

FUSION PROTEINS FOR INHIBITING ANGIOGENESIS

FIELD OF THE INVENTION

5 The present invention relates to a biologic that inhibits angiogenesis. In particular, the present invention relates to fusion proteins that inhibit angiogenic factor-activated pathways, the compositions of these fusion proteins, as well as methods for producing and using the same.

10 REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS WEB

 The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 479685SEQLIST.TXT, created on June 17, 2016, and having a size of 37kb, and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of
15 the specification and is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

 Angiogenesis is the process of growing new blood vessels from the existing vasculature. It plays an important role in several physiological processes, including
20 embryonic development, as well as tissue and wound repair (Folkman J et al., Angiogenic factors. Science 1987; 235:442-7). The physiologic steps of angiogenesis are well characterized, and involve proteolysis of the extracellular matrix, proliferation, migration, and assembly of the endothelial cells into a tubular channel, mural cell recruitment and differentiation, and extracellular matrix production (Carmeliet P et al., Nature. 2011;
25 473:298–307). Pathologic angiogenesis may occur in tumor formation, ocular disorders (*e.g.*, diabetic retinopathy, diabetic macular edema or macular degeneration) arthritis, psoriasis, fibrotic diseases, inflammatory diseases, and arteriosclerosis (Polverini PJ. Crit Rev Oral Biol Med. 1995; 6(3):230-47).

 Pathologic angiogenesis is more heterogeneous and chaotic, often demonstrating
30 tortuous vessel organization, hypoxic voids of various sizes, uneven and imperfect vessel walls and linings, and ineffective perfusion (Jain RK., Nat Med. 2003; 9(6):685–93). These distinct characteristics of new blood vessel formation in diseases have made

therapeutic targeting of angiogenesis a challenge. Although anti-VEGF therapies such as Lucentis®, Eylea®, or off-label use of Avastin® can generally stabilize or improve visual function, sub-retinal scarring (fibrosis) can develop in approximately half of all treated eyes within two years after anti-VEGF treatment and has been identified as one cause of

5 unsuccessful outcomes (Daniel E et al., *Ophthalmology*. 2014;121(3):656-66). Many of the critical players in sub-retinal fibrosis are likely to be the growth factors and the matricellular proteins that are involved in the fibrotic process (cell proliferation, migration and ECM remodeling). Despite its complexity, with our increasing knowledge of the angiogenic process, anti-angiogenic drug development remains an area of great interest.

10 . Currently, many key players in the neovascularization process have been identified, and the vascular endothelial growth factor (VEGF) family has a predominant role. The human VEGF family consists of 6 members: VEGF-A VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). In addition, multiple isoforms of VEGF-A, VEGF-B, and PlGF are generated through alternative RNA splicing (Sullivan et al., *MAbs*, 2002, 2(2): 165 - 75). VEGF-A is the primary factor involved with angiogenesis; it binds to both VEGFR-1 and VEGFR-2. The strategy of inhibiting angiogenesis by obstructing VEGF-A signaling has established successful therapies for treatment of specific cancers as well as retinal neovascular and ischemic diseases. (Major et al., *J Pharmacol Exp Ther.*, 1997, 283(1):402-10; Willet et al., *Nat. Med.* 2004, 10:145-7; Papadopoulos et al.,

15 2002, 2(2): 165 - 75). VEGF-A is the primary factor involved with angiogenesis; it binds to both VEGFR-1 and VEGFR-2. The strategy of inhibiting angiogenesis by obstructing VEGF-A signaling has established successful therapies for treatment of specific cancers as well as retinal neovascular and ischemic diseases. (Major et al., *J Pharmacol Exp Ther.*, 1997, 283(1):402-10; Willet et al., *Nat. Med.* 2004, 10:145-7; Papadopoulos et al.,

20 *Angiogenesis*, 2012, 15(2):171-85; Aiello et al., *PNAS*, 1995, 92:10457-61).

Other growth factors, cytokines, chemokines include Platelet Derived Growth Factors (PDGFs), Transforming Growth Factors beta (TGF- β), Epidermal Growth Factors (EGFs), Nerve Growth Factors (NGFs), Hypoxia-Induced Factor (HIF), Connective-Tissue Growth Factor (CTGF), Granulocyte– Macrophage Colony-Stimulating Factor (GM-CSF),

25 Insulin-Like Growth Factor (IGF), Hepatocyte Growth Factors/Scatter Factor (HGF/SF), Tumor Necrosis Factor alpha (TNF- α), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 17 (IL-17), Interleukin 18 (IL-18), Interleukin 20 (IL-20), Interleukin 23 (IL-23), Chemoattractants such as C-C motif Ligand (CCL28, CCL21) and C-X-C motif Ligand (CXCL1, CXCL5), Macrophage migration Inhibitory Factor (MIF), and immune cell

30 surface proteins such as Clusters of Differentiation (CDs). These factors are reported to be overexpressed and play key roles in angiogenesis-related diseases (Elshabrawy et al., *Angiogenesis* (2015) 18:433–448; Brian P. Eliceiri, *Circ Res.* 2001 Dec 7;89(12):1104-10). Targeting these factors to reduce their downstream pathway activation may decrease angiogenesis-related diseases.

35 Integrins, a family of cell surface receptors, are also found to be overexpressed on the endothelial cell surface and are believed to facilitate the growth and survival of newly forming vessels during angiogenesis. Integrins are heterodimeric cell surface receptors

that interact with extracellular matrix proteins and are critical for many biological processes. The expression of integrins in various cell types are involved in tumor progression, and their ability to crosstalk with growth factor receptors has made them attractive therapeutic targets. (Staunton DE, et al., *Adv Immunol.* 2006;91:111-57; Avraamides, C. J., et al., *Nat Rev Cancer* 2008;8:604-617.) In particular, the integrin $\alpha v \beta 3$ is upregulated in both tumor cells and angiogenic endothelial cells, and is important for tumor cell migration, angiogenesis, and dysregulated cell signaling. Therefore, antagonists of the integrin $\alpha v \beta 3$ are intensively studied for their anti-angiogenic and anti-tumor properties (Desgrosellier JS et al., *Nat Rev Cancer.* 2010 10:9-22).

Disintegrins are the peptides found in snake venom of the viper family and mainly inhibit the function of $\beta 1$ - and $\beta 3$ -associated integrins. They were first identified as inhibitors of integrin $\alpha IIb \beta 3$ and were subsequently shown to bind with high affinity to other integrins, blocking the interaction of integrins with RGD-containing proteins. They contain 47 to 84 amino acids with about 4 to 7 disulfide bonds and carry the same RGD motif (McLane MA, et al., *Proc Soc Exp Biol Med* 1998 219: 109-119; Niewiarowski S, et al., *Semin Hematol* 1994 31: 289-300; Calvete JJ., *Curr Pharm Des.* 2005 11: 829-835; Blobel CP et al., *Curr Opin Cell Biol* 1992 4: 760-765). The conserved RGD sequence in the disintegrin family plays the most important role in recognizing the integrins. Disintegrins were found to interact with eight out of twenty-four integrins and inhibited integrin-mediated cell adhesion, migration, and angiogenesis (McLane MA, et al., *Front Biosci.* 2008 13: 6617-6637; Swenson S, et al., *Curr Pharm Des.* 2007 13: 2860-2871). Animal studies showed that disintegrins targeted neovascular endothelium and metastatic tumors, indicating their potential use in cancer therapy. The specific binding of RGD-containing proteins to integrin is a function of both the conformation and the local sequence surrounding the RGD motif. Many studies have shown that the residues flanking the RGD motif of RGD-containing proteins affect their binding specificities and affinities to integrins (Scarborough RM et al., *J Biol Chem* 1993 268: 1058-1065; Rahman S et al., *Biochem J* 1998 335: 247-257).

Angiogenesis is a complex biological process which involves various growth factors and signaling receptors, and targeting single molecules in the signaling cascade may not provide an effective clinical treatment for uncontrolled angiogenesis in diseases such as cancer. Therefore, there is a growing need to develop innovative therapeutics capable of binding several key angiogenic factors in a cooperative manner to effectively inhibit angiogenesis and progression of the disease.

BRIEF SUMMARY OF THE INVENTION

The invention provided herein discloses polypeptides for specifically binding to multiple targets so as to antagonize several angiogenic factors. The invention also provides fusion proteins that inhibit a selective integrin pathway and other angiogenic pathways. The invention further provides compositions having these fusion proteins. The invention further provides compositions having a vector that comprises a nucleic acid encoding the fusion proteins. The invention further describes methods for producing and using these fusion proteins for the treatment or prevention of angiogenic diseases, ocular diseases, autoimmune diseases, inflammatory diseases, fibrotic diseases, and/or cancer.

In accordance with the present invention, a fusion protein comprising an integrin binding peptide selected from a group consisting of disintegrin, anti-integrin $\alpha v \beta x$ antibody, anti-integrin $\alpha 5 \beta 1$ antibody, fibronectin targeting integrin $\alpha v \beta x$ or $\alpha 5 \beta 1$ and their integrin binding fragments, other protein binding peptide targeting an angiogenic factor and a Fc domain, wherein x is 1, 3, 5, 6 or 8. In some embodiments, the angiogenic factor comprises Angiopoietin (ANG), Ephrin (Eph), Fibroblast Growth Factor (FGF), Neuropilin (NRP), Plasminogen Activators, Platelet-Derived Growth Factor (PDGF), Tumor Growth Factor beta (TGF- β), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial cadherin (VE-cadherin), Epidermal Growth Factors (EGFs), Nerve Growth Factors (NGFs), Connective-Tissue Growth Factor (CTGF), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), Insulin-like Growth Factor (IGF), Hepatocyte Growth Factors/Scatter Factor (HGF/SF), Tumor Necrosis Factor alpha (TNF- α), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 17 (IL-17), Interleukin 18 (IL-18), Interleukin 20 (IL-20), Interleukin 23 (IL-23), Chemoattractants such as C-C motif Ligand (CCL28, CCL21), C-X-C motif Ligand (CXCL1, CXCL5), Macrophage migration Inhibitory Factor (MIF), immune cell surface protein such as Clusters of Differentiation (CDs), and receptors thereof.

According to one aspect, the invention provides a fusion protein comprising an integrin binding peptide having an amino acid sequence selected from a group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, or amino acid sequence having at least 85% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, other protein binding peptide comprising extracellular domains of VEGF receptors, and a Fc domain, wherein the integrin binding peptide has at least one mutation on or adjacent to a RGD motif.

According to another aspect, the invention provides a fusion protein comprising an integrin binding peptide that includes disintegrin and its integrin binding fragments, other protein binding peptide comprising extracellular domains of VEGF receptors and a Fc

domain, wherein the integrin binding peptide comprises at least one mutation on or adjacent to the RGD motif. In some embodiments, the fusion protein comprises the integrin binding peptide, the Fc domain, and the other protein binding peptide from C-terminus to N-terminus. In some embodiments, the fusion protein comprises the integrin binding peptide, the Fc domain, and the other protein binding peptide from N-terminus to C-terminus. In further embodiments of the fusion protein, the integrin binding peptide has at least 85% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 or SEQ ID NO: 7.

In accordance with other embodiments, the invention also provides a fusion protein comprising an integrin binding peptide that includes an amino acid sequence of SEQ ID NO:1 with at least one mutation on or adjacent to the RGD motif, an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 1 or an amino acid sequence of SEQ ID NO: 2, a human or humanized constant sub-region comprising an immunoglobulin CH2 domain and a CH3 domain, and other protein binding peptide having an Ig-like domain D2 of a VEGFR1 and an Ig-like domain D3 of a VEGFR2. In a further embodiment of the fusion protein, the integrin binding peptide has at least 85% sequence identity to SEQ ID NO: 2.

In accordance with one aspect, the invention provides a nucleic acid sequence encoding a fusion protein of the invention disclosed herein.

In accordance with another aspect, the invention provides a dimer of fusion proteins of the invention disclosed herein.

In accordance with another aspect, the invention provides a method of producing a fusion protein of the invention, comprising culturing a host cell transfected with a vector comprising a nucleic acid sequence of the invention, under a condition that produces the fusion protein and recovering the fusion protein produced by the host cell.

In accordance with another aspect, the invention provides a method of treating or preventing an angiogenic disease comprising administering an effective amount of the fusion protein to a subject in need thereof. In some embodiments, the angiogenic disease comprises rheumatoid arthritis, inflammatory arthritis, osteoarthritis, ocular and systemic cancer, tumor related metastasis and invasion, systemic fibrotic diseases including idiopathic lung fibrosis (IPF), nonalcoholic steatohepatitis (NASH) or liver fibrosis, diabetic nephropathy and/or kidney fibrosis, dermal fibrosis or keloid, wound healing, cardio-fibrosis, and ischemia-induced stroke; ocular disease characterized by neovascularization or ischemia (such as choroidal neovascularization), uveitis, retinitis pigmentosa, age-related macular degeneration (AMD), diabetic retinopathy and diabetic macular edema (DME), central and branch retinal vein occlusion (CRVO and BRVO), human retinopathy of prematurity (ROP), polypoidal choroidal vasculopathy (PCV),

symptomatic vitreomacular adhesion, and glaucoma. In some embodiments, the fusion protein of the invention comprises a linker sequence between the integrin binding peptide and the other protein binding peptide. In some embodiments, the fusion protein of the invention comprises the signal peptide sequence upstream of the other binding domain.

5 In accordance with another aspect, the invention provides a composition comprising a fusion protein of the invention and a pharmaceutically acceptable carrier.

In accordance with another aspect, the invention provides a polypeptide targeting to multiple angiogenic factors comprising: an integrin binding peptide comprising an amino acid sequence selected from a group consisting of SEQ ID NO: 1, SEQ ID NO: 2, 10 SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, or amino acid sequence having at least 85% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7; other protein binding peptide selected from a group consisting of extracellular domains of VEGF receptors, anti-VEGF antibody, anti-PDGF antibody and their fragments; and a Fc 15 domain; wherein the integrin binding peptide comprising at least one mutation on or adjacent to the RGD motif.

In some embodiments, the extracellular domains of VEGF receptors in the polypeptide of the invention comprise the Ig-like domains D1-D7 of the VEGF receptors. In some embodiments, the extracellular domains of VEGF receptors in the polypeptide of 20 the invention comprise: i) the Ig-like domain 2 (D2) of a VEGFR1 and an Ig-like domain 3 (D3) of a VEGFR2; ii) the amino acid sequence of SEQ ID NO: 10; or iii) an amino acid sequence having at least 90% identity to SEQ ID NO: 10. In some embodiments, the extracellular domains of PDGF receptors in the polypeptide of the invention comprise the Ig-like domains 1-5 of the PDGF receptors. In some embodiments, the extracellular 25 domains of PDGF receptors in the polypeptide of the invention comprise: i) the Ig-like domains 1-3 of a PDGFR β ; ii) the amino acid sequence of SEQ ID NO: 11; or iii) an amino acid sequence having at least 90% identity to SEQ ID NO: 11. In some embodiments, the polypeptide of the invention further comprises a Gly-Ser (GS) or Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly (G₉) linker between the Fc domain and the integrin 30 binding peptide or the extracellular domains of VEGF or PDGF receptors.

The specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. All publications, patents and 35 patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects, features, and advantages of exemplary embodiments will become more apparent and may be better understood by referring to the following description taken in conjunction with the accompanying drawings.

5 Figure 1 is a schematic illustrating the composition of an exemplary fusion protein according to one embodiment of the invention.

Figure 2 is a schematic illustrating the purified Fusion Protein 1 analyzed by SDS-PAGE.

Figure 3 is a schematic illustrating a VEGF-ligand binding assay.

10 Figure 4 is a schematic illustrating a $\alpha v\beta 3$ competitive binding assay.

Figure 5 is a schematic illustrating VEGF-induced HUVEC proliferation.

Figure 6 is a schematic illustrating the inhibition of CHO- $\alpha v\beta 3$ cell adhesion.

Figures 7A-G is a schematic illustrating tube formation inhibitory effect by Fusion Proteins.

15 Figures 8A-D provide one embodiment showing the dose-response inhibition of VEGF-induced leakage in Dutch Belted Rabbits by Fusion Proteins.

Figures 9A-C provide one embodiment showing the reduction of lesion size in laser-induced choroidal neovascularization (CNV) in rats by Fusion Proteins. An arrow (▼) denotes location of laser lesion.

20 DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person skilled in the art to which this invention belongs.

As used herein, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a binding domain” includes a plurality of binding domains and equivalents thereof known to those skilled in the art.

As used herein, the term “polypeptide” and “protein” may be used interchangeably to refer to a long chain of peptide having an amino acid sequence of the native protein or the amino acid sequence with one or more mutations such as deletion, additions, and/or substitutions of one or more amino acid residues.

A “fusion protein” refers to a protein having two or more portions covalently linked together, where each of the portions is derived from different proteins.

35 The present invention provides a fusion protein comprising an integrin binding peptide selected from a group consisting of disintegrin (see U.S. Pat. Nos. 7,943,728 and PCT Application No. PCT/US 15/46322 for the description of amino acid sequences, each

of which is incorporated by reference in its entirety), anti-integrin $\alpha\beta x$ antibody (see U.S. Pat. Nos. 6,160, 099 and 8, 350, 010 for the description of amino acid sequences, each of which is incorporated by reference in its entirety), anti-integrin $\alpha 5\beta 1$ antibody, fibronectin (see U.S. Pub. No. 2015/0218251 for the description of amino acid sequences, which is incorporated by reference in its entirety) targeting integrin isoform $\alpha\beta x$ or $\alpha 5\beta 1$ and their integrin binding fragments, other protein binding peptide targeting an angiogenic factor and a Fc domain, wherein x is 1, 3, 5, 6 or 8.

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy chains and two light chains inter-connected by disulfide bonds. A full-length heavy chain includes a variable region domain, VH, and three constant region domains, CH1, CH2 and CH3. The VH domain is at the amino-terminus of the polypeptide, and the CH3 domain is at the carboxy-terminus. A full-length light chain includes a variable region domain, VL, and a constant region domain, CL. An antigen binding fragment (Fab) is comprised of one light chain and the CH1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. A Fab' fragment contains one light chain and one heavy chain that contains more of the constant region, between the CH1 and CH2 domains, such that an interchain disulfide bond can be formed between two heavy chains to form diabodies. A variable fragment (Fv) region comprises the variable regions from both the heavy and light chains, but lacks the constant regions. Single-chain fragments (scFv) are Fv molecules in which the heavy and light chain variable regions have been connected by a flexible linker to form a single polypeptide chain which forms an antigen-binding region. Single chain antibodies are discussed in detail in WO88/01649 and U.S. Pat. Nos. 4,946,778 and 5,260,203. As used herein, the term "antibody" includes the an immunoglobulin molecule with two full length L-chains and two full length H-chains, and fragments thereof, such as an antigen binding fragment (Fab), a Fv region, a scFv, etc.

The term "Fc domain" refers to a molecule or sequence comprising the sequence of a non-antigen binding portion of an antibody, whether in monomeric or multimeric form. The original immunoglobulin source of an Fc is preferably of human origin and can be from any isotype, e.g., IgG, IgA, IgM, IgE or IgD. A full-length Fc consists of the following Ig heavy chain regions: CH1, CH2 and CH3, wherein the CH1 and CH2 regions are typically connected by a flexible hinge region.

The present invention provides a fusion protein comprising an integrin binding peptide that includes disintegrin and its integrin binding fragments, other protein binding peptide comprising extracellular domains of VEGF receptor and a Fc domain, wherein the integrin binding peptide comprises at least one mutation on or adjacent to the RGD motif.

In accordance with embodiments of the present invention, the disintegrin and its integrin binding fragments have an amino acid sequence selected from a group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, or amino acid sequence having at least 85% sequence identity to
5 SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7

As used herein, "disintegrin" refers to a class of cysteine-rich proteins or polypeptides that are potent soluble ligands of integrins. The RGD motif is a tri-peptide (Arg-Gly-Asp) conserved in most of monomeric disintegrins and is located at an integrin-
10 binding loop. The disintegrins described herein are isolated from snake venom or derived from wild-type forms and have at least one mutation on or adjacent to a RGD motif to selectively bind to or target to various integrin isoforms. The term "adjacent to a RGD motif" as used herein means any mutation which occurs at any amino acid residue within 15-20 amino acids from the RGD motif in a given peptide or polypeptide sequence.

15 Other amino acid sequence variants of the disintegrin are also contemplated. For example, binding affinity and/or other biological properties of a disintegrin can be improved by altering the amino acid sequence encoding the protein. Disintegrin mutants can be prepared by introducing appropriate modifications into the nucleic acid sequence encoding the protein or by introducing modification by peptide synthesis. Such
20 modifications include mutations such as deletions from, insertions into, and/or substitutions within the nucleic or amino acid sequence of the disintegrin. Any combination of deletion, insertion, and substitution can be made to arrive at the final amino acid construct of the disintegrin provided that the final construct possesses the desired characteristics such as binding to an integrin superfamily member and/or
25 inhibiting the integrin activated pathway.

Substantial modifications in the biological properties of the proteins or polypeptides are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or
30 hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

A useful method for identifying certain residues or regions of the fusion protein that are preferred locations for mutagenesis is known as "alanine scanning mutagenesis" as described in Science, 1989, 244:1081-1085. For example, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys and Glu) and
35 replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the target binding partner. Those amino acid locations demonstrating functional sensitivity to the substitutions then

are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed fusion polypeptide variants are screened for desired activity. For example, cysteine bond(s) may be added to the fusion protein or protein components to improve its stability.

Accordingly, provided herein are disintegrin mutants that can be a component of any fusion protein disclosed herein. In some embodiments, the disintegrin comprises an amino acid sequence with at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to the amino acid sequence of disintegrins selected from a group consisting of Rhodostomin (SEQ ID NO: 1), Triflavin (SEQ ID NO: 3), Echistatin (SEQ ID NO: 4), Trimucrin (SEQ ID NO: 5), Elegantin (SEQ ID NO: 6) and Trigramin (SEQ ID NO: 7). In some embodiments, a disintegrin comprises an amino acid sequence having at least one mutation on or adjacent to the RGD motif of Rhodostomin (SEQ ID NO: 1), Triflavin (SEQ ID NO: 3), Echistatin (SEQ ID NO: 4), Trimucrin (SEQ ID NO: 5), Elegantin (SEQ ID NO: 6) or Trigramin (SEQ ID NO: 7). In some embodiments, the disintegrin comprises an amino acid sequence with at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to the amino acid sequence of a disintegrin mutant (SEQ ID NO: 2). The Xaa in SEQ ID NO: 2 indicates various positions that can be modified by either insertion, substitution or deletion to produce amino acid sequence variants that are different from the wild type form of disintegrin. According to some examples, the Xaa at position 50 of SEQ ID NO: 2 which corresponds to glycine (Gly) in the RGD motif of wild type Rhodostomin (SEQ ID NO: 1) may be substituted with naturally occurring amino acids other than glycine to generate Rhodostomin mutants. In other examples, one or more Xaa in SEQ ID NO: 2 may also be substituted with naturally occurring amino acids other than those originally found in corresponding positions of wild type Rhodostomin (SEQ ID NO: 1) to generate various Rhodostomin mutants. It is further noted that the disintegrin mutants are not limited to include only single mutation at any Xaa in SEQ ID NO: 2, multiple mutations that occur at several locations of Xaa in SEQ ID NO: 2 or corresponding locations in other consensus sequences of disintegrin (such as SEQ ID NOs: 3-7) may also be encompassed by the scope of the invention.

Although the variants of disintegrins are discussed mostly with reference to the amino acid sequences discussed above, polypeptide sequences or nucleotide sequence encoding the snake venom such as Albolabrin, Applagin, Basilicin, Batroxostatin, Bitistatin, Cereberin, Cerastin, Crotatroxin, Durissin, Flavoridin, Flavostatin, Halysin, Halystatin, Jararacin, Jarastatin, Kistrin, Lachesin, Lutosin, Molossin, Salmosin, Saxatilin,

Tergeminin, Trimestatin, Trimutase, Ussuristatin, Viridian and their mutants having at least one mutation on or adjacent to the RGD motif may also be encompassed by the scope of the present invention.

Without being bound by theory, it is contemplated herein that disintegrins inhibit
5 the integrin activated pathway by binding to an integrin superfamily member to block its interaction with a multivalent integrin receptor. In some aspects, the disintegrin binds to an integrin superfamily member which includes but is not limited to the integrin isoforms $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 5\beta 1$ and/or $\alpha v\text{IIb}\beta 3$.

According to the present invention, the other protein binding peptide of the fusion
10 protein may be receptor protein that binds to a target selected from the group consisting of a tumor antigen, a TNF receptor superfamily member, a Hedgehog family member, a receptor tyrosine kinase, a proteoglycan-related molecule, a TGF-beta superfamily member, a Wnt-related molecule and an angiogenesis target.

According to some embodiments of the invention, the other protein binding
15 peptide may specifically bind to an angiogenesis target which includes but is not limited to Angiopoietin (ANG), Ephrin (Eph), Fibroblast Growth Factor (FGF), Neuropilin (NRP), Plasminogen Activators, Platelet-Derived Growth Factor (PDGF), Tumor Growth factor beta (TGF- β), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial cadherin (VE-cadherin) and their receptors. Therefore, in accordance with embodiments of the
20 invention, the other protein binding peptide may include extracellular portions of a receptor protein that binds to and antagonizes the angiogenesis target. In other embodiments, the other protein binding peptide may bind to extracellular portions of angiogenic factor receptors.

In some embodiments, the other protein binding peptide may be an anti-VEGF
25 antibody (see WO2015/200905 for the description of its amino acid sequence, which is incorporated herein by reference in its entirety) that binds to the VEGF ligand or an anti-VEGFR1 or anti-VEGFR2 antibody (see U.S. Pat. No. 5,874,542 for the description of its amino acid sequence, which is incorporated herein by reference in its entirety) that binds to VEGF receptor. In other embodiments, the other protein binding peptide may also be
30 an anti-PDGF antibody (see U.S. Pat. No. 5,094,941 for the description of its amino acid sequence, which is incorporated herein by reference in its entirety) that binds to the PDGF ligand or an anti-PDGFR beta antibody (see U.S. Pat. No. 9,265,827 for the description of its amino acid sequence, which is incorporated herein by reference in its entirety for all purposes) that binds to PDGF receptor.

35 In certain embodiments, the other protein binding peptide binds to the same VEGF as any one of VEGF receptors (VEGFR): VEGFR1, VEGFR2 and VEGFR3. In some embodiments, the other protein binding peptide comprises at least one extracellular

portion of a VEGFR of any of the VEGFRs described herein. For example, the other protein binding peptide comprises at least one extracellular portion of VEGFR1 or one extracellular portion of VEGFR2. In another example, the other protein binding peptide comprises one extracellular portion of VEGFR1 such as Ig-like domain 2 (D2) and one
5 extracellular portion of VEGFR2 such as Ig-like domain 3 (D3). In some aspect, the other protein binding peptide comprises one extracellular portion of a VEGFR1 comprising amino acid sequence of SEQ ID NO: 8 and one extracellular portion of a VEGFR2 comprising amino acid sequence of SEQ ID NO: 9. In some aspect, the other protein binding peptide comprises a fusion of extracellular portions of VEGFR1 and VEGFR2
10 comprising an amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 10.

In other embodiments, the other protein binding peptide binds to the same PDGF as any one of PDGF receptors (PDGFR): PDGFR- α and PDGFR- β . In some embodiments, the other protein binding peptide comprises at least one extracellular
15 portion of a PDGFR of any of the PDGFRs described herein. For example, the other protein binding peptide comprises at least one extracellular portion of PDGFR- α or one extracellular portion of PDGFR- β . In another example, the other protein binding peptide comprises one extracellular portion of PDGFR- β such as Ig-like domain 1 – 3. In some aspect, the other protein binding peptide comprises an extracellular portion of a PDGFR
20 comprising an amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 11.

In accordance with other embodiments, the invention also provides a fusion protein comprising an integrin binding peptide that includes an amino acid sequence of SEQ ID NO:1 with at least one mutation on or adjacent to the RGD motif, an amino acid
25 sequence having at least 85% sequence identity to SEQ ID NO: 1 or an amino acid sequence of SEQ ID NO: 2, a human or humanized constant sub-region comprising an immunoglobulin CH2 domain and a CH3 domain, and other protein binding peptide having an Ig-like D2 of a VEGFR1 and an Ig-like D3 of a VEGFR2. In a further embodiment of the fusion protein, the integrin binding peptide has at least 85% sequence
30 identity to SEQ ID NO: 2.

The term “percent (%) sequence identity” with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the
35 sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic

acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, as those described in Current Protocols in Molecular Biology (Ausubel et al., eds., 1987), and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) Software. Those skilled in
5 the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with or against a given amino acid sequence B is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues
10 score as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

15 The present invention provides a dimeric fusion protein comprising two fusion proteins, wherein each fusion protein comprises any fusion protein disclosed herein. In one embodiment, the dimeric fusion protein comprises two identical fusion proteins. In another embodiment, the dimeric fusion protein may comprise two different fusion proteins. The fusion proteins disclosed herein may form multimers of two or more
20 identical fusion proteins or form heterologous fusion proteins through a multimerization domain which includes a constant sub-region of a human or humanized antibody. In some embodiments, the constant sub-region of a human or humanized antibody is selected from the group consisting of an IgG Fc region, IgA Fc region, IgM Fc region, IgD Fc region and IgE Fc region. In the further embodiment, the constant sub-region of a
25 human or humanized antibody is selected from the group consisting of an IgG1 Fc region, IgG2 Fc region, IgG3 Fc region and IgG4 Fc region. In some aspect, the sub-region comprises a CH2 region and a CH3 region of IgG1, IgG2, IgG3, or IgG4. Amino acid sequences encoding immunoglobulins that comprise Fc regions are well known in the art.

The components of the fusion protein may be connected directly to each other or
30 be connected via linkers. Generally, the term "linker" means one or more molecules e.g., nucleic acids, amino acids or non-peptide moieties which may be inserted between one or more component domains. For example, linkers may be used to provide a desirable site of interest between components for ease of manipulation. A linker may also be provided to enhance expression of the fusion protein from a host cell, to decrease steric
35 hindrance such that the component may assume its optimal tertiary structure and/or interact appropriately with its target molecule. A linker sequence may include one or more amino acids naturally connected to a receptor component, or may be an added sequence

used to enhance expression of the fusion protein, to provide specifically desired sites of interest, to allow component domains to form optimal tertiary structures and/or to enhance the interaction of a component with its target molecule.

Preferably, the linker increases flexibility of the fusion protein components without
5 interfering with the structure of each functional component within the fusion protein. In some embodiments, the linker moiety is a peptide linker with a length of 2 to 100 amino acids. Exemplary linkers include linear peptides having at least two amino acid residues such as Gly-Gly, Gly-Ala-Gly, Gly-Pro-Ala, Gly (G)_n and Gly-Ser (GS) linker. The GS linker described herein includes but is not limited to (GS)_n, (GSGSG)_n, (G₂S)_n, G₂S₂G, (G₂SG)_n, (G₃S)_n, (G₄S)_n, (GGSGG)_nGn and GSG₄SG₄SG, wherein n is 1 or more. One
10 example of the (G)_n linker includes a G₉ linker. Suitable linear peptides include polyglycine, polyserine, polyproline, polyalanine and oligopeptides consisting of alanyl and/or serinyl and/or prolinyl and/or glycyl amino acid residues. The linker moieties may be used to link any of the components of the fusion proteins disclosed herein. In some
15 embodiments, a linker is used between an extracellular portion of a receptor protein and a constant sub-region of an immunoglobulin. In other embodiments, a linker is used between disintegrin or its variant and a constant sub-region of an immunoglobulin. In certain embodiments, the fusion protein comprises a linker between an extracellular portion of a receptor protein and disintegrin or its variant, and a linker between disintegrin
20 or its variant and a constant sub-region of an immunoglobulin. As embodied in the present invention, a fusion protein may comprise at least one linker but no more than four linkers.

The fusion protein described herein may or may not comprise a signal peptide that functions for secreting the fusion protein from a host cell. A nucleic acid sequence
25 encoding the signal peptide can be operably linked to a nucleic acid sequence encoding the protein of interest. In some embodiments, the fusion protein comprises a signal peptide. In some embodiment, the fusion protein does not comprise a signal peptide.

Moreover, the fusion proteins described in the present invention may comprise modified forms of the protein binding peptides. For example, the fusion protein
30 components may have post-translational modifications, including for example, glycosylation, sialylation, acetylation, and phosphorylation to any of the protein binding peptides.

Although the embodiments are generally described with reference to two protein binding peptides included in the fusion protein, the invention also contemplates a fusion
35 protein which incorporates more than two protein binding peptides to provide any additional or synergistic effects in terms of inhibiting the process of angiogenesis. For example, there may be an additional protein binding peptide that binds to other

angiogenesis targets or acts as angiogenic factor antagonists to be linked to the existing two protein binding peptides.

The present invention provides a nucleic acid encoding a fusion protein of the invention disclosed herein. The invention further provides a method of producing a fusion protein disclosed herein. The method involves culturing a host cell transfected with a
5 vector which comprises a nucleic acid sequence of the invention and recovering the fusion protein produced by the host cell under suitable conditions.

The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus,
10 this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine or pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

Isolated nucleic acid molecules encoding a fusion protein or a component of a
15 fusion protein can be in the form of RNA, such as mRNA, hnRNA, tRNA or any other form, or in the form of DNA, including, but not limited to cDNA and genomic DNA obtained by cloning or produced synthetically, or any combination thereof. The DNA can be triple-stranded, double-stranded or single-stranded, or any combination thereof. Any portion of at least one strand of the DNA or RNA can be the coding strand, known as the sense strand, or it can be the non-coding strand, also referred to as the anti-sense strand.
20 The isolated nucleic acids encoding a fusion protein or fusion protein component can be prepared by a variety of methods known in the art including, but not limited to, isolation from a natural source or preparation by oligonucleotide-mediated mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the fusion protein or fusion protein component. *See Molecular Cloning: A Laboratory Manual*
25 (Sambrook et al., 4th ed., Cold Spring Harbor, N.Y. 2012) and *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds., 2003).

A "vector" refers to a recombinant plasmid that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

The present invention contemplates the use of a nucleic acid delivery vehicle for
30 introduction of one or more nucleic acid sequences encoding a fusion protein or fusion protein component into a cell for expression of said protein. Examples of nucleic acid delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides;
35 artificial viral envelopes; metal particles; and bacteria, viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombinant vehicles typically used in the art which have been described in a variety of

eukaryotic and prokaryotic hosts. In some embodiments, the nucleic acid delivery vehicle is an expression vector such as a plasmid. The vector may include any element to establish a conventional function of an expression vector (for example, a promoter, ribosome binding element, terminator, enhancer, selection marker), and an origin of replication. The promoter can be a constitutive, inducible or repressible promoter. A number of expression vectors capable of delivering nucleic acids to a cell are known in the art and may be used herein for production of a fusion protein or fusion protein component in the cell. Expressed fusion proteins or fusion protein components can be harvested from the cells and purified according to conventional techniques known in the art and as described herein.

Provided herein are host cells comprising a nucleic acid encoding a fusion protein described herein. Nucleic acids encoding fusion proteins or fusion protein components (e.g., an extracellular portion of an angiogenic factor receptor, a disintegrin or its variant and/or a constant sub-region of an immunoglobulin) can be provided to a target cell by any means known in the art. In some embodiments, the nucleic acid encoding a protein of interest is in an expression vector such as a plasmid. In other embodiments, the nucleic acid encoding a protein of interest is in a viral vector and the vector has been packaged, then the virions can be used to infect cells. Transfection and transformation procedures are known to be appropriately used to introduce a nucleic acid encoding a protein of interest into a target cell. Formulations utilizing polymers, liposomes, or nanospheres can be used for delivery of nucleic acids encoding a protein of interest. Cells which are transformed or transfected with recombinant constructs according to the invention may be any which are convenient to one skilled in the art. Exemplary cell types which may be used include bacteria, yeast, fungi, insect, plant, and mammalian cells. In some embodiments, transformed or transfected cells can be provided to a cell or mammalian host. Suitable cells for delivery to a cell or mammalian host include any mammalian cell type from any origin, tumor, or cell line.

Cells used to produce the fusion proteins or fusion protein components of the invention are grown in media known to those skilled in the art and suitable for culture of the selected host cells. A given medium is generally supplemented as necessary with hormones and/or other growth factors, DHFR, salts, buffers, nucleosides, antibiotics, trace elements, and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the cell selected for expression, and will be apparent to one skilled in the art.

Proteins may be purified and identified using commonly known methods such as fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; hydrophobic affinity resins, ligand affinity using a suitable binding partner immobilized on a matrix, centrifugation, Enzyme-Linked Immunosorbent Assay (ELISA), BIAcore, Western blot assay, amino acid and nucleic acid sequencing, and biological activity. In some embodiments, the fusion protein is expressed in host cells and purified therefrom using a combination of one or more standard purification techniques, including, but not limited to Protein A affinity chromatography, Protein G affinity chromatography, buffer exchange, size exclusion chromatography, ultrafiltration, and dialysis. Accordingly, the recovered fusion protein is substantially pure. In a further embodiment, the recovered fusion protein is at least any of 90%, 95%, 96%, 97%, 98% or 99% pure.

The fusion proteins or fusion protein components disclosed herein may be characterized or assessed for biological activities including, but not limited to, affinity to a target binding partner, competitive binding, inhibitory activity, inhibition of cell proliferation, inhibition of tumor growth, and inhibition of angiogenesis. In some embodiments, the fusion proteins or fusion protein components disclosed herein can be assessed for biological activity in vitro and in vivo. Many methods for assessing binding affinity are known in the art and can be used to identify the binding affinities of fusion proteins or fusion protein components to a binding partner. Binding affinities can be expressed as dissociation constant (K_d) values or half maximal effective concentration (EC_{50}) values.

In any of the embodiments herein, a fusion protein has an EC_{50} of less than or equal to 1 μ M, 100 nM, 10 nM, 1 nM, 0.1 nM, 0.01 nM, or 0.001 nM for inhibition of an activity (e.g., inhibition of angiogenic factor activity and/or integrin activity). In any of the embodiments herein, a fusion protein has a K_d for a binding partner (angiogenic factor and/or integrin) of less than about 1.0 mM, 500 μ M, 100 μ M, 50 μ M, 10 μ M, 5 μ M, 1 μ M, 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM, 50 pM, 10 pM or 5 pM, including any values in between these numbers.

The invention also provides a pharmaceutical composition comprising a fusion protein comprising an integrin binding peptide selected from a group consisting of disintegrin, anti-integrin $\alpha v \beta x$ antibody, anti-integrin $\alpha 5 \beta 1$ antibody, fibronectin targeting integrin $\alpha v \beta x$ or $\alpha 5 \beta 1$ and their integrin binding fragments, other protein binding peptide targeting an angiogenic factor and a Fc domain, wherein x is 1, 3, 5, 6 or 8. Compositions of the invention comprise a therapeutically effective amount of the fusion protein.

The term "therapeutically effective amount" means an amount of a therapeutically active compound needed to elicit the desired biological or clinical effect. According to embodiments of the invention, "a therapeutically effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. A therapeutically effective amount can be administered in one or more administrations. In terms of a disease state, a therapeutically effective amount is an amount sufficient to ameliorate, stabilize, or delay development of a disease. According to specific embodiments of the invention, a therapeutically effective amount is an amount of a fusion protein needed to treat or prevent a disorder characterized by abnormal angiogenesis, such as a disease characterized by neovascularization, vascular permeability, edema, inflammation, retinopathies, fibrosis or cancer.

In some embodiments, the pharmaceutical composition comprising a fusion protein comprises a fusion protein formulated in a buffer at a protein concentration from about 0.5 to about 100 mg/mL, preferably about 40 to about 80 mg/mL, such as about 40, 50, 60, 70 or 80 mg/mL, most preferably about $40 \pm$ about 5 mg/mL. In other preferred embodiments, the fusion protein is formulated in a buffer at a protein concentration of more than about 40 mg/mL.

In particular embodiments, the buffer is a phosphate buffer with a pH of about 6.5 to 8, more preferably about 7 to 7.5, even more preferably about 7.2. The phosphate buffer comprises about 5 to 20 mM sodium phosphate, such as 5, 10, 15 or 20 mM sodium phosphate, more preferably about 10 mM sodium phosphate; about 20 to 60 mM sodium chloride, more preferably about 40 mM sodium chloride; about 1 to 10% weight-per-volume (w/v) sucrose, more preferably about 5% w/v sucrose; and about 0.01 to 0.05% w/v of a surfactant, more preferably about 0.03% w/v polysorbate 20.

In other particular embodiments, the buffer is a histidine buffer with a pH of about 5 to 8, more preferably about 6 to 7, most preferably about 6.8. The histidine buffer comprises about 10 to 50 mM histidine, such as 10, 20, 30, 40 or 50 mM histidine, more preferably about 25 mM histidine; about 10 to 30 mM sodium chloride, such as 10, 20 or 30 mM sodium chloride, more preferably about 20 mM sodium chloride; about 1 to 10% w/v sucrose, such as 1, 2, 4, 6, 8 or 10% w/v sucrose, more preferably about 6% w/v sucrose; and about 0.01 to 0.05% w/v of a surfactant, more preferably about 0.03% w/v polysorbate 20.

The present invention also relates to a use of the composition according to the present invention to treat or prevent an integrin-associated disease in an individual or a subject. An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits and rodents (e.g., mice and

rats). In some embodiments, a method of treating or preventing one or more aspects or symptoms of a disease comprises administering an effective amount of a composition comprising the fusion protein to an individual.

The methods described herein can be used for the treatment of a variety of diseases, including but not limited to, inflammatory disease, ocular disease, autoimmune disease, or cancer. In some embodiments, the disease to be treated includes, but is not limited to, rheumatoid arthritis, inflammatory arthritis, osteoarthritis, cancer, fibrosis, retinitis pigmentosa, uveitis (such as anterior uveitis or posterior uveitis and ocular disease characterized by neovascularization or ischemia (such as choroidal neovascularization, diabetic retinopathy, diabetic macular edema, age-related macular degeneration (AMD), retinal vein occlusion, polypoidal choroidal vascularization.

The compositions described herein can be administered to an individual via any route, including, but not limited to, intravenous, intraperitoneal, ocular, intra-arterial, intrapulmonary, oral, inhalation, intravesicular, intramuscular, intratracheal, subcutaneous, intrathecal, transdermal, transpleural, intraarterial, topical, inhalational, mucosal, subcutaneous, transdermal, gastrointestinal, intraarticular, intracisternal, intraventricular, intracranial, intraurethral, intrahepatic and intratumoral. In some embodiments, the compositions are administered systemically (for example by intravenous injection). In some embodiments, the compositions are administered locally (for example by intraarterial or intraocular injection).

In some embodiments, the compositions are administered directly to the eye or the eye tissue. In some embodiments, the compositions are administered topically to the eye, for example, in eye drops. In some embodiments, the compositions are administered by injection to the eye (intraocular injection) or to the tissues associated with the eye. The compositions can be administered, for example, by intraocular injection, periorbital injection, subretinal injection, intravitreal injection, superchoroidal injection, trans-septal injection, subscleral injection, intrachoroidal injection, intracameral injection, subconjunctival injection, sub-Tenon's injection, retrobulbar injection, peribulbar injection, or posterior juxtасcleral delivery. These methods are known in the art. The compositions may be administered, for example, to the vitreous, aqueous humor, sclera, conjunctiva, the area between the sclera and conjunctiva, the retina choroids tissues, macula, or other area in or proximate to the eye of an individual.

The optimal effective amount of the compositions can be determined empirically and will depend on the type and severity of the disease, route of administration, disease progression and health, mass and body area of the individual. Such determinations are within the skill of one in the art. Compositions comprising a fusion protein can also be administered six times a week, five times a week, four times a week, three times a week,

twice a week, once a week, once every two weeks, once every three weeks, once a month, once every two months, once every three months, once every six months, once every nine months, or once every year.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to describe the methods and/or materials in connection with which the publications are cited.

The present invention is further illustrated by the following Examples, which are provided for the purpose of demonstration rather than limitation.

Example 1: Production of Rhodostomin Mutant Proteins

Method of disintegrin expression

Overlap extension polymerase chain reaction (PCR) techniques were used to create Rhodostomin (Rho) mutant constructs. The expression kit and the yeast transfer vector, pPICZaA, were purchased from Invitrogen. The gene mutant constructs were created by PCR-amplifying the gene sequence using a sense primer containing a complementary sequence to the gene mutant plus an EcoR1 recognition site and six histidine residues for facilitating purification, and the antisense primer designed to contain a Sac II recognition site and TTA stop codon. The PCR product was purified and then cloned into the EcoR1 and Sac II sites of yeast recombination vector, pPICZaA. The recombinant plasmid was transformed into E coli competent cell DH5a, and colonies were selected by agar plate with low salt Luria Broth (LB, 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar at pH 7.0) and 25 µg/mL of the antibiotic Zeocin. A single colony was selected to amplify the plasmid for sequencing. After the clones had been confirmed by DNA sequencing, 10 µg of the plasmid was prepared and then linearized by digesting with Sac I enzyme. The linearized construct was transformed into the Pichia strain X33 using a kit (Pichia EasyComp; Invitrogen), and colonies were selected by agar plate using YPDS (1% yeast extract, 2% peptone, 2% glucose, 2% agar, and 1 M Sorbitol) and 100 µg/mL of Zeocin. PCR analysis was used to analyze Pichia integrants to determine whether the gene had integrated into the Pichia genome. From a number of clones with multiple copies of gene insertion, clones with the highest protein expression were chosen.

Method of disintegrin purification

Rho mutants were produced as follows: 100 µL of cell stock were grown at 30°C for 48 hours in 200 mL of yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose) and 100 µg/mL Zeocin. Cells were then
5 transferred into 800 mL of YPD medium. After another 48 hours, the cells were collected by centrifugation and grown in 1 L of minimal methanol medium (1.34% yeast nitrogen base (YNB) with ammonium sulfate without amino acids, 4×10^{-5} % biotin, and 1% Methanol). Once every 24 hours, 1% methanol was added to induce protein expression for 2 days. The supernatant was collected after centrifugation and dialyzed twice against
10 5 L of H₂O and once against 5 L of 20 mM Tris-HCl and 200 mM NaCl at pH 8. The final solution was loaded into a nickel-chelating column and eluted with a gradient of 200 mM imidazole. The recombinant Rho proteins produced in *P. pastoris* were further purified by reverse-phase C18 HPLC with a gradient of 15% to 18% acetonitrile. A tricine-SDS-PAGE analysis confirmed that the purification of proteins was greater than 95%.

15 Example 2: Generation of VEGFR/Rho Mutant Fusion Proteins

The extracellular domain D2 of the Flt-1 receptor (VEGFR1) of SEQ ID NO: 8 and the extracellular domain D3 of the Flt-1 receptor (VEGFR2) of SEQ ID NO: 9, collectively known as VEGF trap, provided the protein component for binding to VEGF ligand. In order to bind to both VEGF ligand and integrins, fusion proteins comprising VEGFR1
20 D2/VEGFR2 D3 of SEQ ID NO: 10 and Rho mutant of SEQ ID NO: 13 were generated.

A previously generated VEGF trap linked to human immunoglobulin G1 CH2 and CH3 domains (IgG1 Fc) was used to generate DNA constructs encoding the VEGFR1 D2/VEGFR2 D3-Rho mutant hybrid. See Holash, J., et al., PNAS, 2002, 99 (17): 11393-11398 for a description of the VEGF trap which is incorporated herein by reference in its
25 entirety. A fusion protein of SEQ ID NO: 16 was constructed by linking the VEGFR1 D2/VEGFR2 D3 of SEQ ID NO: 10 and Rho mutant of SEQ ID NO: 13 via at least one peptide linker of Gly-Gly-Gly-Gly-Ser (G₄S).

Similarly, Fusion Proteins 2 and 3 of SEQ ID NOs: 15 and 17 were also generated by fusing Rho mutants of SEQ ID NO: 12 and SEQ ID NO: 14 respectively with the
30 VEGFR1 D2/VEGFR2 D3 of SEQ ID NO: 10. Fusion Protein 4 of SEQ ID NO: 18 was generated by fusing a commercially available VEGF Trap, aflibercept (Eylea®, Regeneron) and Rho mutant of SEQ ID NO: 13. Figure 1 provides a schematic showing the structure of a fusion protein according to one embodiment of the invention. SS is a signal peptide sequence of SEQ ID NO: 20 which helps secretion of protein from the
35 CHO cell. VEGF trap is made up of the extracellular Ig-like domains 2 and 3 of human VEGFR1 and VEGFR2. An Fc fragment is the CH2 and CH3 region of human IgG1. A

GS is a linker. Rho mutants have been described in U.S. Pat. Nos. 7,943,728 and PCT Application No. PCT/US 15/46322 and their sequences are incorporated herein by reference.

To generate Fusion Protein 1, which has the amino acid sequence of SEQ ID NO: 16, the DNA sequence encoding Fusion Protein 1 was codon-optimized for expression in CHO cells. The synthesized codon-optimized DNA, with a nucleic acid sequence of SEQ ID NO: 19, together with the signal peptide having a nucleic acid sequence of SEQ ID NO: 21 was cloned into an expression vector. CHO K1 host cells were seeded at 2×10^5 cells/mL in CD CHO (Gibco 12490-003) containing 4 mM Glutamine (J.T Baker 2078-06) and 1% HT Supplement (Gibco 11067-030) 72 hours before transfection. The host cells were incubated in an Infors shaker (36.5°C, 75% humidity, 6% CO₂, 110 RPM) and counted for cell density before use. A suitable amount of expression plasmid DNA was added into the host cells (1L or 5L working volume), and polymer-based transfection reagent was added. The transfected cultures were incubated in an Infors shaker (36.5°C, 75% humidity, 6% CO₂, 110 RPM) for 4 hours and a proprietary feed solution was added. The transfected cultures were then incubated in an Infors shaker (32°C, 75% humidity, 6% CO₂, 110 RPM). The transfected cultures were harvested on day 10 after transfection. The supernatants were purified for generation of research materials. The purification process included clarification, Protein A affinity chromatography, concentration by Amicon Ultracel, size exclusion chromatography, dialysis by Slide-A-Lyzer, and final concentration by Amicon Ultracel in the formulation buffer.

Fusion proteins having simultaneous anti-VEGF and anti-integrin activities were constructed, expressed, purified and characterized. Figure 2 provides a schematic characterizing the purified Fusion Protein 1 by SDS-PAGE. The molecular marker was loaded in Lane M. Non-reduced and reduced forms of engineered VEGF-trap (Positive Control 1; SEQ ID NO: 22) consisting of portions of human VEGFR1 and VEGFR2 fused to the Fc portion of human IgG1 were loaded in Lanes 1 and 2, respectively. Non-reduced and reduced forms of commercially available VEGF-trap, aflibercept (Eylea®, Regeneron) (Positive Control 2) were loaded in Lanes 3 and 4, respectively. Non-reduced and reduced forms of Fusion Protein 1 were loaded in Lanes 5 and 6. Each protein sample was loaded in the volume of 3 µg in each lane. As shown in Figure 2, the results indicate that both the positive control and Fusion Protein 1 exhibited high integrity and purity under reducing and non-reducing conditions. The final concentration of Fusion Protein 1 was approximately 40 mg/mL in the designated formulation buffer (based on the absorption at 280 nm).

Production of protein homodimers by cell transfection with their respective constructs was confirmed by SDS-PAGE and bioactivity analysis.

Example 3: Binding Affinity of Fusion Protein to Human VEGF₁₆₅

A direct binding enzyme-linked immunosorbent assay (ELISA) was used to measure the binding affinity of fusion proteins of the invention to human VEGF₁₆₅, a splice variant of VEGF-A.

5 VEGF Trap is a soluble VEGF receptor that was engineered for therapeutic use and is currently approved by FDA to treat AMD. The VEGF Trap contains the second Ig-like domain 2 (D2) of VEGFR1 fused to the third Ig-like domain 3 (D3) of VEGFR2 fused to the Fc region of human IgG1 (Holash, J., et al, Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11393-8). VEGF Trap targets VEGF-A, VEGF-B, and PlGF. The commercially
10 available VEGF Trap, aflibercept (Eylea®, Regeneron), was included as Positive Control 2.

100 µL of a coating solution (1 µg/mL VEGF₁₆₅ in 1x phosphate buffered saline (PBS), pH 7.2) were added to each well of a 96-well ELISA plate, and the plate was incubated overnight at 4°C. The wells were washed twice with 400 µL of 1x PBS buffer,
15 and excess liquid was carefully removed with a paper towel.

400 µL of a blocking solution (5 g non-fat skim milk in 100 mL 1x PBS) were added to each well, and the plate was incubated at room temperature for 1 hour. The wells were washed twice with 1x PBS buffer.

Fusion protein and control samples were serially diluted three-fold in blocking
20 solution, with a highest protein concentration of 10 nM. 100 µL of the serially diluted samples were added to each well. The plate was covered and incubated on a plate shaker (~100 rpm) for 1 hour at room temperature. The wells were washed three times with wash buffer (1x PBS, 0.05% Tween-20).

100 µL of 1:2500 diluted horseradish peroxidase-conjugated goat anti-human IgG
25 Fc specific antibodies in blocking solution were added to each well. The plates were sealed and incubated on a plate shaker for 1 hour at room temperature. The plates were washed three times with wash buffer.

100 µL of 3,5,3',5'-Tetramethylbenzidine (TMB) were added to each well, and the plates were incubated for 3 to 5 minutes to allow for the reaction to take place. To stop
30 the reaction, 100 µL of stop solution (1N HCl) were added to each well.

The optical density (OD) of each well was determined using an ELISA plate reader at an absorbance wavelength of 450 nm. The absorbance was plotted against the protein concentration of the fusion protein or the control, and the concentration at which the signal was half the maximal effective concentration (EC₅₀) was determined.

35 The binding affinity, expressed as the EC₅₀ value, was between 0.10 and 0.21 nM for the tested fusion proteins of the invention. The ELISA results are shown in **Table 1**.

Table 1

Test Material	EC ₅₀ (nM)
Positive Control 2 (afibercept)	0.088-0.195
Fusion Protein 1 (SEQ ID NO: 16)	0.106-0.207

Results from Example 3 showed that fusion proteins according to embodiments of the invention, bind VEGF₁₆₅ with high affinity. This is also illustrated in Figure 3.

5 **Example 4 – Competitive Binding of Fusion Protein to Integrin $\alpha v \beta 3$**

The competitive binding was used to measure the binding affinity of Fusion Protein 1 of the invention to integrin $\alpha v \beta 3$. Wild type Rho (SEQ ID NO: 1) and Rho variant KG (SEQ ID NO: 13) were synthesized in accordance with example 1 and included as Positive Control 3 and Positive Control 4 respectively in the competitive
10 binding assay.

1 μ g/mL vitronectin dissolved in coating solution were added to each well of a 96-well ELISA plate, and the plate was incubated overnight at 4°C. The wells were washed twice with PBS buffer, and excess liquid was carefully removed with a paper towel.

Blocking solution (5 g non-fat skim milk in 100 mL 1x PBS) were added to each
15 well, and the plate was incubated at room temperature for 1 hour. The wells were washed twice with 1x PBS buffer.

Various concentrations of Positive Control 3, Positive Control 4, and Fusion Protein 1 were mixed with certain concentration of soluble integrin $\alpha v \beta 3$. One hundred microliters (100 μ L) of the serially diluted samples were added to each well. The plate
20 was covered and incubated on a plate shaker (~100 rpm) for 1 hour at room temperature. The wells were washed three times with wash buffer (1x PBS, 0.05% Tween-20).

Diluted primary anti-integrin αv antibody was added and incubated, then washed. Horseradish peroxidase-conjugated goat anti-human IgG Fc specific antibodies in blocking solution were added to each well. The plates were sealed and incubated on a
25 plate shaker for 1 hour at room temperature. The plates were washed three times with wash buffer.

100 μ L of 3,5,3',5'-Tetramethylbenzidine (TMB) were added to each well, and the plates were incubated for 3 to 5 minutes to allow for the reaction to take place. To stop the reaction, 100 μ L of stop solution (1N HCl) were added to each well.

30 The optical density (OD) of each well was determined using an ELISA plate reader at an absorbance wavelength of 450 nm. The absorbance was plotted against the protein concentration of the fusion protein or the control, and the concentration at which the signal was half the maximal inhibition concentration (IC₅₀) was determined.

The competitive binding, expressed as the IC_{50} value, was between 2.2 and 16 nM for the tested fusion protein of the invention. The results are shown in Table 2 and also illustrated in Figure 4

Table 2

Test Material	IC_{50} (nM)
Positive Control 3 (wild type Rho, SEQ ID NO: 1)	2.6-3.2
Positive Control 4 (Rho variant KG, SEQ ID NO: 13)	2.2-2.9
Fusion Protein 1 (SEQ ID NO: 16)	2.2-16.0

5

Example 5 – Inhibition of HUVEC Proliferation by Fusion Protein

A human umbilical vein endothelial cell (HUVEC) proliferation assay was carried out to test the functionality of the fusion proteins of the invention. A commercially available VEGF Trap, aflibercept (Eylea®, Regeneron), was included as Positive Control 2.

10 100 μ L of a coating solution (1% gelatin in double distilled water) were added to each well of a 96-well ELISA plate, and the plate was incubated for 2 hours or overnight at 37°C. The wells were washed twice with 1x PBS buffer.

3500 counts of human umbilical vein endothelial cells in endothelial cell growth medium were added to each well, and the plate was incubated overnight at 37°C.

15 Fusion protein samples were diluted in assay buffer (Medium-199 1x Earle's Salts, 10% fetal bovine serum, 10 mM HEPES, 1x antibiotic/antimycotic), with a highest protein concentration of 300 nM. The fusion protein samples were mixed with VEGF₁₆₅ (8 ng/mL), and the mixtures were incubated overnight at room temperature. The wells were then washed with 200 μ L of 1x PBS.

20 100 μ L of the VEGF₁₆₅/sample mixture were added to each well, and the plates were incubated for 72 hours at 37°C with 5% CO₂. Following incubation, 10 μ L MTS detection reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) + phenazine methosulfate in distilled PBS) were added to each well, and the plates were incubated at 37°C for 2.5 hours.

25 The OD of each well was determined using an ELISA plate reader at an absorbance wavelength of 490 nm. The absorbance was plotted against the protein concentration of the fusion protein or the control, and the concentration at which the cell proliferation was inhibited by 50% (IC_{50}) was determined.

30 The inhibition of cell proliferation (IC_{50}) was determined to be between 0.039 and 0.103 nM for the tested fusion protein of the invention. The results of the proliferation assay are shown in Table 3 and they are also illustrated in Figure 5. Both Positive Control 2 and Fusion Protein 1 exhibited similar activity on the inhibition of VEGF dependent HUVEC proliferation.

Table 3

Test Material	IC ₅₀ (nM)
Positive Control 2 (afibercept)	0.026-0.136
Fusion Protein 1 (SEQ ID NO: 16)	0.039-0.103

Example 6 – Inhibition of $\alpha\beta 3$ and $\alpha 5\beta 1$ Cell Adhesion by Fusion Protein

The adhesion of $\alpha\beta 3$ -overexpressing CHO cells to fibrinogen and $\alpha 5\beta 1$ -
 5 expressing K562 cells to fibronectin were evaluated in the presence of fusion proteins.

96-well Immulon-2 microtiter plates (Costar, Corning, NY) were coated with 100
 μ L of phosphate-buffered saline (PBS: 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4)
 containing substrates at a concentration of 50-500 nM, and incubated overnight at 4°C.
 The substrates and their coating concentrations were fibrinogen (Fg) at 200 μ g/mL and
 10 fibronectin (Fn) at 25 μ g/mL. Non-specific protein binding sites were blocked by
 incubating each well with 200 μ L of heat-denatured 1% bovine serum albumin (BSA)
 (Calbiochem) at room temperature for 1.5 hours. The heat-denatured BSA was discarded
 and each well was washed twice with 200 μ L of PBS.

CHO cells that expressed the integrin $\alpha\beta 3$ (CHO- $\alpha\beta 3$) from the same source
 15 of Patent Application No. 62/US2015/46322 were maintained in DMEM. Human
 erythroleukemia K562 was maintained as written in Patent Application No.
 62/US2015/46322 from ATCC and cultured in Roswell Park Memorial Institute (RPMI)-
 1640 medium containing 5% FCS. Harvested K562 was washed in PBS buffer containing
 1 mM EDTA and resuspended in Tyrode's buffer (150 mM NaCl, 5 mM KCl, and 10
 20 mM HEPES, pH 7.35) containing 1 mM MgSO₄, 2 mM CaCl₂, and 500 μ M MnCl₂.

Cells (CHO and K562) were diluted to 3×10^5 cells/mL, and 100 μ L of the cells were
 used for the assay. Rho and its mutants were added to the cultured cells and incubated
 at 37°C, 5% CO₂ for 15 minutes. Rho and its variants were used as inhibitors at the
 concentrations of 0.001-500 μ M. The treated cells were then added into the coated plate
 25 and incubated at 37°C, 5% CO₂ for 1 hour. The incubation solution was then discarded
 and non-adhered cells were removed by washing twice with 200 μ L PBS. Bound cells
 were quantified by crystal violet staining. Briefly, the well was fixed with 100 μ L of 10%
 formalin for 10 minutes and dried. Fifty microliters (50 μ L) of 0.05% crystal violet were
 then added into the well at room temperature for 20 minutes. Each well was washed with
 30 200 μ L of distilled water four times and dried. Colorization was carried out by adding 150
 μ L of colorizing solution (50% alcohol and 0.1% acetic acid). The resulting absorbance
 was read at 600 nm and the readings were correlated with the number of adhering cells.

Inhibition calculation of cell adhesion was conducted according to the following
 equation.

$$\% \text{ Inhibition} = 100 - \frac{OD_{600}(TA) - OD_{600}(NC)}{OD_{600}(PC) - OD_{600}(NC)} \times 100$$

In the above equation, TA refers to a “test article”; NC refers to “negative control”, and PC refers to “positive control”.

The IC₅₀ was defined as the concentration (nM) of a disintegrin variant required for 50% inhibition of the cell adhesion mediated by a particular integrin. Therefore, lower IC₅₀ indicates greater specificity or potency of the disintegrin variant in inhibiting the cell adhesion activity of the respective integrin, thus higher binding activity (or selectivity) of the disintegrin variant to the respective integrin. The IC₅₀ results are summarized in **Table 4**. Figure 6 provides a schematic showing the inhibition of CHO- $\alpha v\beta 3$ cell adhesion by Fusion Protein 1.

Table 4

Test Material	$\alpha v\beta 3$, IC ₅₀ (nM)	$\alpha 5\beta 1$, IC ₅₀ (nM)
Positive Control 3 (SEQ ID NO: 1)	24.7	252.5
Positive Control 4 (SEQ ID NO: 13)	19.6	17.5
Fusion Protein 1 (SEQ ID NO: 16)	4.76	3.4

Example 7: Inhibition of HUVEC Tube Formation by Fusion Protein

HUVECs (1.5×10^4 cells/well) were placed in a 96-well matrigel-coated plate. Six doses of Fusion Protein 1 (0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, and 30 μ M) and vehicle were added to each well in growth media under an atmosphere of 5% CO₂ at 37°C. After an 18-hour incubation period, morphology of the endothelial cell tubes, which resemble a capillary-like network, were evaluated by photomicroscopy. Disruption (anti-angiogenesis) of total tube length was measured from each photograph and determined relative to the vehicle control group. Minimum inhibitory concentration (MIC $\geq$ 30%) of tube formation was then determined and used to assess the degree of anti-angiogenesis. Suramin was used as an anti-angiogenic positive control (Positive Control 5) for all studies.

The inhibition of tube formation (IC₅₀) was determined to be less than 0.1 μ M for the tested Fusion Protein 1 which showed a higher potency than Positive Control 4. The results of the tube formation assay are shown in **Table 5**. Figures 7A-G provide a schematic showing the inhibitory effect of Fusion Protein 1 on tube formation when Fusion Protein 1 was added at a dose of 0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, and 30 μ M, respectively.

Table 5

Test Material	IC ₅₀ (μM)
Positive Control 5 (Suramin®)	15.8
Positive Control 4 (SEQ ID NO: 13)	5.9
Fusion Protein 1 (SEQ ID NO: 16)	<0.1

Example 8 – Inhibition of VEGF-Induced Leakage in Dutch Belted Rabbits by**5 Fusion Proteins**

Fusion proteins of the invention were tested in an *in vivo* model of vascular permeability to determine their efficacy in preventing vascular leakage. In this model, VEGF was intravitreally injected to the vitreous of rabbit eyes to induce uncontrolled retinal leakage. Aflibercept (Eylea®, Regeneron), a recombinant fusion protein consisting of portions of human VEGFR1 and VEGFR2 fused to the Fc portion of human IgG1, and bevacizumab (Avastin®, Roche), a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A were included as Positive Controls 2 and 6, respectively.

In one embodiment, a pharmaceutical composition contained 20,000 μg/mL of Fusion Protein 1 in a formulation buffer comprising 25 mM histidine buffer, 20mM NaCl, 6% (w/v) sucrose, 0.03% (w/v) polysorbate, pH 6.0.

Dutch Belted rabbits were anesthetized using isoflurane (3-5%), and their eyes were treated with ophthalmic Betadine solution. The rabbits' eyes were then washed with sterile saline, and lidocaine hydrochloride (2% injectable) or proparacaine (0.5%) was applied to the ocular surface.

On Day 1, Dutch Belted rabbits were intravitreally injected with fusion proteins of the invention, vehicle (negative) control, or reference (positive) controls at predetermined doses using a BD 300 μL insulin syringe (31 ga x 5/16 inch). The needle was inserted through the dorso-temporal quadrant of the eye, approximately 3-4 mm posterior to the limbus and 3-4 mm lateral to the dorsal rectus muscles, and 50 μL of solution was delivered. Vascular leakage was induced by injecting exogenous VEGF₁₆₅ into the same eyes on Day 3.

Fluorescein angiography (FA) was conducted on all dosage groups 3 days after VEGF injection to assess leakiness and tortuosity using a scale from 0 (normal) to 4 (severe).

Signs of ocular irritation were scored using the Draize scoring system prior to fusion protein dosing, VEGF induction, and assessments. According to the Draize analysis, all of the rabbit eyes were normal prior to the initiation of dosing. Transient signs

of minimal ocular inflammation were observed in all treatment groups after intravitreal dose administration, and were attributed to the intravitreal procedure. There were no drug-related findings evident during the course of the study.

FAs associated with the vehicle control group had the highest mean score (2.58) associated with retinal vasculature leakiness and tortuosity. The two reference positive control groups 2 and 6 had mean scores of 0 and 0.25, indicating a significant reduction in retinal vasculature leakiness and tortuosity. The tested fusion proteins of the invention had a mean score of 0.167, showing effectiveness in reducing VEGF-induced retinal leakiness and tortuosity comparable to the positive controls. The results of the *in vivo* assay are shown in Table 6. Figures 8A-D provide the representative FAs showing the inhibition of VEGF-induced leakage in Dutch Belted Rabbits by the Fusion Protein 1 and positive controls.

Table 6

Test Material	Dose (µg)	No. of Scores	Day 6 Mean Leakage Score
Vehicle	0	12	2.583
Positive Control 6 (bevacizumab)	1250	12	0.250
Positive Control 2 (aflibercept)	625	12	0
Fusion Protein 1 (SEQ ID NO: 16)	1000	12	0.417

Example 9 – Dose-Response Inhibition of VEGF-Induced Leakage in Dutch Belted Rabbits by Fusion Protein

Fusion proteins of the invention were tested in an *in vivo* model of retinal vascular permeability at varying doses to determine their dose-response effectiveness in preventing vascular leakage. In this model, human VEGF₁₆₅ was intravitreally injected to the vitreous of rabbit eyes to induce retinal leakage.

On Day 1, Dutch Belted rabbits were intravitreally injected with Fusion Protein 1 according to an embodiment of the invention at various doses, vehicle (negative) control, or reference (positive) controls. Vascular leakage was induced by injecting exogenous VEGF₁₆₅ into the same eyes on Day 3.

FAs were conducted on all dosage groups 3 days after the VEGF-induction (Day 6) to assess leakiness and tortuosity using a scale from 0 (normal) to 4 (severe).

Signs of ocular irritation were scored using the Draize scoring system prior to fusion protein dosing, VEGF induction, and assessments. According to the Draize analysis, all of the rabbit eyes were normal prior to the initiation of dosing. Transient

signs of minimal ocular inflammation were observed in all treatment groups after intravitreal dose administration, and were attributed to the intravitreal procedure. There were no drug-related findings evident during the course of the study.

For the first exogenous VEGF injection, FAs associated with vehicle control group had the highest mean score (3.4) associated with retinal vasculature leakiness and tortuosity. The two reference positive control groups had mean scores of 0, indicating a significant reduction in retinal vasculature leakiness and tortuosity. The tested fusion protein of the invention (Fusion Protein 1) had scores of 0.08, 0.42, and 0.17 at doses of 100, 500 and 1000 μ g, respectively, showing effectiveness in reducing VEGF-induced retinal leakiness and tortuosity comparable to the positive controls.

The results of the dose-response in vitro assay are shown in Table 7.

Table 7

Test Material	Dose (μ g)	No. of Scores	Day 6 Mean Leakage Score
Vehicle	0	10	3.400
Positive Control 6 (bevacizumab)	1250	12	0.17
Positive Control 2 (aflibercept)	625	12	0
Fusion Protein 1 (SEQ ID NO: 16)	1000	12	0.33
Fusion Protein 1 (SEQ ID NO: 16)	500	10	0.40
Fusion Protein 1 (SEQ ID NO: 16)	100	10	0.60

Example 10 – Reduction of Lesion Size in Laser-induced Choroidal Neovascularization (CNV) in Rats by Fusion Proteins

The eyes of Brown Norway rats were dilated with a 1% Cyclogyl solution and protected from light. Following the dilation, the rats were anesthetized using a ketamine and xylazine mixture. Three lesion burns were introduced to the retina of each eye using a laser at 532 nm on Day 1.

On Day 3, the animals were anesthetized with a ketamine and xylazine mixture, their eyes were dilated, and 5 μ L of Fusion Protein 1, vehicle (negative control), or Positive Control 2 (reference) at predetermined doses were intravitreally injected into both eyes of an animal using a Hamilton syringe with 33 gauge needle.

To confirm successful lesion formation, fundus images were taken using a Micron III small animal funduscope (Phoenix Research) prior to lesion introduction, after the lesion burns, and on Day 22. The animals received an IP injection of 10% fluorescein sodium at 1 μ L/g of body weight on Day 22 to assess neovascularization of the lesion burns. From fluorescein angiograms, lesion size were determined and compared across dosage groups as shown in Figures 9A-C, wherein Figure 9A shows a schematic of

vehicle, Figure 9B shows a schematic of Positive Control 2, and Figure 9C shows a schematic of Fusion Protein 1. The arrows (▼) in Figures 9A-C indicated laser lesion spots.

Example 11 – Reduction of Lesion Size in Laser-induced CNV in Monkeys by

5 Fusion Proteins

The fusion proteins are tested in a laser-induced CNV model established in monkeys. Six to nine burns are introduced around the macula of each eye using 532 nm diode laser photocoagulation, and 0.5-4 mg of fusion proteins of the invention are intravitreally injected on the same day.

10 The animals are sedated with intravenous 2.5% soluble pentobarbitone (1 mL/kg) 20 days later. The eyelids are fixed to keep the eyes open, and color photographs are taken using a fundus camera.

Fluorescein dye (20% fluorescein sodium; 0.05mL/kg) is injected into a vein of a lower extremity. Photographs are taken at several time points after injection of the dye, 15 including the arterial phase, early arteriovenous phase, and several late arteriovenous phases, to monitor leakage of fluorescein associated with CNV lesions.

Example 12 – Inhibition of Orthotopic Human Glioblastoma Tumor Growth in Xenograft Mice by Fusion Proteins

20 The human glioblastoma cell line U87-MG (2.5×10^5 cells/2 μ L, Luciferase cells) is implanted into BALB/c nude mice to establish an orthotopic xenograft model.

In order to assess the inhibitory effects of the fusion proteins of the invention on the tumor growth, tumor cells are implanted into nude mice, and various concentrations of fusion proteins according to embodiments of the invention, ranging from 3 to 30 mg/kg, 25 are administered to the mice intravenously twice weekly. The tumor growth, and survival rate of animals are measured weekly for up to 8 weeks.

Example 13 – Inhibition of Lung Fibrosis in Bleomycin-Induced Fibrosis Mice by the Fusion Proteins

30 Male C57BL/6 mice weighing 20 ± 2 g are used to induce lung fibrosis and the effect of the fusion proteins on inhibiting fibrosis. Animals are anesthetized with isoflurane and then a single dose of bleomycin at 1.5 IU/kg (dissolved in 25 μ L of saline) is administered intratracheally on Day 1. This dose of bleomycin is known to reproducibly generate pulmonary fibrosis and may induce a mortality of 10-20%.

35 Nintedanib is administered each day by oral gavage at 10-50 mg/kg per day as a positive control. The administration volume is 10 mL/kg body weight. As a negative control, the fusion protein vehicle is administered to the animals by IV injection. A no

treatment control is used to monitor the lung pathology changes after bleomycin administration. Various doses of Fusion Protein 1 and vehicle are administered intravenously once a day, starting from Day 1 to Day 21. Clinical observations including body weight are monitored daily. Animals are sacrificed on Day 22. Bronchoalveolar

5 lavage fluid is collected to investigate cell count and TGF- β 1.

In order to assess the inhibitory effects of the fusion proteins of the invention on fibrosis formation, lung tissues are removed and homogenized in buffer to measure the change of hydroxyproline compared to a standard curve. Other histopathology experiments are performed to gather expression of fibrosis and inflammatory specific

10 biomarkers.

While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and

15 scope of the invention.

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<120> Fusion Proteins for Inhibiting Angiogenesis

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<140>105120174

<141>2016-6-27

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20

25

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35

40

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20

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15

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20

25

30

Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro

35

40

45

Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser

50

55

60

Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val

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35 40 45

Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu

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Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr

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35 40 45

Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser

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Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val

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Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp			
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 20 25 30
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 35 40 45
 Gly Thr Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp
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 Thr Gly Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr
 65 70 75 80
 Asp Glu Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly
 85 90 95
 Phe Leu Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile
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 Thr Glu Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val
 115 120 125
 Thr Leu His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp
 130 135 140
 His Gln Arg Gly Phe Phe Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys
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 Lys Thr Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val
 165 170 175
 Tyr Arg Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln
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Thr Val Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile
 195 200 205
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 210 215 220
 Gly Arg Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr
 225 230 235 240
 His Ile Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser
 245 250 255
 Gly Thr Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp
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Cys Glu Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Gly

35 40 45

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Cys Glu Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Gly

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35 40 45

Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser

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Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val

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Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn

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130	135	140	
Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met			
145	150	155	160
Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp			
165	170	175	
Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys			
180	185	190	
Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr			
195	200	205	
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser			
210	215	220	
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg			
225	230	235	240
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro			
245	250	255	
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala			
260	265	270	
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val			
275	280	285	

Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr

290

295

300

Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr

305

310

315

320

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu

325

330

335

Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys

340

345

350

Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser

355

360

365

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp

370

375

380

Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser

385

390

395

400

Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala

405

410

415

Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys

420

425

430

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly

435

440

445

Lys Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Ala Ala

450

455

460

Thr Cys Lys Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys Cys

465

470

475

480

Glu Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Gly Arg

485

490

495

Gly Asp Asn Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys Pro

500

505

510

Arg Trp Asn Asp Leu

515

<210> 16

<211> 516

<212> PRT

<213>Artificial Sequence

<220>

<223> Fusion of VEGFR1 D2/VEGFR2 D3 and Rhodostomin variant KG

<400> 16

Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile

1

5

10

15

Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr

20

25

30

Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu

35

40

45

Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile

50

55

60

Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala				
65	70	75	80	
Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln				
	85	90	95	
Thr Asn Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile Glu				
	100	105	110	
Leu Ser Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu				
	115	120	125	
Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His				
	130	135	140	
Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser				
145	150	155	160	
Glu Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg				
	165	170	175	
Ser Asp Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr				
	180	185	190	
Lys Lys Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Pro Gly Asp				
	195	200	205	
Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly				
	210	215	220	
Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile				
225	230	235	240	
Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu				
	245	250	255	

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His

260

265

270

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg

275

280

285

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys

290

295

300

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu

305

310

315

320

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr

325

330

335

Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu

340

345

350

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp

355

360

365

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val

370

375

380

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp

385

390

395

400

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His

405

410

415

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro

420

425

430

Gly Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser

435

440

445

Gly Lys Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Ala

450

455

460

Ala Thr Cys Lys Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys

465

470

475

480

Cys Glu Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Gly

485

490

495

Arg Gly Asp Asn Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys

500

505

510

Pro Arg Tyr His

515

<210> 17

<211> 515

<212> PRT

<213>Artificial Sequence

<220>

<223> Fusion of VEGFR1 D2/VEGFR2 D3 and Rhodostomin variant KA

<400> 17

Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His

1

5

10

15

Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro

20

25

30

Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro			
35	40	45	
Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser			
50	55	60	
Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val			
65	70	75	80
Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn			
85	90	95	
Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser			
100	105	110	
Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn			
115	120	125	
Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His			
130	135	140	
Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met			
145	150	155	160
Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp			
165	170	175	
Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys			
180	185	190	
Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr			
195	200	205	
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser			
210	215	220	

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg			
225	230	235	240
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro			
	245	250	255
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala			
	260	265	270
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val			
	275	280	285
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr			
	290	295	300
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr			
305	310	315	320
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu			
	325	330	335
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys			
	340	345	350
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser			
	355	360	365
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp			
	370	375	380
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser			
385	390	395	400
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala			
	405	410	415

Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 420 425 430
 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
 435 440 445
 Lys Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Ala Ala
 450 455 460
 Thr Cys Lys Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys Cys
 465 470 475 480
 Glu Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Ala Arg
 485 490 495
 Gly Asp Asn Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys Pro
 500 505 510
 Arg Tyr His
 515

<210> 18

<211> 514

<212> PRT

<213> Artificial Sequence

<220>

<223> Fusion of Eylea and Rhodostomin variant KG

<400> 18

Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu

1 5 10 15

Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val

20 25 30

Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr

35 40 45

Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe

50 55 60

Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu

65 70 75 80

Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg

85 90 95

Gln Thr Asn Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile

100 105 110

Glu Leu Ser Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr

115 120 125

Glu Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys

130 135 140

His Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly

145 150 155 160

Ser Glu Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr

165 170 175

Arg Ser Asp Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met

180 185 190

Thr Lys Lys Asn Ser Thr Phe Val Arg Val His Glu Lys Asp Lys Thr

195

200

205

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser

210

215

220

Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg

225

230

235

240

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro

245

250

255

Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala

260

265

270

Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val

275

280

285

Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr

290

295

300

Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr

305

310

315

320

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu

325

330

335

Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys

340

345

350

Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser

355

360

365

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp

370

375

380

Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
 385 390 395 400
 Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
 405 410 415
 Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Gly
 420 425 430
 Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Lys
 435 440 445
 Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Ala Ala Thr
 450 455 460
 Cys Lys Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys Cys Glu
 465 470 475 480
 Gln Cys Lys Phe Lys Lys Ala Arg Thr Ile Cys Ala Arg Gly Arg Gly
 485 490 495
 Asp Asn Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys Pro Arg
 500 505 510
 Tyr His

<210> 19

<211> 1551

<212> DNA

<213> Artificial Sequence

<220>

<223> nucleotide sequence of fusion protein of SEQ ID NO: 16

<400> 19

agcggcggaa ggccttctgt cgagatgtac agcgagatcc ccgagatcat tcacatgacc	60
gaaggcaggg agctcgtgat cccttgcagg gtcacatccc ccaacatcac cgtcacccctc	120
aagaagtcc ccctggatac cctcatcccc gacggcaagc ggatcatttg ggatagccgg	180
aagggttca tcattccaa cgctacctac aaagaaattg gactgctgac ctgcgaggct	240
accgtcaacg gccacctcta taagaccaac tacctgacct acaggcagac caataccatc	300
atcgatgtgg tcctcagccc cagccacgga atcgaactgt ccgtgggcga gaagctggtc	360
ctgaactgta cagccaggac agaactcaac gtgggcatcg acttcaactg ggagtacct	420
agctccaagc accagcaca gaagctggtc aaccgggacc tgaagacca gtccggctcc	480
gaaatgaaga agttcctgtc caccctcacc atcgatggag tcacccggag cgatcaggga	540
ctgtatacct gcgccgctc ctccggcctg atgacaaaga agaacagcac ctctgtgcgg	600
gtgcacgaga aaggccccgg cgacaagaca cacacctgcc ctccctgccc cgcccccgag	660
ctgctcggcg gaccagcgt gtctctgttc cccctaagc ccaaggacac cctcatgac	720
agcaggacct ctgaggtgac atgcgtcgtc gtggacgtga gccatgaaga ccccgagggtg	780
aagtcaact ggtatgtgga cggcgtcgag gtgcataacg ccaagacaa accccgggag	840
gagcaataca acagcacata cagggtgggtg tccgtgctga ccgtcctgca ccaggattgg	900
ctgaacggca aagagtataa gtgcaagggtg agcaacaaag ccctgcccgc tccatcgag	960
aagacaatct ccaaggccaa gggccaacct agggagcctc aggtgtacac actgcctcct	1020
tcccgggacg agctgacaaa aaaccaagtg agcctgacct gcctcgtcaa gggcttctac	1080
ccttccgata tcgccgtgga gtgggagtc aacggccagc ctgagaacaa ctacaagacc	1140
accctcccg tgctcgattc cgacggctcc ttcttctct acagcaagct cacagtggac	1200
aatcccggt ggcagcaggg caatgtgttc agctgttccg tgatgcacga ggccctccac	1260

aatcactaca cccagaagag cctgtccctg tccccggcg gaggcggcgg ctccggcgga 1320
ggcggctccg gcggcggcgg atccggaaaa gagtgcgatt gcagctcccc cgagaacccc 1380
tgctgcgatg ccgctacatg caaactgcgg cctggagccc agtgtggaga aggcctgtgc 1440
tgcgagcagt gcaagttaa gaaggcccgg accatttgtg ctaggggccc gggagacaac 1500
cctgacgacg ggtgcaccgg ccaaagcgt gactgtcccc ggtaccactg a 1551

<210> 20

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> A Signal Peptide

<400> 20

Met Ala Trp Ala Leu Leu Leu Leu Thr Leu Leu Thr Arg Asp Thr Gly

1

5

10

15

Ser Trp Ala

<210> 21

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> Nucleotide Sequence of a Signal Peptide

<400> 21

atggcctggg ctctcctgct gctgaccctg ctgacacggg acacaggatc ctgggcc 57

<210>22

<211>26

<212>PRT

<213>Artificial Sequence

<220>

<223> Signaling Peptide of Fusion Protein of SEQ ID NO: 18

<400> 22

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser

1

5

10

15

Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly

20

25

<210>23

<211>431

<212>PRT

<213>Artificial Sequence

<220>

<223> VEGFR1 extracellular domain D2/VEGFR2 extracellular domain D3
fusion with human IgG 1 Fc region

<400>23

Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile

1 5 10 15

Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr

20 25 30

Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu

35 40 45

Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile

50 55 60

Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala

65 70 75 80

Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln

85 90 95

Thr Asn Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile Glu

100 105 110

Leu Ser Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu

115 120 125

Leu Asn Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His

130	135	140	
Gln His Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser			
145	150	155	160
Glu Met Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg			
	165	170	175
Ser Asp Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr			
	180	185	190
Lys Lys Asn Ser Thr Phe Val Arg Val His Glu Lys Asp Lys Thr His			
	195	200	205
Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val			
	210	215	220
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr			
225	230	235	240
Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu			
	245	250	255
Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys			
	260	265	270
Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser			
	275	280	285
Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys			
	290	295	300
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile			
305	310	315	320
Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro			

325	330	335	
Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu			
340	345	350	
Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn			
355	360	365	
Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser			
370	375	380	
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg			
385	390	395	400
Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu			
405	410	415	
His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys			
420	425	430	

WHAT IS CLAIMED IS:

1. A fusion protein comprising:

a disintegrin binding to integrins $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$, which has an amino acid sequence selected from a group consisting of SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14,

5 other protein binding peptide binding to an angiogenic factor,

the angiogenic factor comprising Angiopoietin (ANG), Ephrin (Eph), Fibroblast Growth Factor (FGF), Neuropilin (NRP), Plasminogen Activators, urokinase-type plasminogen activator receptor (uPAR), Platelet-Derived Growth Factor (PDGF), Placental growth factor (PlGF), Tumor Growth Factor beta (TGF- β), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial cadherin (VE-cadherin), Insulin-like Growth Factor (IGF), Connective-Tissue Growth Factor (CTGF), Tumor Necrosis Factor alpha (TNF- α), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), and receptors thereof; and

a Fc domain.

15 2. The fusion protein of claim 1, wherein the other protein binding peptide comprises extracellular domains of VEGF receptors.

3. The fusion protein of claim 1, wherein

20 the Fc domain is a human or humanized constant sub-region comprising an immunoglobulin CH2 domain and a CH3 domain; and

the other protein binding peptide having an Ig-like domain D2 of a VEGFR1 and an Ig-like domain D3 of a VEGFR2.

25 4. The fusion protein according to claim 1 or 2 comprising the disintegrin, the Fc domain and the other protein binding peptide from C-terminus to N-terminus.

5. The fusion protein according to claim 1 or 2 comprising the disintegrin, the Fc domain and the other protein binding peptide from N-terminus to C-terminus.

30 6. The fusion protein of any of claims 1-3 further comprising a linker sequence between the disintegrin and the other protein binding peptide.

7. The fusion protein of any of claims 1-3 further comprising a signal peptide sequence upstream of the other protein binding peptide.
- 5 8. The fusion protein according to claim 2, wherein the extracellular domains of VEGF receptors comprise an Ig-like domains D1-D7 of the VEGF receptors.
9. The fusion protein according to claim 2, wherein extracellular domains of VEGF receptors comprising i) an Ig-like domain D2 of a VEGFR1 and an Ig-like domain D3 of a
10 VEGFR2; or ii) the amino acid sequence of SED ID NO: 10.
10. The fusion protein of claim 1 or 2 further comprising a GS or G₉ linker between the Fc domain and the disintegrin or the other protein binding peptide.
- 15 11. A nucleic acid encoding the fusion protein of any of claims 1-3.
12. A dimer of the fusion protein of any of claims 1-3.
13. A vector comprising a nucleotide sequence encoding the fusion protein of any of
20 claims 1-3.
14. A method of producing a fusion protein, comprising culturing a host cell comprising a nucleic acid encoding the fusion protein of any of claims 1-3 under a condition that produces the fusion protein, and recovering the fusion protein produced by the host cell.
- 25 15. A composition comprising the fusion protein according to any of claims 1-3 and a pharmaceutically acceptable adjuvant, carrier or excipient.
16. Use of the fusion protein of any one of claims 1-3 in the manufacture of a
30 medicament for treating an angiogenic disease.
17. The use of claim 16, wherein the angiogenic disease comprises rheumatoid arthritis, inflammatory arthritis, osteoarthritis, ocular and systemic cancer, tumor related metastasis and invasion, ocular disease characterized by neovascularization or ischemia, uveitis,
35 retinitis pigmentosa, age-related macular degeneration (AMD), diabetic retinopathy and diabetic macular edema (DME).

18. The use of claim 17, wherein the ocular disease characterized by neovascularization or ischemia is choroidal neovascularization.
- 5 19. Use of the fusion protein of any one of claims 1-3 in the manufacture of a medicament for wound healing.