verify that VEGF and VEGF-C are integral to the induction of ephrin B2 expression we treated HUVEC with VEGF, VEGF-C or EGF. HUVECs were grown in EBM-2 media containing 5 % FBS with two different concentration of individual growth factor (10 ng, 100 ng/ml) for 48 h. Only VEGF-A or VEGF-C induced ephrin B2 expression in a dose dependent manner (Figure 48B). In contrast, EGF had no effect on expression of ephrin B2.

E. Ephrin B2 siRNA inhibits the expression of Ephrin B2 in KS

Three ephrin B2 siRNA were synthesized as described in the methods section. KS-SLK cells were transfected with siRNA and 48 h later ephrin B2 expression was determined by Western Blot. Ephrin B2 siRNAs 137 or 254 inhibited about 70% of ephrin B2 expression compared to control siRNA such as siRNA Eph B4 50 or siRNA GFP. Ephrin B2 63 siRNA was less effective than the above two siRNA Ephrin B2 (Figure 49A).

F. Ephrin B2 is necessary for full KS and EC viability, cord formation and in vivo angiogenesis activities

The most effective ephrin B2 siRNA (254) was then used to determine whether inhibiting expression of ephrin B2 has any effect on the growth of KS-SLK or HUVEC cells. The viability of KS-SLK cells was decreased by the same siRNAs that inhibited ephrin B2 protein levels (figure 49B). KS-SLK express high levels of ephrin B2 and this

result shows maintenance of ephrin B2 expression is integral to cell viability in this setting. HUVECs do not express ephrin B2, except when stimulated by VEGF as shown in Fig. 48B. Ephrin B2 siRNA 264 dramatically reduced growth of HUVECs cultured with VEGF as the sole growth factor. In contrast, no significant effect was seen when HUVECs were cultured with IGF, EGF and bFGF. As a control, EphB4 siRNA 50 had no detrimental effect on HUVECs in either culture condition (figure 49C).In addition to inhibition of viability of KS and primary endothelial cells, EphB4-ECD inhibits cord formation in HUVEC and KS-SLK and in vivo angiogenesis in the Matrigel™ plug assay (Figure 50).

G. Methods and Materials

A detailed description of the materials and methods for this example may be found in U.S. Patent Publication No. 20050084873.

Example 7. Expression of EphB4 in Bladder cancer: a candidate target for therapy

Figure 51 shows expression of EPHB4 in bladder cancer cell lines (A), and regulation of EPHB4 expression by EGFR signaling pathway (B).

Figure 52 shows that transfection of p53 inhibit the expression of EPHB4 in 5637 cell.

Figure 53 shows growth inhibition of bladder cancer cell line (5637) upon treatment with EPHB4 siRNA 472.

Figure 54 shows results on apoptosis study of 5637 cells transfected with EPHB4 siRNA 472.

Figure 55 shows effects of EPHB4 antisense probes on cell migration. 5637 cells were treated with EPHB4AS10 (10 μM).

Figure 56 shows effects of EPHB4 siRNA on cell invasion. 5637 cells were transfected with siRNA 472 or control siRNA.

Example 8. Inhibition of EphB4 Gene Expression by EphB4 antisense probes and RNAi probes

Cell lines expressing EphB4 were treated with the synthetic phosphorothioate modified oligonucleotides and harvested after 24 hr. Cell lysates were prepared and probed by western blot analysis for relative amounts of EphB4 compared to untreated control cells.

Studies on inhibition of cell proliferation were done in HNSCC cell lines characterized to express EphB4. Loss of cell viability was shown upon knock-down of EphB4 expression. Cells were treated in vitro and cultured in 48-well plates, seeded with 10

thousand cells per well. Test compounds were added and the cell viability was tested on day 3. The results on EphB4 antisense probes were summarized below in Table 6. The results on EphB4 RNAi probes were summarized below in Table 7.

A detailed description of the antisense and siRNA constructs for this example may be found in U.S. Patent Publication No. 20050084873.

Example 9. Inhibition of Ephrin B2 Gene Expression by Ephrin B2 antisense probes and RNAi probes

KS SLK, a cell line expressing endogenous high level of ephrin B2. Cell viability was tested using fixed dose of each oligonuceotide (5uM). Gene expression downregulation was done using cell line 293 engineered to stably express full-length ephrin B2. KS SLK expressing EphrinB2 were also used to test the viability in response to RNAi probes tested at the fixed dose of 50 nM. Protein expression levels were measured using 293 cells stably expressing full-length EphrinB2, in cell lysates after 24 hr treatment with fixed 50 nM of RNAi probes.

The results on Ephrin B2 antisense probes were summarized below in Table 8. The results on Ephrin B2 RNAi probes were summarized below in Table 9.

A detailed description of the antisense and siRNA constructs for this example may be found in U.S. Patent Publication No. 20050084873.

Example 10. EphB4 antibodies inhibit tumor growth

Figure 57 shows results on comparison of EphB4 monoclonal antibodies by G250 and in Pull-down assay.

Figure 58 shows that EphB4 antibodies, in the presence of matrigel and growth factors, inhibit the *in vivo* tumor growth of SCC15 cells.

BalbC nude mice were injected subcutaneously with 2.5 x 10^6 viable tumor cells SCC15 is a head and neck squamous cell carcinoma line. Tumors were initiated in nu/nu mice by injecting 2.5-5x10^6 cells premixed with matrigel and Growth factors, and Ab's subcutaneously to initiate tumor xenografts. Mice were opened 14 days after injections. SCC15 is a head and neck squamous cell carcinoma line, B16 is a melanoma cell line, and MCF-7 is a breast carcinoma line. The responses of tumors to these treatments were compared to control treated mice, which receive PBS injections. Animals were observed daily for tumor growth and subcutaneous tumors were measured using a caliper every 2

days. Antibodies #1 and #23 showed significant regression of SCC15 tumor size compared to control, especially with no additional growth factor added.

Figure 59 shows that EphB4 antibodies cause apoptosis, necrosis and decreased angiogenesis in SCC15, head and neck carcinoma tumor type.

Angiogenesis was assessed by CD-31 immunohistochemistry. Tumor tissue sections from treated and untreated mice were stained for CD31. Apoptosis was assessed by immunohistochemical TUNNEL, and proliferation by BrdU assay. Following surgical removal, tumors were immediately sliced into 2 mm serial sections and embedded in paraffin using standard procedures. Paraffin embedded tissue were sectioned at 5 μm, the wax removed and the tissue rehydrated. The rehydrated tissues were microwave irradiated in antigen retreival solution. . Slides were rinsed in PBS, and TUNNEL reaction mixture (Terminal deoxynucleotidyl transferase and flourescein labeled nucleotide solution), and BrdU were added in a humidity chamber completely shielded from light. The TUNNEL and BrdU reaction mixture were then removed, slides were rinsed and anti-flourescein antibody conjugated with horseradish peroxidase was added. After incubation and rinsing, 3, 3'diaminobenzidine was added. Masson's Trichrome and Hematoxylin and Eosin were also used to stain the slides to visualize morphology. Masson's Trichrome allows to visualize necrosis and fibrosis. The tumor gets blood support from tumor/skin, muscle boundary. As tumor grows, inner regions get depleted of nutrients. This leads to necrosis (cell death), preferably at the tumor center. After cells die, (tumor) tissue gets replaced with fibroblastic tissue. Slides were visualized under 20-fold magnification with digital images acquired. A different morphology was obtained on SCC tumors with each antibody administered. Ab #1 showed an increase in necrosis and fibrosis but not apoptosis. Ab #23 showed an increase in apoptosis, necrosis and fibrosis and a decrease in vessel infiltration. Ab #35 showed an increase in necrosis and fibrosis, and a small increase in apoptosis and a decrease in vessel infiltration. Ab #79 showed a large increase in apoptosis, and necrossis and fibrosis. Ab #91 showed no change in apoptosis but an increase in proliferation. And Ab #138 showed an increase in apoptosis, necrosis, fibrosis and a decrease in proliferation and vessel infiltration. Tumors treated with control PBS displayed abundant tumor density and a robust angiogenic response. Tumors treated with EphB4 antibodies displayed a decrease in tumor cell density and a marked inhibition of tumor angiogenesis in regions with viable tumor cells, as well as tumor necrosis and apoptosis.

Figure 60 shows that systemic administration of antibodies on xenografts leads to tumor regression in SCC15 tumor xenografts.

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

Example 11. HSA-EphB4 ectodomain fusion and PEG-modified EphB4 Ectodomain

A. Generation of HSA-EphB4 ectodomain fusion

Human serum albumin fragment in XbaI-NotI form was PCR-amplified out for creating a fusion with GCF2, and TA-cloned into pEF6. In the next step, the resulting vector was cut with Xba I (partial digestion) and the HSA fragment (1.8 kb) was cloned into Xba I site of pEF6-GCF2-Xba to create fusion expression vector. The resulting vector had a point mutation C to T leading to Thr to Ile substitution in position 4 of the mature protein. It was called pEF6-GCF2-HSAmut. In the next cloning step, the mutation was removed by substituting wild type KpnI fragment from pEF6-GCF2-IF (containing piece of the vector and N-terminal part of GCF2) for the mutated one, this final vector was called pEF6-GCF2. The DNA sequence of pEF6-GCF2 was confirmed.

The predicted amino acid of the HSA-EphB4 precursor protein was as follows (SEQ ID NO:18):

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHS
VRTYEVCDVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCK
ETFTVFYYESDADTATALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKVNVKT
LRLGPLSKAGFYLAFQDQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPV
AGSCVVDAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQG
TFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVV
SRLNGSSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLV
EPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVT
RSSPSSLSLAWAVPRAPSGAVLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKR
GASYLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQSRDAHKSEVAHRFKDL

GEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLF
GDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMC
TAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP
KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLV
TDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAE
VENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLL
LRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFKQLGEYK
FQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVL
NQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADIC
TLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFA
EEGKKLVAASQAALGL

The predicted amino acid sequence of the mature form of the HSA-EphB4 protein was as follows (SEQ ID NO:19):

LEETLLNTKLETADLKWVTFPQVDGQWEELSGLDEEQHSVRTYEVCDVQRAPGQ
AHWLRTGWVPRRGAVHVYATLRFTMLECLSLPRAGRSCKETFTVFYYESDADTAT
ALTPAWMENPYIKVDTVAAEHLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQ
DQGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVPAPGPS
PSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGEGSCQPCP
ANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRLNGSSLHLEWSA
PLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPRDLVEPWVVVRGLRPDFT
YTFEVTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSPSSLSLAWAVPR
APSGAVLDYEVKYHEKGAEGPSSVRFLKTSENRAELRGLKRGASYLVQVRARSEA
GYGPFGQEHHSQTQLDESEGWREQSRDAHKSEVAHRFKDLGEENFKALVLIAFAQ
YLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETY
GEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKY
LYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSA
KQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGD
LLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLA
ADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCC
AAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFKQLGEYKFQNALLVRYTKKVP
QVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSD
RVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTA
LVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAA
LGL

The nucleic acid sequence of the pEF6-GCF2 plasmid was as follows (SEQ ID NO: 20):

aatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaatagggttccgcg
cacatttccccgaaaagtgccacctgacgtcgacggatcgggagatctcccgatcccctatggtcgactctcagtacaatctgctctg
atgccgcatagttaagccagtatctgctccctgcttgtgtgttggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaag
gcaaggcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgtacgggccagatatacgcgt

tgacattgattattgactaggcttttgcaaaaagctttgcaaagatggataaagttttaaacagagaggaatctttgcagctaatggacct
tctaggtcttgaaaggagtgcctcgtgaggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttg
gggggaggggtcggcaattgaaccggtgcctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgc
ctttttcccgagggtgggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttcgcaacgggtttgccgccagaacac
aggtaagtgccgtgtgtggttcccgcgggcctggcctctttacgggttatggcccttgcgtgccttgaattacttccacctggctgcag
tacgtgattcttgatcccgagcttcgggttggaagtgggtgggagagttcgaggccttgcgcttaaggagccccttcgcctcgtgctt
gagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctc
tagccatttaaaatttttgatgacctgctgcgacgctttttttctggcaagatagtcttgtaaatgcgggccaagatctgcacactggtatt
tcggtttttggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggc
caccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggcctcgcgccgccgtgtatcgccccgccctg
ggcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgcagggagctcaaaat
ggaggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggcctttccgtcctcagccgtcgcttca
tgtgactccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggga g
gggttttatgcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaattt
gccctttttgagtttggatcttggttcattctcaagcctcagacagtggttcaaagttttttttcttccatttcaggtgtcgtgaggaattagctt
ggtactaatacgactcactatagggagacccaagctggctaggtaagcttggtaccgagctcggatccactagtccagtgtggtgg
aattgcccttCAAGCTTGCCGCCACCATGGAGCTCCGGGTGCTGCTCTGCTGGGCTTC
GTTGGCCGCAGCTTTGGAAGAGACCCTGCTGAACACAAAATTGGAAACTGCTG
ATCTGAAGTGGGTGACATTCCCTCAGGTGGACGGGCAGTGGGAGGAACTGAGC
GGCCTGGATGAGGAACAGCACAGCGTGCGCACCTACGAAGTGTGTGACGTGCA
GCGTGCCCCGGGCCAGGCCCACTGGCTTCGCACAGGTTGGGTCCCACGGCGGG
GCGCCGTCCACGTGTACGCCACGCTGCGCTTCACCATGCTCGAGTGCCTGTCCC
TGCCTCGGGCTGGGCGCTCCTGCAAGGAGACCTTCACCGTCTTCTACTATGAGA
GCGATGCGGACACGGCCACGGCCCTCACGCCAGCCTGGATGGAGAACCCCTAC
ATCAAGGTGGACACGGTGGCCGCGGAGCATCTCACCCGGAAGCGCCCTGGGGC
CGAGGCCACCGGGAAGGTGAATGTCAAGACGCTGCGCCTGGGACCGCTCAGCA
AGGCTGGCTTCTACCTGGCCTTCCAGGACCAGGGTGCCTGCATGGCCCTGCTAT
CCCTGCACCTCTTCTACAAAAAGTGCGCCCAGCTGACTGTGAACCTGACTCGAT
TCCCGGAGACTGTGCCTCGGGAGCTGGTTGTGCCCGTGGCCGGTAGCTGCGTGG
TGGATGCCGTCCCCGCCCCTGGCCCCAGCCCCAGCCTCTACTGCCGTGAGGATG
GCCAGTGGGCCGAACAGCCGGTCACGGGCTGCAGCTGTGCTCCGGGGTTCGAG
GCAGCTGAGGGGAACACCAAGTGCCGAGCCTGTGCCCAGGGCACCTTCAAGCC
CCTGTCAGGAGAAGGGTCCTGCCAGCCATGCCCAGCCAATAGCCACTCTAACA
CCATTGGATCAGCCGTCTGCCAGTGCCGCGTCGGGTACTTCCGGGCACGCACAG
ACCCCCGGGGTGCACCCTGCACCACCCCTCCTTCGGCTCCGCGGAGCGTGGTTT
CCCGCCTGAACGGCTCCTCCCTGCACCTGGAATGGAGTGCCCCCCTGGAGTCTG
GTGGCCGAGAGGACCTCACCTACGCCCTCCGCTGCCGGGAGTGTCGACCCGGA
GGCTCCTGTGCGCCCTGCGGGGGAGACCTGACTTTTGACCCCGGCCCCCGGGAC
CTGGTGGAGCCCTGGGTGGTGGTTCGAGGGCTACGTCCTGACTTCACCTATACC
TTTGAGGTCACTGCATTGAACGGGGTATCCTCCTTAGCCACGGGGCCCGTCCCA
TTTGAGCCTGTCAATGTCACCACTGACCGAGAGGTACCTCCTGCAGTGTCTGAC
ATCCGGGTGACGCGGTCCTCACCCAGCAGCTTGAGCCTGGCCTGGGCTGTTCCC
CGGGCACCCAGTGGGGCTGTGCTGGACTACGAGGTCAAATACCATGAGAAGGG
CGCCGAGGGTCCCAGCAGCGTGCGGTTCCTGAAGACGTCAGAAAACCGGGCAG
AGCTGCGGGGGCTGAAGCGGGGAGCCAGCTACCTGGTGCAGGTACGGGCGCGC
TCTGAGGCCGGCTACGGGCCCTTCGGCCAGGAACATCACAGCCAGACCCAACT
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TCTTCGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTG
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GCCCCGGAACTCCTTTTCTTTGCTAAAAGGTATAAAGCTGCTTTTACAGAATGT
TGCCAAGCTGCTGATAAAGCTGCCTGCCTGTTGCCAAAGCTCGATGAACTTCGG
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GATTTCCCAAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCA
AAGTCCACACGGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGG
GCGGACCTTGCCAAGTATATCTGTGAAAATCAGGATTCGATCTCCAGTAAACTG
AAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGT
GGAAAATGATGAGATGCCTGCTGACTTGCCTTCATTAGCTGCTGATTTTGTTGA
AAGTAAGGATGTTTGCAAAAACTATGCTGAGGCAAAGGATGTCTTCCTGGGCA
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CAACTCCAACTCTTGTAGAGGTCTCAAGAAACCTAGGAAAAGTGGGCAGCAAA
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aaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttc

B. Cell culture and transfections:

The human embryonic kidney cell line, 293T cells, was maintained in DMEM with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Transfections of plasmids encoding EphB4 ectodomain, fragments thereof, and EphB4-HSA fusions were performed using Lipofectamine 2000 reagent (Invitrogen) according to suggested protocol. One day before transfections, 293T cells were seeded at a high density to reach 80% confluence at the time of transfection. Plasmid DNA and Lipofectamine reagent at 1:3 ratio were diluted in Opti-MEM I reduced serum medium (Invitrogen) for 5 min and mixed together to form DNA-Lipofectamine complex. For each 10 cm culture dish, 10 μg of plasmid DNA was used. After 20 min, the above complex was added directly to cells in culture medium. After 16 hours of transfection, medium was aspirated, washed once with serum free DMEM and replaced with serum free DMEM. Secreted proteins were harvested after 48 hours by collecting conditional medium. Conditional medium was clarified by centrifugation at 10,000 g for 20 min and filtered through 0.2 μ filter and used for purification.

C. Chromatographic separation of EphB4 ectodomain and EphB4 ectodomain-HSA fusion protein

The EphB4 ectodomain fused to HSA was purified as follows: 700 ml of media was harvested from transiently transfected 293 cells grown in serum free media and concentrated up to final volume of 120 ml. Membrane: (Omega, 76 mm), 50 kDa C/O. After concentration, pH of the sample was adjusted by adding 6 ml of 1M NaAc, pH 5.5. Then sample was dialyzed against starting buffer (SB): 20 mM NaAc, 20 mM NaCl, pH 5.5 for O/N. 5 ml of SP-Sepharose was equilibrated with SB and sample was loaded. Washing: 100 ml of SB. Elution by NaCl: 12 ml/fraction and increment of 20 mM. Most of the EphrinB2 binding activity eluted in the 100mM and 120mM fractions.

Fractions, active in EphrinB2 binding assay (See SP chromatography, fractions # 100-120 mM) were used in second step of purification on Q-column. Pulled fractions were dialyzed against starting buffer#2 (SB2): 20 mM Tris-HCl, 20 mM NaCl, pH 8 for O/N and loaded onto 2 ml of Q-Sepharose. After washing with 20 ml of SB2, absorbed protein was eluted by NaCl: 3 ml/fraction with a concentration increment of 25 mM. Obtained fractions were analyzed by PAGE and in Ephrin-B2 binding assay. The 200mM and 225mM fractions were found to contain the most protein and the most B2 binding activity.

Soluble EphB4 ectodomain protein was purified as follows: 300 ml of conditional medium (see: *Cell culture and transfections*) were concentrated up to final volume of 100 ml, using ultrafiltration membrane with 30 kDa C/O. After concentration, pH of the sample was adjusted by adding 5 ml of 1 M Na-Acetate, pH 5.5. Then sample was dialyzed against starting buffer (StB): 20 mM Na-Acetate, 20 mM NaCl, pH 5.5 for O/N. 5 ml of SP-Sepharose was equilibrated with StB and sample was loaded. After washing the column with 20 ml of StB, absorbed proteins were eluted by linear gradient of concentration of NaCl (20-250 mM and total elution volume of 20 column's volumes). Purity of the proteins was analyzed by PAGE.

D. Biotinylation of sB4 and sB4-HSA fusion protein.

Both soluble EphB4 ectodomain protein (sB4) and EphB4 ectodomain fused to HSA (HSA-sB4) were biotin labeled through carbohydrate chains using sodium meta-periodate as an oxidant and EZ-Link Biotin Hydrazide (PIERCE, Cat. # 21339) according to manufacture's protocol. The *in vitro* stability of the biotinylated sB4 protein was tested

by incubating $2.0x10^{-9}$ with 40 μL of mouse serum at 37°C for 0, 0.5, 1, 2 and 3 days. Two μL of magnetic beads and B2-AP was added for an extra hour at room temperature. After washing twice with buffer, pnPP was added for 1 hour. Biotinylated sB4 protein was found to very stable over three days, with less than 10% of the B2 binding activity being lost over that time.

E. Ephrin-B2 Binding Properties of B4-HSA

To test whether the B4-HSA fusion property retained the ability of the EphB4 extracellular domain to bind to EphrinB2, the ability of the purified B4-HSA fusion was compared to that of GCF2F, GCF2, GC, CF and B4-Fc fusion, which comprises the extracellular domain of B4 fused to hIgG1 Fc as described in Example 1. Biotinylated or His-tag protein samples were inoculated with the corresponding affinity magnetic beads and B2-AP for an hour at room temperature, before addition of PnPP. Results of binding assays are shown on Figure 67. B4-HSA was found to retain most of its binding activity towards EphrinB2. Surprisingly, the B4-HSA protein was superior to the B4-Fc fusion in binding to EphrinB2.

An EphB4 ectodomain fusion to the C-terminus of HSA was also generated, and found to retain the ability to bind to EphrinB2 and was found to have enhanced stability *in vivo* over the EphB4 ectodomain.

F. Stability of B4-HSA vs. sB4 in Mice

The stability of the purified biotinylated sB4 and sB4-HSA were assayed *in vivo*. Each of the proteins were intravenously injected into the tail of mice in the amount of 0.5 nmoles per mouse. Blood from the eye of each mouse was taken in time frames of 15 min (0 days), 1, 2, 3 and 6 days. 10 ml of obtained serum was used in binding assay with Ephrin-B2-Alkaline Phosphatase fusion protein and Streptavidin-coated magnetic beads as a solid phase. The stability of the two proteins is shown on Figure 68. sB4-HSA was found to have superior stability relative to sB4. For example, one day after injection, the levels of sB4-HSA in the blood of the mice were 5-fold greater than those of sB4.

G. PEGylation of biotinylated sB4

Prior to PEGylation, biotinylated sB4 protein generated as described above was concentrated up to final concentration of 2 mg/ml using a 30kDa MWCO ultra membrane.

Sample was dialyzed O/N against coupling buffer: 30mM phosphate, 75mM NaCl, pH 8.00. Coupling to PEG was performed at 4°C for 18 hours in 10 fold molar excess of reactive linear PEG unless otherwise indicated. The reactive PEG used was PEG-succinimidyl propionate, having a molecular weight of about 20kda. Coupling to PEG may be similarly performed using branches PEGs, such as of 10kDa, 20kDa or 40kDa. Other linear PEG molecules of 10 or 40 kDa may also be used.

After PEGylation, the protein sample containing EphB4 ectodomain was dialyzed against StB O/N. Three ml of SP-Sepharose was equilibrated with StB and sample was loaded. Washing and elution of absorbed proteins was performed as above (see: *Purification of soluble EphB4 ectodomain and its fusion to HSA*) with just one modification: total elution volume was 40 volumes of column. Figure 69 shows chromatographic separation of PEG derivatives of EphB4 protein on SP-Sepharose columns. Purity of the PEG-modified EphB4 protein was analyzed by SDS-PAGE.

Double modified (PEGylated Biotinylated) sB4 was used on ion-exchange chromatography to separate non-PEGylated, mono-PEGylated and poly-PEGylated proteins from each other. Pegylated sample was dialyzed O/N against 20 mM Na-acetate, 20 mM NaCl, pH 5.5 and loaded onto 2 ml of SP-Sepharose. After washing with 10 ml of buffer, absorbed proteins were separated by gradual elution of NaCl: 3 ml/fraction and increment of 25 mM NaCl. Obtained fractions were analyzed by PAGE and in Ephrin-B2 binding assay.

H. Effect of PEGylation conditions on sB4 binding to EphrinB2

The effects of pegylating biotinylated sB4 under different pH conditions was determined. sB4 was pegylated at pH 6, 7 or 8, and the pegylated products were tested for binding to EphrinB2 as shown in Figure 69. Ephrin2B binding activity was retained when PEGylation was performed at pH 6 and pH 7, but was partially lost at pH 8.

Additional combinations of parameters were tested, including temperature, pH and molar ratio of pegylation agent to sB4 protein, and the ability of the products of the pegylation reaction to bind to Ephrin-B2. The results of the optimization experiment are shown in Figure 70. These results confirm the gradual decrease in B2 binding activity at basic pH.

I. Purification of Pegylated sB4 Species

Biotinylated sB4 protein was concentrated up to final concentration of 2 mg/ml using a 30kDa MWCO ultra membrane. Sample was dialyzed O/N against coupling buffer: 30mM phosphate, 75mM NaCl, pH 8.00. Coupling to PEG was performed at 4°C for 18 hours in 10 fold molar excess of reactive PEG. Double modified (PEGylated Biotinylated) sB4 was used on ion-exchange chromatography to separate non-PEGylated, mono-PEGylated and poly-PEGylated proteins from each other. Sample was dialyzed for O/N against 20 mM Na-Acetate, 20 mM NaCl, pH 5.5 and loaded onto 2 ml of SP-Sepharose. After washing with 10 ml of buffer, absorbed proteins were separated by gradual elution of NaCl: 3 ml/fraction and increment of 25 mM NaCl. Obtained fractions were analyzed by PAGE as shown in Figure 71. Fractions 1, 2 and 3 were found to correspond to polypegylated, monopegylated and unpegylated biotinylated sB4.

J. *In vitro* properties of PEGylated EphB4 derivatives

Fractions 1, 2 and 3 of biotinylated and PEGylated sB4 from the SP column purification, corresponding to polypegylated, monopegylated and unpegylated biotinylated sB4, were tested for their ability to bind EphrinB2 using the standard assay. Results of this experiment are shown on Figure 72. The order of binding activity was found to be Unpegylated > monopegylated > polypegylated.

The fractions were also tested for their stability *in vitro*. The fractions were tested for retention of EphrinB2 binding activity after incubation in mouse serum at 37°C for three days. The results of this experiment are shown in Figure 73. The order of *in vitro* stability was found to be monopegylated > unpegylated > polypegylated.

K. In vivo stability analysis of PEGylated derivatives of EphB4 ectodomain in mice

Fractions 1, 2 and 3 of biotinylated and PEGylated sB4 from the SP column purification, corresponding to polypegylated, monopegylated and unpegylated biotinylated sB4, were introduced by intravenous injection into mice in the amount of 0.5 nMoles/mouse. Blood from each mouse was taken in time frame of 10 min, 1, 2 and 3 days. 10 ml of obtained serum was used in binding assay with Ephrin-B2-Alkaline Phosphatase fusion protein and Streptavidin-coated magnetic beads as a solid phase. Signals, obtained at 10 min were taken as 100%. The two mice for each protein were of a different strain.

Results are shown in Figure 74. Pegylation was found to increase the stability of EphB4 *in vivo* relative to unpegylated EphB4.

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.

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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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. An isolated soluble polypeptide comprising an amino acid sequence of an extracellular domain of an EphB4 protein fused to an albumin protein or a fragment thereof, wherein the polypeptide is a monomer and binds specifically to an Ephrin B2 polypeptide, and has an *in vivo* serum half-life at least 50% greater than that of the extracellular domain of the EphB4 alone, and wherein the polypeptide inhibits signaling that results from the interaction between EphB4 and EphrinB2.

2. The polypeptide of claim 1, comprising a globular domain of an EphB4 protein or a sequence that is at least 90% identical to a globular domain of EphB4.

3. The polypeptide of claim 1, comprising a sequence at least 90% identical to residues 29-197 of the amino acid sequence defined by Figure 65 (SEQ ID NO:10).

4. The polypeptide of claim 1, comprising a sequence at least 90% identical to residues 29-526 of the amino acid sequence defined by Figure 65 (SEQ ID NO:10).

5. The polypeptide of claim 1, wherein said polypeptide has a serum half-life *in vivo* at least 100% greater than that of an unmodified EphB4 polypeptide.

6. The polypeptide of claim 1, wherein said albumin protein is selected from a human serum albumin (HSA) and bovine serum albumin (BSA).

7. The polypeptide of claim 1 or claim 6, wherein the albumin is a naturally occurring variant.

8. The polypeptide of any one of claims 1 to 7, wherein the polypeptide has one or more activities selected from:

(a) inhibition of EphrinB2 activity;

(b) inhibition of EphrinB2 kinase activity;

(c) inhibition of EphB4 kinase activity;

(d) inhibition of clustering of Ephrin B2; and

(e) inhibition of clustering of EphB4.

9. A pharmaceutical composition comprising a polypeptide of any one of claims 1 to 8, and a pharmaceutically acceptable carrier.

10. Use of a polypeptide of any one of claims 1 to 8 for preparing a medicament for inhibiting signaling through Ephrin B2/EphB4 pathway.

11. Use of a polypeptide of any one of claims 1 to 8 for preparing a medicament for reducing the growth rate of the tumor.

12. Use of a polypeptide of any one of claims 1 to 8 for preparing a medicament for treating cancer in a patient.

13. Use of a polypeptide of any one of claims 1 to 8 for preparing a medicament for inhibiting angiogenesis in a patient.

14. Use of a polypeptide of any one of claims 1 to 8 for preparing a medicament for treating a patient suffering from an angiogenesis-associated disease.

15. The polypeptide of any one of claims 1 to 8, wherein the polypeptide comprises one or more modified amino acid residues.

16. A cosmetic composition comprising the polypeptide of any of claims 1 to 8 or 15, and a pharmaceutically acceptable carrier.

17. The use of claim 12, wherein the cancer comprises cancer cells expressing EphrinB2 and/or EphB4 at a higher level than noncancerous cells of a comparable tissue.

18. The use of claim 12 or claim 17, wherein the cancer is metastatic cancer.

19. The use of any one of claims 12, 17 or 18, wherein the cancer is selected from the group consisting of colon carcinoma, breast tumor, mesothelioma, prostate tumor, squamous cell carcinoma, Kaposi sarcoma, and leukemia.

20. The use of any one of claims 12 or 17 to 19, wherein the cancer is an angiogenesis-dependent cancer.

21. The use of any one of claims 12 or 17 to 19, wherein the cancer is an angiogenesis-independent cancer.

22. A method of inhibiting signaling through Ephrin B2/EphB4 pathway comprising administering a polypeptide of any one of claims 1 to 8.

23. A method of reducing the growth rate of a tumor comprising administering a polypeptide of any one of claims 1 to 8.

24. A method of treating cancer in a patient comprising administering a polypeptide of any one of claims 1 to 8.

25. A method of inhibiting angiogenesis in a patient comprising administering a polypeptide of any one of claims 1 to 8.

26. A method of treating a patient suffering from an angiogenesis-associated disease comprising administering a polypeptide of any one of claims 1 to 8.

27. An isolated soluble polypeptide according to claim 1; a pharmaceutical composition according to claim 9; use according to any one of claims 10-14; a cosmetic composition according to claim 16; or a method according to any one of claims 22-26, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.

Amino acid sequence of the B4ECv3 protein

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

Fig. 1

Amino acid sequence of the B4ECv3NT protein

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEELSGL
DEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATLRFTMLE
CLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPYIKVDTVAAE
HLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQDQGACMALLSLHL
FYKKCAQLTVNLTRFPETVPRELVVPVAGSCVVDAVPAPGPSPSLYCR
EDGQWAEQPVTGCSCAPGFEAAEGNTKCRACAQGTFKPLSGEGSCQPC
PANSHSNTIGSAVCQCRVGYFRARTDPRGAPCTTPPSAPRSVVSRLNG
SSLHLEWSAPLESGGREDLTYALRCRECRPGGSCAPCGGDLTFDPGPR
DLVEPWVVVRGLRPDFTYTFEVTALNGVSSLATGPVPFEPVNVTTDRE
VPPAVSDIRVTRSSPSSLSLAWAVPRAPSGAWLDYEVKYHEKGAEGPS
SVRFLKTSENRAELRGLKRGASYLVQVRARSEAGYGPFGQEHHSQTQL
DESEGWREQGSKRAILQISSTVAAARV

Fig. 2

Amino acid sequence of the B2EC protein

MAVRRDSVWKYCWGVLMVLCRTAISKSIVLEPIYWNSSNSKFLPGQGL
VLYPQIGDKLDIICPKVDSKTVGQYEYYKVYMVDKDQADRCTIKKENT
PLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIISTSNGSLEG
LDNQEGGVCQTRAMKILMKVGQDASSAGSTRNKDPTRRPELEAGTNGR
SSTTSPFVKPNPGSSTDGNSAGHSGNNILGSEVGSHHHHHH

Fig. 3

Amino acid sequence of the B4ECv3-FC protein

MELRVLLCWASLAAALEETLLNTKLETADLKWVTFPQVDGQWEEL
SGLDEEQHSVRTYEVCEVQRAPGQAHWLRTGWVPRRGAVHVYATL
RFTMLECLSLPRAGRSCKETFTVFYYESDADTATALTPAWMENPY
IKVDTVAAEHLTRKRPGAEATGKVNVKTLRLGPLSKAGFYLAFQD
QGACMALLSLHLFYKKCAQLTVNLTRFPETVPRELVVPVAGSCVV
DAVPAPGPSPSLYCREDGQWAEQPVTGCSCAPGFEAAEGNTKCRA
CAQGTFKPLSGEGSCQPCPANSHSNTIGSAVCQCRVGYFRARTDP
RGAPCTTPPSAPRSVVSRLNGSSLHLEWSAPLESGGREDLTYALR
CRECRPGGSCAPCGGDLTFDPGPRDLVEPWVVVRGLRPDFTYTFE
VTALNGVSSLATGPVPFEPVNVTTDREVPPAVSDIRVTRSSPSSL
SLAWAVPRAPSGAWLDYEVKYHEKGAEGPSSVRFLKTSENRAELR
GLKRGASYLVQVRARSEAGYGPFGQEHHSQTQLDESEGWREQDPE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL
PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

Fig. 4

Amino acid sequence of the B2EC-FC protein

MAVRRDSVWKYCWGVLMVLCRTAISKSIVLEPIYWNSSNSKFLPGQ
GLVLYPQIGDKLDIICPKVDSKTVGQYEYYKVYMVDKDQADRCTIK
KENTPLLNCAKPDQDIKFTIKFQEFSPNLWGLEFQKNKDYYIISTS
NGSLEGLDNQEGGVCQTRAMKILMKVGQDASSAGSTRNKDPTRRPE
LEAGTNGRSSTTSPFVKPNPGSSTDGNSAGHSGNNILGSEVDPEPK
SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fig. 5

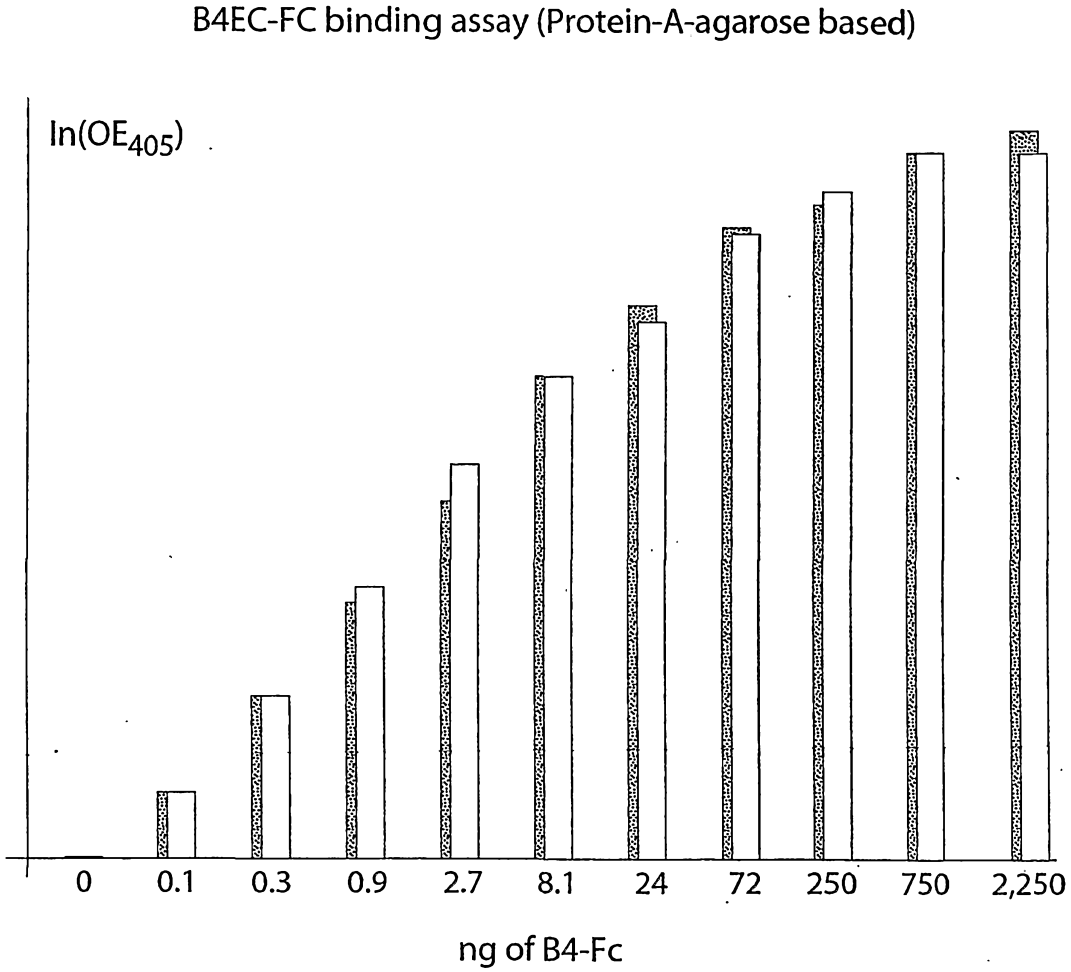

B4EC-FC binding assay (Protein-A-agarose based)
ln(OE405)
0
0.1
0.3
0.9
2.7
8.1
24
72
250
750
2,250
ng of B4-Fc

Fig. 6

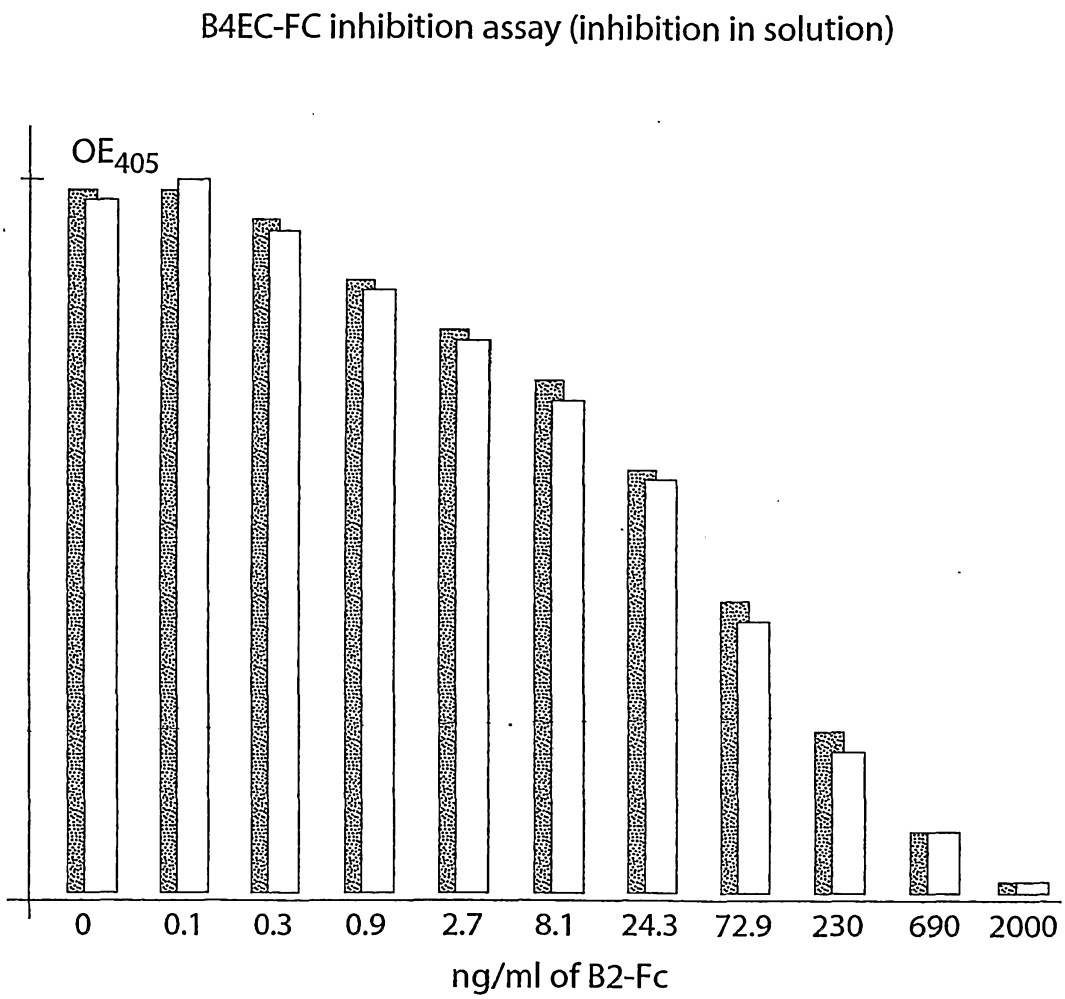

B4EC-FC inhibition assay (inhibition in solution)
OE405
0
0.1
0.3
0.9
2.7
8.1
24.3
72.9
230
690
2000
ng/ml of B2-Fc

Fig. 7

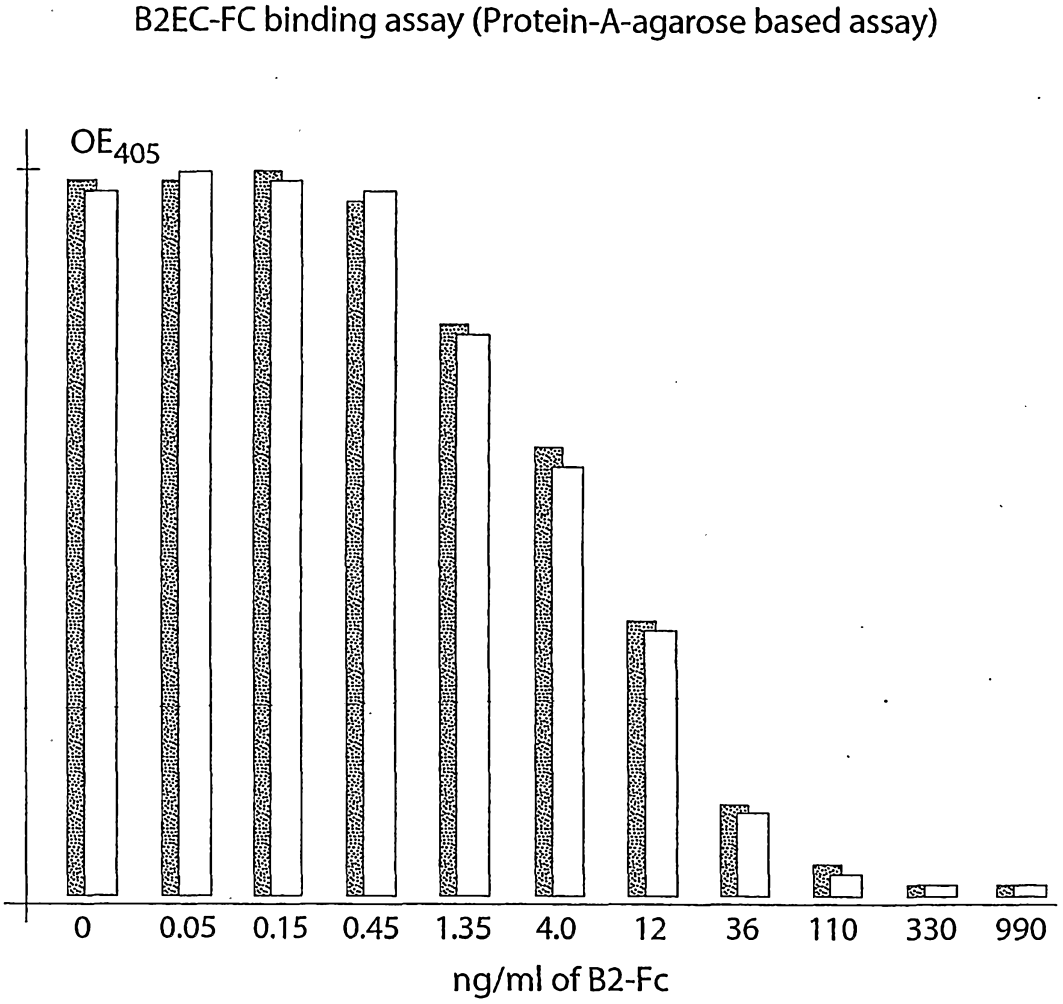

B2EC-FC binding assay (Protein-A-agarose based assay)
OE405
0
0.05
0.15
0.45
1.35
4.0
12
36
110
330
990
ng/ml of B2-Fc

Fig. 8

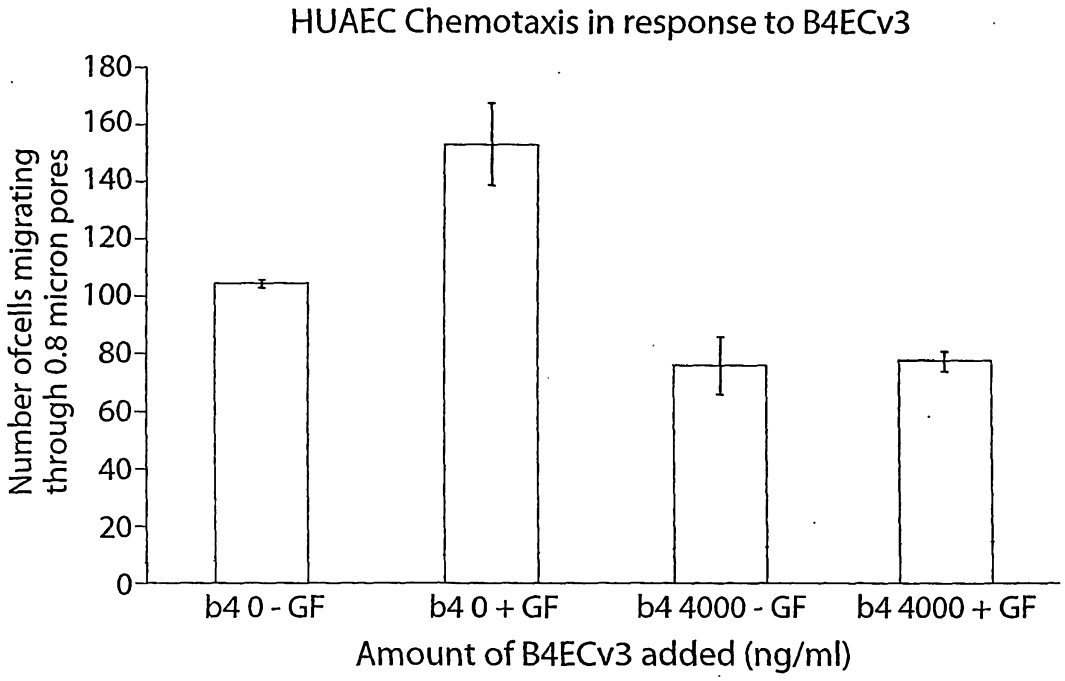

HUAEC Chemotaxis in response to B4ECv3
Number ofcells migrating through 0.8 micron pores
180
160
140
120
100
80
60
40
20
0
b4 0 - GF
b4 0 + GF
b4 4000 - GF
b4 4000 + GF
Amount of B4ECv3 added (ng/ml)

Fig. 9

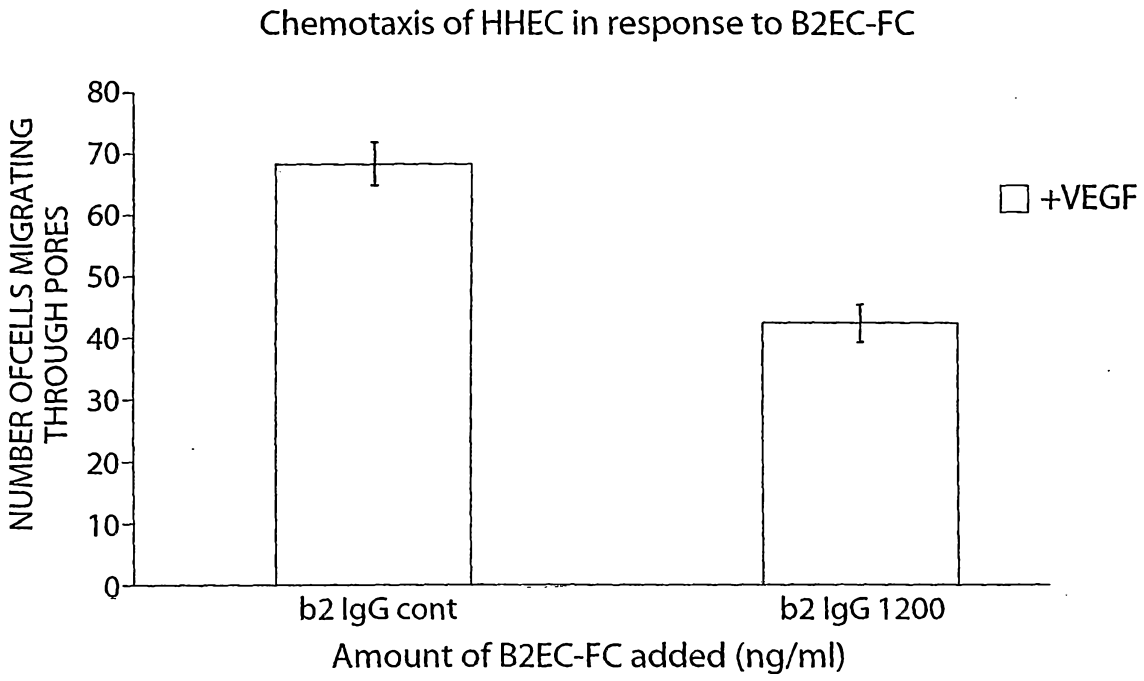

Chemotaxis of HHEC in response to B2EC-FC
NUMBER OFCELLS MIGRATING THROUGH PORES
80
70
60
50
40
30
20
10
0
+VEGF
b2 IgG cont
b2 IgG 1200
Amount of B2EC-FC added (ng/ml)

Fig. 10

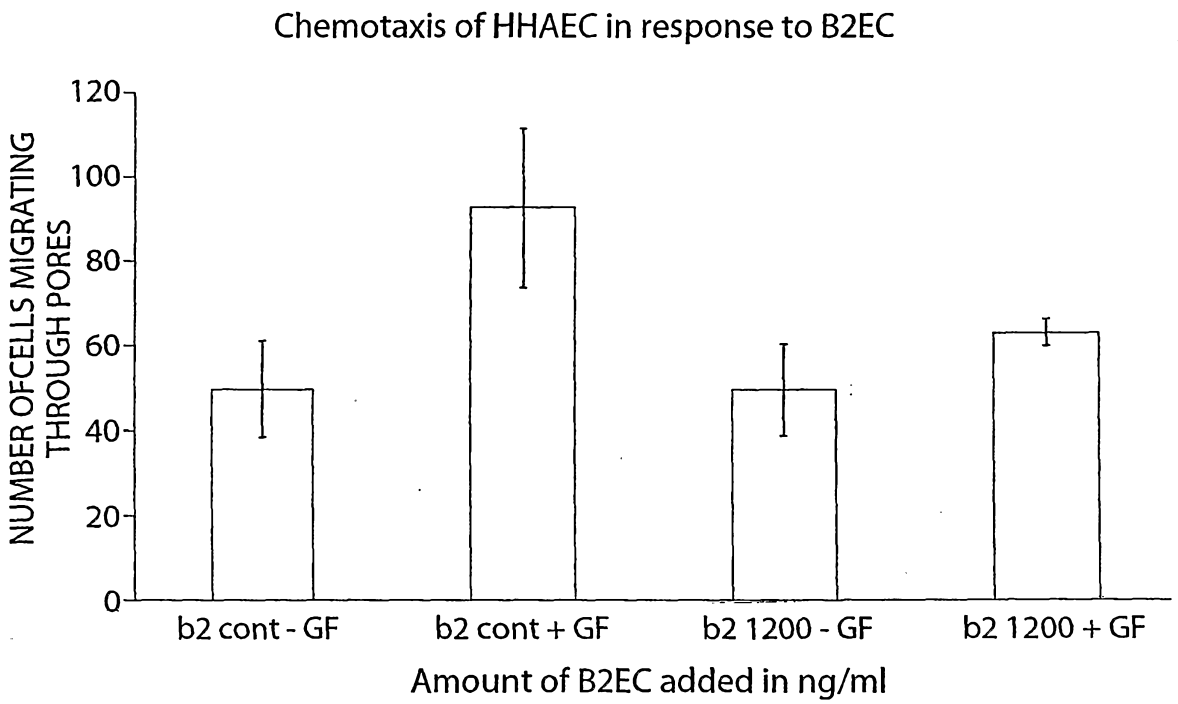

Chemotaxis of HHAEC in response to B2EC
NUMBER OFCELLS MIGRATING THROUGH PORES
120
100
80
60
40
20
0
b2 cont - GF
b2 cont + GF
b2 1200 - GF
b2 1200 + GF
Amount of B2EC added in ng/ml

Fig. 11

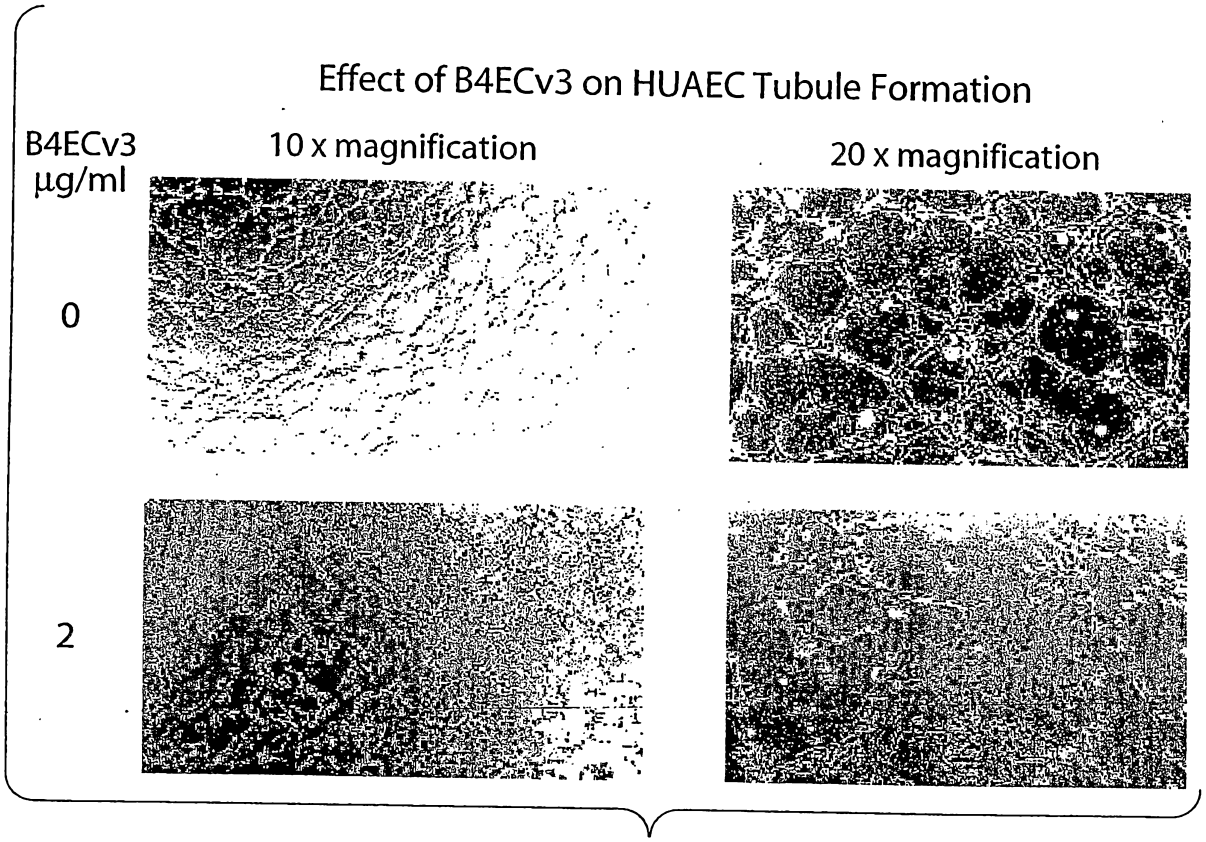

Effect of B4ECv3 on HUAEC Tubule Formation
B4ECv3 µg/ml
10 x magnification
20 x magnification
0
2

Fig. 12

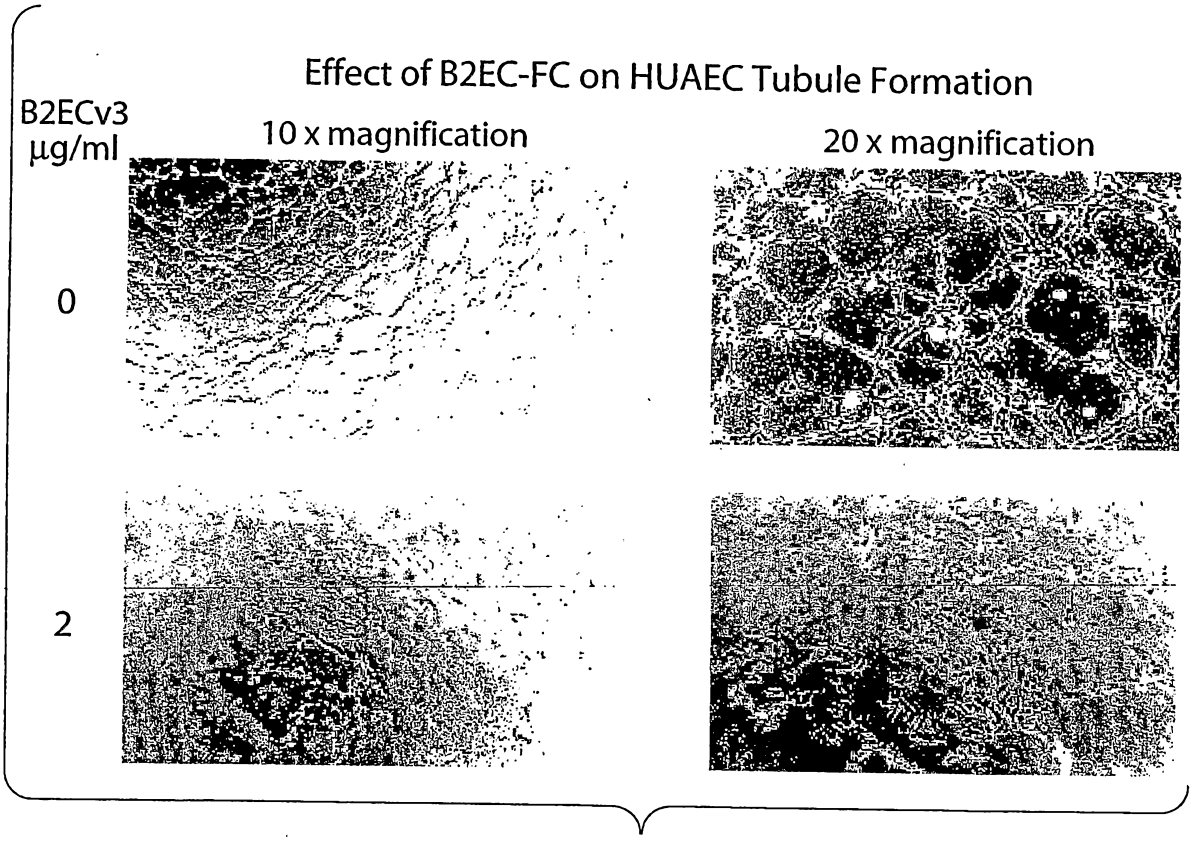

Effect of B2EC-FC on HUAEC Tubule Formation
B2ECv3
µg/ml
10 x magnification
20 x magnification
0
2

Fig. 13

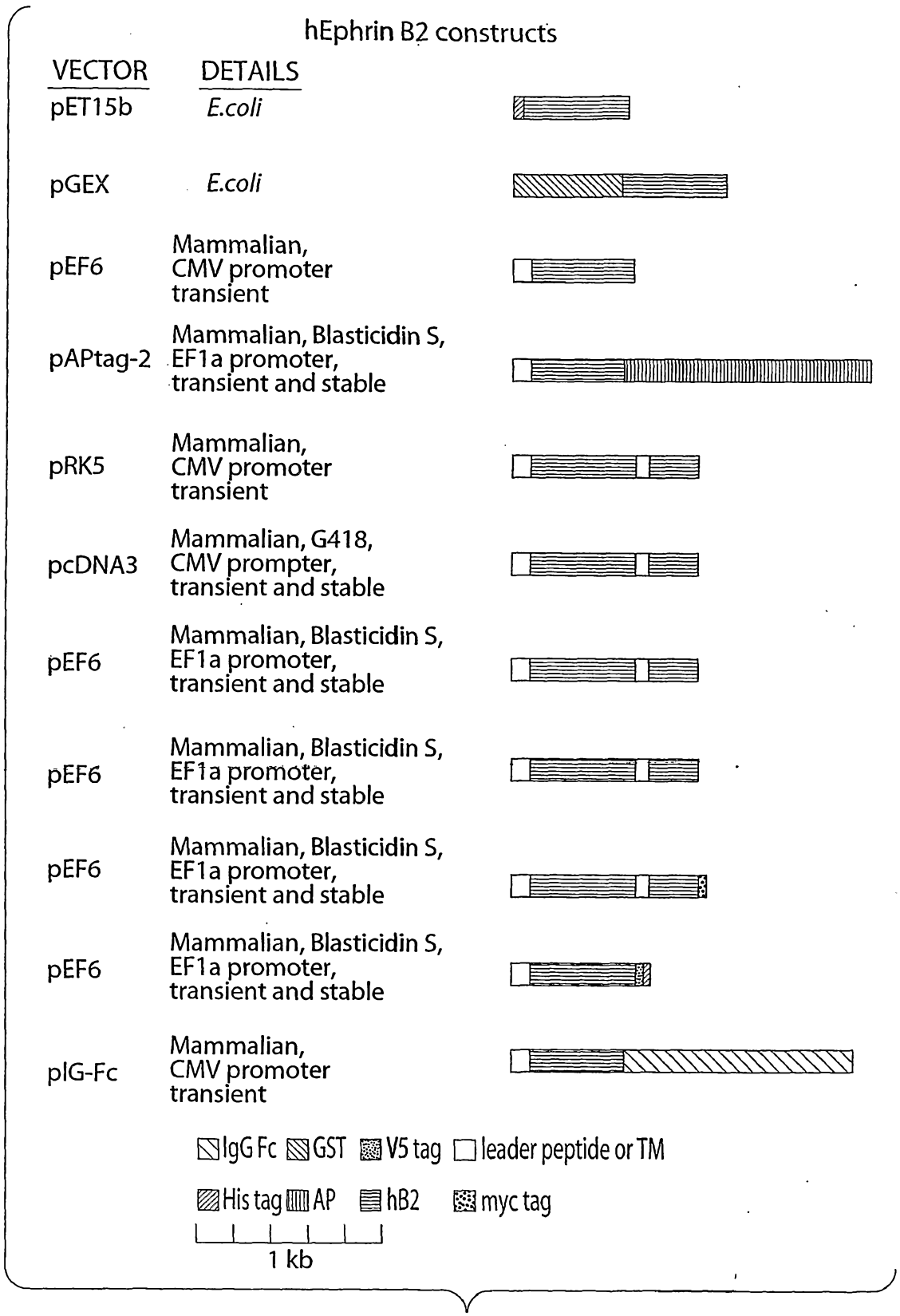

hEphrin B2 constructs
VECTOR
DETAILS
pET15b
E.coli
pGEX
E.coli
pEF6
Mammalian, CMV promoter transient
pAPtag-2
Mammalian, Blasticidin S, EF1a promoter, transient and stable
pRK5
Mammalian, CMV promoter transient
pcDNA3
Mammalian, G418, CMV prompter, transient and stable
pEF6
Mammalian, Blasticidin S, EF1a promoter, transient and stable
pEF6
Mammalian, Blasticidin S, EF1a promoter, transient and stable
pEF6
Mammalian, Blasticidin S, EF1a promoter, transient and stable
pEF6
Mammalian, Blasticidin S, EF1a promoter, transient and stable
pIG-Fc
Mammalian, CMV promoter transient
IgG Fc
GST
V5 tag
leader peptide or TM
His tag
AP
hB2
myc tag
1 kb

Fig. 14

hEph B4 constructs

VECTOR	DETAILS
pET15b	*E.coli*
pET15b	*E.coli*
pGEX	*E.coli*
pGEX	*E.coli*
pEF6	Mammalian, Blasticidin S, EF1a promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pAPtag-2	Mammalian, CMV promoter
pAPtag-2	Mammalian, CMV promoter
pAPtag-2	Mammalian, CMV promoter
pRK5	Mammalian, CMV promoter
pcDNA3	Mammalian, G418 CMV promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pEF6	Mammalian, Blasticidin S, EF1a promoter
pIG-Fc	Mammalian, CMV promoter transient
pIG-Fc	Mammalian, CMV promoter transient

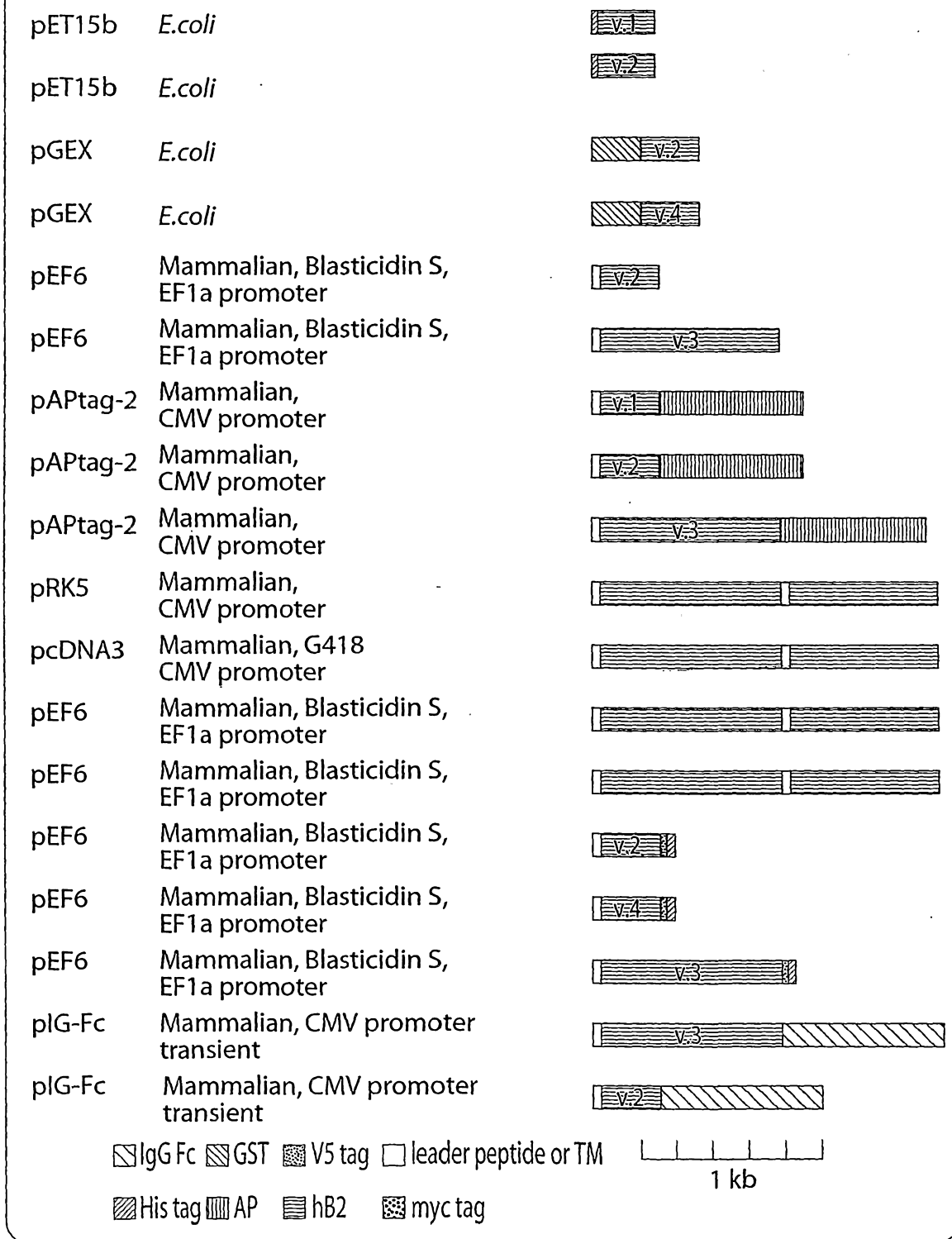


Fig. 15

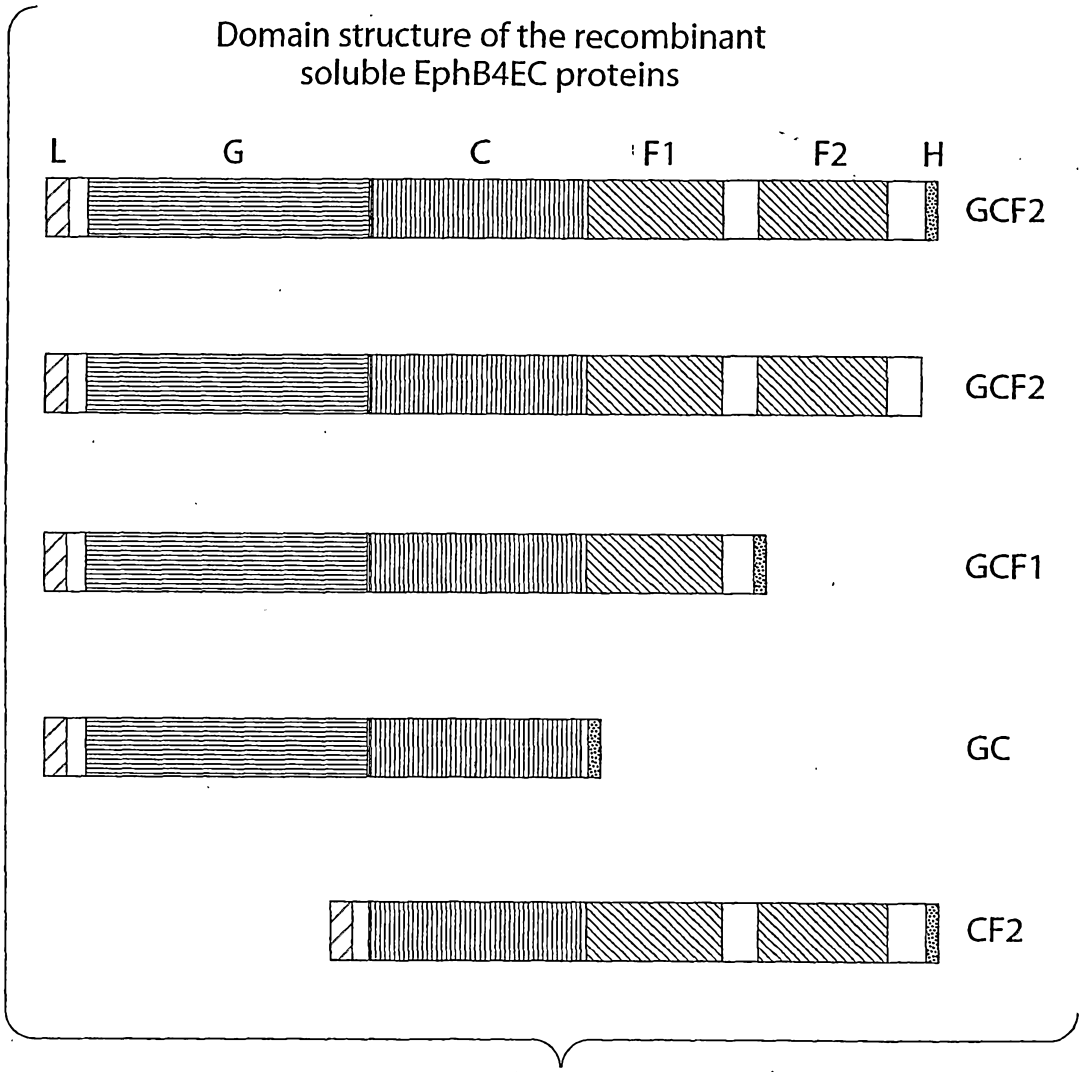

Domain structure of the recombinant soluble EphB4EC proteins
L
G
C
F1
F2
H
GCF2
GCF2
GCF1
GC
CF2

Fig. 16

Purification and ligand binding properties of the EphB4EC proteins

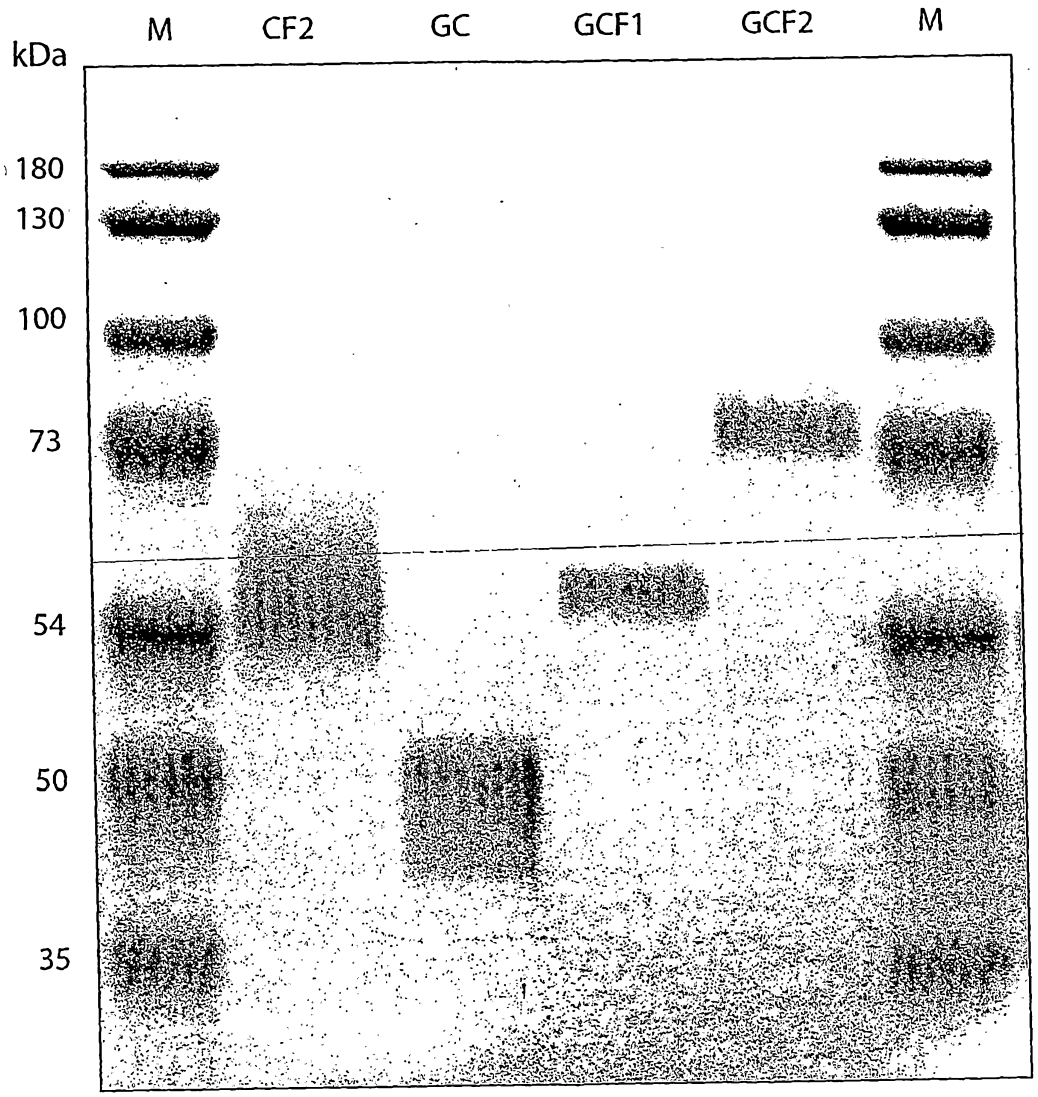


Fig. 17A

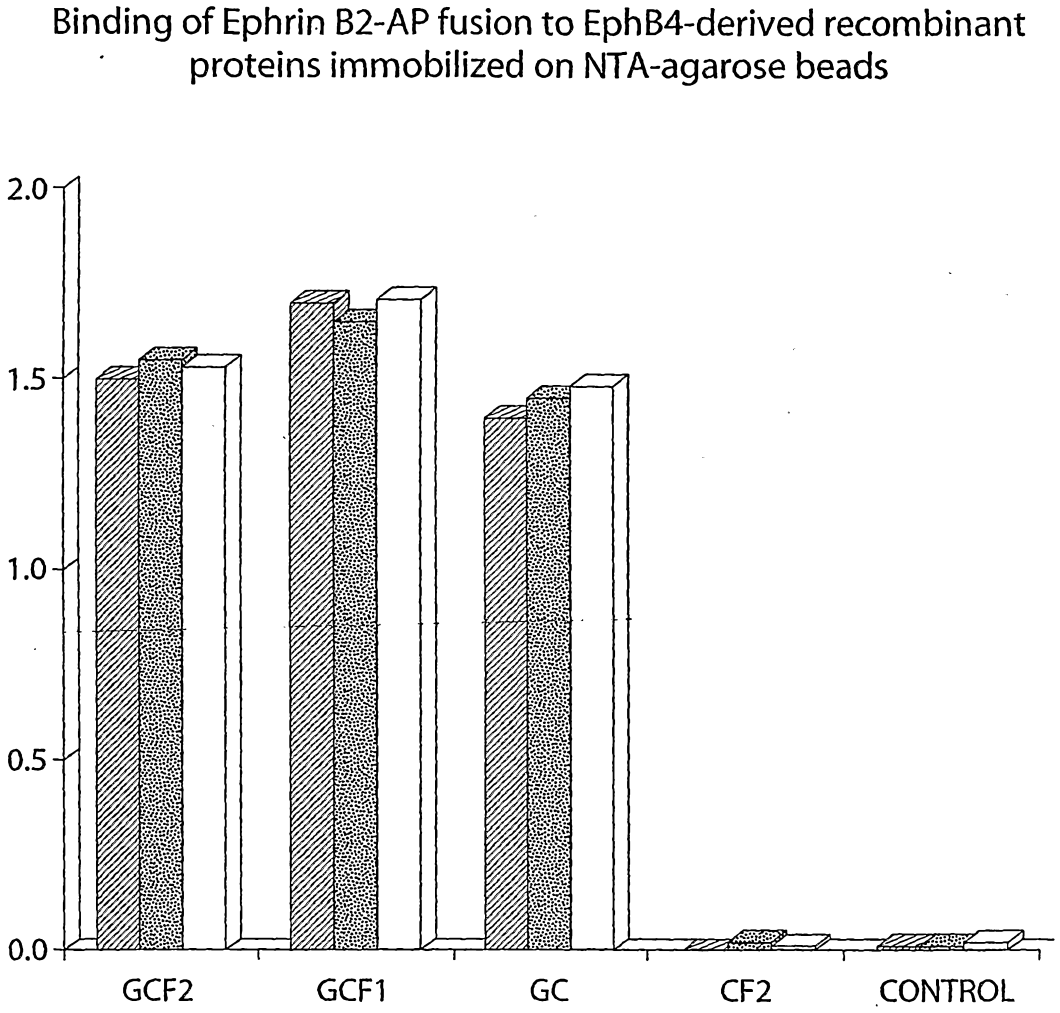

Binding of Ephrin B2-AP fusion to EphB4-derived recombinant proteins immobilized on NTA-agarose beads
2.0
1.5
1.0
0.5
0.0
GCF2
GCF1
GC
CF2
CONTROL

Fig. 17B

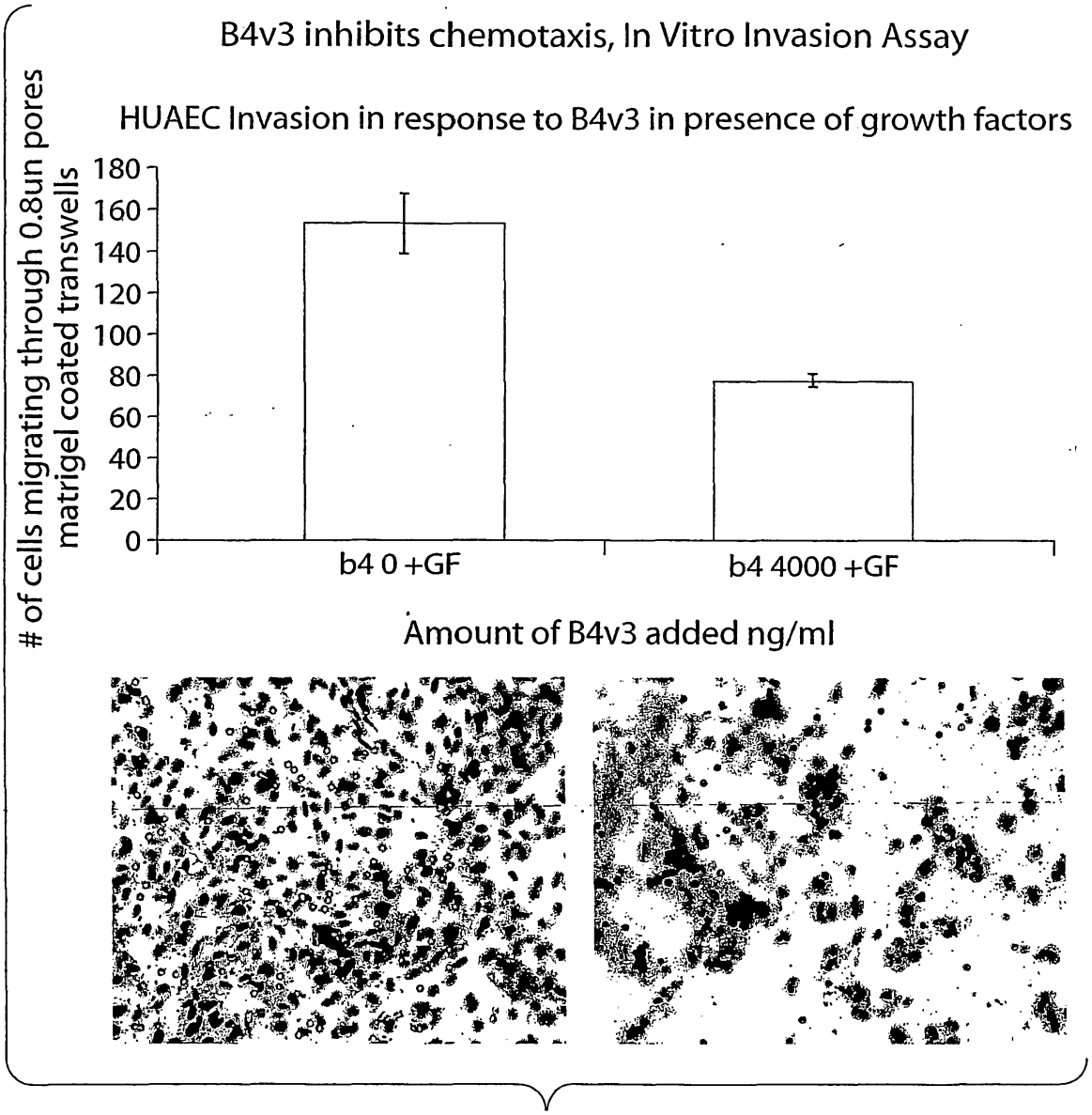

B4v3 inhibits chemotaxis, In Vitro Invasion Assay
HUAEC Invasion in response to B4v3 in presence of growth factors
# of cells migrating through 0.8un pores matrigel coated transwells
180
160
140
120
100
80
60
40
20
0
b4 0 +GF
b4 4000 +GF
Amount of B4v3 added ng/ml

Fig. 18

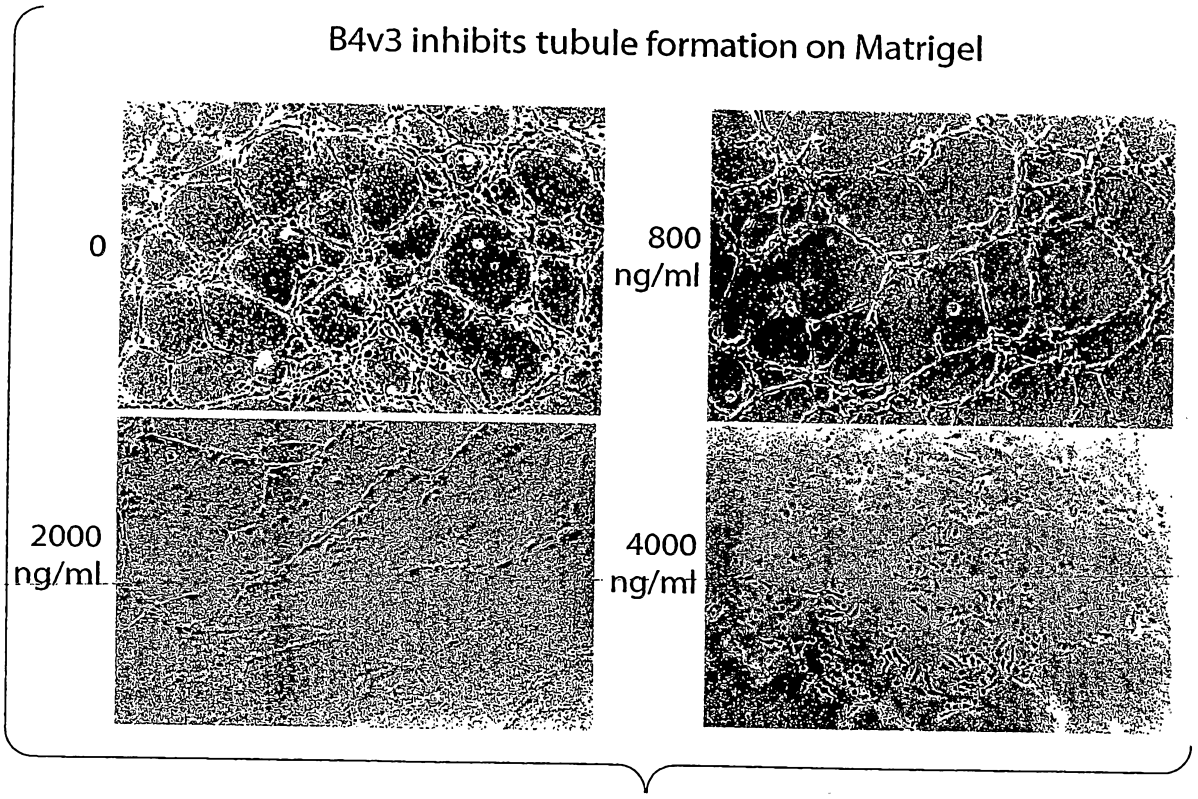

B4v3 inhibits tubule formation on Matrigel
0
800 ng/ml
2000 ng/ml
4000 ng/ml

Fig. 19A

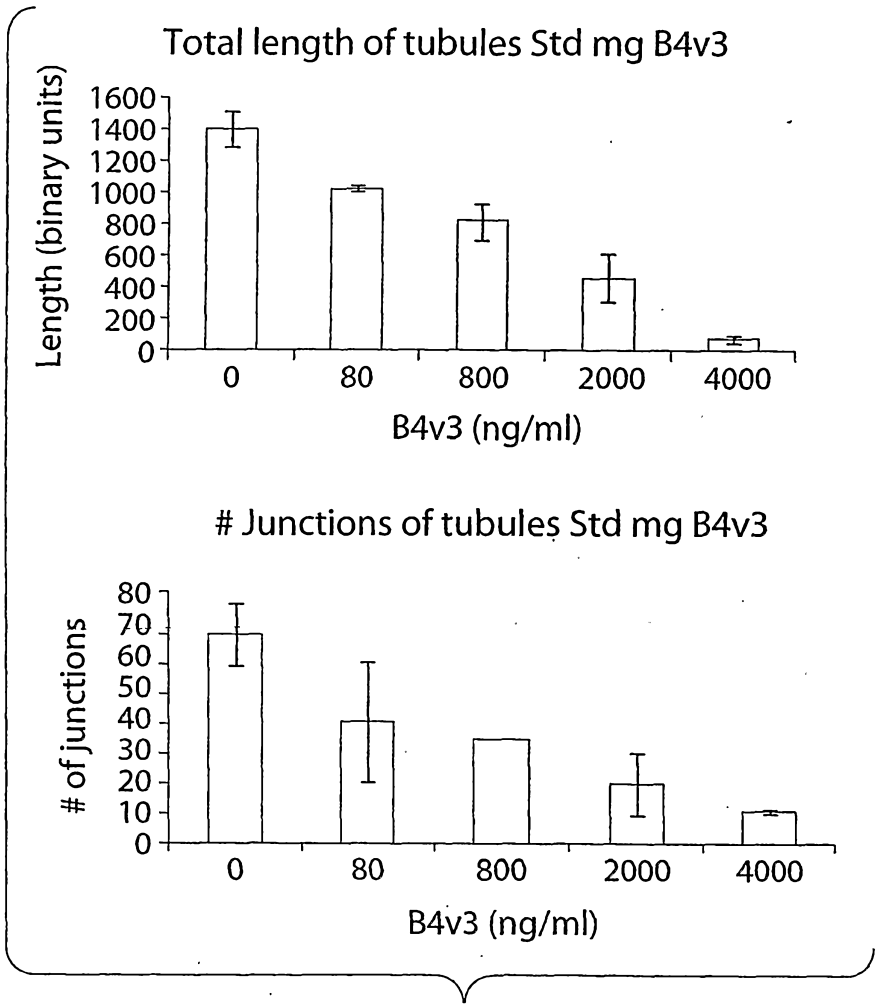

Total length of tubules Std mg B4v3
Length (binary units)
1600
1400
1200
1000
800
600
400
200
0
0
80
800
2000
4000
B4v3 (ng/ml)
Junctions of tubules Std mg B4v3
of junctions
80
70
60
50
40
30
20
10
0
0
80
800
2000
4000
B4v3 (ng/ml)

Fig. 19B

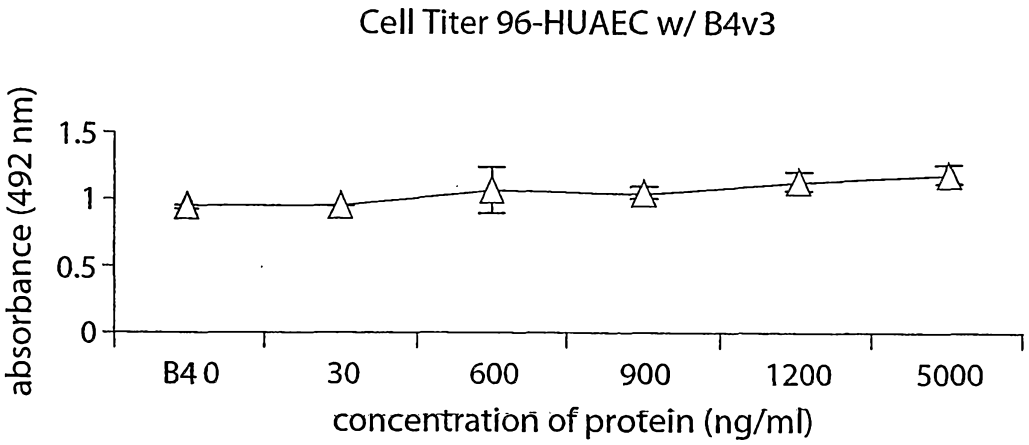

Cell Titer 96-HUAEC w/ B4v3
absorbance (492 nm)
1.5
1
0.5
0
B4 0
30
600
900
1200
5000
concentration of protein (ng/ml)

Fig. 20

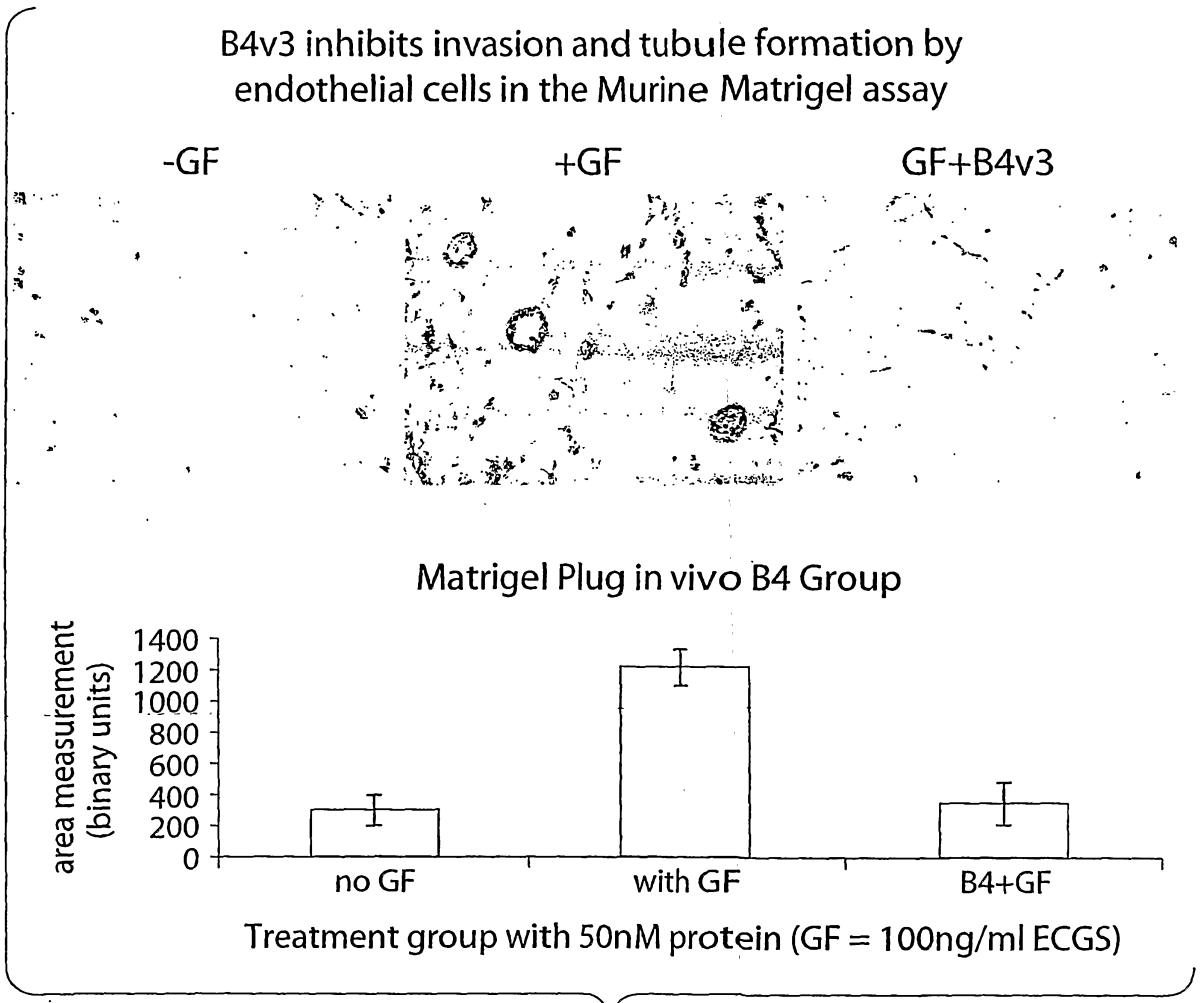

B4v3 inhibits invasion and tubule formation by endothelial cells in the Murine Matrigel assay
-GF
+GF
GF+B4v3
Matrigel Plug in vivo B4 Group
area measurement (binary units)
1400
1200
1000
800
600
400
200
0
no GF
with GF
B4+GF
Treatment group with 50nM protein (GF = 100ng/ml ECGS)

Fig. 21

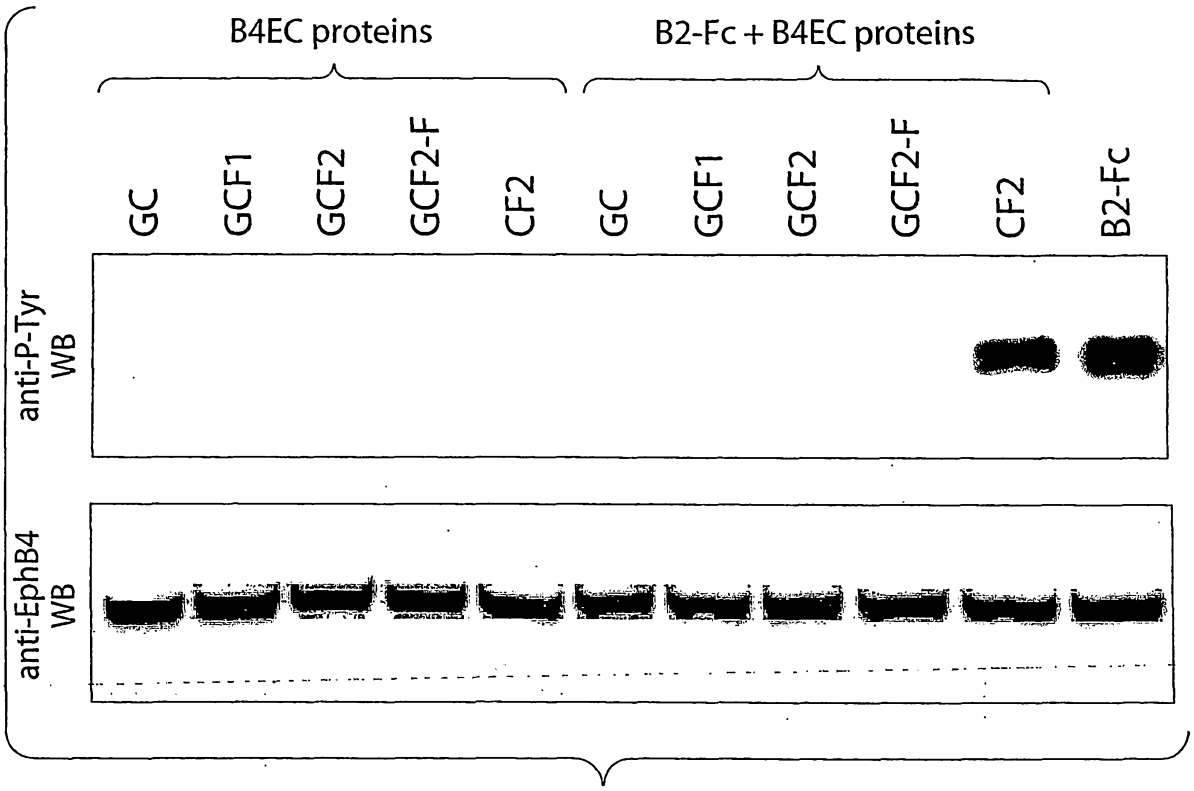

B4EC proteins
B2-Fc + B4EC proteins
GC
GCF1
GCF2
GCF2-F
CF2
GC
GCF1
GCF2
GCF2-F
CF2
B2-Fc
anti-P-Tyr WB
anti-EphB4 WB

Fig. 22

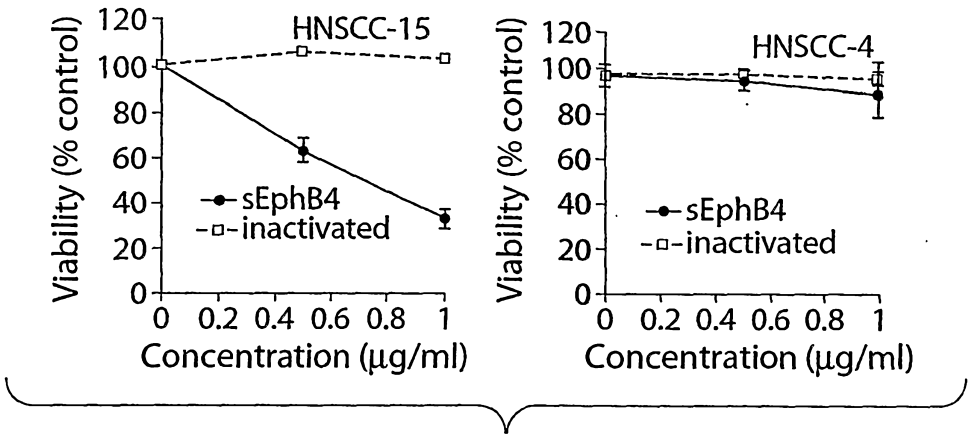


Fig. 23A

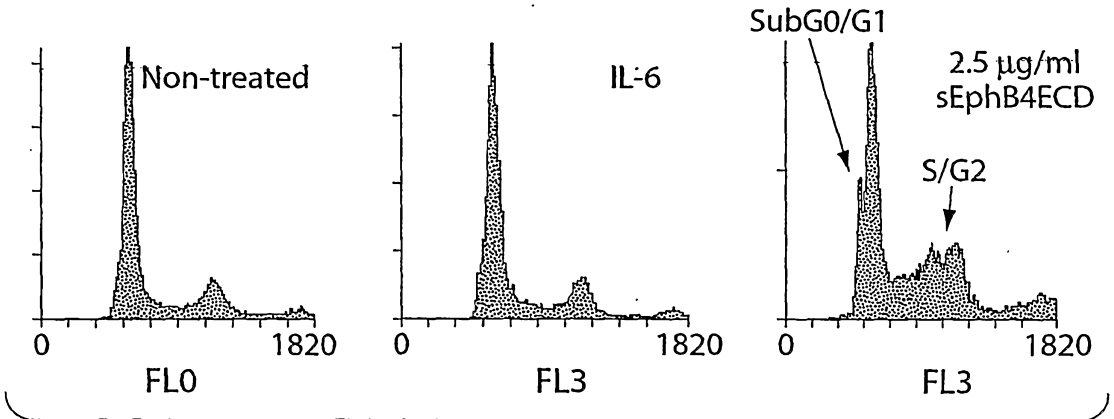


Fig. 23B

B4v3 inhibitis neovascular response in a murine corneal hydron micropocket assay

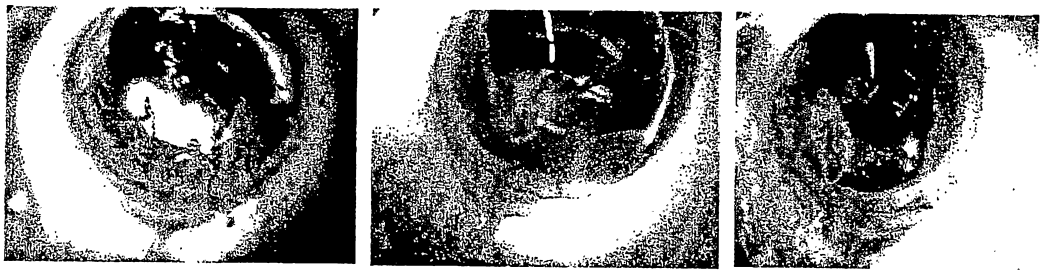
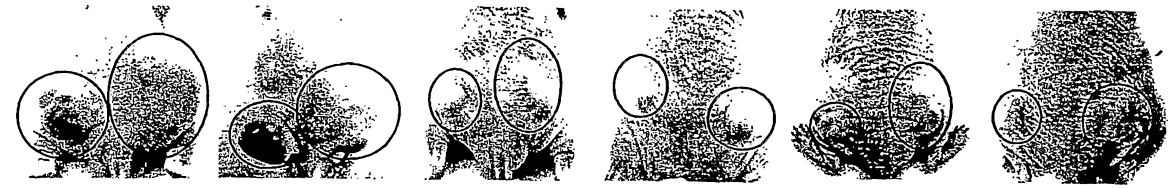
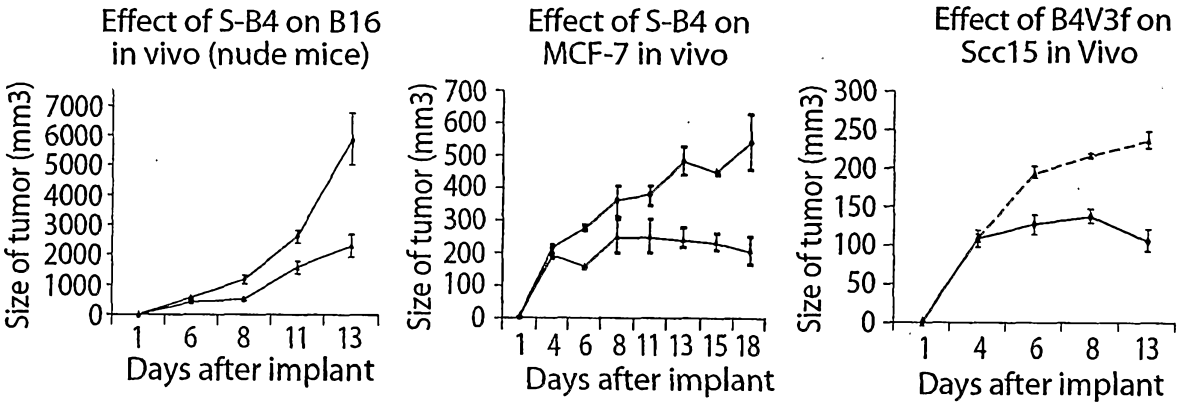


Fig. 24




Effect of S-B4 on B16 in vivo (nude mice)
Size of tumor (mm3)
7000
6000
5000
4000
3000
2000
1000
0
1 6 8 11 13
Days after implant
Effect of S-B4 on MCF-7 in vivo
Size of tumor (mm3)
700
600
500
400
300
200
100
0
1 4 6 8 11 13 15 18
Days after implant
Effect of B4V3f on Scc15 in Vivo
Size of tumor (mm3)
300
250
200
150
100
50
0
1 4 6 8 13
Days after implant

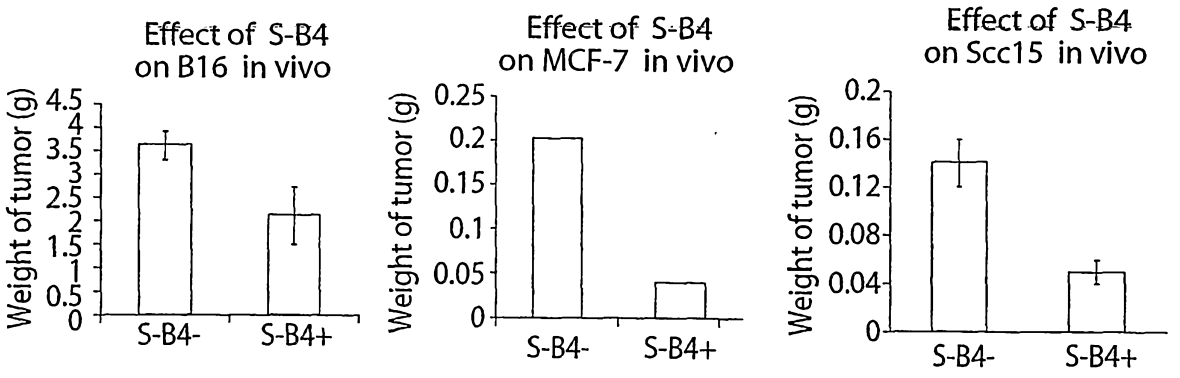

Effect of S-B4 on B16 in vivo
Weight of tumor (g)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
S-B4- S-B4+
Effect of S-B4 on MCF-7 in vivo
Weight of tumor (g)
0.25
0.2
0.15
0.1
0.05
0
S-B4- S-B4+
Effect of S-B4 on Scc15 in vivo
Weight of tumor (g)
0.2
0.16
0.12
0.08
0.04
0
S-B4- S-B4+

Fig. 25

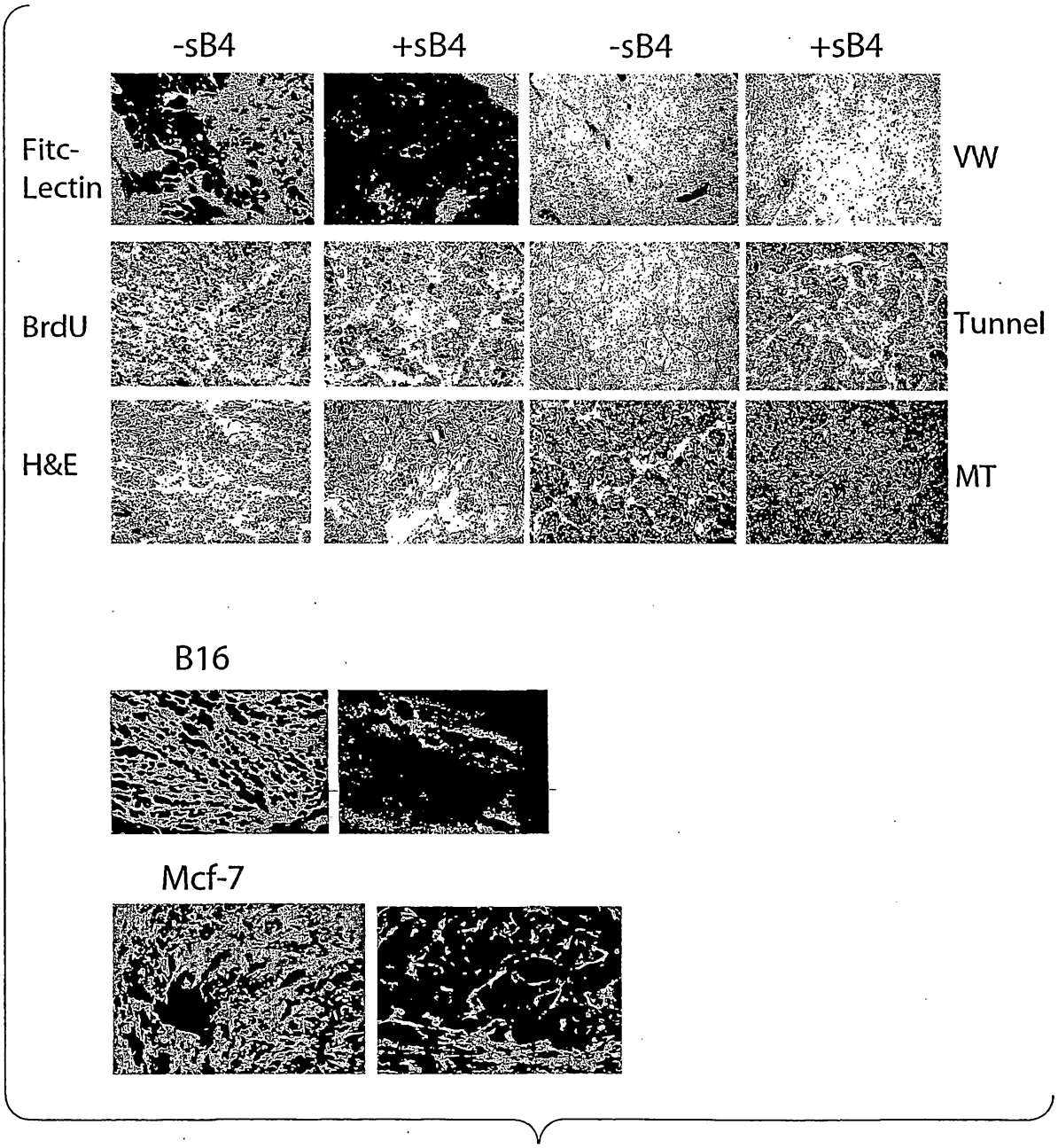


Fig. 26

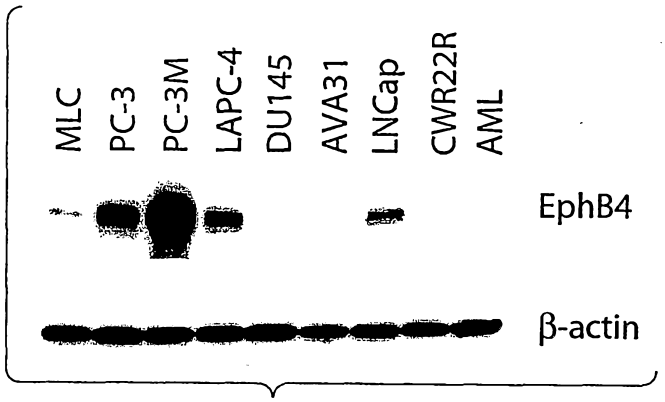


Fig. 27A

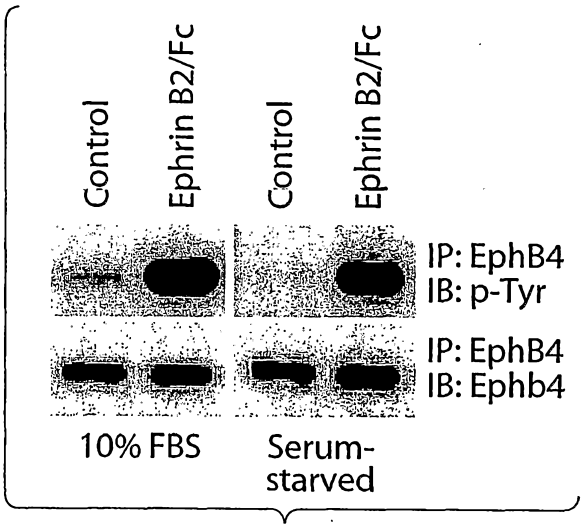
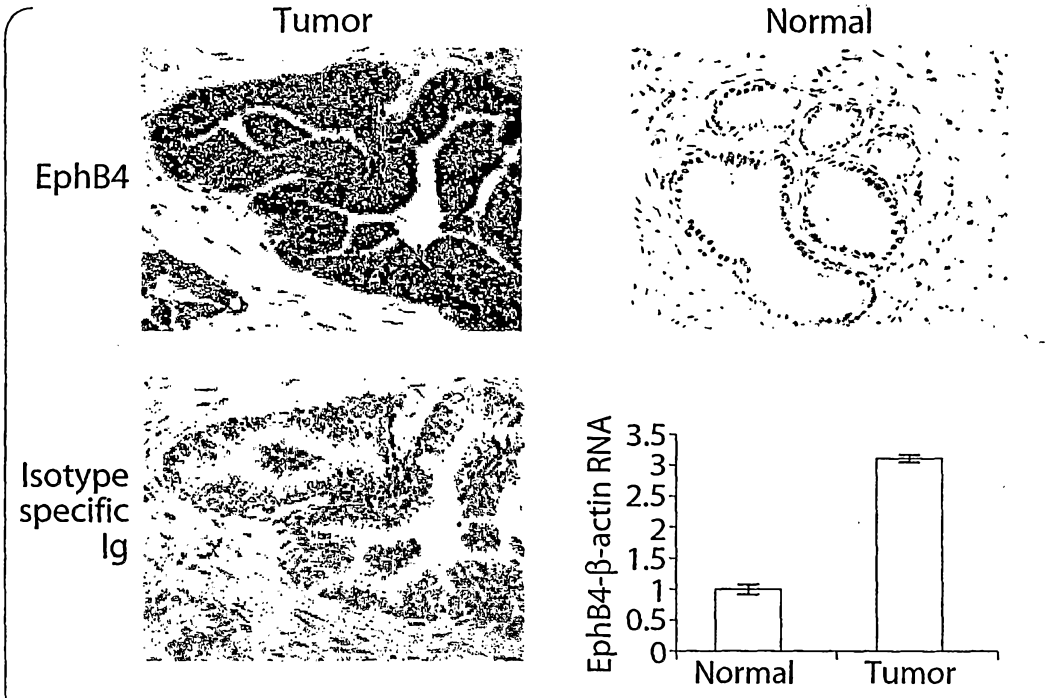


Fig. 27B



EPHB4 staining in prostate tissues array

	negative	positive
Normal (n = 20)	17	3
Tumor (n = 32)	8	24

$P = 3.8 \times 10^{-5}$ χ^2 analysis

Fig. 28

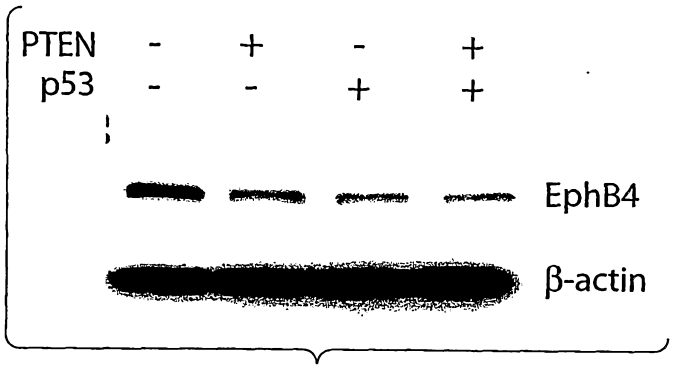


Fig. 29A

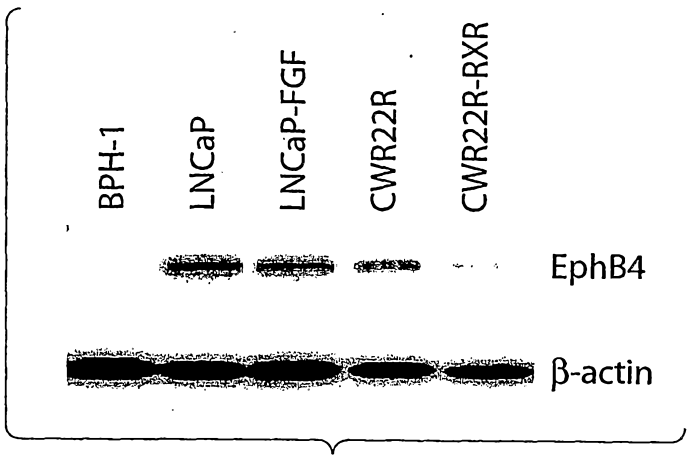


Fig. 29B

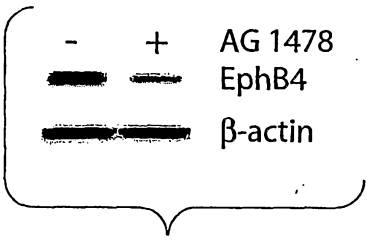


Fig. 30A

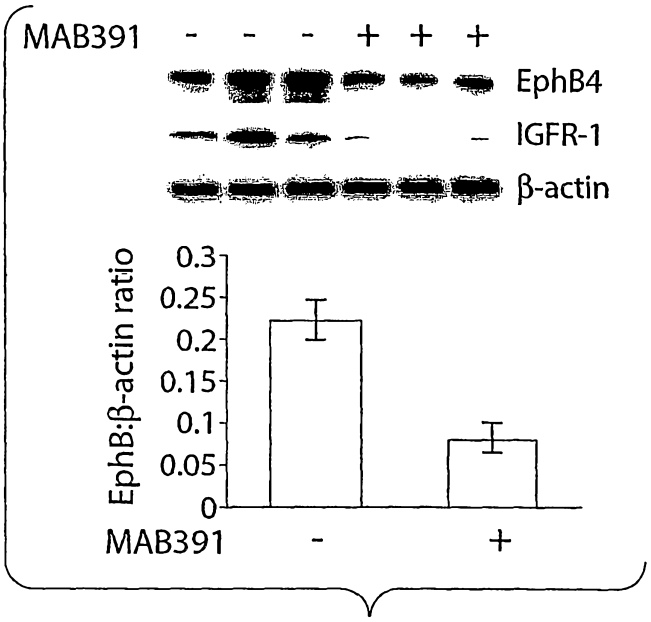


Fig. 30B

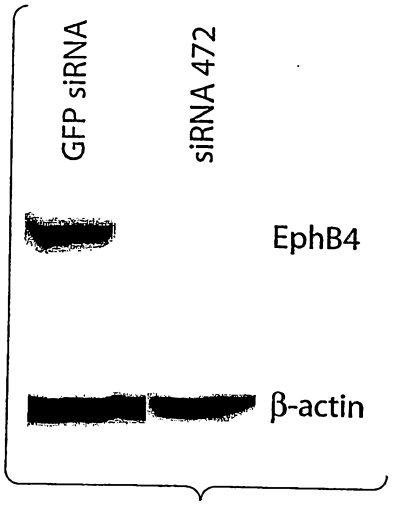


Fig. 31A

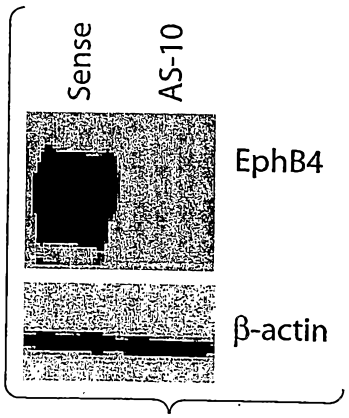


Fig. 31B

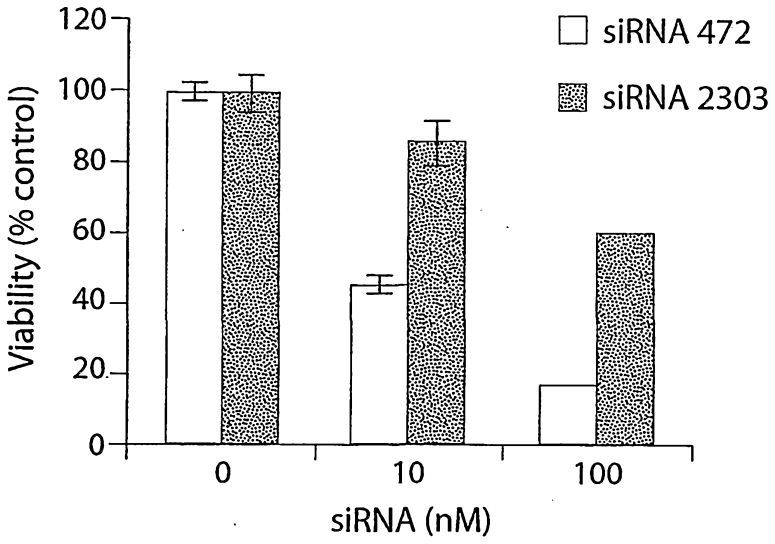


Fig. 31C

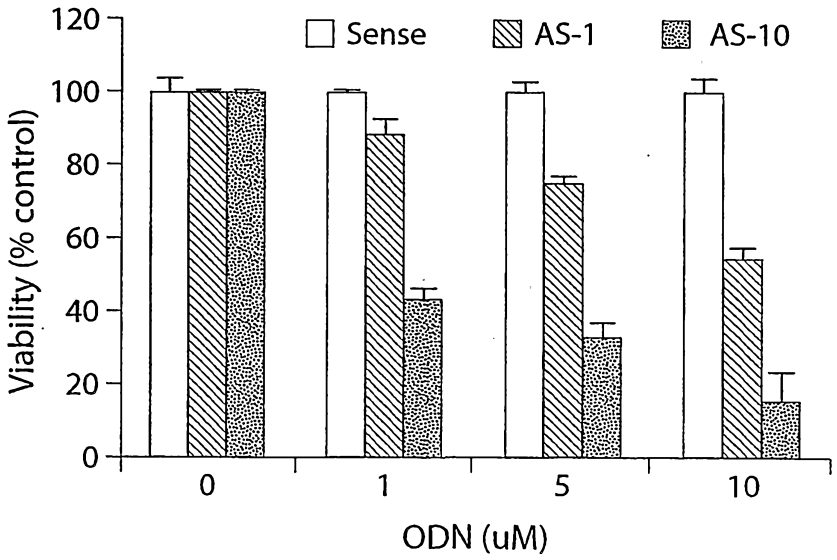


Fig. 31D

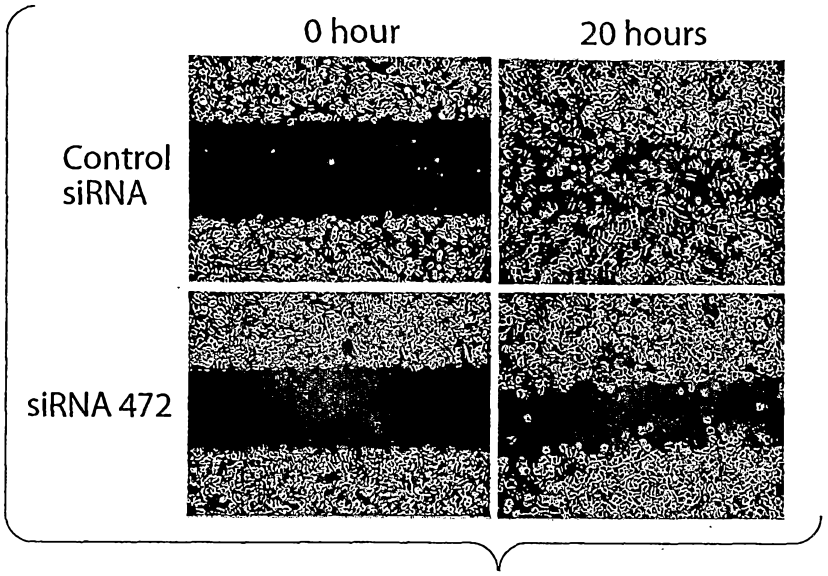


Fig. 31E

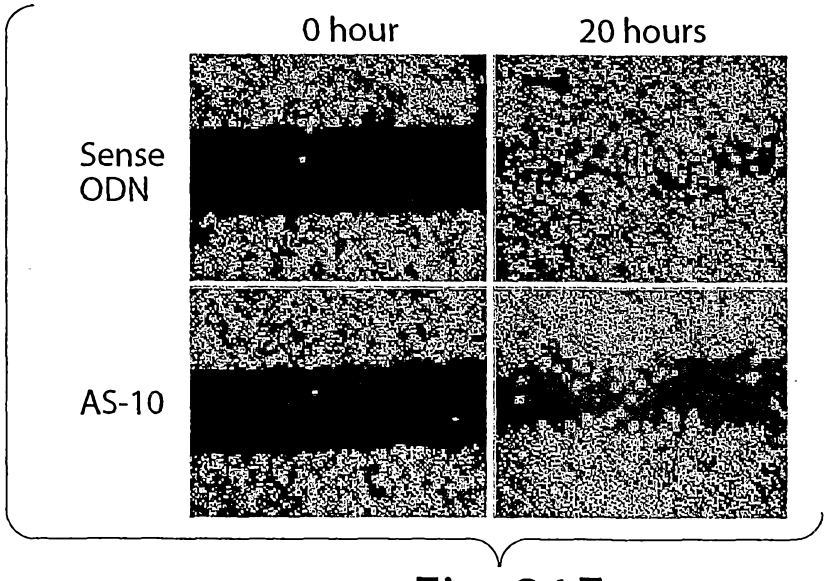


Fig. 31F

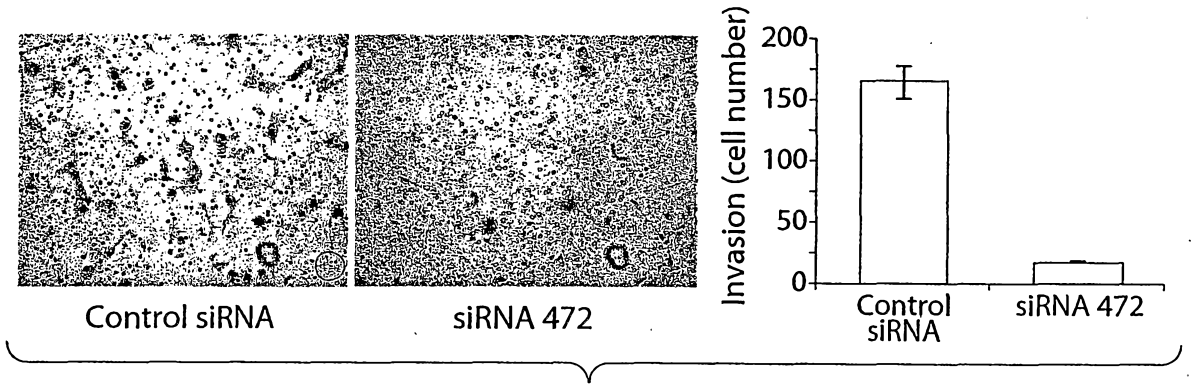

Control siRNA
siRNA 472
Invasion (cell number)
200
150
100
50
0
Control siRNA
siRNA 472

Fig. 31G

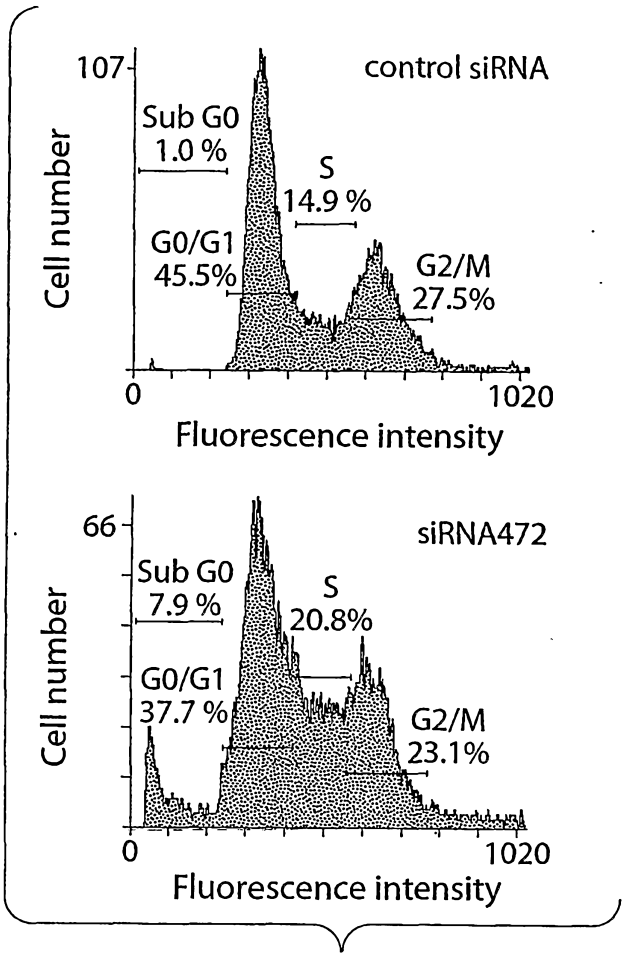


Fig. 32A

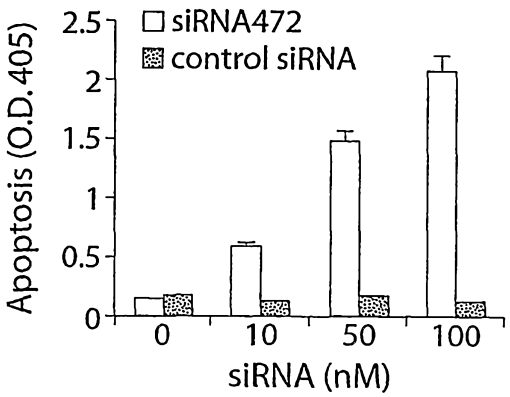


Fig. 32B

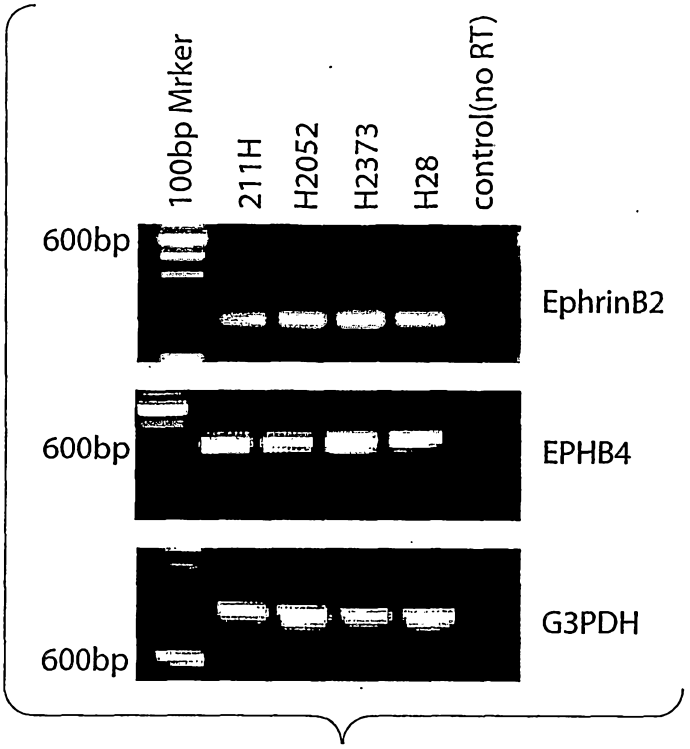


Fig. 33A

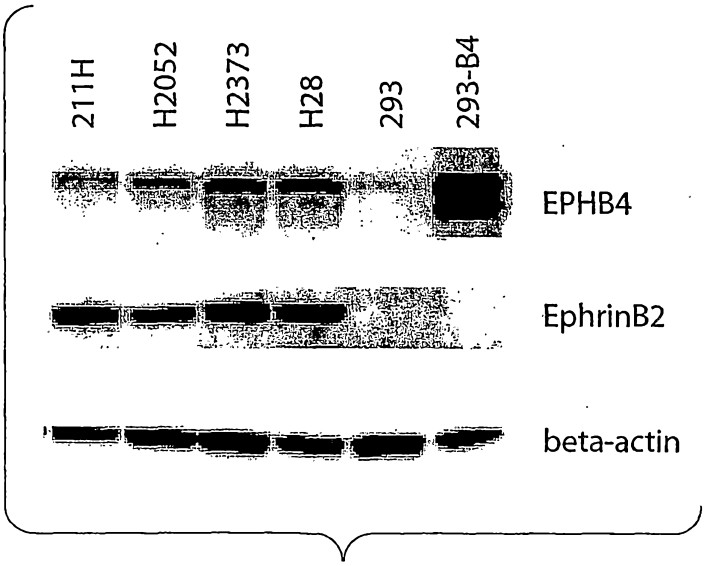


Fig. 33B

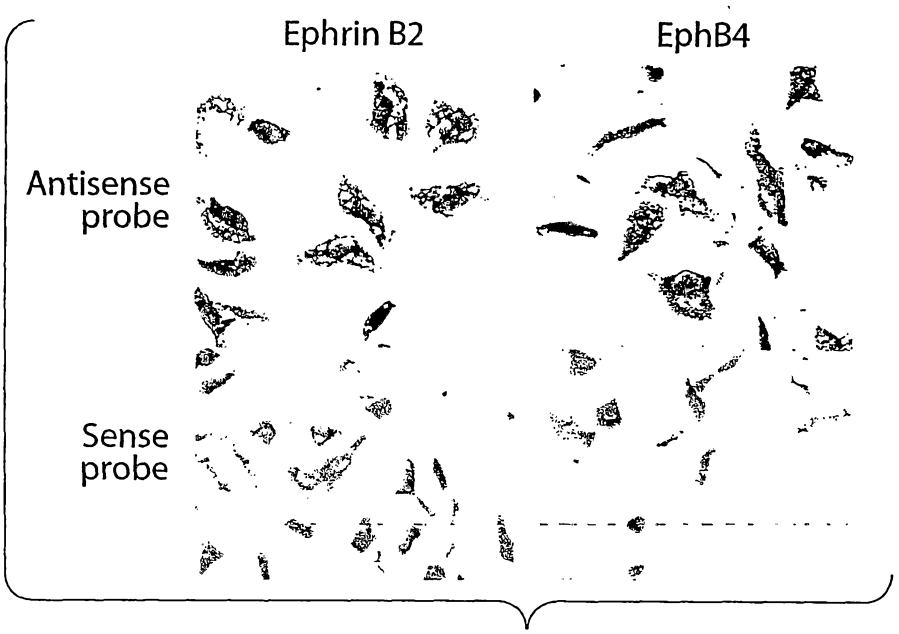


Fig. 34

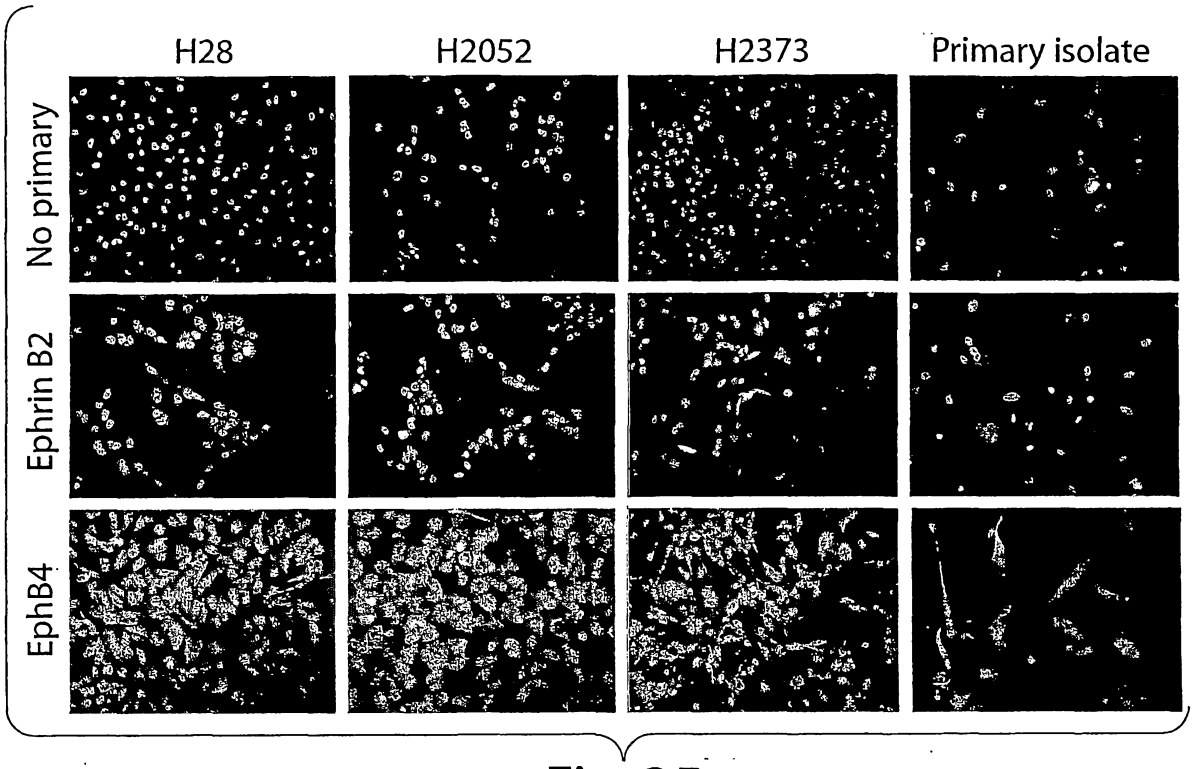


Fig. 35

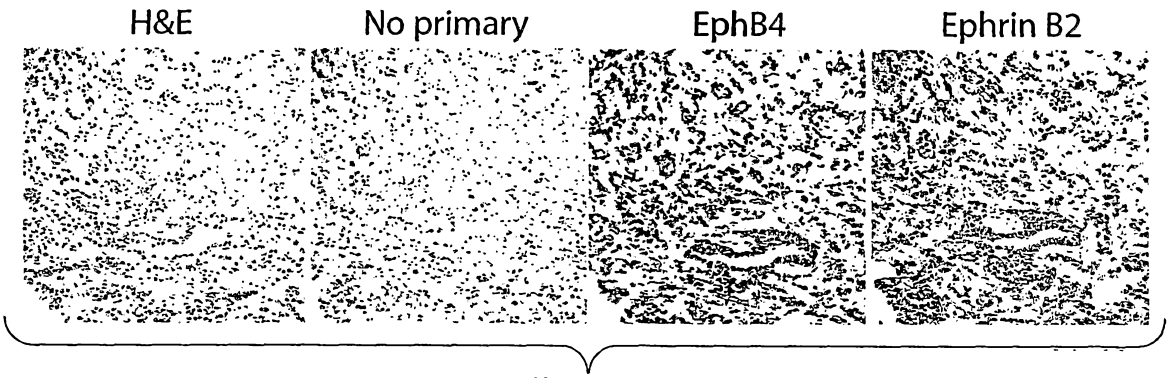


Fig. 36

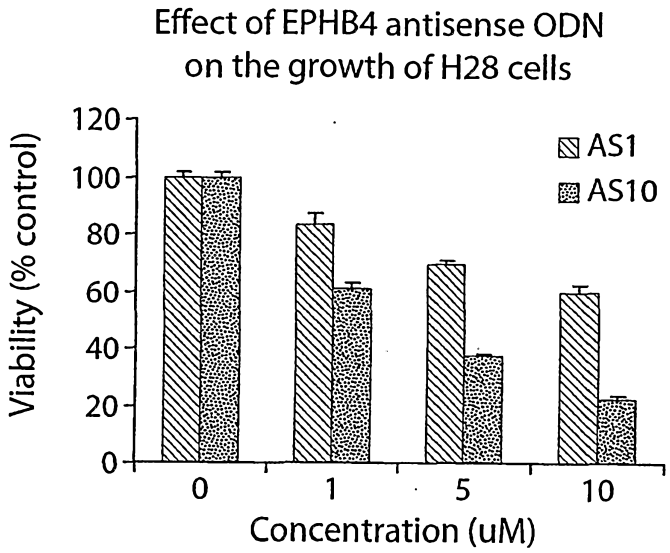


Fig. 37A

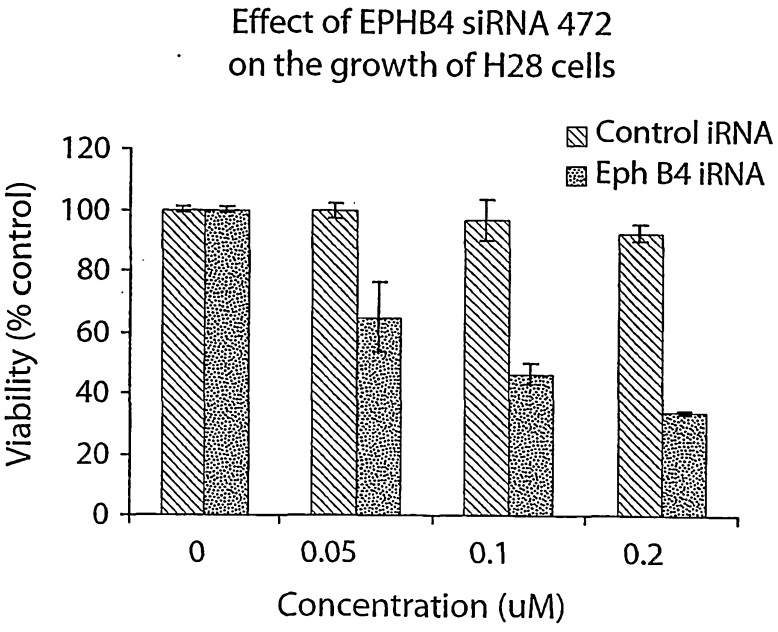


Fig. 37B

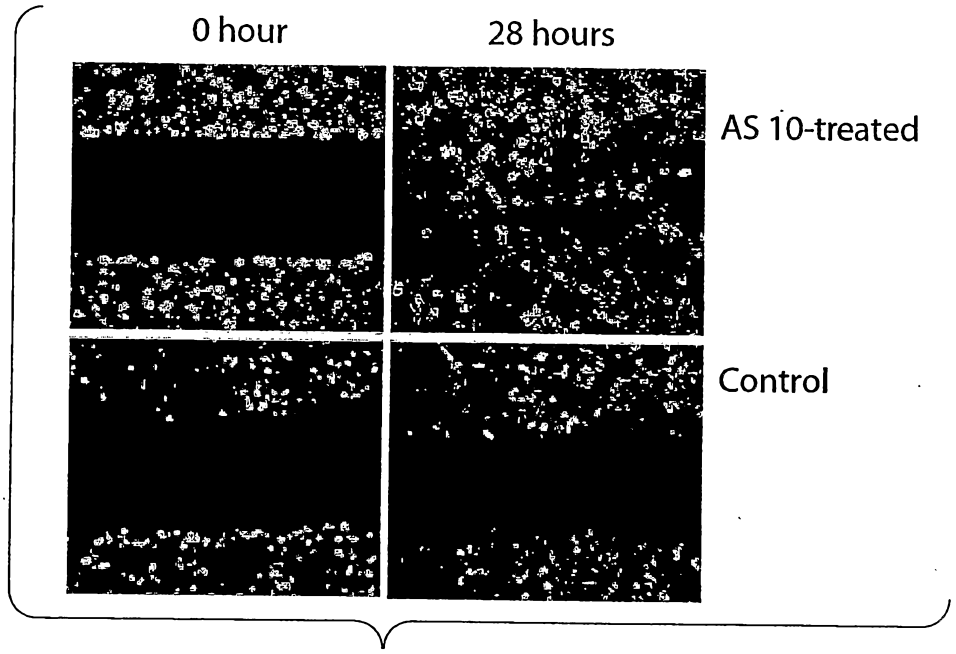


Fig. 38A

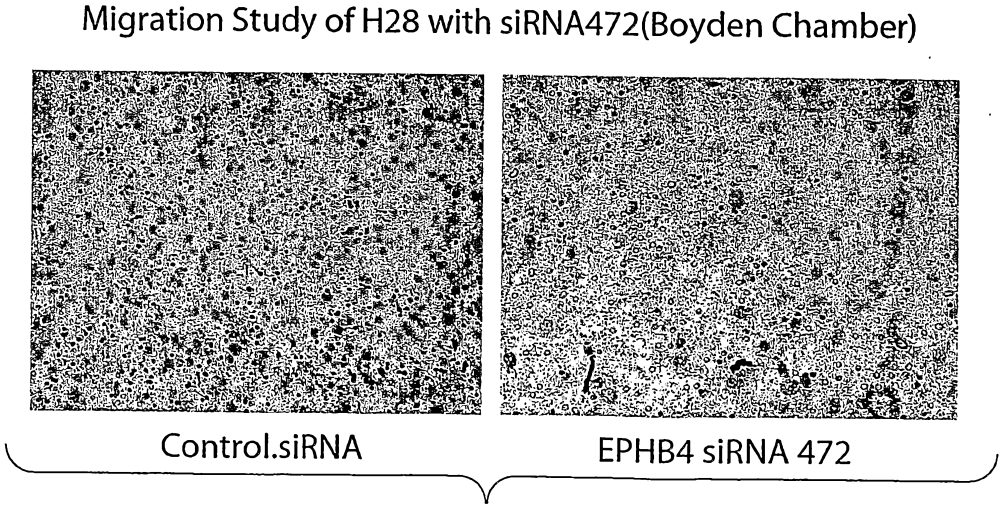


Fig. 38B

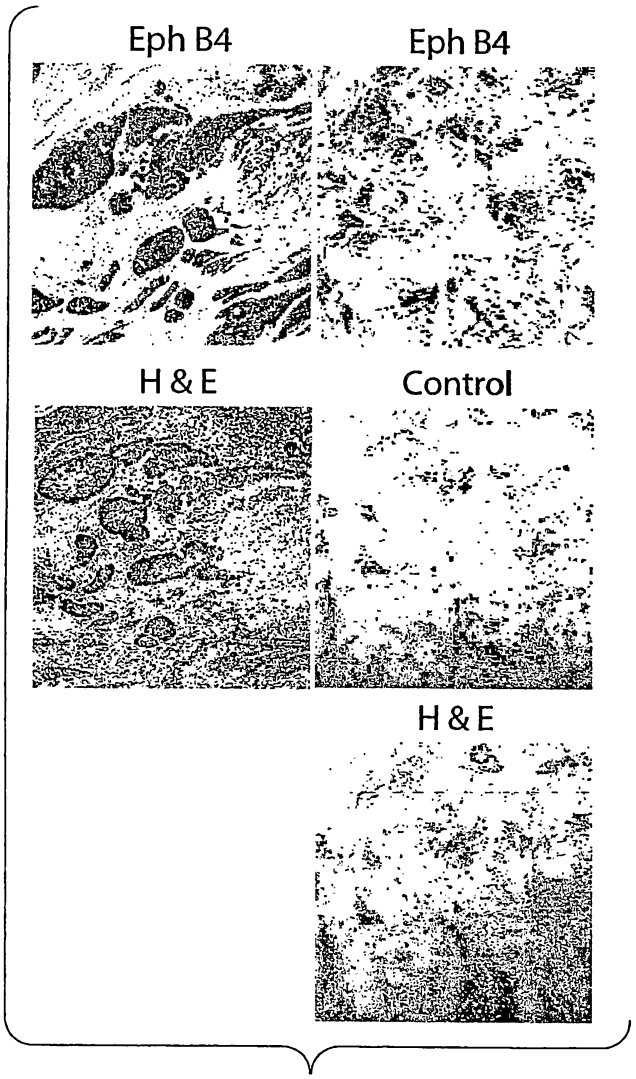


Fig. 39A

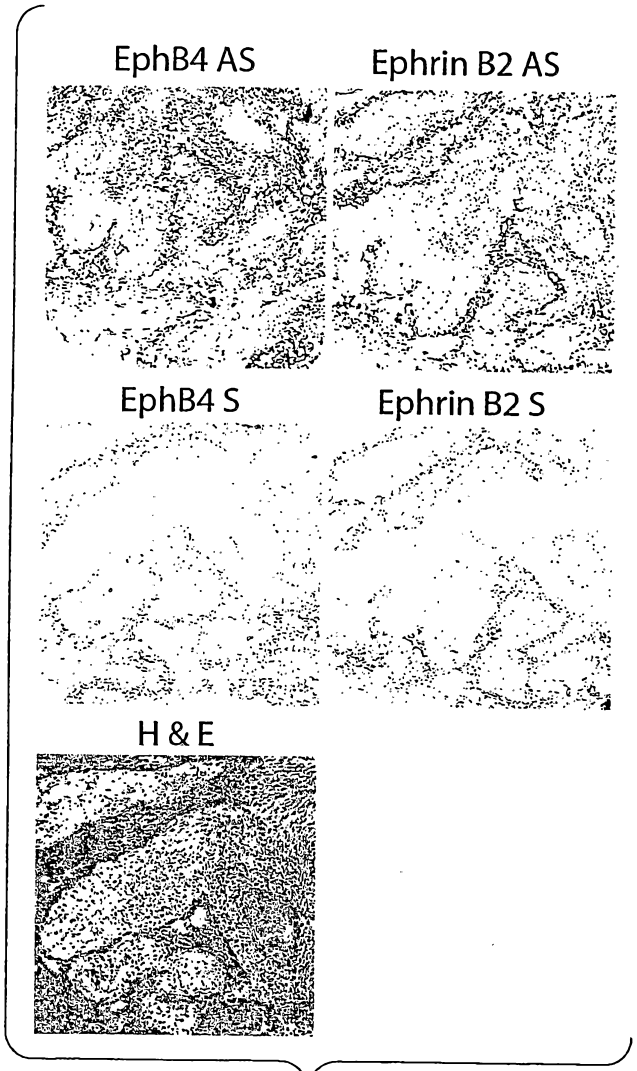


Fig. 39B

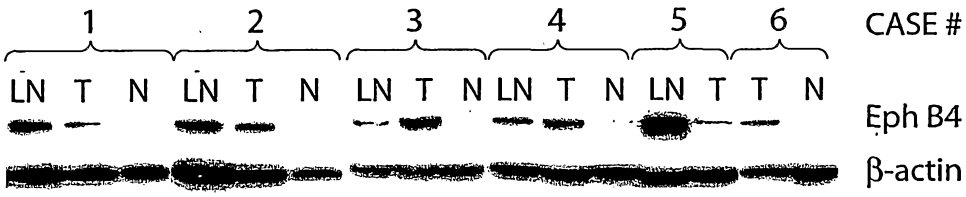


Fig. 39C

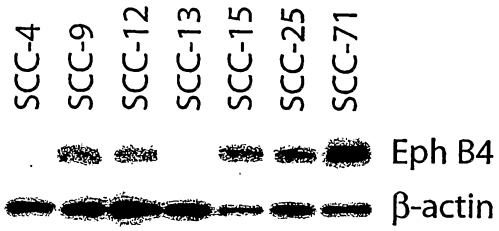


Fig. 40A

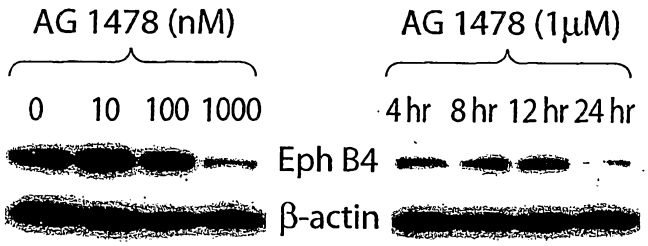


Fig. 40B

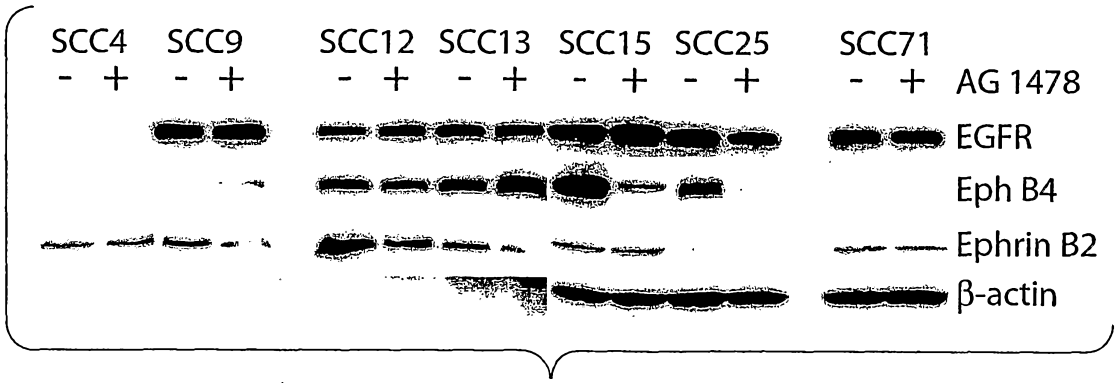


Fig. 40C

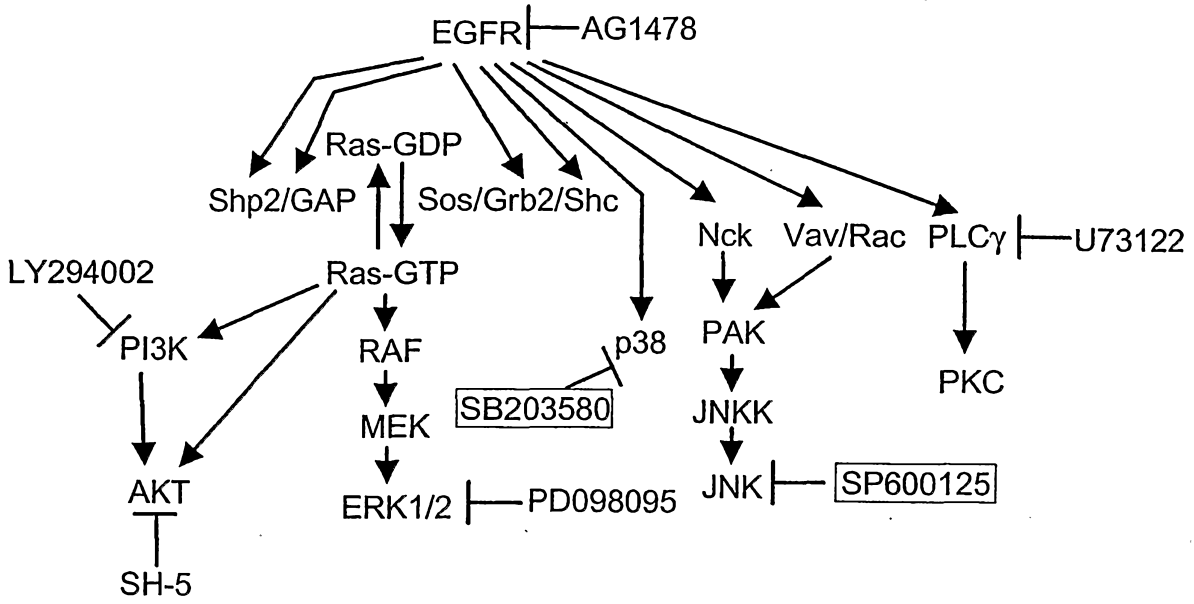


Fig. 41A

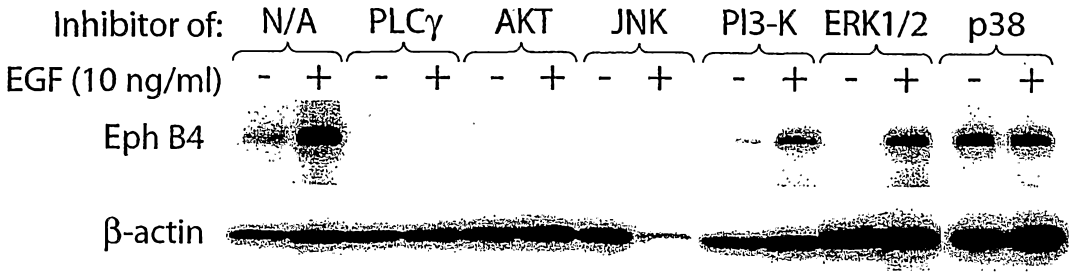


Fig. 41B

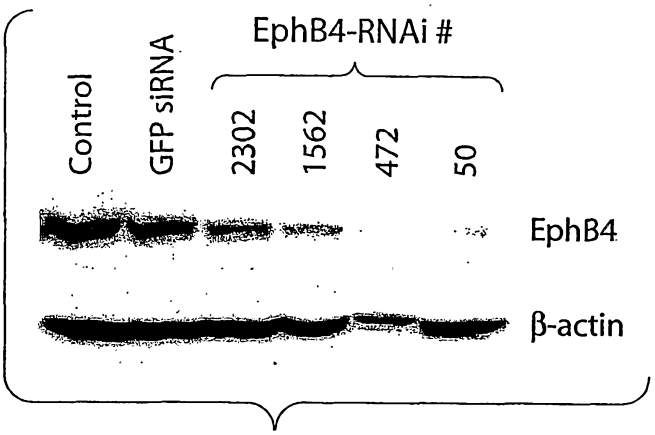


Fig. 42A

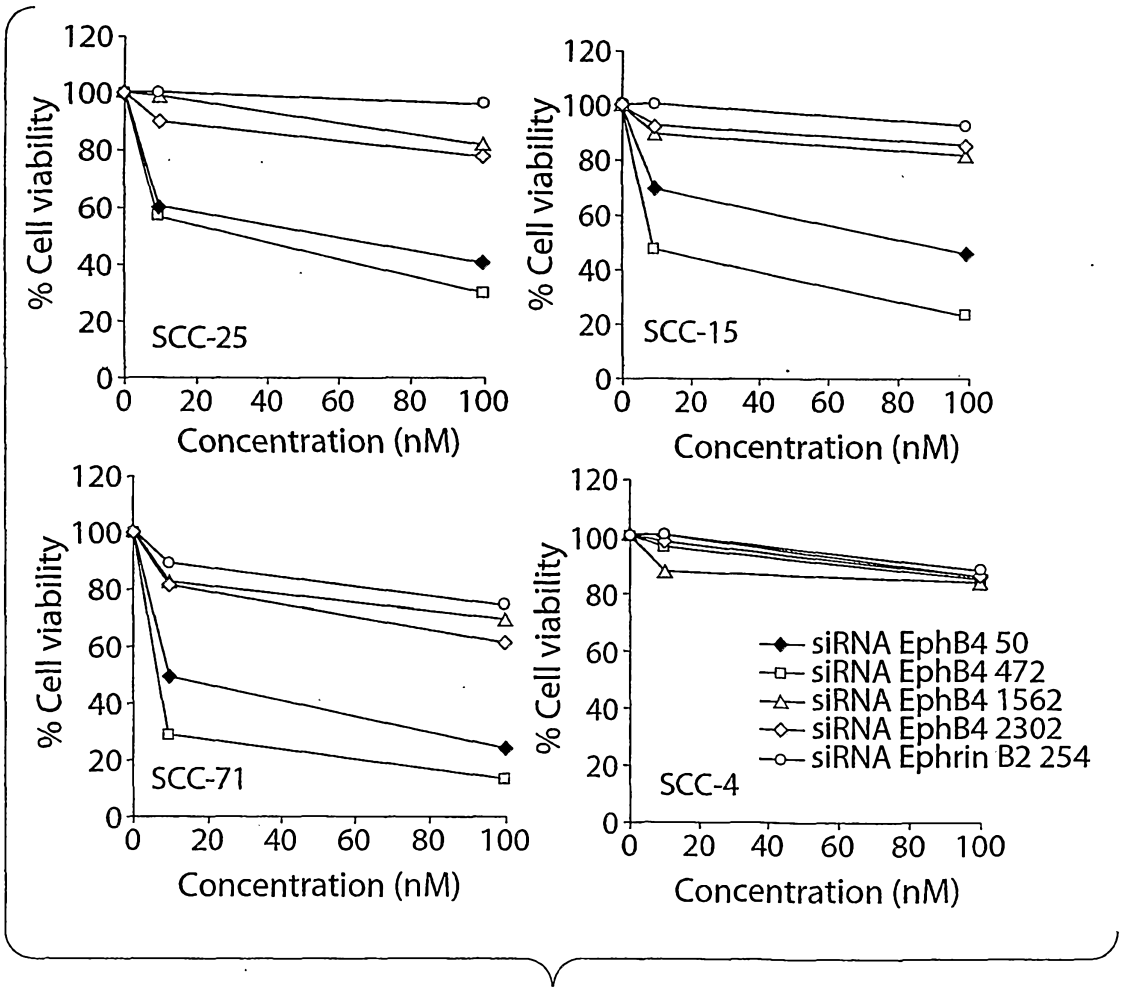


Fig. 42B

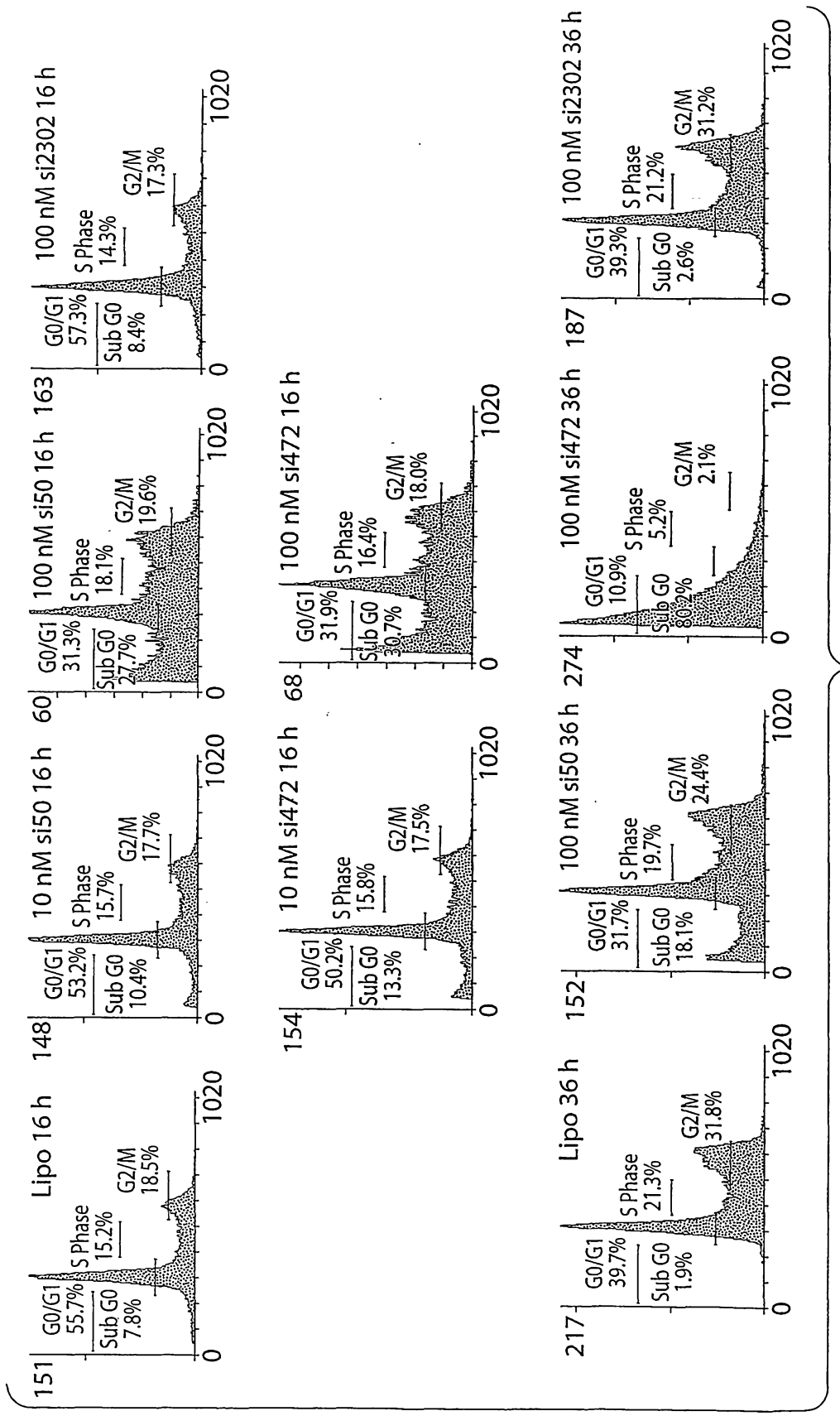

Lipo 16 h
151
G0/G1 55.7%
S Phase 15.2%
Sub G0 7.8%
G2/M 18.5%
0
1020
10 nM si50 16 h
148
G0/G1 53.2%
S Phase 15.7%
Sub G0 10.4%
G2/M 17.7%
100 nM si50 16 h
60
G0/G1 31.3%
S Phase 18.1%
Sub G0 27.7%
G2/M 19.6%
100 nM si2302 16 h
163
G0/G1 57.3%
S Phase 14.3%
Sub G0 8.4%
G2/M 17.3%
10 nM si472 16 h
154
G0/G1 50.2%
S Phase 15.8%
Sub G0 13.3%
G2/M 17.5%
100 nM si472 16 h
68
G0/G1 31.9%
S Phase 16.4%
Sub G0 30.7%
G2/M 18.0%
Lipo 36 h
217
G0/G1 39.7%
S Phase 21.3%
Sub G0 1.9%
G2/M 31.8%
100 nM si50 36 h
152
G0/G1 31.7%
S Phase 19.7%
Sub G0 18.1%
G2/M 24.4%
100 nM si472 36 h
274
G0/G1 10.9%
S Phase 5.2%
Sub G0 80.2%
G2/M 2.1%
100 nM si2302 36 h
187
G0/G1 39.3%
S Phase 21.2%
Sub G0 2.6%
G2/M 31.2%

Fig. 42C

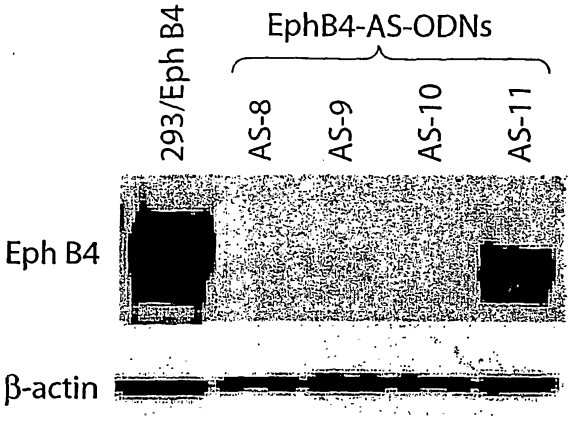


Fig. 43A

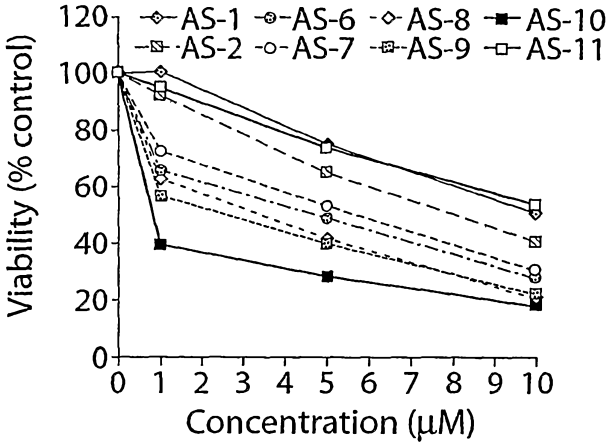


Fig. 43B

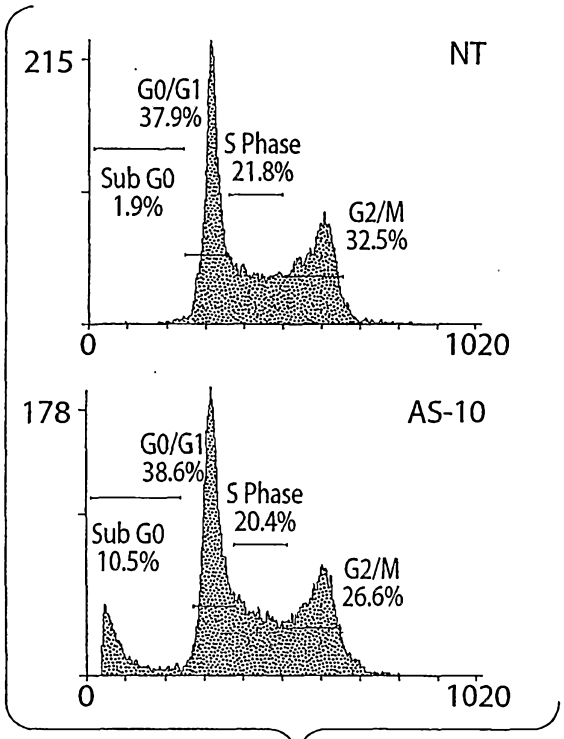

215
NT
G0/G1
37.9%
S Phase
21.8%
Sub G0
1.9%
G2/M
32.5%
0
1020
178
AS-10
G0/G1
38.6%
S Phase
20.4%
Sub G0
10.5%
G2/M
26.6%
0
1020

Fig. 43C

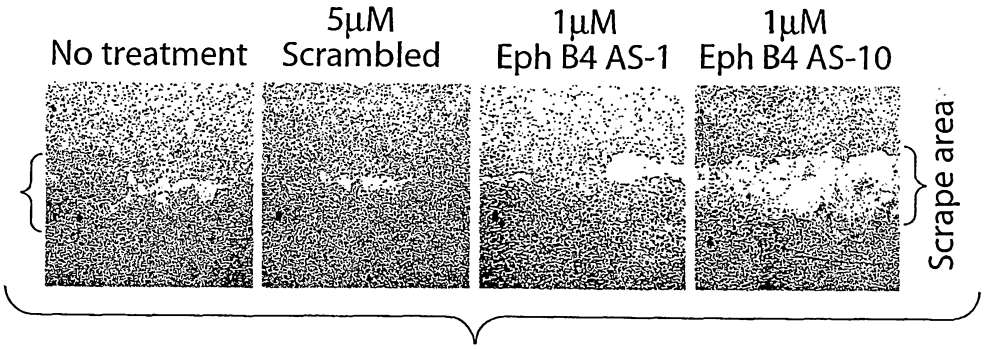


Fig. 43D

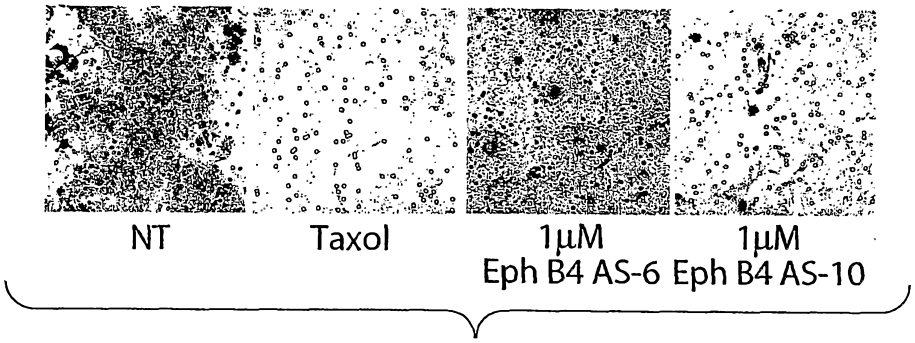


Fig. 43E

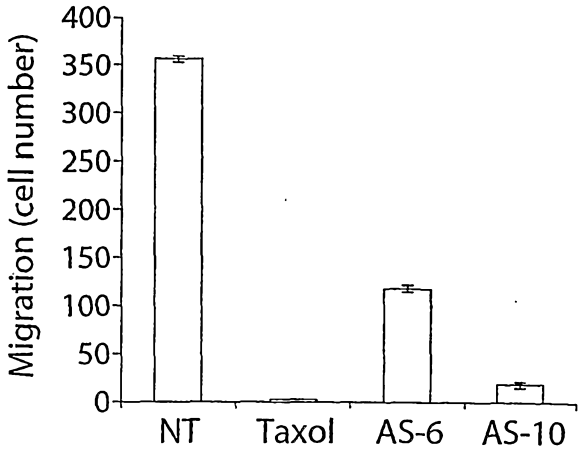


Fig. 43F

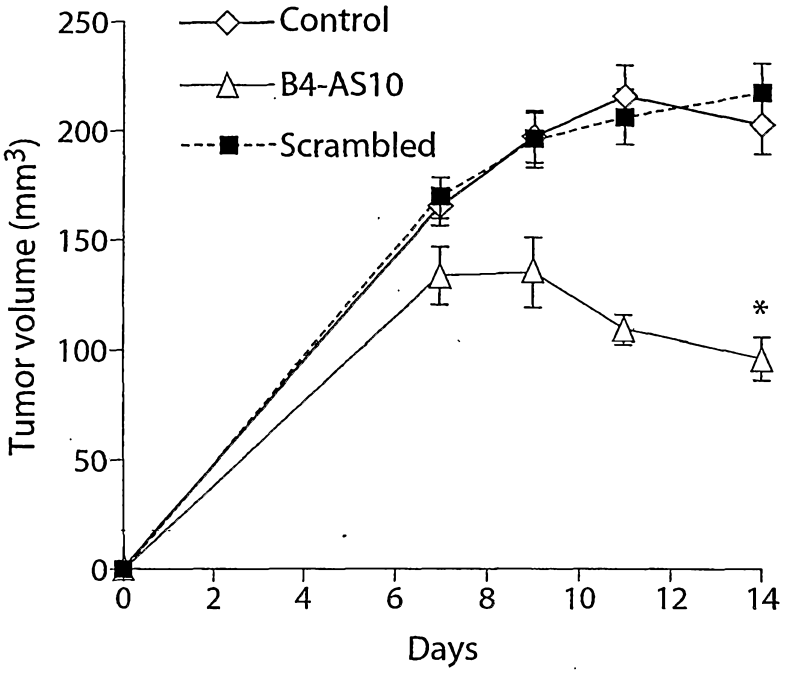

Control
B4-AS10
Scrambled
Tumor volume (mm3)
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Days
*

Fig. 44

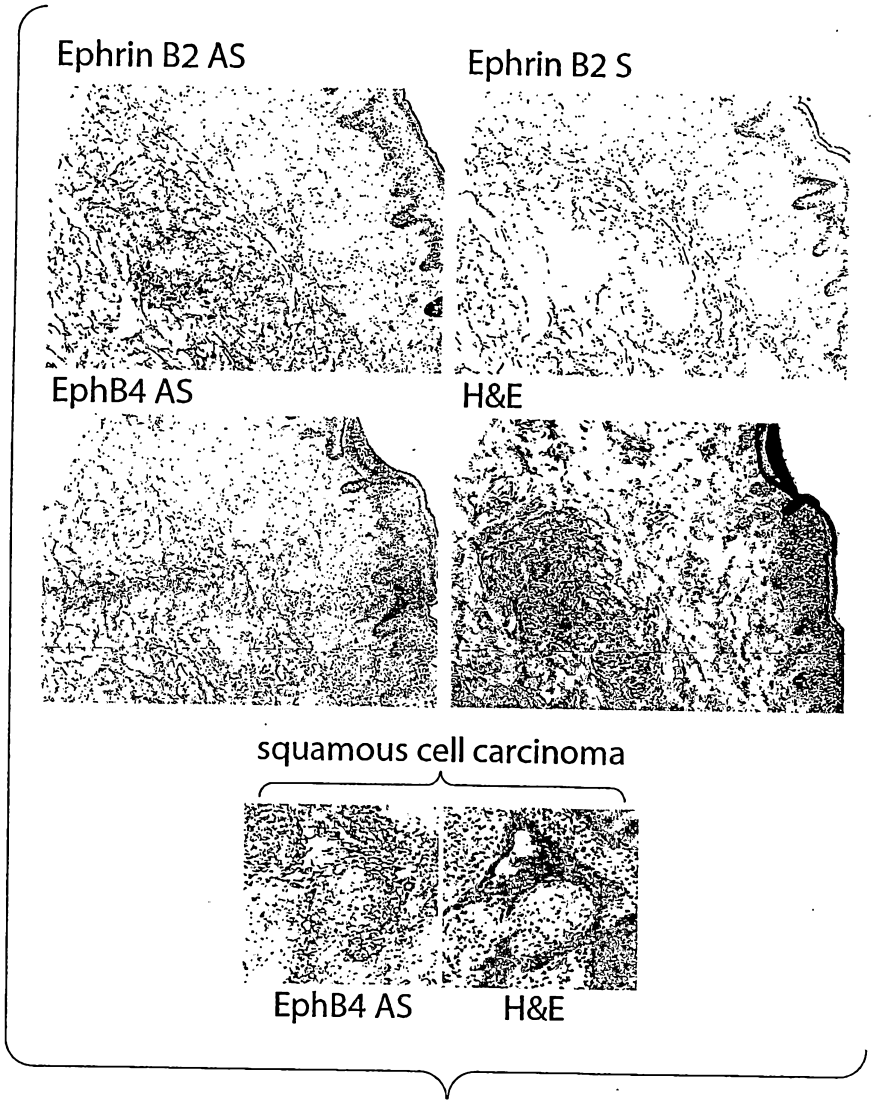

Ephrin B2 AS
Ephrin B2 S
EphB4 AS
H&E
squamous cell carcinoma
EphB4 AS
H&E

Fig. 45A

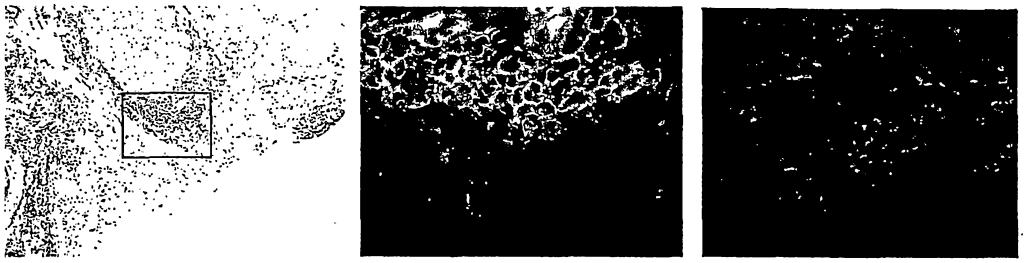


Fig. 45B

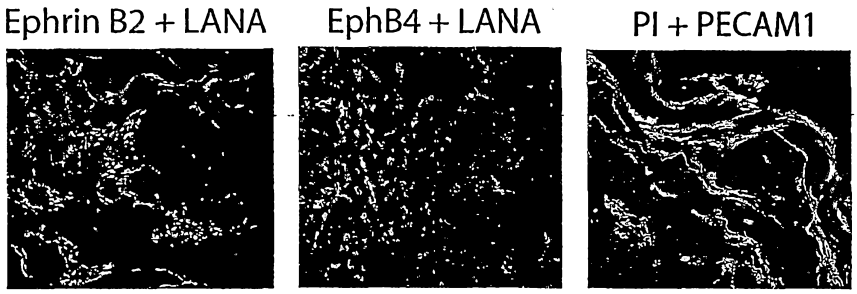


Fig. 45C

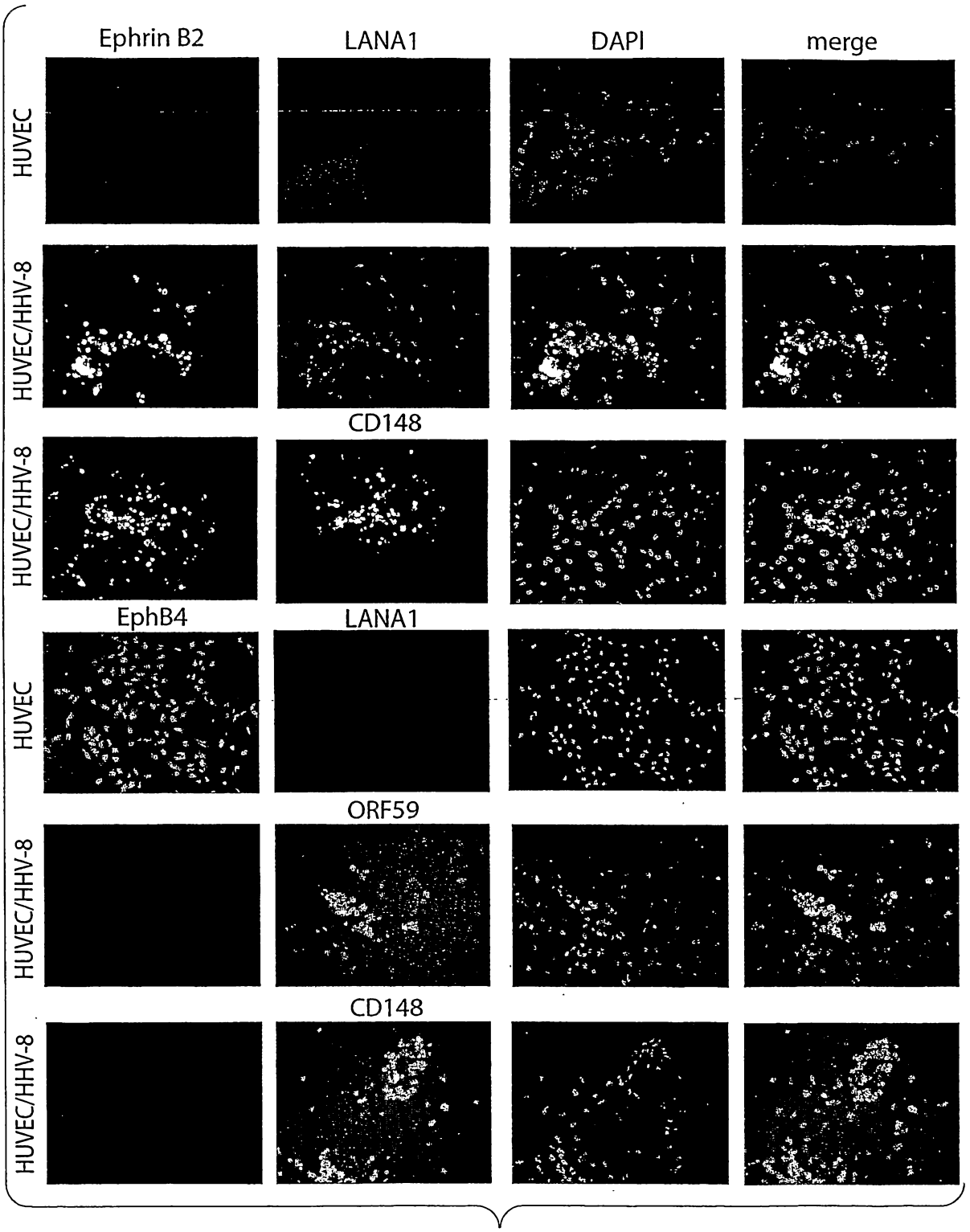

Ephrin B2
LANA1
DAPI
merge
HUVEC
HUVEC/HHV-8
CD148
HUVEC/HHV-8
EphB4
LANA1
HUVEC
ORF59
HUVEC/HHV-8
CD148
HUVEC/HHV-8

Fig. 46A

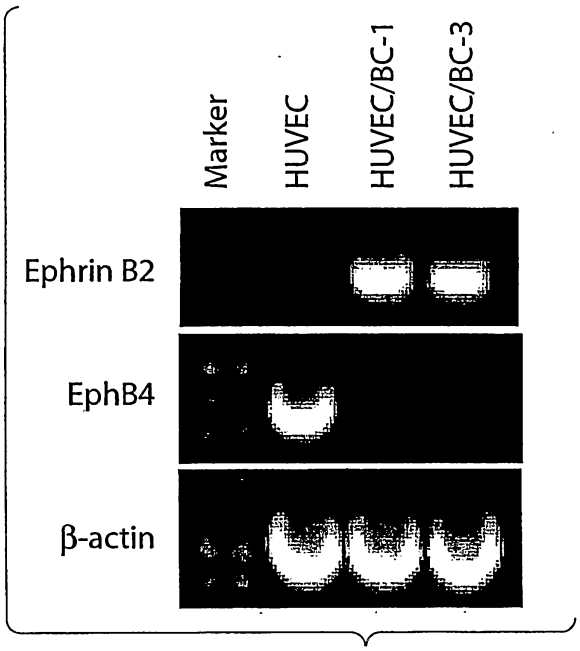


Fig. 46B

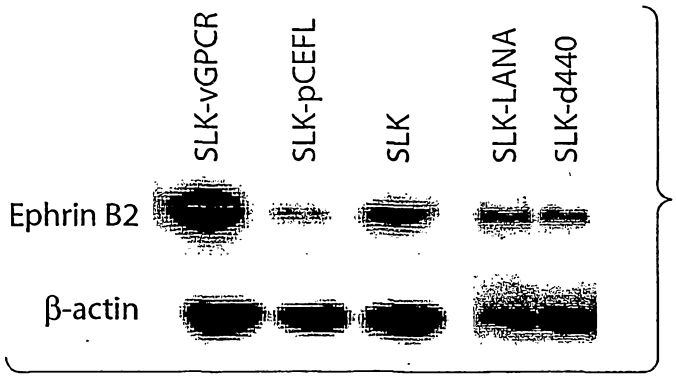


Fig. 47A

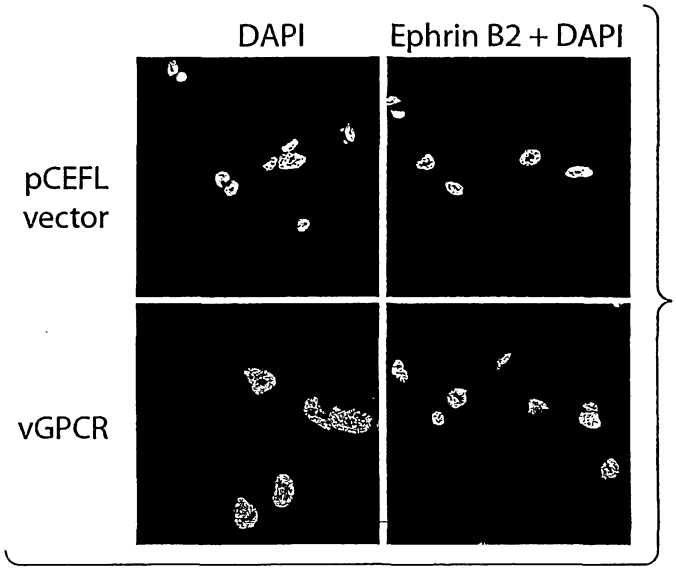


Fig. 47B

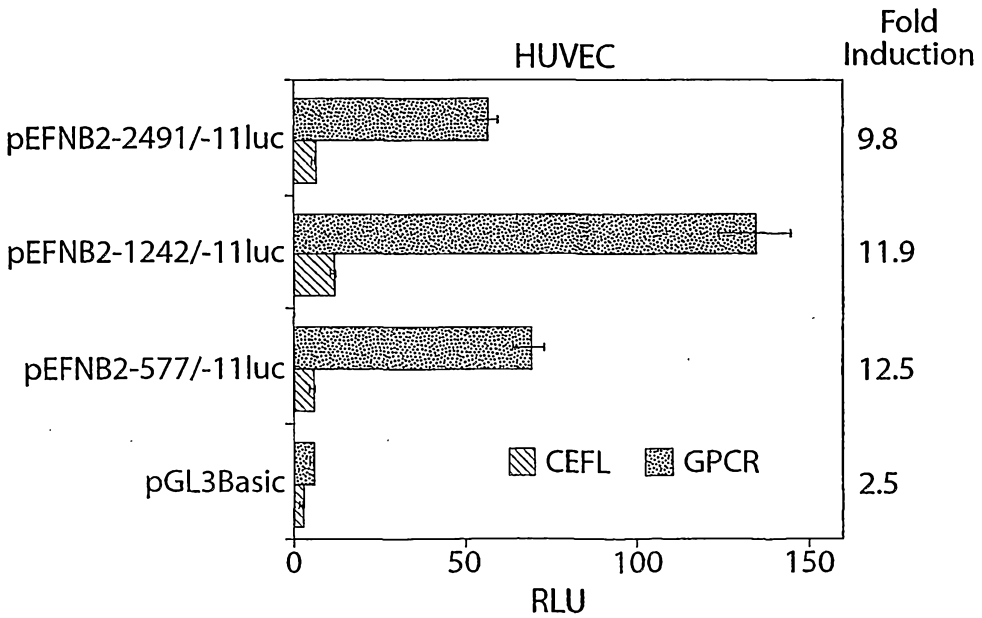


Fig. 47C

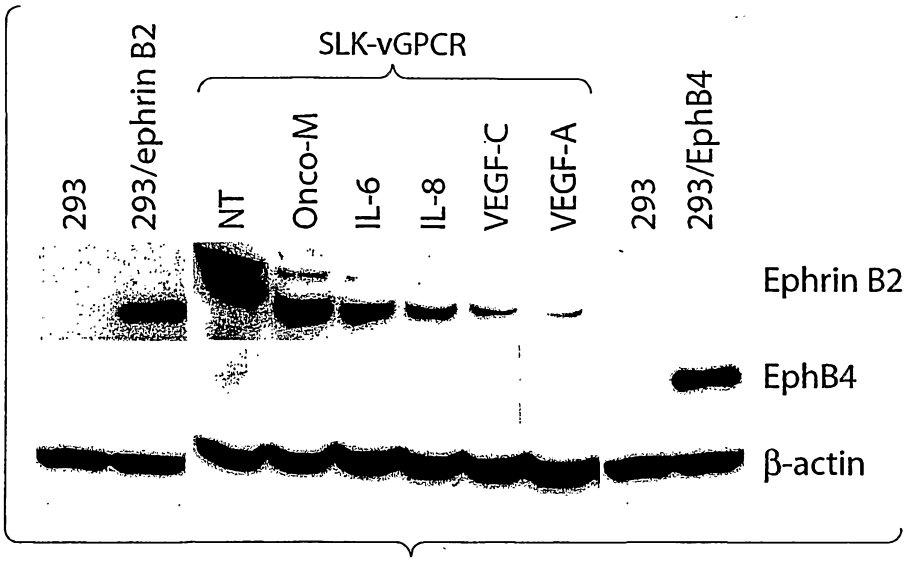


Fig. 48A

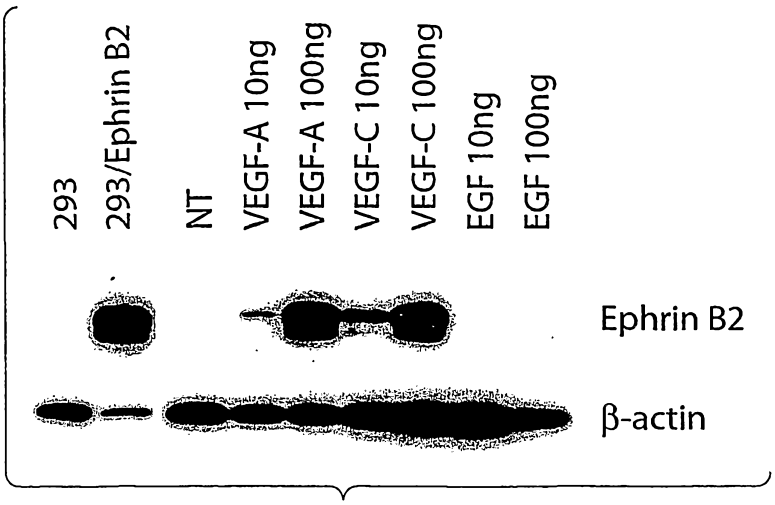


Fig. 48B

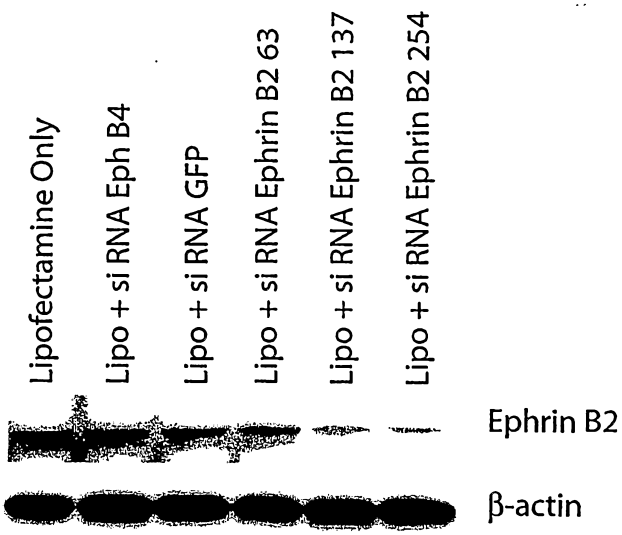


Fig. 49A

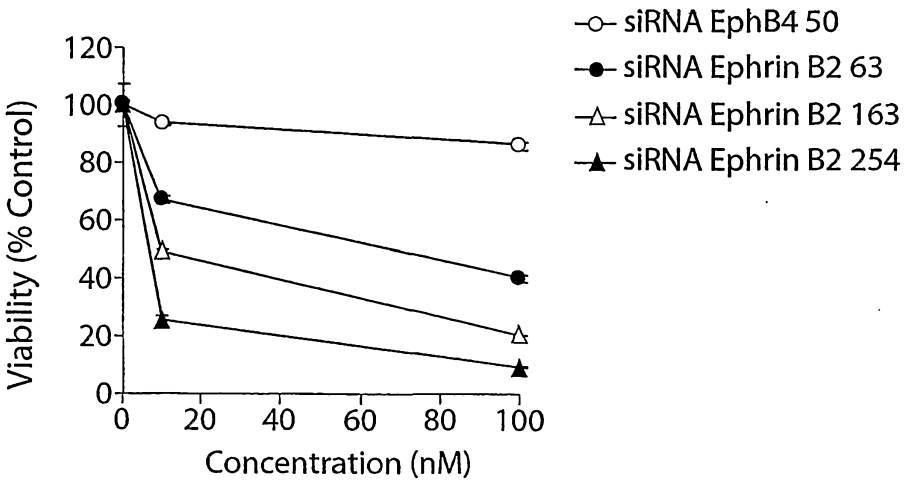


Fig. 49B

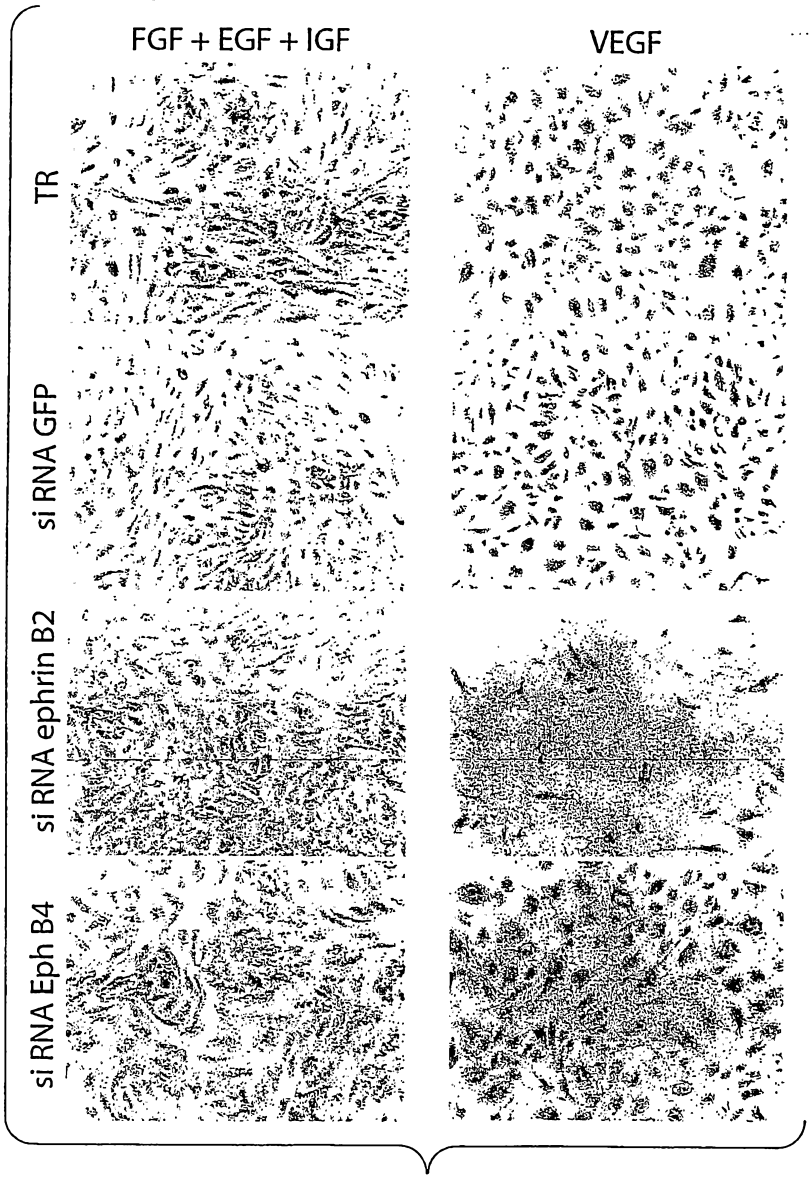


Fig. 49C

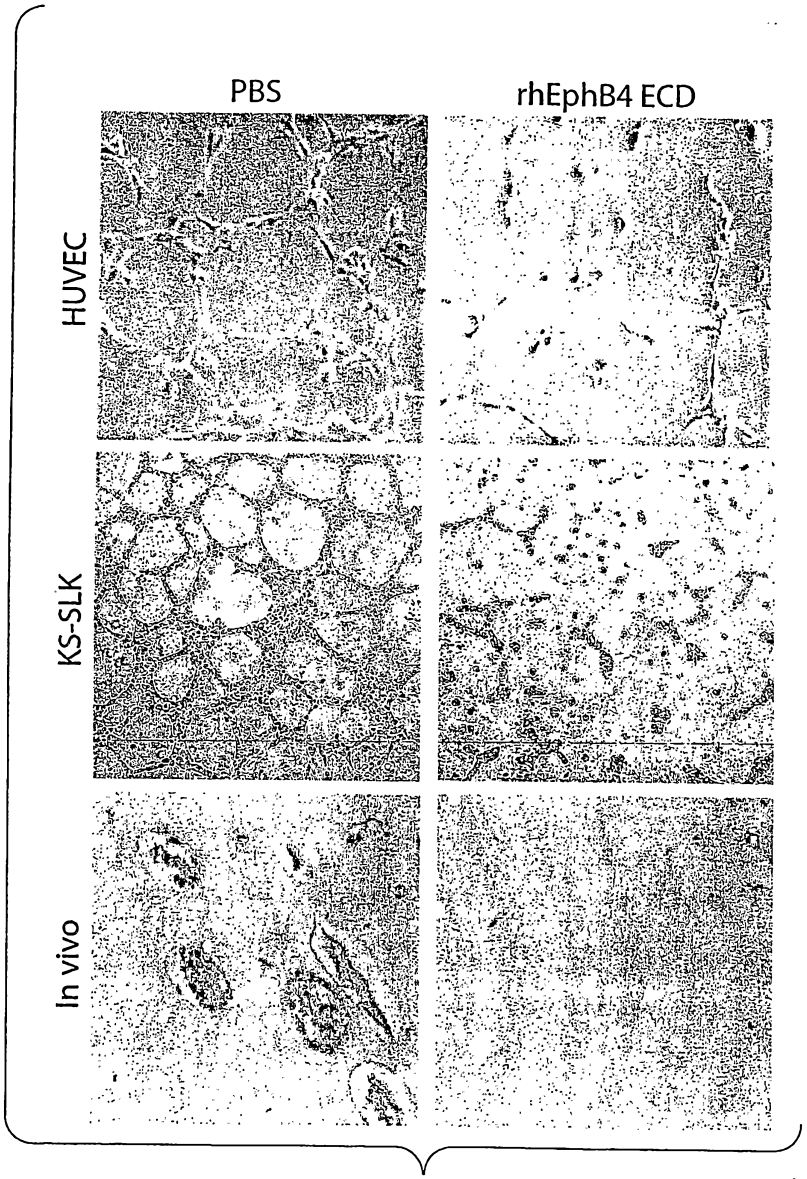


Fig. 50

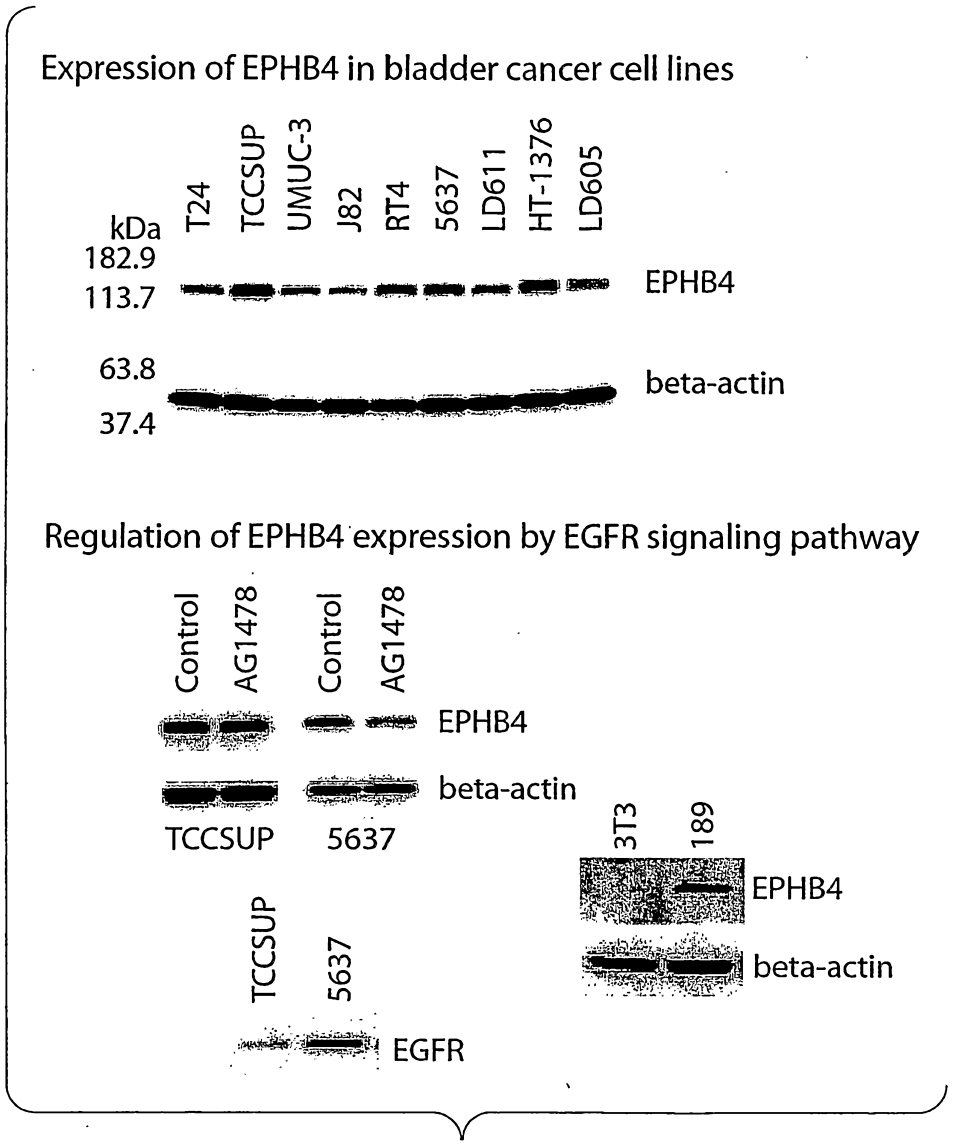

Expression of EPHB4 in bladder cancer cell lines
kDa
182.9
113.7
63.8
37.4
T24
TCCSUP
UMUC-3
J82
RT4
5637
LD611
HT-1376
LD605
EPHB4
beta-actin
Regulation of EPHB4 expression by EGFR signaling pathway
Control
AG1478
Control
AG1478
EPHB4
beta-actin
TCCSUP
5637
3T3
189
EPHB4
beta-actin
TCCSUP
5637
EGFR

Fig. 51

Transfection of p53 inhibit the expression of EPHB4 in 5637 cell

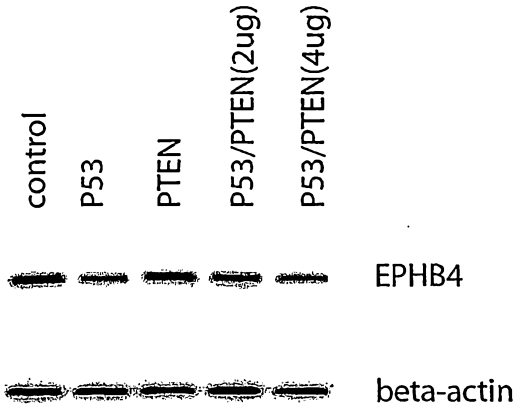


Fig. 52

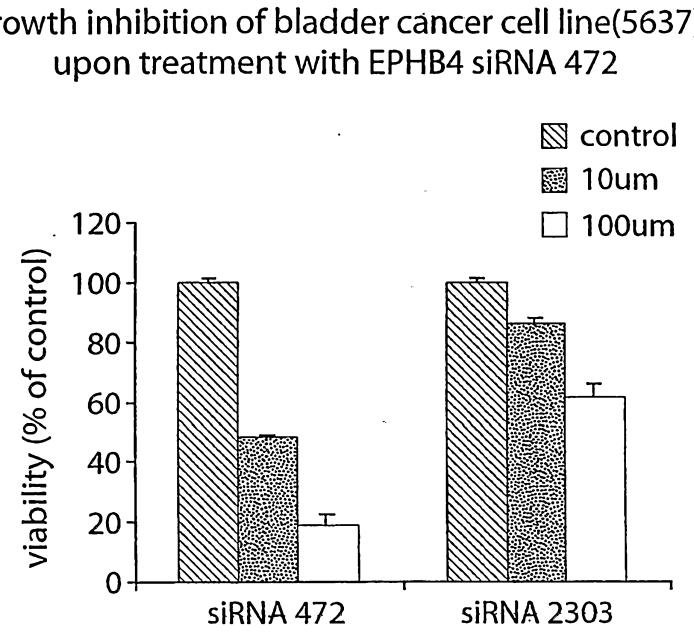

Growth inhibition of bladder cancer cell line(5637)
upon treatment with EPHB4 siRNA 472
control
10um
100um
viability (% of control)
120
100
80
60
40
20
0
siRNA 472
siRNA 2303

Fig. 53

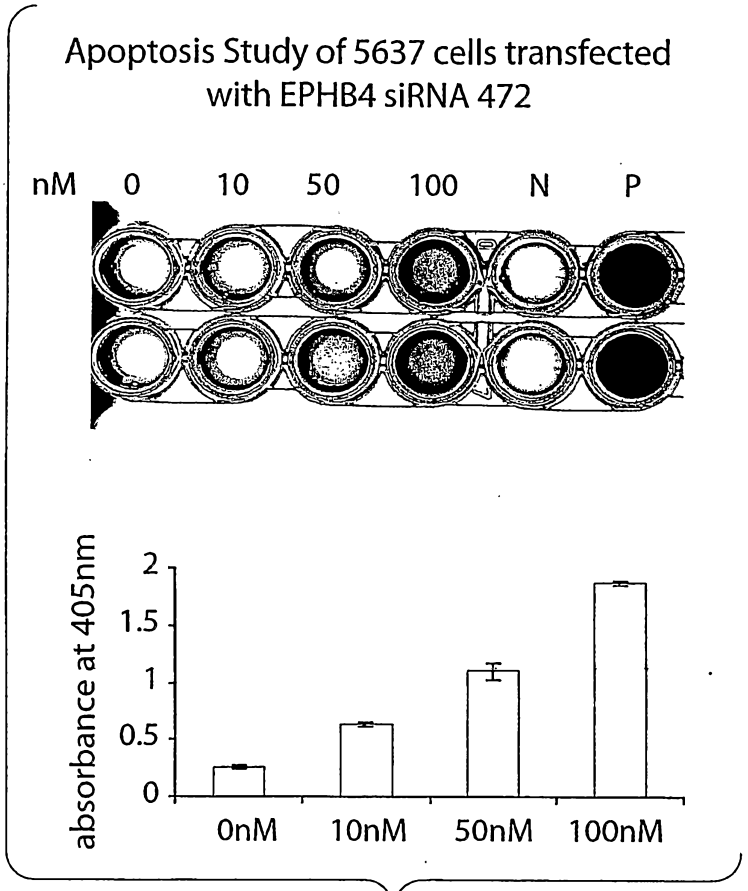

Apoptosis Study of 5637 cells transfected with EPHB4 siRNA 472
nM
0
10
50
100
N
P
absorbance at 405nm
2
1.5
1
0.5
0
0nM
10nM
50nM
100nM

Fig. 54

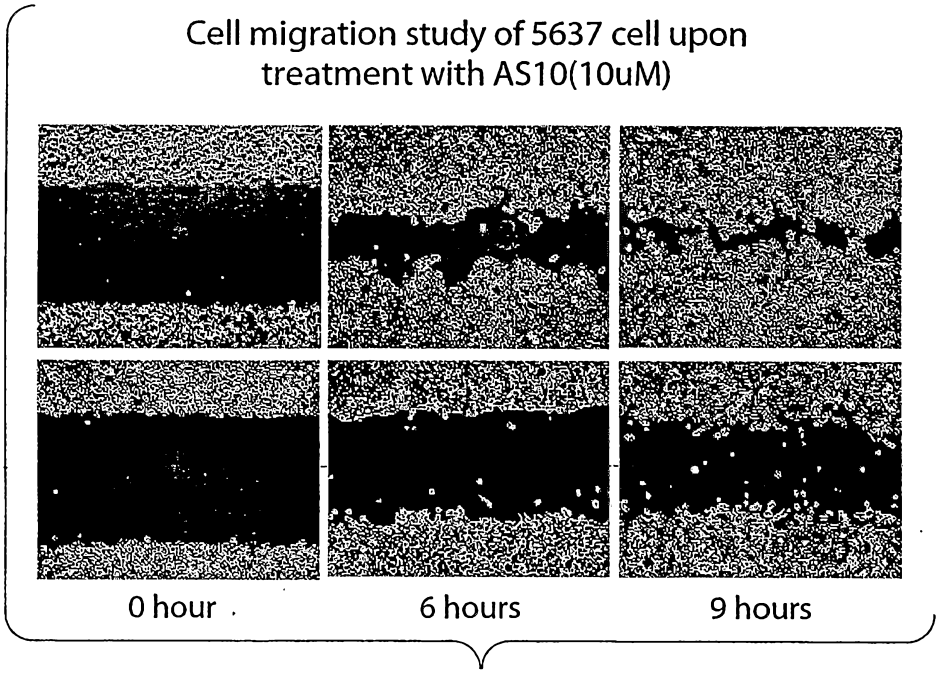
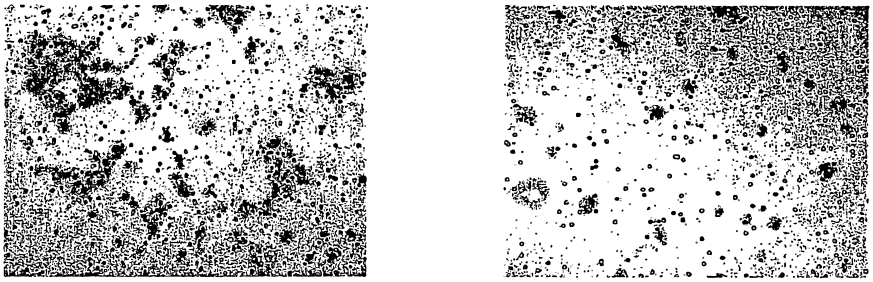

Cell migration study of 5637 cell upon treatment with AS10(10uM)
0 hour
6 hours
9 hours

Fig. 55

Invasion study of 5637 cell transfected with siRNA 472 or control siRNA



Control siRNA472

Fig. 56

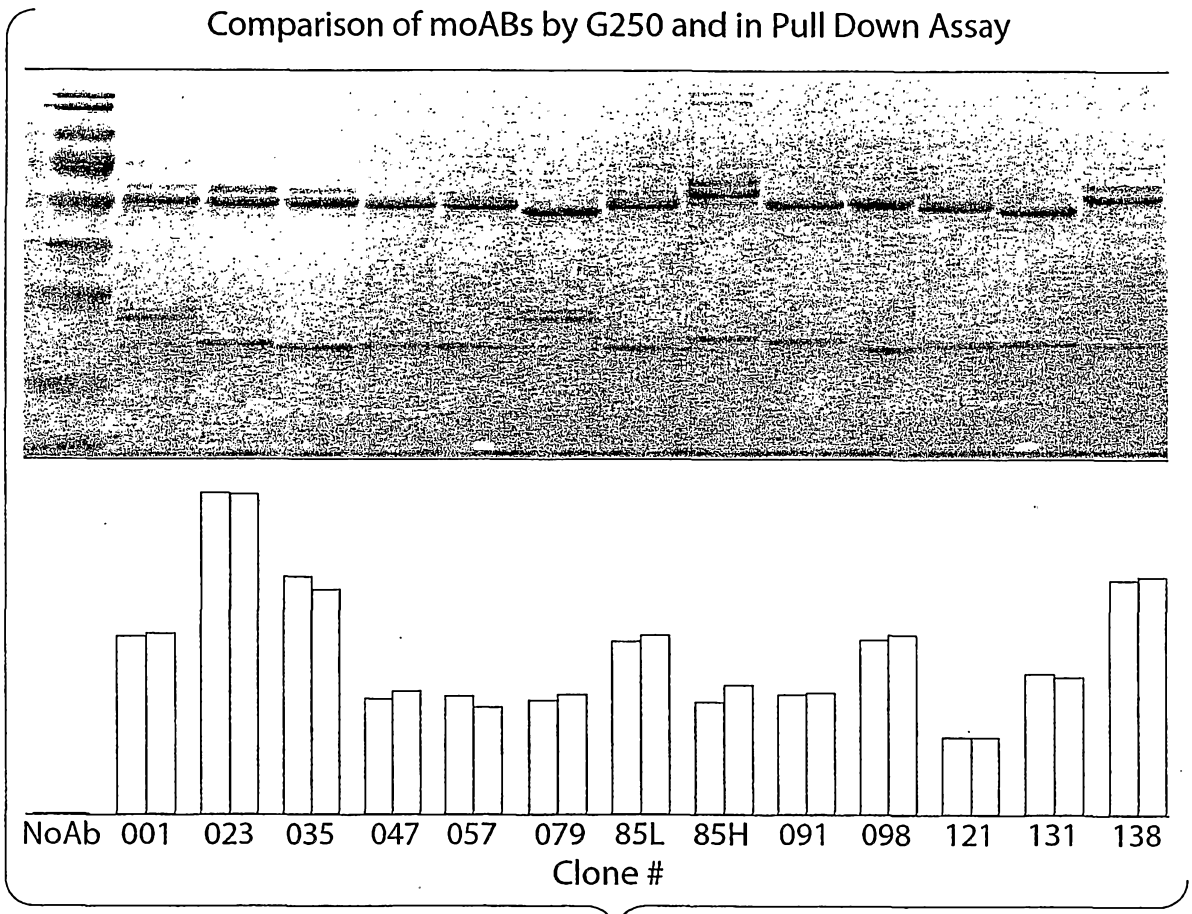

Comparison of moABs by G250 and in Pull Down Assay
NoAb
001
023
035
047
057
079
85L
85H
091
098
121
131
138
Clone #

Fig. 57

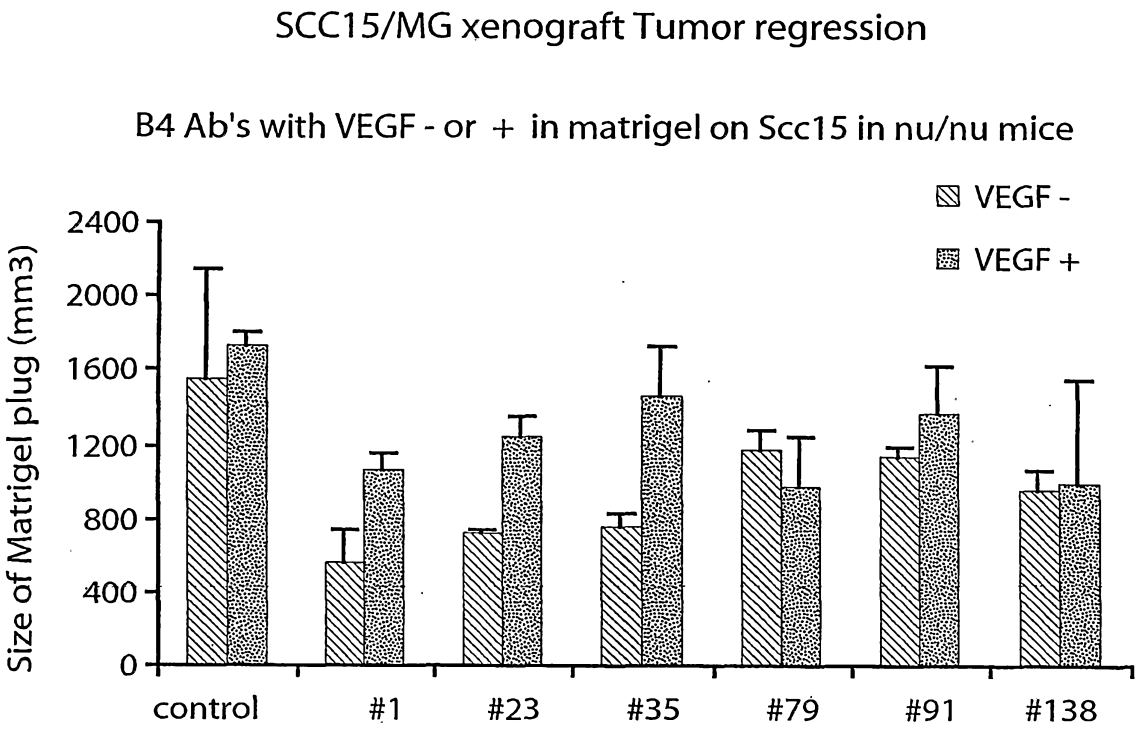

SCC15/MG xenograft Tumor regression
B4 Ab's with VEGF - or + in matrigel on Scc15 in nu/nu mice
VEGF -
VEGF +
Size of Matrigel plug (mm3)
2400
2000
1600
1200
800
400
0
control
#1
#23
#35
#79
#91
#138

Fig. 58

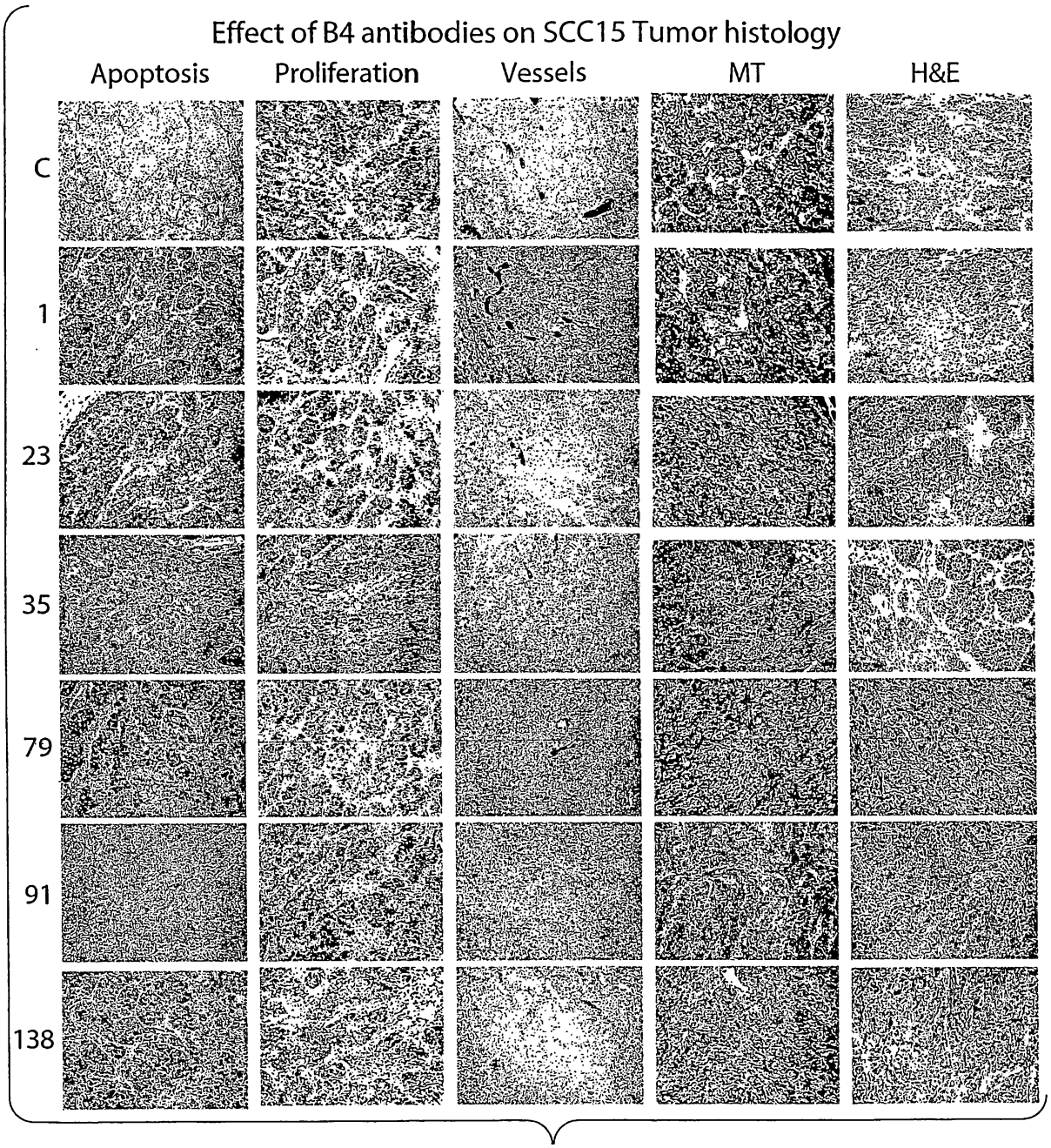

Effect of B4 antibodies on SCC15 Tumor histology
Apoptosis
Proliferation
Vessels
MT
H&E
C
1
23
35
79
91
138

Fig. 59

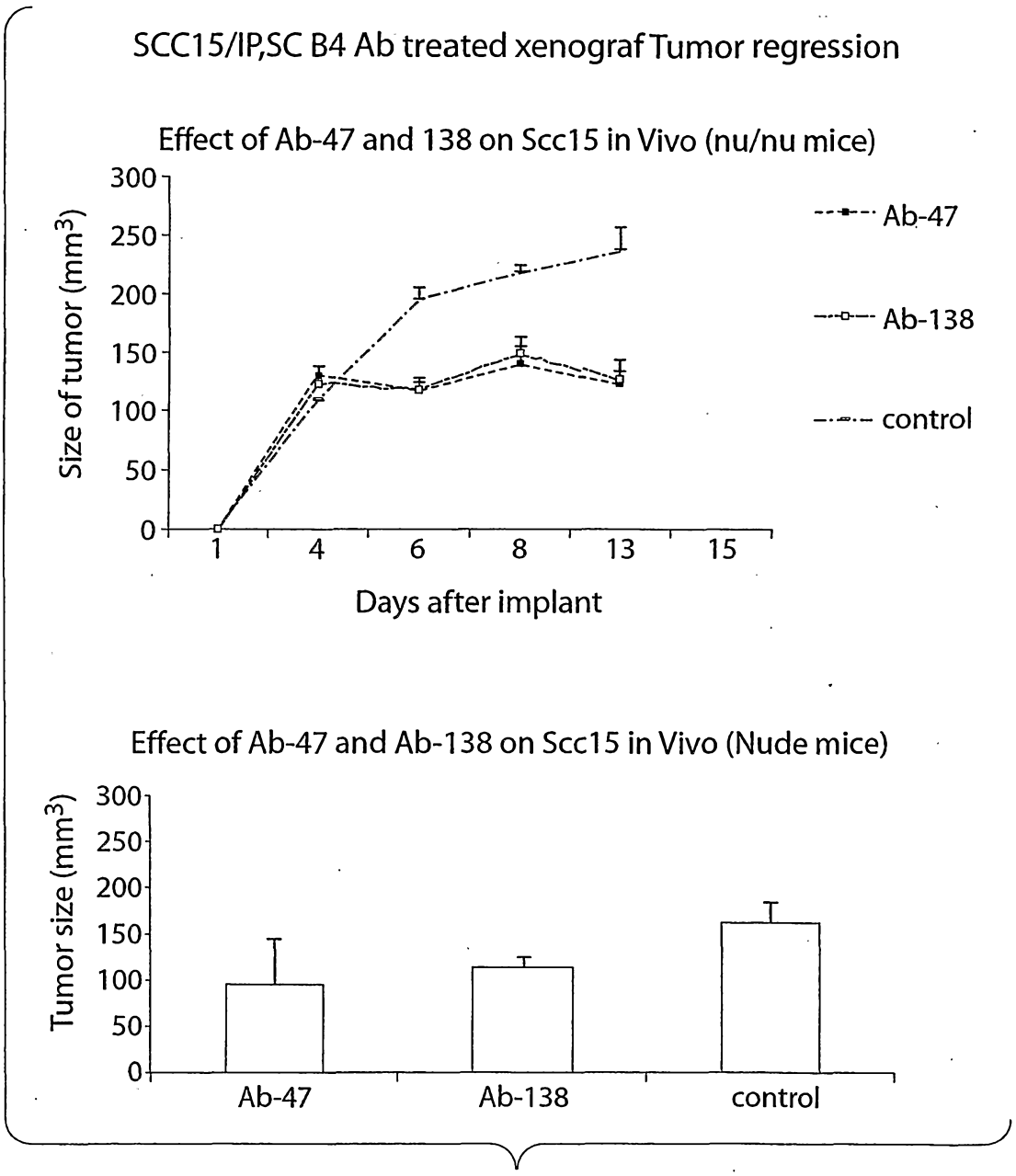

SCC15/IP,SC B4 Ab treated xenograf Tumor regression
Effect of Ab-47 and 138 on Scc15 in Vivo (nu/nu mice)
Size of tumor (mm3)
300
250
200
150
100
50
0
1
4
6
8
13
15
Days after implant
Ab-47
Ab-138
control
Effect of Ab-47 and Ab-138 on Scc15 in Vivo (Nude mice)
Tumor size (mm3)
300
250
200
150
100
50
0
Ab-47
Ab-138
control

Fig. 60

EphB4 gene

```
   1 ggggtttcat catgttggcc aggctggtct tgaactcctg acctcaaatg atccgcctgc
  61 ctctgcctcc caaaatgctg ggactacagg cgtgagccac cgcgcccgcc acacccacct
 121 tttctttacc gttgtttcct cgatttttct ctactcccta gcgcagctta gtgcgcgcct
 181 cctctggaca tttttcaggg cttggttgcg cgcacagtag gtccccaaca ctgaatgttt
 241 atggggtgac tgtgtgaacg ttcgctgcaa ggctatccaa actgggattg ctccttgagg
 301 ccccctgggc ggccgtcaat tctccaaagc ttctactccc ttttccttcc ttttccccca
 361 aaacgcagtc cctgcgccca ctagagggtg gtgggcgcat ccaagagcgg catctagagt
 421 ccgcagcaag gtcagagcgg gctttgtgtg cgcggtgaac atttacgtgc acgcctgggc
 481 ggccctccgt gttgctgctg ggtgtgtgtt ttctctgctc cctggtgcca gccgggttcg
 541 ggcctgtccc gggggtccct gggccccagc cccgacatgc tcggtcctgg acagcgcgca
 601 ccgccacggc gcacatctgg gcggtcccgg ggttcctcac ccgccgcccc tccccttct
 661 ccaaactttc tctcaacttc ccgacctgct ccactcggtg cccctctccg cttccctcat
 721 gaattattca gtagcgtgag ctccaatcag cgcgcccggg gctcactcgc ggagcccccg
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2461 gggaacaaaa accatgaacg aggggaagag gaaggccaaa ggggtggaaa aaccacgagg
2521 acgaggtgtg gtgagaagga aagacgcaaa gaggaaatgg tgattgtgac acctattacc
2581 tgagtgtttc caagcaccag gcctgtgctg agcgccttac aaatattaat ttcacccatc
```

Fig. 61A

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Fig. 61B

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Fig. 61C

```
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9481  cccatgtcta ctaaaaataa aaaaattagc caggcatggt ggtgggcacc tgtaatccac
9541  ttgggaagca accagaagaa ttgcttgaac ccaggaggcg gaggttgcag taagctgaga
9601  ctgcgccact gcactccagc ctgggtgata gagcacgact ccgtctcgaa aaaaaaaatt
9661  ttttttaagt taagggacag agctaccatg cacaagggtt ccctgtgtct ctgcctctca
9721  cagtacctcc tgcagtgtct gacatccggg tgacgcggtc ctcacccagc agcttgagcc
9781  tggcctgggc tgttccccgg gcacccagtg gggctgtgct ggactacgag gtcaaatacc
9841  atgagaaggt aaggccatcc cccagccctg gggtgggtgg gcaatgggtt gtgctctcct
9901  ggctgggaca cctgggttgc aggcacctgg caggcatttg aattccagct ctgccatgga
9961  ttccctgggc agccttgggt aagccccttg gcctgtctga gcctcagact cttcatctat
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10381 ggctgaggca ggagaatggc gtgaacccag gaggcggagc ttgcagtgag ccgagatagc
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Fig. 61D

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10681 gtgtgttggc gggcccctgt aatcccagct actcaggagg ctgagacagg agaatcactg
10741 gaacccaagt gattctcgac ttgaggtcga ggctgcagtg agtcgtgttt gcaccattgc
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Fig. 61E

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15721 acctcctcca gccttttcct cttgggctaa gccctggtcc tctgcctttt ctttttttta
15781 agacagagcc tcgctctgtc gcccaggctg gagtgcagtg gcgcgatctc ggctcattgc

Fig. 61F

15841 tgtctccacc tccagggttc aagcgattct cctgcctcag tctcccaagt agctggtact
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18361 cacagtgctg ggattacagg catgagccac tatgcccggc taatttttgt atttttagta

Fig. 61G

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Fig.61H

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22861 atggcgggtg aggactgcag agaatgggcc ctccttcccg ctctctgccc ccactccttg
22921 cccagaagtg tccgttcatt ggtgttgggt gggagggcct ctgtccgcct ctgcaaggct
22981 gggttccacc tcctcccccg gacctgggcc tggtactcag cattcctccc catccttgcc
23041 ccctagggcc tcacaccctc tcctggacca gcggcagcct cactactcag cttttggctc
23101 tgtgggcgag tggcttcggg ccatcaaaat gggaagatac gaagaaagtt tcgcagccgc
23161 tggctttggc tccttcgagc tggtcagcca gatctctgct gagtaagcag tggcaggagc
23221 tggagtgggg ctgggagagc ggggcagctg gagtcaggcc cacggggtct ccaggggctt
23281 ttggggtcag cttcgggtgc caatgctgtc ttcttgcact gcgctcatgc catgcctaga
23341 agggccccag aggagcagtc acagccccat ggagctgagg acccaaggac tctttggggc
23401 cagcctgccc gcctcacctc ctcctgccat cacagccctg ggccatcgcg cttccgcctc
23461 tcacttctag ctatctttgt gcatctatct gcattccagg cccggctctc acggtaacaa
23521 tgtgtcaact cgggttctct ttttccaacc ataaaaggag aagattgggc taggttttgg
23581 agatcctctt cagcttttat gtgaaatggt tttatgattc cttgcctccc aaaggctgcg
23641 tatccccact tggcctttgt ctgctactcc ccctttctgc cttcccgttc ctctcccaag
23701 atctcctctc accccaggtt gaataacaga aatagaagga atagaaatct gaaggccggg
23761 catggtggct catgcctgta atgccagcac tttgggaggc cgaggtgggc agatcacttg

Fig. 61l

23821 aggttaggag ttcgagacca ttgtggacaa cttggtgaaa ccttatgtct actaaaaata
23881 caaaaattag ctgggcatgg tggtgcgtgc ctgtaatacc agctactgag gaggctgagg
23941 caggagaatc gcttgaaccc gggaggtgga ggttgcagtg agccgagatc gcaccactgc
24001 actccagcct ggatgacaga gtgaaattcc atctcaaaaa aaaaaaaaaa aaaaaaaaag
24061 aaatgtgaag gccaggtggt ggctcacgcc tgtaatctca gcactttggg aggctcaggt
24121 ggaccgattg cttgagccca ggagtttgag agcagcctgg ccaaaatagc aaaaccccat
24181 ctctacaaaa caaaaacaaa aaaattagct gggcatggtg gtgcgtgcct gtggtcccag
24241 ctactcagga ggctagagcc agagggtctc aggccagtct gcccctgccc cacggggcct
24301 gggcacatcc ctccctaatt cttcccagcc tctctctgac ccaggggccc tcctctccct
24361 tttttccccc tatctcagcc tccagccatc agcaacctcc tcttcctctc cacccagctc
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24541 cccaagccaa aggccctgcc tctggcctcc agccctaccc ccttctgcgg ttgcacagaa
24601 ggatggctgc ccagctctta aaaaaactgc ccgggaactg ttgacatctg ttctccctcc
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25561 aggggcccat catcatgtct gtttccagaa cagtgccttg gtcatcccac atccccggac
25621 cccgcctggg acccccaagc tgtgtcctat gaaggggtgt ggggtgaggt agtgaaaagg
25681 gcggtagttg gtggtggaac ccagaaacgg acgccggtgc ttggaggggt tcttaaatta
25741 tatttaaaaa agtaactttt tgtataaata aaagaaaatg ggacgtgtcc cagctccagg
25801 ggtgatgggg gtgatggact agatttctaa ggagagtggg gctgggtagg gagggctttg
25861 tggctgaccg agaggtgtca gaggtctgga ggctgcaggg ctgtaggggc tggaacttgg
25921 ttatcagccc cagggtatgt ttgaggtggt ggggtggggg ccgagcgaga tgaatcattc
25981 gcagctgctt ctaacgtctc

Fig. 61J

EphB4, mRNA

```
   1 ctcggcccgg cggcgcgagc agagccactc cagggagggg gggagaccgc gagcggccgg
  61 ctcagccccc gccacccggg gcgggacccc gaggccccgg agggacccca actccagcca
 121 cgtcttgctg cgcgcccgcc cggcgcggcc actgccagca cgctccgggc ccgccgcccg
 181 cgcgcgcggc acagacgcgg ggccacactt ggcgccgccg cccggtgccc cgcacgctcg
 241 catgggcccg cgctgagggc cccgacgagg agtcccgcgc ggagtatcgg cgtccacccg
 301 cccagggaga gtcagacctg gggggggcgag ggccccccaa actcagttcg gatcctaccc
 361 gagtgaggcg gcgccatgga gctccgggtg ctgctctgct gggcttcgtt ggccgcagct
 421 ttggaagaga ccctgctgaa cacaaaattg gaaactgctg atctgaagtg ggtgacattc
 481 cctcaggtgg acgggcagtg ggaggaactg agcggcctgg atgaggaaca gcacagcgtg
 541 cgcacctacg aagtgtgtga cgtgcagcgt gccccgggcc aggcccactg gcttcgcaca
 601 ggttgggtcc cacggcgggg cgccgtccac gtgtacgcca cgctgcgctt caccatgctc
 661 gagtgcctgt ccctgcctcg ggctgggcgc tcctgcaagg agaccttcac cgtcttctac
 721 tatgagagcg atgcggacac ggccacggcc ctcacgccag cctggatgga gaacccctac
 781 atcaaggtgg acacggtggc cgcggagcat ctcacccgga agcgccctgg ggccgaggcc
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1681 gcagtgtctg acatccgggt gacgcggtcc tcacccagca gcttgagcct ggcctgggct
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1921 gggcccttcg gccaggaaca tcacagccag acccaactgg atgagagcga gggctggcgg
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2101 gacaaacacg gacagtatct catcggacat ggtactaagg tctacatcga ccccttcact
2161 tatgaagacc ctaatgaggc tgtgagggaa tttgcaaaag agatcgatgt ctcctacgtc
2221 aagattgaag aggtgattgg tgcaggtgag tttggcgagg tgtgccgggg gcggctcaag
2281 gccccaggga agaaggagag ctgtgtggca atcaagaccc tgaagggtgg ctacacggag
2341 cggcagcggc gtgagtttct gagcgaggcc tccatcatgg gccagttcga gcaccccaat
2401 atcatccgcc tggagggcgt ggtcaccaac agcatgcccg tcatgattct cacagagttc
2461 atggagaacg gcgccctgga ctccttcctg cggctaaacg acggacagtt cacagtcatc
2521 cagctcgtgg gcatgctgcg gggcatcgcc tcgggcatgc ggtaccttgc cgagatgagc
2581 tacgtccacc gagacctggc tgctcgcaac atcctagtca acagcaacct cgtctgcaaa
```

Fig. 62A

2641 gtgtctgact ttggcctttc ccgattcctg gaggagaact cttccgatcc cacctacacg
2701 agctccctgg gaggaaagat tcccatccga tggactgccc cggaggccat tgccttccgg
2761 aagttcactt ccgccagtga tgcctggagt tacgggattg tgatgtggga ggtgatgtca
2821 tttggggaga ggccgtactg ggacatgagc aatcaggacg tgatcaatgc cattgaacag
2881 gactaccggc tgcccccgcc cccagactgt cccacctccc tccaccagct catgctggac
2941 tgttggcaga aagaccggaa tgcccggccc cgcttccccc aggtggtcag cgccctggac
3001 aagatgatcc ggaaccccgc cagcctcaaa atcgtggccc gggagaatgg cggggcctca
3061 caccctctcc tggaccagcg gcagcctcac tactcagctt ttggctctgt gggcgagtgg
3121 cttcgggcca tcaaaatggg aagatacgaa gaaagtttcg cagccgctgg ctttggctcc
3181 ttcgagctgg tcagccagat ctctgctgag gacctgctcc gaatcggagt cactctggcg
3241 ggacaccaga agaaaatctt ggccagtgtc cagcacatga agtcccaggc caagccggga
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3361 agggacaccg cctccccatt ttccggggca gagtggggac tcacagaggc ccccagccct
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3781 tgtcaccacc aaactcaatc attttttcc cttgtaaatg cccctccccc agctgctgcc
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4081 atgaaggggt gtggggtgag gtagtgaaaa gggcggtagt tggtggtgga acccagaaac
4141 ggacgccggt gcttggaggg gttcttaaat tatatttaaa aaagtaactt tttgtataaa
4201 taaaagaaaa tgggacgtgt cccagctcca ggggt

Fig. 62B

EphrinB2 Gene

```
   1 gcgcctcgga gctgcctgcg ggcgcacgcc gtcttccccg ccagtctgcc ccggaggatt
  61 gggggtccca gcctgcgtcc cgtcagtccc ttcttggccc ggagtgcgcg gagctgggag
 121 tggcttcgcc atggctgtga gaagggactc cgtgtggaag tactgctggg gtgttttgat
 181 ggttttatgc agaactgcga tttccaaatc gatagtttta gagcctatct attggaattc
 241 ctcgaactcc aagtaagtgg cgtccgcgat ccccctatgt ccccgccccg gggtccgccg
 301 cgccgtccgg gcgggaggag gggtcagtcc gcggggcctc ggagcctgtt tctggaacct
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 421 cggctttcca acccgagccc cgctggaacg gacggtctct ccgcctttcc tcccccgaac
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 661 cgggcggcgt ggtccggggc ccgcgggcgg gcgaccgggg tggcaggacg ctggcagcga
 721 agcgcgttct ggagagggga gcctggagtc gctacgctgc ccgcagagcc ctggagccgg
 781 ggcgccttgg caccgcgccg ccagcccgag ggtgcgcggg gagctcgcct gcttcgcagg
 841 agaactcggg cgtcgagccc tttcctccgc gccggggaga cgggccttag gcttctccct
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 961 ggtctcgatg gccgacctga ttgtaatgca cttcctataa aagcttaggg ccctgcccag
1021 tcgacactgc tcctgaagcc ttctccctcg ggaccctggt aggaatggga tccttaggat
1081 cagatttgct cttaccggac tctacagccg ggagcgagcc aggccttgtg gagagtaact
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2461 ggtctccctt gcagctgaat gctagctcca gagatcagaa agatttcttc ctgtaggagc
2521 cataggaaag agtcctctct aagtttttga gaatgcatac aacccccctga tgacaggggg
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```

Fig. 63A

2641 gatgtggcag gaaggtaatc aaatgcattg aagtttcaca tcagttccta tgaactgtgg
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2761 ctttttagc aggtggtcga cacagggagc atgcagcagc tgtttggata cagggtccag
2821 aaaacccttt gtaaattcag cgtctccgta actactttaa tcacattgtc ggctctcccg
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5161 taggcaaatc aaccctggtc atagttaata aggggattac aactcagact aggtctttac
5221 agatgtgatg taaatcaagg gcagagtata aagaaactga tccctttga ttgaagtata

Fig. 63B

5281 gtaaaaaggc atagagaaac tagcagcagt aatctgattg tatggcaata aaaccaccat
5341 tttctgtctt tcagataaaa ataatgtggt aaatccatgc agttcataag atgtaaaggc
5401 agataaaggg tgaagccatg gcaacatata gattagcttg atgttagaaa tgacacgtct
5461 ctgaaaaggg cgcgggacga aggcccttgc ctccaggctg ttgggcatta tgtgagaacc
5521 acacagactt ggaaactggg attaggaagt atgaaagctc tacttgtggt ctgggatggc
5581 tgaggcagta aagaaaagct gctcagttct tgctcattgg tggtggataa tatggcaaag
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5701 cactgcagtt gttagggcct gggagatttt ccttttaac tcctggccta acagcagcag
5761 ccgttctgta ggattaactg cacttcgcgg tcgttgcctt aatctatttg ggcttcaggc
5821 agggacatgc tgggaaggaa cagagaccag aggggatagg tagggctggg gttatctgaa
5881 aagaaaacag agacctttg atttcagcca tcttttcaga cccagctccc tctcccgctg
5941 catgggagaa gcaaaggtaa acaggacaca ttgtccctct ccctcagcca cagagctctt
6001 ctgtgagttt tgtctttccc accctggaaa aaaagataaa atacaatttt taaaagggga
6061 gggaggaatt tagttttaat tcaaatgagt agtaatccaa tatgccaaaa gcagtgggct
6121 ctacctagat gtaattttac tcgtaaatgt gagtcttaaa ctttgagttg aatggggcag
6181 gctgttagag gtggtgtaaa ttacaggatt ataaaaatgt tagtgctgcc cagccttaaa
6241 gtcaaaaaca gaaaaatctc tgtgctgttg agtcttcccg ccctctctcc tgaacaacct
6301 tgtaagtaag ctagactttt gttttgcct tccatacttt ccatttcagc cattaaacaa
6361 aataagccat tgaaaccacg attgggttcc atgcagagtg acatccgcaa tcgggtcaag
6421 ccagaaggaa atacttgctc gattgccccc tatttggcat tacaggaaag tctccacact
6481 ttggaagagt ctgaactctc aagacattga aaatgccaaa ggctgcaaac accctgtgtc
6541 tttcttgatg gagtgcatct tggtgtgttt tacaaagggg aattcagtgc tgtttttttg
6601 ttgttgttgt tgtttttttt ttttaaagag cagcataggg cccttctaga ctcttggatt
6661 ctgtgtctga caaaaatggt cattaaatga gcaatattat aatttagacc catttcactg
6721 attttgttcc aaattctcaa ctgacttgag catctgtttg gggctgtaga tacattgccc
6781 ttgttgactg tttttctcgt ttctatggga attactgtag ccattactat gtagctttca
6841 tagactcaaa acatttttaa agtattgcat ataggctggc catatccagt gcctgttact
6901 ttaccttctt tttctaactt aatgcagcag tctgtattaa cagatccatt tcatttgtct
6961 agcttcatca gagagaggct acccccctgat ttacaggctg ctcacatcca agcaccttgc
7021 attctacact tgacagtgat tgctaatggc ccattcaact aaagtatttg cttgttaaca
7081 gggaacagaa catgataaat gtccagcaag cttgctgcct ccttcagctt ttcaaacgca
7141 gactggtgca tatttatggc aggcaaatga caaaagaaaa agctgaattg ccctggcctc
7201 cagctttcta tcagaaacag ggttaaagtg attaaagcaa tcattcaaga aagccctgcc
7261 gtttgtttac taaccttcat ccaacattta gctttgtagt ctacctgtga gaagatattt
7321 cagaagtatt agagataagg aaggaggatc tagcaaacca gtgaaaagag taggtgacca
7381 gttataaaat gctttccatg cacattgaat gccaggcgaa cctatttctg ttattccagc
7441 agacaatcag cagtggctct agattattaa catattttcc tttcatgtat aaattcaaat
7501 atgtaattct agtccaaagc attctgtggc tggtaagcac atacttgctg atttcaaata
7561 agaaaacata gcaagggaaa gctccattaa acaagttgtt tctgccctta gtaattctct
7621 aaacaagata ggaagaaaaa gtggacagta gtggagtatt aatagtgtgc tcttttcatt
7681 ctctaaagca cgagtaagta agcgttcaaa ctactctgtg gtgggcatac atttagagcg
7741 ctgtgaatga accactgctg ttctgccata cttaatttat ttatattatt atttttattt
7801 tattgttgtt tttatgtatt attataatta tttatttata ttactaattt attttctcaa
7861 tttaaatcct gttgcatcca attttaatta cagtttttgt atctgccttc ccatacttgc

Fig. 63C

7921 tacccacgtc cccattgcca ctgcggcctt atccatgttt tctgtgtaca ccactctcgt
7981 atcaccccag aataattatg agtgctaccc agacttttga aaccactaga gtcaacatgt
8041 ttgtctttga ggaaagccaa tgatgcttta gcatttttgg caggggtgga tgtgtgttta
8101 agtggggtgg gtgcagctcc ttattgtctg cctattctac tgttgttccc aatccacatt
8161 ccctgcgggg cacctaacct gtgtgcatag caaagaattt ccgaccttca gagccagaag
8221 tgtttctcaa ttgatctctt ccagcctagg gttatagctg atgaattata atccttgctc
8281 tttccacacc tttacctggg cttaccatgg ccctaaaaca tttgcccaga atcagaattg
8341 tctcatgagt gagtggggca aggcaaatcc tgttccagac cagctgagaa tgtacctagc
8401 tgcagaagaa gttagaaagt gtcatctttt acttatctac cagaactata ttcgaggtac
8461 attttagatt taaaaaaaaa gcaagttctc gtaggccttg aatccccccc ttgctatggg
8521 aaaatggatc attattataa tggactgtcc agtaaagttc atgatttctc ctagacatgt
8581 tctctctctt tatgacctag atcaagagtg atctctttaa gtcttttctt cataatccca
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8701 atgcaagcct tcaccactct acctgtccta aaagtcaggg acacaccttc ttcatttcat
8761 cagtccctac ttctatccag cattggcatc cagtaagtat tagtggaatg gacagacaac
8821 ccgaatttgt gctgatggca gtttaccctg ttttaactgt catccttctg ctactagaca
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8941 ccagaatccc ctgcagggct tgagaaaaca ggggtttctg ggccccaccc ccagagttcc
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9061 cgctggcaac tgctgcctca gggccatgct gagaaccctc gccctacaca aacctttctg
9121 ggaaaacaac tcaacattaa agctgtttgg ggatctctga agaaatctgt agtccttgcc
9181 ttgttggggg agcatcaggg atctaaccat tgatggtgga gtatttgttg ttaattcagc
9241 aagcaactat taagtgttag gcctgttact cggctctaac aatacaaggc agagtgacct
9301 gtaccctcga gatttaaagt ctaagtcctg tagagagaag cccaggtggg agcaagcaca
9361 tttagagtta ggtgcttggt gcaaggtggg gacacagaag aagggaatgg catttgcctc
9421 tggaggggtc cggaaacagc ctagggagga ggagcttgag tcttgaaata ctgtgggcat
9481 ctctaagcaa agtcacagta gacagctgaa ataaagaaaa tagtaagcaa gccaaagaaa
9541 cagtatttca gccaagggca gcgtgtgtct atcacgtcca cctgtgaaca cgtcccagga
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9661 ttcagctacc tgttcacgtg agaattatca gtacctactg cttttcaaaa tgagtatgat
9721 catggatagg tgaggcaatt cagtttcgca gagacagtag ggcaagtgcc actgtagttt
9781 agttaagggc acatgcttta gagtttggct atgtgagtcc aatcccagtt tagccattta
9841 ttagctgggt agctttagga gcagtagcct tagtgtctct cagttgtccc atctctataa
9901 tagggacaat aacataatag tgctgaataa aagagtaaca aaattttggt caacatttaa
9961 tgtatttaaa gagctaagct ccgtgattgg cacaatgaac caatcaatca aacaccagtt
10021 gttattaata aaagtcagtt gaatatgtac tgtgtgcctg gccgtggttc aatttgcctt
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10141 cttaaacttc ttgatgttaa tttattttgt ttacctgcca aacttctact cattccttat
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10321 ttcagctgtg caactgtgga ctgagaggag gctctttgaa ttcattttgt atattctagt
10381 agagagtact gtgagcagtt gggttgttga atgaatacat taattcaacc tggagggatg
10441 ggcagtattg catttttttac attgatatta catgatattt agaaaactgc ttaactggtg
10501 gacgttgttt tattaacagc attttgtgta tagcactcac tatgtgccag ctgctattct

Fig. 63D

10561 aactgcctga caaatactcc tgaaaccttc atggtaacca tatgagggaa gcacttttaa
10621 tatatccata ataccaacgg ggagactgtg gccaaattgg ttaattaact tagccaaagt
10681 catattgaac taataagtgg atttaaaccc agctagtctg gggccagggt ccctctttta
10741 atcttctgcc tcctgcttat gctgttgcat ggagtagtct ttatcatata actaaattaa
10801 gcatgcattt gcttaaagca gtgcatacat gatggatcaa aaagtttgtg gtataattgg
10861 tttaattctg tcattatcca ttttgattta tagtcacttt cttatgatgg tcgtgtagtt
10921 ttaaatggaa cctttgaatc tttgatataa taaggttatg tcaaatcttg ggtataataa
10981 ggttataccc aatggaaaca gaataatgat cagcccattt aaaggatgac tggagagtta
11041 ttacaataca taatagtcat gcatatattg agtagtattc ctttggtaac attttccttt
11101 taaaaattgt aacatttgat tgttccttgt tgggagaaaa ggaggtcaga tttttgaggg
11161 gagatccatt tggtgagatg ctgagtgtgt gtcaagctaa ggagatagta tgacatcttt
11221 tttagagtct agtcacaatt aaatgccatt ttattttgga ttttgggatc cgtgccagct
11281 tccagcttgt cagagctgag aagactcaaa tcaagtccag gcttatttct acagcaaact
11341 gggattctgg cttcttgccg gtggattcat tcagtacagc ccatctggct tttgatgttc
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11461 tctcttgact ccaagtggtg ccccttggtt tgcattttca ccatgcttag catctgctta
11521 cctggagacc atgcagccgc cggccagagg tctccaacaa ccaaatcttc atgcctttta
11581 gaactcagag tccccagcac atcctccttc ctcctccttg tccaattact ttcatgcagt
11641 tctcagtagc tgcttgtttg aatcacttat agtatttaac ttctagggtg tttttgggtt
11701 ttggtcaagg taattccagg ctgaatgtgg tgactaagca ggaaataaat gggtcgtcct
11761 caaagttaca gtggagcgct gtttctattt tcctaaggta cacagttgtg ggggcgatcc
11821 gtatggaagt caggaaccca gtctgatttt gcttcctttt gatggtagca gtacagacct
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11941 caaagccctg agacgtgcag gaaaatgtct cctgtcatac gcccacagca gacctagccc
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12061 tgcacacagc cttgtaaact gtgttactgg gctgattata cttgattggc aaggtgaatc
12121 tcttatagca aaagagaact tggagagttt tatctcatct tatgccttat taatttgttc
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12421 gaaggaaagg gatgggcaga gggttaaaac aaaagatatg ctctccctaa ctctagattg
12481 tagtattgtt atgcttgtca ctgtagctga attccatttc tttgagtttt ttcaatgcca
12541 aggcattccc tgtatgactt acgtgagcct ttcatctccg cgatttttcc cattcaggta
12601 aatgagcaaa tggatttgaa cactcatatc taaaacaaga gagaaccagc tggaaatgcc
12661 ctttgaattt ctttctctat gtaaaccatt tttctttctg gtgcctcacc tataaataac
12721 aggagttcca ccttccttta tagactcttg ctgaaagcat ggtttggaac aagaccgtac
12781 aggtgcacac aaattacagt tgggaaagaa gcctgcagtg catcttgtct ctgaaggtta
12841 tgaaatcctc cttttagtaa tggagctggc gtgatcaagc cagcaggatg aaatttggca
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12961 caaatctcca catttttttct tatctagcta caaaattcat aggctgattt ttttggggtg
13021 cgtgtgtggt tttttttttg ttttttttggt aaataaagac ctgcattttt attttgatat
13081 aggtggttga gttttgtctt taatttcatg acagagattt aactagtctc aacttttgaa
13141 aagacaacaa tgatatttgg ggatcacaca cttaaagtta gatttctaga tgattaatac

Fig. 63E

13201 caaagtagat gatttttag cctcagccat ttataggtat gcccttctgt gaatttttta
13261 tgacagtgaa aatcatggca cagataaaaa ttaaataaat acttctgtta ttttcctgaa
13321 gaaaaaaaaa aaaagcttaa actatgagaa tactgtcttt gagcacttta aaataaaatt
13381 gacttcagcc agcaggattt tgagcattac atcacaaata aaaaacaaga ttaacatcaa
13441 aaggagtcag ttttcattca attgtgcagc actgtgggct gtgaaattta atattatttt
13501 gactcatatg ctaattgtag actgacagag gaaaatggat tgtgtttaaa taaaaggata
13561 cacagcatca cacgcagctg tatcaaatac aagttgaggt ctttgggcca ggaactgggg
13621 gccctctagc tctgttattg cagattcaag tttgacaaat aaaactttcc tttagactgt
13681 agtttaatta ctttttttca aaggtatgcg tgatgaagag gcacaaatac acctcacctt
13741 gaagagttgc taaactggtt tgtgtgccga tcagttcacc gtgtgtttga atttctgtgc
13801 ttctcatctt tccttttctt gaaaagattt tgcttgtcat tggtgtgaat tgtacccccc
13861 acccccaccc atctagtctt tgctctcaga tttataacac tttaatggtt ccaaattgta
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14041 acacatatac atccaaatta aagcagaaaa tgtcaaagcc tccaatcact accttatttc
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14161 acttttttact ctaagcagta gtatctggta acctaattcc gtataaacct gacaccctat
14221 cgctacaccc cagtatttct ctgatttcag aataagtctg cgtagaaact tgttctgatg
14281 ttaaagtgca aaaggggggca gtaaagtgct atccacaaaa aaggaaaaac attttccaag
14341 tatttcttat tactgcctgt gtctttcgta ggccctgcct ttatttattc attttataac
14401 aaaactctta tgtttggggc attcagagaa taccttatta agctgttgca gcaatctagc
14461 attaaatgga agacatgcaa gactgaagat cctgcctgtt tatgaagtgt gccatcaaat
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14641 gcaggcccaa agtacatggt ggggtgaagg gtcaagggtt tgggcttatc cagagcagcg
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14881 atggggtaaa ttcgaagtat acttgataca gaacactggc ttgggaataa acctgtgata
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15121 ttaaataggc cacaacttca actaggataa ttttatttat ctgcttgtta gggaattgca
15181 tcaaaagttt aagtctgtag gcattaaata ttttaaatgc ttatttttaa agtcaattat
15241 gaaagatagc acaaagtttt tctgaaacta cattaaaaaa ataatgtttt aatcttatca
15301 caaaagcatt gactatttat tgcaaagaaa acacagaaag ctaaaaatca ttctaagtcc
15361 accattcagt agcccaaagt ggtctcaggt aaaggcggtg tgtgtgacca tttgtttatg
15421 gttgtctccg tgcagtcagc aaaataaaca gaacaacatg ccatatatta ttgatgtgta
15481 tattttcaac tgaaattagc catctgctta caatgatcat atacactaat ggtataattt
15541 tgaaatgaaa agaaaaataa aataattctt tgtggagagt aatgcgaatt gacttatgaa
15601 tctcgccctg cttggcagtt tgctctagag gtagaagagc tttatgtgtg ggcctcctcc
15661 ccccccacac atttattctg ctcacacttg caccagcatc catgtcagga ctcaccttgt
15721 cctgttacat gagtaacatg gccctgattc tcaagtgcat gataactgcc ataattacac
15781 ataaatatta aatatttaaa tagatcttta cgtgtgtaat attaggtaga agtggctctg

Fig. 63F

15841 gatcgaatct gatgcttttt aaatagaagc tttcccacaa catttccaag cactgtcatc
15901 gtgtctgtct cgatttgggg tttacctggc ctagttatct gtctgggtgt agaaactggt
15961 agttcctgtt tgtatctttt ttgttctgat ctctttattc tgtgtcagct aaatattctt
16021 gcagtcagtt actaacatat taactcatcc ttgtttggaa actttggcat atccttccat
16081 ggtttccttc cgtggacctg tcgcgtctct caggagagcc accaggtata ttgtcacaca
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16321 tggccgtggg tgggagggga agcagggagt gcaatttcgg gttcactaca cagctctcca
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16441 atagatcagg cgaccactgc ctccgtccat ttcccaggca ccctgtgaga cccgataatg
16501 caatacaggt cagcagaaaa gtccagactt gacatcccaa cgtgccatgg tctggtctgt
16561 gaatgaaaat cacatgaggt gacctctgaa ctctaagtgg ctggtttatg ttttcagtgt
16621 attaggcccg tgttttaaac aagcatgtgc tcgtagtgta ggttaaaact ttctgttgtc
16681 ttcattaatt atgctgtgtt ctagtctatt aatattaaag aatattgtgt tgcataatga
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16861 ctccgagtaa ctaggactac aggcgcccgc caccatgccc agctaagtgt tgtatttta
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16981 acccgcctca gcctcccaaa gtgctgggat tataggcgtg agccaccacg cctggcaaca
17041 taaggactat tttttaaagt ttttacaatt atgactgtga agttgaaatg tctaaattat
17101 tagagatcca gtttagatta ctaaatattt atgtctaatt gagatgatta gacttagcca
17161 aagtatccat gtagaagtat tagagtctag attggtgaaa aacttgaaaa agcttggctt
17221 aagttcaata ggtaatccaa gagtaaaaac agattccaat atcagatctt ttcaccatag
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17701 cagactttat taaatgacac ctctctctaa ccactctctg tctgggcgaa gtttaacaag
17761 aacagcctcc ccccatgtgg tatgggttgt aactgtggcg gtttccctct gctgtttttg
17821 gttacaagat gaacattatc tgaacacaca gaaagaaatc tgtatttggc atccataatg
17881 gaaagtcagt ttagtaattt aaacttagcc agttatcatc atcataattc tttttaacac
17941 tttcaaagtc agcataggag aagtgtattg ttgaatatta caaaatattt agggcataga
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18061 aatataaacc ccatcacttc caaaatagga actctgttta ctgacttgat tataacatat
18121 ggaactcaat tgttttccat taaaaaatga tactattagg aaactcaccc cattttcttt
18181 tcatatatat tctgctattt gcataattgt ctggagtcca tatgtaatat taaatgtaaa
18241 acacaaatgc catgtagctg gtctgtttct tcctcacctt ttggttcctg gcctcctggg
18301 gaagggttgc acatctgagc cgtggtctca gatgactgcc tcggaagaag cctcttccct
18361 tcaggcacca ctgatgtgtg cttggtgtgg agctagactt tccctggctc tccatgtgac
18421 gctcacatgt gcgtgtcttg atttccctta acttcatggc ttatctatga acagcttgat

Fig. 63G

18481 ttgggggaaa aaaatgtgtt tcccaatgct ggagttataa ttgaatgtgc tgcagtcaaa
18541 actgaaatgt gtgcagagaa agggggcttt tcctgtcatg ctcattgggc accagtgtgt
18601 cttcacctgt tttgtgtgtt aggtccatgc gtcatgctga aatgaagaac atgggatgta
18661 tggggctttg gacagtgctg agccaaaagc aagtgctcaa aagcagctgt gtttgtatta
18721 ttagtggttc tggaggtggc tgattgcctt gcattttaag tagagaggga ttgtagaaga
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18841 cttgctctcc agaaatgcca gtgtgcctgg gagggcatct tcagaaatcc agtctctcct
18901 cctcagtgtg tcctgtaccg actcagtggt tctgtcttca gaattcctat catgtctgtg
18961 atctgcaaat agtggtattt aatttgactt caatttgtat aaatgttagc ttctatttgt
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19201 acataggtta cttgagttgt tacactcaca gtactgttgt ttgctgccat ggtgctttag
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19321 ggccaggaga gctgtagttc aggctctcag ctcaactgca ctctgtccac tgagaagcca
19381 taatttcttc acttaaagtg actgtgcgct atggctgttt atatatacgc ttaaaaagta
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19501 cattgtttta atgagctcta gaaacaatta cttttgaaga gccgaggatc aaattcttgc
19561 ctcacgtttt gccacagtgt gttctgaaag gtgaattaat gcttttggaa tcatcaggaa
19621 tagtgagctt tgtcacgatt tacttttttac aagcgtatct aatatgcata ttgaaatgtg
19681 agcctcccca ccacacttcc gctttgataa gcatcccccg gattgccgtc actgaccatt
19741 atagattttt aacaaagttg gacagtacac actgaatgaa aactttacat caaggaaggc
19801 ctggcgtgtt tgtaaaatga attaaaaggc tcattaaatg atttatatga cttacgcctt
19861 ctgaaaatat ggcctcaaac acagagatcc ccaaagccac accgacccct gcgtcccatg
19921 ttctcgacct caccgcatca gcaccagcaa gacctgtcgc tgagacggtg agtgatgaga
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20761 gtgatttgca tttgtcaaaa gttctagaga attcttcaac agtacacaca tggttgtttt
20821 aaatatatca ttgttataaa aattcgtttt gagttctgtt tcacagaaag ttttttgaa
20881 tgaatgaatg tcatatatcc ttgctaaagg agctcagtta aaaaaaaagg gaccatcctt
20941 ctcttttggg ggttgtacag taacacattc ccaagaaaga ggtaacagcc acatacattt
21001 ttcttcccaa taaagagtgt gggtttttaa tatgaatcca tagtatgatt tctgttatgt
21061 tttgtgctgc ttcataacca cactcatgca cttttcagaa aattaatacc attcattagc

Fig. 63H

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21241 aagaaagggt tggattggaa ggattcagag gcgattcttt gttcttaggc tttaatattt
21301 taatgagcct gcaggcttgg ctgcttacga acgagctgag atttctaagt gtgttgttag
21361 tgttagcact tgtagaagga tgttcattag gaagttcttg tttcagtttt tcagagaaac
21421 tccccattaa gaaagatcat tcaggaacat ggctaccaag aaagaggaaa gggaggaggg
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21841 aagtattagt ggatccactg ttccctccaa ttgacgagct ttctctgtgg catgcccaat
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22201 ggcattgcct ggctatcttg taactgtcat taatactgtt aatttttatc aactcaatgg
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23641 ggctggcgtg cagtggcatg atcacagctc actgtagcct tgaactccag ggctcaagtg
23701 atcctcccac ctcagcctcc aagtagctcg gactacaggc atgtgccact gcacccagct
23761 caagagctac acttcaaagc acagaatgaa aacctatttt taaagccaac ttgatacata
```

Fig. 63I

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24061 aagactttat ttttttattt tattttattt tatttttgag acggagtctc tctctttcgt
24121 caggctggag tgcagtggcg tgatctcggc tcactgcaac ctccacctcc tgggttcaag
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24841 tttataatta taatattaat ctacacaata acgacatcta ttattttctt tttttggaaa
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26281 tatgcatcct cattcattct tctcatagat ggttatttgt tttttaactt gtggcaaaat
26341 tatatatgaa tggtcaccga cttaaaatag ttccacttaa atttttcaac tttctgatgg

Fig. 63J

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26701 taatttacct aacctctagt tattttatta ttgtctttaa agtttttttt aatgttcatg
26761 aaataactgt tctgaaattg cctattttca agggaagctg tgtcttagac ttactaaatg
26821 ctccagttga tactgggaaa gccttcttgt gttcgtagcc tttatccgta gagttttctt
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28981 ttcccagtgt gtgtcagtga gactcctgat tgaatttaat atgaataaag ataaattctt
29041 tacatttaag gattaaagtc tcagcttctg cttaacttga gattgcactg agaaactcct

Fig. 63K

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31621 gactctccac attggccact cccggattcc ctagctcagg acacatattc aggtgtgtct
31681 aaggctggct cttctatgtg aagttactta ttcttttacc attgactctc atgttcccac
31741 tatattaagt ttttctgaat tactgtggca ataagaaacg gtcccttaaa ttatactaga

Fig. 63L

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33241 tttgggaggc cgaggcgggc ggatcacgag gtcaggagat cgagaccaaa ctggctaaca
33301 ctgtgaaacc ccgtctctac taaaaatata aaaaatgagc cgggtgtggt ggcgggctcc
33361 tgtagtccca gctactcggg aggctgaggc aggagaatgg cgtgaacctg ggaggcagag
33421 cttgtggtga gccaagatca caccactgca ctccagcctg ggtgacagag cgagactccg
33481 tctcaaaaaa aaaaaaaagg aaaataaatt cttctgtatt tttctttctt caagtgaggc
33541 catttagggg aaagtatacc ataaaacttg ctctaagata aggcaaattt ggtattatag
33601 gatgaagtgc tatgtgattt gaagtaatgc tgaatttttt aaatatatta aactaaacaa
33661 gaataatgag gccctcggaa agtcatgatt atatttctca tttttctcat tttaaagcca
33721 cagtgaaaaa cacataaaag gaagaagtta gaaaaaaaaa tgaatgaaat tctttttttc
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33841 aacagtcatt tatttttggc attcagtgaa cactatcatt tccatgttta gaacttttct
33901 tctaagttag catcttaaaa gataactgtg aaactcaagg cattcaacta cattaatttg
33961 agtttcagaa attgaattct tgtttctaga gtacatagtt tgaattgatg tcagggtgtt
34021 aaatagataa atcttagctt cctaggttgt atattcacac taattatttt tttatcagcc
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34141 tgtggtagaa gaaaacagta atacagtttg gtttgttgtt gtgtttgtgt ttattttaaa
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34261 caagataaca gtcttccgat cttcagaagt ctgtatcaag cttaaggaaa ctgatgtgta
34321 ggaagactcg cctaagaagt ccaaattagc aaggctagca tgtgaggaca tgctggaaaa
34381 gaatagttcc catagatatt gacagagaat gttcataaaa tgctacttgt tttgtggtta
34441 catgagagta acttgtgtcc agtgcagctg tatgtaaggg caacgttttt attctgacga

Fig. 63M

34501 ctctgtggtt ttcatgaccc tggatgctta tcatgtctct ctgttggact tcttcaacgg
34561 agttgataca aatacttgct tccaagtgtc catctgccct ctcctccatc ctggccccat
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34681 tgcttcattc tttccataag aactgtgata ccattattct gtaggatttt tttgtgcttc
34741 cccgtttcac atctctgtgc cagtgagacc catatatcgg tgcaaatcca gaagtttgat
34801 tgtccatctg attagcacac tgttagcaat gtggtggact aaacacagcc aagatgtggg
34861 gctggagctt agcctcctgg gagcagagcg gtgaacatca gatgaagaca tgtgaaaatg
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37081 gattccacct gaatgaagta aaccaaacat tttaaactat cagccagata gagacatcag
37141 cctttcactt ctttctatat gcagacatat cctaattttt tagaaaaatc aaataggaaa

Fig. 63N

37201 attctcaaca attaattgaa gattatagct ctgctctgaa atggtccaga aataggatct
37261 gctcatagaa actcatagtt tgaagcctct gggaggaaag gatactttaa aatttagtca
37321 catatttgga ggagggaaaa gggaaagagc agaatgaaga actgaaaaaa atcacacacc
37381 ggggcctgtc gtgaggtggg ggactggggg agggatagca ttaggagata tacctaatgt
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39661 aaaatatcca gaatgaaaac taaaagcttg tgcagttttg ctcatttctg aatcttgact
39721 acagaagagt tttgttcatt gtgacttttc caatatagat aacctattgt gcagaaagaa
39781 ataattattc ttctaattaa aaattggtat agtagtcaat caacttgctc agttaaattg
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Fig. 63O

39901 gcctcacgtt ctaaacctct gaaataacta gtataaccat tttgttttaa aagaaaaatt
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42481 taccggactt gtcacacgga cctcgggcta gttaaggtgt gcaaagatct ctagagttta
42541 gtccttactg tctcactcgt tctgttaccc agggctctgc agcacctcac ctgagacctc

Fig. 63P

42601 cactccacat ctgcatcact catggaacac tcatgtctgg agtcccctcc tccagccgct
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42721 tgccctttag ccagatgcta ggctgtctgc gaagaaggct aggagttcat agaagggagt
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43921 tgccttatgg gctgaagtgt tctctaga

Fig. 63Q

EphrinB2, mRNA

```
   1 gcgcggagct gggagtggct tcgccatggc tgtgagaagg gactccgtgt ggaagtactg
  61 ctggggtgtt ttgatggttt tatgcagaac tgcgatttcc aaatcgatag ttttagagcc
 121 tatctattgg aattcctcga actccaaatt tctacctgga caaggactgg tactataccc
 181 acagatagga gacaaattgg atattatttg ccccaaagtg gactctaaaa ctgttggcca
 241 gtatgaatat tataaagttt atatggttga taaagaccaa gcagacagat gcactattaa
 301 gaaggaaaat acccctctcc tcaactgtgc caaaccagac caagatatca aattcaccat
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 421 ttacattata tctacatcaa atgggtcttt ggagggcctg gataaccagg agggaggggt
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 541 atcaaccagg aataaagatc caacaagacg tccagaacta gaagctggta caaatggaag
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```

Fig. 64A

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2761 cctccgaagg catctcacgg aaccgtagac taggaagtac gagccccaca gagcaggaag
2821 ccgatgtgac tgcatcatat atttaacaat gacaagatgt tccggcgttt atttctgcgt
2881 tgggttttcc cttgccttat gggctgaagt gttctctaga atccagcagg tcacactggg
2941 ggcttcaggt gacgatttag ctgtggctcc ctcctcctgt cctcccccgc acccccctccc
3001 ttctgggaaa caagaagagt aaacaggaaa cctacttttt atgtgctatg caaaatagac
3061 atctttaaca tagtcctgtt actatggtaa cactttgctt tctgaattgg aagggaaaaa
3121 aaatgtagcg acagcatttt aaggttctca gacctccagt gagtacctgc aaaaatgagt
3181 tgtcacagaa attatgatcc tctatttcct gaacctggaa atgatgttgg tccaaagtgc
3241 gtgtgtgtat gtgtgagtgg gtgcgtggta tacatgtgta catatatgta taatatatat
3301 ctacaatata tattatatat atctatatca tatttctgtg gagggttgcc atggtaacca
3361 gccacagtac atatgtaatt ctttccatca ccccaacctc tcctttctgt gcattcatgc
3421 aagagtttct tgtaagccat cagaagttac ttttaggatg ggggagaggg gcgagaaggg
3481 gaaaaatggg aaatagtctg attttaatga aatcaaatgt atgtatcatc agttggctac
3541 gttttggttc tatgctaaac tgtgaaaaat cagatgaatt gataaaagag ttccctgcaa
3601 ccaattgaaa agtgttctgt gcgtctgttt tgtgtctggt gcagaatatg acaatctacc
3661 aactgtccct ttgtttgaag ttggtttagc tttggaaagt tactgtaaat gccttgcttg
3721 tatgatcgtc cctggtcacc cgactttgga atttgcacca tcatgtttca gtgaagatgc
3781 tgtaaatagg ttcagatttt actgtctatg gatttggggt gttacagtag ccttattcac
3841 ctttttaata aaaatacaca tgaaaacaag aaagaaatgg cttttcttac ccagattgtg
3901 tacatagagc aatgttggtt ttttataaag tctaagcaag atgttttgta taaaatctga
3961 attttgcaat gtatttagct acagcttgtt taacggcagt gtcattcccc tttgcactgt
4021 aatgaggaaa aaatggtata aaaggttgcc aaattgctgc atatttgtgc cgtaattatg
4081 taccatgaat atttatttaa aatttcgttg tccaatttgt aagtaacaca gtattatgcc
4141 tgagttataa atatttttct ctttctttgt tttattttaa tagcctgtca taggttttaa
4201 atctgcttta gtttcacatt gcagttagcc ccagaaaatg aaatccgtga agtcacattc
4261 cacatctgtt tcaaactgaa tttgttctta aaaaaataaa atatttttct cctatggaaa
4321 aaaaaaaaaa aaaaa

Fig. 64B

EphB4 Precursor Protein

```
  1 melrvllcwa slaaaleetl lntkletadl kwvtfpqvdg qweelsglde eqhsvrtyev
 61 cdvqrapgqa hwlrtgwvpr rgavhvyatl rftmleclsl pragrscket ftvfyyesda
121 dtataltpaw menpyikvdt vaaehltrkr pgaeatgkvn vktlrlgpls kagfylafqd
181 qgacmallsl hlfykkcaql tvnltrfpet vprelvvpva gscvvdavpa pgpspslycr
241 edgqwaeqpv tgcscapgfe aaegntkcra caqgtfkpls gegscqpcpa nshsntigsa
301 vcqcrvgyfr artdprgapc ttppsaprsv vsrlngsslh lewsaplesg gredltyalr
361 crecrpggsc apcggdltfd pgprdlvepw vvvrglrpdf tytfevtaln gvsslatgpv
421 pfepvnvttd revppavsdi rvtrsspssl slawavprap sgavldyevk yhekgaegps
481 svrflktsen raelrglkrg asylvqvrar seagygpfgq ehhsqtqlde segwreqlal
541 iagtavvgvv lvlvvivvav lclrkqsngr eaeysdkhgq ylighgtkvy idpftyedpn
601 eavrefakei dvsyvkieev igagefgevc rgrlkapgkk escvaiktlk ggyterqrre
661 flseasimgq fehpniirle gvvtnsmpvm iltefmenga ldsflrlndg qftviqlvgm
721 lrgiasgmry laemsyvhrd laarnilvns nlvckvsdfg lsrfleenss dptytsslgg
781 kipirwtape aiafrkftsa sdawsygivm wevmsfgerp ywdmsnqdvi naieqdyrlp
841 pppdcptslh qlmldcwqkd rnarprfpqv vsaldkmirn paslkivare nggashplld
901 qrqphysafg svgewlraik mgryeesfaa agfgsfelvs qisaedllri gvtlaghqkk
961 ilasvqhmks qakpgtpggt ggpapqy
```

Fig. 65

EphrinB2

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

Fig. 66

Fig. 67

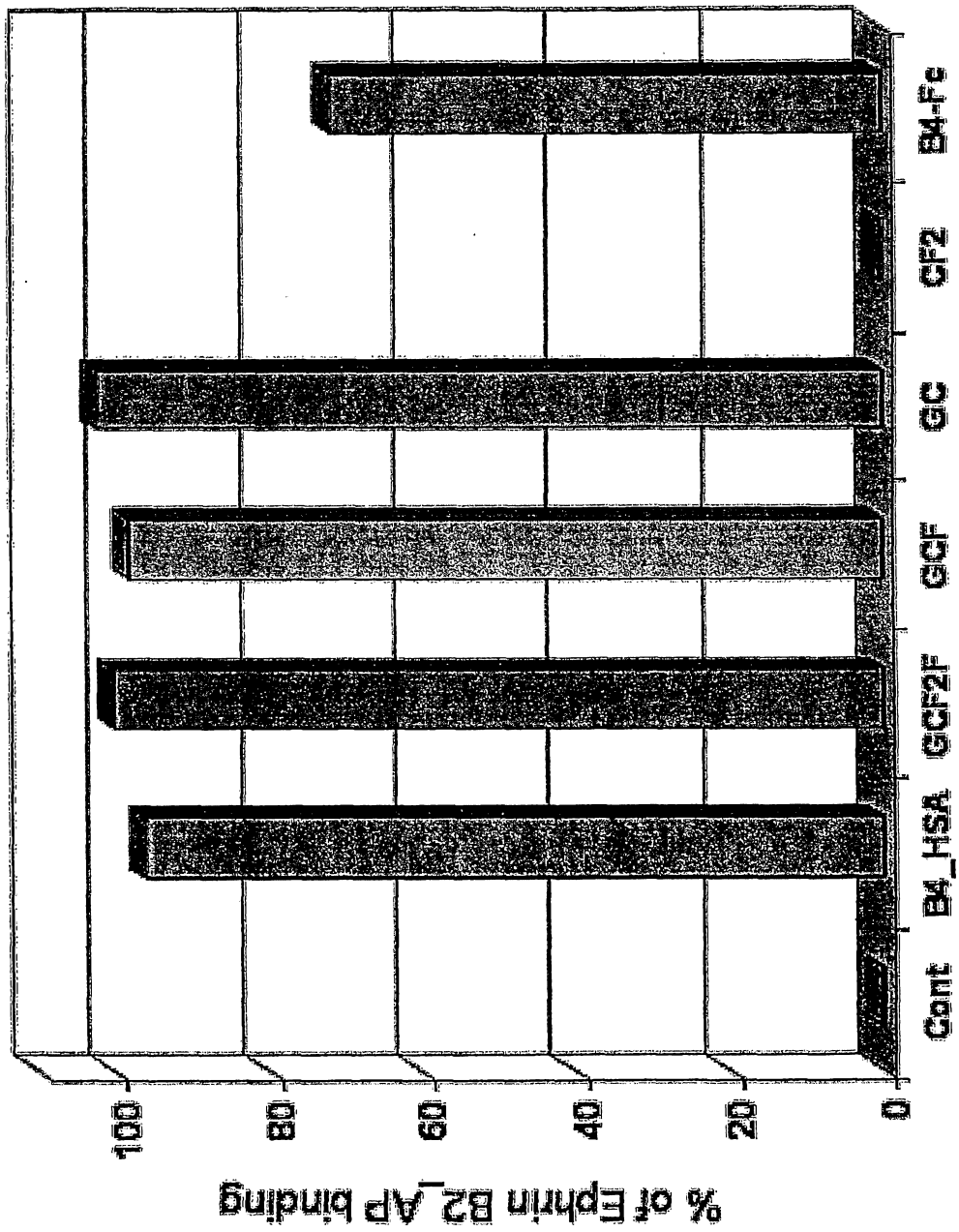

% of Ephrin B2_AP binding
100
80
60
40
20
0
Cont
B4_HSA
GCF2F
GCF
GC
CF2
B4-Fc

Fig. 68

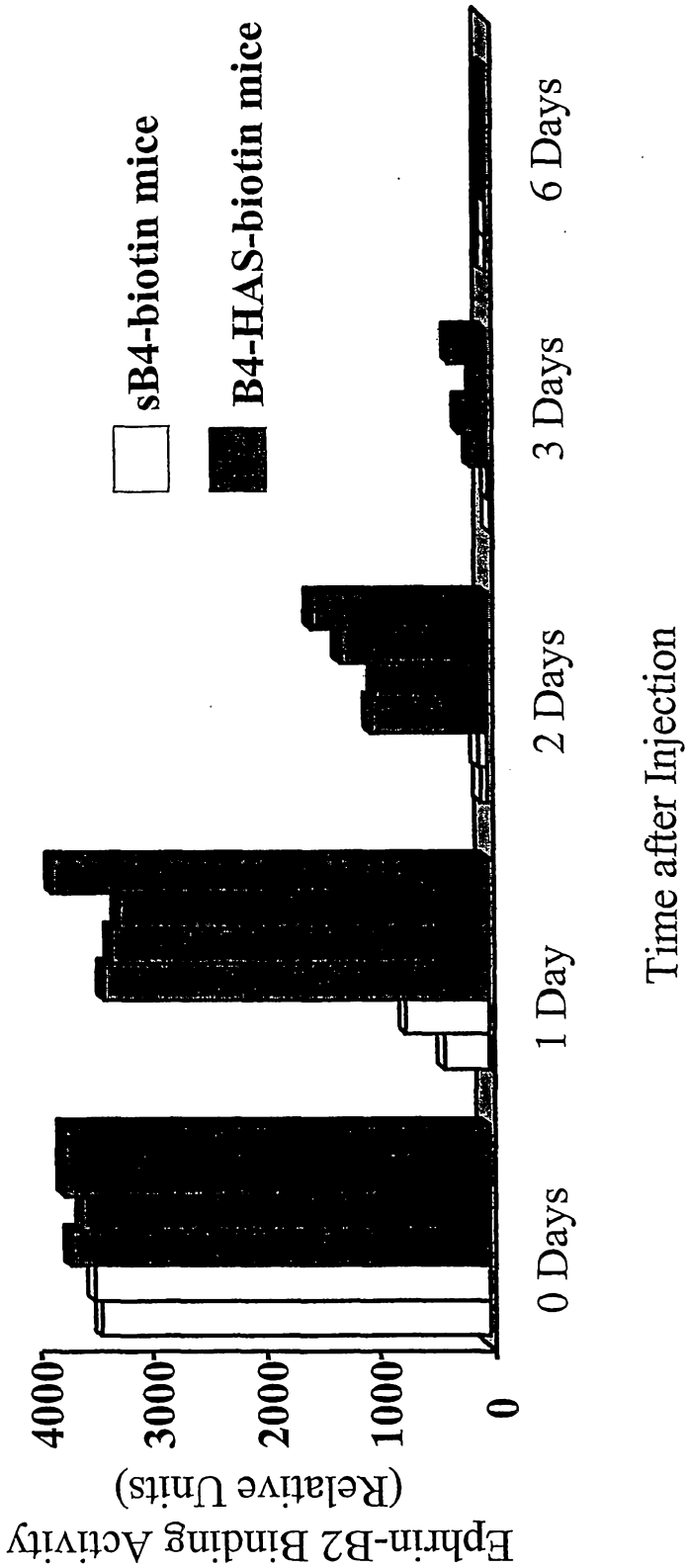

sB4-biotin mice
B4-HAS-biotin mice
Ephrin-B2 Binding Activity
(Relative Units)
4000
3000
2000
1000
0
0 Days
1 Day
2 Days
3 Days
6 Days
Time after Injection

Fig. 69

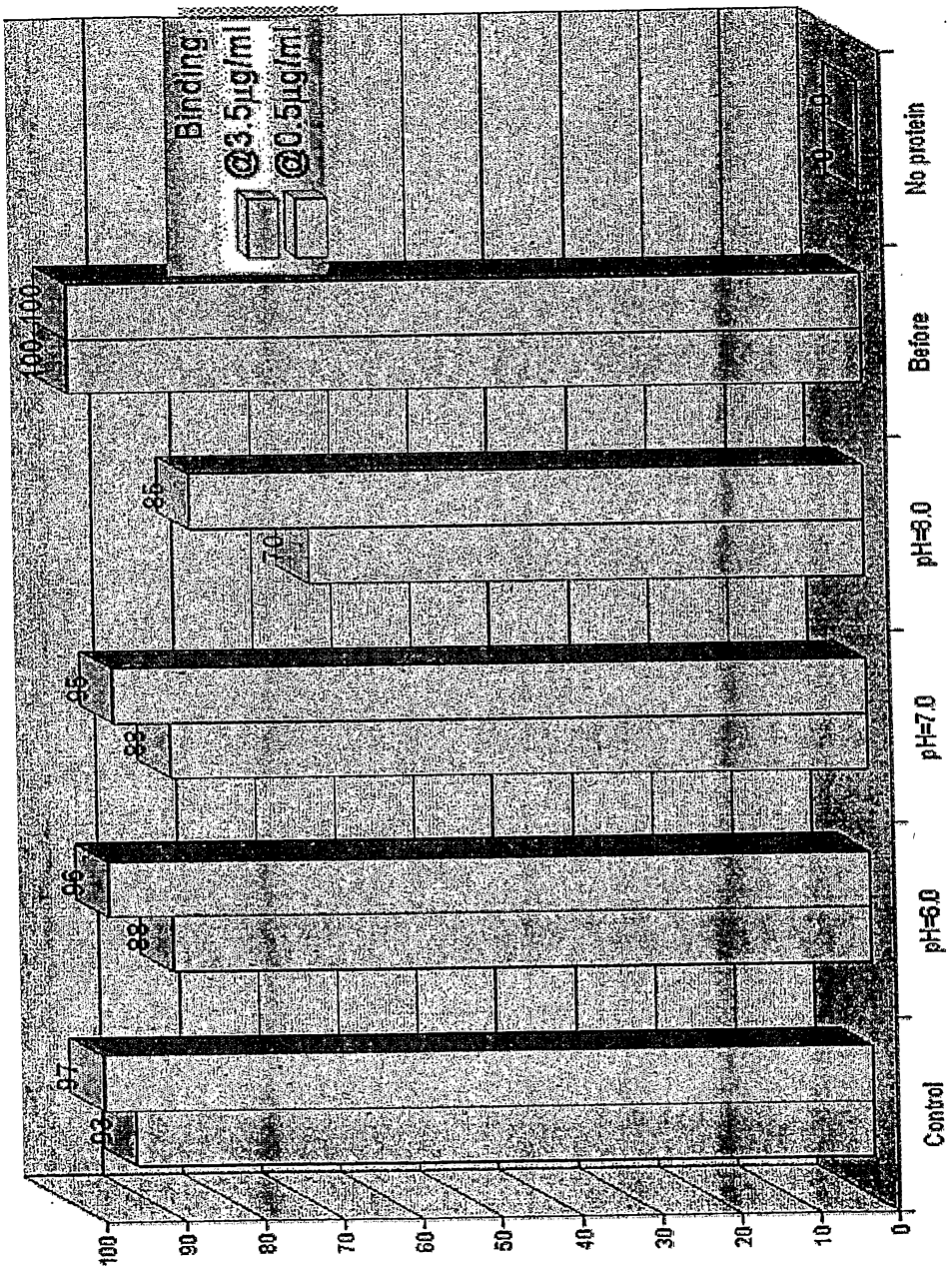

Binding
@3.5µg/ml
@0.5µg/ml
100
100
85
70
95
88
96
88
97
93
100
90
80
70
60
50
40
30
20
10
0
Control
pH=6.0
pH=7.0
pH=8.0
Before
No protein

Fig. 70

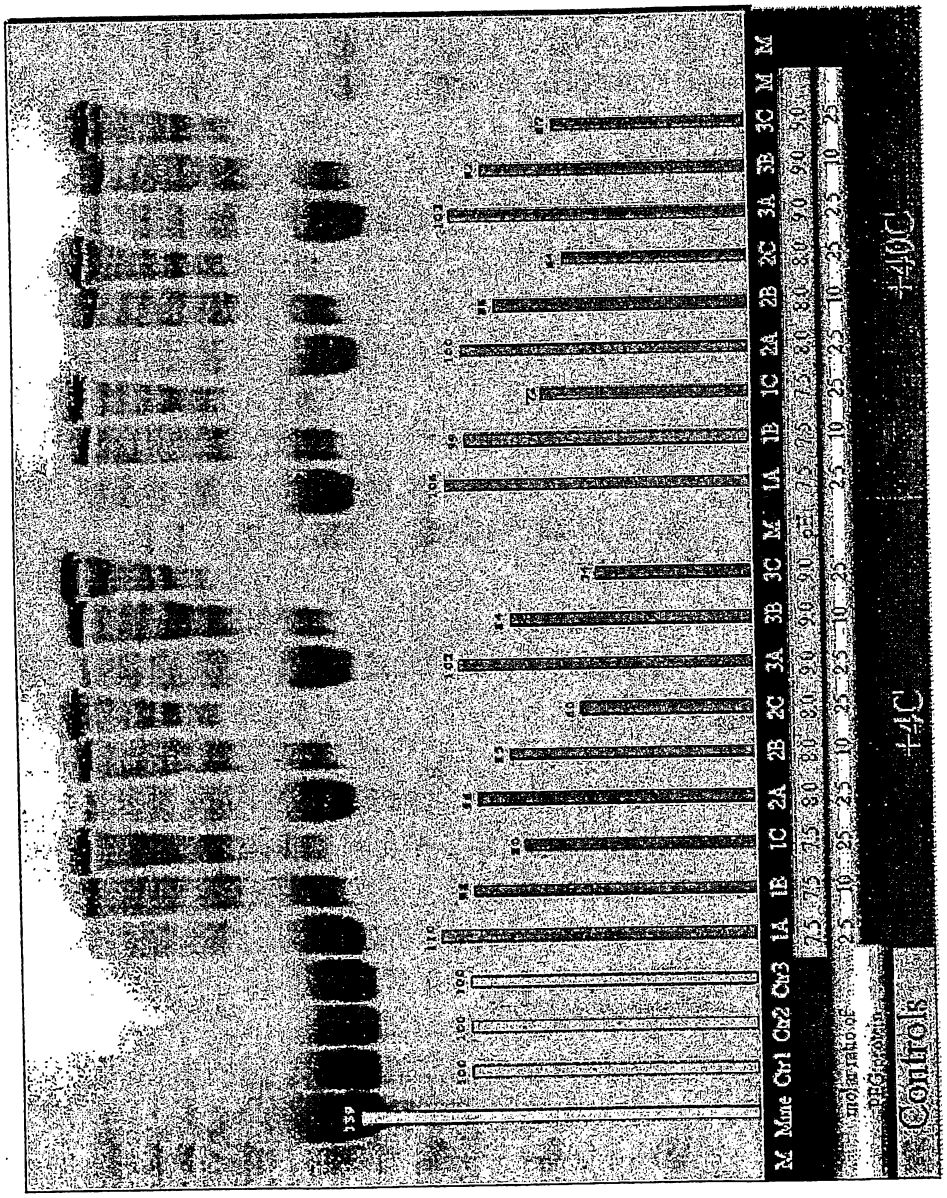

M Mme Ctr1 Ctr2 Ctr3 1A 1B 1C 2A 2B 2C 3A 3B 3C M 1A 1B 1C 2A 2B 2C 3A 3B 3C M
Control
+4C
+40C

Fig. 71

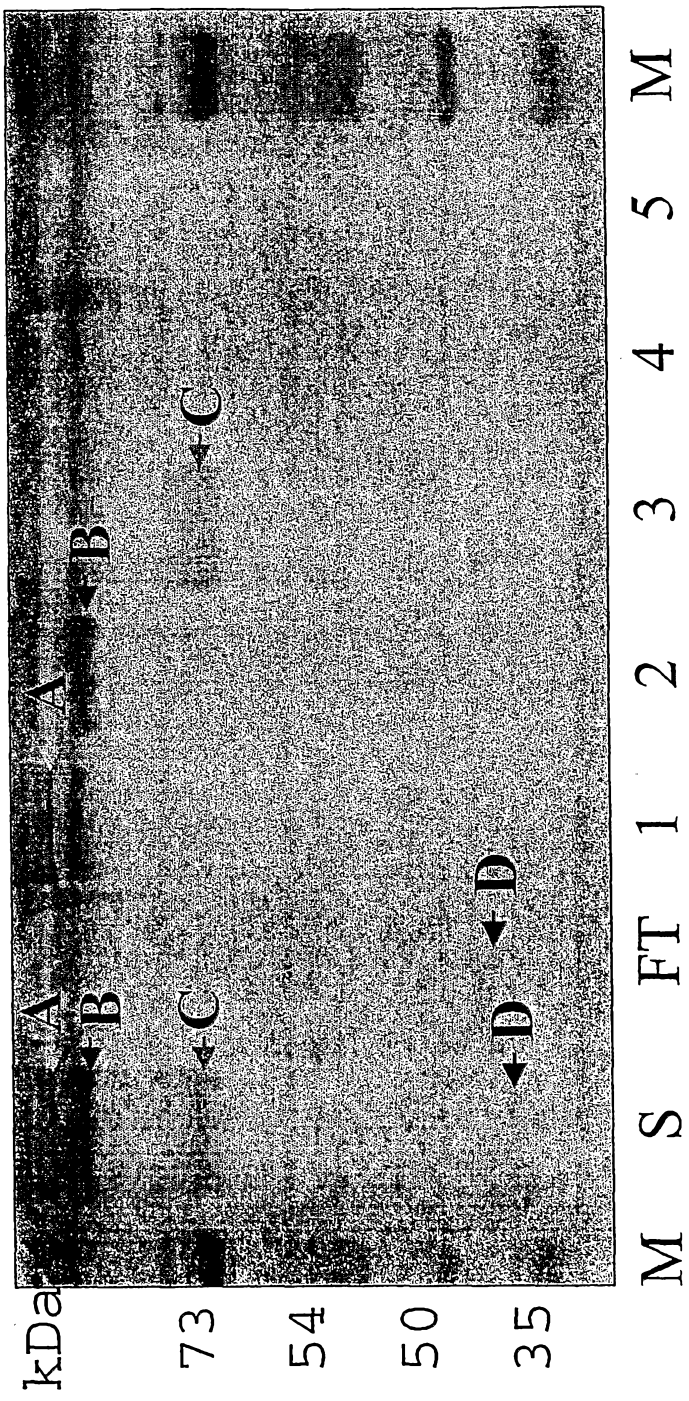

kDa
73
54
50
35
M S FT 1 2 3 4 5 M
A
B
C
D
B4_Biotin +2 and more PEGs ◄ A
B4_Biotin+1PEG ◄ B
Free (Non modified) B4_Biotin ◄ C
Free PEG ◄ D

Fig. 72

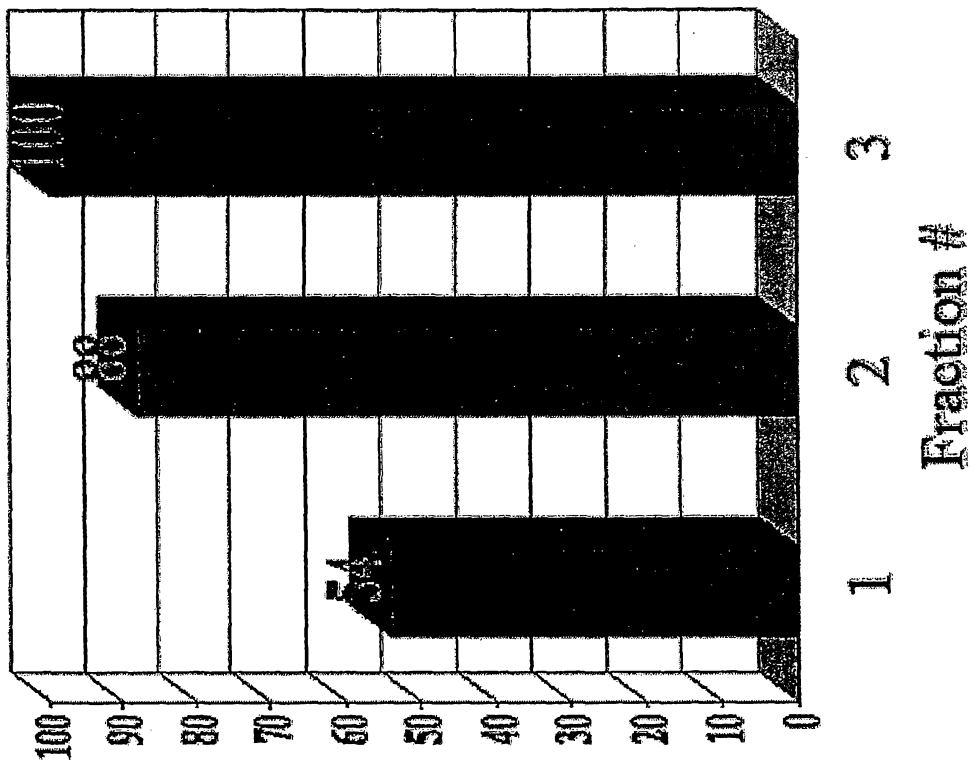


Fig. 73

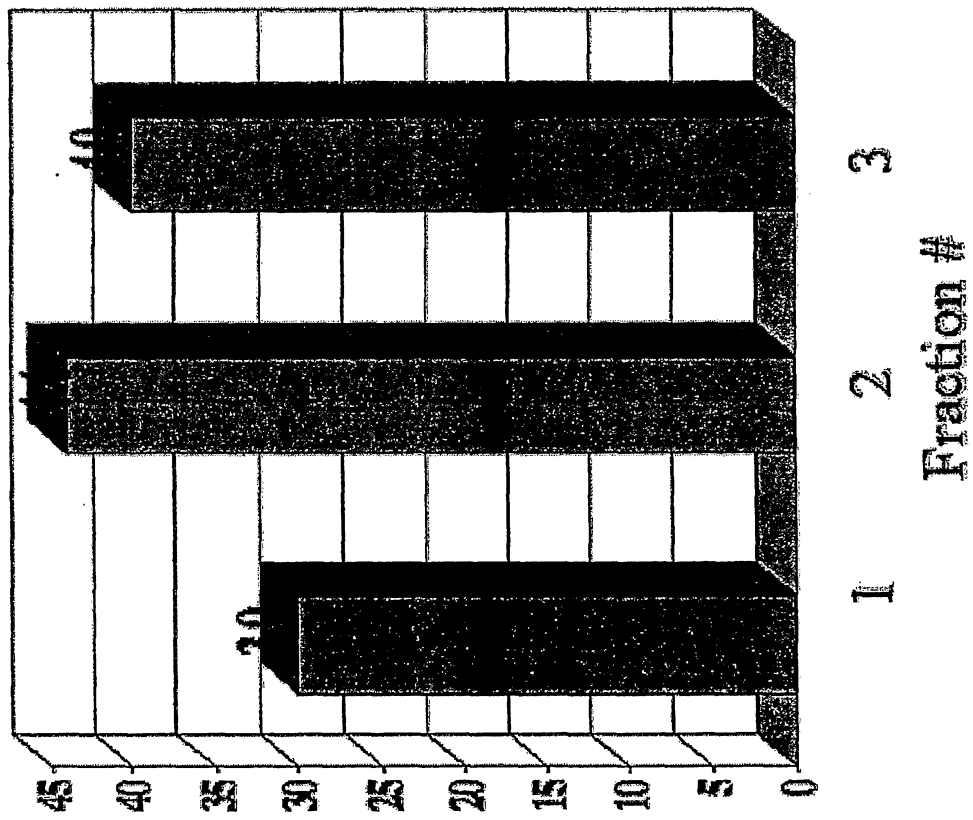
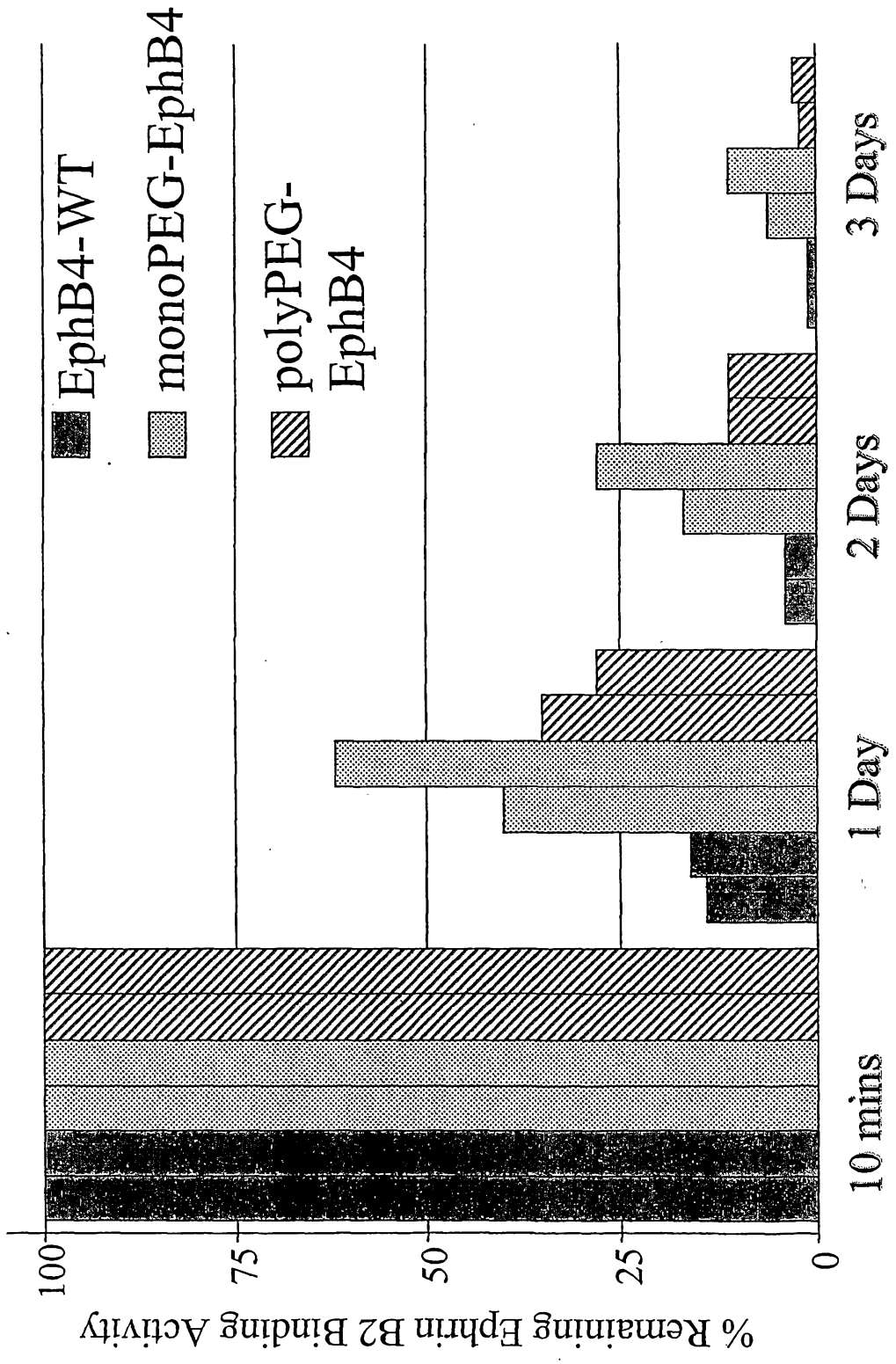


Fig. 74


% Remaining Ephrin B2 Binding Activity
100
75
50
25
0
10 mins
1 Day
2 Days
3 Days
EphB4-WT
monoPEG-EphB4
polyPEG-EphB4