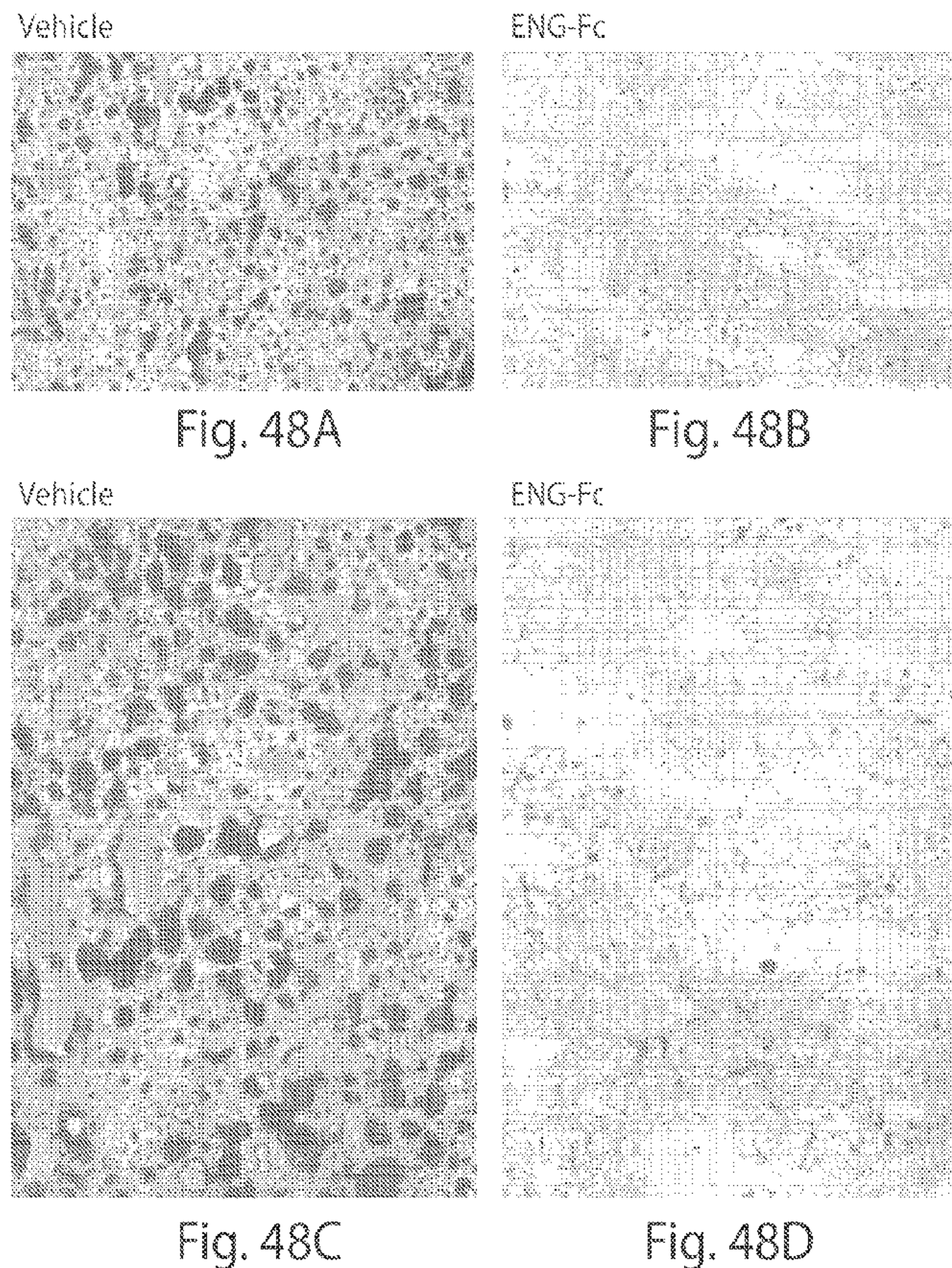




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(54) **Titre : PEPTIDES D'ENDOGLINE POUR TRAITER DES MALADIES FIBROTIQUES**  
 (54) **Title: ENDOGLIN PEPTIDES TO TREAT FIBROTIC DISEASES**



(57) **Abrégé/Abstract:**

In certain aspects, the present disclosure relates to the insight that a polypeptide comprising a truncated, ligand-binding portion of the extracellular domain of endoglin (ENG) polypeptide may be used to treat fibrotic disorders.

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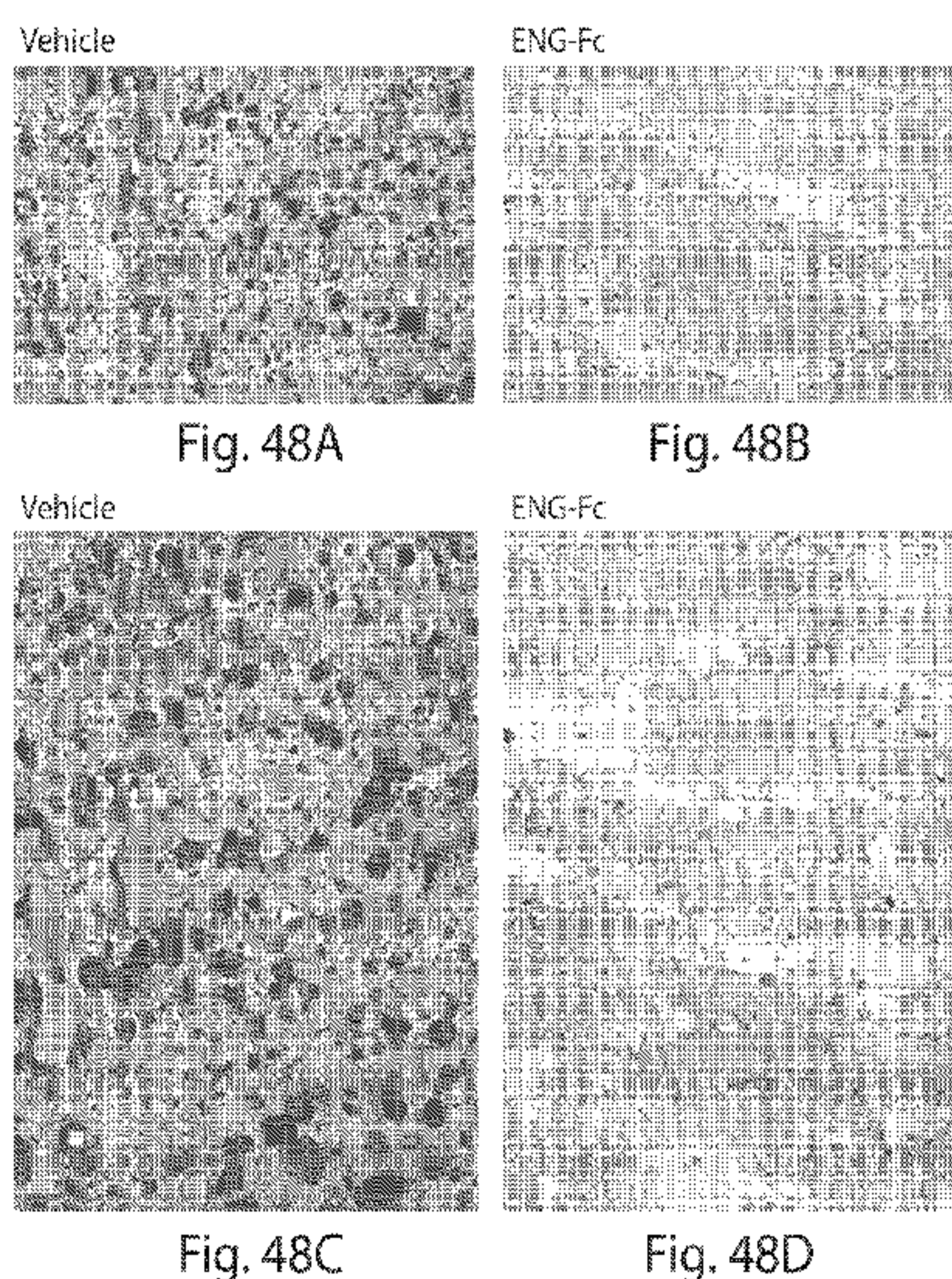
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(54) Title: ENDOGLIN PEPTIDES TO TREAT FIBROTIC DISEASES



(57) Abstract: In certain aspects, the present disclosure relates to the insight that a polypeptide comprising a truncated, ligand-binding portion of the extracellular domain of endoglin (ENG) polypeptide may be used to treat fibrotic disorders.

## ENDOGLIN PEPTIDES TO TREAT FIBROTIC DISEASES

### RELATED APPLICATIONS

This application claims the benefit of the filing date under 35 U.S.C. §119 of United States Provisional Application Serial Number 61/896,002, filed October 25, 2013, and entitled *Endoglin Peptides To Treat Fibrotic Diseases*, the entire contents of which are incorporated herein by reference.

### BACKGROUND

Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue. Fibrosis may occur in response to physical or chemical injury as part of a reparative or reactive process, also referred to as scarring. Fibrosis may also arise from a pathological aberration in a cell or tissue without external injury. Fibrosis results in the deposition of connective tissue, which can support tissue homeostasis and healing after trauma. Excessive fibrosis, however, can obliterate the architecture and impede the function of the underlying organ or tissue, leading to fibrotic disorders, such as, for example, liver fibrosis, pulmonary fibrosis, and cystic fibrosis. Fibrotic tissue can typically not carry out the specialized functions of the respective organ, and cannot be repaired. Treatment options for fibrotic disorders are, thus, limited to tissue replacement approaches, such as organ transplantation, and palliative care.

It is desirable that effective compositions and methods for inhibiting and treating fibrosis be developed. These include methods and compositions which can inhibit and/or reverse excessive fibrosis associated with fibrotic disorders.

### SUMMARY

Some aspects of this disclosure provide endoglin (ENG) polypeptides and the use of such endoglin polypeptides to treat or prevent fibrotic disorders. Some embodiments of this disclosure provide methods of treating or preventing a fibrotic disorder in a patient in need thereof. In some embodiments, the method comprises administering to the patient an effective amount of an endoglin polypeptide provided herein. In some embodiments, the endoglin polypeptide used comprises an amino acid sequence that is at least 95% identical to amino acids 42-333 of SEQ ID NO: 1.

In some embodiments, the fibrotic disorder is liver fibrosis, vascular fibrosis, pulmonary fibrosis, pancreatic fibrosis, renal fibrosis, musculoskeletal fibrosis, cardiac fibrosis, skin fibrosis, eye fibrosis, progressive systemic sclerosis (PSS), chronic graft-versus-host disease, Peyronie's disease, post-cystoscopic urethral stenosis, retroperitoneal fibrosis, mediastinal fibrosis, progressive massive fibrosis, proliferative fibrosis, nephrogenic systemic fibrosis, neoplastic fibrosis, Dupuytren's disease, strictures, radiation induced fibrosis, cystic fibrosis, pleural fibrosis, sarcoidosis, scleroderma, spinal cord injury/fibrosis, myelofibrosis, vascular restenosis, atherosclerosis, injection fibrosis (which can occur as a complication of intramuscular injections, especially in children), or complications of coal workers' pneumoconiosis. In some embodiments, the fibrotic disorder is not myelofibrosis. In some embodiments, the liver fibrosis is liver cirrhosis, alcohol-induced liver fibrosis, biliary duct injury, primary biliary cirrhosis, infection-induced liver fibrosis, congenital hepatic fibrosis or autoimmune hepatitis. In some embodiments, the infection-induced liver fibrosis is bacterial-induced or viral-induced. In some embodiments, the pulmonary fibrosis is idiopathic, pharmacologically-induced, radiation-induced, chronic obstructive pulmonary disease (COPD), or chronic asthma. In some embodiments, the cardiac fibrosis is endomyocardial fibrosis or idiopathic myocardial pathology. In some embodiments, the skin fibrosis is scleroderma, post-traumatic, operative cutaneous scarring, keloids, or cutaneous keloid formation. In some embodiments, the eye fibrosis is glaucoma, sclerosis of the eyes, conjunctival scarring, corneal scarring, or pterygium. In some embodiments, the retroperitoneal fibrosis is idiopathic, pharmacologically-induced or radiation-induced. In some embodiments, the cystic fibrosis is cystic fibrosis of the pancreas or cystic fibrosis of the lungs. In some embodiments, the injection fibrosis occurs as a complication of an intramuscular injection.

In some embodiments, the endoglin polypeptide used to treat a fibrotic disorder as provided herein does not include a sequence consisting of amino acids 379-430 of SEQ ID NO: 1. In some embodiments, the endoglin polypeptide comprises an amino acid sequence at least 95% identical to a sequence beginning at an amino acid corresponding to any of positions 26-42 of SEQ ID NO: 1 and ending at an amino acid corresponding to any of positions 333-378 of SEQ ID NO: 1. In some embodiments, the endoglin polypeptide comprises an amino acid sequence at

least 95% identical to amino acids 26-346 of SEQ ID NO: 1, amino acids 26-359 of SEQ ID NO: 1, or amino acids 26-378 of SEQ ID NO: 1. In some embodiments, the endoglin polypeptide consists of a first portion consisting of an amino acid sequence at least 95% identical to amino acids 26-346 of SEQ ID NO: 1, amino acids 26-359 of SEQ ID NO: 1, or amino acids 26-378 of SEQ ID NO: 1, and a second portion that is heterologous to SEQ ID NO: 1. In some embodiments, the second portion of the endoglin polypeptide comprises an Fc portion of an IgG. In some embodiments, the endoglin polypeptide does not include more than 50 consecutive amino acids from a sequence consisting of amino acids 379-586 of SEQ ID NO: 1. In some 5 10 15 20 25 30

embodiments the endoglin polypeptide is a dimer or higher order multimer comprising two or more endoglin polypeptides, and may optionally be a homodimer, heterodimer, homomultimer or heteromultimer.

In some embodiments, the endoglin polypeptide used to treat a fibrotic disorder as provided herein binds human BMP-9 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $1 \times 10^{-3} \text{ s}^{-1}$ . In some embodiments, the endoglin polypeptide binds human BMP-9 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $5 \times 10^{-4} \text{ s}^{-1}$ . In some embodiments, the endoglin polypeptide binds human BMP-10 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $5 \times 10^{-3} \text{ s}^{-1}$ . In some 20 25 30

embodiments, the endoglin polypeptide binds human BMP-10 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $2.5 \times 10^{-3} \text{ s}^{-1}$ . Optionally the endoglin polypeptide characterized by any of the above BMP-9 or BMP-10 binding properties is a dimer or higher order multimer.

In some embodiments, the endoglin polypeptide does not bind human TGF- $\beta$ 1, human TGF- $\beta$ 3, human VEGF, or human basic fibroblast growth factor (FGF-2). In some embodiments, the endoglin polypeptide is a fusion protein including, in addition to a portion comprising an endoglin amino acid sequence, one or more polypeptide portions that enhance one or more of: in vivo stability, in vivo half-life, uptake/administration, tissue localization or distribution, formation of protein complexes, such as dimers or multimers, and/or purification. In some embodiments, the endoglin polypeptide includes a portion of a constant domain of an immunoglobulin and/or a portion of a serum albumin. In some embodiments, the

endoglin polypeptide comprises an immunoglobulin Fc domain. In some embodiments, the immunoglobulin Fc domain is joined to the ENG polypeptide portion by a linker. In some embodiments, the linker consists of an amino acid sequence consisting of SEQ ID NO: 31 (TGGG) or GGG. In some embodiments the Fc domains form a dimer. In some embodiments, the endoglin polypeptide includes one or more modified amino acid residues selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, and an amino acid conjugated to an organic derivatizing agent.

10 In some embodiments, the endoglin polypeptide is administered intravenously, intramuscularly, intraarterially, subcutaneously, or orally.

In part, the present disclosure provides endoglin polypeptides and the use of such endoglin polypeptides as selective antagonists for BMP9 and/or BMP10. As described herein, polypeptides comprising part or all of the endoglin extracellular domain (ECD) bind to BMP9 and BMP10 while not exhibiting substantial binding to other members of the TGF-beta superfamily. This disclosure demonstrates that polypeptides comprising part or all of the endoglin ECD are effective antagonists of BMP9 and BMP10 signaling and act to inhibit angiogenesis and tumor growth in vivo. Thus, in certain aspects, the disclosure provides endoglin polypeptides as antagonists of BMP9 and/or BMP10 for use in inhibiting angiogenesis as well as other disorders associated with BMP9 or BMP10 described herein.

In certain aspects, the disclosure provides polypeptides comprising a truncated extracellular domain of endoglin for use in inhibiting angiogenesis and treating other BMP9 or BMP10-associated disorders. While not wishing to be bound to any particular mechanism of action, it is expected that such polypeptides act by binding to BMP9 and/or BMP10 and inhibiting the ability of these ligands to form signaling complexes with receptors such as ALK1, ALK2, ActRIIA, ActRIIB and BMPRII. In certain embodiments, an endoglin polypeptide comprises, consists of, or consists essentially of, an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of amino acids 42-333, 26-346, 26-359 or 26-378 of the human endoglin sequence of SEQ ID NO:1. An endoglin polypeptide may comprise, consist of, or consist essentially of an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%

identical to the sequence of amino acids beginning at any of positions 26-42 of SEQ ID NO:1 and ending at any of positions 333-378 of the human endoglin sequence of SEQ ID NO:1. An endoglin polypeptide may comprise, consist of, or consist essentially of, a polypeptide encoded by a nucleic acid that hybridizes under less stringent, stringent or highly stringent conditions to a complement of a nucleotide sequence selected from a group consisting of: nucleotides 537-1412 of SEQ ID NO: 2, nucleotides 121-1035 of SEQ ID NO: 30, nucleotides 121-1074 of SEQ ID NO: 26, nucleotides 121-1131 of SEQ ID NO: 24, nucleotides 73-1035 of SEQ ID NO: 30, nucleotides 73-1074 of SEQ ID NO: 26, and nucleotides 73-1131 of SEQ ID NO: 24.

In each of the foregoing, an endoglin polypeptide may be selected such that it does not include a full-length endoglin ECD (e.g., the endoglin polypeptide may be chosen so as to not include the sequence of amino acids 379-430 of SEQ ID NO:1, or a portion thereof or any additional portion of a unique sequence of SEQ ID NO:1). An endoglin polypeptide may be used as a monomeric protein or in a dimerized form. An endoglin polypeptide may also be fused to a second polypeptide portion to provide improved properties, such as an increased half-life or greater ease of production or purification. A fusion may be direct or a linker may be inserted between the endoglin polypeptide and any other portion. A linker may be a structured or unstructured and may consist of 1, 2, 3, 4, 5, 10, 15, 20, 30, 50 or more amino acids, optionally relatively free of secondary structure. A linker may be rich in glycine and proline residues and may, for example, contain a sequence of threonine/serine and glycines (e.g., TGGG (SEQ ID NO: 31)) or simply one or more glycine residues, (e.g., GGG (SEQ ID NO: 32)). Fusions to an Fc portion of an immunoglobulin or linkage to a polyoxyethylene moiety (e.g., polyethylene glycol) may be particularly useful to increase the serum half-life of the endoglin polypeptide in systemic administration (e.g., intravenous, intraarterial and intra-peritoneal administration). In certain embodiments, an endoglin-Fc fusion protein comprises a polypeptide comprising, consisting of, or consisting essentially of, an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence of amino acids starting at any of positions 26-42 of SEQ ID NO:1 and ending at any of positions 333-378 of the human endoglin sequence of SEQ ID NO:1, and optionally may not include a full-length endoglin ECD (e.g., the endoglin polypeptide may be chosen so as to not include the sequence of amino acids 379-430 of SEQ ID NO:1, or a portion thereof, or so as not to include any 5, 10, 20, 30, 40, 50, 52, 60, 70, 100, 150

or 200 or more other amino acids of any part of endoglin or any part of amino acids 379 to 581 of SEQ ID NO:1), which polypeptide is fused, either with or without an intervening linker, to an Fc portion of an immunoglobulin. An endoglin polypeptide, including an endoglin-Fc fusion protein, may bind to BMP9 and/or BMP10 with a  $K_D$  of less than  $10^{-8}M$ ,  $10^{-9}M$ ,  $10^{-10}M$ ,  $10^{-11}M$  or less, or a dissociation constant ( $k_d$ ) of less than  $10^{-3}s^{-1}$ ,  $3 \times 10^{-3}s^{-1}$ ,  $5 \times 10^{-3}s^{-1}$  or  $1 \times 10^{-4}s^{-1}$ . The endoglin polypeptide may be selected to have a  $K_D$  for BMP9 that is less than the  $K_D$  for BMP10, optionally less by 5-fold, 10-fold, 20-fold, 30-fold, 40-fold or more. The endoglin polypeptide may have little or no substantial affinity for any or all of TGF- $\beta$ 1, - $\beta$ 2 or - $\beta$ 3, and may have a  $K_D$  for any or all of TGF- $\beta$ 1, - $\beta$ 2 or - $\beta$ 3 of greater than  $10^{-9}M$ ,  $10^{-8}M$ ,  $10^{-7}M$  or  $10^{-6}M$ . The endoglin polypeptide may be a dimer or higher order multimer.

An Fc portion may be selected so as to be appropriate to the organism. Optionally, the Fc portion is an Fc portion of a human IgG1. Optionally, the endoglin-Fc fusion protein comprises the amino acid sequence of any of SEQ ID NOs: 33, 34, 35, or 36. Optionally, the endoglin-Fc fusion protein is the protein produced by expression of a nucleic acid of any of SEQ ID Nos: 17, 20, 22, 24, 26, 28 or 30 in a mammalian cell line, particularly a Chinese Hamster Ovary (CHO) cell line. An endoglin polypeptide may be formulated as a pharmaceutical preparation that is substantially pyrogen free. The pharmaceutical preparation may be prepared for systemic delivery (e.g., intravenous, intramuscular, intraarterial or subcutaneous delivery) or local delivery (e.g., to the eye).

The endoglin polypeptides disclosed herein may be used in conjunction or sequentially with one or more additional therapeutic agents, including, for example, anti-angiogenesis agents, VEGF antagonists, anti-VEGF antibodies, anti-neoplastic compositions, cytotoxic agents, chemotherapeutic agents, anti-hormonal agents, and growth inhibitory agents. Further examples of each of the foregoing categories of molecules are provided herein.

In certain aspects, the disclosure provides methods for inhibiting angiogenesis in a mammal by administering any of the endoglin polypeptides described generally or specifically herein. The endoglin polypeptide may be delivered locally (e.g., to the eye) or systemically (e.g., intravenously, intramuscularly, intraarterially or subcutaneously). In certain embodiments, the disclosure provides a method for inhibiting angiogenesis in the eye of a mammal by administering an endoglin



polypeptide to the mammal at a location distal to the eye, e.g. by systemic administration.

In certain aspects the disclosure provides methods for treating a tumor in a mammal. Such a method may comprise administering to a mammal that has a tumor an effective amount of an endoglin polypeptide. A method may further comprise administering one or more additional agents, including, for example, anti-angiogenesis agents, VEGF antagonists, anti-VEGF antibodies, anti-neoplastic compositions, cytotoxic agents, chemotherapeutic agents, anti-hormonal agents, and growth inhibitory agents. A tumor may also be one that utilizes multiple pro-angiogenic factors, such as a tumor that is resistant to anti-VEGF therapy.

In certain aspects, the disclosure provides methods for treating patients having a BMP9 or BMP10 related disorder. Examples of such disorders are provided herein, and may include, generally, disorders of the vasculature, hypertension, and fibrotic disorders.

In certain aspects the disclosure provides ophthalmic formulations. Such formulations may comprise an endoglin polypeptide disclosed herein. In certain aspects, the disclosure provides methods for treating a fibrotic disease of the eye or an angiogenesis related disease of the eye. Such methods may comprise administering systemically or to said eye a pharmaceutical formulation comprising an effective amount of an endoglin polypeptide disclosed herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows the native amino acid sequence of human ENG, isoform 1 (L-ENG). The leader (residues 1-25) and predicted transmembrane domain (residues 587-611) are each underlined.

**Figure 2** shows the native nucleotide sequence encoding human ENG, isoform 1 (L-ENG). Sequences encoding the leader (nucleotides 414-488) and predicted transmembrane domain (nucleotides 2172-2246) are each underlined.

**Figure 3** shows the native amino acid sequence of human ENG, isoform 2 (S-ENG). The leader (residues 1-25) and predicted transmembrane domain (residues 587-611) are each underlined. Compared to isoform 1, isoform 2 has a shorter and

distinct C-terminus, but the sequence of the extracellular domain (see Figure 9) is identical.

**Figure 4** shows the native nucleotide sequence encoding human ENG, isoform 2 (S-ENG). Sequences encoding the leader (nucleotides 414-488) and  
5 predicted transmembrane domain (nucleotides 2172-2246) are each underlined.

**Figure 5** shows the native amino acid sequence of murine ENG, isoform 1 (L-ENG). The leader (residues 1-26) and predicted transmembrane domain (residues 582-606) are underlined and bracket the extracellular domain of the mature peptide (see Figure 10). Isoform 3 of murine ENG (GenBank accession NM\_001146348)  
10 differs from the depicted sequence only in the leader, where the threonine at position 23 (highlighted) is deleted and there is a glycine-to-serine substitution at position 24 (also highlighted).

**Figure 6** shows the native nucleotide sequence encoding murine ENG, isoform 1 (L-ENG). Sequences encoding the leader (nucleotides 364-441) and  
15 predicted transmembrane domain (nucleotides 2107-2181) are underlined. The nucleotide sequence encoding isoform 3 of murine ENG (GenBank accession NM\_001146348) differs from the depicted sequence only in the leader, specifically at positions 430-433 (highlighted).

**Figure 7** shows the native amino acid sequence of murine ENG, isoform 2 (S-ENG). The leader (residues 1-26) and predicted transmembrane domain (residues  
20 582-606) are underlined. Compared to isoform 1, isoform 2 has a shorter and distinct C-terminus, but the sequence of the extracellular domain (see Figure 10) is identical.

**Figure 8** shows the native nucleotide sequence encoding murine ENG, isoform 2 (S-ENG). Sequences encoding the leader (nucleotides 364-441) and  
25 predicted transmembrane domain (nucleotides 2107-2181) are underlined.

**Figure 9** shows the amino acid sequence of the extracellular domain of human ENG. The extracellular domains of the two human isoforms are identical in both amino-acid and nucleotide sequence.

**Figure 10** shows the amino acid sequence of the extracellular domain of  
30 murine ENG, which is 69% identical to its human counterpart. The extracellular domains of the two murine isoforms are identical in both amino-acid and nucleotide sequence.

**Figure 11** shows an amino acid sequence of the human IgG1 Fc domain. Underlined residues are optional mutation sites as discussed in the text.

**Figure 12** shows an N-terminally truncated amino acid sequence of the human IgG1 Fc domain. Underlined residues are optional mutation sites as discussed in the  
5 text.

**Figure 13** shows the amino acid sequence of hENG(26-586)-hFc. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 14** shows a nucleotide sequence encoding hENG(26-586)-hFc.  
10 Nucleotides encoding the ENG domain are underlined, those encoding the TPA leader sequence are double underlined, and those encoding linker sequences are bold and highlighted.

**Figure 15** shows the amino acid sequence of hENG(26-586)-hFc with an N-terminally truncated Fc domain. The ENG domain is underlined, the TPA leader  
15 sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 16** shows the amino acid sequence of mENG(27-581)-mFc. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 17** shows a nucleotide sequence encoding mENG(27-581)-mFc.  
20 Nucleotides encoding the ENG domain are underlined, those encoding the TPA leader sequence are double underlined, and those encoding linker sequences are bold and highlighted.

**Figure 18** shows characterization of BMP-9 binding to hENG(26-586)-hFc, as determined in a surface plasmon resonance (SPR)-based assay. BMP-9 binding to  
25 captured hENG(26-586)-hFc was assessed at ligand concentrations of 0 and 0.01-0.625 nM (in two-fold increments, excluding 0.3125 nM), and non-linear regression was used to determine the  $K_D$  as 29 pM.

**Figure 19** shows characterization of BMP-10 binding to hENG(26-586)-hFc, as determined in an SPR-based assay. BMP-10 binding to captured hENG(26-586)-  
30 hFc was assessed at ligand concentrations of 0 and 0.01-1.25 nM (in two-fold increments), and non-linear regression was used to determine the  $K_D$  as 400 pM.

**Figure 20** shows the effect of soluble human ENG extracellular domain, hENG(26-586), on binding of BMP-9 to ALK1. Concentrations of hENG(26-586) from 0-50 nM were premixed with a fixed concentration of BMP-9 (10 nM), and BMP-9 binding to captured ALK1 was determined by an SPR-based assay. The uppermost trace corresponds to no hENG(26-586), whereas the lowest trace corresponds to an ENG:BMP-9 ratio of 5:1. Binding of BMP-9 to ALK1 was inhibited by soluble hENG(26-586) in a concentration-dependent manner with an IC<sub>50</sub> of 9.7 nM.

**Figure 21** shows the effect of soluble human ENG extracellular domain, hENG(26-586), on binding of BMP-10 to ALK1. Concentrations of hENG(26-586) from 0-50 nM were premixed with a fixed concentration of BMP-10 (10 nM), and BMP-10 binding to captured ALK1 was measured by an SPR-based assay. The uppermost trace corresponds to no hENG(26-586), and the lowest trace corresponds to an ENG:BMP-10 ratio of 5:1. Binding of BMP-10 to ALK1 was inhibited by soluble hENG(26-586) in a concentration-dependent manner with an IC<sub>50</sub> of 6.3 nM.

**Figure 22** shows the effect of mENG(27-581)-hFc on cord formation by human umbilical vein endothelial cells (HUVEC) in culture. Data are means of duplicate cultures  $\pm$  SD. The inducer endothelial cell growth substance (ECGS) doubled mean cord length compared to no treatment, and mENG(27-581)-hFc cut this increase by nearly 60%. In the absence of stimulation (no treatment), mENG(27-581)-hFc had little effect.

**Figure 23** shows the effect of mENG(27-581)-hFc on VEGF-stimulated angiogenesis in a chick chorioallantoic membrane (CAM) assay. Data are means  $\pm$  SEM; \*,  $p < 0.05$ . The number of additional blood vessels induced by VEGF treatment was decreased by 65% with concurrent mENG(27-581)-hFc treatment.

**Figure 24** shows the effect of mENG(27-581)-mFc treatment for 11 days on angiogenesis stimulated by a combination of the growth factors (GF) vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) in a mouse angioreactor assay. Angiogenesis in units of relative fluorescence  $\pm$  SEM; \*,  $p < 0.05$ . mENG(27-581)-mFc completely blocked GF-stimulated angiogenesis in this in vivo assay.

**Figure 25** shows the domain structure of hENG-Fc fusion constructs. Full-length ENG extracellular domain (residues 26-586 in top structure) consists of an orphan domain and N-terminal and C-terminal zona pellucida (ZP) domains. Below it are shown structures of selected truncated variants and whether they exhibit high-affinity binding (+/-) to BMP-9 and BMP-10 in an SPR-based assay.

**Figure 26** shows the amino acid sequence of hENG(26-437)-hFc. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 27** shows a nucleotide sequence encoding hENG(26-437)-hFc. Nucleotides encoding the ENG domain are underlined, those encoding the TPA leader sequence are double underlined, and those encoding linker sequences are bold and highlighted.

**Figure 28** shows the amino acid sequence of hENG(26-378)-hFc with an N-terminally truncated Fc domain. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 29** shows a nucleotide sequence encoding hENG(26-378)-hFc with an N-terminally truncated Fc domain. Nucleotides encoding the ENG domain are underlined and those encoding linker sequences are bold and highlighted.

**Figure 30** shows the amino acid sequence of hENG(26-359)-hFc. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 31** shows a nucleotide sequence encoding hENG(26-359)-hFc. Nucleotides encoding the ENG domain are underlined, those encoding the TPA leader sequence are double underlined, and those encoding linker sequences are bold and highlighted.

**Figure 32** shows the amino acid sequence of hENG(26-359)-hFc with an N-terminally truncated Fc domain. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 33** shows a nucleotide sequence encoding hENG(26-359)-hFc with an N-terminally truncated Fc domain. Nucleotides encoding the ENG domain are

underlined, those encoding the TPA leader sequence are double underlined, and those encoding linker sequences are bold and highlighted.

**Figure 34** shows the amino acid sequence of hENG(26-346)-hFc with an N-terminally truncated Fc domain. The ENG domain is underlined, the TPA leader sequence is double underlined, and linker sequences are bold and highlighted.

**Figure 35** shows a nucleotide sequence encoding hENG(26-346)-hFc with an N-terminally truncated Fc domain. Nucleotides encoding the ENG domain are underlined and those encoding linker sequences are bold and highlighted.

**Figure 36** shows size-exclusion chromatograms for hENG(26-586)-hFc (A), hENG(26-359)-hFc (B), and hENG(26-346)-hFc (C) after the respective CHO-cell-derived proteins were purified by protein A affinity chromatography. Percent recovery of monomeric hENG(26-346)-hFc was equal to that of hENG(26-586)-hFc. In contrast, recovery of monomeric hENG(26-359)-hFc was reduced by the presence of additional high-molecular-weight aggregates, thus requiring additional procedures to obtain purity equivalent to that of the other constructs.

**Figure 37** shows kinetic characterization of BMP-9 binding to hENG(26-586)-hFc (A), hENG(26-359)-hFc (B), and hENG(26-346)-hFc (C), as determined in an SPR-based assay. BMP-9 binding to captured CHO-cell-derived proteins was assessed at ligand concentrations of 0.0195-0.625 nM in two-fold increments. RU, response units. Note slower off-rates for the truncated variants compared to hENG(26-586)-hFc.

**Figure 38** shows the effect of hENG(26-359)-hFc on VEGF-stimulated angiogenesis in a CAM assay. Data are means  $\pm$  SEM; \*,  $p < 0.05$ . The number of additional blood vessels induced by VEGF treatment was decreased by 75% with concurrent hENG(26-359)-hFc treatment, even though hENG(26-359)-hFc does not bind VEGF.

**Figure 39** shows the effect of hENG(26-346)-hFc treatment for 11 days on angiogenesis stimulated by a combination of the growth factors (GF) VEGF and FGF-2 in a mouse angioreactor assay. A. Angiogenesis in units of relative fluorescence  $\pm$  SEM; \*,  $p < 0.05$ . B. Photographs of individual angioreactors (four per mouse) arranged by treatment group, with blood vessel formation visible as darkened

contents. Although unable to bind VEGF or FGF-2 itself, hENG(26-346)-hFc completely blocked GF-stimulated angiogenesis in this in vivo assay.

**Figure 40** shows the effect of mENG(27-581)-mFc on growth of 4T1 mammary tumor xenografts in mice. Data are means  $\pm$  SEM. By day 24 post implantation, tumor volume was 45% lower ( $p < 0.05$ ) in mice treated with mENG(27-581)-mFc compared to vehicle.

**Figure 41** shows the effect of mENG(27-581)-mFc on growth of Colon-26 tumor xenografts in mice. mENG(27-581)-mFc treatment inhibited tumor growth in a dose-dependent manner, with tumor volume in the high-dose group nearly 70% lower than vehicle by day 58 post implantation.

**Figure 42** shows liver as % body weight in a mouse CCl<sub>4</sub> model of liver fibrosis with or without endoglin (mENG(27-581)-mFc) treatment.

**Figure 43** shows H&E staining of liver tissue in mock-injected (PBS) mice.

**Figure 44** shows H&E staining of liver tissue in mENG(27-581)-mFc injected mice.

**Figure 45** shows Masson's Trichrome staining of liver tissue in CCl<sub>4</sub>-induced mice.

**Figure 46** shows Oil Red O staining of liver tissue in CCl<sub>4</sub>-induced mice injected with PBS or with mENG(27-581)-mFc. mENG(27-581)-mFc treated animals had the lowest percentage of livers with extensive positive oil red O staining

**Figure 47** shows serum alkaline phosphatase levels in CCl<sub>4</sub>-induced and mock-induced (olive oil) mice treated with mENG(27-581)-mFc or with PBS. Serum AP was lower in the endoglin-treated cohorts.

**Figure 48** shows the effect of ENG-Fc treatment on hepatic lipid deposition in MCDD mice, a model of liver fibrosis caused by methionine and choline dietary deficiency. Compared to vehicle (A,C), treatment with mENG(27-581)-mFc for 3 weeks markedly reduced hepatic lipid deposits (B,D) in MCDD mice. Lipid deposits were identified by intense staining with Oil Red O, a lipid-soluble diazo dye. Magnification, 100 $\times$  (A,B) and 200 $\times$  (C,D).

## DETAILED DESCRIPTION

1. Overview

5           In certain aspects, the present invention relates to ENG polypeptides. ENG  
(also known as CD105) is referred to as a coreceptor for the transforming growth  
factor- $\beta$  (TGF- $\beta$ ) superfamily of ligands and is implicated in normal and pathological  
fibrosis and angiogenesis. ENG expression is low in quiescent vascular endothelium  
but upregulated in endothelial cells of healing wounds, developing embryos,  
10 inflammatory tissues, and solid tumors (Dallas et al, 2008, Clin Cancer Res 14:1931-  
1937). Mice homozygous for null ENG alleles die early in gestation due to defective  
vascular development (Li et al, 1999, Science 284:1534-1537), whereas heterozygous  
null ENG mice display angiogenic abnormalities as adults (Jerkic et al, 2006,  
Cardiovasc Res 69:845-854). In humans, ENG gene mutations have been identified  
15 as the cause of hereditary hemorrhagic telangiectasia (Osler-Rendu-Weber  
syndrome) type-1 (HHT-1), an autosomal dominant form of vascular dysplasia  
characterized by arteriovenous malformations resulting in direct flow  
(communication) from artery to vein (arteriovenous shunt) without an intervening  
capillary bed (McAllister et al, 1994, Nat Genet 8:345-351; Fernandez-L et al, 2006,  
20 Clin Med Res 4:66-78). Typical symptoms of patients with HHT include recurrent  
epistaxis, gastrointestinal hemorrhage, cutaneous and mucocutaneous telangiectases,  
and arteriovenous malformations in the pulmonary, cerebral, or hepatic vasculature.

          Although the specific role of ENG in fibrosis and angiogenesis remains to be  
determined, it is likely related to the prominent role of the TGF- $\beta$  signaling system in  
25 this process (Cheifetz et al, 1992, J Biol Chem 267:19027-19030; Pardali et al, 2010,  
Trends Cell Biol 20:556-567). Significantly, ENG expression is upregulated in  
proliferating vascular endothelial cells within tumor tissues (Burrows et al, 1995, Clin  
Cancer Res 1:1623-1634; Miller et al, 1999, Int J Cancer 81:568-572), and the  
number of ENG-expressing blood vessels in a tumor is negatively correlated with  
30 survival for a wide range of human tumors (Fonsatti et al, 2010, Cardiovasc Res  
86:12-19). Thus, ENG is a promising target for antiangiogenic therapy generally, and  
for cancer in particular (Dallas et al, 2008, Clin Cancer Res 14:1931-1937; Bernabeu  
et al, 2009, Biochim Biophys Acta 1792:954-973).



Structurally, ENG is a homodimeric cell-surface glycoprotein. It belongs to the zona pellucida (ZP) family of proteins and consists of a short C-terminal cytoplasmic domain, a single hydrophobic transmembrane domain, and a long extracellular domain (ECD) (Gougos et al, 1990, J Biol Chem 265:8361-8364). As determined by electron microscopy, monomeric ENG ECD consists of two ZP regions and an orphan domain located at the N-terminus (Llorca et al, 2007, J Mol Biol 365:694-705). In humans, alternative splicing of the primary transcript results in two ENG isoforms, one consisting of 658 residues (long, L, SEQ ID NO: 1) and the other 625 residues (short, S, SEQ ID NO: 3), which differ only in their cytoplasmic domain (Bellon et al, 1993, 23:2340-2345; ten Dijke et al, 2008, Angiogenesis 11:79-89). Murine ENG exists as three isoforms: L-ENG (SEQ ID NO: 5), S-ENG (SEQ ID NO: 7), and a third variant (isoform 3) of unknown functional significance identical to L-ENG except for changes at two positions within the leader sequence (Perez-Gomez et al, 2005, Oncogene 24:4450-4461). The ECD of murine ENG displays 69% amino acid identity with that of human ENG and lacks the Arg-Gly-Asp (RGD) integrin interaction motif found in the human protein. Recent evidence suggests that the L-ENG and S-ENG isoforms may play different functional roles in vivo (Blanco et al, 2008, Circ Res 103:1383-1392; ten Dijke et al, 2008, Angiogenesis 11:79-89).

As a coreceptor, ENG is thought to modulate responses of other receptors to TGF- $\beta$  family ligands without direct mediation of ligand signaling by itself. Ligands in the TGF- $\beta$  family typically signal by binding to a homodimeric type II receptor, which triggers recruitment and transphosphorylation of a homodimeric type I receptor, thereby leading to phosphorylation of Smad proteins responsible for transcriptional activation of specific genes (Massague, 2000, Nat Rev Mol Cell Biol 1:169-178). Based on ectopic cellular expression assays, it has been reported that ENG cannot bind ligands on its own and that its binding to TGF- $\beta$ 1, TGF- $\beta$ 3, activin A, bone morphogenetic protein-2 (BMP-2), and BMP-7 requires the presence of an appropriate type I and/or type II receptor (Barbara et al, 1999, J Biol Chem 274:584-594). Nevertheless, there is evidence that ENG expressed by a fibroblast cell line can bind TGF- $\beta$ 1 (St.-Jacques et al, 1994, Endocrinology 134:2645-2657), and recent results in COS cells indicate that transfected full-length ENG can bind BMP-9 in the absence of transfected type I or type II receptors (Scharpfenecker et al, 2007, J Cell Sci 120:964-972).

In addition to the foregoing, ENG can occur in a soluble form in vivo under certain conditions after proteolytic cleavage of the full-length membrane-bound protein (Hawinkels et al, 2010, Cancer Res 70:4141-4150). Elevated levels of soluble ENG have been observed in the circulation of patients with cancer and preeclampsia (Li et al, 2000, Int J Cancer 89:122-126; Calabro et al, 2003, J Cell Physiol 194:171-175; Venkatesha et al, 2006, Nat Med 12:642-649; Levine et al, 2006, N Engl J Med 355:992-1005). Although the role of endogenous soluble ENG is poorly understood, a protein corresponding to residues 26-437 of the ENG precursor (amino acids 26-437 of SEQ ID NO: 1) has been proposed to act as a scavenger or trap for TGF- $\beta$  family ligands (Venkatesha et al, 2006, Nat Med 12:642-649; WO-2007/143023), of which only TGF- $\beta$ 1 and TGF- $\beta$ 3 have specifically been implicated.

The present disclosure provides polypeptides comprising a truncated portion of the extracellular domain of ENG bind selectively to BMP9 and/or BMP10 and can act as BMP9 and/or BMP10 antagonists, provide advantageous properties relative to the full-length extracellular domain, and may be used to inhibit fibrosis. In part, the disclosure provides the identity of physiological, high-affinity ligands for soluble ENG polypeptides. Surprisingly, soluble ENG polypeptides are shown herein to have highly specific, high affinity binding for BMP-9 and BMP-10 while not exhibiting any meaningful binding to TGF- $\beta$ 1, TGF- $\beta$ 2 or TGF- $\beta$ 3, and moreover, soluble ENG polypeptides are shown herein to inhibit BMP9 and BMP10 interaction with type II receptors, thereby inhibiting cellular signal transduction. The disclosure further demonstrates that ENG polypeptides inhibit fibrosis. The data also demonstrate that an ENG polypeptide can exert an anti-angiogenic effect despite the finding that ENG polypeptide does not exhibit meaningful binding to TGF- $\beta$ 1, TGF- $\beta$ 3, VEGF, or FGF-2.

Thus, in certain aspects, the disclosure provides endoglin polypeptides as antagonists of BMP-9 or BMP-10 for use in inhibiting any BMP-9 or BMP-10 disorder generally, and particularly for inhibiting fibrosis and/or angiogenesis, including both VEGF-dependent angiogenesis and VEGF-independent angiogenesis. However, it should be noted that antibodies directed to ENG itself are expected to have different effects from an ENG polypeptide. A pan-neutralizing antibody against ENG (one that inhibits the binding of all strong and weak ligands) would be expected to inhibit the signaling of such ligands through ENG but would not be expected to

inhibit the ability of such ligands to signal through other receptors (e.g., ALK-1, ALK-2, BMPRII, ActRIIA or ActRIIB in the case of BMP-9 or BMP-10). It should further be noted that, given the existence of native, circulating soluble ENG polypeptides that, based on the data presented here, presumably act as natural BMP-9/10 antagonists, it is not clear whether a neutralizing anti-ENG antibody would primarily inhibit the membrane bound form of ENG (thus acting as an ENG/BMP-9/10 antagonist) or the soluble form of ENG (thus acting as an ENG/BMP-9/10 agonist). On the other hand, based on this disclosure, an ENG polypeptide would be expected to inhibit all of the ligands that it binds to tightly (including, for constructs such as those shown in the Examples, BMP-9 or BMP-10) but would not affect ligands that it binds to weakly. So, while a pan-neutralizing antibody against ENG would block BMP-9 and BMP-10 signaling through ENG, it would not block BMP-9 or BMP-10 signaling through another receptor. Also, while an ENG polypeptide may inhibit BMP-9 signaling through all receptors (including receptors besides ENG) it would not be expected to inhibit a weakly binding ligand signaling through any receptor, even ENG.

Proteins described herein are the human forms, unless otherwise specified. Genbank references for the proteins are as follows: human ENG isoform 1 (L-ENG), NM\_001114753; human ENG isoform 2 (S-ENG), NM\_000118; murine ENG isoform 1 (L-ENG), NM\_007932; murine ENG isoform 2 (S-ENG), NM\_001146350; murine ENG isoform 3, NM\_001146348. Sequences of native ENG proteins from human and mouse are set forth in Figures 1-8.

The terms used in this specification generally have their ordinary meanings in the art, within the context of this disclosure and in the specific context where each term is used. Certain terms are discussed in the specification, to provide additional guidance to the practitioner in describing the compositions and methods disclosed herein and how to make and use them. The scope or meaning of any use of a term will be apparent from the specific context in which the term is used.

## 2. Therapeutic Methods and Uses of ENG polypeptides

### *Fibrosis and fibrotic disorders*

Some aspects of this disclosure are based on the surprising recognition that ENG polypeptides can be used to inhibit and/or treat fibrotic disorders. The

disclosure provides methods of inhibiting fibrosis in a mammal by administering an effective amount of an ENG polypeptide, e.g., an ENG polypeptide comprising an amino acid sequence that is at least 95% identical to amino acids 42-333 of SEQ ID NO: 1, including an ENG-Fc fusion protein or nucleic acid antagonists (e.g., antisense or siRNA) of the foregoing. These ENG polypeptides, ENG-Fc fusion proteins, and nucleic acid antagonists are hereafter collectively referred to as “therapeutic agents.”

In some embodiments, the instant disclosure provides ENG polypeptides and methods of using such polypeptides that are useful in the treatment, inhibition, or prevention of fibrosis. As used herein, the term "fibrosis" refers to the aberrant formation or development of excess fibrous connective tissue by cells in an organ or tissue. Although processes related to fibrosis can occur as part of normal tissue formation or repair, dysregulation of these processes can lead to altered cellular composition and excess connective tissue deposition that progressively impairs to tissue or organ function. The formation of fibrous tissue can result from a reparative or reactive process.

Fibrotic disorders or conditions that can be treated with ENG polypeptides and therapeutic methods using such polypeptides as provided herein include, but are not limited to, fibroproliferative disorders associated with vascular diseases, such as cardiac disease, cerebral disease, and peripheral vascular disease, as well as tissues and organ systems including the heart, skin, kidney, lung, peritoneum, gut, and liver (as disclosed in, e.g., Wynn, 2004, Nat Rev 4:583-594, incorporated herein by reference). Exemplary disorders that can be treated include, but are not limited to, renal fibrosis, including nephropathies associated with injury/fibrosis, e.g., chronic nephropathies associated with diabetes (e.g., diabetic nephropathy), lupus, scleroderma, glomerular nephritis, focal segmental glomerular sclerosis, and IgA nephropathy; lung or pulmonary fibrosis, e.g., idiopathic pulmonary fibrosis, radiation induced fibrosis, chronic obstructive pulmonary disease (COPD), scleroderma, and chronic asthma; gut fibrosis, e.g., scleroderma, and radiation-induced gut fibrosis; liver fibrosis, e.g., cirrhosis, alcohol-induced liver fibrosis, biliary duct injury, primary biliary cirrhosis, infection or viral induced liver fibrosis, congenital hepatic fibrosis and autoimmune hepatitis; and other fibrotic conditions, such as cystic fibrosis, endomyocardial fibrosis, mediastinal fibrosis, pleural fibrosis, sarcoidosis, scleroderma, spinal cord injury/fibrosis, myelofibrosis, vascular restenosis,

atherosclerosis, cystic fibrosis of the pancreas and lungs, injection fibrosis (which can occur as a complication of intramuscular injections, especially in children), endomyocardial fibrosis, idiopathic pulmonary fibrosis of the lung, mediastinal fibrosis, myofibrosis, retroperitoneal fibrosis, progressive massive fibrosis, a  
5 complication of coal workers' pneumoconiosis, and nephrogenic systemic fibrosis.

As used herein, the terms "fibrotic disorder", "fibrotic condition," and "fibrotic disease," are used interchangeably to refer to a disorder, condition or disease characterized by fibrosis. Examples of fibrotic disorders include, but are not limited to vascular fibrosis, pulmonary fibrosis (e.g., idiopathic pulmonary fibrosis),  
10 pancreatic fibrosis, liver fibrosis (e.g., cirrhosis), renal fibrosis, musculoskeletal fibrosis, cardiac fibrosis (e.g., endomyocardial fibrosis, idiopathic cardiomyopathy), skin fibrosis (e.g., scleroderma, post-traumatic, operative cutaneous scarring, keloids and cutaneous keloid formation), eye fibrosis (e.g., glaucoma, sclerosis of the eyes, conjunctival and corneal scarring, and pterygium), progressive systemic sclerosis  
15 (PSS), chronic graft-versus-host disease, Peyronie's disease, post-cystoscopic urethral stenosis, idiopathic and pharmacologically induced retroperitoneal fibrosis, mediastinal fibrosis, progressive massive fibrosis, proliferative fibrosis, and neoplastic fibrosis.

As used herein, the term "cell" refers to any cell prone to undergoing a fibrotic  
20 response, including, but not limited to, individual cells, tissues, and cells within tissues and organs. The term cell, as used herein, includes the cell itself, as well as the extracellular matrix (ECM) surrounding a cell. For example, inhibition of the fibrotic response of a cell, includes, but is not limited to the inhibition of the fibrotic response of one or more cells within the lung (or lung tissue); one or more cells within  
25 the liver (or liver tissue); one or more cells within the kidney (or renal tissue); one or more cells within muscle tissue; one or more cells within the heart (or cardiac tissue); one or more cells within the pancreas; one or more cells within the skin; one or more cells within the bone, one or more cells within the vasculature, one or more stem cells, or one or more cells within the eye.

30 The methods and compositions of the present invention can be used to treat and/or prevent fibrotic disorders. Exemplary types of fibrotic disorders include, but are not limited to, vascular fibrosis, pulmonary fibrosis (e.g., idiopathic pulmonary fibrosis), pancreatic fibrosis, liver fibrosis (e.g., cirrhosis), renal fibrosis,

musculoskeletal fibrosis, cardiac fibrosis (e.g., endomyocardial fibrosis, idiopathic  
myocardiopathy), skin fibrosis (e.g., scleroderma, post-traumatic, operative cutaneous  
scarring, keloids and cutaneous keloid formation), eye fibrosis (e.g., glaucoma,  
sclerosis of the eyes, conjunctival and corneal scarring, and pterygium), progressive  
5 systemic sclerosis (PSS), chronic graft versus-host disease, Peyronie's disease, post-  
cystoscopic urethral stenosis, idiopathic and pharmacologically induced  
retroperitoneal fibrosis, mediastinal fibrosis, progressive massive fibrosis,  
proliferative fibrosis, neoplastic fibrosis, Dupuytren's disease, strictures, and radiation  
induced fibrosis. In a particular embodiment, the fibrotic disorder is not  
10 myelofibrosis.

The methods and compositions of the present invention can be used to treat  
and/or prevent liver disorders that manifest as or result in liver fibrosis, including non-  
alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) and  
acquired fibrotic disorders that may result from long-term excessive alcohol  
15 consumption, cholestasis, autoimmune liver diseases, iron or copper overload and  
chronic viral hepatitis. NAFLD results from the metabolic conditions of obesity and  
type 2 diabetes. Patients with NAFLD may exhibit a range of histopathologic  
findings including steatosis alone (fatty liver), to necroinflammation, which is often  
termed NASH. NAFLD and NASH patients may progress to more advanced states of  
20 fibrosis including advanced fibrosis and cirrhosis. Patients with NASH develop  
progressive fibrosis in 25%- 50% over a period of 4 to 6 years and 15% to 25% of  
individuals with NASH can progress to cirrhosis. NASH cirrhosis is an important  
cause of liver transplantation in the United States and it is associated with an  
increased risk for hepatocellular carcinoma and mortality in patients awaiting liver  
25 transplant. Alcoholism and viral infection can also cause liver damage that progresses  
to liver fibrosis and cirrhosis. A variety of tools may be used to assess liver health  
and the progression of fibrotic disease. Liver biopsy permits the assessment of  
histological features of the liver tissue, including staining for and quantitation of  
collagen levels in the tissue and well as lipid levels in the case of fatty liver diseases.  
30 The NAFLD Activity Score (NAS) provides a numerical score and is the sum of the  
separate scores for steatosis (0–3), hepatocellular ballooning (0–2) and lobular  
inflammation (0–3), with the majority of patients with NASH having a NAS score of  
 $\geq 5$ . See Kleiner et al.. Design and validation of a histological scoring system for

nonalcoholic fatty liver disease. *Hepatology* 41(6), 1313–1321 (2005). Serum markers include markers of liver function, ALT and AST, and markers of extracellular matrix formation, markers of the fibrolytic process, markers of extracellular matrix degradation and certain cytokines.

5           The present invention contemplates the use of ENG polypeptides in combination with one or more other therapeutic modalities. Thus, in addition to the use of ENG polypeptides, one may also administer to the subject one or more "standard" therapies for treating fibrotic disorders. For example, the ENG polypeptides can be administered in combination with (i.e., together with) cytotoxins,  
10 immunosuppressive agents, radiotoxic agents, and/or therapeutic antibodies. Particular co-therapeutics contemplated by the present invention include, but are not limited to, steroids (e.g., corticosteroids, such as Prednisone), immune-suppressing and/or anti-inflammatory agents (e.g., gamma-interferon, cyclophosphamide, azathioprine, methotrexate, penicillamine, cyclosporine, colchicines, antithymocyte  
15 globulin, mycophenolate mofetil, and hydroxychloroquine), cytotoxic drugs, calcium channel blockers (e.g., nifedipine), angiotensin converting enzyme inhibitors (ACE) inhibitors, para-aminobenzoic acid (PABA), dimethyl sulfoxide, transforming growth factor-beta (TGF- $\beta$ ) inhibitors, interleukin-5 (IL-5) inhibitors, and pan caspase inhibitors.

20           Additional anti-fibrotic agents that may be used in combination with ENG polypeptides include, but are not limited to, lectins (as described in, for example, U.S. Patent No.: 7,026,283, the entire contents of which is incorporated herein by reference), as well as the anti-fibrotic agents described by Wynn et al (2007, *J Clin Invest* 117:524-529, the entire contents of which is incorporated herein by reference).  
25 For example, additional anti-fibrotic agents and therapies include, but are not limited to, various anti-inflammatory/ immunosuppressive/ cytotoxic drugs (including colchicine, azathioprine, cyclophosphamide, prednisone, thalidomide, pentoxifylline and theophylline), TGF- $\beta$  signaling modifiers (including relaxin, SMAD7, HGF, and BMP7, as well as TGF- $\beta$ 1, TGF $\beta$ RI, TGF $\beta$ RII, EGR-I, and CTGF inhibitors),  
30 cytokine and cytokine receptor antagonists (inhibitors of IL-1 $\beta$ , IL-5, IL-6, IL-13, IL-21, IL-4R, IL-13R $\alpha$ 1, GM-CSF, TNF- $\alpha$ , oncostatin M, WISP-I, and PDGFs), cytokines and chemokines (IFN- $\gamma$ , IFN- $\alpha/\beta$ , IL-12, IL-10, HGF, CXCL10, and CXCL11), chemokine antagonists (inhibitors of CXCL1, CXCL2, CXCL12, CCL2,

CCL3, CCL6, CCL17, and CCL18), chemokine receptor antagonists (inhibitors of CCR2, CCR3, CCR5, CCR7, CXCR2, and CXCR4), TLR antagonists (inhibitors of TLR3, TLR4, and TLR9), angiogenesis antagonists (VEGF-specific antibodies and adenosine deaminase replacement therapy), antihypertensive drugs (beta blockers and inhibitors of ANG 11, ACE, and aldosterone), vasoactive substances (ET-1 receptor antagonists and bosentan), inhibitors of the enzymes that synthesize and process collagen (inhibitors of prolyl hydroxylase), B cell antagonists (rituximab), integrin/adhesion molecule antagonists (molecules that block  $\alpha\beta1$  and  $\alpha v\beta6$  integrins, as well as inhibitors of integrin-linked kinase, and antibodies specific for ICAM-I and VCAM-I), proapoptotic drugs that target myofibroblasts, MMP inhibitors (inhibitors of MMP2, MMP9, and MMP12), and TIMP inhibitors (antibodies specific for TIMP-1).

The ENG polypeptide and the co-therapeutic agent or co-therapy can be administered in the same formulation or separately. In the case of separate administration, the ENG polypeptide can be administered before, after, or concurrently with the co-therapeutic or co-therapy. One agent may precede or follow administration of the other agent by intervals ranging from minutes to weeks. In embodiments where two or more different kinds of therapeutic agents are applied separately to a subject, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that these different kinds of agents would still be able to exert an advantageously combined effect on the target tissues or cells.

### *Angiogenesis*

Angiogenesis, the process of forming new blood vessels, is critical in many normal and abnormal physiological states. Under normal physiological conditions, humans and animals undergo angiogenesis in specific and restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development and formation of the corpus luteum, endometrium and placenta.

Undesirable or inappropriately regulated angiogenesis occurs in many disorders, in which abnormal endothelial growth may cause or participate in the pathological process. For example, angiogenesis participates in the growth of many tumors. Deregulated angiogenesis has been implicated in pathological processes such as rheumatoid arthritis, retinopathies, hemangiomas, and psoriasis. The diverse



pathological disease states in which unregulated angiogenesis is present have been categorized as angiogenesis-associated diseases.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Capillary blood vessels are composed primarily of endothelial cells and pericytes, surrounded by a basement membrane. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic factors induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" protruding from the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. Endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

Agents that inhibit angiogenesis have proven to be effective in treating a variety of disorders. Avastin<sup>TM</sup> (bevacizumab), a monoclonal antibody that binds to vascular endothelial growth factor (VEGF), is used in the treatment of a variety of cancers. Macugen<sup>TM</sup>, an aptamer that binds to VEGF has proven to be effective in the treatment of neovascular (wet) age-related macular degeneration. Antagonists of the SDF/CXCR4 signaling pathway inhibit tumor neovascularization and are effective against cancer in mouse models (Guleng et al. Cancer Res. 2005 Jul 1;65(13):5864-71). A variety of so-called multitargeted tyrosine kinase inhibitors, including vandetanib, sunitinib, axitinib, sorafenib, vatalanib, and pazopanib are used as anti-angiogenic agents in the treatment of various tumor types. Thalidomide and related compounds (including pomalidomide and lenalidomide) have shown beneficial effects in the treatment of cancer, and although the molecular mechanism of action is not clear, the inhibition of angiogenesis appears to be an important component of the anti-tumor effect (see, e.g., Dredge et al. Microvasc Res. 2005 Jan;69(1-2):56-63).

Although many anti-angiogenic agents have an effect on angiogenesis regardless of the tissue that is affected, other angiogenic agents may tend to have a tissue-selective effect.

The disclosure provides methods and compositions for treating or preventing conditions of dysregulated angiogenesis, including both neoplastic and non-neoplastic disorders. Also provided are methods and compositions for treating or preventing

certain cardiovascular disorders. In addition the disclosure provides methods for treating disorders associated with BMP9 and/or BMP10 activity.

The disclosure provides methods of inhibiting angiogenesis in a mammal by administering to a subject an effective amount of an ENG polypeptide, including an  
5 ENG-Fc fusion protein or nucleic acid antagonists (e.g., antisense or siRNA) of the foregoing, hereafter collectively referred to as "therapeutic agents". The data presented indicate specifically that the anti-angiogenic therapeutic agents disclosed herein may be used to inhibit tumor-associated angiogenesis. It is expected that these therapeutic agents will also be useful in inhibiting angiogenesis in the eye.

10 Angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque  
15 neovascularization; telangiectasia; hemophiliac joints; and angiofibroma.

In particular, polypeptide therapeutic agents of the present disclosure are useful for treating or preventing a cancer (tumor), and particularly such cancers as are known to rely on angiogenic processes to support growth. Unlike most anti-angiogenic agents, ENG polypeptides affect angiogenesis induced by multiple factors.  
20 This is highly relevant in cancers, where a cancer will frequently acquire multiple factors that support tumor angiogenesis. Thus, the therapeutic agents disclosed herein will be particularly effective in treating tumors that are resistant to treatment with a drug that targets a single angiogenic factor (e.g., bevacizumab, which targets VEGF), and may also be particularly effective in combination with other anti-angiogenic  
25 compounds that work by a different mechanism.

Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both neoplastic and non-neoplastic conditions. The terms "cancer" and "cancerous" refer to, or describe, the physiological condition in mammals that is typically characterized  
30 by unregulated cell growth/proliferation. Examples of cancer, or neoplastic disorders, include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer,

small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer, including squamous cell head and neck cancer. Other examples of neoplastic disorders and related conditions include esophageal carcinomas, thecomas, arrhenoblastomas, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendroglioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, and Meigs' syndrome. A cancer that is particularly amenable to treatment with the therapeutic agents described herein may be characterized by one or more of the following: the cancer has angiogenic activity, elevated ENG levels detectable in the tumor or the serum, increased BMP-9 or BMP-10 expression levels or biological activity, is metastatic or at risk of becoming metastatic, or any combination thereof.

Non-neoplastic disorders with dysregulated angiogenesis that are amenable to treatment with ENG polypeptides useful in the invention include, but are not limited to, undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis, psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ ARDS, sepsis, primary

pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/ closed head injury/ trauma), synovial inflammation, pannus formation in RA, myositis ossificans, hypertrophic bone formation, osteoarthritis, refractory ascites, polycystic ovarian disease, endometriosis, 3rd  
5 spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osler-Weber syndrome, pyogenic  
10 granuloma retroental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion. Further examples of such disorders include an epithelial or cardiac disorder.

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

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

A wide array of conventional compounds have been shown to have anti-neoplastic activities. These compounds have been used as pharmaceutical agents in chemotherapy to shrink solid tumors, prevent metastases and further growth, or  
30 decrease the number of malignant cells in leukemic or bone marrow malignancies. Although chemotherapy has been effective in treating various types of malignancies, many anti-neoplastic compounds induce undesirable side effects. It has been shown that when two or more different treatments are combined, the treatments may work

synergistically and allow reduction of dosage of each of the treatments, thereby reducing the detrimental side effects exerted by each compound at higher dosages. In other instances, malignancies that are refractory to a treatment may respond to a combination therapy of two or more different treatments.

5           When a therapeutic agent disclosed herein is administered in combination with another conventional anti-neoplastic agent, either concomitantly or sequentially, such therapeutic agent may enhance the therapeutic effect of the anti-neoplastic agent or overcome cellular resistance to such anti-neoplastic agent. This allows decrease of dosage of an anti-neoplastic agent, thereby reducing the undesirable side effects, or  
10 restores the effectiveness of an anti-neoplastic agent in resistant cells.

          According to the present disclosure, the antiangiogenic agents described herein may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with the ENG polypeptide, and then the  
15 ENG polypeptide may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize any residual primary tumor.

          Many anti-angiogenesis agents have been identified and are known in the arts, including those listed herein and, e.g., listed by Carmeliet and Jain, *Nature* 407:249-257 (2000); Ferrara et al., *Nature Reviews:Drug Discovery*, 3:391-400 (2004); and  
20 Sato *Int. J. Clin. Oncol*, 8:200-206 (2003). See also, US Patent Application US20030055006. In one embodiment, an ENG polypeptide is used in combination with an anti-VEGF neutralizing antibody (or fragment) and/or another VEGF antagonist or a VEGF receptor antagonist including, but not limited to, for example, soluble VEGF receptor (e.g., VEGFR-1, VEGFR-2, VEGFR-3, neuropillins (e.g.,  
25 NRPI, NRP2)) fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti-VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases (RTK), antisense strategies for VEGF, ribozymes against VEGF or VEGF receptors, antagonist variants of VEGF; and any combinations thereof. Alternatively, or additionally, two or more angiogenesis inhibitors may optionally be  
30 co-administered to the patient in addition to VEGF antagonist and other agent. In certain embodiment, one or more additional therapeutic agents, e.g., anti-cancer agents, can be administered in combination with an ENG polypeptide, the VEGF antagonist, and an anti-angiogenesis agent.

The terms "VEGF" and "VEGF-A" are used interchangeably to refer to the 165-amino acid vascular endothelial cell growth factor and related 121-, 145-, 183-, 189-, and 206- amino acid vascular endothelial cell growth factors, as described by Leung et al. *Science*, 246:1306 (1989), Houck et al. *Mol Endocrinol*, 5:1806 (1991), and, Robinson & Stringer, *J Cell Sci*, 144(5):853-865 (2001), together with the naturally occurring allelic and processed forms thereof.

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases, and fusions proteins, e.g., VEGF-Trap (Regeneron), VEGF121-gelonin (Peregrine). VEGF antagonists also include antagonist variants of VEGF, antisense molecules directed to VEGF, RNA aptamers, and ribozymes against VEGF or VEGF receptors.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. The anti-VEGF antibody can be used as a therapeutic agent in targeting and interfering with diseases or conditions wherein the VEGF activity is involved. See, e.g., U.S. Patents 6,582,959, 6,703,020; WO98/45332; WO 96/30046; WO94/10202, WO2005/044853; ; EP 0666868B1; US Patent Applications 20030206899, 20030190317, 20030203409, 20050112126, 20050186208, and 20050112126; Popkov et al, *Journal of Immunological Methods* 288:149-164 (2004); and WO2005012359. An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF. The anti-VEGF antibody "Bevacizumab (BV)", also known as "rhuMAb VEGF" or "Avastin®", is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. *Cancer Res.* 57:4593-4599 (1997). It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of Bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from

the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated. Bevacizumab and other humanized anti-VEGF antibodies, including the anti-VEGF antibody fragment "ranibizumab", also known as "Lucentis®", are further described in U.S. Pat. No. 6,884,879 issued February 26, 5 2005.

The term "anti-neoplastic composition" refers to a composition useful in treating cancer comprising at least one active therapeutic agent, e.g., "anti-cancer agent". Examples of therapeutic agents (anti-cancer agents, also termed "anti-neoplastic agent" herein) include, but are not limited to, e.g., chemotherapeutic 10 agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, toxins, and other agents to treat cancer, e.g., anti-VEGF neutralizing antibody, VEGF antagonist, anti-HER-2, anti-CD20, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor, erlotinib, a COX-2 inhibitor (e.g., 15 celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the ErbB2, ErbB3, ErbB4, or VEGF receptor(s), inhibitors for receptor tyrosine kinases for platelet-derived growth factor (PDGF) and/or stem cell factor (SCF) (e.g., imatinib mesylate (Gleevec® Novartis)), TRAIL/ Apo2L, and other bioactive and organic chemical agents, etc.

20 An "angiogenic factor or agent" is a growth factor which stimulates the development of blood vessels, e.g., promotes angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis, etc. For example, angiogenic factors, include, but are not limited to, e.g., VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands 25 (Angiopoietins), ephrins, ANGPTL3, ALK-1, etc. It would also include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of its family, and TGF- $\alpha$  and TGF- $\beta$ . See, e.g., Klagsbrun and D'Amore, *Annu. Rev. Physiol*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003); Ferrara & Alitalo, 30 *Nature Medicine* 5(12): 1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 1 listing angiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003).

An "anti-angiogenesis agent" or "angiogenesis inhibitor" refers to a small molecular weight substance, a polynucleotide (including, e.g., an inhibitory RNA (RNAi or siRNA)), a polypeptide, an isolated protein, a recombinant protein, an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF, antibodies to VEGF receptors, small molecules that block VEGF receptor signaling (e.g., PTK787/ZK2284, SU6668, SUTENT®/SU 11248 (sunitinib malate), AMG706, or those described in, e.g., international patent application WO 2004/113304). Anti-angiogenesis agents also include native angiogenesis inhibitors, e.g., angiostatin, endostatin, etc. See, e.g., Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003) (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo, *Nat Med* 5(12): 1359-1364 (1999); Tonini et al, *Oncogene*, 22:6549-6556 (2003) (e.g., Table 2 listing antiangiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003) (e.g., Table 1 lists Anti-angiogenesis agents used in clinical trials).

In certain aspects of the invention, other therapeutic agents useful for combination tumor therapy with an ENG polypeptide include other cancer therapies: e.g., surgery, cytotoxic agents, radiological treatments involving irradiation or administration of radioactive substances, chemotherapeutic agents, anti-hormonal agents, growth inhibitory agents, anti-neoplastic compositions, and treatment with anti-cancer agents listed herein and known in the art, or combinations thereof.

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



antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scoplectin, and 9-aminocamptothecin); bryostatin; callistatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994))); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin,

tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP- 16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5 -FU and leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVISTA® raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY1 17018, onapristone, and FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin acetate, busserelin acetate and triptorelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestane, fadrozole, RIVIS OR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole. In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), DIDROC AL® etidronate, NE-58095, ZOMET A® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREDIA® pamidronate, SKELID® tiludronate, or ACTONEL® risedronate; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle

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

Angiogenesis-inhibiting agents can also be given prophylactically to individuals known to be at high risk for developing new or re-current cancers. Accordingly, an aspect of the disclosure encompasses methods for prophylactic prevention of cancer in a subject, comprising administering to the subject an effective  
20 amount of an ENG polypeptide and/or a derivative thereof, or another angiogenesis-inhibiting agent of the present disclosure.

Certain normal physiological processes are also associated with angiogenesis, for example, ovulation, menstruation, and placentation. The angiogenesis inhibiting proteins of the present disclosure are useful in the treatment of disease of excessive or  
25 abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochela minalia quintosa*) and ulcers (*Helicobacter pylori*).

30 General angiogenesis-inhibiting proteins can be used as birth control agents by reducing or preventing uterine vascularization required for embryo implantation. Thus, the present disclosure provides an effective birth control method when an amount of the inhibitory protein sufficient to prevent embryo implantation is

administered to a female. In one aspect of the birth control method, an amount of the inhibiting protein sufficient to block embryo implantation is administered before or after intercourse and fertilization have occurred, thus providing an effective method of birth control, possibly a "morning after" method. While not wanting to be bound by this statement, it is believed that inhibition of vascularization of the uterine endometrium interferes with implantation of the blastocyst. Similar inhibition of vascularization of the mucosa of the uterine tube interferes with implantation of the blastocyst, preventing occurrence of a tubal pregnancy. Administration methods may include, but are not limited to, pills, injections (intravenous, subcutaneous, intramuscular), suppositories, vaginal sponges, vaginal tampons, and intrauterine devices. It is also believed that administration of angiogenesis inhibiting agents of the present disclosure will interfere with normal enhanced vascularization of the placenta, and also with the development of vessels within a successfully implanted blastocyst and developing embryo and fetus.

In the eye, angiogenesis is associated with, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, and retrolental fibroplasias. The therapeutic agents disclosed herein may be administered intra-ocularly or by other local administration to the eye. Other diseases associated with angiogenesis in the eye include, but are not limited to, epidemic keratoconjunctivitis, vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phlyctenulosis, syphilis, mycobacteria infections, lipid degeneration, chemical burns, bacterial ulcers, fungal ulcers, herpes simplex infections, herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, mariginal keratolysis, rheumatoid arthritis, systemic lupus, polyarteritis, trauma, Wegeners sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, corneal graft rejection, sickle cell anemia, sarcoid, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme disease, systemic lupus erythematosus, retinopathy of prematurity, Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications. Other

diseases include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy.

5 Conditions of the eye can be treated or prevented by, e.g., systemic, topical, intraocular injection of a therapeutic agent, or by insertion of a sustained release device that releases a therapeutic agent. A therapeutic agent may be delivered in a pharmaceutically acceptable ophthalmic vehicle, such that the compound is maintained in contact with the ocular surface for a sufficient time period to allow the compound to penetrate the corneal and internal regions of the eye, as for example the  
10 anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically-acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material. Alternatively, the therapeutic agents of the disclosure may be injected directly into the vitreous and aqueous humour. In a further alternative, the  
15 compounds may be administered systemically, such as by intravenous infusion or injection, for treatment of the eye.

One or more therapeutic agents can be administered. The methods of the disclosure also include co-administration with other medicaments that are used to treat conditions of the eye. When administering more than one agent or a combination  
20 of agents and medicaments, administration can occur simultaneously or sequentially in time. The therapeutic agents and/or medicaments may be administered by different routes of administration or by the same route of administration. In one embodiment, a therapeutic agent and a medicament are administered together in an ophthalmic pharmaceutical formulation.

25 In one embodiment, a therapeutic agent is used to treat a disease associated with angiogenesis in the eye by concurrent administration with other medicaments that act to block angiogenesis by pharmacological mechanisms. Medicaments that can be concurrently administered with a therapeutic agent of the disclosure include, but are not limited to, pegaptanib (Macugen<sup>TM</sup>), ranibizumab (Lucentis<sup>TM</sup>), squalamine  
30 lactate (Evizon<sup>TM</sup>), heparinase, and glucocorticoids (e.g. Triamcinolone). In one embodiment, a method is provided to treat a disease associated with angiogenesis is treated by administering an ophthalmic pharmaceutical formulation containing at least one therapeutic agent disclosed herein and at least one of the following medicaments:

pegaptanib (Macugen<sup>TM</sup>), ranibizumab (Lucentis<sup>TM</sup>), squalamine lactate (Evizon<sup>TM</sup>), heparinase, and glucocorticoids (e.g. Triamcinolone).

#### *Other Diseases or Disorders*

In some embodiments, ENG polypeptides can be used to treat a patient who suffers from a cardiovascular disorder or condition associated with BMP-9 or BMP-10 but not necessarily accompanied by angiogenesis. Exemplary disorders of this kind include, but are not limited to, heart disease (including myocardial disease, myocardial infarct, angina pectoris, and heart valve disease); renal disease (including chronic glomerular inflammation, diabetic renal failure, and lupus-related renal inflammation); disorders of blood pressure (including systemic and pulmonary types); disorders associated with atherosclerosis or other types of arteriosclerosis (including stroke, cerebral hemorrhage, subarachnoid hemorrhage, angina pectoris, and renal arteriosclerosis); thrombotic disorders (including cerebral thrombosis, pulmonary thrombosis, thrombotic intestinal necrosis); complications of diabetes (including diabetes-related retinal disease, cataracts, diabetes-related renal disease, diabetes-related neuropathology, diabetes-related gangrene, and diabetes-related chronic infection); vascular inflammatory disorders (systemic lupus erythematosus, joint rheumatism, joint arterial inflammation, large-cell arterial inflammation, Kawasaki disease, Takayasu arteritis, Churg-Strauss syndrome, and Henoch-Schoenlein pupura); and cardiac disorders such as congenital heart disease, cardiomyopathy (e.g., dilated, hypertrophic, restrictive cardiomyopathy), and congestive heart failure. The ENG polypeptide can be administered to the subject alone, or in combination with one or more agents or therapeutic modalities, e.g., therapeutic agents, which are useful for treating BMP-9/10 associated cardiovascular disorders and/or conditions. In one embodiment, the second agent or therapeutic modality is chosen from one or more of: angioplasty, beta blockers, anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, endothelin antagonists, calcium channel blockers, phosphodiesterase inhibitors, angiotensin type 2 antagonists and/or cytokine blockers/inhibitors.

In still other embodiments, ENG polypeptides may be useful in the treatment of inflammatory disorders or conditions likely to be BMP9-related but not already noted above. Exemplary disorders include liver disease (including acute hepatitis, chronic hepatitis, and cirrhosis); thoracic or abdominal edema; chronic pancreatic

disease; allergies (including nasal allergy, asthma, bronchitis, and atopic dermatitis); Alzheimer's disease; Raynaud's syndrome; and diffuse sclerosis.

### 3. Formulations and Effective Doses

5 The therapeutic agents described herein may be formulated into pharmaceutical compositions. Pharmaceutical compositions for use in accordance with the present disclosure may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Such formulations will generally be substantially pyrogen free, in compliance with most regulatory  
10 requirements.

In certain embodiments, the therapeutic method of the disclosure includes administering the composition systemically, or locally as an implant or device. When administered, the therapeutic composition for use in this disclosure is in a pyrogen-free, physiologically acceptable form. Therapeutically useful agents other than the  
15 ENG signaling antagonists which may also optionally be included in the composition as described above, may be administered simultaneously or sequentially with the subject compounds (e.g., ENG polypeptides) in the methods disclosed herein.

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

In one embodiment, the ENG polypeptides disclosed herein are administered



in an ophthalmic pharmaceutical formulation. In some embodiments, the ophthalmic pharmaceutical formulation is a sterile aqueous solution, preferable of suitable concentration for injection, or a salve or ointment. Such salves or ointments typically comprise one or more ENG polypeptides disclosed herein dissolved or suspended in a sterile pharmaceutically acceptable salve or ointment base, such as a mineral oil-white petrolatum base. In salve or ointment compositions, anhydrous lanolin may also be included in the formulation. Thimerosal or chlorobutanol are also preferably added to such ointment compositions as antimicrobial agents. In one embodiment, the sterile aqueous solution is as described in U.S. Pat. No. 6,071,958.

10 The disclosure provides formulations that may be varied to include acids and bases to adjust the pH; and buffering agents to keep the pH within a narrow range. Additional medicaments may be added to the formulation. These include, but are not limited to, pegaptanib, heparinase, ranibizumab, or glucocorticoids. The ophthalmic pharmaceutical formulation according to the disclosure is prepared by aseptic  
15 manipulation, or sterilization is performed at a suitable stage of preparation.

The compositions and formulations may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for  
20 administration.

#### 4. Soluble ENG Polypeptides

Except under certain conditions, naturally occurring ENG proteins are transmembrane proteins, with a portion of the protein positioned outside the cell (the extracellular portion) and a portion of the protein positioned inside the cell (the intracellular portion). Aspects of the present disclosure encompass polypeptides comprising a portion of the extracellular domain (ECD) of ENG.

In certain embodiments, the disclosure provides ENG polypeptides. ENG polypeptides may include a polypeptide consisting of, or comprising, an amino acid  
30 sequence at least 90% identical, and optionally at least 95%, 96%, 97%, 98%, 99%, or 100% identical to a truncated ECD domain of a naturally occurring ENG polypeptide, whose C-terminus occurs at any of amino acids 333-378 of SEQ ID NO: 1 and which polypeptide does not include a sequence consisting of amino acids 379-430 of SEQ

ID NO:1. Optionally, an ENG polypeptide does not include more than 5 consecutive amino acids, or more than 10, 20, 30, 40, 50, 52, 60, 70, 80, 90, 100, 150 or 200 or more consecutive amino acids from a sequence consisting of amino acids 379-586 of SEQ ID NO: 1 or from a sequence consisting of amino acids 379-581 of SEQ ID NO:1. The unprocessed ENG polypeptide may either include or exclude any signal sequence, as well as any sequence N-terminal to the signal sequence. As elaborated herein, the N-terminus of the mature (processed) ENG polypeptide may occur at any of amino acids 26-42 of SEQ ID NO: 1. Examples of mature ENG polypeptides include amino acids 25-377 of SEQ ID NO: 23, amino acids 25-358 of SEQ ID NO: 25, and amino acids 25-345 of SEQ ID NO: 29. Likewise, an ENG polypeptide may comprise a polypeptide that is encoded by nucleotides 73-1131 of SEQ ID NO: 24, nucleotides 73-1074 of SEQ ID NO: 26, or nucleotides 73-1035 of SEQ ID NO: 30, or silent variants thereof or nucleic acids that hybridize to the complement thereof under stringent hybridization conditions (generally, such conditions are known in the art but may, for example, involve hybridization in 50% v/v formamide, 5x SSC, 2% w/v blocking agent, 0.1% N-lauroylsarcosine, and 0.3% SDS at 65°C overnight and washing in, for example, 5x SSC at about 65°C). The term “ENG polypeptide” accordingly encompasses isolated extracellular portions of ENG polypeptides, variants thereof (including variants that comprise, for example, no more than 2, 3, 4, 5, 10, 15, 20, 25, 30, or 35 amino acid substitutions in the sequence corresponding to amino acids 26-378 of SEQ ID NO: 1), fragments thereof, and fusion proteins comprising any of the preceding, but in each case preferably any of the foregoing ENG polypeptides will retain substantial affinity for BMP-9 and/or BMP-10. Generally, an ENG polypeptide will be designed to be soluble in aqueous solutions at biologically relevant temperatures, pH levels, and osmolarity.

Data presented here show that Fc fusion proteins comprising shorter C-terminally truncated variants of ENG polypeptides display no appreciable binding to TGF- $\beta$ 1 and TGF- $\beta$ 3 but instead display higher affinity binding to BMP-9, with a markedly slower dissociation rate, compared to either ENG(26-437)-Fc or an Fc fusion protein comprising the full-length ENG ECD. Specifically, C-terminally truncated variants ending at amino acids 378, 359, and 346 of SEQ ID NO: 1 were all found to bind BMP-9 with substantially higher affinity (and to bind BMP-10 with undiminished affinity) compared to ENG(26-437) or ENG(26-586). However,

binding to BMP-9 and BMP-10 was completely disrupted by more extensive C-terminal truncations to amino acids 332, 329, or 257. Thus, ENG polypeptides that terminate between amino acid 333 and amino acid 378 are all expected to be active, but constructs ending at, or between, amino acids 346 and 359 may be most active.

5 Forms ending at, or between, amino acids 360 and 378 are predicted to trend toward the intermediate ligand binding affinity shown by ENG(26-378). Improvements in other key parameters are expected with certain constructs ending at, or between, amino acids 333 and 378 based on improvements in protein expression and elimination half-life observed with ENG(26-346)-Fc compared to fusion proteins

10 comprising full-length ENG ECD (see Examples). Any of these truncated variant forms may be desirable to use, depending on the clinical or experimental setting.

At the N-terminus, it is expected that an ENG polypeptide beginning at amino acid 26 (the initial glutamate), or before, of SEQ ID NO: 1 will retain ligand binding activity. As disclosed herein, an N-terminal truncation to amino acid 61 of SEQ ID

15 NO: 1 abolishes ligand binding, as do more extensive N-terminal truncations. However, as also disclosed herein, consensus modeling of ENG primary sequences indicates that ordered secondary structure within the region defined by amino acids 26-60 of SEQ ID NO: 1 is limited to a four-residue beta strand predicted with high confidence at positions 42-45 of SEQ ID NO: 1 and a two-residue beta strand

20 predicted with very low confidence at positions 28-29 of SEQ ID NO: 1. Thus, an active ENG polypeptide will begin at (or before) amino acid 26, preferentially, or at any of amino acids 27-42 of SEQ ID NO: 1.

Taken together, an active portion of an ENG polypeptide may comprise amino acid sequences 26-333, 26-334, 26-335, 26-336, 26-337, 26-338, 26-339, 26-340, 26-

25 341, 26-342, 26-343, 26-344, 26-345, or 26-346 of SEQ ID NO: 1, as well as variants of these sequences starting at any of amino acids 27-42 of SEQ ID NO: 1. Exemplary ENG polypeptides comprise amino acid sequences 26-346, 26-359, and 26-378 of SEQ ID NO: 1. Variants within these ranges are also contemplated, particularly those having at least 80%, 85%, 90%, 95%, or 99% identity to the corresponding portion of

30 SEQ ID NO: 1. An ENG polypeptide may not include the sequence consisting of amino acids 379-430 of SEQ ID NO:1.

As described above, the disclosure provides ENG polypeptides sharing a specified degree of sequence identity or similarity to a naturally occurring ENG

polypeptide. To determine the percent identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The amino acid residues at corresponding amino acid positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid "identity" is equivalent to amino acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm. (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

In one embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J Mol. Biol. (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>). In a specific embodiment, the following parameters are used in the GAP program: either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (Devereux, J., *et al.*, Nucleic Acids Res. 12(1):387 (1984)) (available at <http://www.gcg.com>). Exemplary parameters include using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. Unless otherwise specified, percent identity between two amino acid sequences is

to be determined using the GAP program using a Blosum 62 matrix, a GAP weight of 10 and a length weight of 3, and if such algorithm cannot compute the desired percent identity, a suitable alternative disclosed herein should be selected.

In another embodiment, the percent identity between two amino acid  
5 sequences is determined using the algorithm of E. Myers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

Another embodiment for determining the best overall alignment between two  
10 amino acid sequences can be determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.*, 6:237-245 (1990)). In a sequence alignment the query and subject sequences are both amino acid sequences. The result of said global sequence alignment is presented in terms of percent identity. In one embodiment, amino acid sequence identity is performed using the FASTDB  
15 computer program based on the algorithm of Brutlag *et al.* (*Comp. App. Biosci.*, 6:237-245 (1990)). In a specific embodiment, parameters employed to calculate percent identity and similarity of an amino acid alignment comprise: Matrix=PAM 150, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5 and Gap Size Penalty=0.05.

20 In certain embodiments, an ENG polypeptide binds to BMP-9 and BMP-10, and the ENG polypeptide does not show substantial binding to TGF- $\beta$ 1 or TGF- $\beta$ 3. Binding may be assessed using purified proteins in solution or in a surface plasmon resonance system, such as a Biacore<sup>TM</sup> system. ENG polypeptides may be selected to exhibit an anti-angiogenic activity. Bioassays for angiogenesis inhibitory activity  
25 include the chick chorioallantoic membrane (CAM) assay, the mouse angioreactor assay, and assays for measuring the effect of administering isolated or synthesized proteins on implanted tumors. The CAM assay, the mouse angioreactor assay, and other assays are described in the Examples.

ENG polypeptides may additionally include any of various leader sequences at  
30 the N-terminus. Such a sequence would allow the peptides to be expressed and targeted to the secretion pathway in a eukaryotic system. See, e.g., Ernst *et al.*, U.S. Pat. No. 5,082,783 (1992). Alternatively, a native ENG signal sequence may be used to effect extrusion from the cell. Possible leader sequences include honeybee mellitin,

TPA, and native leaders (SEQ ID NOs. 13-15, respectively). Examples of ENG-Fc fusion proteins incorporating a TPA leader sequence include SEQ ID NOs: 23, 25, 27, and 29. Processing of signal peptides may vary depending on the leader sequence chosen, the cell type used and culture conditions, among other variables, and therefore actual N-terminal start sites for mature ENG polypeptides may shift by 1, 2, 3, 4 or 5 amino acids in either the N-terminal or C-terminal direction. Examples of mature ENG-Fc fusion proteins include SEQ ID NOs: 33-36, as shown below with the ENG polypeptide portion underlined.

10 Human ENG(26-378)-hFc (truncated Fc)

ETVHCD LQPVGPERDE VTYTTSQVSK GCVAQAPNAI LEVHVLFLFLEF  
PTGPSQLELT LQASKQNGTW PREVLLVLSV NSSVFLHLQA LGIPLHLAYN  
SSLVTFQEPP GVNTTELPSF PKTQILEWAA ERGPITSAAE LNDPQSILLR  
LGQAQGSLSF CMLEASQDMG RTLEWRP RTP ALVRGCHLEG VAGHKEAHIL  
15 RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL ILQGPPYVSW LIDANHNMQI  
WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG EARMLNASIV ASFVELPLAS  
IVSLHASSCG GRLQTSPAPI QTTPPKDTCS PELLMSLIQT KCADDAMTLV  
LKKELVATGG GTHTCPPCPA PELLGGPSVF LFPPKPKDTL MISRTPEVTC  
VVDVSHEDP EVKFNWYVDG VEVHNAKTKP REEQYNSTYR VVSVLTVLHQ  
20 DWLNGKEYKC KVS NKALPAP IEKTISKAKG QPREPQVYTL PPSREEMTKN  
QVSLTCLVKG FYPSDIAVEW ESNGQPENNY KTPPVLDSD GSFFLYSKLT  
VDKSRWQQGN VFSCSVMHEA LHNHYTQKSL SLSPGK (SEQ ID NO: 33)

Human ENG(26-359)-hFc

25 ETVHCD LQPVGPERDE VTYTTSQVSK GCVAQAPNAI LEVHVLFLFLEF  
PTGPSQLELT LQASKQNGTW PREVLLVLSV NSSVFLHLQA LGIPLHLAYN  
SSLVTFQEPP GVNTTELPSF PKTQILEWAA ERGPITSAAE LNDPQSILLR  
LGQAQGSLSF CMLEASQDMG RTLEWRP RTP ALVRGCHLEG VAGHKEAHIL  
RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL ILQGPPYVSW LIDANHNMQI  
30 WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG EARMLNASIV ASFVELPLAS  
IVSLHASSCG GRLQTSPAPI QTTPPKDTCS PELLMSLITG GGPKSCDKTH  
TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK

FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS  
 NKALPAPIEK TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP  
 SDIAVEWESN GQPENNYKTT PPVLDS DGSF FLYSKLTVDK SRWQQGNVFS  
 CSVMHEALHN HYTQKSLSLS PGK (SEQ ID NO: 34)

5

## Human ENG(26-359)-hFc (truncated Fc)

ETVHCD LQPVGPERDE VTYTTSQVSK GCVAQAPNAI LEVHVLFLFLEF  
PTGPSQLELT LQASKQNGTW PREVLLVLSV NSSVFLHLQA LGIPLHLAYN  
SSLVTFQEPP GVNTTELPSF PKTQILEWAA ERGPITSAAE LNDPQSILLR  
 10 LGQAQGSLSF CMLEASQDMG RTLEWRP RTP ALVRGCHLEG VAGHKEAHIL  
RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL ILQGPPYVSW LIDANHNMQI  
WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG EARMLNASIV ASFVELPLAS  
IVSLHASSCG GRLQTSPAPI QTTPPKDTCS PELLMSLITG GGTHTCPPCP  
APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD  
 15 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA  
PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE  
WESNGQPENN YKTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE  
ALHNHYTQKS LSLSPGK (SEQ ID NO: 35)

## 20 Human ENG(26-346)-hFc (truncated Fc)

ETVHCD LQPVGPERDE VTYTTSQVSK GCVAQAPNAI LEVHVLFLFLEF  
PTGPSQLELT LQASKQNGTW PREVLLVLSV NSSVFLHLQA LGIPLHLAYN  
SSLVTFQEPP GVNTTELPSF PKTQILEWAA ERGPITSAAE LNDPQSILLR  
LGQAQGSLSF CMLEASQDMG RTLEWRP RTP ALVRGCHLEG VAGHKEAHIL  
 25 RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL ILQGPPYVSW LIDANHNMQI  
WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG EARMLNASIV ASFVELPLAS  
IVSLHASSCG GRLQTSPAPI QTTPPTGGGT HTCPCPCPAPE LLGGPSVFLF  
PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE VHNAKTKPRE  
EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAKGQP  
 30 REPQVYTLPP SREEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT  
TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL  
SPGK (SEQ ID NO: 36)

In certain embodiments, the present disclosure contemplates specific mutations of the ENG polypeptides so as to alter the glycosylation of the polypeptide. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, such as O-linked or N-linked glycosylation sites. Asparagine-linked glycosylation recognition sites generally comprise a tripeptide sequence, asparagine-X-threonine (or asparagines-X-serine) (where "X" is any amino acid) which is specifically recognized by appropriate cellular glycosylation enzymes. The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the wild-type ENG polypeptide (for O-linked glycosylation sites). A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Another means of increasing the number of carbohydrate moieties on an ENG polypeptide is by chemical or enzymatic coupling of glycosides to the ENG polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine; (b) free carboxyl groups; (c) free sulfhydryl groups such as those of cysteine; (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (1981) *CRC Crit. Rev. Biochem.*, pp. 259-306, incorporated by reference herein. Removal of one or more carbohydrate moieties present on an ENG polypeptide may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure of the ENG polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the amino acid sequence intact. Chemical deglycosylation is further described by Hakimuddin et al. (1987) *Arch. Biochem. Biophys.* 259:52 and by Edge et al. (1981) *Anal. Biochem.* 118:131. Enzymatic cleavage of carbohydrate moieties on ENG polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (1987) *Meth. Enzymol.* 138:350. The sequence of an ENG polypeptide may be



adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce differing glycosylation patterns that can be affected by the amino acid sequence of the peptide. In general, ENG polypeptides for use in humans will be expressed in a mammalian cell line that provides proper glycosylation, such as HEK293 or CHO cell lines, although other mammalian expression cell lines, yeast cell lines with engineered glycosylation enzymes, and insect cells are expected to be useful as well.

This disclosure further contemplates a method of generating mutants, particularly sets of combinatorial mutants of an ENG polypeptide, as well as truncation mutants; pools of combinatorial mutants are especially useful for identifying functional variant sequences. The purpose of screening such combinatorial libraries may be to generate, for example, ENG polypeptide variants which can act as either agonists or antagonist, or alternatively, which possess novel activities all together. A variety of screening assays are provided below, and such assays may be used to evaluate variants. For example, an ENG polypeptide variant may be screened for ability to bind to an ENG ligand, to prevent binding of an ENG ligand to an ENG polypeptide or to interfere with signaling caused by an ENG ligand. The activity of an ENG polypeptide or its variants may also be tested in a cell-based or in vivo assay, particularly any of the assays disclosed in the Examples.

Combinatorially-derived variants can be generated which have a selective or generally increased potency relative to an ENG polypeptide comprising an extracellular domain of a naturally occurring ENG polypeptide. Likewise, mutagenesis can give rise to variants which have serum half-lives dramatically different than the corresponding wild-type ENG polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other processes which result in destruction of, or otherwise elimination or inactivation of, a native ENG polypeptide. Such variants, and the genes which encode them, can be utilized to alter ENG polypeptide levels by modulating the half-life of the ENG polypeptides. For instance, a short half-life can give rise to more transient biological effects and can allow tighter control of recombinant ENG polypeptide levels within the patient. In an Fc fusion protein, mutations may be made in the linker (if any) and/or the Fc portion to alter the half-life of the protein.

A combinatorial library may be produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential ENG polypeptide sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential ENG polypeptide nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display).

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

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, ENG polypeptide variants can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) *Biochemistry* 33:1565-1572; Wang et al., (1994) *J. Biol. Chem.* 269:3095-3099; Balint et al., (1993) *Gene* 137:109-118; Grodberg et al., (1993) *Eur. J. Biochem.* 218:597-601; Nagashima et al., (1993) *J. Biol. Chem.* 268:2888-2892; Lowman et al., (1991) *Biochemistry* 30:10832-10838; and Cunningham et al., (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) *Virology* 193:653-660; Brown et al., (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight et al., (1982) *Science* 232:316); by saturation mutagenesis (Meyers et al., (1986) *Science* 232:613); by PCR mutagenesis (Leung et al., (1989) *Method Cell Mol Biol* 1:11-19); or by random mutagenesis, including chemical

mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of ENG polypeptides.

5 A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of ENG polypeptides. The most widely  
10 used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Preferred assays include ENG  
15 ligand binding assays and ligand-mediated cell signaling assays.

In certain embodiments, the ENG polypeptides of the disclosure may further comprise post-translational modifications in addition to any that are naturally present in the ENG polypeptides. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, pegylation  
20 (polyethyleneglycol) and acylation. As a result, the modified ENG polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of an ENG polypeptide may be tested as described herein for other ENG polypeptide variants. When an ENG polypeptide is produced in cells by cleaving a  
25 nascent form of the ENG polypeptide, post-translational processing may also be important for correct folding and/or function of the protein. Different cells (such as CHO, HeLa, MDCK, 293, WI38, NIH-3T3 or HEK293) have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the ENG  
30 polypeptides.

In certain aspects, functional variants or modified forms of the ENG polypeptides include fusion proteins having at least a portion of the ENG polypeptides and one or more fusion domains. Well known examples of such fusion domains

include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt-conjugated resins are used. Many of such matrices are available in “kit” form, such as the Pharmacia GST purification system and the QIAexpress<sup>TM</sup> system (Qiagen) useful with (HIS<sub>6</sub>) fusion partners. As another example, a fusion domain may be selected so as to facilitate detection of the ENG polypeptides. Examples of such detection domains include the various fluorescent proteins (e.g., GFP) as well as “epitope tags,” which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus hemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain preferred embodiments, an ENG polypeptide is fused with a domain that stabilizes the ENG polypeptide in vivo (a “stabilizer” domain). By “stabilizing” is meant anything that increases serum half-life, regardless of whether this is because of decreased destruction, decreased clearance by the kidney, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (e.g., dimerizing, tetramerizing) domains and functional domains.

As specific examples, the present disclosure provides fusion proteins comprising variants of ENG polypeptides fused to one of two Fc domain sequences (e.g., SEQ ID NOs: 11, 12). Optionally, the Fc domain has one or more mutations at residues such as Asp-265, Lys-322, and Asn-434 (numbered in accordance with the corresponding full-length IgG). In certain cases, the mutant Fc domain having one or

more of these mutations (e.g., Asp-265 mutation) has reduced ability of binding to the Fc $\gamma$  receptor relative to a wildtype Fc domain. In other cases, the mutant Fc domain having one or more of these mutations (e.g., Asn-434 mutation) has increased ability of binding to the MHC class I-related Fc-receptor (FcRN) relative to a wildtype Fc domain.

It is understood that different elements of the fusion proteins may be arranged in any manner that is consistent with the desired functionality. For example, an ENG polypeptide may be placed C-terminal to a heterologous domain, or, alternatively, a heterologous domain may be placed C-terminal to an ENG polypeptide. The ENG polypeptide domain and the heterologous domain need not be adjacent in a fusion protein, and additional domains or amino acid sequences may be included C- or N-terminal to either domain or between the domains.

As used herein, the term "immunoglobulin Fc domain" or simply "Fc" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, preferably an immunoglobulin heavy chain constant region, or a portion thereof. For example, an immunoglobulin Fc region may comprise 1) a CH1 domain, a CH2 domain, and a CH3 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of two or more domains and an immunoglobulin hinge region. In a preferred embodiment the immunoglobulin Fc region comprises at least an immunoglobulin hinge region a CH2 domain and a CH3 domain, and preferably lacks the CH1 domain.

In one embodiment, the class of immunoglobulin from which the heavy chain constant region is derived is IgG (Ig $\gamma$ ) ( $\gamma$  subclasses 1, 2, 3, or 4). Other classes of immunoglobulin, IgA (Ig $\alpha$ ), IgD (Ig $\delta$ ), IgE (Ig $\epsilon$ ) and IgM (Ig $\mu$ ), may be used. The choice of appropriate immunoglobulin heavy chain constant region is discussed in detail in U.S. Pat. Nos. 5,541,087, and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. The portion of the DNA construct encoding the immunoglobulin Fc region preferably comprises at least a portion of a hinge domain, and preferably at least a portion of a CH<sub>3</sub> domain of Fc gamma or the homologous domains in any of IgA, IgD, IgE, or IgM.

Furthermore, it is contemplated that substitution or deletion of amino acids within the immunoglobulin heavy chain constant regions may be useful in the practice of the methods and compositions disclosed herein. One example would be to introduce amino acid substitutions in the upper CH2 region to create an Fc variant with reduced affinity for Fc receptors (Cole *et al.* (1997) *J. Immunol.* 159:3613).

In certain embodiments, the present disclosure makes available isolated and/or purified forms of the ENG polypeptides, which are isolated from, or otherwise substantially free of (e.g., at least 80%, 90%, 95%, 96%, 97%, 98%, or 99% free of), other proteins and/or other ENG polypeptide species. ENG polypeptides will generally be produced by expression from recombinant nucleic acids.

In certain embodiments, the disclosure includes nucleic acids encoding soluble ENG polypeptides comprising the coding sequence for an extracellular portion of an ENG protein. In further embodiments, this disclosure also pertains to a host cell comprising such nucleic acids. The host cell may be any prokaryotic or eukaryotic cell. For example, a polypeptide of the present disclosure may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art. Accordingly, some embodiments of the present disclosure further pertain to methods of producing the ENG polypeptides. It has been established that ENG-Fc fusion proteins set forth in SEQ ID NOs: 25 and 29 and expressed in CHO cells have potent anti-angiogenic activity.

#### 5. Nucleic Acids Encoding ENG Polypeptides

In certain aspects, the disclosure provides isolated and/or recombinant nucleic acids encoding any of the ENG polypeptides, including fragments, functional variants and fusion proteins disclosed herein. For example, SEQ ID NOs: 2 and 4 encode long and short isoforms, respectively, of the native human ENG precursor polypeptide, whereas SEQ ID NO: 30 encodes one variant of ENG extracellular domain fused to an IgG1 Fc domain. The subject nucleic acids may be single-stranded or double stranded. Such nucleic acids may be DNA or RNA molecules. These nucleic acids may be used, for example, in methods for making ENG polypeptides or as direct therapeutic agents (e.g., in an antisense, RNAi or gene therapy approach).

In certain aspects, the subject nucleic acids encoding ENG polypeptides are further understood to include nucleic acids that are variants of SEQ ID NOs: 24, 26, 28, or 30. Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants.

5 In certain embodiments, the disclosure provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NOs: 24, 26, 28, or 30. One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to SEQ ID NOs: 24, 26, 28, or 30, and variants of SEQ ID NOs: 24, 26, 28, or 30 are also within the scope of  
10 this disclosure. In further embodiments, the nucleic acid sequences of the disclosure can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In other embodiments, nucleic acids of the disclosure also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequences  
15 designated in SEQ ID NOs: 24, 26, 28, or 30, complement sequences of SEQ ID NOs: 24, 26, 28, or 30, or fragments thereof. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of  
20 2.0 x SSC at 50°C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or  
25 temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the disclosure provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the nucleic acids as set forth in SEQ  
30 ID NOs: 24, 26, 28, or 30 due to degeneracy in the genetic code are also within the scope of the disclosure. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for

example, CAU and CAC are synonyms for histidine) may result in “silent” mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this disclosure.

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

15 Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the disclosure. The promoters may be either

20 naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known

25 in the art and will vary with the host cell used.

In certain aspects disclosed herein, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding an ENG polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the ENG polypeptide.

30 Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control



sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding an ENG polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, RSV promoters, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast  $\alpha$ -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

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

Some mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or

Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and in transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 3rd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 2001). In some instances, it may be desirable to express the recombinant polypeptides by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

In a preferred embodiment, a vector will be designed for production of the subject ENG polypeptides in CHO cells, such as a Pcmv-Script vector (Stratagene, La Jolla, Calif.), pcDNA4 vectors (Invitrogen, Carlsbad, Calif.) and pCI-neo vectors (Promega, Madison, Wisc.). As will be apparent, the subject gene constructs can be used to cause expression of the subject ENG polypeptides in cells propagated in culture, e.g., to produce proteins, including fusion proteins or variant proteins, for purification.

This disclosure also pertains to a host cell transfected with a recombinant gene including a coding sequence (e.g., SEQ ID NOs: 24, 26, 28, or 30) for one or more of the subject ENG polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, an ENG polypeptide disclosed herein may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present disclosure further pertains to methods of producing the subject ENG polypeptides. For example, a host cell transfected with an expression vector encoding an ENG polypeptide can be cultured under appropriate conditions to allow expression of the ENG polypeptide to occur. The ENG polypeptide may be secreted and isolated from a mixture of cells and medium containing the ENG polypeptide. Alternatively, the ENG polypeptide may be

retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The subject ENG polypeptides can be isolated from cell culture medium, host cells, or both, using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, immunoaffinity purification with antibodies specific for particular epitopes of the ENG polypeptides and affinity purification with an agent that binds to a domain fused to the ENG polypeptide (e.g., a protein A column may be used to purify an ENG-Fc fusion). In a preferred embodiment, the ENG polypeptide is a fusion protein containing a domain which facilitates its purification. As an example, purification may be achieved by a series of column chromatography steps, including, for example, three or more of the following, in any order: protein A chromatography, Q sepharose chromatography, phenylsepharose chromatography, size exclusion chromatography, and cation exchange chromatography. The purification could be completed with viral filtration and buffer exchange.

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant ENG polypeptide, can allow purification of the expressed fusion protein by affinity chromatography using a Ni<sup>2+</sup> metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified ENG polypeptide (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a

chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

Examples of categories of nucleic acid compounds that are antagonists of ENG, BMP-9, or BMP-10 include antisense nucleic acids, RNAi constructs and catalytic nucleic acid constructs. A nucleic acid compound may be single or double stranded. A double stranded compound may also include regions of overhang or non-complementarity, where one or the other of the strands is single stranded. A single stranded compound may include regions of self-complementarity, meaning that the compound forms a so-called “hairpin” or “stem-loop” structure, with a region of double helical structure. A nucleic acid compound may comprise a nucleotide sequence that is complementary to a region consisting of no more than 1000, no more than 500, no more than 250, no more than 100 or no more than 50, 35, 30, 25, 22, 20 or 18 nucleotides of the full-length ENG nucleic acid sequence or ligand nucleic acid sequence. The region of complementarity will preferably be at least 8 nucleotides, and optionally at least 10 or at least 15 nucleotides, and optionally between 15 and 25 nucleotides. A region of complementarity may fall within an intron, a coding sequence, or a noncoding sequence of the target transcript, such as the coding sequence portion. Generally, a nucleic acid compound will have a length of about 8 to about 500 nucleotides or base pairs in length, and optionally the length will be about 14 to about 50 nucleotides. A nucleic acid may be a DNA (particularly for use as an antisense), RNA, or RNA:DNA hybrid. Any one strand may include a mixture of DNA and RNA, as well as modified forms that cannot readily be classified as either DNA or RNA. Likewise, a double stranded compound may be DNA:DNA, DNA:RNA or RNA:RNA, and any one strand may also include a mixture of DNA and RNA, as well as modified forms that cannot readily be classified as either DNA or RNA. A nucleic acid compound may include any of a variety of modifications, including one or modifications to the backbone (the sugar-phosphate portion in a natural nucleic acid, including internucleotide linkages) or the base portion (the purine or pyrimidine portion of a natural nucleic acid). An antisense nucleic acid compound will preferably have a length of about 15 to about 30 nucleotides and will often contain one or more modifications to improve characteristics such as stability in the serum, in a cell or in a place where the compound is likely to be delivered, such as the stomach in the case of orally delivered compounds and the lung for inhaled

compounds. In the case of an RNAi construct, the strand complementary to the target transcript will generally be RNA or modifications thereof. The other strand may be RNA, DNA, or any other variation. The duplex portion of double stranded or single stranded “hairpin” RNAi construct will preferably have a length of 18 to 40  
5 nucleotides in length and optionally about 21 to 23 nucleotides in length, so long as it serves as a Dicer substrate. Catalytic or enzymatic nucleic acids may be ribozymes or DNA enzymes and may also contain modified forms. Nucleic acid compounds may inhibit expression of the target by about 50%, 75%, 90%, or more when contacted with cells under physiological conditions and at a concentration where a nonsense or  
10 sense control has little or no effect. Preferred concentrations for testing the effect of nucleic acid compounds are 1, 5 and 10 micromolar. Nucleic acid compounds may also be tested for effects on, for example, angiogenesis.

#### 6. Alterations in Fc-fusion proteins

The application further provides ENG-Fc fusion proteins with engineered or  
15 variant Fc regions. Such antibodies and Fc fusion proteins may be useful, for example, in modulating effector functions, such as, antigen-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Additionally, the modifications may improve the stability of the antibodies and Fc fusion proteins. Amino acid sequence variants of the antibodies and Fc fusion proteins are prepared by  
20 introducing appropriate nucleotide changes into the DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibodies and Fc fusion proteins disclosed herein. Any combination of deletion, insertion, and  
25 substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibodies and Fc fusion proteins, such as changing the number or position of glycosylation sites.

Antibodies and Fc fusion proteins with reduced effector function may be produced by introducing changes in the amino acid sequence, including, but are not  
30 limited to, the Ala-Ala mutation described by Bluestone et al. (see WO 94/28027 and WO 98/47531; also see Xu et al. 2000 Cell Immunol 200; 16-26). Thus in certain embodiments, antibodies and Fc fusion proteins of the disclosure with mutations within the constant region including the Ala-Ala mutation may be used to reduce or

abolish effector function. According to these embodiments, antibodies and Fc fusion proteins may comprise a mutation to an alanine at position 234 or a mutation to an alanine at position 235, or a combination thereof. In one embodiment, the antibody or Fc fusion protein comprises an IgG4 framework, wherein the Ala-Ala mutation would describe a mutation(s) from phenylalanine to alanine at position 234 and/or a mutation from leucine to alanine at position 235. In another embodiment, the antibody or Fc fusion protein comprises an IgG1 framework, wherein the Ala-Ala mutation would describe a mutation(s) from leucine to alanine at position 234 and/or a mutation from leucine to alanine at position 235. The antibody or Fc fusion protein may alternatively or additionally carry other mutations, including the point mutation K322A in the CH2 domain (Hezareh et al. 2001 J Virol. 75: 12161-8).

In particular embodiments, the antibody or Fc fusion protein may be modified to either enhance or inhibit complement dependent cytotoxicity (CDC). Modulated CDC activity may be achieved by introducing one or more amino acid substitutions, insertions, or deletions in an Fc region (see, e.g., U.S. Pat. No. 6,194,551).

Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved or reduced internalization capability and/or increased or decreased complement-mediated cell killing. See Caron et al., J. Exp Med. 176:1191-1195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992), WO99/51642, Duncan & Winter Nature 322: 738-40 (1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821; and WO94/29351.

## EXAMPLES

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

**Example 1: Expression of fusion protein comprising full-length extracellular domain of human ENG**

Applicants constructed a soluble endoglin (ENG) fusion protein (hENG(26-586)-hFc) in which the full-length extracellular domain (ECD) of human ENG (**Figure 9**, SEQ ID NO: 9) was attached to a human IgG<sub>1</sub> Fc domain (**Figure 11**, SEQ ID NO: 11) with a minimal linker between these domains. hENG(26-586)-hFc was expressed by transient transfection in HEK 293 cells. In brief, HEK 293 cells were set up in a 500-ml spinner at  $6 \times 10^5$  cells/ml in a 250 ml volume of Freestyle media (Invitrogen) and grown overnight. Next day, these cells were treated with DNA:PEI (1:1) complex at 0.5 ug/ml final DNA concentration. After 4 hrs, 250 ml media was added and cells were grown for 7 days. Conditioned media was harvested by spinning down the cells and concentrated. For expression in CHO cells, ENG polypeptide constructs were transfected into a CHO DUKX B11 cell line. Clones were selected in methotrexate (MTX), typically at an initial concentration of 5 nM or 10 nM, and optionally followed by amplification in 50nM MTX to increase expression. A high expressing clone could be identified by dilution cloning and adapted to serum-free suspension growth to generate conditioned media for purification. Optionally, a ubiquitous chromatin opening element (UCOE) may be included in the vector to facilitate expression. See, e.g., Cytotechnology. 2002 Jan;38(1-3):43-6.

Three different leader sequences may be used:

- (i) Honey bee mellitin (HBML): MKFLVNVALVFMVVYISYIYA (SEQ ID NO: 13)
- (ii) Tissue plasminogen activator (TPA): MDAMKRGLCCVLLLCGAVFVSP (SEQ ID NO: 14)
- (iii) Native human ENG: MDRGTLPLAVALLASCSLSPTSLA (SEQ ID NO: 15)

The selected form of hENG(26-586)-hFc uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 13** (SEQ ID NO: 16), and is encoded by the nucleotide sequence shown in **Figure 14** (SEQ ID NO: 17).

Applicants also envision an alternative hENG(26-586)-hFc sequence with TPA leader (**Figure 15**, SEQ ID NO: 18) comprising an N-terminally truncated hFc domain (**Figure 12**, SEQ ID NO: 12) attached to hENG(26-586) by a TGGG linker.

Purification was achieved using a variety of techniques, including, for example, filtration of conditioned media, followed by protein A chromatography, elution with low-pH (3.0) glycine buffer, sample neutralization, and dialysis against PBS. Purity

of samples was evaluated by analytical size-exclusion chromatography, SDS-PAGE, silver staining, and Western blot. Analysis of mature protein confirmed the expected N-terminal sequence.

5 **Example 2: Expression of fusion protein comprising full-length extracellular domain of murine ENG**

Applicants constructed a soluble murine ENG fusion protein (mENG(27-581)-mFc) in which the full-length extracellular domain of murine ENG (**Figure 10**, SEQ ID NO: 10) was fused to a murine IgG<sub>2a</sub> Fc domain with a minimal linker between these domains. mENG(27-581)-mFc was expressed by transient transfection in HEK 293 cells.

The selected form of mENG(27-581)-mFc uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 16** (SEQ ID NO: 19), and is encoded by the nucleotide sequence shown in **Figure 17** (SEQ ID NO: 20).

15 Purification was achieved by filtration of conditioned media from transfected HEK 293 cells, followed by protein A chromatography. Purity of samples was evaluated by analytical size-exclusion chromatography, SDS-PAGE, silver staining, and Western blot analysis.

20 **Example 3: Selective binding of BMP-9 / BMP-10 to proteins comprising full-length extracellular ENG domain**

Considered a co-receptor, ENG is widely thought to function by facilitating the binding of TGF- $\beta$ 1 and -3 to multiprotein complexes of type I and type II receptors. To investigate the possibility of direct ligand binding by isolated ENG, Applicants used surface plasmon resonance (SPR) methodology (Biacore™ instrument) to screen for binding of captured proteins comprising the full-length extracellular domain of ENG to a variety of soluble human TGF- $\beta$  family ligands.

Ligand	Construct Binding		
	hENG(26-586)-hFc*	hENG(26-586)**	mENG(27-581)-hFc***
hBMP-2	—	—	—
hBMP-2/7	—	—	—
hBMP-7	—	—	—
hBMP-9	++++	++++	++++



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hBMP-10	++++	++++	++++
hTGF- $\beta$ 1	—	—	—
hTGF- $\beta$ 2	—	—	—
hTGF- $\beta$ 3	—	—	—
hActivin A	—	—	—

\* [hBMP-9], [hBMP-10] = 2.5 nM; all other ligands tested at 100 nM

\*\* [hBMP-9], [hBMP-10] = 2.5 nM; all other ligands tested at 25 nM

\*\*\* [hBMP-9], [hBMP-10] = 0.5 nM; [hTGF- $\beta$ 1], [hTGF- $\beta$ 2], [hTGF- $\beta$ 3] = 10 nM; all other ligands tested at 25 nM

5

As shown in this table, binding affinity to hENG(26-586)-hFc was high (++++,  $K_D < 1$  nM) for hBMP-9 and hBMP-10 as evaluated at low ligand concentrations. Even at concentrations 40-fold higher, binding of TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, activin A, BMP-2, and BMP-7 to hENG(26-586)-hFc was undetectable (—). For this latter group of ligands, lack of direct binding to isolated ENG fusion protein is noteworthy because multiprotein complexes of type I and type II receptors have been shown to bind most of them better in the presence of ENG than in its absence.

As also shown in the table above, similar results were obtained when ligands were screened for their ability to bind immobilized hENG(26-586) (R&D Systems, catalog #1097-EN), a human variant with no Fc domain, or their ability to bind captured mENG(27-581)-hFc (R&D Systems, catalog #1320-EN), consisting of the extracellular domain of murine ENG (residues 27-581) attached to the Fc domain of human IgG<sub>1</sub> via a six-residue linker sequence (IEGRMD). Characterization by SPR (**Figures 18, 19**) determined that captured hENG(26-586)-hFc binds soluble BMP-9 with a  $K_D$  of 29 pM and soluble BMP-10 with a  $K_D$  of 400 pM. Thus, selective high-affinity binding of BMP-9 and BMP-10 is a previously unrecognized property of the ENG extracellular domain that is generalizable across species.

25

#### **Example 4: Soluble extracellular domain of hENG inhibits binding of BMP-9 / BMP-10 to ALK1 and other cognate receptors**

BMP-9 and BMP-10 are high-affinity ligands at the type I receptor ALK1 (activin receptor-like kinase 1). An SPR-based assay was used to determine the effect of soluble hENG(26-586) (R&D Systems, catalog #1097-EN) on binding of BMP-9 and BMP-10 to ALK1. ALK1-hFc was captured and then exposed to solutions

30

containing soluble hENG(26-586) premixed with BMP-9 in various ratios. As shown in **Figure 20**, soluble hENG(26-586) inhibited binding of BMP-9 to ALK1-Fc in a concentration-dependent manner with an  $IC_{50}$  less than 10 nM. Similar results were obtained with BMP-10 (**Figure 21**). Separate experiments have demonstrated that soluble hENG(26-586) does not bind ALK1 and therefore does not inhibit ligand binding to ALK1 by this mechanism. Indeed, additional SPR-based experiments indicate that soluble hENG(26-586) binds neither type I receptors ALK2-ALK7 nor type II receptors such as activin receptor IIA, activin receptor IIB, bone morphogenetic protein receptor II, and TGF- $\beta$  receptor II. These results provide further evidence that ENG inhibits binding of BMP-9 and BMP-10 to ALK1 primarily through a direct interaction with these ligands.

Taken together, these data demonstrate that soluble ENG-Fc chimeric proteins as well as non-chimeric soluble ENG can be used as antagonists of BMP-9 and BMP-10 signaling through multiple signaling pathways, including ALK1.

15

#### **Example 5: Effect of mENG(27-581)-hFc on human umbilical vein endothelial cells (HUVEC) in culture**

Applicants investigated the angiogenic effect of mENG(27-581)-hFc in a HUVEC-based culture system. HUVECs were cultured on a polymerized Matrigel substrate, and the effect of test articles on formation of endothelial-cell tubes (cords) was assessed by phase-contrast microscopy after 12 h exposure. Cords possessing single-cell width and at least three branches were identified visually, and computer-assisted image analysis was used to determine the total length of such cords. Mean values are based on duplicate culture wells per experimental condition, with each well characterized as the average of three fields of observation. Compared to basal conditions (no treatment), the strong inducing agent endothelial cell growth substance (ECGS, 0.2  $\mu$ g/ml) doubled mean cord length (**Figure 22**). mENG(27-581)-hFc (R&D Systems, catalog #1320-EN; 10  $\mu$ g/ml) cut this increase by nearly 60%, an effect specific for stimulated conditions because the same concentration of mENG(27-581)-hFc had little effect in the absence of ECGS (**Figure 22**). These results demonstrate that ENG-Fc fusion protein can inhibit endothelial cell aggregation under otherwise stimulated conditions in a cell-culture model of angiogenesis.

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**Example 6: ENG-Fc inhibits VEGF-inducible angiogenesis in a chick chorioallantoic membrane (CAM) assay**

A chick chorioallantoic membrane (CAM) assay system was used to investigate effects of ENG-Fc fusion protein on angiogenesis. In brief, nine-day-old fertilized chick embryos were maintained in an egg incubator at controlled temperature (37°C) and humidity (60%). The egg shell was softened with alcohol, punctured with a tiny hole to create a “blister” between the shell membrane and CAM, and removed to create a window overlying prominent blood vessels. Small filter disks were treated with VEGF (50 ng daily) in the presence or absence of mENG(27-581)-hFc protein (R&D Systems, catalog #1320-EN; 14 µg daily) dissolved in buffer (pH 7.4) containing 0.01 M HEPES, 0.5 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20, and 0.5 mg/ml bovine serum albumin. Filter disks containing test article were then inserted through the opening and apposed to the CAM. Eggs (n = 8 per group) were treated with fresh test article daily for three days, and on the fourth day the number of blood vessels associated with the filter disk was determined by visual inspection with the assistance of an egg lamp.

As expected, VEGF treatment in the CAM assay system increased the number of blood vessels markedly over that of vehicle. The number of additional blood vessels induced by VEGF treatment was decreased by 65% with concurrent mENG(27-581)-hFc treatment (**Figure 23**). SPR-based studies indicate that VEGF does not bind mENG(27-581)-mFc, and thus effects of mENG(27-581)-hFc on angiogenesis in the present CAM experiment were not due to a direct interaction between the fusion protein and VEGF. The foregoing results indicate that ENG-Fc can significantly inhibit the well-established angiogenic effect of VEGF in an in vivo model without contacting VEGF itself.

**Example 7: Effect of mENG(27-581)-mFc on angiogenesis in a mouse angioreactor assay**

Effects of ENG-Fc fusion protein on angiogenesis were further investigated in a mouse angioreactor assay, also known as a directed in vivo angiogenesis assay (DIVAA™; Guedez et al., 2003, Am J Pathol 162:1431-1439), which was performed according to instructions of the manufacturer (Trevigen®). In brief, hollow cylinders

made of implant-grade silicone and closed at one end were filled with 20  $\mu$ l of basement membrane extract (BME) premixed with or without a combination of basic fibroblast growth factor (FGF-2, 1.8  $\mu$ g) and VEGF (600 ng). After the BME had gelled, angioreactors were implanted subcutaneously in athymic nude mice (four per mouse). Mice were treated daily with mENG(27-581)-mFc (10 mg/kg, s.c.) or vehicle (Tris-buffered saline) for 11 days, at which time mice were injected with fluorescein isothiocyanate (FITC)-labeled dextran (20 mg/kg, i.v.) and euthanized 20 min later. Angioreactors were removed, and the amount of FITC-dextran contained in each was quantified with a fluorescence plate reader (Infinite® M200, Tecan) at 485 nm excitation / 520 nm emission as an index of blood vessel formation. As shown in **Figure 24**, addition of FGF-2 and VEGF to the BME led to a significant increase in vascularization within the angioreactors at study completion, whereas the concurrent administration of mENG(27-581)-mFc prevented this increase completely. These results obtained in a mammalian system complement those obtained with the CAM assay described above and demonstrate the in vivo anti-angiogenic activity of ENG-Fc fusion proteins incorporating a full-length ENG extracellular domain.

#### **Example 8: Expression of variants with truncated hENG extracellular domain**

Applicants generated soluble ENG fusion proteins in which truncated variants of the human ENG ECD were fused to a human IgG<sub>1</sub> Fc domain with a minimal linker. These variants are listed below, and the structures of selected variants are shown schematically in **Figure 25**.

	Human Construct	Transient Expression	Purified	Stable Expression (CHO Cells)
Full Length	hENG(26-586)-hFc	HEK 293	Yes	Yes
Carboxy-Terminal	hENG(26-581)-hFc	HEK 293	Yes	No
Truncations	hENG(26-437)-hFc	HEK 293	Yes	No
	hENG(26-378)-hFc	HEK 293	Yes	No
	hENG(26-359)-hFc	HEK 293	Yes	Yes
	hENG(26-346)-hFc	HEK 293	Yes	Yes
	hENG(26-332)-hFc	HEK 293	Yes	No
	hENG(26-329)-hFc	HEK 293	Yes	No
	hENG(26-257)-hFc	HEK 293	Yes	No
Amino-Terminal	hENG(360-586)-hFc	HEK 293	Yes	No

Terminal	hENG(438-586)-hFc	HEK 293	Yes	No
Truncations	hENG(458-586)-hFc	COS	No	No
Double	hENG(61-346)-hFc	HEK 293	Yes	No
Truncations	hENG(129-346)-hFc	HEK 293	Yes	No
	hENG(133-346)-hFc	HEK 293	Yes	No
	hENG(166-346)-hFc	HEK 293	Yes	No
	hENG(258-346)-hFc	HEK 293	Yes	No
	hENG(360-581)-hFc	HEK 293	Yes	No
	hENG(360-457)-hFc	COS	No	No
	hENG(360-437)-hFc	COS	No	No
	hENG(458-581)-hFc	COS	No	No

These variants were expressed by transient transfection in HEK 293 cells or COS cells, as indicated.

The selected form of hENG(26-437)-hFc uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 26** (SEQ ID NO: 21), and is encoded by the nucleotide sequence shown in **Figure 27** (SEQ ID NO: 22). The selected form of hENG(26-378)-hFc also uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 28** (SEQ ID NO: 23), and is encoded by the nucleotide sequence shown in **Figure 29** (SEQ ID NO: 24). The selected form of hENG(26-359)-hFc also uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 30** (SEQ ID NO: 25), and is encoded by the nucleotide sequence shown in **Figure 31** (SEQ ID NO: 26). Applicants also envision an alternative hENG(26-359)-hFc sequence with TPA leader (**Figure 32**, SEQ ID NO: 27) comprising an N-terminally truncated hFc domain (**Figure 12**, SEQ ID NO: 12) attached to hENG(26-359) by a TGGG linker. The nucleotide sequence encoding this alternative hENG(26-359)-hFc protein is shown in **Figure 33** (SEQ ID NO: 28). The selected form of hENG(26-346)-hFc uses the TPA leader, has the unprocessed amino acid sequence shown in **Figure 34** (SEQ ID NO: 29) comprising an N-terminally truncated hFc domain, and is encoded by the nucleotide sequence shown in **Figure 35** (SEQ ID NO: 30).

Selected hENG-hFc variants, each with an N-terminally truncated Fc domain (SEQ ID NO: 12), were stably expressed in CHO cells (using methodology described above) and purified from conditioned media by filtration and protein A chromatography. Analysis of mature protein expressed in CHO cells confirmed the N-terminal sequences of hENG(26-359)-hFc and hENG(26-346)-hFc to be as

expected. On the basis of protein yield (uncorrected for differences in theoretical molecular weight), hENG(26-346)-hFc (90 mg/liter) was superior to both hENG(26-359)-hFc (9 mg/liter) and full-length hENG(26-586)-hFc (31 mg/liter). As shown in **Figure 36**, analysis of these purified samples by size-exclusion chromatography revealed the quality of hENG(26-346)-hFc protein (96% monomeric) to be superior to that of hENG(26-359)-hFc protein (84% monomeric) and equivalent to that of hENG(26-586)-hFc protein (96% monomeric). Thus, greater levels of high-molecular-weight aggregates require the use of additional purification steps for hENG(26-359)-hFc compared to hENG(26-346)-hFc.

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### Example 9: High-affinity binding of BMP-9 / BMP-10 to truncated hENG-hFc variants

Applicants used SPR methodology to screen the following hENG-hFc protein variants for high-affinity binding to human BMP-9 and BMP-10. In these experiments, captured hENG-hFc proteins were exposed to soluble BMP-9 or BMP-10 at 100 nM each.

15

	Human Construct	Binding to hBMP-9 and hBMP-10
Full Length Carboxy-Terminal Truncations	hENG(26-586)-hFc	++++
	hENG(26-581)-hFc	++++
	hENG(26-437)-hFc	++++
	hENG(26-378)-hFc	++++
	hENG(26-359)-hFc	++++
	hENG(26-346)-hFc	++++
	hENG(26-332)-hFc	—
	hENG(26-329)-hFc	—
Amino-Terminal Truncations	hENG(26-257)-hFc	—
	hENG(360-586)-hFc	—
	hENG(438-586)-hFc	—
Double Truncations	hENG(458-586)-hFc	—
	hENG(61-346)-hFc	—
	hENG(129-346)-hFc	—
	hENG(133-346)-hFc	—
	hENG(166-346)-hFc	—
	hENG(258-346)-hFc	—
	hENG(360-581)-hFc	—
hENG(360-457)-hFc	—	
hENG(360-437)-hFc	—	
hENG(458-581)-hFc	—	

++++ KD &lt; 1 nM

— Binding undetectable

As indicated in the table above, high-affinity binding to BMP-9 and BMP-10 was observed only for the full-length construct and for C-terminally truncated variants as short as hENG(26-346)-hFc. High-affinity binding to BMP-9 and BMP-10 was lost for all N-terminal truncations of greater than 61 amino acids that were tested.

A panel of ligands were screened for potential binding to the C-terminal truncated variants hENG(26-346)-hFc, hENG(26-359)-hFc, and hENG(26-437)-hFc. High-affinity binding of these three proteins was selective for BMP-9 and BMP-10. Neither hENG(26-346)-hFc, hENG(26-359)-hFc, nor hENG(26-437)-hFc displayed detectable binding to BMP-2, BMP-7, TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, or activin A, even at high ligand concentrations.

Ligand	Construct Binding		
	hENG(26-346)-hFc*	hENG(26-359)-hFc**	hENG(26-437)-hFc**
hBMP-2	—	—	—
hBMP-2/7	—	—	—
hBMP-7	—	—	—
hBMP-9	++++	++++	++++
hBMP-10	++++	++++	++++
hTGF- $\beta$ 1	—	—	—
hTGF- $\beta$ 2	—	—	—
hTGF- $\beta$ 3	—	—	—
hActivin A	—	—	—

\* [hBMP-9], [hBMP-10] = 5 nM; [hTGF- $\beta$ 3] = 50 nM; all other ligands tested at 100 nM

\*\* [hBMP-9], [hBMP-10] = 5 nM; [hTGF- $\beta$ 3] = 50 nM; all other ligands tested at 100 nM

++++ KD < 1 nM

— Binding undetectable

Applicants used SPR methodology to compare the kinetics of BMP-9 binding by five constructs: hENG(26-586)-hFc, hENG(26-437)-hFc, hENG(26-378)-hFc, hENG(26-359)-hFc, and hENG(26-346)-hFc. **Figure 37** shows binding curves for several of the constructs, and the table below lists calculated values for the equilibrium dissociation constants and dissociation rate constants ( $k_d$ ). The affinity of human BMP-9 for hENG(26-359)-hFc or hENG(26-346)-hFc (with  $K_D$ s in the low picomolar range) was nearly an order of magnitude stronger than for the full-length construct. It is highly desirable for ligand traps such as ENG-Fc to exhibit a relatively slow rate of ligand dissociation, so the ten-fold improvement (decrease) in the BMP-9

dissociation rate for hENG(26-346)-hFc compared to the full-length construct is particularly noteworthy.

Ligand	Construct	$K_D$ ( $\times 10^{-12}$ M)	$k_d$ ( $\times 10^{-4}$ s $^{-1}$ )
hBMP-9	hENG(26-586)-hFc *	33	25
	hENG(26-437)-hFc **	19	14
	hENG(26-378)-hFc **	6.7	3.4
	hENG(26-359)-hFc *	4.2	3.5
	hENG(26-346)-hFc *	4.3	2.4

\* CHO-cell-derived protein

\*\* HEK293-cell-derived protein

5

As shown below, each of the truncated variants also bound BMP-10 with higher affinity, and with better kinetics, compared to the full-length construct. Even so, the truncated variants differed in their degree of preference for BMP-9 over BMP-10 (based on  $K_D$  ratio), with hENG(26-346)-hFc displaying the largest differential and hENG(26-437)-hFc the smallest. This difference in degree of ligand preference among the truncated variants could potentially translate into meaningful differences in their activity in vivo.

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Ligand	Construct	$K_D$ ( $\times 10^{-12}$ M)	$k_d$ ( $\times 10^{-4}$ s $^{-1}$ )
hBMP-10	hENG(26-586)-hFc *	490	110
	hENG(26-437)-hFc **	130	28
	hENG(26-378)-hFc **	95	19
	hENG(26-359)-hFc *	86	23
	hENG(26-346)-hFc *	140	28

\* CHO-cell-derived protein

\*\* HEK293-cell-derived protein

15

The foregoing results indicate that fusion proteins comprising certain C-terminally truncated variants of the hENG ECD display high-affinity binding to BMP-9 and BMP-10 but not to a variety of other TGF- $\beta$  family ligands, including TGF- $\beta$ 1 and TGF- $\beta$ 3. In particular, the truncated variants hENG(26-359)-hFc, hENG(26-346)-hFc, and hENG(26-378)-hFc display higher binding affinity at equilibrium and improved kinetic properties for BMP-9 compared to both the full-length construct hENG(26-586)-hFc and the truncated variant hENG(26-437)-hFc.

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### Example 10: Prediction of secondary structure for ENG N-terminal region



As disclosed above, N-terminal truncations as short as 36 amino acids (hENG(61-346)-hFc) were found to abolish ligand binding to ENG polypeptides. To anticipate the effect of even shorter N-terminal truncations on ligand binding, the secondary structure for the human endoglin orphan domain was predicted computationally with a modified Psipred version 3 (Jones, 1999, J Mol Biol 292:195-202). The analysis indicates that ordered secondary structure within the ENG polypeptide region defined by amino acids 26-60 of SEQ ID NO: 1 is limited to a four-residue beta strand predicted with high confidence at positions 42-45 of SEQ ID NO: 1 and a two-residue beta strand predicted with very low confidence at positions 28-29 of SEQ ID NO: 1. Accordingly, ENG polypeptide variants beginning at amino acids 27 or 28 and optionally those beginning at any of amino acids 29-42 of SEQ ID NO: 1 are likely to retain important structural elements and ligand binding.

#### **Example 11: Potency of ENG-Fc variants in a cell-based assay**

A reporter-gene assay in A204 cells was used to determine the potency with which hENG-hFc fusion proteins inhibit signaling by BMP-9 and BMP-10. This assay is based on a human rhabdomyosarcoma cell line transfected with a pGL3 BRE-luciferase reporter plasmid (Korchynskyi et al, 2002, J Biol Chem 277: 4883-4891), as well as a Renilla reporter plasmid (pRLCMV-luciferase) to control for transfection efficiency. BRE motifs are present in BMP-responsive genes (containing a Id1 promoter), so this vector is of general use for factors signaling through Smad1 and/or Smad5. In the absence of ENG-Fc fusion proteins, BMP-9 and BMP-10 dose-dependently stimulate signaling in A204 cells.

On the first day of the assay, A204 cells (ATCC® number: HTB-82™; depositor: DJ Giard) were distributed in 48-well plates at  $10^5$  cells per well. On the next day, a solution containing 12  $\mu$ g pGL3 BRE-luciferase, 0.1  $\mu$ g pRLCMV-luciferase, 30  $\mu$ l Fugene 6 (Roche Diagnostics), and 970  $\mu$ l OptiMEM (Invitrogen) was preincubated for 30 min at room temperature before addition to 24 ml of assay buffer (McCoy's medium supplemented with 0.1% BSA). This mixture was applied to the plated cells (500  $\mu$ l/well) for incubation overnight at 37 °C. On the third day, medium was removed and replaced with test substances (250  $\mu$ l/well) diluted in assay buffer. After an overnight incubation at 37°C, the cells were rinsed and lysed with

passive lysis buffer (Promega E1941) and frozen at -70°C. Prior to assay, the plates were warmed to room temperature with gentle shaking. Cell lysates were transferred in duplicate to a chemoluminescence plate (96-well) and analyzed in a luminometer with reagents from a Dual-Luciferase Reporter Assay system (Promega E1980) to  
 5 determine normalized luciferase activity.

Results indicate that hENG-hFc proteins are potent inhibitors of cellular signaling mediated by BMP-9 and BMP-10. As shown in the table below, the full-length construct hENG(26-586)-hFc inhibits signaling by BMP-9 and BMP-10 with IC<sub>50</sub> values in the sub-nanomolar and low-nanomolar ranges, respectively. Moreover,  
 10 truncated variants hENG(26-359)-hFc and hENG(26-346)-hFc were both more potent than hENG(26-586)-hFc.

Construct	IC <sub>50</sub> (nM)	
	hBMP-9	hBMP-10
hENG(26-586)-hFc	0.26	7.9
hENG(26-359)-hFc	0.16	3.5
hENG(26-346)-hFc	0.19	4.6

### **Example 12: Truncated variant hENG(26-359)-hFc inhibits VEGF-inducible angiogenesis in a CAM assay**

15 Applicants investigated effects of the truncated variant hENG(26-359)-hFc on angiogenesis in the same CAM assay system described in Example 6, in which VEGF is used to induce angiogenesis. The number of additional blood vessels induced by VEGF treatment (50 ng daily) was decreased by 75% with concurrent hENG(26-359)-hFc (SEQ ID NO: 25; 20 µg daily) (**Figure 38**). SPR-based studies confirmed that  
 20 VEGF does not bind hENG(26-359)-hFc, and thus effects of this variant on angiogenesis in the present CAM experiment were not due to a direct interaction between the fusion protein and VEGF. Note that, for hENG(26-359)-hFc, a dose of 10 µg corresponds to the dose of 14 µg used for the longer ENG-Fc constructs tested in Example 6, based on the theoretical molecular weight of each construct. Thus, the  
 25 truncated variant hENG(26-359)-hFc displayed equivalent, if not greater, effectiveness in inhibiting VEGF-inducible angiogenesis compared to ENG constructs with full-length ECD (**Figure 23**) in this same assay system.

**Example 13: Truncated variant hENG(26-346)-hFc inhibits angiogenesis in a mouse angioreactor assay**

Truncated variant hENG(26-346)-hFc was tested in the same mouse angioreactor assay described in Example 7. Angioreactors were implanted subcutaneously in athymic nude mice (four per mouse), and mice were treated daily with hENG(26-346)-hFc (10 mg/kg, s.c.) or vehicle (Tris-buffered saline) for 11 days, at which time the mice were injected with fluorescein isothiocyanate (FITC)-labeled dextran (20 mg/kg, i.v.) and euthanized 20 min later. The quantity of FITC-dextran contained in each angioreactor was then measured as an index of blood vessel formation. As shown in **Figure 39**, addition of the growth factors (GF) FGF-2 and VEGF to the angioreactors led to a significant increase in vascularization, whereas concurrent administration of hENG(26-346)-hFc prevented this increase completely. SPR-based studies confirmed that hENG(26-346)-hFc binds neither FGF-2 nor VEGF, thereby excluding the possibility that effects of hENG(26-346)-hFc on inducible angiogenesis in the present experiment were due to a direct interaction between the fusion protein and either FGF-2 or VEGF. The present results in this mammalian assay system complement those obtained for the truncated variant hENG(26-359)-hFc in a CAM assay (Example 12). Together, they demonstrate anti-angiogenic activity in vivo of ENG-Fc fusion proteins incorporating preferred truncations of the ENG extracellular domain.

**Example 14: Longer in vivo half-life of truncated variant hENG(26-346)-hFc**

Applicants conducted a modified pharmacokinetic study to determine the whole-body elimination half-life of hENG(26-346)-hFc and compared it to that of the full-length protein mENG(27-581)-mFc. hENG(26-346)-hFc protein was fluorescently labeled with Alexa Fluor® 750 dye using a SAIVI™ (small animal in vivo imaging) Rapid Antibody Labeling kit according to instructions of the manufacturer (Invitrogen™). Labeled protein was separated from free label by size exclusion chromatography. Athymic nude mice (n = 3, 17-20 g) were injected with labeled hENG(26-346)-hFc (2 mg/kg, s.c.), and whole-body imaging was performed with an IVIS imaging system (Xenogen®/Caliper Life Sciences) to determine fusion protein levels at 2, 4, 6, 8, 24, 32, 48, and 72 h post injection. The mean elimination

half-life of hENG(26-346)-hFc was 26.5 h, which is 20% longer than the 22 h half-life of mENG(27-581)-mFc determined in a similar study.

### 5 **Example 15: Effect of ENG-Fc proteins on tumor growth in mouse xenograft models**

ENG-Fc proteins were tested in two different mouse xenograft models to determine whether these proteins can inhibit tumor growth. In the first experiment, athymic nude mice were injected subcutaneously at 6 weeks of age with  $10^6$  4T1 mammary carcinoma cells (ATCC® number: CRL-2539™; depositor: BA Pulaski).  
10 Mice (n = 10 per group) were dosed daily (s.c.) with mENG(27-581)-mFc (10 mg/kg) or vehicle (Tris-buffered saline). Tumors were measured manually with digital calipers, and tumor volume was calculated according to the formula: volume =  $0.5(\text{length})(\text{width}^2)$ . As shown in **Figure 40**, treatment with mENG(27-581)-mFc reduced tumor volume by 45% compared to vehicle by day 24 post implantation.

15 ENG-Fc fusion proteins were also tested in a Colon-26 carcinoma xenograft model. BALB/c mice were injected subcutaneously at 7 weeks of age with  $1.5 \times 10^6$  Colon-26 carcinoma cells (ATCC® number: CRL-2638™; depositor: N Restifo). Mice (n = 10 per group) were dosed daily (s.c.) with mENG(27-581)-mFc (at 1, 10, or 30 mg/kg) or vehicle (Tris-buffered saline). Tumor volume was determined as  
20 described above. As shown in **Figure 41**, mENG(27-581)-mFc treatment caused a dose-dependent reduction in tumor volume, with decreases of 55% and nearly 70% compared to vehicle at doses of 10 mg/kg and 30 mg/kg, respectively, by day 58 post implantation. Thus, mENG(27-581)-mFc markedly slowed the growth of two  
25 different tumor types in mouse xenograft models, consistent with the aforementioned antiangiogenic activity of fusion proteins incorporating the full-length murine ENG extracellular domain (Examples 5-7). In a preliminary experiment, the truncated variant hENG(26-346) also slowed tumor growth compared to vehicle in the Colon-26 xenograft model, consistent with the antiangiogenic activity of this variant in the mouse angioreactor assay (Example 13).

30 Taken together, the aforementioned results demonstrate that fusion proteins comprising the full-length ENG ECD, and certain truncated variants thereof, display high-affinity binding to BMP-9 and BMP-10 but not a variety of other TGFβ-family

ligands, including TGF $\beta$ -1 and TGF $\beta$ -3. These ENG polypeptides can inhibit angiogenesis and tumor growth in model systems and thus have the potential to treat patients with unwanted angiogenesis, including those with cancer. Compared to constructs comprising the full-length ENG ECD, the truncated ENG polypeptides hENG(26-346)-hFc and/or hENG(26-359)-hFc displayed higher potency and improved performance on several other key parameters (see summary table below).

Parameter		ECD Polypeptide in Fusion Protein (CHO cell derived)		
		Full length ECD – Human 26-586 or Murine 27-581	Human 26-359	Human 26-346
Expression	Quantity	31 mg/L	9 mg/L	90 mg/L
	Quality	96% monomeric	84% monomeric	96% monomeric
Binding affinity (K <sub>D</sub> )	BMP-9	33 pM	4.2 pM	4.3 pM
	BMP-10	490 pM	86 pM	140 pM
Dissociation rate (k <sub>d</sub> )	BMP-9	25 x 10 <sup>-4</sup> s <sup>-1</sup>	3.5 x 10 <sup>-4</sup> s <sup>-1</sup>	2.4 x 10 <sup>-4</sup> s <sup>-1</sup>
	BMP-10	110 x 10 <sup>-4</sup> s <sup>-1</sup>	23 x 10 <sup>-4</sup> s <sup>-1</sup>	28 x 10 <sup>-4</sup> s <sup>-1</sup>
Potency (cell-based IC <sub>50</sub> )	BMP-9	0.26 nM	0.16 nM	0.19 nM
	BMP-10	7.9 nM	3.5 nM	4.6 nM
Elimination half-life		22 h	---	26.5 h
Anti-angiogenesis activity	HUVEC	Yes	---	---
	CAM	65% inhibition	75% inhibition	---
	Angioreactor	100% inhibition	---	100% inhibition
Anti-tumor activity	4T1 tumor	Yes	---	---
	Colon-26 tumor	Yes	---	Yes
		Dose-dependent		

--- Not investigated

Variant hENG(26-346)-hFc, in particular, possessed a superior combination of attributes, with higher potency, stronger binding affinity, slower dissociation rate, longer elimination half-life, and better protein production than full-length ENG ECD constructs. As ligand traps, truncated ENG polypeptides should preferably exhibit a slow rate of ligand dissociation, so the ten-fold reduction in the BMP-9 dissociation rate for hENG(26-346)-hFc compared to the full-length construct is highly desirable. The variant hENG(26-378)-hFc displayed BMP-9 binding properties (affinity and dissociation rate) intermediate between hENG(26-346)-hFc and hENG(26-359)-hFc, on one hand, and hENG(26-437)-hFc, on the other, with hENG(26-378) more closely resembling the shorter constructs.

**Example 16: Treatment of a mouse model of liver fibrosis with ENG-Fc proteins**

The effectiveness of ENG-Fc proteins in the treatment of fibrosis was evaluated in the mouse CCL4 (carbon tetrachloride) model of liver fibrosis. Fifty mice were used in this study. Male and female A/J mice of approximately 14 weeks of age at the start (day 0) of the experiment were acclimated in the laboratory for at least 48 hours. Animals were monitored daily during the course of the experiment and were sacrificed if any signs of morbidity, mortality and Test Article Toxicity were observed.

Animals received a dose of 1ml/kg of 50% CCl<sub>4</sub> in olive oil via oral gavage twice a week to induce liver fibrosis. Animals were dosed for 13 weeks with mENG(27-581)-mFc as described in the table below.

Group	N	Liver Fibrosis	Treatment	Dose	Frequency	Admin.
1	20	CCl <sub>4</sub> +Olive Oil	PBS	Isovolume	B.I.W.	I.P.
2	20	CCl <sub>4</sub> +Olive Oil	mu-Endoglin	10 mg/kg	T.I.W.	I.P.
3	5	Olive Oil	PBS	Isovolume	B.I.W.	I.P.
4	5	Olive Oil	mu-Endoglin	10 mg/kg	T.I.W.	I.P.

Animals were analyzed for changes in body weight (BW), liver weight, liver performance, and histology. On day 0, day 28, day 56, and day 90, animals were NMR scanned. Animals were euthanized on Day 45 or 90 using CO<sub>2</sub>. For serum analysis, animals were fasted 12 hrs prior to sacrifice and serum sampling. Whole blood was collected for liver function analysis, and the liver from each animal was collected and weighed. Half of the liver was put in a cartridge in 10% Formalin, and a lobe of the liver was flash frozen in liquid nitrogen.

Treatment with mENG(27-581)-mFc did not affect liver weight (measured as a percentage of body weight) over a period of 13 weeks (Figure 42). After the 13-week dosing period, animals were sacrificed and liver sections were stained with H&E and Masson's Trichrome staining (Figures 43-45). Treatment animals exhibited markedly reduced fibrosis relative to untreated animals (Figure 45). Additionally, staining with Oil Red O revealed that mENG(27-581)-mFc treatment resulted in decreased accumulation of fatty deposits in liver tissue, which are often a precursor of liver damage and fibrotic deposition (Figure 46). Additionally, mENG(27-581)-mFc treatment appeared to reduce ballooning degeneration of hepatocytes, which is associated with apoptosis and is seen in connection with inflammation of the liver.

Serum alkaline phosphatase levels were lower in the endoglin-treated cohorts as compared to the untreated ones (Figure 47). Collectively, these data indicate that mENG(27-581)-mFc treatment can decrease liver damage in this mouse model of liver fibrosis, and thus ENG-Fc proteins are likely to be useful in the treatment of fibrotic disorders of the liver, including cirrhosis and the eventual hepatocellular carcinomas.

### **Example 17: Effect of ENG-Fc protein in a mouse dietary model of liver fibrosis**

Effectiveness of ENG-Fc proteins was also evaluated in a mouse model of nonalcoholic steatohepatitis (NASH) caused by methionine and choline dietary deficiency (MCDD). Wild-type C57BL/6 mice were fed either a standard chow diet or a diet containing high sucrose (40%) and fat (10%) but lacking methionine and choline, which are essential for hepatic  $\beta$ -oxidation and production of very low density lipoprotein (Takahashi et al., 2012, World J Gastroenterol 18:2300-2308). As a result, MCDD mice exhibit fatty deposits considered to be a precursor of liver damage and fibrotic deposition (Corbin et al., 2012, Curr Opin Gastroenterol 28:159-165). At 12 weeks of age, mice were placed on their respective diets and began intraperitoneal treatment with either mENG(27-581)-mFc (10 mg/kg) or vehicle (n = 10 per group) twice weekly for 3 weeks. At the conclusion of dosing, mice were killed and liver sections were stained with Oil Red O, a lipid-soluble diazo dye, to assess the extent of lipid deposition.

As expected, mice fed the chow diet exhibited only tiny lipid deposits in liver tissue (data not shown), whereas MCDD mice exhibited many large lipid deposits that collectively occupied a considerable fraction of total tissue area (Figure 48A,C). In MCDD mice, mENG(27-581)-mFc treatment markedly reduced hepatic lipid deposits compared with vehicle (Figure 48). Although endogenous TGF $\beta$  is heavily implicated in progression of liver disease (Dooley et al., 2012, Cell Tissue Res 347:245-256), an Fc fusion protein which comprises TGF $\beta$  receptor type II and binds TGF $\beta$  with high affinity had little effect on the accumulation of hepatic lipid deposits (data not shown). As disclosed in Example 3, mENG(27-581)-mFc and other ENG-Fc proteins bind neither TGF $\beta$ 1, TGF $\beta$ 2, nor TGF $\beta$ 3, so the bioactivity of mENG(27-581)-mFc in MCDD mice is not due to inhibition of signaling by these ligands.

Together, these results indicate that mENG(27-581)-mFc can markedly reduce deposition of lipids in a mouse model in which dietary deficiency leads eventually to fibrosis and nonalcoholic steatohepatitis, thereby providing additional evidence that ENG-Fc proteins are likely to be useful in the treatment of liver fibrosis.

5

#### INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

10

#### EQUIVALENTS

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

15



## CLAIMS

We claim:

1. A method of treating or preventing a fibrotic disorder in a patient in need  
5 thereof, the method comprising administering to the patient an effective amount of an  
endoglin polypeptide comprising an amino acid sequence at least 95% identical to  
amino acids 42-333 of SEQ ID NO: 1.
2. The method of claim 1, wherein the fibrotic disorder is liver fibrosis.  
10
3. The method of claim 2, wherein the liver fibrosis is liver cirrhosis, alcohol-  
induced liver fibrosis, biliary duct injury, primary biliary cirrhosis, infection-induced  
liver fibrosis, congenital hepatic fibrosis or autoimmune hepatitis.
- 15 4. The method of claim 4, wherein the infection-induced liver fibrosis is  
bacterial-induced or viral-induced.
5. The method of any one of claims 1-4, wherein the endoglin polypeptide does  
not include a sequence consisting of amino acids 379-430 of SEQ ID NO: 1.  
20
6. The method of any one of claims 1-5, wherein the endoglin polypeptide  
comprises an amino acid sequence at least 95% identical to a sequence beginning at  
an amino acid corresponding to any of positions 26-42 of SEQ ID NO: 1 and ending  
at an amino acid corresponding to any of positions 333-378 of SEQ ID NO: 1.  
25
7. The method of any one of claims 1-6, wherein the endoglin polypeptide  
comprises an amino acid sequence at least 95% identical to a sequence selected from  
a group consisting of:
  - a. amino acids 26-346 of SEQ ID NO: 1,
  - 30 b. amino acids 26-359 of SEQ ID NO: 1, and
  - c. amino acids 26-378 of SEQ ID NO: 1.

8. The method of any one of claims 1-7, wherein the endoglin polypeptide consists of a first portion consisting of an amino acid sequence at least 95% identical to a sequence selected from a group consisting of:
- a. amino acids 26-346 of SEQ ID NO: 1,
  - 5 b. amino acids 26-359 of SEQ ID NO: 1, and
  - c. amino acids 26-378 of SEQ ID NO: 1
- and a second portion that is heterologous to SEQ ID NO: 1.
9. The method of claim 8, wherein the second portion of the endoglin polypeptide  
10 comprises an Fc portion of an IgG.
10. The method of any of claims 1-9, wherein the endoglin polypeptide is a dimer.
11. The method of any of claims 1-10, wherein the endoglin polypeptide is a  
15 homodimer.
12. The method of any one of claims 1-11, wherein the endoglin polypeptide does not include more than 50 consecutive amino acids from a sequence consisting of amino acids 379-586 of SEQ ID NO: 1.  
20
13. The method of any one of claims 1-12, wherein the endoglin polypeptide binds human BMP-9 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $1 \times 10^{-3} \text{ s}^{-1}$ .
- 25 14. The method of any one of claims 1-13, wherein the endoglin polypeptide binds human BMP-9 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $5 \times 10^{-4} \text{ s}^{-1}$ .
- 30 15. The method of any one of claims 1-14, wherein the endoglin polypeptide binds human BMP-10 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $5 \times 10^{-3} \text{ s}^{-1}$ .

16. The method of any one of claims 1-15, wherein the endoglin polypeptide binds human BMP-10 with an equilibrium dissociation constant (KD) less than  $1 \times 10^{-9}$  M or a dissociation rate constant (kd) less than  $2.5 \times 10^{-3} \text{ s}^{-1}$ .
- 5 17. The method of any one of claims 1-16, wherein the endoglin polypeptide does not bind human TGF- $\beta$ 1, human TGF- $\beta$ 3, human VEGF, or human basic fibroblast growth factor (FGF-2).
18. The method of any one of claims 1-17, wherein the endoglin polypeptide is a  
10 fusion protein including, in addition to a portion comprising an endoglin amino acid sequence, one or more polypeptide portions that enhance one or more of: in vivo stability, in vivo half-life, uptake/administration, tissue localization or distribution, formation of protein complexes, and/or purification.
- 15 19. The method of any one of claims 1-18, wherein the endoglin polypeptide includes a portion selected from the group consisting of: a constant domain of an immunoglobulin and a serum albumin.
20. The method of any one of claims 1-19, wherein the endoglin polypeptide  
20 comprises an immunoglobulin Fc domain.
21. The method of claim 20, wherein the immunoglobulin Fc domain is joined to the ENG polypeptide portion by a linker.
- 25 22. The method of claim 21, wherein the linker consists of an amino acid sequence consisting of SEQ ID NO: 31 (TGGG) or GGG.
23. The method of any one of claims 1-22, wherein the endoglin polypeptide  
30 includes one or more modified amino acid residues selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, and an amino acid conjugated to an organic derivatizing agent.

24. The method of any one of claims 1-23, wherein the endoglin polypeptide is administered intravenously, intramuscularly, intraarterially, subcutaneously, or orally.

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**Amino acid sequence of human ENG, isoform 1 (L-ENG)**

(GenBank NM\_001114753)

1 MDRGTLPLAV ALLLASCCLS PTSLAETVHC DLQVGPPERG EVTYTTSQVS KGCVAQAPNA  
61 ILEVHVLFLFLE FPTGPSQLEL TLQASKQNGT WPREVLLVLS VNSSVFLHLQ ALGIPLHLAY  
121 NSSLVTFQEP PGVNTTELPS FPKTQILEWA AERGPITSAA ELNDPQSILL RLGQAQGSLS  
181 FCMLEASQDM GRTLEWRPRT PALVRGCHLE GVAGHKEAHI LRVLPGHSAG PRTVTVKVEL  
241 SCAPGDLDV LILQGPPYVS WLIDANHNMQ IWTGGEYSFK IFPEKNIRGF KLPDTPQGLL  
301 GEARMLNASI VASFVEFLA SIVSLHASSC GGRLQTSPAP IQTTPFKDTC SPELLMSLIQ  
361 TKCADDAMTL VLKKELVAHL KCTITGLTFW DPSCEAEDRG DKFVLRSAVS SCGMQVSASM  
421 ISNEAVVNIL SSSSPQRKKV HCLNMDLSLF QGLYLSPHF LQASNTIEPG QQSFVQVRVS  
481 PSVSEFLLQL DSCHLDLGPE GGTVELIQGR AAKGNCVSLI SPSPEGDPFR SFLHFYTVP  
541 IPKTGTLSCV VALRPKTGSQ DQEVHRTVFM RLNIISPDLI GCTSKGLVLP AVLGITFGAF  
601 LIGALLTAAL WYIYSHTRSP SKREPVVAVA APASSESSST NHSIGSTQST PCSTSSMA  
(SEQ ID NO: 1)

Fig. 1

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**Nucleotide sequence encoding human ENG, isoform 1 (L-ENG)**  
 (GenBank NM\_001114753)

361 CCTGCCACTG GACACAGGAT AAGGCCCAGC GCACAGGCC CCACGTGGAC AGCATGGACC  
 421 GCGGCACGCT CCCTCTGGCT GTTGCCCTGC TGCTGGCCAG CTGCAGCCTC AGCCCCACAA  
 481 GTCTTGCAGA AACAGTCCAT TGTGACCTTC AGCCTGTGGG CCCCAGAGAG GCGGAGGTGA  
 541 CATATACCAC TAGCCAGGTC TCGAAGGGCT GCGTGGCTCA GGCCCCAAT GCCATCCTTG  
 601 AAGTCCATGT CCTCTTCCTG GAGTTCCCAA CGGGCCCGTC ACAGCTGGAG CTGACTCTCC  
 661 AGGCATCCAA GCAAAATGGC ACCTGGCCCC GAGAGGTGCT TCTGGTCCTC AGTGTAACA  
 721 GCAGTGTCTT CCTGCATCTC CAGGCCCTGG GAATCCCCT GCACTTGGCC TACAATTCCA  
 781 GCCTGGTCAC CTCCAAGAG CCCCCGGGG TCAACACCAC AGAGCTGCCA TCCTTCCCCA  
 841 AGACCCAGAT CCTTGAGTGG GCAGCTGAGA GGGGCCCCAT CACCTCTGCT GCTGAGCTGA  
 901 ATGACCCCCA GAGCATCCTC CTCCGACTGG GCCAAGCCCA GGGGTCCTG TCCTTCTGCA  
 961 TGCTGGAAGC CAGCCAGGAC ATGGGCCGCA CGCTCGAGTG GCGGCCGGT ACTCCAGCCT  
 1021 TGGTCCGGGG CTGCCACTTG GAAGGCGTGG CCGGCCACAA GGAGGCGCAC ATCCTGAGGG  
 1081 TCCTGCCGGG CCACTCGGCC GGGCCCCGGA CGGTGACGGT GAAGGTGGAA CTGAGCTGG  
 1141 CACCCGGGGA TCTCGATGCC GTCCCTCATCC TCCAGGGTCC CCCCTACGTG TCCTGGCTCA  
 1201 TCGACGCCAA CCACAACATG CAGATCTGGA CCACTGGAGA ATACTCCTTC AAGATCTTTC  
 1261 CAGAGAAAAA CATTCGTGGC TTCAAGCTCC CAGACACACC TCAAGGCCTC CTGGGGGAGG  
 1321 CCCGGATGCT CAATGCCAGC ATTGTGGCAT CCTTCGTGGA GCTACCGCTG GCCAGCATTC  
 1381 TCTCACTTCA TGCCCTCAGC TCGGGTGGTA GGCTGCAGAC CTCACCCGCA CCGATCCAGA  
 1441 CCACTCCTCC CAAGGACACT TGTAGCCCGG AGCTGCTCAT GTCCTTGATC CAGACAAAGT  
 1501 GTGCCGACGA CGCCATGACC CTGGTACTAA AGAAAGAGCT TGTGCGCAT TTGAAGTGCA  
 1561 CCATCACGGG CCTGACCTTC TGGGACCCCA GCTGTGAGGC AGAGGACAGG GGTGACAAGT  
 1621 TTGTCTTGCG CAGTGCTTAC TCCAGCTGTG GCATGCAGGT GTCAGCAAGT ATGATCAGCA  
 1681 ATGAGGCGGT GGTCAATATC CTGTCGAGCT CATCACCACA GCGGAAAAG GTGCACTGCC  
 1741 TCAACATGGA CAGCCTCTCT TTCCAGCTGG GCCTCTACCT CAGCCCACAC TTCTCCAGG  
 1801 CCTCCAACAC CATCGAGCCG GGGCAGCAGA GCTTTGTGCA GGTCAGAGTG TCCCCATCCG  
 1861 TCTCCGAGTT CCTGCTCCAG TTAGACAGCT GCCACCTGGA CTTGGGGCCT GAGGGAGGCA  
 1921 CCGTGGAACT CATCCAGGGC CGGGCGGCCA AGGGCAACTG TGTGAGCCTG CTGTCCCCAA  
 1981 GCCCCGAGGG TGACCCCGGC TTCAGCTTCC TCCTCCACTT CTACACAGTA CCCATACCCA  
 2041 AAACCGGCAC CCTCAGCTGC ACGGTAGCCC TCGGTCCCAA GACCGGGTCT CAAGACCAGG  
 2101 AAGTCCATAG GACTGTCTTC ATGCGCTTGA ACATCATCAG CCCTGACCTG TCTGGTTGCA  
 2161 CAAGCAAAGG CCTCGTCCTG CCCGCCGTGC TGGGCATCAC CTTTGGTGCC TTCCTCATCG  
 2221 GGGCCCTGCT CACTGCTGCA CTCTGGTACA TCTACTCGCA CACGCGTTCC CCCAGCAAGC  
 2281 GGGAGCCCGT GGTGGCGGTG GCTGCCCGG CCTCCTCGGA GAGCAGCAGC ACCAACCACA  
 2341 GCATCGGGAG CACCCAGAGC ACCCCCTGCT CCACCAGCAG CATGGCATAG

(SEQ ID NO: 2)

Fig. 2

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**Amino acid sequence of human ENG, isoform 2 (S-ENG)**

(GenBank NM\_000118)

1 MDRGTLPLAV ALLLASCCLS PTSLAETVHC DLQVGPPERG EVTYTTSQVS KGCVAQAPNA  
61 ILEVHVLFLFLE FPTGPSOLEL TLQASKQNGT WPREVLLVLS VNSSVFLHLQ ALGIPLHLAY  
121 NSSLVTFQEP PGNVTEELPS FPKTQILEWA AERGPITSAA ELNDPQSILL RLGQAQGSLS  
181 FCMLEASQDM GRTLEWRPRT PALVRGCHLE GVAGHKEAHI LRVLPGHSAG PRTVTVKVEL  
241 SCAPGDLDV LILQGPYVS WLIDANHNMQ IWTTGEYSFK IFPEKNIRGF KLPDTPQGLL  
301 GEARMLNASI VASFVELFLA SIVSLHASSC GGRLQTSPAP IQTTPFKDTC SPELLMSLIQ  
361 TKCADDAMTL VLKKELVAHL KCTITGLTFW DPSCEAEDRG DKFVLRSAVS SCGMQVSASM  
421 ISNEAVVNIL SSSSPQRKKV HCLNMDLSLF QGLYLSPHF LQASNTIEPG QQSFVQVRVS  
481 PSVSEFLLQL DSCHLDLGPE GGTVELIQGR AAKGNCVSLI SPSPEGDPRE SFLHFYTVP  
541 IPKTGTLSCV VALRPKTGSQ DQEVHRTVFM RLNIISPDLS GCTSKGLVLP AVLGITFGAF  
601 LIGALLTAAL WYIYSH TREY PRPPQ  
(SEQ ID NO: 3)

Fig. 3

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**Nucleotide sequence encoding human ENG, isoform 2 (S-ENG)**  
(GenBank NM\_000118)

```

361 CCTGCCACTG GACACAGGAT AAGGCCCAGC GCACAGGCC CCACGTGGAC AGCATGGACC
421 GCGGCACGCT CCCTCTGGCT GTTGCCCTGC TGCTGGCCAG CTGCAGCCTC AGCCCCACAA
481 GTCTTGCAGA AACAGTCCAT TGTGACCTTC AGCCTGTGGG CCCCAGAGAG GCGGAGGTGA
541 CATATACCAC TAGCCAGGTC TCGAAGGGCT GCGTGGCTCA GGCCCCAAT GCCATCCTTG
601 AAGTCCATGT CCTCTTCCTG GAGTTCCCAA CGGGCCCGTC ACAGCTGGAG CTGACTCTCC
661 AGGCATCCAA GCAAAATGGC ACCTGGCCCC GAGAGGTGCT TCTGGTCCTC AGTGTAACA
721 GCAGTGTCTT CCTGCATCTC CAGGCCCTGG GAATCCCCT GCACTTGGCC TACAATTCCA
781 GCCTGGTCAC CTCCAAGAG CCCCCGGGG TCAACACCAC AGAGCTGCCA TCCTTCCCCA
841 AGACCCAGAT CCTTGAGTGG GCAGCTGAGA GGGGCCCAT CACCTCTGCT GCTGAGCTGA
901 ATGACCCCCA GAGCATCCTC CTCCGACTGG GCCAAGCCCA GGGGTCCTG TCCTTCTGCA
961 TGCTGGAAGC CAGCCAGGAC ATGGGCCGCA CGCTCGAGTG GCGGCCGGT ACTCCAGCCT
1021 TGGTCCGGGG CTGCCACTTG GAAGGCGTGG CCGGCCACAA GGAGGCGCAC ATCCTGAGGG
1081 TCCTGCCGGG CCACTCGGCC GGGCCCCGGA CGGTGACGGT GAAGGTGGAA CTGAGCTGG
1141 CACCCGGGGA TCTCGATGCC GTCTCATCC TCAGGGTCC CCCCTAGGTG TCCTGGCTCA
1201 TCGACGCCAA CCACAACATG CAGATCTGGA CCACTGGAGA ATACTCCTTC AAGATCTTTC
1261 CAGAGAAAAA CATTCGTGGC TTCAAGCTCC CAGACACACC TCAAGGCCTC CTGGGGGAGG
1321 CCCGGATGCT CAATGCCAGC ATTGTGGCAT CCTTCGTGGA GCTACCGCTG GCCAGCATTC
1381 TCTCACTTCA TGCTCCAGC TCGGGTGGTA GGCTGCAGAC CTCACCCGCA CCGATCCAGA
1441 CCACTCCTCC CAAGGACACT TGTAGCCCGG AGCTGCTCAT GTCCTTGATC CAGACAAAGT
1501 GTGCCGACGA CGCCATGACC CTGGTACTAA AGAAAGAGCT TGTTCGCAT TTGAAGTGCA
1561 CCATCACGGG CCTGACCTTC TGGGACCCA GCTGTGAGGC AGAGGACAGG GGTGACAAGT
1621 TTGTCTTGGC CAGTGCTTAC TCCAGCTGTG GCATGCAGGT GTCAGCAAGT ATGATCAGCA
1681 ATGAGGCGGT GGTCAATATC CTGTCGAGCT CATCACCACA GCGGAAAAG GTGCACTGCC
1741 TCAACATGGA CAGCCTCTCT TTCCAGCTGG GCCTCTACCT CAGCCCACAC TTCTCCAGG
1801 CCTCCAACAC CATCGAGCCG GGGCAGCAGA GCTTTGTGCA GGTCAGAGTG TCCCCATCCG
1861 TCTCCGAGTT CCTGCTCCAG TTAGACAGCT GCCACCTGGA CTTGGGGCCT GAGGGAGGCA
1921 CCGTGGAACT CATCCAGGGC CGGGCGGCCA AGGGCAACTG TGTGAGCCTG CTGTCCCCAA
1981 GCCCCGAGGG TGACCCCGGC TTCAGCTTCC TCCTCCACTT CTACACAGTA CCCATACCCA
2041 AAACCGGCAC CCTCAGCTGC ACGGTAGCCC TCGTCCCAA GACCGGGTCT CAAGACCAGG
2101 AAGTCCATAG GACTGTCTTC ATGCGCTTGA ACATCATCAG CCCTGACCTG TCTGGTTGCA
2161 CAAGCAAAGG CCTCGTCCTG CCCGCCGTGC TGGGCATCAC CTTTGGTGCC TTCCTCATCG
2221 GGGCCCTGCT CACTGCTGCA CTCTGGTACA TCTACTCGCA CACGCGTGAG TACCCAGGC
2281 CCCCACAGTG A
(SAQ ID NO: 4)

```

Fig. 4

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**Amino acid sequence of murine ENG, isoform 1 (L-ENG)**

(GenBank NM\_007932)

1 MDRGVLPPLPI TLLFVIYSFV PTTGLAERVG CDLQPVDPTR GEVTFFTTSQV SEGCVAQAAN  
 61 AVREVHVLFL DFPGMLSHLE LTLQASKQNG TETQEVFLVL VSNKNVFKF QAPEIPLHLA  
 121 YDSSLVIFQG QPRVNITVLP SLTSRKQILD WAATKGAIIS IAALDDPQSI VLQLGQDPKA  
 181 PFLCLPEAHK DMGATLEWQP RAQTPVQSCR LEGVSGHKEA YILRILPGSE AGPRTVTVM  
 241 ELSCTSGDAI LILHGPPYVS WFIDINHSMQ ILTTGEYSVK IFPGSKVKGV ELPDTPQGLI  
 301 AEARKLNASI VTSFVELEFLV SNVSLRASSC GGVFQTPAP VVTTPFRDTC SPVLLMSLIQ  
 361 PKCGNQVMTL ALNKKHVQTL QCTITGLTFW DSSCOAEDTD DHLVLSSAYS SCGMKVTAHV  
 421 VSNEVIISFP SGSPPLRKKV QCIDMDSLFS QGLYLSPHF LQASNTIELG QQAFVQVSVS  
 481 PLTSEVTVQL DSCHLDLGPE GDMVELIQSR TAKGSCVTLL SPSPEGDPFR SFLLRVYMVP  
 541 TPTAGTLSCN LALRPSTLSQ EVYKTVSMRL NIVSPDLSGK GLVLPSVLGI TFGAFLIGAL  
 601 LTAALWYIYS HTRGFSKREP VVAVAAPASS ESSSTNHSIG STQSTPCSTS SMA  
 (SEQ ID NO: 5)

Fig. 5

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**Nucleotide sequence encoding murine ENG, isoform 1 (L-ENG)**  
(GenBank NM\_007932)

```

361 AGCATGGACC GTGGCGTGCT CCCTCTGCCC ATTACCCTGC TGTTCGTTCAT CTATAGCTTT
421 GTACCCACA CAGGTCTCGC AGAAAGAGTC GGCTGTGATC TACAGCCTGT GGACCCACACA
481 AGGGGTGAGG TGACGTTTAC CACCAGCCAG GTCTCCGAGG GCTGTGTAGC TCAGGCTGCC
541 AATGCTGTGC GTGAAGTCCA CGTTCTCTTC CTGGATTTTC CCGGAATGCT GTCACATCTG
601 GAGCTGACTC TTCAGGCATC CAAGCAAAAT GGCACGGAGA CCCAGGAGGT GTTCCTGGTC
661 CTCGTTTCGA AAAAAATGT CTTCGTGAAG TTCCAGGCCC CGGAAATCCC ATTGCACTTG
721 GCCTACGACT CCAGCCTGGT CATCTTCCAA GGACAGCCAA GAGTCAACAT CACAGTGCTA
781 CCATCCCTTA CCTCCAGGAA ACAGATCCTC GACTGGGCAG CCACCAAGGG CGCCATCACC
841 TCGATAGCAG CACTGGATGA CCCCCAAAGC ATCGTCCTCC AGTTGGGCCA AGACCCAAAG
901 GCACCATTCT TGTGCTTGCC AGAAGCTCAC AAGGACATGG GCGCCACACT TGAATGGCAA
961 CCACGAGCCC AGACCCAGT CCAAAGCTGT CGCTTGAAG GTGTGTCTGG CCACAAGGAG
1021 GCCTACATCC TGAGGATCCT GCCAGGTTCT GAGGCCGGGC CCCGGACGGT GACCGTAATG
1081 ATGGAACTGA GTTGCACATC TGGGGACGCC ATTCTCATCC TGCATGGTCC TCCATATGTC
1141 TCCTGGTTCA TCGACATCAA CCACAGCATG CAGATCTTGA CCACAGGTGA ATACTCCGTC
1201 AAGATCTTTC CAGGAAGCAA GGTCAAAGGC GTGGAGCTCC CAGACACACC CCAAGGCCTG
1261 ATAGCGGAGG CCCGCAAGCT CAATGCCAGC ATTGTCACCT CCTTTGTAGA GCTCCCTCTG
1321 GTCAGCAATG TCTCCCTGAG GGCTCCAGC TGCGGTGGTG TGTTCAGAC CACCCCTGCA
1381 CCCGTTGTGA CCACACCTCC CAAGGACACA TGCAGCCCCG TGCTACTCAT GTCCCTGATC
1441 CAGCCAAAGT GTGGCAATCA GGTCAATGACT CTGGCACTCA ATAAAAACA CGTGCAGACT
1501 CTCAGTGCA CCATCACAGG CCTGACTTTC TEGGACTCCA GCTGCCAGGC TGAAGACACT
1561 GACGACCATC TTGTCCTGAG TAGCGCCTAC TCCAGCTGCG GCATGAAAGT GACAGCCCAT
1621 GTGGTCAGCA ATGAGGTGAT CATCAGTTTC CCGTCAGGCT CACCACCACT TCGGAAAAG
1681 GTACAGTGCA TCGACATGGA CAGCCTCTCC TTCAGCTGG GCCTCTACCT CAGCCCGCAC
1741 TTCCTCCAGG CATCCAACAC CATCGAACTA GGCCAGCAGG CCTTCGTACA GGTGAGCGTG
1801 TCTCCATTGA CCTCTGAGGT CACAGTCCAG CTAGATAGCT GCCATCTGGA CTTGGGGCCC
1861 GAAGGGGACA TGGTGGAACT CATCCAGAGC CGAACAGCCA AGGGCAGCTG TGTGACCTTG
1921 CTGTCTCCAA GCCCTGAAGG TGACCCACGC TTCAGCTTCC TCCTCCGGGT CTACATGGTG
1981 CCCACACCCA CCGCTGGCAC CCTCAGTTC AACTTAGCTC TCGCCCTAG CACCTTGTCC
2041 CAGGAAGTCT ACAAGACAGT CTCCATGCGC CTGAACATCG TCAGCCCTGA CCTGTCTGGT
2101 AAAGGCCTTG TCCTGCCCTC TGTACTGGGT ATCACCTTTG GTGCCTTCCT GATTGGGGCC
2161 CTGCTCACAG CTGCACTCTG GTACATCTAT TCTCACACAC GTGGCCCCAG CAAGCGGGAG
2221 CCCGTGGTGG CAGTGGCTGC CCCGGCCTCC TCTGAGAGCA GCAGTACCAA CCACAGCATC
2281 GGGAGCACCC AGAGCACCCC CTGCTCCACC AGCAGCATGG CGTAG

```

(SEQ ID NO: 6)

Fig. 6

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**Amino acid sequence of murine ENG, isoform 2 (S-ENG)**

(GenBank NM\_001146350)

1 MDRGVLPPLPI TLLFVIYSFV PTTGLAERVG CDLQPVDPTR GEVTFTTSQV SEGCVAQAAN  
61 AVREHVHVLFL DFPGMLSHLE LTLQASKQNG TETQEVFLVL VSNKNVFKF QAPEIPLHLA  
121 YDSSLVIFQG QPRVNITVLP SLTSRKQILD WAATKGAIIS IAALDDPQSI VLQLGQDPKA  
181 PFLCLPEAHK DMGATLEWQP RAQTPVQSCR LEGVSGHKEA YILRILPGSE AGPRTVTVM  
241 ELSCTSGDAI LILHGPPYVS WFIDINHSMQ ILTTGEYSVK IFPGSKVKGV ELPDTPQGLI  
301 AEARKLNASI VTSFVELEFLV SNVSLRASSC GGVFQTPAP VVTPPKDTC SPVLLMSLIQ  
361 PKCGNQVMTL ALNKKHVQTL QCTITGLTFW DSSCOAEDTD DHLVLSSAYS SCGMKVTAHV  
421 VSNEVIISFP SGSPPLRKKV QCIDMDSLFS QGLYLSPHF LQASNTIELG QQAFVQVSVS  
481 PLTSEVTVQL DSCHLDLGPE GDMVELIQSR TAKGSCVTLL SPSPEGDPRE SFLLRVYMVP  
541 TPTAGTLSCN LALRPSTLSQ EVYKTVSMRL NIVSPDLSGK GLVLPSVLGI TFGAFLIGAL  
601 LTAALWYIYS HTREYPKPPP HSHSKRSGPV HTTPGHTQWS L  
(SEQ ID NO: 7)

Fig. 7

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**Nucleotide sequence encoding murine ENG, isoform 2 (S-ENG)**  
(GenBank NM\_001146350)

```

361 AGCATGGACC GTGGCGTGCT CCCTCTGCCC ATTACCCTGC TGTTTGTCAT CTATAGCTTT
421 GTACCCACAA CAGGTCTCGC AGAAAGAGTC GGCTGTGATC TACAGCCTGT GGACCCACAA
481 AGGGGTGAGG TGACGTTTAC CACCAGCCAG GTCTCCGAGG GCTGTGTAGC TCAGGCTGCC
541 AATGCTGTGC GTGAAGTCCA CGTTCTCTTC CTGGATTTTC CCGGAATGCT GTCACATCTG
601 GAGCTGACTC TTCAGGCATC CAAGCAAAAT GGCACGGAGA CCCAGGAGGT GTTCCTGGTC
661 CTCGTTTCGA AAAAAATGT CTTCGTGAAG TTCCAGGCCC CGGAAATCCC ATTGCACTTG
721 GCCTACGACT CCAGCCTGGT CATCTTCCAA GGACAGCCAA GAGTCAACAT CACAGTGCTA
781 CCATCCCTTA CCTCCAGGAA ACAGATCCTC GACTGGGCAG CCACCAAGGG CGCCATCACC
841 TCGATAGCAG CACTGGATGA CCCCCAAAGC ATCGTCCTCC AGTTGGGCCA AGACCCAAAG
901 GCACCATTCT TGTGCTTGCC AGAAGCTCAC AAGGACATGG GCGCCACACT TGAATGGCAA
961 CCACGAGCCC AGACCCCACT CCAAAGCTGT CGCTTGGAAG GTGTGTCTGG CCACAAGGAG
1021 GCCTACATCC TGAGGATCCT GCCAGGTTCT GAGGCCGGGC CCCGGACGGT GACCGTAATG
1081 ATGGAAGTGA GTTGCACATC TGGGGACGCC ATTCTCATCC TGCATGGTCC TCCATATGTC
1141 TCCTGGTTCA TCGACATCAA CCACAGCATG CAGATCTTGA CCACAGGTGA ATACTCCGTC
1201 AAGATCTTTC CAGGAAGCAA GGTCAAAGGC GTGGAGCTCC CAGACACACC CCAAGGCCTG
1261 ATAGCGGAGG CCCGCAAGCT CAATGCCAGC ATTGTCACCT CCTTTGTAGA GCTCCCTCTG
1321 GTCAGCAATG TCTCCCTGAG GGCTCCAGC TGCGGTGGTG TGTTCAGAC CACCCCTGCA
1381 CCCGTTGTGA CCACACCTCC CAAGGACACA TGCAGCCCCG TGCTACTCAT GTCCCTGATC
1441 CAGCCAAAGT GTGGCAATCA GGTCAATGACT CTGGCACTCA ATAAAAACA CGTGCAGACT
1501 CTCCAGTGCA CCATCACAGG CCTGACTTTC TEGGACTCCA GCTGCCAGGC TGAAGACACT
1561 GACGACCATC TTGTCCTGAG TAGCGCCTAC TCCAGCTGCG GCATGAAAGT GACAGCCCAT
1621 GTGGTCAGCA ATGAGGTGAT CATCAGTTTC CCGTCAGGCT CACCACCACT TCGGAAAAG
1681 GTACAGTGCA TCGACATGGA CAGCCTCTCC TTCCAGCTGG GCCTCTACCT CAGCCCGCAC
1741 TTCTCCAGG CATCCAACAC CATCGAATA GGCCAGCAGG CCTTCGTACA GGTGAGCGTG
1801 TCTCCATTGA CCTCTGAGGT CACAGTCCAG CTAGATAGCT GCCATCTGGA CTTGGGGCCC
1861 GAAGGGGACA TGGTGGAACT CATCCAGAGC CGAACAGCCA AGGGCAGCTG TGTGACCTTG
1921 CTGTCTCCAA GCCCTGAAGG TGACCCACGC TTCAGCTTCC TCCTCCGGGT CTACATGGTG
1981 CCCACACCCA CCGCTGGCAC CCTCAGTTGC AACTTAGCTC TCGCCCTAG CACCTTGTCC
2041 CAGGAAGTCT ACAAGACAGT CTCCATGCGC CTGAACATCG TCAGCCCTGA CCTGTCTGGT
2101 AAAGGCCTTG TCCTGCCCTC TGTACTGGGT ATCACCTTTG GTGCCTTCCT GATTGGGGCC
2161 CTGCTCACAG CTGCACTCTG GTACATCTAT TCTCACACAC GTGAGTATCC CAAGCCTCCA
2221 CCCCATPCCC ACAGCAAGCG CTCAGGGCCC GTCCACACCA CCCCAGGGCA CACCCAGTGG
2281 AGCCTCTGA

```

(SEQ ID NO: 8)

Fig. 8

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**Amino acid sequence for human ENG extracellular domain**

ETVHC DLQPVGPERG EVTYTTSQVS KGCVAQAPNA  
ILEVHVLFLFLE FFTGPSOLEL TLQASKQNGT WPREVLLVLS VNSSVFLHLQ ALGIPLHLAY  
NSSLVTFQEP PGVNTTELPS FPKTQILEWA AERGPITSAA ELNDPQSILL RLGQAQGSLS  
FCMLEASQDM GRTLEWRPRT PALVRGCHLE GVAGHKEAHI LRVLPGHSAG PRTVTVKVEL  
SCAPGDLDV LILQPPYVS WLIDANHNMQ IWTGGEYSFK IFPEKNIRGF KLPDTPQGLL  
GEARMLNASI VASFVELPLA SIVSLHASSC GGRLQTSPPAP IQTTPPKDTC SPELLMSLIQ  
TKCADDAMTL VLKKELVAHL KCTITGLTFW DPSCEAEDRG DKFVLRSAVS SCGMQVSASM  
ISNEAVVNIL SSSSPQRKKV HCLNMDLSLF QLGLYLSPHF LQASNTIEPG QQSFVQVRVS  
PSVSEFLLQL DSCHLDLGPE GGTVELIQGR AAKGNCVSLI SPSPEGDPFR SPLLHFYTVF  
IPKTGTLSCV VALRPKTGSQ DQEVHRTVFM RLNIISPDLI GCTSKG (SEQ ID NO: 9)

Fig. 9

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**Amino acid sequence of murine ENG extracellular domain**

ERVG CDLQPVDPTR GEVTFTTSQV SEGCVAQAAN  
AVREHVHVLFL DFPGLSHLE LTLQASKQNG TETREVFLVL VSNKNVFKF QAPEIFLHLA  
YDSSLVIFQG QPRVNITVLP SLTSRKQILD WAATKGAIITS IAALDDPQSI VLQLGQDPKA  
PFLCLPEAHK DMGATLEWQP RAQTPVQSCR LEGVSGHKEA YILRILPGSE AGPRTVTVMM  
ELSCTSGDAI LILHGPPYVS WFIDINHSMQ ILTTGEYSVK IFPGSKVKGV ELPDTPQGLI  
AEARKLNASI VTSFVELPLV SNVSLRASSC GGVFQTFPAP VVTTPPKDTC SPVLLMSLIQ  
PKCGNQVMTL ALNKKHVQTL QCTITGLTFW DSSCOAEDTD DHLVLSSAYS SCGMKVTAHV  
VSNEVIISFP SGSPPLRKKV QCIDMDSLFS QLGLYLSPHF LQASNTIELG QQAFVQVSVS  
PLTSEVTVQL DSCHLDLGPE GDMVELIQSR TAGSCVTLL SPSPEGDPFR SFLLRVYMVP  
TPTAGTILSCN LALRPSTLSQ EVYKTVSMRL NVVSPDLGK G (SEQ ID NO: 10)

**Fig. 10**

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**Amino acid sequence of human IgG1 Fc domain**

1 GGP<sup>K</sup>SCDKTH TCP<sup>P</sup>CPAFEL LGG<sup>P</sup>SVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK  
 61 FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK  
 121 TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT  
 181 PPVLDSGSE FLYSKLTVDK SRWQOGNVFS CSVMHEALHN HYTQKSLSL S PGK  
 (SEQ ID NO: 11)

Fig. 11

**Amino acid sequence of N-terminally truncated human IgG1 Fc domain**

1 THTCP<sup>P</sup>CPAP ELLGG<sup>P</sup>SVFL FPPKPKDTLM ISRTPEVTCV VDVSHEDPE VKFNWYVDGV  
 61 EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ  
 121 PREPQVYTL PPSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG  
 181 SFFLYSKLTV DKSRWQOGNV FSCSVMHEAL HNHYTQKSL S LSPGK  
 (SEQ ID NO: 12)

Fig. 12

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## Amino acid sequence of hENG(26-586)-hFc

1 MDAMKRGLCC VLLLCGAVFV SPGAETVHCD LOPVGPERDE VTYTTSQVSK  
 51 GCVAQAPNAI LEVHVLFLEF PTGPSOLELT LOASKQNGTW PREVLLVLSV  
 101 NSSVFLHLQA LGIPLHLAYN SSLVTFQEPG GVNTTELPSE PKTOILEWAA  
 151 ERGPITSAAE LNDPOSILLR LGOAOGSLSF CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL  
 251 ILOGPPYVSW LIDANHNMQI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLQTSFAPL QTFPPKDTCS  
 351 PELLMSLIQT KCADDAMTLV LKKELVAHLK CTITGLTFWD PSCEAEDRGD  
 401 KFVLRSAYSS CGMQVSASMI SNEAVVNILS SSSPQRKKVH CLNMDSLSEFQ  
 451 LGLYLSPHFL QASNTIEPGQ QSEVQVRVSP SVSEFLLQLD SCHLDDLGPEG  
 501 GTVELIQGRA AKGNCVSLLS PSPEGDPRES FLLHFYTVPI PKTGTLSCTV  
 551 ALRPKTGSQD QEVHRTVFMK LNIISPDLSE CTSKGTGGP KSCDKTHTCP  
 601 PCPAPELLGG PSVFLFPPKF KDTLMISRTP EVTCVVVDVS HEDPEVKFNW  
 651 YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA  
 701 LPAPIEKTIS KARGQPREFQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI  
 751 AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV  
 801 MHEALHNHYT QKSLSLSPGK

(SEQ ID NO: 16)

Fig. 13



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## Nucleotide sequence encoding hENG(26-586)-hFc

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC  
 AGTCTTCGTT TCGCCCGGGC CCGAAACAGT CCATTGTGAC CTTCAGCCTG  
 101 TGGGCCCCGA GAGGGACGAG GTGACATATA CCACTAGCCA GGTCTCGAAG  
 GGCTGCGTGG CTCAGGCCCC CAATGCCATC CTTGAAGTCC ATGTCTCTTT  
 201 CCTGGAGTTC CCAACGGGOC CGTCACAGCT GGAGCTGACT CTCCAGGCAT  
 CCAAGCAAAA TGGCACCTGG CCCCAGAGAGG TGCTTCTGGT CCTCAGTGTA  
 301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACTT  
 GGCCTACAAT TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA  
 401 CCACAGAGCT GCCATCCTTC CCCAAGACCC AGATCCTTGA GTGGGCAGCT  
 GAGAGGGGCC CCATCACCTC TGCTGCTGAG CTGAATGACC CCCAGAGCAT  
 501 CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCCTTC TGCATGCTGG  
 AAGCCAGCCA GGACATGGGC CGCACGCTCG AGTGGCGGCC GCGTACTCCA  
 601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC  
 GCACATCCTG AGGGTCCTGC CGGGCCACTC GGCCGGGCCC CGGACGGTGA  
 701 CGGTGAAGGT GGAAC TGAGC TGGCACCCCG GGGATCTCGA TGCCGTCCTC  
 ATCCTGCAGG GTCCCCCTA CGTGTCTTGG CTCATCGACG CCAACCACAA  
 801 CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC TTTCCAGAGA  
 AAAACATTCG TGGCTTCAAG CTCCCAGACA CACCTCAAGG CCTCCTGGGG  
 901 GAGGCCCGGA TGCTCAATGC CAGCATTGTG GCATCCTTCG TGGAGCTACC  
 GCTGGCCAGC ATTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC  
 1001 AGACCTCACC CGCACCGATC CAGACCACTC CTCCAAGGA CACTTGTAGC  
 CCGGAGCTGC TCATGTCCTT GATCCAGACA AAGTGTGCCG ACGACGCCAT  
 1101 GACCCTGGTA CTAAAGAAAG AGCTTGTGTC GCATTTGAAG TGCACCATCA  
 CGGGCCTGAC CTTCTGGGAC CCCAGCTGTG AGGCAGAGGA CAGGGGTGAC  
 1201 AAGTTTGTCT TGGCCAGTGC TTA CTCCAGC TGTGGCATGC AGGTGTCAGC  
 AAGTATGATC AGCAATGAGG CCGTGGTCAA TATCCTGTCG AGCTCATCAC  
 1301 CACAGCGGAA AAAGGTGCAC TGCCTCAACA TGGACAGCCT CTCTTTCCAG  
 CTGGGCCTCT ACCTCAGCCC ACACTTCCTC CAGGCCTCCA ACACCATCGA  
 1401 GCCGGGGCAG CAGAGCTTTG TGCAGGTCAG AGTGTCCCCA TCCGTCTCCG  
 AGTTCCTGCT CCAGTTAGAC AGCTGCCACC TGGACTTGGG GCCTGAGGGA  
 1501 GGCACCGTGG AACTCATCCA GGGCCGGGCG GCCAAGGGCA ACTGTGTGAG  
 CCTGCTGTCC CCAAGCCCCG ABBGTGACCC GCGCTTCAGC TTCCTCCTEC  
 1601 ACTTCTACAC AGTACCCATA CCCAAAACCG GCACCCCTCAG CTGCACGGTA  
 GCCCTGCGTC CCAAGACCGG GTCTCAAGAC CAGGAAGTCC ATAGGACTGT  
 1701 CTTTATGCGC TTGAACATCA TCAGCCCTGA CCTGTCTGGT TGCACAAGCA  
 AAGGCACCGG TGGTGGACCC AAATCTTGTG ACAA AACTCA CACATGCCCA  
 1801 CCGTGCCAG CACCTGAACT CCTGGGGGGA CCGTCAGTCT TCCTCTTCCC  
 CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT GAGGTCACAT

Fig. 14

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```
1901 GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG
      TACGTGGACG GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA
2001 GCAGTACAAC AGCACGTACC GTGTGGTCAG CGTCCTCACC GTCCTGCACC
      AGGACTGGCT GAATGGCAAG GAGTACAAGT GCAAGGTCTC CAACAAAGCC
2101 CTCCCAGCCC CCATCGAGAA AACCATCTCC AAAGCCAAAG GGCAGCCCCG
      AGAACCACAG GTGTACACCC TGCCCCCATC CCGGGAGGAG ATGACCAAGA
2201 ACCAGGTCAG CCTGACCTGC CTGGTCAAAG GCTTCTATCC CAGCGACATC
      GCCGTGGAGT GGGAGAGCAA TGGGCAGCCG GAGAACAAC TACAAGACCAC
2301 GCCTCCCCTG CTGGACTCCG ACGGCTCCTT CTTCCCTCTAT AGCAAGCTCA
      CCGTGGACAA GAGCAGGTGG CAGCAGGGGA ACGTCTTCTC ATGCTCCGTC
2401 ATGCATGAGG CTCTGCACAA CCACTACACG CAGAAGAGCC TCTCCCTGTC
      CCCGGGTAAA TGA
```

(SEQ ID NO: 17)

Fig. 14 continued

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**Amino acid sequence of hENG(26-586)-hFc with N-terminally truncated Fc domain**

1 MDAMKRGLCC VLLLCGAVFV SPGAETVHCD LOPVQPERDE VTYTTSQVSK  
 51 GCVAQAPNAI LEVHVLFLFET PTGPSOLELT LOASKONGTW PREVLLVLSV  
 101 NSSVFLHLQA LGIPLHLAYN SSLVTFQEPF GVNTTELPSE PKTQILEWAA  
 151 ERGPITSAE LNDPOSILLR LGQAQGSLSF CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL  
 251 ILOGPPYVSW LIDANHNMOI WTTGEYSFKI FPEKNIRGFK LPDTPOGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLQTSAPAI QTPPKDTCF  
 351 PELLMSLIQF KCADDAMTLV LKKELVAHLK CTITGLTFWD PSCEAEDRGD  
 401 KFVLRSAYSS CGMQVSASMI SNEAVVNILS SSSPQRKKVH CLNMDSLSFQ  
 451 LGLYLSPHFL QASNTIEPGQ QSFVQVRVSP SVSEFLLQLD SCHLDLGPEG  
 501 GTVELIQGRA AKGNCVSLLS PSPEGDPFRFS FLLHFYTVPI PKTGTLSCFV  
 551 ALRFKTSQD QEVHRTVFMR LNIISFDLSG CTSKGTGGGT HTCPCPCAPE  
 601 LLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV KFNWYVDGVE  
 651 VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE  
 701 KTISKAKGQP REPQVYTLPP SREEMTKNQV SLTCLVKGFY PSDIAVEWES  
 751 NGQFENNYKT TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH  
 801 NHYTQKSLSL SPGK

(SEQ ID NO: 18)

Fig. 15

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**Amino acid sequence of mENG(27-581)-mFc**

1 MDAMKRGLCC VLLLCGAVFV SPGGERVGCD LOPVDPTRGE VTFTTSQVSE  
 51 GCVAQAANAV REVHVLFLDF PGMLSHLELT LOASKONGTE TQEVFLVLVS  
 101 NKNVFVKFOA PEIPLHLAYD SSLVIFOGQP RVNITVLPSL TSRKQILDWA  
 151 ATKGAITSIA ALDDPQSIVL QLGQDPKAPF LCLPEAHKDM GATLEWQPRRA  
 201 QTPVQSCRLE GVS~~GH~~KEAYI LRILPGSEAG PRTVTVM~~MEL~~ SCTSGDAILI  
 251 LHGPPYVSWF IDINHSMOIL TTGEYSVKIF PGSKVKGVEL PDTPOGLIAE  
 301 ARKLNASIVT SFVELPLVSN VSLRASSCGG VFQTPAPVV TTPPKDTCSP  
 351 VLLMS~~LI~~QPK CGNQVMTLAL NKKHVQTLQC TITGLTFWDS SQAEDTDDH  
 401 LVLSSAYSSC GMKVTAHVVS NEVIISFPSG SPPLRKKVQC IDMDSL~~SF~~Q~~L~~  
 451 GLYLSPHFLO ASNTIELGQQ AFVQVS~~VS~~PL TSEVTVQ~~LD~~S CHLDLGPEGD  
 501 MVELIQSR~~TA~~ KGSCVTLLSP SPEGDPRFSF LLRVY~~VM~~VPTP TAGT~~LS~~CNLA  
 551 LRPSTLSQEV YKTVSMRLNI VSPDLSGKGT GGGEPRVPIT QN~~PC~~PLKEC  
 601 PPCAAPDLLG GPSVFIFPPK IKDVL~~MIS~~LS PMVTCV~~VVD~~V SEDDPDVQIS  
 651 WVNNVEVHT AQTQTHREDY NSTLRVVSAL PIQH~~QD~~WMSG KEFKCKVNNR  
 701 ALPSPIEKTI SKPRGPVRAP QVYVLPPPAE EMTKKEFSLT CMITGFLPAE  
 751 IAVDWTSNGR TEQNYKNTAT VLDS~~DG~~SYFM YSKLRVQKST WERGSLFACS  
 801 VVHEGLHNHL TTKTISRSLG K  
 (SEQ ID NO: 19)

Fig. 16

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## Nucleotide sequence encoding mENG(27-581)-mFc

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC  
 AGTCTTCGTT TCGCCCGGCG GGEAAAGAGT CCGCTGTGAT CTACAGCCTG  
 101 TGGACCCAC AAGGGGTGAG GTGACGTTTA CCACCAGCCA GGTCTCCGAG  
 GGCTGTGTAG CTCAGGCTGC CAATGCTGTG CGTGAAGTCC ACGTTCCTCT  
 201 CCTGGATTTT CCCGGAATGC TGTCACATCT GGAGCTGACT CTTCAGGCAT  
 CCAAGCAAAA TGGCACGGAG ACCCAGGAGG TGTTCCTGGT CCTCGTTTCG  
 301 AACAAAAATG TCTTCGTGAA GTTCCAGGCC CCGGAAATCC CATTGCACTT  
 GGCTTACGAC TCCAGCCTGG TCATCTTCCA AGGACAGCCA AGAGTCAACA  
 401 TCACAGTGCT ACCATCCCTT ACCTCCAGGA AACAGATCCT CGACTGGGCA  
 GCCACCAAGG GCGCCATCAC CTCGATAGCA GCACTGGATG ACCCCCAAAG  
 501 CATCGTCTTC CAGTTGGGCC AAGACCCAAA GGCACCATTG TTGTGCTTGC  
 CAGAAGCTCA CAAGGACATG GCGCCACAC TTGAATGGCA ACCACGAGCC  
 601 CAGACCCAG TCCAAAGCTG TCGCTTGGAA GGTGTGTCTG GCCACAAGGA  
 GGCTTACATC CTGAGGATCC TGCCAGGTTC TGAGGCCGGG CCCCAGGAGG  
 701 TGACCGTAAT GATGGAACTG AGTTGCACAT CTGGGGACGC CATTCTCATC  
 CTGCATGGTC CTCCATATGT CTCCTGGTTC ATCGACATCA ACCACAGCAT  
 801 GCAGATCTTG ACCACAGGTG AATACTCCGT CAAGATCTTT CCAGGAAGCA  
 AGGTCAAAGG CGTGGAGCTC CCAGACACAC CCCAAGGCCT GATAGCGGAG  
 901 GCCCGCAAGC TCAATGCCAG CATTGTCACC TCCTTTGTAG AGCTCCCTCT  
 GGTCAGCAAT GTCTCCCTGA GGCCTCCAG CTGCGGTGGT GTGTTCCAGA  
 1001 CCACCCCTGC ACCCGTTGTG ACCACACCTC CCAAGGACAC ATGCAGCCCC  
 GTGCTACTCA TGTCCTTGAT CCAGCCAAAG TGTGGCAATC AGGTTCATGAC  
 1101 TCTGGCACTC AATAAAAAAC ACGTGCAGAC TCTCCAGTGC ACCATCACAG  
 GCCTGACTTT CTGGGACTCC AGCTGCCAGG CTGAAGACAC TGACGACCAT  
 1201 CTTGTCTGA GTAGCGCCTA CTCCAGCTGC GGCATGAAAG TGACAGCCA  
 TGTGGTCAGC AATGAGGTGA TCATCAGTTT CCCGTCAGGC TCACCACCAC  
 1301 TTCGGAAAAA GGTACAGTGC ATCGACATGG ACAGCCTCTC CTTCAGCTG  
 GGCTCTACC TCAGCCCGCA CTTCCTCCAG GCATCCAACA CCATCGAACT  
 1401 AGGCCAGCAG GCCTTCGTAC AGGTGAGCGT GTCTCCATTG ACCTCTGAGG  
 TCACAGTCCA GCTAGATAGC TGCCATCTGG ACTTGGGGCC CGAAGGGGAC  
 1501 ATGGTGGAAC TCATCCAGAG CCGAACAGCC AAGGGCAGCT GTGTGACCTT  
 GCTGTCTCCA AGCCCTGAAG GTGACCCACG CTTTCAGCTT CTCCTCCGGG  
 1601 TCTACATGGT GCCACACCC ACEGCTGGCA CCTTCAGTTG CAACTTAGCT  
 CTGCGCCCTA GCACCTTGTC CCAGGAAGTC TACAAGACAG TCTCCATGCG  
 1701 CCTGAACATC GTCAGCCCTG ACCTGTCTGG TAAAGGCACC GGTGGGGGTG  
 AGCCAGAGT GCCATAACA CAGAACCCTT GTCCTCCACT CAAAGAGTGT  
 1801 CCCCATGCG CAGCTCCAGA CCTCTGGGT GGACCATCCG TCTTCATCTT  
 CCCTCCAAAG ATCAAGGATG TACTCATGAT CTCCTGAGC CCCATGGTCA

Fig. 17

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1901 CATGTGTGGT GGTGGATGTG AGCGAGGATG ACCCAGACGT CCAGATCAGC  
TGGTTTGTGA ACAACGTGGA AGTACACACA GCTCAGACAC AAACCCATAG  
2001 AGAGGATTAC AACAGTACTC TCCGGGTGGT CAGTGCCCTC CCCATCCAGC  
ACCAGGACTG GATGAGTGGC AAGGAGTTCA AATGCAAGGT CAACAACAGA  
2101 GCCCTCCCAT CCCCATCGA GAAAACCATC TCAAAACCCA GAGGGCCAGT  
AAGAGCTCCA CAGGTATATG TCTTGCCTCC ACCAGCAGAA GAGATGACTA  
2201 AGAAAGAGTT CAGTCTGACC TGCATGATCA CAGGCTTCTT ACCTGCCGAA  
ATTGCTGTGG ACTGGACCAG CAATGGGCGT ACAGAGCAAA ACTACAAGAA  
2301 CACCGCAACA GTCCTGGACT CTGATGGTTC TTACTTCATG TACAGCAAGC  
TCAGAGTACA AAAGAGCACT TGGGAAAGAG GAAGTCTTTT CGCCTGCTCA  
2401 GTGGTCCACG AGGGTCTGCA CAATCACCTT ACCACTAAGA CCATCTCCCG  
GTCTCTGGGT AAATGA

(SEQ ID NO: 20)

Fig. 17 continued

High-affinity binding of hENG(26-586)-hFc to BMP-9

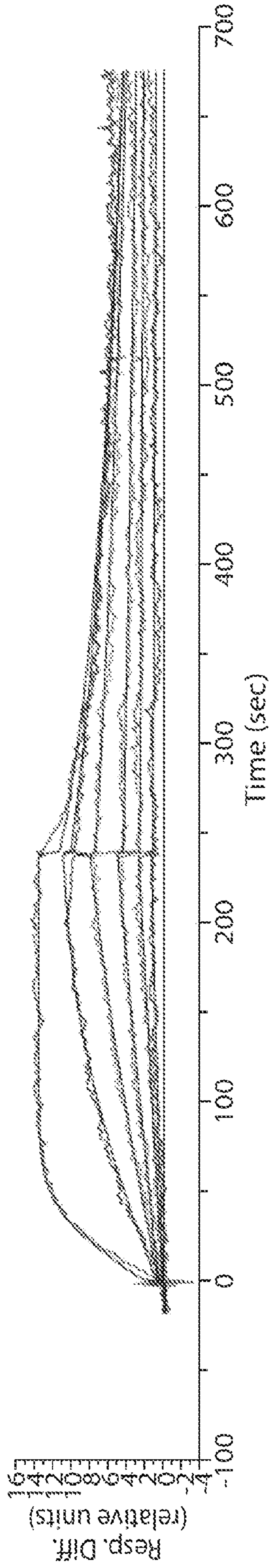


Fig. 18

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High-affinity binding of hENG(26-586)-hFc to BMP-10

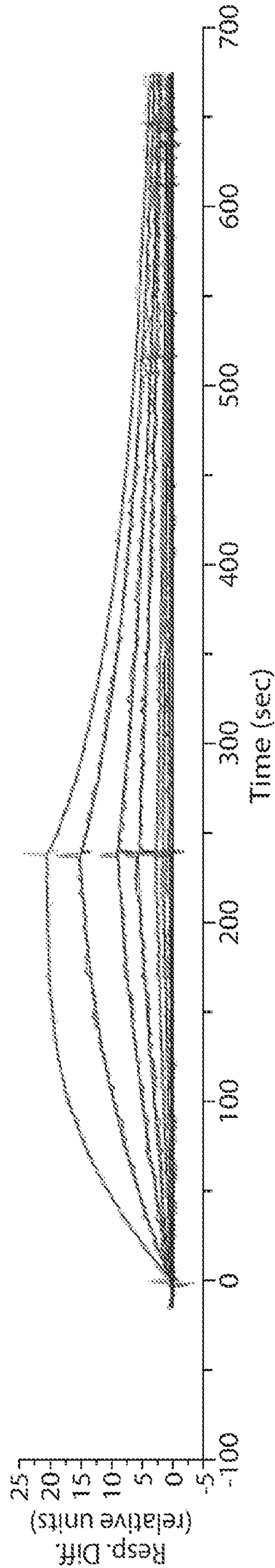
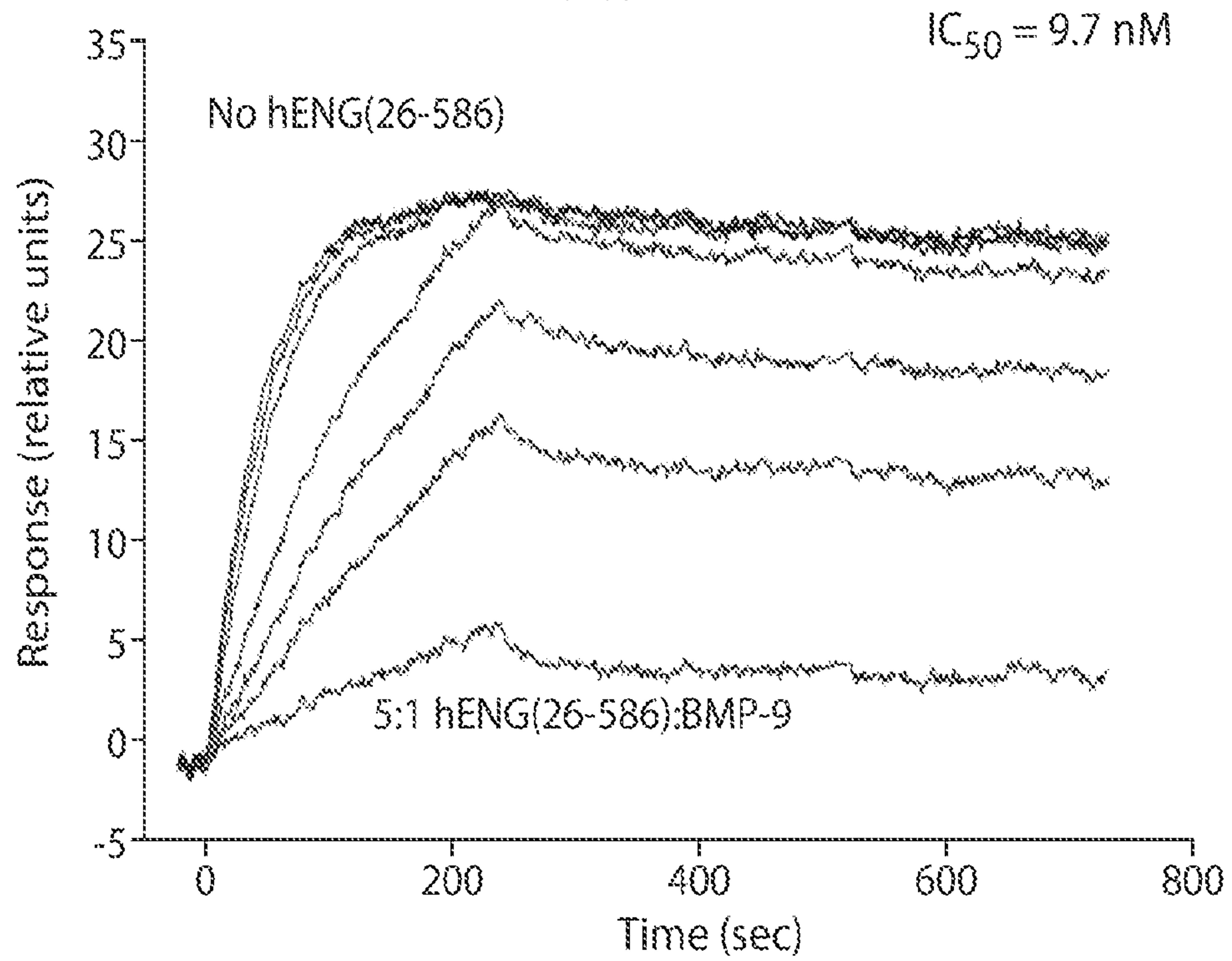
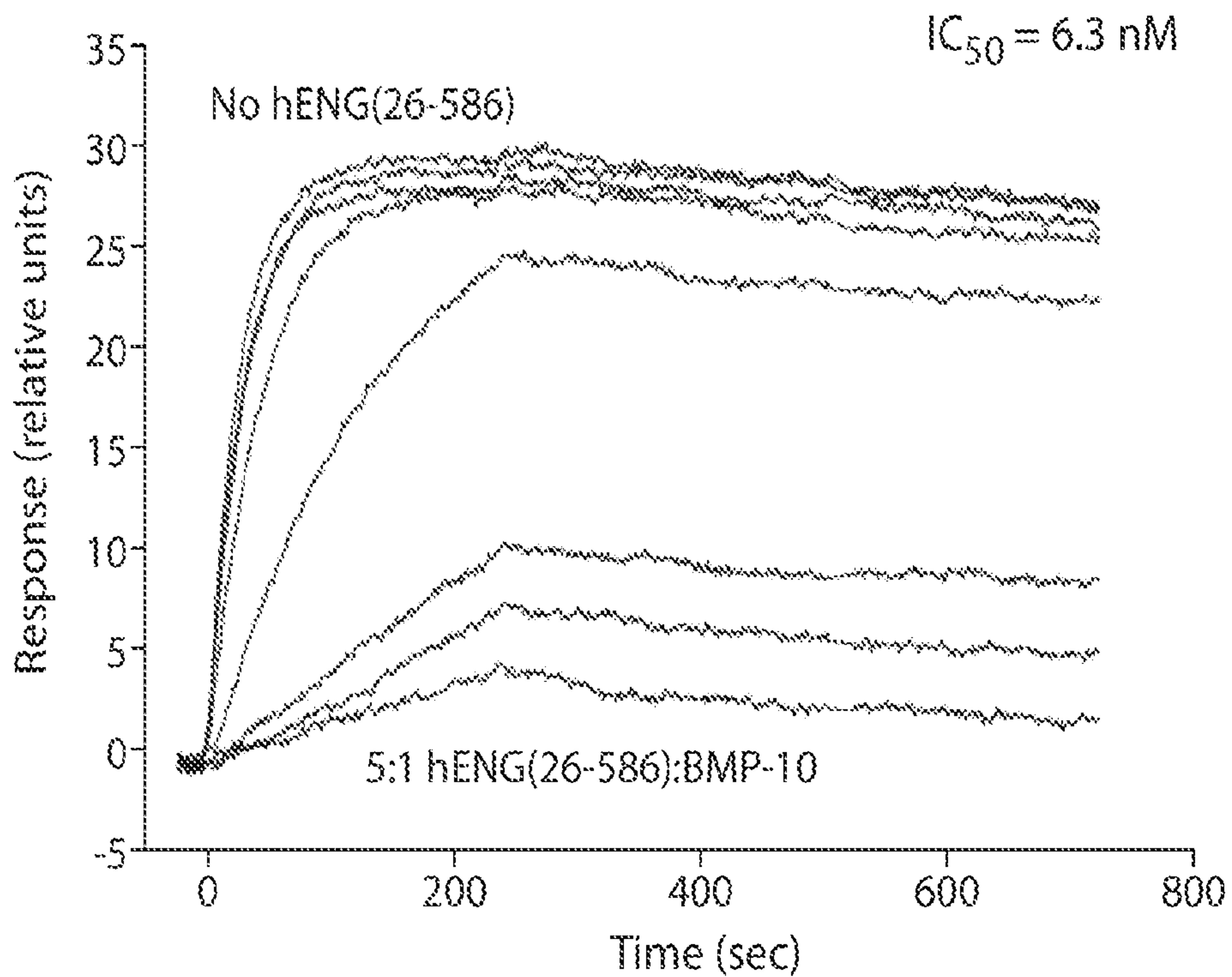


Fig. 19

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**Effect of soluble hENG extracellular domain, hENG(26-586), on binding of BMP-9 to ALK1****Fig. 20****Effect of soluble hENG extracellular domain, hENG(26-586), on binding of BMP-10 to ALK1****Fig. 21**



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Effect of mENG(27-581)-hFc on cord formation by human umbilical vein endothelial cells (HUVEC) in culture

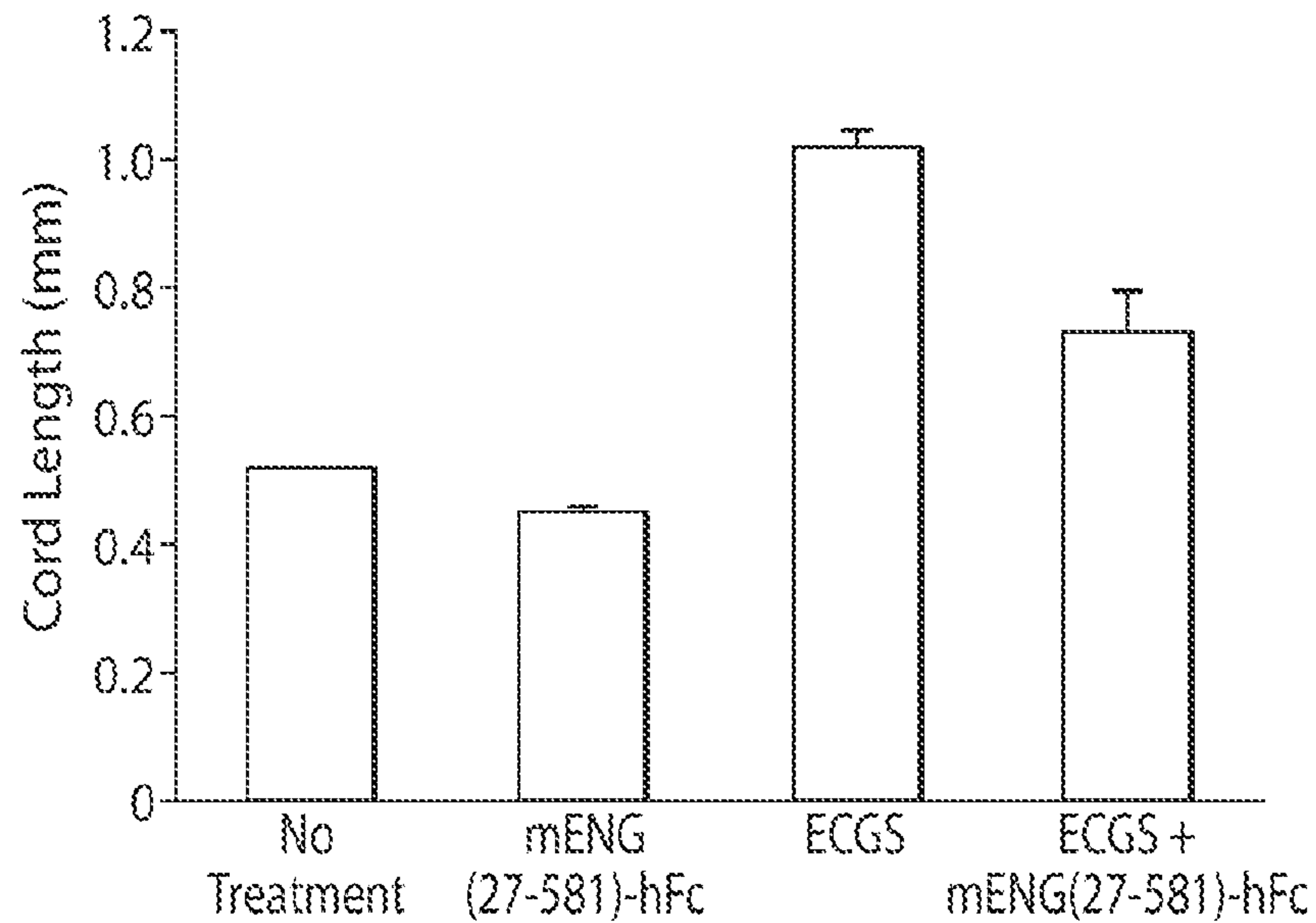


Fig. 22

mENG(27-581)-hFc inhibits VEGF-stimulated angiogenesis in a CAM assay

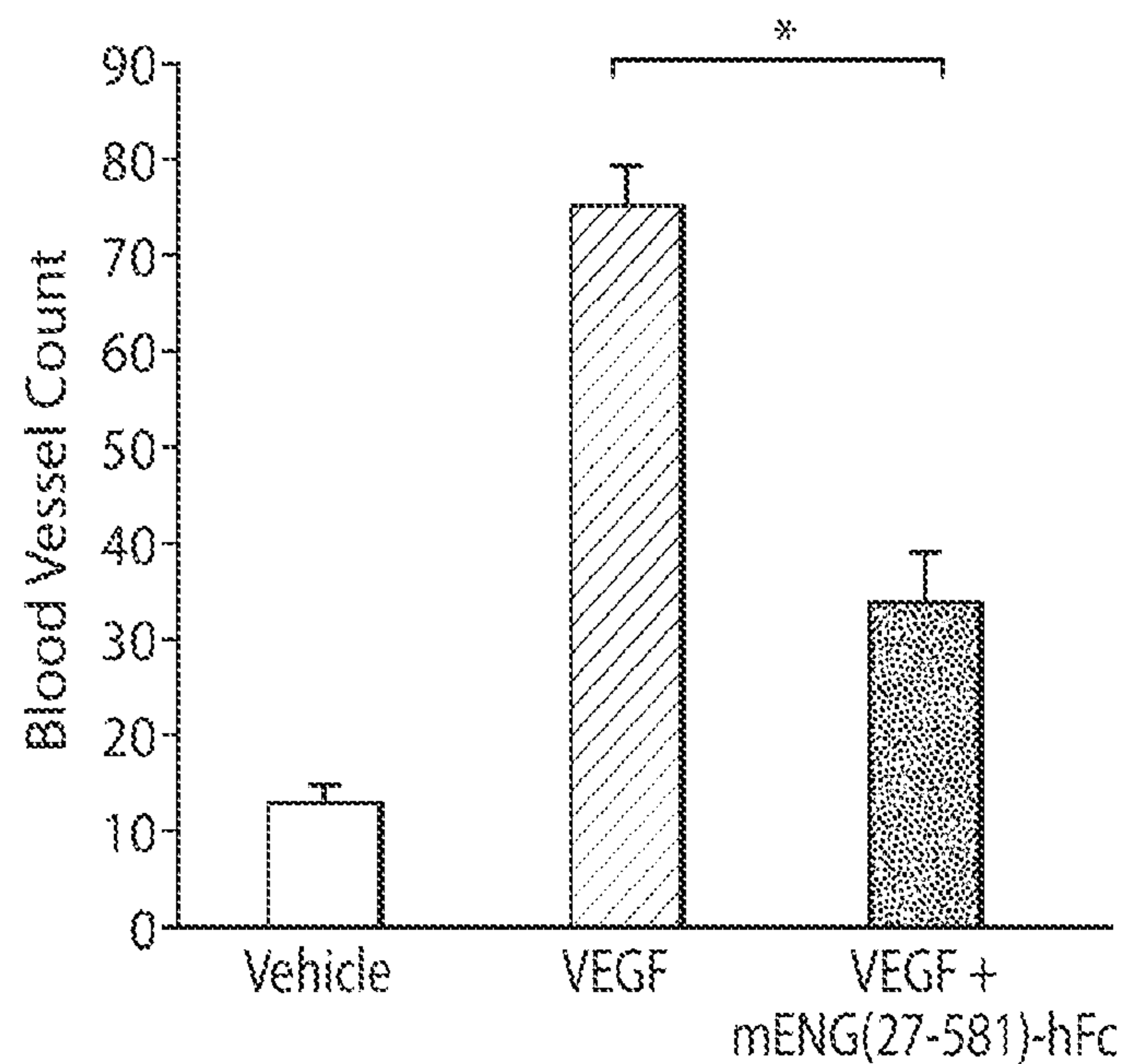


Fig. 23

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Effect of mENG(27-581)-mFc on growth-factor stimulated angiogenesis in a mouse angioreactor assay

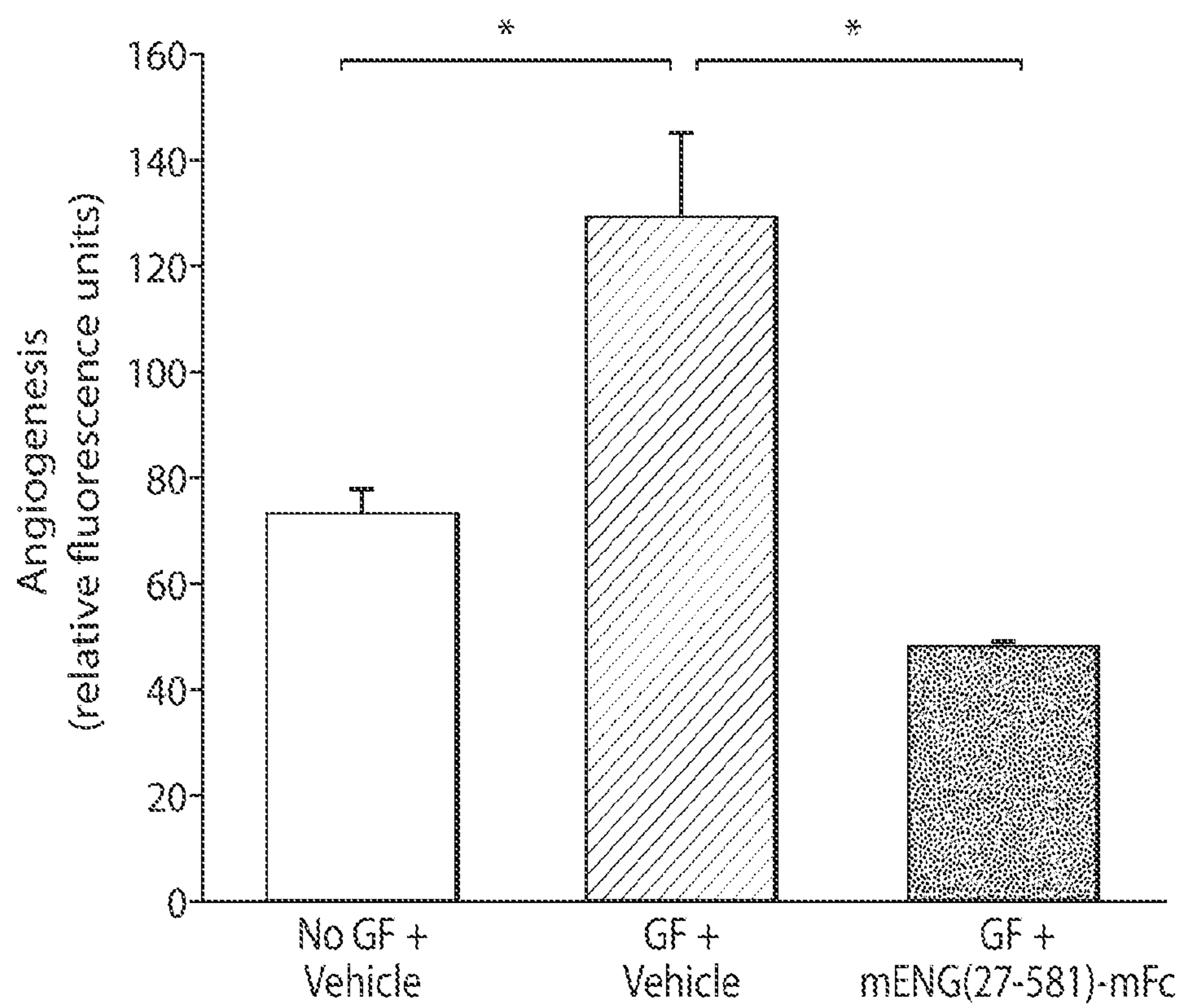


Fig. 24

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Schematic comparison of selected truncated hENG constructs

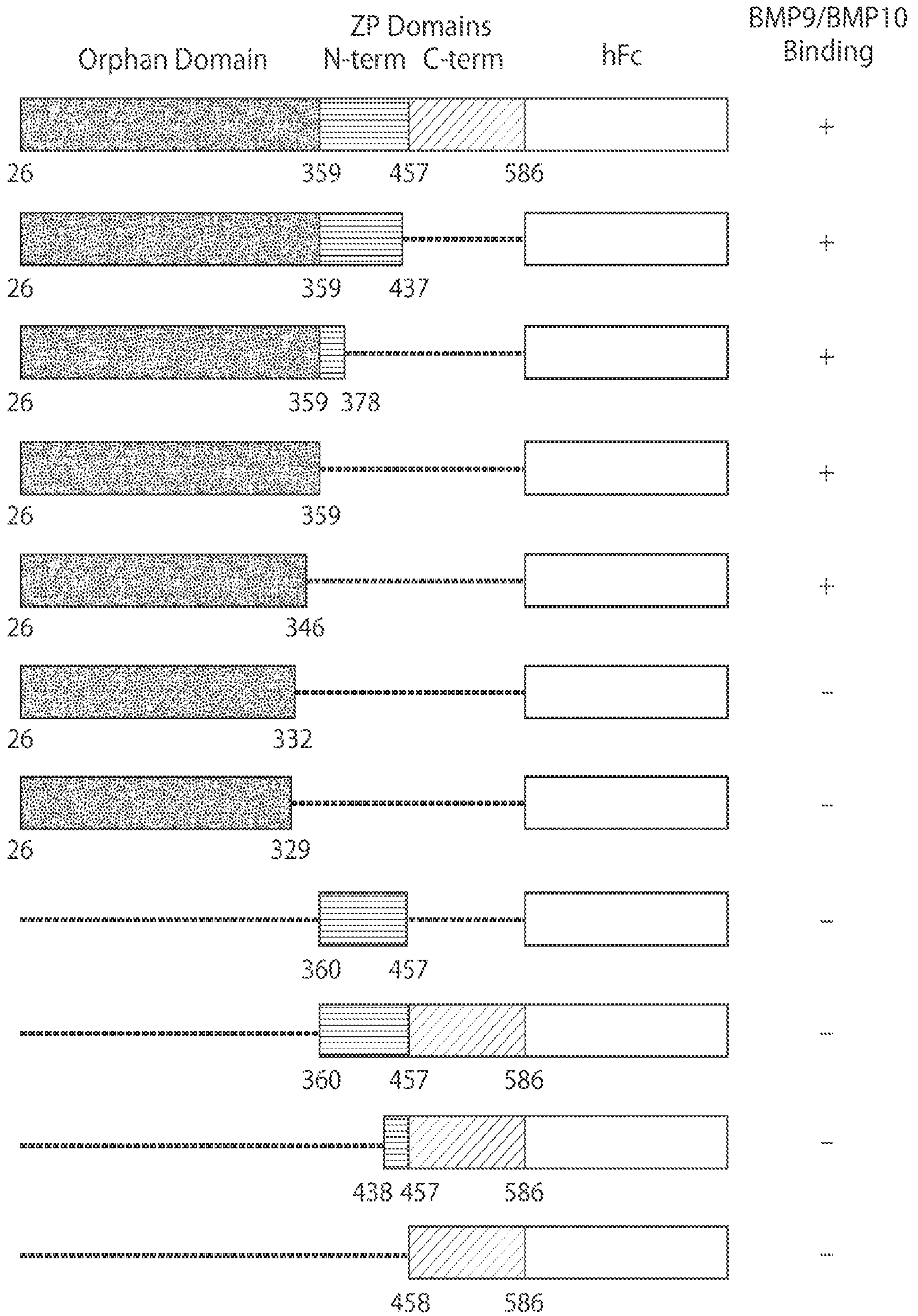


Fig. 25

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**Amino acid sequence of hENG(26-437)-hFc**

1 MDAMKRG LCC VLLLCGAVFV SP<sup>GA</sup>ETVHCD LQVGPPERDE VTYTTSQVSK  
 51 GCVAQAPNAI LEVHVLFLEF PTGPSOLELT LOASKONGTW PREVLLVLSV  
 101 NSSVFLHLOA LGIPLHLAYN SSLVTFQEPP GVNTTELPSE PKTQILEWAA  
 151 ERGPITSAAE LNDPOSILLR LGOAOGSLSF CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL  
 251 ILOGPPYVSW LIDANHNMOI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLQTSAPAI QTPPKDTCG  
 351 PELLMSLIQT KCADDAMTLV LKKELVAHLK CTITGLTFWD PSCEAEDRGD  
 401 KFVLRSAISS CGMOVSASMI SNEAVVNILS SSSPQ<sup>RT</sup>GGG PKSCDKTHTC  
 451 PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN  
 501 WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK  
 551 ALPAPIEKTI SKAKGQPREP QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD  
 601 IAVEWESNGQ PENNYKTPP VLSDSGSFFL YSKLTVDKSR WQQGNVFSCS  
 651 VMHEALHNHY TQKSLSLSPG K  
 (SEQ ID NO: 21)

Fig. 26

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**Nucleotide sequence encoding hENG(26-437)-hFc**

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC  
 AGTCTTCGTT TCGCCCGGCG CCGAAACAGT CCATTGTGAC CTTCAGCCTG  
 101 TGGGCCCCGA GAGGGACGAG GTGACATATA CCACTAGCCA GGTCTCGAAG  
 GGCTGCGTGG CTCAGGCCCC CAATGCCATC CTTGAAGTCC ATGTCCTCTT  
 201 CCTGGAGFTC CCAACGGGCC CGTCACAGCT GGAGCTGACT CTCCAGGCAT  
 CCAAGCAAAA TGGCACCTGG CCCCAGAGAG TGCTTCTGGT CCTCAGTGTA  
 301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACCTT  
 GGCTACAAT TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA  
 401 CCACAGAGCT GCCATCCTTC CCCAAGACCC AGATCCTTGA GTGGGCAGCT  
 GAGAGGGGCC CCATCACCTC TGCTGCTGAG CTGAATGACC CCCAGAGCAT  
 501 CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCCTTC TGCATGCTGG  
 AAGCCAGCCA GGACATGGGC CGCACGCTCG AGTGECGGCC GCCTACTCCA  
 601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC  
 GCACATCCTG AGGGTCCTGC CGGGCCACTC GGCCGGGCCC CGGACGGTGA  
 701 CGGTGAAGGT GGAAGTGAAG TCGCCACCCG GGGATCTCGA TGCCGTCCTC  
 ATCCTGCAGG GTCCCCCTA CGTGTCCTGG CTCATCGACG CCAACCACAA  
 801 CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC TTTCCAGAGA  
 AAAACATTCG TGGCTTCAAG CTCCAGACA CACCTCAAGG CCTCCTGGGG  
 901 GAGGCCCGGA TGCTCAATGC CAGCATTGTG GCATCCTTCG TGGAGCTACC  
 GCTGGCCAGC ATTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC  
 1001 AGACCTCACC CGCACCGATC CAGACCACTC CTCCCAAGGA CACTTGTAGC  
 CCGGAGCTGC TCATGTCTCT GATCCAGACA AAGTGTGCCG ACGACGCCAT  
 1101 GACCCTGGTA CTAAAGAAAG AGCTTGTGTC GCATTTGAAG TGCACCATCA  
 CGGGCCTGAC CTTCTGGGAC CCCAGCTGTG AGGCAGAGGA CAGGGGTGAC  
 1201 AAGTTTGTCT TGCGCAGTGC TTAATCCAGC TGTGGCATGC AGGTGTCAGC  
 AAGTATGATC AGCAATGAGG CGGTGGTCAA TATCCTGTGC AGCTCATCAC  
 1301 CACAGCGGAC CCGTGGTGA CCCAAATCTT GTGACAAAAC TCACACATGC  
 CCACCGTGCC CAGCACCTGA ACTCCTGGGG GGACCGTCAG TCTTCCTCTT  
 1401 CCCCCCAAAA CCCAAGGACA CCCTCATGAT CTCCCGGACC CCTGAGGTCA  
 CATGCGTGGT GGTGGACGTG AGCCACGAAG ACCCTGAGGT CAAGTTCAAC  
 1501 TGGTACGTGG ACGGCGTGGG GGTGCATAAT GCCAAGACAA AGCCGCGGGA  
 GGAGCAGTAC AACAGCACGT ACCGTGTGGT CAGCGTCCTC ACCGTCTCTG  
 1601 ACCAGGACTG GCTGAATGGC AAGGAGTACA AGTGCAAGGT CTCCAACAAA  
 GCCCTCCCAG CCCCATCGA GAAAACCATC TCCAAAGCCA AAGGGCAGCC  
 1701 CCGAGAACCA CAGGTGTACA CCCTGCCCCC ATCCCGGGAG GAGATGACCA  
 AGAACCAGGT CAGCCTGACC TGCCTGGTCA AAGGCTTCTA TCCCAGCGAC  
 1801 ATCGCCGTGG AGTGGGAGAG CAATGGGCAG CCGGAGAACA ACTACAAGAC  
 CACGCCTCCC GTGCTGGACT CCGACGGCTC CTTCTTCTC TATAGCAAGC  
 1901 TCACCGTGGG CAAGAGCAGG TGGCAGCAGG GGAACGTCTT CTCATGCTCC  
 GTGATGCATG AGGCTCTGCA CAACCACTAC ACGCAGAAGA GCCTCTCCCT  
 2001 GTCCCCGGGT AAATGA

(SEQ ID NO: 22)

Fig. 27

SUBSTITUTE SHEET (RULE 26)

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**Amino acid sequence of hENG(26-378)-hFc**

1 MDAMKRG LCC VLLLCGAVFV SP<sup>GA</sup>ETVHCD LQVGPPERDE VTYTTSQVSK  
 51 GCVAQAPNAI LEVHVLFLEF PTGPSOLELT LOASKONGTW PREVLLVLSV  
 101 NSSVFLHLQA LGIPLHLAYN SSLVTFQEPP GVNTTELPSE PKTQILEWAA  
 151 ERGPITSAAE LNDPQSILLR LGOAOGSLSE CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGDLDVAVL  
 251 ILOGPPYVSW LIDANHNMOI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLOTSPAPI QTPPKDTCG  
 351 PELLMSLIQT KCADDAMTLV LKELVA<sup>TGG</sup> ~~Q~~<sup>G</sup>THTCPPCPA PELLGGPSVF  
 401 LFPPKPKDTL MISRTPEVTC VVVDVSHEDP EVKFNWYVDG VEVHNAKTKP  
 451 REEQYNSTYR VSVLTVLHQ DWLNGKEYKC KVSNKALPAP IEKTISKAKG  
 501 QPREPQVYTL PPSREEMTKN QVSLTCLVKG FYPSDIAVEW ESNGQPENNY  
 551 KTPPVLDSD GSFFLYSKLT VDKSRWQOGN VFSCSVMHEA LHNHYTQKSL  
 601 SLSPGK

(SEQ ID NO: 23)

Fig. 28

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**Nucleotide sequence encoding hENG(26-378)-hFc**

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC AGTCTTCGTT  
 61 TCGCCC**GGCG** **CC**GAAACAGT CCATTGTGAC CTTCAGCCTG TGGGCCCCGA GAGGGACGAG  
 121 GTGACATATA CCACTAGCCA GGTCTCGAAG GGCTGCGTGG CTCAGGCCCC CAATGCCATC  
 181 CTTGAAGTCC ATGTCCTCTT CCTGGAGTTC CCAACGGGCC CGTCACAGCT GGAGCTGACT  
 241 CTCCAGGCAT CCAAGCAAAA TGGCACCTGG CCCCAGAGAG TGCTTCTGGT CCTCAGTGTA  
 301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACTT GGCTACAAT  
 361 TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA CCACAGAGCT GCCATCCTTC  
 421 CCCAAGACCC AGATCCTTGA GTGGGCAGCT GAGAGGGGCC CCATCACCTC TGCTGCTGAG  
 481 CTGAATGACC CCCAGAGCAT CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCTTTC  
 541 TGCATGCTGG AAGCCAGCCA GGACATGGGC CGCACGCTCG AGTGGCGGCC GCGTACTCCA  
 601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC GCACATCCTG  
 661 AGGGTCCTGC CGGGCCACTC GGCCGGGCCC CGGACGGTGA CGGTGAAGGT GGAAGTGAAG  
 721 TGGCACCACG GGGATCTCGA TGCCGTCTTC ATCCTGCAGG GTCCCCCTTA CGTGTCTTGG  
 781 CTCATCGACG CCAACCACAA CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC  
 841 TTTCCAGAGA AAAACATTCG TGGCTTCAAG CTCCCAGACA CACCTCAAGG CCTCCTGGGG  
 901 GAGGCCCGGA TGCTCAATGC CAGCATTGTG GCATCCTTCG TGGAGCTACC GCTGGCCAGC  
 961 ATTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC AGACCTCACC CGCACCGATC  
 1021 CAGACCACTC CTCCCAAGGA CACTTGTAGC CCGGAGCTGC TCATGTCTTT GATCCAGACA  
 1081 AAGTGTGCCG ACGACGCCAT GACCCTGGTA CTAAAGAAAG AGCTTGTTCG **GACCGGTGGT**  
 1141 **GGA**ACTCACA CATGCCACC GTGCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC  
 1201 CTCTTCCCCC CAAAACCCAA GGACACCCTC ATGATCTCCC GGACCCCTGA GGTCACATGC  
 1261 GTGGTGGTGG ACGTGAGCCA CGAAGACCCT GAGGTCAAGT TCAACTGGTA CGTGGACGGC  
 1321 GTGGAGGTGC ATAATGCCAA GACAAAGCCG CGGGAGGAGC AGTACAACAG CAGGTACCGT  
 1381 GTGGTCAGCG TCCTCACCGT CCTGCACCAG GACTGGCTGA ATGGCAAGGA GTACAAGTGC  
 1441 AAGGTCTCCA ACAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCATCTCCAA AGCCAAAGGG  
 1501 CAGCCCCGAG AACCACAGGT GTACACCCTG CCCCCATCCC GGGAGGAGAT GACCAAGAAC  
 1561 CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC CGTGGAGTGG  
 1621 GAGAGCAATG GGCAGCCGGA GAACAACACT AAGACCACGC CTCCCGTGCT GGAATCCGAC  
 1681 GGCTCCTTCT TCCTCTATAG CAAGCTCACC GTGGACAAGA GCAGGTGGCA GCAGGGGAAC  
 1741 GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC  
 1801 TCCCTGTCCC CGGGTAAATG A

(SEQ ID NO: 24)

Fig. 29

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**Amino acid sequence of hENG(26-359)-hFc**

1 MDAMKRGLCC VLLLCGAVFV SPGAETVHCD LQVGPPERDE VTYTTSQVSK  
51 GCVAQAPNAI LEVHVLFLEF PTGPSOLELT LQASKQNGTW PREVLLVLSV  
101 NSSVFLHLOA LGIPLHLAYN SSLVTFQEPP GVNTTELPSF PKTQILEWAA  
151 ERGPITSAAE LNDPOSILLR LGQAOGSLSF CMLEASQDMG RTLEWRPRTP  
201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGDLDAVL  
251 ILOGPPYVSW LIDANHNMOI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLOTSPAPI QTTPPKDTCS  
351 PELLMSLITC GGPKSCDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS  
401 RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS  
451 VLTVLHQDWL NGKEYKCKVS NKALPAPIEK TISKAKGQPR EPQVYTLPPS  
501 REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF  
551 FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK

(SEQ ID NO: 25)

Fig. 30



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## Nucleotide sequence encoding hENG(26-359)-hFc

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC  
 AGTCTTCGTT TCGCCC**GGCG** CCGAAACAGT CCATTGTGAC CTCAGCCTG  
 101 TGGGCCCCGA GAGGGACGAG GTGACATATA CCACTAGCCA GGTCTCGAAG  
 GGCTGCGTGG CTCAGGCCCC CAATGCCATC CTTGAAGTCC ATGTCCTCTT  
 201 CCTGGAGTTC CCAACGGGGC CGTCACAGCT GGAGCTGACT CTCCAGGCAT  
 CCAAGCAAAA TGGCACCTGG CCCCAGAGAG TGCTTCTGGT CCTCAGTGTA  
 301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACTT  
 GGCCTACAAT TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA  
 401 CCACAGAGCT GCCATCCTTC CCCAAGACCC AGATCCTTGA GTGGGCAGCT  
 GAGAGGGGCC CCATCACCTC TGCTGCTGAG CTGAATGACC CCCAGAGCAT  
 501 CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCCTTC TGCATGCTGG  
 AAGCCAGCCA GGACATGGGC CECACGCTCG AGTGGCGGCC GCGTACTCCA  
 601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC  
 GCACATCCTG AGGGTCCTGC CGGGCCACTC GGCCGGGCC CCGACGGTGA  
 701 CGGTGAAGGT GGAAGTGAAG TCGCACCCCG GGGATCTCGA TGCCGTCCTC  
 ATCCTGCAGG GTCCCCCCTA CGTGTCTTGG CTCATCGACG CCAACCACAA  
 801 CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC TTCCAGAGA  
 AAAACATTCG TGGCTTCAAG CTCCCAGACA CACCTCAAGG CCTCCTGGGG  
 901 GAGGCCCGGA TGCTCAATGC CAGCATTGTG GCATCCTTCG TGGAGCTACC  
 GCTGGCCAGC APTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC  
 1001 AGACCTCACC CGCACCGATC CAGACCACTC CTCCCAAGGA CACTTGTAGC  
 CCGGAGCTGC TCATGTCCTT GATC**ACCGGT** GGTGGACCCA AATCTTGTGA  
 1101 CAAAACTCAC ACATGCCCAC CGTGCCCAGC ACCTGAACTC CTGGGGGGAC  
 CGTCAGTCTT CCTCTTCCCC CAAAACCCA AGGACACCCT CATGATCTCC  
 1201 CGGACCCCTG AGGTACATG CGTGGTGGTG GACGTGAGCC ACGAAGACCC  
 TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG CATAATGCCA  
 1301 AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG TGTGGTCAGC  
 GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG AGTACAAGTG  
 1401 CAAGGTCTCC AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACCAFTCTCA  
 AAGCCAAAGG GCAGCCCCGA GAACCACAGG TGTACACCCT GCCCCATCC  
 1501 CGGGAGGAGA TGACCAAGAA CCAGGTCAGC CTGACCTGCC TGGTCAAAGG  
 CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT GGGCAGCCGG  
 1601 AGAACAACTA CAAGACCAGC CCTCCCCTGC TGGACTCCGA CGGCTCCTTC  
 TTCCTCTATA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC AGCAGGGGAA  
 1701 CGTCTTCTCA TGCTCCGTGA TGCATGAGGC TCTGCACAAC CACTACACGC  
 AGAAGAGCCT CTCCCTGTCC CCGGGTAAAT GA

(SEQ ID NO: 26)

Fig. 31

SUBSTITUTE SHEET (RULE 26)

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**Amino acid sequence of hENG(26-359)-hFc with N-terminally truncated Fc domain**

1 MDAMKRGLCC VLLLCGAVFV SPCAETVHCD LQVGP~~PERDE~~ VTYTTSQVSK  
 51 GCVAQAFNAI LEVHVLFLFEP PTGPSOLELT LOASKONGTW PREVLLVLSV  
 101 NSSVFLHLOA LGIPLHLAYN SSLVTFQ~~EPP~~ GVNTE~~ELPSF~~ PKTQILEWAA  
 151 ERGPITSAAE LNDPOSILLR LGQAQGSLSF CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGD~~LDAVL~~  
 251 ILOGPPYVSW LIDANHNMQI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLQTS~~PAPI~~ QTTPPKDTCS  
 351 PELLMSLITG GGTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT  
 401 CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH  
 451 QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK  
 501 NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTTPVLDS DGSFFLYSKL  
 551 TVDKSRWQQG NVFSCSV~~MHE~~ ALHNHYTQKS LSLSPGK

(SEQ ID NO: 27)

Fig. 32

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**Nucleotide sequence encoding hENG(26-359)-hFc with  
N-terminally truncated Fc domain**

```

1  ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC
   AGTCTTCGTT TCGCCCGGCG CCGAAACAGT CCATTGTGAC CTCAGCCTG
101 TGGGCCCCGA GAGGGACGAG GTGACATATA CCACTAGCCA GGTCTCGAAG
   GGCTCCGTGG CTCAGGCCCC CAATGCCATC CTTGAAGTCC ATGTCCTCTT
201 CCTGGAGTTC CCAACGGGCC CGTCACAGCT GGAGCTGACT CTCCAGGCAT
   CCAAGCAAAA TGGCACCTGG CCCCAGAGAG TGCTTCTGGT CCTCAGTGTA
301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACTT
   GGCCTACAAT TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA
401 CCACAGAGCT GCCATCCTTC CCCAAGACCC AGATCCTTGA GTGGGCAGCT
   GAGAGGGGCC CCATCACCTC TGCTGCTGAG CTGAATGACC CCCAGAGCAT
501 CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCCTTC TGCATGCTGG
   AAGCCAGCCA GGACATGGGC CGCACGCTCG AGTGGCGGCC GCGTACTCCA
601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC
   GCACATCCTG AGGGTCCTGC CGGGCCACTC GGCCGGGCC CCGACGGTGA
701 CGGTGAAGGT GGAAGTGAAG TGCGCACCCG GGGATCTCGA TGCCGTCCTC
   ATCCTGCAGG GTCCCCCCTA CGTGTCTCTG CTCATCGACG CCAACCACAA
801 CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC TTCCAGAGA
   AAAACATTCG TGGCTTCAAG CTCCCAGACA CACCTCAAGG CCTCCTGGGG
901 GAGGCCCGGA TGCTCAATGC CAGCATTGTG GCATCCTTCG TGGAGCTACC
   GCTGGCCAGC APTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC
1001 AGACCTCACC CGCACCGATC CAGACCACTC CTCCCAAGGA CACTTGTAGC
   CCGGAGCTGC TCATGTCCTT GATCACCGGT GGTGGAAACTC ACACATGCCC
1101 ACCGTGCCCA GCACCTGAAC TCCTGGGGGG ACCGTCAGTC TTCTCTTCC
   CCCCAAAACC CAAGGACACC CTCATGATCT CCGGACCCC TGAGGTCACA
1201 TGGGTGGTGG TGGACGTGAG CCACGAAGAC CCTGAGGTCA AGTTCAACTG
   GTACGTGGAC GGCCTGGAGG TGCATAATGC CAAGACAAAG COGCGGGAGG
1301 AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC
   CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC
1401 CCTCCCAGCC CCCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC
   GAGAACCACA GGTGTACACC CTGCCCCCAT CCGGGGAGGA GATGACCAAG
1501 AACCAGGTCA GCCTGACCTG CCTGGTCAA GECTTCTATC CCAGCGACAT
   CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC TACAAGACCA
1601 CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA TAGCAAGCTC
   ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT
1701 GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT
   CCCCGGGTAA ATGA

```

(SEQ ID NO: 28)

Fig. 33

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**Amino acid sequence of hENG(26-346)-hFc with N-terminally truncated hFc domain**

1 MDAMKRGLCC VLLLCGAVFV SPCAETVHCD LQVGP~~PERDE~~ VTYTTSQVSK  
 51 GCVAQAFNAI LEVHVLFLFPTGPSOLELT LOASKONGTW PREVLLVLSV  
 101 NSSVFLHLOA LGIPLHLAYN SSLVTFQEPP GVNTELP~~SPF~~ PKTQILEWAA  
 151 ERGPITSAAE LNDPOSILLR LGQAQGSLSF CMLEASQDMG RTLEWRPRTF  
 201 ALVRGCHLEG VAGHKEAHIL RVLPGHSAGP RTVTVKVELS CAPGD~~LDAVL~~  
 251 ILOGPPYVSW LIDANHNMQI WTTGEYSFKI FPEKNIRGFK LPDTPQGLLG  
 301 EARMLNASIV ASFVELPLAS IVSLHASSCG GRLQTS~~PAPI~~ Q~~TTPT~~TGGGT  
 351 HTCPPCPAPE LLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV  
 401 KFNWYVDGVE VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV  
 451 SNKALPAPIE KTISKAKGQP REPQVYTLPP SREEMTKNQV SLTCLVKGFY  
 501 PSDIAVEWES NGOPENNYKT TPPVLDS~~DGS~~ FFLYSKLTVD KSRWQOGN~~VF~~  
 551 SCSVMHEALH NHYTQKSLSL SPGK

(SEQ ID NO: 29)

Fig. 34

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**Nucleotide sequence encoding hENG(26-346)-hFc with  
N-terminally truncated hFc domain**

1 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC AGTCTTCGTT  
 61 TCGCCCGGCG CCGAAACAGT CCATTGTGAC CTTCAGCCTG TGGGCCCCGA GAGGGACGAG  
 121 GTGACATATA CCACTAGCCA GGTCTCGAAG GGCTGCGTGG CTCAGGCCCC CAATGCCATC  
 181 CTTGAAGTCC ATGTCCTCTT CCTGGAGTTC CCAACGGGCC CGTCACAGCT GGAGCTGACT  
 241 CTCCAGGCAT CCAAGCAAAA TGGCACCTGG CCCCAGAGAG TGCTTCTGGT CCTCAGTGTA  
 301 AACAGCAGTG TCTTCCTGCA TCTCCAGGCC CTGGGAATCC CACTGCACTT GGCCTACAAT  
 361 TCCAGCCTGG TCACCTTCCA AGAGCCCCCG GGGGTCAACA CCACAGAGCT GCCATCCTTC  
 421 CCCAAGACCC AGATCCTTGA GTGGGCAGCT GAGAGGGGCC CCATCACCTC TGCTGCTGAG  
 481 CTGAATGACC CCCAGAGCAT CCTCCTCCGA CTGGGCCAAG CCCAGGGGTC ACTGTCCTTC  
 541 TGCATGCTGG AAGCCAGCCA GGACATGGGC CGCACGCTCG AGTGGCGGCC GCGTACTCCA  
 601 GCCTTGGTCC GGGGCTGCCA CTTGGAAGGC GTGGCCGGCC ACAAGGAGGC GCACATCCTG  
 661 AGGGTCCTGC CGGGCCACTC GGCCGGGCCC CGGACGGTGA CGGTGAAGGT GGAAGTGAAGC  
 721 TGCCGACCCG GGGATCTCGA TGCCGTCTTC ATCCTGCAGG GTCCCCCCTA CGTGTCTCTG  
 781 CTCATCGACG CCAACCACAA CATGCAGATC TGGACCACTG GAGAATACTC CTTCAAGATC  
 841 TTTCCAGAGA AAAACATTCG TGGCTTCAAG CTCCAGACA CACCTCAAGG CCTCCTGGGG  
 901 GAGGCCCGGA TGCTCAATGC CAGCATTTGG GCATCCTTCG TGGAGCTACC GCTGGCCAGC  
 961 APTGTCTCAC TTCATGCCTC CAGCTGCGGT GGTAGGCTGC AGACCTCACC CGCACCGATC  
 1021 CAGACCACTC CTCCCACCGG TGGTGGAACT CACACATGCC CACCGTGCCC AGCACCTGAA  
 1081 CTCTTGGGGG GACCGTCAGT CTTCTCTTTC CCCCCAAAAC CCAAGGACAC CCTCATGATC  
 1141 TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA GCCACGAAGA CCCTGAGGTC  
 1201 AAGTTCAACT GGTACGTGGA CGGCGTGGAG GTGCATAATG CCAAGACAAA GCCGCGGGAG  
 1261 GAGCAGTACA ACAGCACGTA CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG  
 1321 CTGAATGGCA AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG  
 1381 AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC CCTGCCCCCA  
 1441 TCCCGGGAGG AGATGACCAA GAACCAGGTC AGCCTGACCT GCCTGGTCAA AGGCTTCTAT  
 1501 CCCAGCGACA TCGCCGTGGA GTGGGAGAGC AATGGGCAGC CGGAGAACAA CTACAAGACC  
 1561 ACGCCTCCCG TGCTGGACTC CGACGGCTCC TTCTTCTCT ATAGCAAGCT CACCGTGGAC  
 1621 AAGAGCAGGT GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC  
 1681 AACCACTACA CGCAGAAGAG CCTCTCCCTG TCCCCGGGTA AATGA

(SEQ ID NO: 30)

Fig. 35

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Size exclusion chromatogram of hENG-hFc proteins after initial purification  
hENG(26-586)-hFc

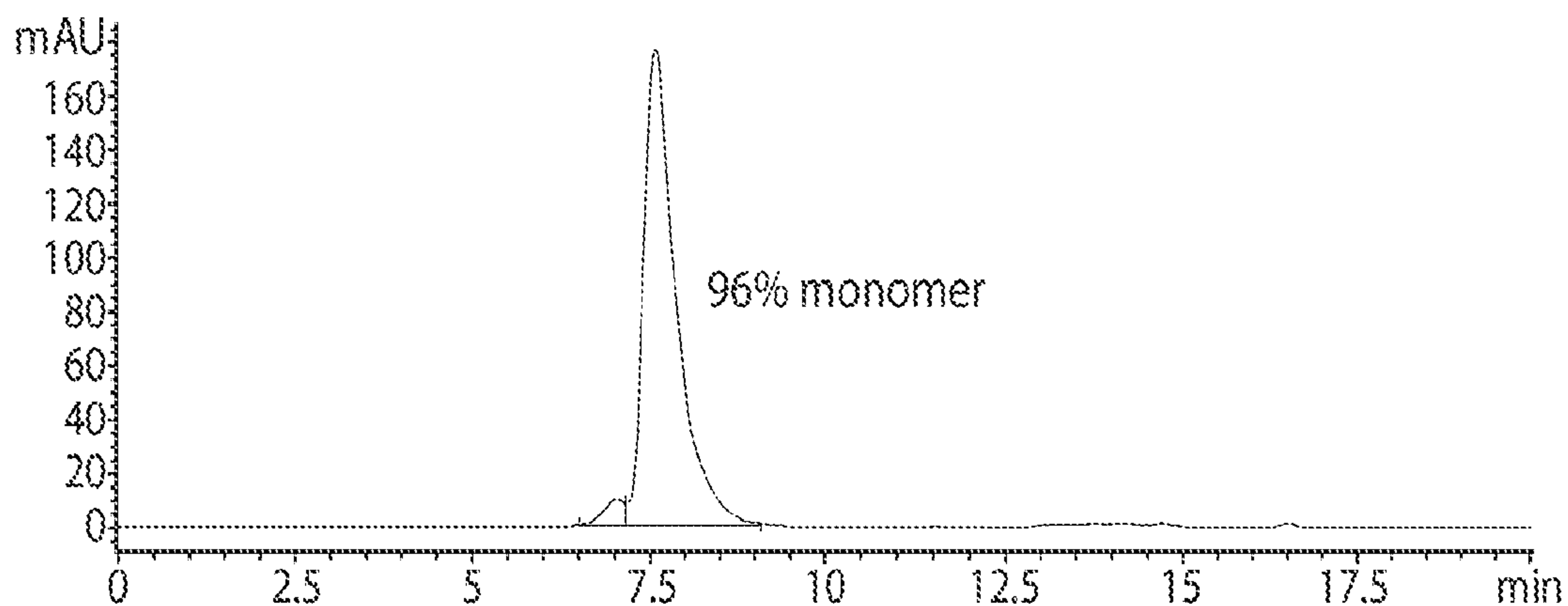


Fig. 36A

Size exclusion chromatogram of hENG-hFc proteins after initial purification  
hENG(26-359)-hFc

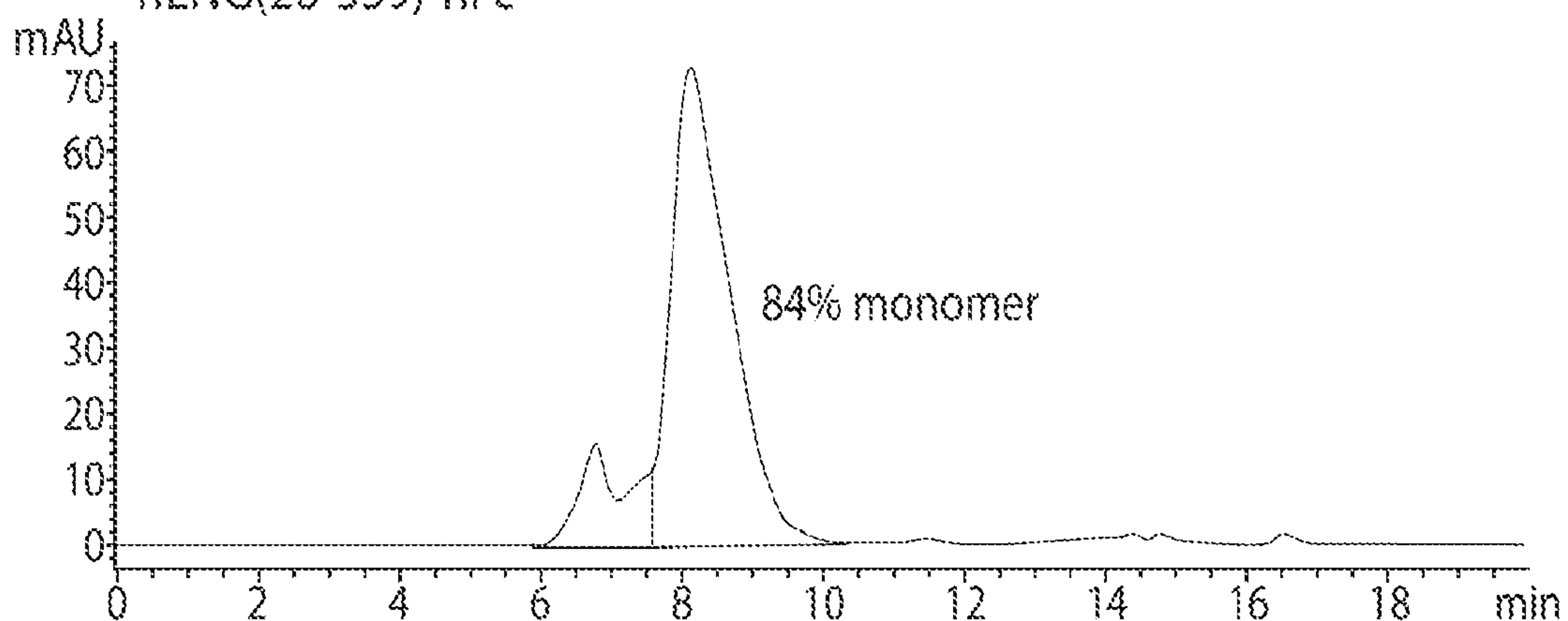


Fig. 36B

Size exclusion chromatogram of hENG-hFc proteins after initial purification

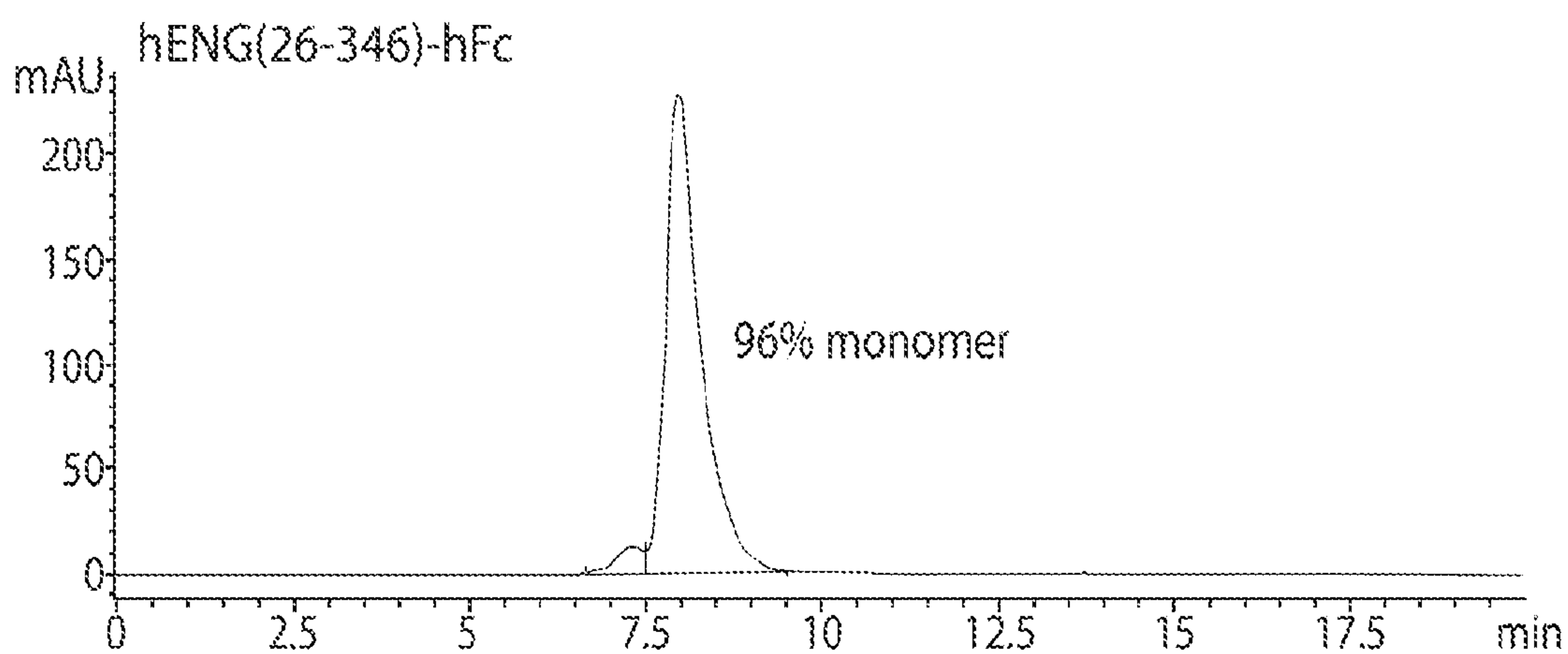


Fig. 36C

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## Characterization of high-affinity binding of BMP-9 to hENG-hFc variants

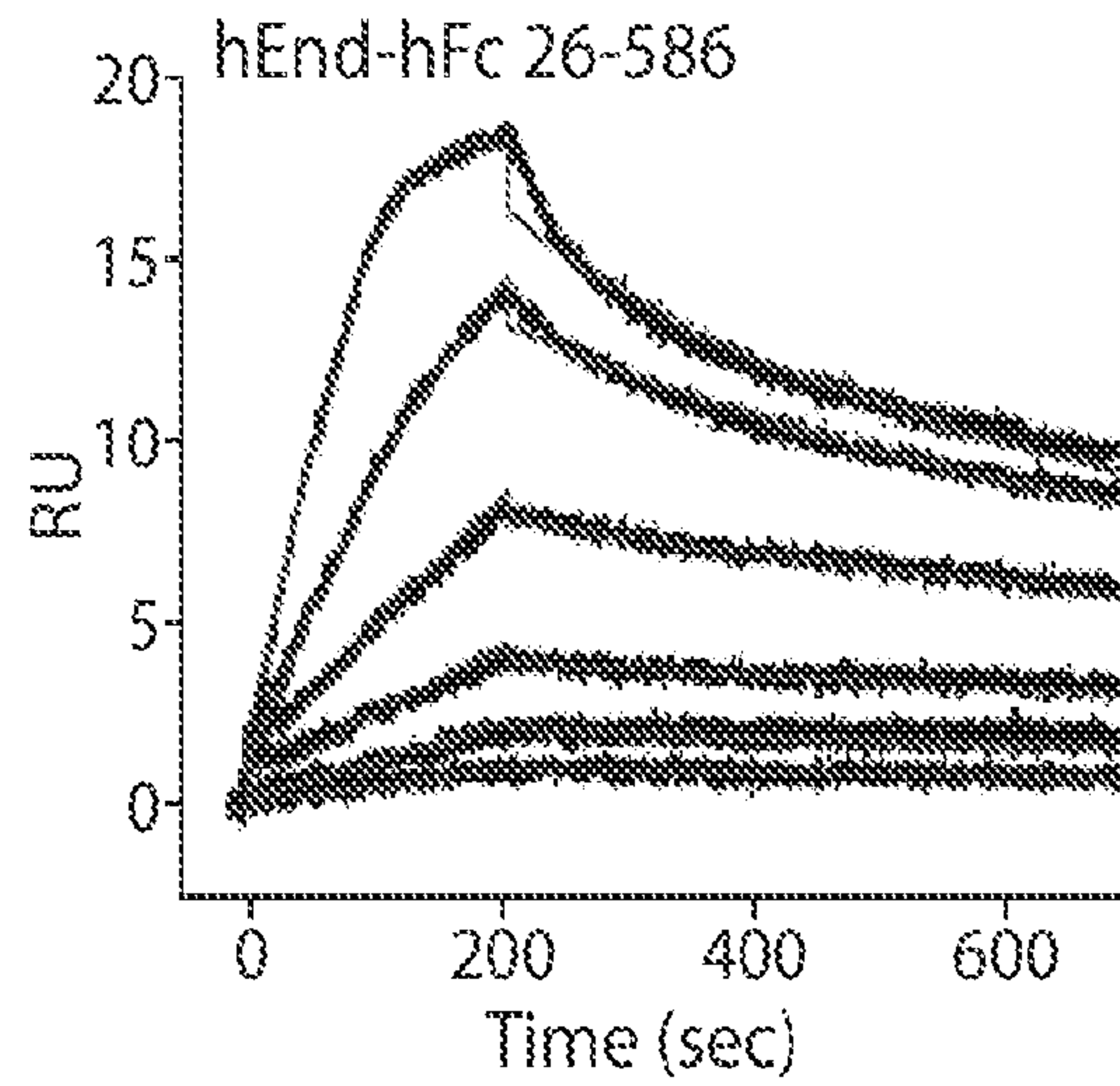


Fig. 37A

## Characterization of high-affinity binding of BMP-9 to hENG-hFc variants

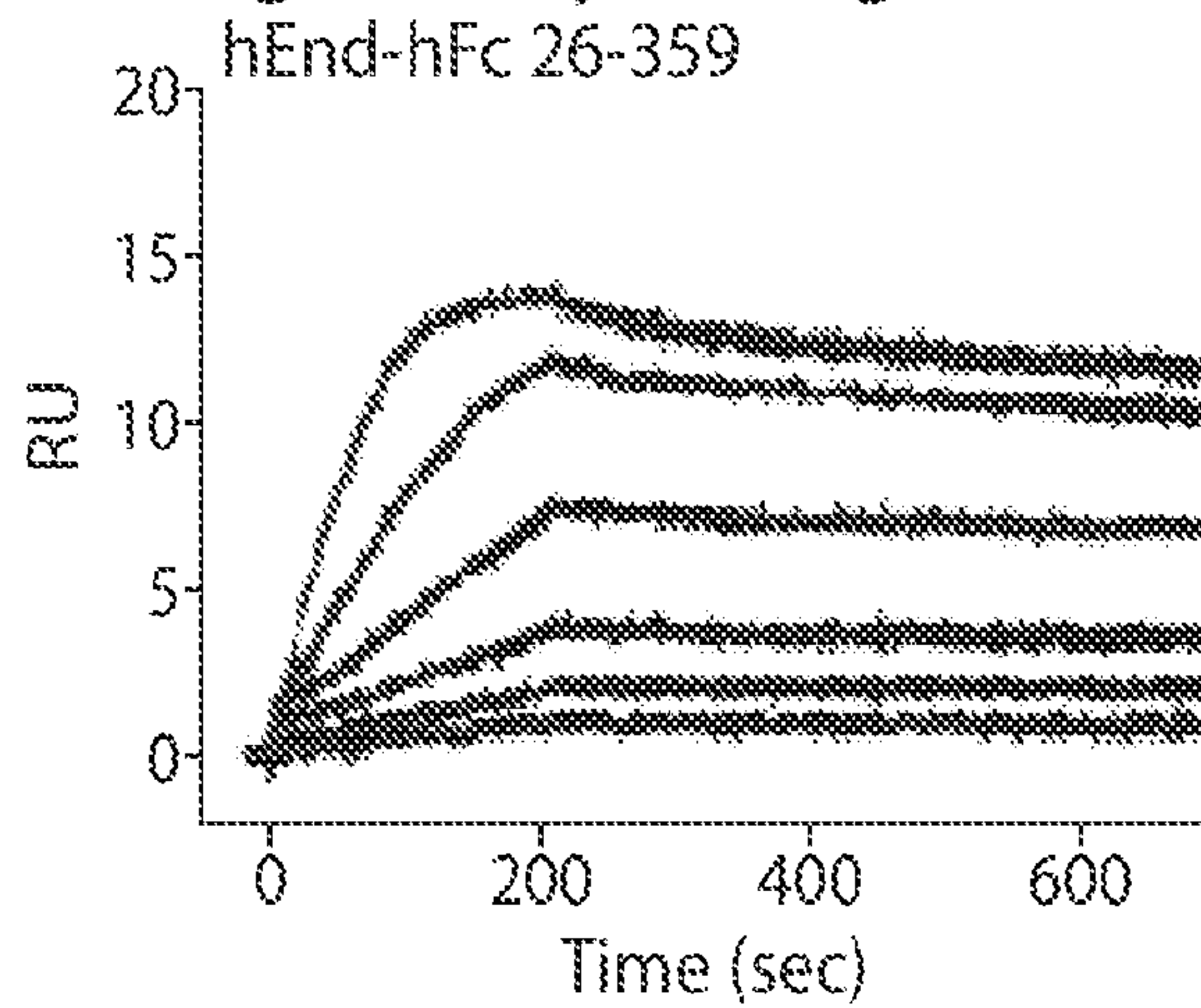


Fig. 37B

## Characterization of high-affinity binding of BMP-9 to hENG-hFc variants

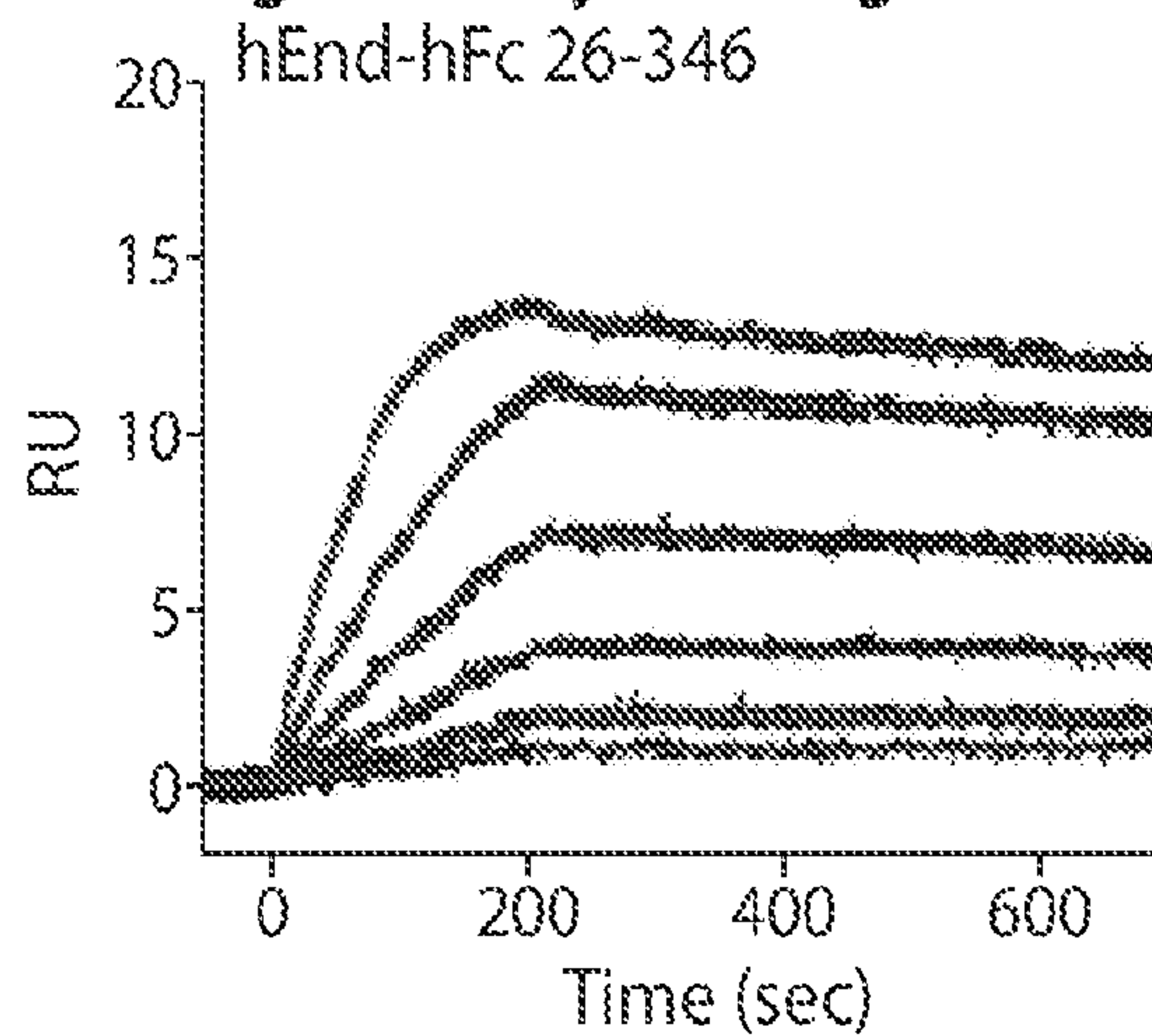
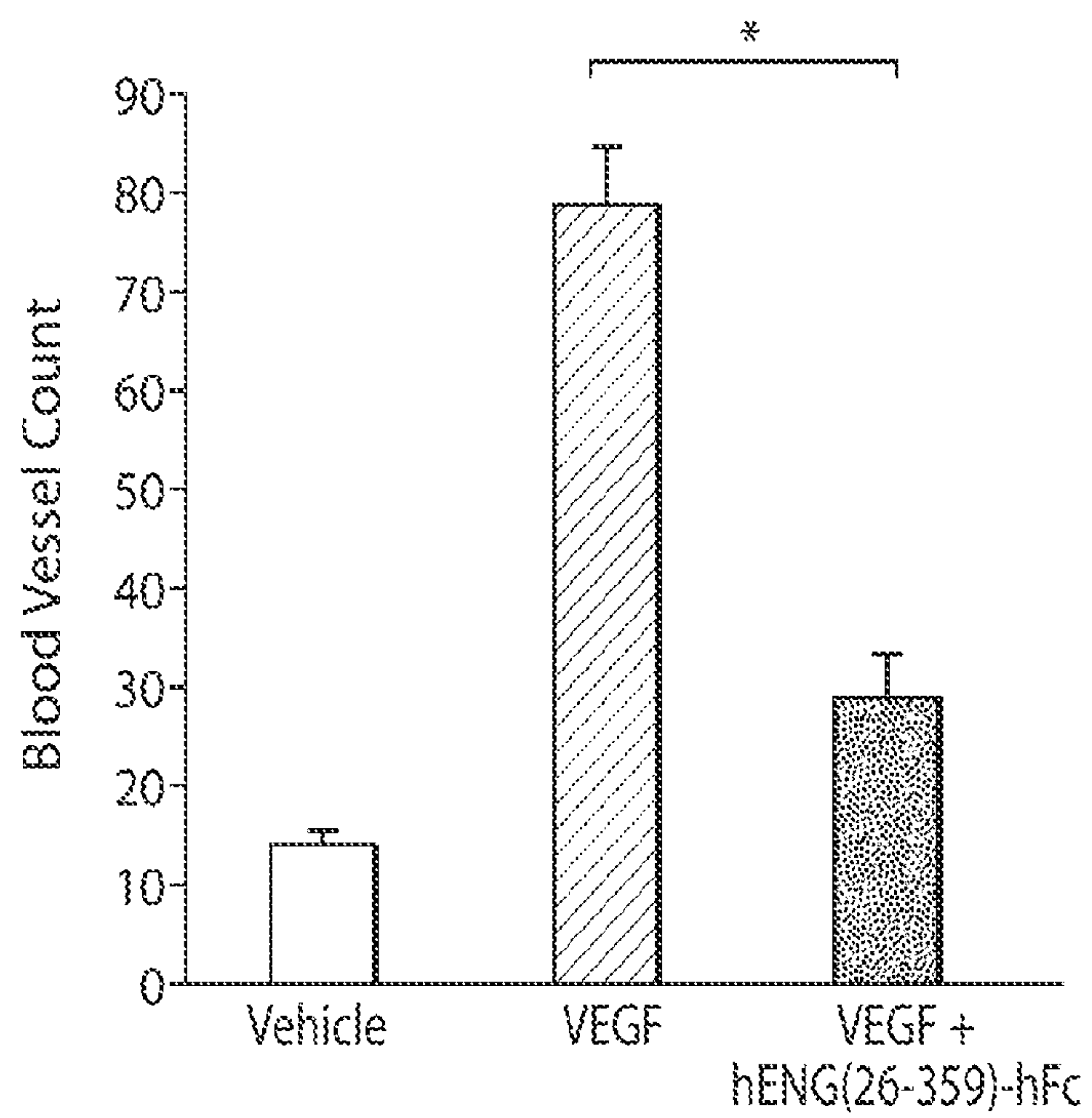


Fig. 37C

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**hENG(26-359)-hFc inhibits VEGF-stimulated angiogenesis  
in a CAM assay**



**Fig. 38**



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Effect of hENG(26-346)-hFc on growth-factor stimulated angiogenesis in a mouse angioreactor assay

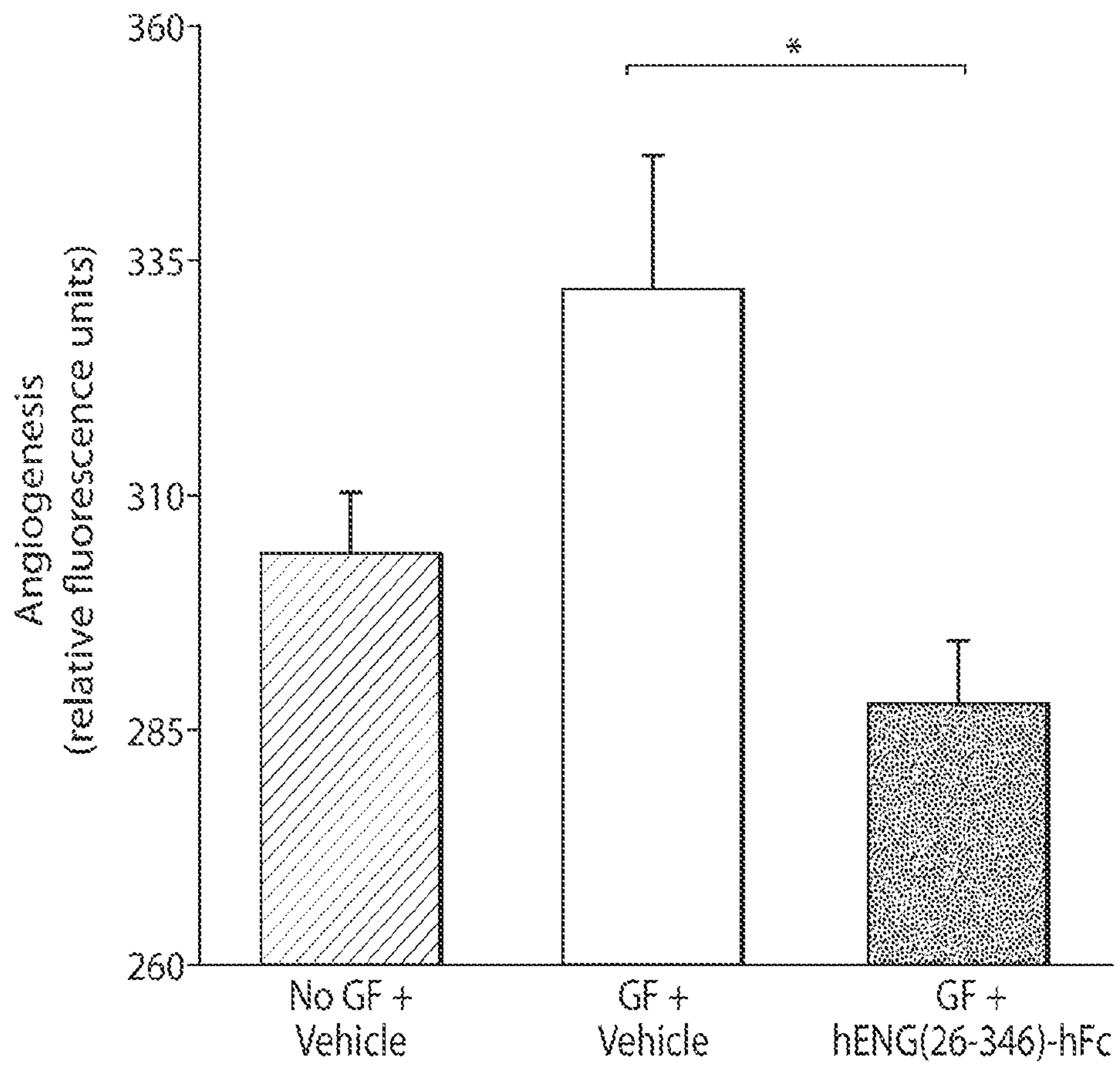


Fig. 39

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Effect of mENG(27-581)-mFc on growth of 4T1 mammary tumors in mice

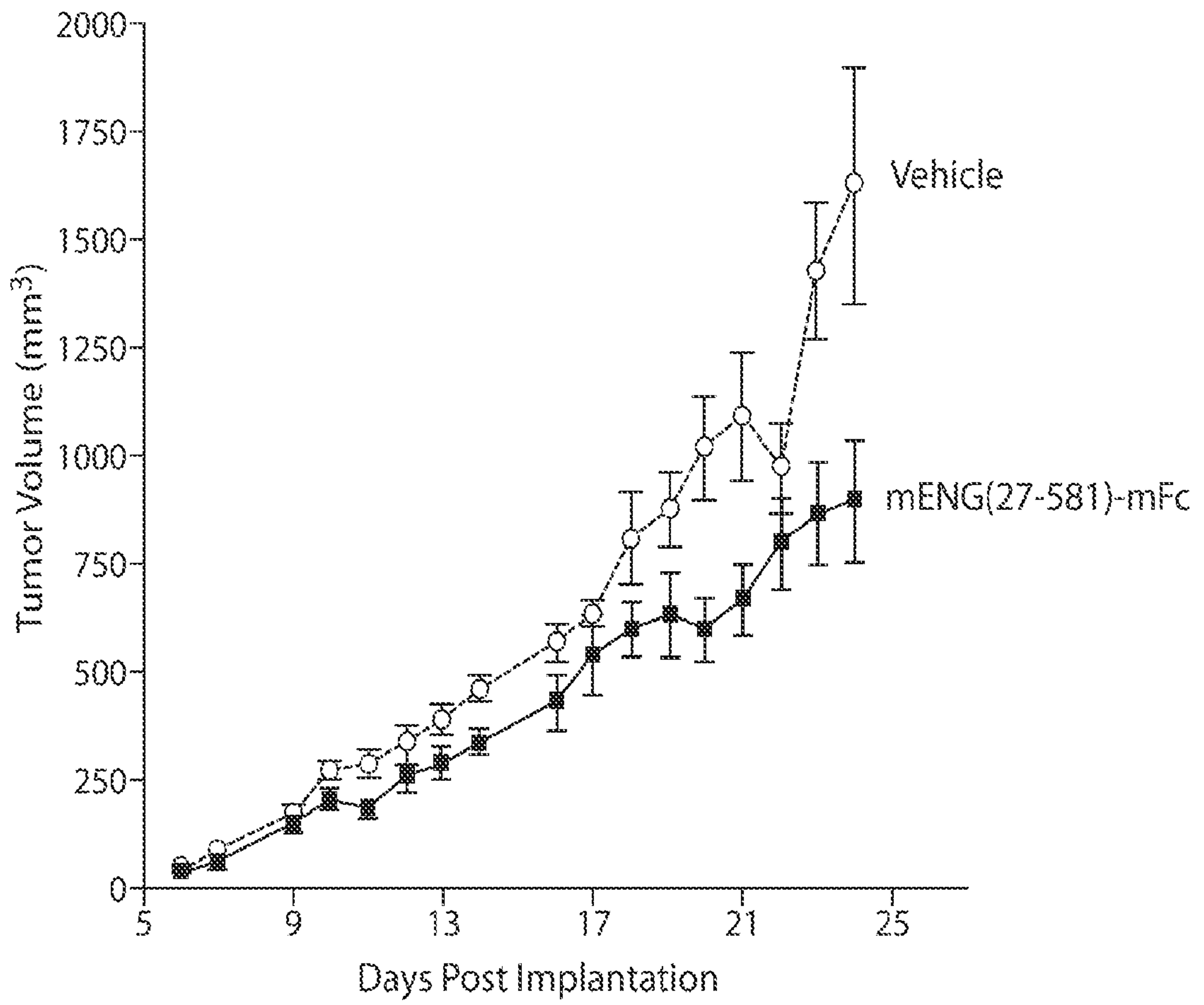


Fig. 40

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Effect of mENG(27-581)-mFc on growth of Colon-26 tumors in mice

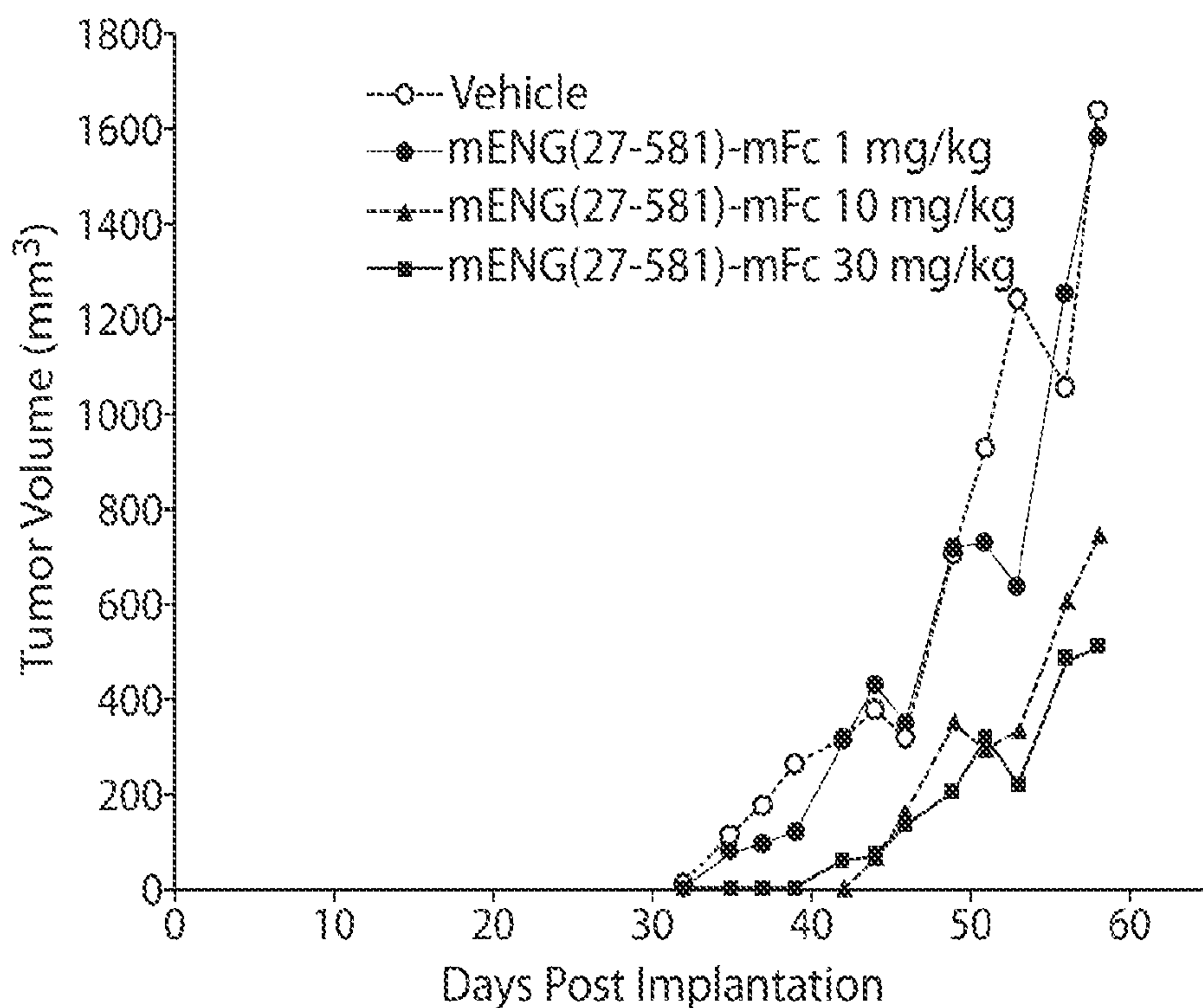


Fig. 41

Liver as %Body Weight

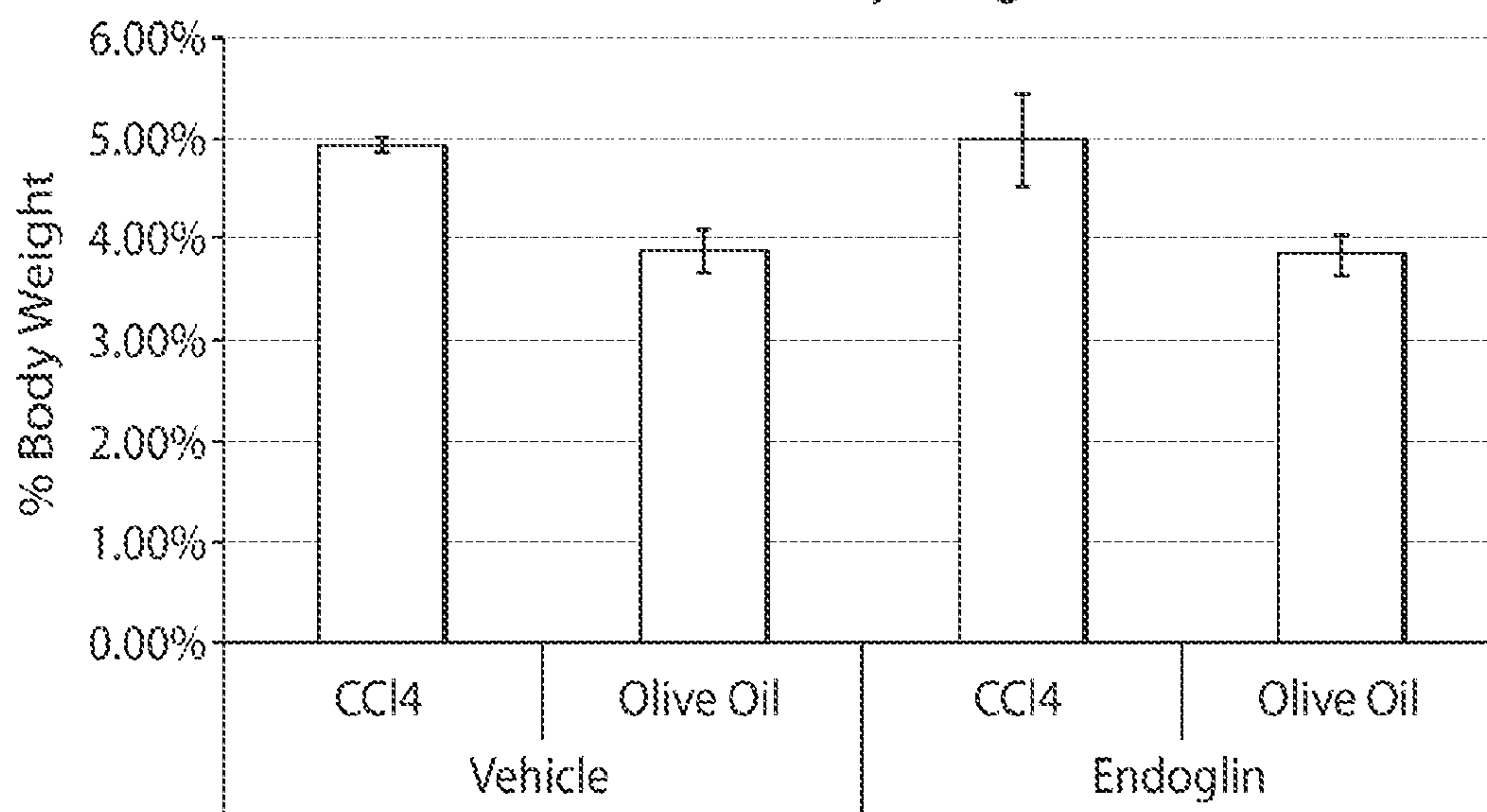


Fig. 42

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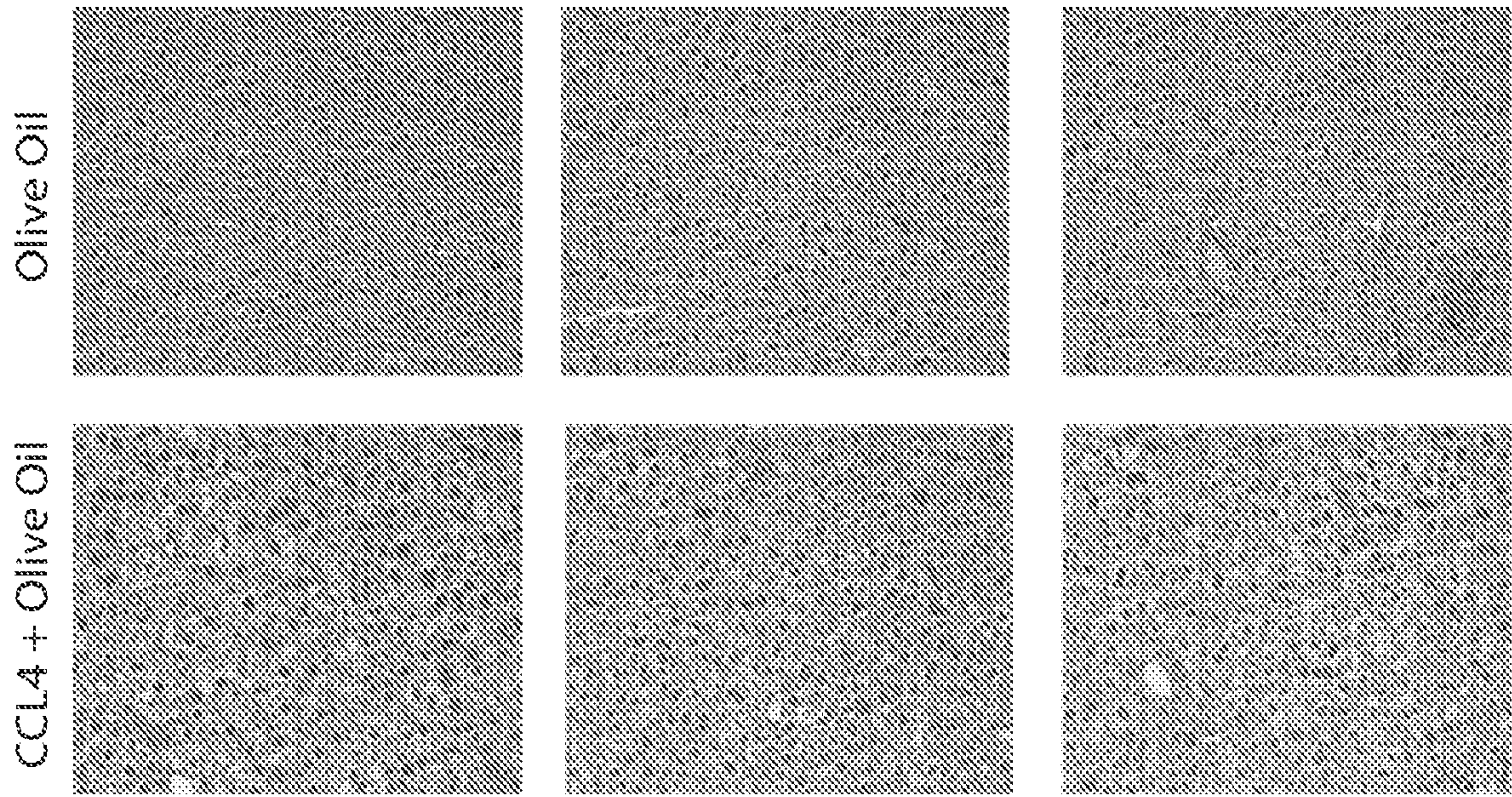


Fig. 43

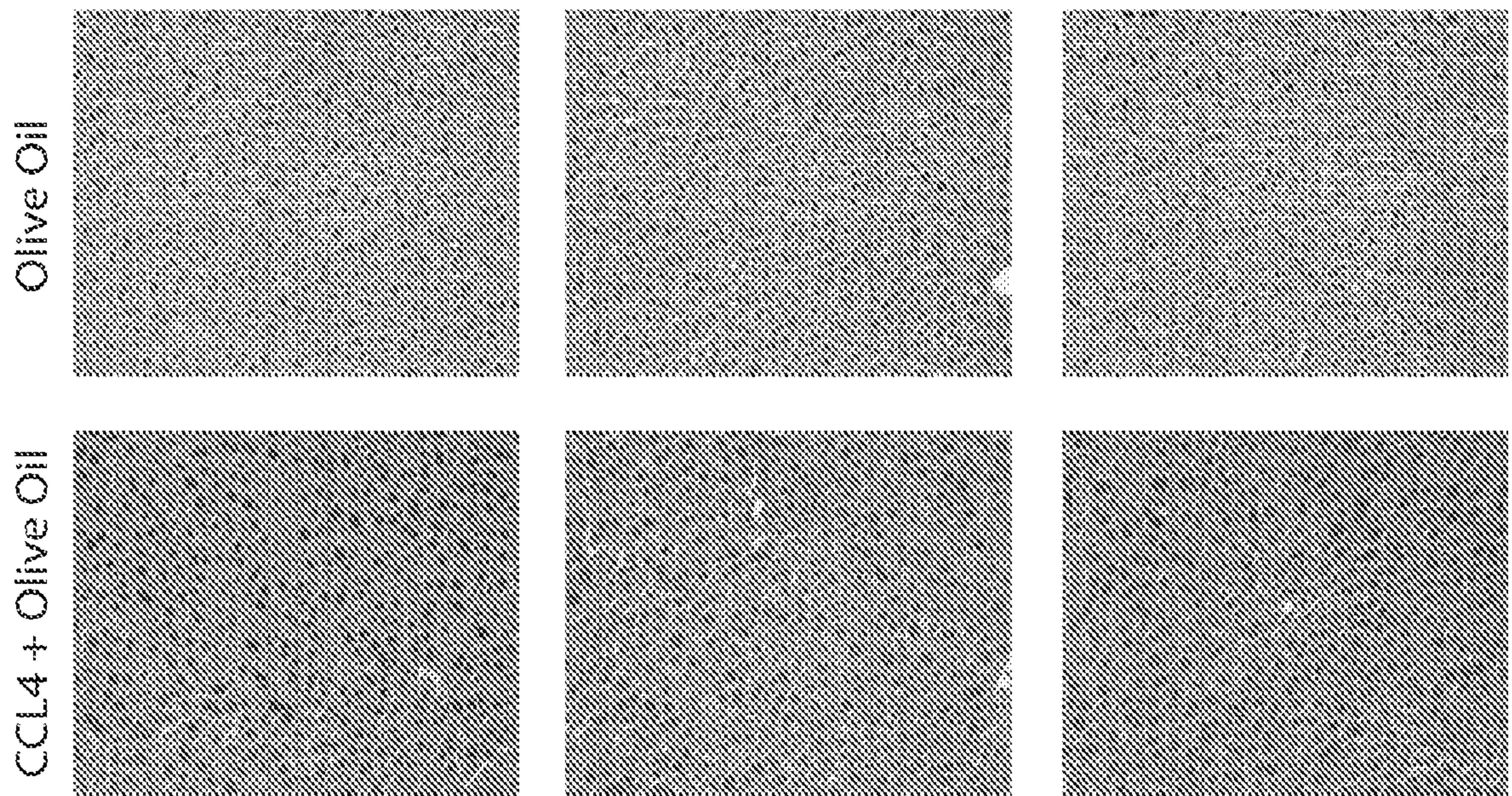


Fig. 44

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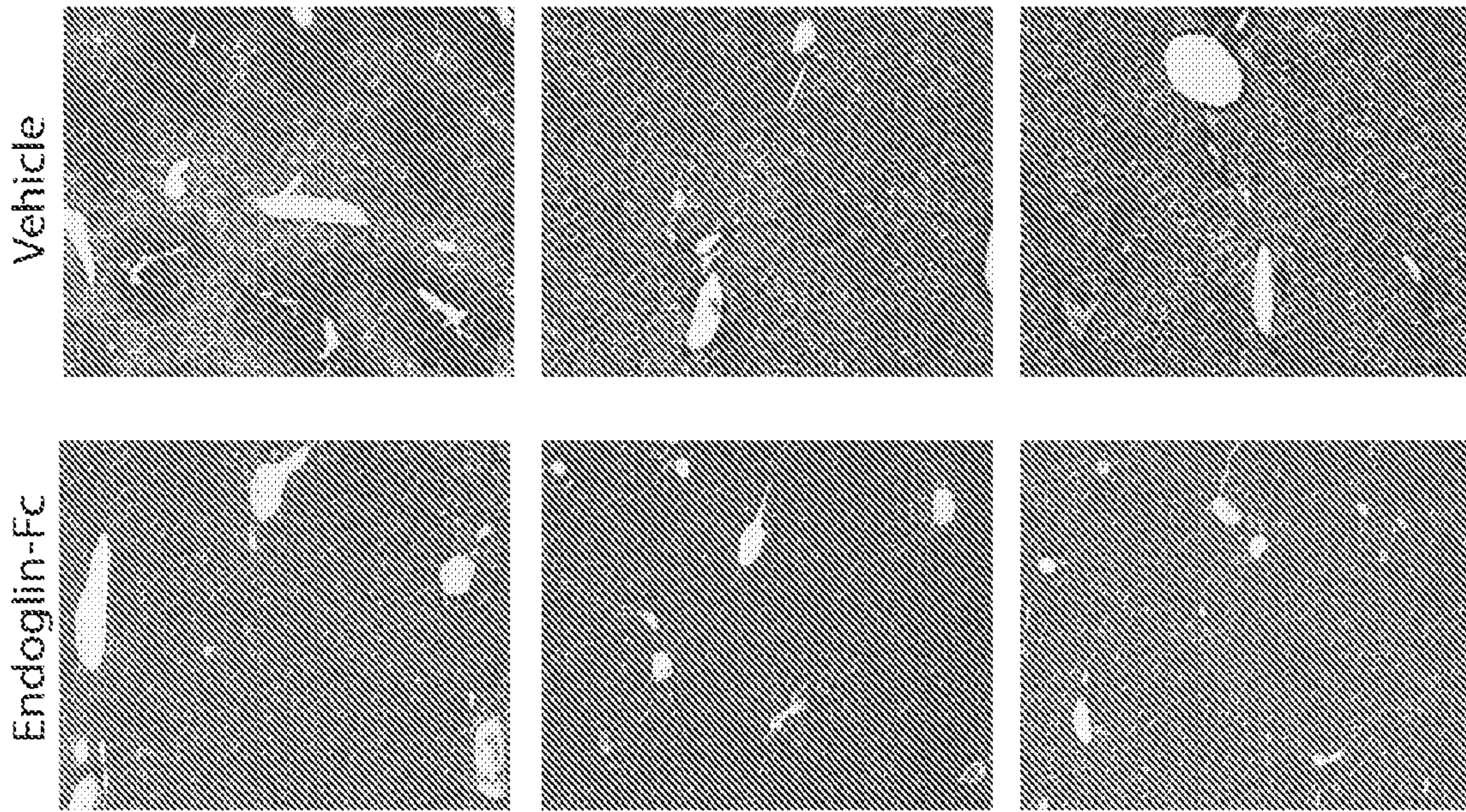
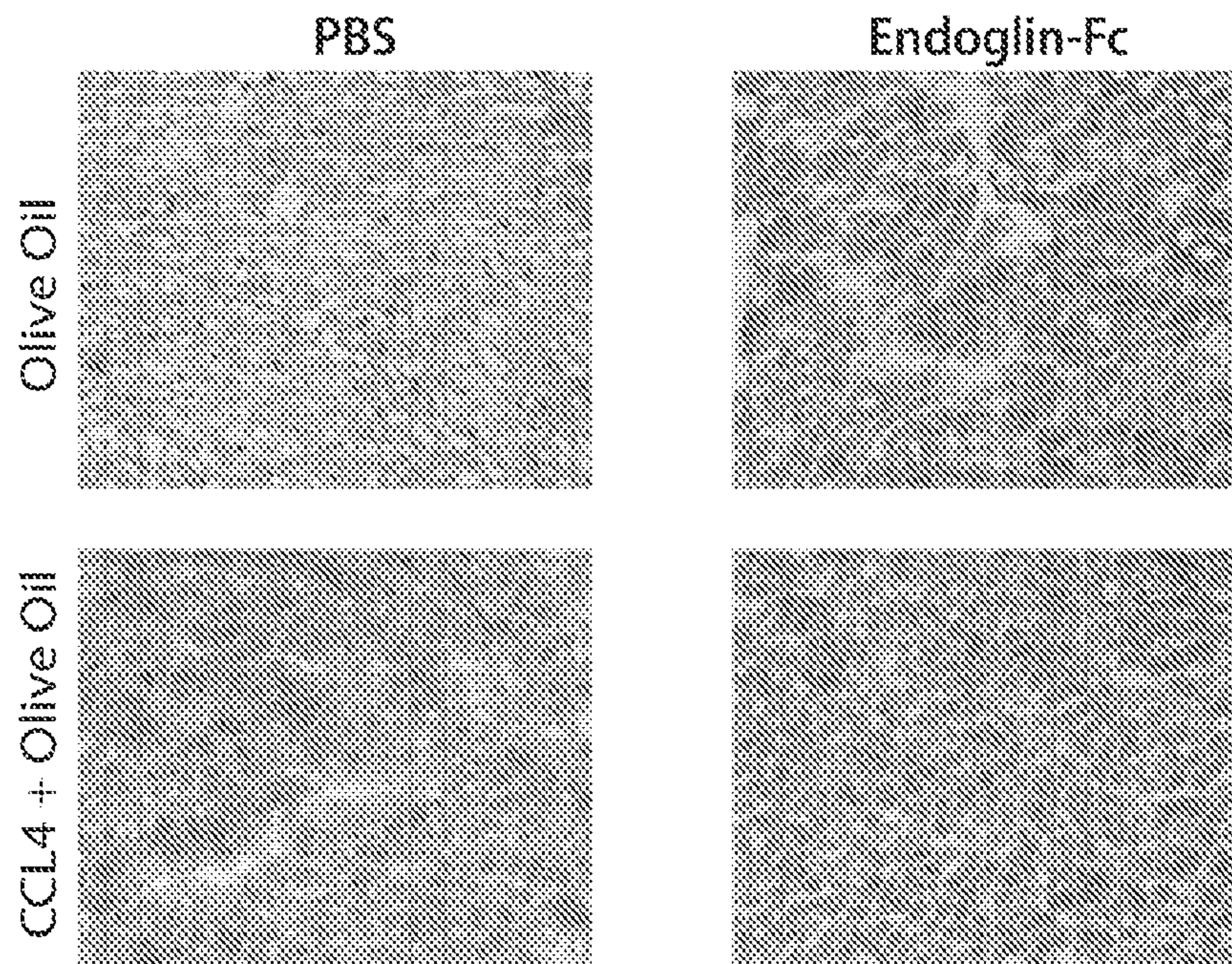


Fig. 45



Endoglin-Fc treated animals had the lowest percentage of livers with extensive positive oil red o staining

Fig. 46

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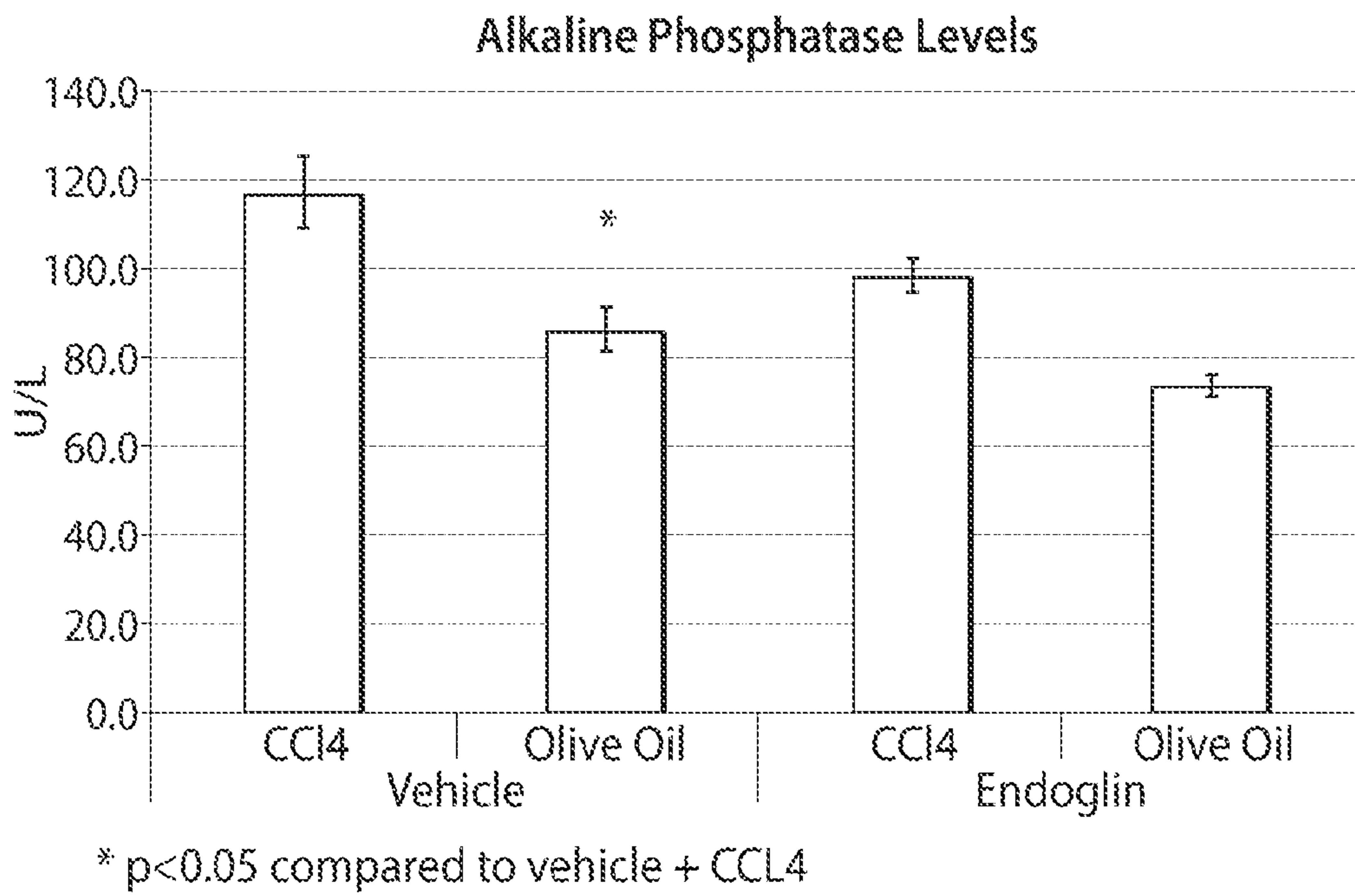


Fig. 47

Vehicle

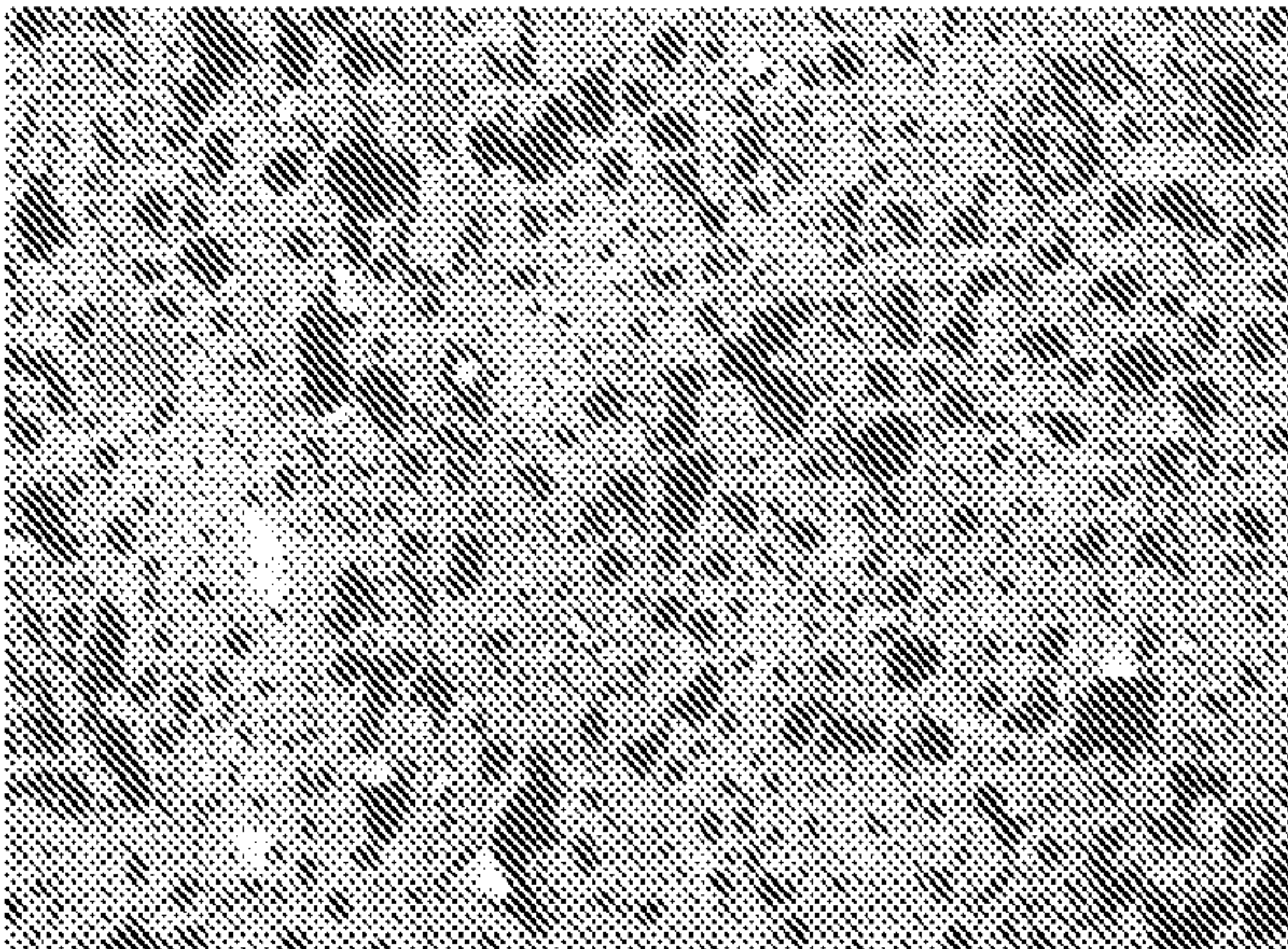


Fig. 48A

ENG-Fc

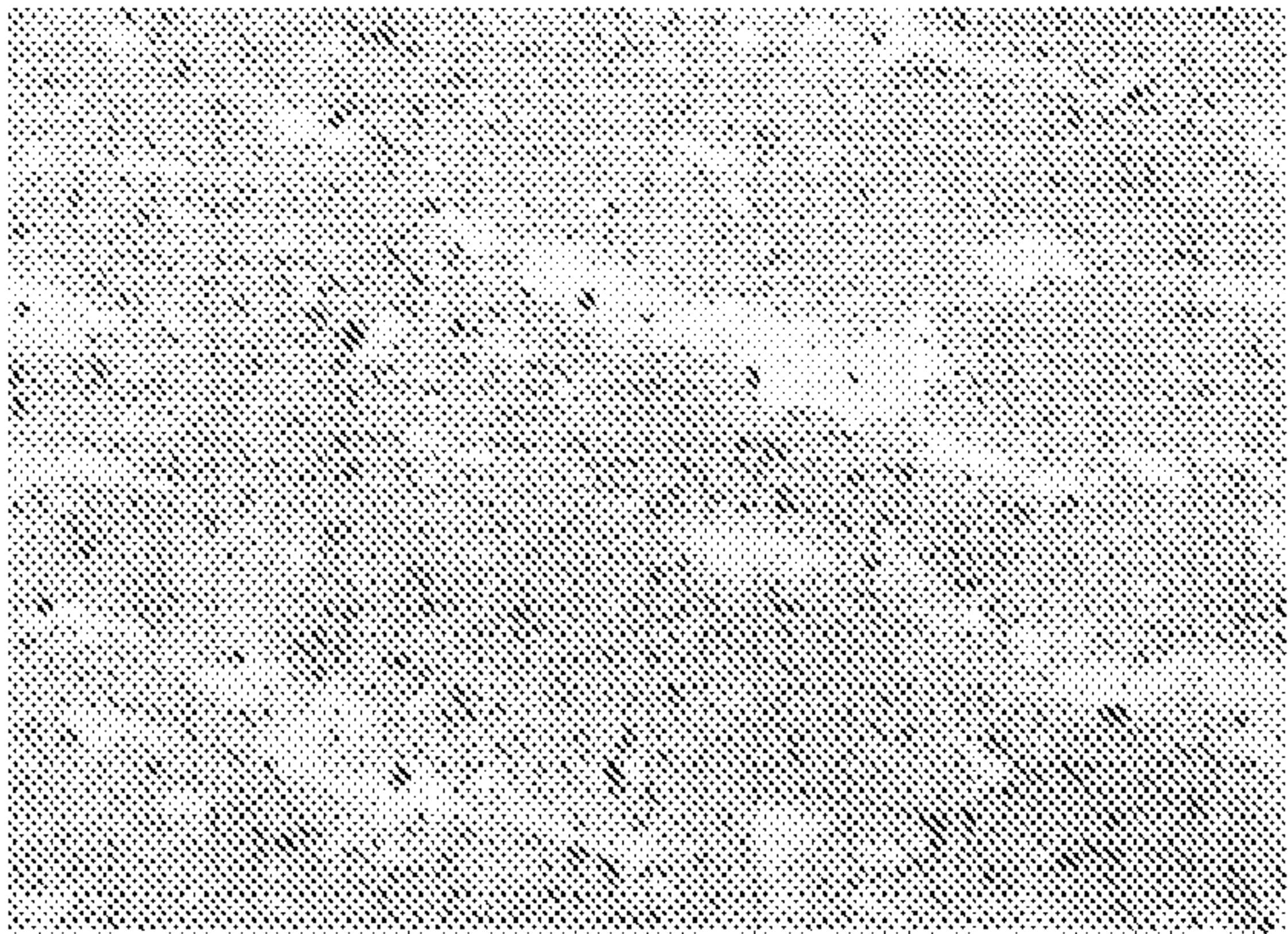


Fig. 48B

Vehicle

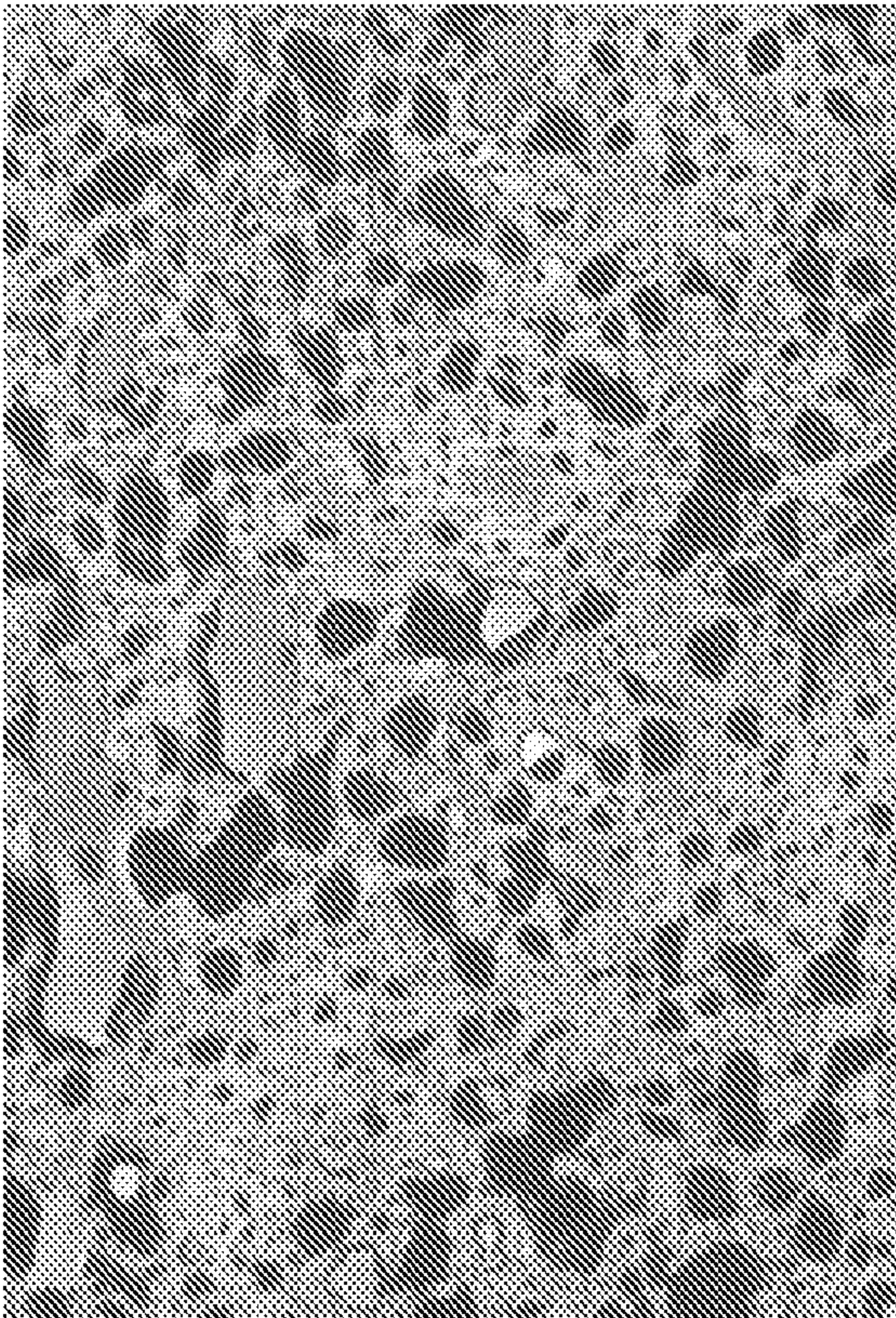


Fig. 48C

ENG-Fc

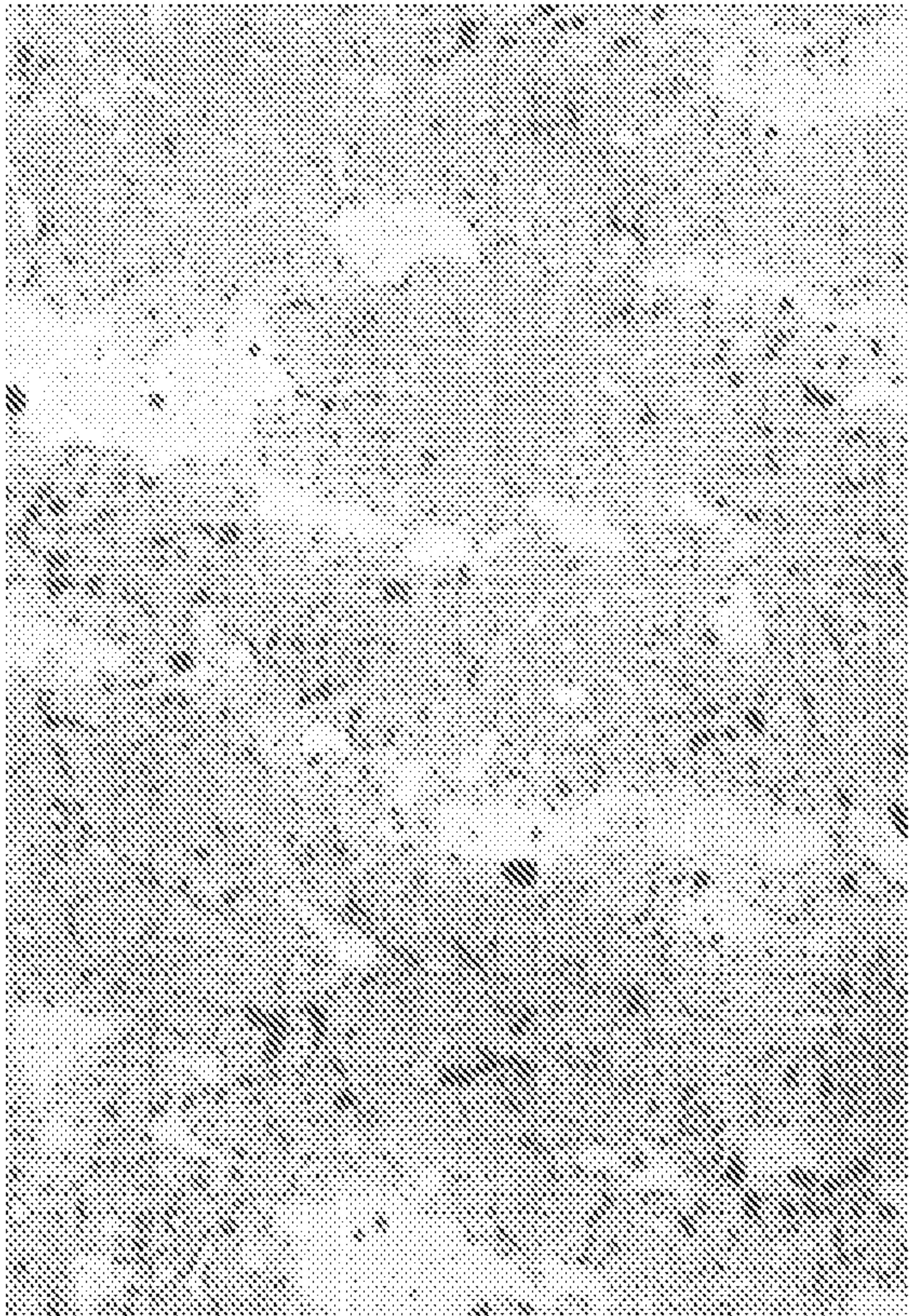


Fig. 48D

Vehicle

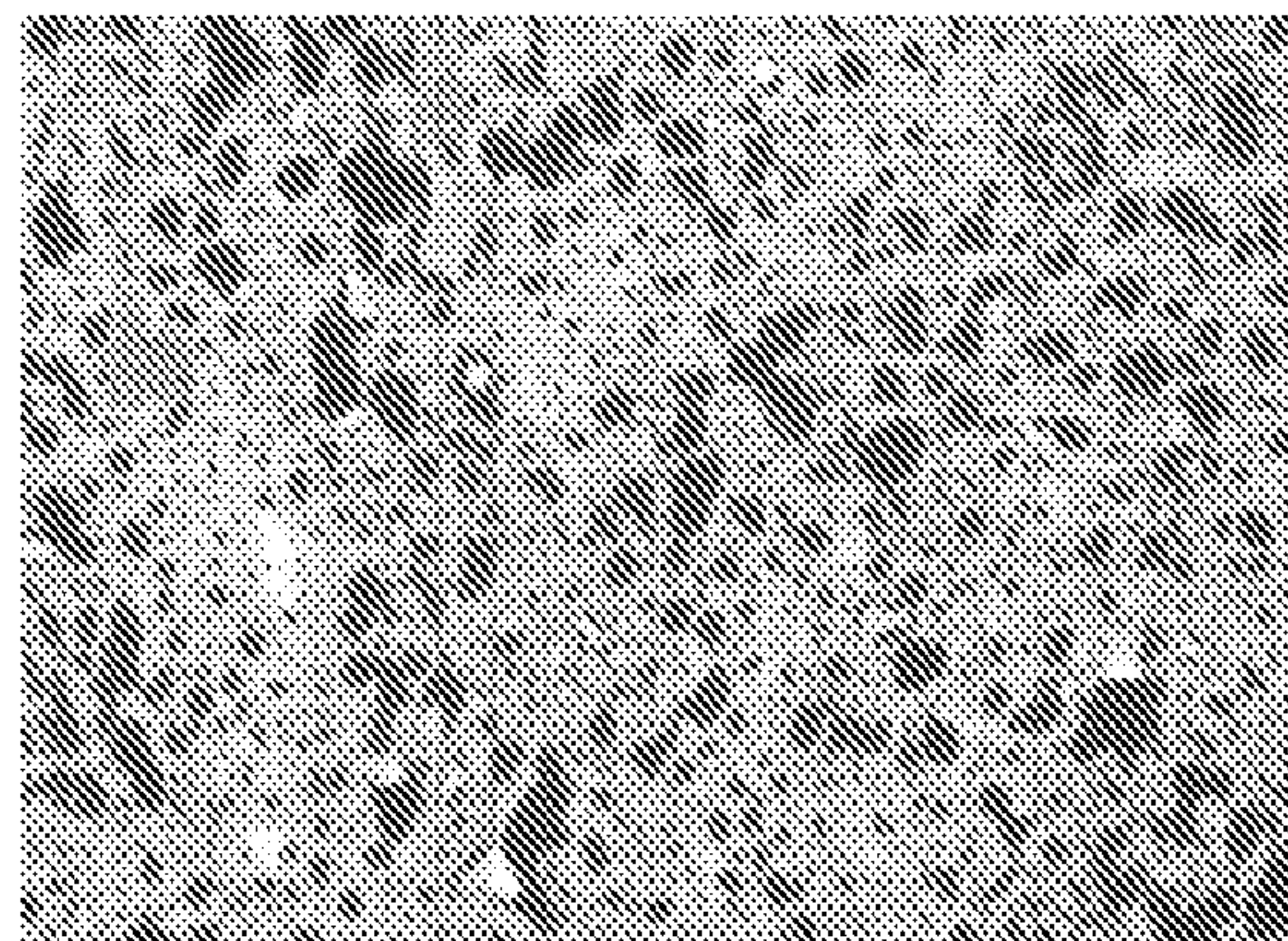


Fig. 48A

ENG-Fc

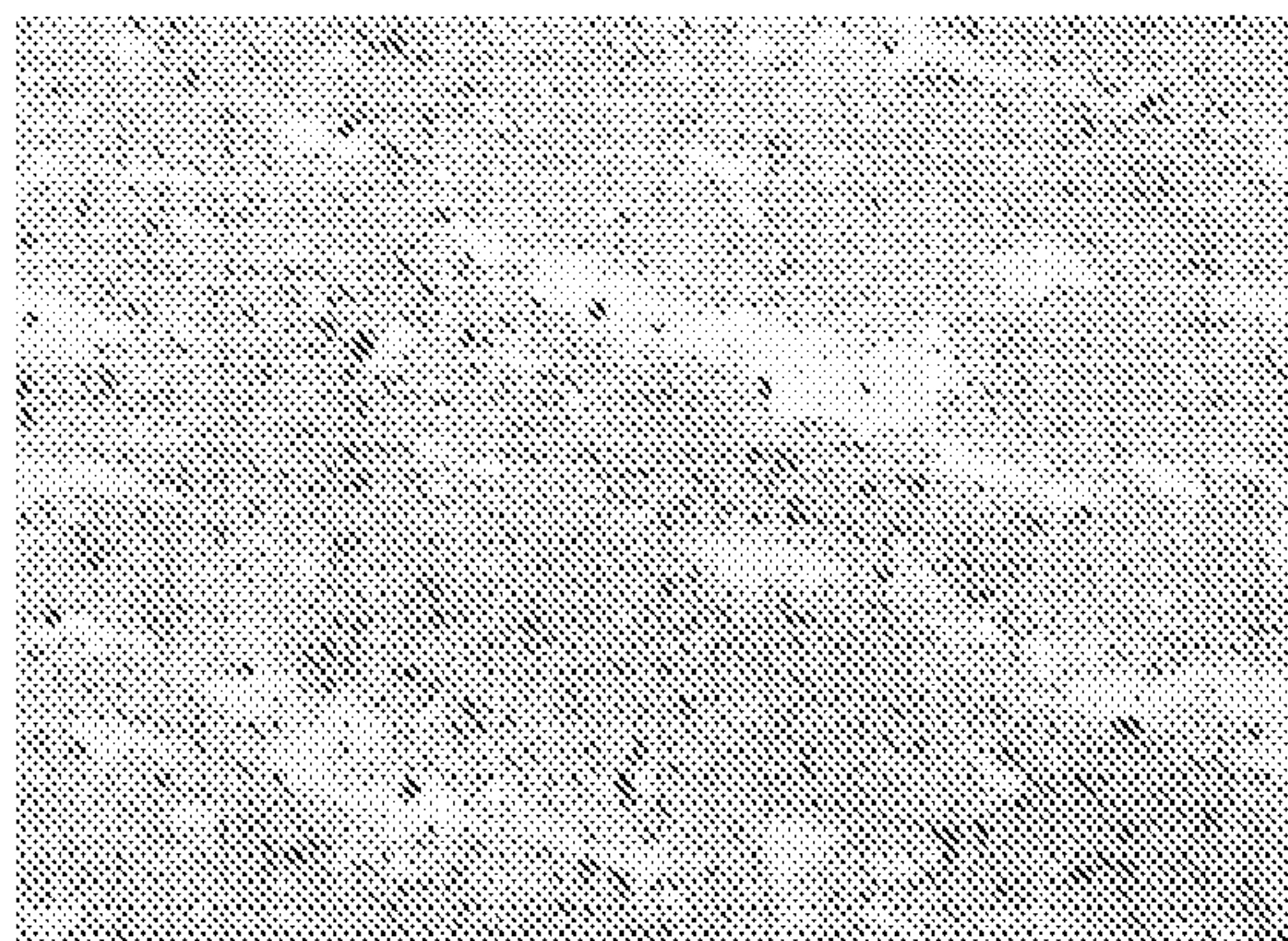


Fig. 48B

Vehicle

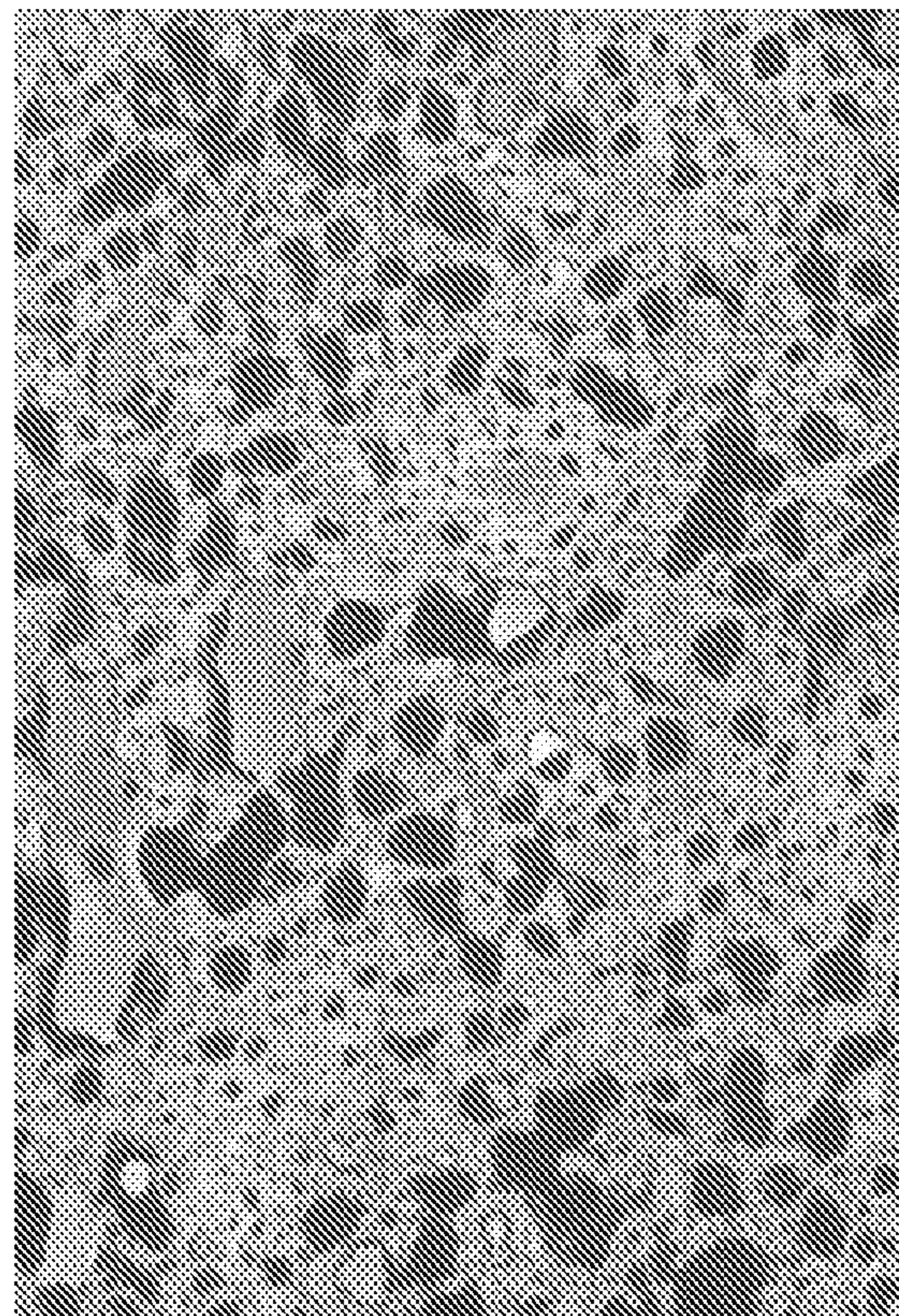


Fig. 48C

ENG-Fc

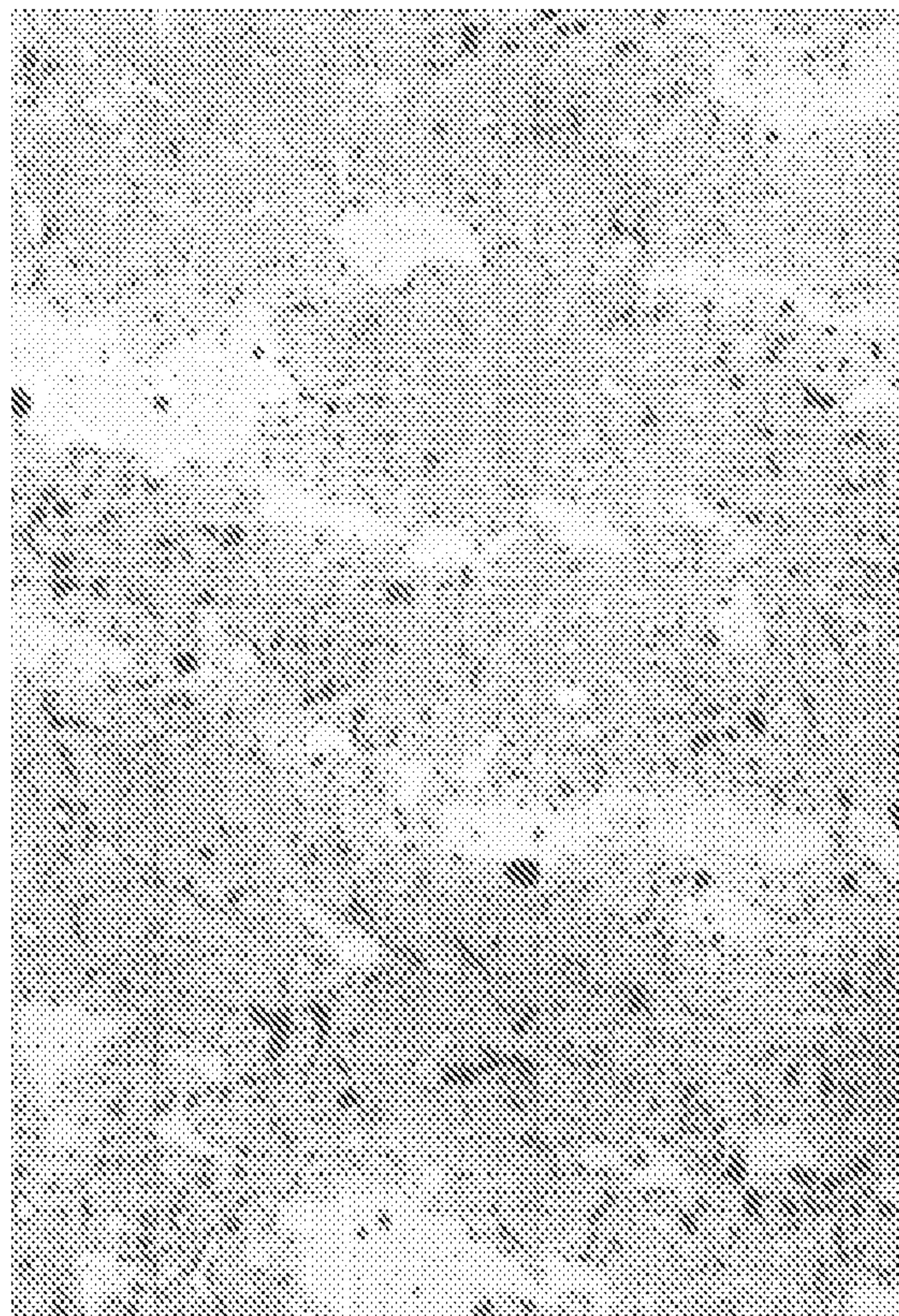


Fig. 48D