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(54) Titre : EXPRESSION ET EXPORTATION D'INHIBITEURS DE L'ANGIOGENESE SOUS FORME
 D'IMMUNOFUSINES

(54) Title: EXPRESSION AND EXPORT OF ANGIOGENESIS INHIBITORS AS IMMUNOFUSINS

(57) **Abrégé/Abstract:**

Disclosed are nucleotide sequences, for example, DNA or RNA sequences, which encode an immunoglobulin Fc-angiogenesis inhibitor fusion protein. The angiogenesis inhibitors can be angiostatin, endostatin, a plasminogen fragment having angiostatin activity, or a collagen XVIII fragment having endostatin activity. The nucleotide sequences can be inserted into a suitable expression vector and expressed in mammalian cells. Also disclosed is a family of immunoglobulin Fc- angiogenesis inhibitor fusion proteins that can be produced by expression of such nucleotide sequences. Also disclosed are methods using such nucleotide sequences and fusion proteins for treating conditions mediated by angiogenesis.

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(54) Title: EXPRESSION AND EXPORT OF ANGIOSTATIN AND ENDOSTATIN AS IMMUNOFUSIS		
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EXPRESSION AND EXPORT OF ANGIOGENESIS INHIBITORS AS IMMUNOFUSINS

Field of the Invention

This invention relates generally to methods and compositions for making and using fusion proteins containing an angiogenesis inhibitor. More particularly, the invention relates to methods and compositions for making and using fusion proteins called immunofusins which contain an immunoglobulin Fc region and an angiogenesis inhibitor.

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Background of the Invention

Two potent angiogenesis inhibitors, angiostatin (O'Reilly et al. (1994) Cell 79:315) and endostatin (O'Reilly et al. (1997) Cell 88:277), were discovered and found to be generated naturally by primary tumors. Both proteins are specific inhibitors of endothelial cell proliferation and inhibit tumor growth by blocking angiogenesis, the formation of new blood vessels that nourish tumors. Studies have shown that these angiogenesis inhibitors are non-toxic even at very high doses and that they may suppressed the growth of metastases and primary tumors may regress to a dormant microscopic state. Both inhibitors were identified as proteolytic fragments of much larger intact molecules. Angiostatin was found to be a fragment of plasminogen, and endostatin a fragment of collagen XVIII.

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These two proteins have generated great interest in the cancer area because they have been shown to suppress the growth of many different types of tumors in mice, with no obvious side effects or drug resistance. Traditional chemotherapy generally leads to acquired drug resistance caused primarily by the genetic instability of cancer cells. Rather than targeting cancer cells, therapies using angiogenesis inhibitors target the normal endothelial cells, which support the growth of the tumor. Because endothelial cells are genetically stable, it is possible that angiogenesis inhibitor therapies may result in less drug resistance. Studies indicate that drug resistance did not develop in mice exposed to prolonged anti-angiogenic therapy using endostatin. Furthermore, repeated cycles of endostatin treatment in mice resulted in prolonged

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tumor dormancy and no recurrence of tumors following discontinuation of therapy (Boehm et al. (1997) Nature 390: 404).

Despite promising results in mice, it has not been possible to produce clinical grade soluble, active angiostatin and endostatin in commercial quantities using *E. coli*, baculoviral, yeast, and mammalian expression systems. Expression in *E. coli* yielded insoluble protein aggregates of undefined composition, which could not be injected into humans. Other production methods, such as baculovirus and mammalian expression systems, yielded very low levels of the recombinant proteins (O'Reilly et al. (1997) Cell 88: 277).

The poor yields of the expression systems to date may be explained by both angiostatin and endostatin being internal fragments of much larger proteins. The truncated proteins may not fold properly in the absence of the residues that are cleaved from the precursor molecules. For example, angiostatin has 26 cysteine residues which form numerous disulfide bonds. Expression of angiostatin by itself may not provide the optimal environment for these numerous disulfide bonds to form correctly in the secretory pathway. Also, the recombinant endostatin protein produced in *E. coli* precipitated during dialysis, possibly due to the hydrophobicity of endostatin (O'Reilly et al. (1997) Cell 88: 277).

A major hurdle with the use of angiostatin and endostatin in their present forms is that relatively large amounts of proteins have to be injected daily for weeks to months to achieve the desired clinical outcome. For example, in current mouse models, dosages of 20 mg/kg/day of endostatin are needed to demonstrate optimal efficacy (Boehm et al. (1997) Nature 390: 404). Given that there is an urgent need to test endostatin and angiostatin clinically, a production method that can generate large quantities of clinical grade material is important.

One expression system that has been used to produce high level expression of fusion proteins in mammalian cells is a DNA construct encoding, a signal sequence, an immunoglobulin Fc region and a target protein. The fusion product of this construct generally is termed an "immunofusin." Several target proteins have been expressed

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successfully as immunofusins which include: IL2, CD26, Tat, Rev, OSF-2, BIG-H3, IgE Receptor, PSMA, and gp120. These expression constructs are disclosed in U.S. Patent No. 5,541,087 and U.S. Patent No. 5,726,044.

5 A major purpose of expressing recombinant fusion proteins in mammalian cells has been to attempt to confer novel or useful properties to the hybrid molecules, e. g., proper folding, increased solubility, targeting of a cytokine or toxin *in vivo*, Fc receptor binding, complement fixation, protein A binding, increased circulation half-life, and increased ability to cross the blood-brain barrier. Examples of recombinant fusion proteins produced in mammalian cells include cytokine immunoconjugates (Gillies et al. 10 (1992) Proc. Natl. Acad. Sci. USA 89: 1428; Gillies et al. (1993) Bioconjugate Chemistry 4: 230), immunoadhesins (Capon et al. (1989) Nature 337: 525), immunotoxins (Chaudhary et al. (1989) Nature 339: 394), and a nerve growth factor conjugate (Friden et al. (1993) Science 259: 373).

15 It is an object of the invention to provide novel DNAs which facilitate efficient production and secretion of angiogenesis inhibitors in a variety of mammalian host cells. It is another object of the invention to provide methods for treating mammals with nucleic acids encoding, or amino acid sequences defining angiogenesis inhibitor proteins, including non-native, biosynthetic, or otherwise artificial proteins such as proteins which have been created by rational design.

20 Summary of the Invention

The present invention features methods and compositions useful in making and using fusion proteins containing an angiogenesis inhibitor protein. The fusion proteins can facilitate a high level expression of biologically active angiogenesis inhibitor proteins. The angiogenesis inhibitor proteins can then be cleaved from the fusion 25 protein and combined with a pharmaceutically acceptable carrier prior to administration to a mammal, for example, a human. Alternatively, nucleic sequences encoding, or amino acid sequences defining the angiogenesis inhibitor containing fusion proteins can be combined with a pharmaceutically acceptable carrier and administered to the mammal.

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In one aspect, the invention provides nucleic acid molecules, for example, DNA or RNA molecules, coding for a signal sequence and a fusion protein of the invention. Thus, the nucleic acid molecule encodes a signal sequence, an immunoglobulin Fc region, and at least one target protein, also referred to herein as the angiogenesis inhibitor protein.

The target protein may be selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment having endostatin activity, and combinations thereof.

In a preferred embodiment, the nucleic acid molecule encodes, serially in a 5' to 3' direction, the signal sequence, the immunoglobulin Fc region and the target protein sequence. In another preferred embodiment, the nucleic acid molecule encodes, serially in a 5' to 3' direction, the signal sequence, the target sequence, and immunoglobulin Fc region.

In another preferred embodiment, the immunoglobulin Fc region comprises an immunoglobulin hinge region and preferably comprises at least one immunoglobulin constant heavy region, for example, an immunoglobulin constant heavy 2 (CH₂) domain, an immunoglobulin constant heavy 3 (CH₃) domain), and depending upon the type of immunoglobulin used to generate the Fc region, optionally an immunoglobulin constant heavy region 4 (CH₄) domain. In a more preferred embodiment, the immunoglobulin Fc region comprises a hinge region, a CH₂ domain and a CH₃ domain. Under certain circumstances, the immunoglobulin Fc region preferably lacks at least the CH₁ domain. Although the immunoglobulin Fc regions may be based on any immunoglobulin class, for example, IgA, IgD, IgE, IgG, and IgM, immunoglobulin Fc regions based on IgG are preferred.

In another embodiment, the nucleic acid of the invention can be incorporated in operative association into a replicable expression vector which can then be transfected into a mammalian host cell. In another preferred embodiment, the invention provides host cells harboring such nucleic acid sequences of the invention.

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Thus, in one aspect there is provided a replicable expression vector for transfecting a mammalian cell, a vector comprising a DNA molecule of the invention.

In another aspect, the invention provides a fusion protein comprising an immunoglobulin Fc region linked, either directly through a polypeptide bond or by means of a polypeptide linker, to a target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, a collagen XVIII fragment having endostatin activity, and combinations thereof. The target protein may be fused via its C-terminal end to an N-terminal end of the immunoglobulin Fc region. However, in a more preferred embodiment the target protein is fused via its N-terminal end to a C-terminal end of the immunoglobulin Fc region.

In another embodiment, the fusion protein may comprise a second target protein selected from the group consisting of angiostatin, endostatin, a plasminogen fragment having angiostatin activity, and a collagen XVIII fragment having endostatin activity. In this type of construct the first and second target proteins can be the same or different proteins. For example, in a preferred embodiment, the fusion protein comprises a first target protein of angiostatin, an immunoglobulin Fc region and a second target protein of endostatin. The first and second target proteins may be linked together, either directly or by means of a polypeptide linker. Alternatively, both target proteins may be linked, either directly or via a polypeptide linker, to the immunoglobulin Fc region. In the latter case, the first target protein is connected to an N-terminal end of the immunoglobulin Fc region and the second target protein is connected to a C-terminal end of the immunoglobulin Fc region.

In another embodiment, two fusion proteins may associate, either covalently, for example, by a disulfide or peptide bond, or non-covalently, to produce a multimeric protein. In a preferred embodiment, two fusion proteins are associated covalently by means of one or more disulfide bonds through cysteine residues, preferably located within immunoglobulin hinge regions disposed within the immunoglobulin Fc regions of both chains.

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In a preferred embodiment, the target protein comprises a plasminogen fragment having a molecular weight of approximately 40 kD and, optionally comprises, an amino acid sequence as set forth in SEQ ID NO: 3. In another preferred embodiment, the target protein comprises a collagen XVIII fragment having an amino acid sequence set forth in SEQ ID NO: 1. Furthermore, the target protein can be full-length angiostatin or endostatin or bioactive fragments thereof. The source of the target protein in generating certain fusion proteins will depend upon the intended use of the target protein. For example, if the target protein is to be administered to a human, the target protein preferably is of human origin.

10 In one aspect, there is provided a homodimeric fusion protein having angiogenesis inhibitor activity comprising an immunoglobulin Fc-region and a target protein, wherein the Fc region comprises a hinge region, a CH2 domain and a CH3 domain, and the target protein has the angiogenesis inhibitor activity of endostatin and is endostatin or a biologically active fragment thereof, and is linked to the N-terminal ends or the C-terminal ends of said immunoglobulin Fc region.

In another aspect, the invention provides methods of producing a fusion protein of the invention, including a homodimeric fusion protein of the invention. The method comprises the steps of (a) transfecting a mammalian cell with a DNA molecule such as a vector encoding the fusion protein of the invention, (b) culturing the mammalian cell to produce the fusion protein, and (c) isolating the fusion protein.

The resulting fusion protein thus can then be harvested, refolded, if necessary, and purified using conventional purification techniques well known and used in the art. Assuming that the fusion protein comprises a proteolytic cleavage site disposed between the immunoglobulin Fc region

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and the target protein, the target can be cleaved from the fusion protein using conventional proteolytic enzymes and if necessary, purified prior to use.

In another aspect, the invention provides methods for treating mammals, for example, a human, in need of an angiogenesis inhibitor based therapy. For example, it is contemplated that
5 the angiogenesis inhibitors of the invention may be administered to a human afflicted with a tumor. Treatment with the angiogenesis inhibitor may slow down or stop tumor growth and, under certain circumstances, may cause tumor regression. Treatment may include administering to the mammal an amount of the angiogenesis inhibitor in an amount sufficient to slow down or stop tumor growth. The angiogenesis inhibitor may be provided in the form of a fusion protein
10 or as a nucleic acid, preferably operatively associated with an expression vector, in combination with a pharmaceutically acceptable carrier.

The foregoing and other objects, features and advantages of the present invention will be made more apparent from the detailed description, drawings, and claims that follow.

Brief Description of the Drawings

Figures 1A-1F are schematic illustrations of exemplary angiogenesis inhibitor fusion proteins constructed in accordance with the invention (see Examples 10-15). The Figures depict, respectively, Figure 1A, Fc-Kringle 1 of Angiostatin; Figure 1B, Fc-inner Kringle 1 of Angiostatin; Figure 1C, Fc-Endostatin-GlySer linker-inner Kringle 1 of Angiostatin; Figure 1D, Fc-Endostatin-GlySer linker-Kringle 1 of Angiostatin; Figure 1E, Fc-Endostatin-GlySer linker-Angiostatin; Figure 1F, Angiostatin-Fc-Endostatin. The vertical lines represent optional disulfide bonds connecting cysteine residues (C) disposed within a hinge region of the Fc molecule.

Detailed Description of the Invention

The invention provides fusion proteins, referred to herein as immunofusins, which were useful in the production of commercial quantities of clinical grade angiogenesis inhibitors. The angiogenesis inhibitors may be cleaved from the immunofusin protein constructs prior to use.
5 However, it is contemplated that the immunofusins or nucleic acids encoding the immunofusins may be administered directly to mammals in need of treatment with an angiogenesis inhibitor.

The invention thus provides fusion proteins comprising an immunoglobulin Fc region and at least one target protein, referred to herein as an angiogenesis inhibitor. The angiogenesis inhibitor preferably is selected from the group consisting of angiostatin, endostatin, a
10 plasminogen fragment angiostatin activity, a collagen XVIII fragment having endostatin activity. It is contemplated, however, that other polypeptides having angiogenesis inhibitor activity, now known or late discovered, may be expressed as fusion proteins of the type described herein.

Six exemplary embodiments of protein constructs embodying the invention are illustrated in the drawing as Figures 1A-1F. Because dimeric constructs are preferred, all are illustrated as
15 dimers cross-linked by a pair of disulfide bonds between cysteines on adjacent subunits. In the drawings, the disulfide bridges are depicted as linking together the portions of two immunoglobulin Fc regions via an immunoglobulin hinge region, and thus are characteristic of native forms of these molecules. While constructs including the hinge region of Fc are preferred and have been shown promise as therapeutic agents, the invention contemplates that the
20 crosslinking at other positions may be chosen as desired. Furthermore, under some circumstances, dimers or multimers useful in the practice of the invention may be produced by non-covalent association, for example, by hydrophobic interaction.

Because homodimeric constructs are important embodiments of the invention, Figure 1 illustrates such constructs. It should be appreciated that heterodimeric structures also are useful
25 but, as is known to those skilled in the art, often can be difficult to purify. However, viable constructs useful to inhibit angiogenesis in various mammalian species, including humans, can be constructed comprising a mixture of homodimers and heterodimers. For example, one chain of the heterodimeric structure may comprise endostatin and the another may comprise angiostatin.

Figure 1A illustrates a dimer construct produced in accordance with the procedure set forth in Example 10. Each monomer of the dimer comprises an immunoglobulin Fc region 1 including a hinge region, a CH₂ domain and a CH₃ domain. Attached directly to the C terminus of the Fc region 1 is the first Kringle region of angiostatin 2, both inner and outer rings. Figure 1B shows a second embodiment of the invention (see Example 11) comprising the same Fc region as in Figure 1A, this time having only the inner ring of Kringle one of angiostatin 3 attached to the C terminal end of the Fc region 1. Figures 1C through 1E depict various embodiments of the protein constructs of the invention, which include as a target protein plural angiogenesis inhibitors arranged in tandem and connected by a linker. In Figure 1C, the target protein comprises full-length endostatin 4, a polypeptide linker 5, and the inner ring of Kringle one of angiostatin 3. Figure 1D depicts a protein comprising an Fc region the same as that of Figure 1A and a target protein comprising a full-length endostatin 4, a polypeptide linker 5, and a full Kringle one region of angiostatin (both inner and outer rings) 2. Figure 1E differs from the construct of Figure 1D in that the most C terminal protein domain comprises a full-length copy of angiostatin 7.

Although Figures 1A-1E represent Fc-X type constructs, where X is the target protein, it is contemplated that X-Fc type constructs may also be useful in the practice of the invention. Furthermore, it is contemplated the useful proteins of the invention may also be depicted by the formula X-Fc-X, wherein the Xs may represent the same or different target proteins. Figure 1F depicts such a construct which comprises in an N- to C-terminal direction, full-length human angiostatin 7, a human immunoglobulin Fc region 6 including a hinge region, and full-length human endostatin domain 4.

The term "angiogenesis inhibitor," as used herein, refers to any polypeptide chain that reduces or inhibits the formation of new blood vessels in a mammal. With regard to cancer therapy, the angiogenesis inhibitor reduces or inhibits the formation of new blood vessels in or on a tumor, preferably in or on a solid tumor. It is contemplated that useful angiogenesis inhibitors may be identified using a variety of assays well known and used in the art. Such assays include, for example, the bovine capillary

endothelial cell proliferation assay, the chick chorioallantoic membrane (CAM) assay or the mouse corneal assay. However, the CAM assay is preferred (see, for example, O'Reilly *et al.* (1994) Cell 79: 315-328 and O'Reilly *et al.* (1997) Cell 88: 277-285). Briefly, embryos with intact yolks are removed from fertilized three day old white eggs and placed in a petri dish. After incubation at 37°C, 3% CO₂ for three days, a methylcellulose disk containing the putative angiogenesis inhibitor is applied to the chorioallantoic membrane of an individual embryo. After incubation for about 48 hours, the chorioallantoic membranes were observed under a microscope for evidence of zones of inhibition.

10 Preferred angiogenesis inhibitors useful in the practice of the invention include, for example, angiostatin (O'Reilly *et al.* (1994) Cell 79: 315-328, and U.S. Patent Nos. 5,733,876; 5,837,682; and 5,885,795), and endostatin (O'Reilly *et al.* (1997) Cell 88: 277-285 and U.S. Patent No. 5,854,205). As stated previously, angiostatin and endostatin are specific inhibitors of endothelial cell proliferation and are capable of
15 inhibiting tumor growth by blocking angiogenesis, the formation of new blood vessels that nourish tumors.

Angiostatin has been identified as a proteolytic fragment of plasminogen (O'Reilly *et al.* (1994) Cell 79: 315-328, and U.S. Patent Nos. 5,733,876; 5,837,682; and 5,885,795). Specifically, angiostatin is a 38 kDa internal fragment of plasminogen containing at least three of the Kringle regions of plasminogen. Endostatin has been
20 identified as a proteolytic fragment of collagen XVIII (O'Reilly *et al.* (1997) Cell 88: 277-285). Specifically, endostatin is a 20 kDa C-terminal fragment of collagen XVIII. The terms "angiostatin" and "endostatin," as used herein, refer not only to the full length proteins, but also to variants and bioactive fragments thereof, as well as to
25 bioactive fragments of plasminogen and collagen XVIII, respectively. The term bioactive fragment, with respect to angiostatin refers to any protein fragment of plasminogen or angiostatin that has at least 30%, more preferably at least 70%, and most preferably at least 90% of the activity of full-length angiostatin as determined by the CAM assay. The term bioactive fragment, with respect to endostatin refers to any
30 protein fragment of collagen XVIII or endostatin that has at least 30%, more preferably

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at least 70% and most preferably at least 90% of the activity of full length endostatin as determined by the CAM assay.

The term variants includes specific and allelic variants, as well as other naturally occurring or non-naturally occurring variants, for example, generated by
5 conventional genetic engineering protocols, that are at least 70% similar or 60% identical, more preferably at least

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75% similar or 65% identical, and most preferably 80% similar or 70% identical to either the naturally-occurring sequences of endostatin or angiostatin disclosed herein.

To determine whether a candidate polypeptide has the requisite percentage similarity or identity to a reference polypeptide, the candidate amino acid sequence and the reference amino acid sequence are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981), *J. Mol. Biol.* 147:195-197, in combination with the BLOSUM62 substitution matrix described in Figure 2 of Henikoff and Henikoff (1992), "Amino acid substitution matrices from protein blocks", *Proc. Natl. Acad. Sci. USA* 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is -12, and an appropriate value for the gap extension penalty is -4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pair-wise similarity score is zero; otherwise the pair-wise similarity score is 1.0. The raw similarity score is the sum of the pair-wise similarity scores of the aligned amino acids. The raw score then is normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence again are compared sequentially. If the amino acids are non-identical, the pair-wise identity score is zero; otherwise the pair-wise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used in the initial alignment.

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The target proteins disclosed herein are expressed as fusion proteins with an Fc region of an immunoglobulin. As is known, each immunoglobulin heavy chain constant region is comprised of four or five domains. The domains are named sequentially as follows: CH₁-hinge-CH₂-CH₃(-CH₄). The DNA sequences of the heavy chain domains have cross-homology among the immunoglobulin classes, e.g., the CH₂ domain of IgG is homologous to the CH₂ domain of IgA and IgD, and to the CH₃ domain of IgM and IgE.

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

The currently preferred class of immunoglobulin from which the heavy chain constant region is derived is IgG (Ig γ) (γ subclasses 1, 2, 3, or 4). Other classes of immunoglobulin, IgA (Ig α), IgD (Ig δ), IgE (Ig ϵ) and IgM (Ig μ), may be used. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Patent 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 CH₃ domain of Fc γ or the homologous domains in any of IgA, IgD, IgE, or IgM.

Depending on the application, constant region genes from species other than human e.g., mouse or rat may be used. The Fc region used as a fusion partner in the immunofusion DNA construct generally may be from any mammalian species. Where it is undesirable to elicit an immune response in the host cell or animal against the Fc region, the Fc region may be derived from the same species as the host cell or animal. For example, human Fc can be used when the

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host animal or cell is human; likewise, murine Fc can be used where the host animal or cell will be a mouse. Further, substitution or deletion of constructs of these constant regions, in which one or more amino acid residues of the constant region domains are substituted or deleted also would be useful. One example would be to introduce amino acid substitutions in the upper CH₂ region to create a Fc variant with reduced affinity for Fc receptors (Cole et al. (1997) J. Immunol. 159:3613). One of ordinary skill in the art can prepare such constructs using well known molecular biology techniques.

The use of human Fcγ1 as the Fc region sequence has several advantages. For example, if the angiogenesis inhibitor Fc fusion protein is to be used as a biopharmaceutical, the Fcγ1 domain may confer the effector function activities to the fusion protein. The effector function activities include the biological activities such as complement fixation, antibody-directed cellular cytotoxicity, placental transfer, and increased serum half-life. The Fc domain also provides for detection by anti-Fc ELISA and purification through binding to *Staphylococcus aureus* protein A ("Protein A"). In certain applications, however, it may be desirable to delete specific effector functions from the Fc region, such as Fc receptor binding or complement fixation.

In the case of angiogenesis inhibitor immunofusins, one function of the immunoglobulin Fc fusion partner is to facilitate proper folding of the angiogenesis inhibitor protein to yield active angiogenesis inhibitor protein and to impact solubility to the active moieties, at least in the extracellular medium. Since the Fc fusion partner is hydrophilic, the angiogenesis inhibitor immunofusin readily is soluble unlike, for example, the recombinant endostatin produced in *E. coli* (O'Reilly (1997) Cell 88:277.) In all of the Examples disclosed herein, high levels of production of the immunofusins were obtained. The angiogenesis inhibitor immunofusins were secreted into media at concentrations typically of about 30 to 100 μg/ml, and could be purified readily to homogeneity by Protein A chromatography. In addition, the angiogenesis inhibitor immunofusins could be cleaved and further purified using conventional purification protocols using, for example, by heparin sepharose, lysine sepharose or affinity purification.

In addition to the high levels of expression, fusion proteins of the invention also exhibit longer serum half-lives, presumably due to their larger molecular sizes. For example, human Fc-human angiostatin has a serum half-life of 33 hours in mouse, as compared to 4-6 hours for human angiostatin (O'Reilly et al. (1996) Nature Medicine 2:689). It is believe that angiostatin

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with a molecular weight of 40 kD, and endostatin with a molecular weight of 20 kD, are small enough to be cleared efficiently by renal filtration. In contrast, the dimeric forms of Fc-angiostatin and dimeric Fc-endostatin are 145 kD and 100 kD, respectively, because there are two immunoglobulin Fc regions attached to either two angiostatin molecules or two endostatin molecules. Such a bivalent structure may exhibit a higher binding affinity to the angiostatin or endostatin receptor. If the angiogenesis inhibiting activity is receptor-mediated, the Fc fusion proteins are potentially more effective to suppress tumors than monovalent angiostatin or monovalent endostatin by themselves. Furthermore, if angiostatin and/or endostatin belong to a class of dimeric protein ligands, the physical constraint imposed by the Fc on angiostatin or endostatin would make the dimerization an intramolecular process, thus shifting the equilibrium in favor of the dimer and enhancing its binding to the receptor. Cysteine residues can also be introduced by standard recombinant DNA technology to the monomer at appropriate places to stabilize the dimer through covalent disulfide bond formation.

As used herein, the term "multivalent" refers to a recombinant molecule that incorporates two or more biologically active segments. The protein fragments forming the multivalent molecule may be linked through a polypeptide peptide linker which attaches the constituent parts without causing a frame shift and permits each to function independently.

As used herein, the term "bivalent" refers to a multivalent recombinant molecule having two target proteins in a fusion construct of the invention, e.g., an Fc-X molecule, where X independently is selected from angiostatin, endostatin, or a variant thereof. Since there are two X moieties fused to an immunoglobulin Fc region (which typically itself is a dimer of the heavy chain fragments including at least a portion of the hinge region and CH₃ domain, and optionally the CH₂ domain), the molecule is bivalent (see, e.g., Figure 1A). If the fusion construct of the invention has the form Fc-X-X, the resulting Fc dimer molecule is tetravalent. The two proteins forming the Fc-X-X molecule may be linked through a peptide linker. A bivalent molecule can increase the apparent binding affinity between the molecule and its receptor. For instance, if one endostatin moiety of an Fc-endostatin can bind to a receptor on a cell with a certain affinity, the second endostatin moiety of the same Fc-endostatin may bind to a second receptor on the same cell with a much higher avidity (apparent affinity). This is because of the physical proximity of the second endostatin moiety to the receptor after the first endostatin moiety is already bound. In the case of an antibody binding to an antigen, the apparent affinity is increased by at least 10⁴.

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As used herein, the terms “multimer” and “multimeric” refers to the stable association of two or more polypeptide chains either covalently, for example, by means of covalent interaction, for example, by a disulfide bond or non-covalently, for example, by hydrophobic interaction. The term multimer is intended to encompass both
5 homomultimers, wherein the polypeptides are the same, as well as heteromultimers, wherein the polypeptides are different.

As used herein, the term “dimeric” refers to a specific multimeric molecule where two protein polypeptide chains are stably associated through covalent or non-covalent interactions. It should be understood that the immunoglobulin Fc region Fc
10 fragment itself typically is a dimer of the heavy chain fragments including at least a portion of the hinge region and CH3 domain, and optionally the CH2 domain. Many protein ligands are known to bind to their receptors as a dimer. If a protein ligand X dimerizes naturally, the X moiety in an Fc-X molecule will dimerize to a much greater extent, since the dimerization process is concentration dependent. The physical
15 proximity of the two X moieties connected by associated immunoglobulin Fc region would make the dimerization an intramolecular process, greatly shifting the equilibrium in favor of the dimer and enhancing its binding to the receptor.

It is understood that the present invention exploits conventional recombinant DNA methodologies for generating the Fc fusion proteins useful in the practice of the
20 invention. The Fc fusion constructs preferably are generated at the DNA level, and the resulting DNAs integrated into expression vectors, and expressed to produce the immunofusins. As used herein, the term “vector” is understood to mean any nucleic acid comprising a nucleotide sequence competent to be incorporated into a host cell and to be recombined with and integrated into the host cell genome, or to replicate
25 autonomously as an episome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors and the like. Non-limiting examples of a viral vector include a retrovirus, an adenovirus and an adeno-associated virus. As used herein, the term “gene expression” or “expression” of a target protein, is understood to mean the transcription of a DNA sequence, translation of the mRNA
30 transcript, and secretion of an Fc fusion protein product.

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A useful expression vector is pdCs (Lo et al. (1988) Protein Engineering 11: 495) in which the transcription of the Fc-X gene utilizes the enhancer/promoter of the human cytomegalovirus and the SV40 polyadenylation signal. The enhancer and promoter sequence of the human cytomegalovirus used was derived from nucleotides-
5 601 to +7 of the sequence provided in Boshart et al., 1985, Cell 41: 521. The vector also contains the mutant dihydrofolate reductase gene as a selection marker (Simonsen and Levinson (1983) Proc. Nat. Acad. Sci. USA 80: 2495).

An appropriate host cell can be transformed or transfected with the DNA sequence of the invention, and utilized for the expression and secretion of a target
10 protein. Currently preferred host cells for use in the invention include immortal hybridoma cells, NS/O myeloma cells, 293 cells, Chinese hamster ovary cells, Hela cells, and COS cells.

The fusion proteins of the invention preferably are generated by conventional recombinant DNA methodologies. The fusion proteins preferably are produced by
15 expression in a host cell of a DNA molecule encoding a signal sequence, an immunoglobulin Fc region and a target protein (also referred to herein as an angiogenesis inhibitor). Preferred constructs may encode in a 5' to 3' direction, the signal sequence, the immunoglobulin Fc region and the target protein. Alternatively, the constructs may encode in a 5' to 3' direction, the signal sequence, the target protein
20 and the immunoglobulin Fc region.

As used herein, the term "signal sequence" is understood to mean a peptide segment which directs the secretion of the angiogenesis inhibitor immunofusin protein and is thereafter cleaved following translation in the host cell. The signal sequence of the invention is a polynucleotide, which encodes an amino acid sequence that initiates
25 transport of a protein across the membrane of the endoplasmic reticulum. Signal sequences which will be useful in the invention include antibody light chain signal sequences, e. g., antibody 14.18 (Gillies et. al., 1989, Jour. of Immunol. Meth., 125: 191-202), antibody heavy chain signal sequences, e. g., the MOPC141 antibody heavy chain signal sequence (Sakano et al., 1980, Nature 286: 5774), and any other signal

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sequences which are known in the art (see for example, Watson, 1984, *Nucleic Acids Research* 12: 5145).

Signal sequences have been well characterized in the art and are known typically to contain 16 to 30 amino acid residues, and may contain greater or fewer amino acid residues. A typical signal peptide consists of three regions: a basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region. The central hydrophobic region contains 4 to 12 hydrophobic residues that anchor the signal peptide across the membrane lipid bilayer during transport of the nascent polypeptide. Following initiation, the signal peptide is usually cleaved within the lumen of the endoplasmic reticulum by cellular enzymes known as signal peptidases. Potential cleavage sites of the signal peptide generally follow the “(-3,- 1) rule.” Thus a typical signal peptide has small, neutral amino acid residues in positions -1 and -3 and lacks proline residues in this region. The signal peptidase will cleave such a signal peptide between the -1 and +1 amino acids. Thus, the portion of the DNA encoding the signal sequence may be cleaved from the amino-terminus of the immunofusin protein during secretion. This results in the secretion of a immunofusin protein consisting of the Fc region and the target protein. A detailed discussion of signal peptide sequences is provided by von Heijne (1986) *Nucleic Acids Res.*, 14: 4683.

As would be apparent to one of skill in the art, the suitability of a particular signal sequence for use in the invention may require some routine experimentation. Such experimentation will include determining the ability of the signal sequence to direct the secretion of an immunofusin and also a determination of the optimal configuration, genomic or cDNA, of the sequence to be used in order to achieve efficient secretion of immunofusins. Additionally, one skilled in the art is capable of creating a synthetic signal peptide following the rules presented by von Heijne, referenced above, and testing for the efficacy of such a synthetic signal sequence by routine experimentation. A signal sequence may also be referred to as a “signal peptide,” “leader sequence,” or “leader peptide.”

The fusion of the signal sequence and the immunoglobulin Fc region is sometimes referred to herein as secretion cassette. An exemplary secretion cassette

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useful in the practice of the invention is a polynucleotide encoding, in a 5' to 3' direction, a signal sequence of an immunoglobulin light chain gene and an Fc γ 1 region of the human immunoglobulin γ 1 gene. The Fc γ 1 region of the immunoglobulin Fc γ 1 gene preferably includes at least a portion of the hinge domain and at least a portion of the CH₃ domain, or alternatively at least portions of the hinge domain, CH₂ domain and CH₃ domain. The DNA encoding the secretion cassette can be in its genomic configuration or its cDNA configuration.

In another embodiment, the DNA sequence encodes a proteolytic cleavage site interposed between the secretion cassette and the angiogenesis inhibitor protein. A cleavage site provides for the proteolytic cleavage of the encoded fusion protein thus separating the Fc domain from the angiogenesis inhibitor protein. As used herein, "proteolytic cleavage site" is understood to mean amino acid sequences which are preferentially cleaved by a proteolytic enzyme or other proteolytic cleavage agents. Useful proteolytic cleavage sites include amino acids sequences which are recognized by proteolytic enzymes such as trypsin, plasmin or enterokinase K. Many cleavage site/cleavage agent pairs are known. See, for example, U.S. Patent No. 5,726,044. Where the target protein sequence is a precursor molecule to angiostatin, endostatin, or an active variant thereof, the desired protein product may be produced by cleavage with the endogenous proteolytic enzyme, such as elastin or plasmin or urokinase.

The present invention also encompasses fusion proteins containing different combinations of recombinant angiostatin and endostatin, or fragments thereof, which can be made in large quantities. Despite the demonstrated efficacy in suppressing tumor growth, the mechanism of how angiostatin and endostatin block angiogenesis is not completely known. Angiostatin has several Kringle structures and endostatin has different structural motifs, each of which may be solely responsible for or assist in binding of the proteins to endothelial cells and exerting an anti-angiogenic effect. Accordingly, this invention includes target proteins which are bioactive fragments of angiostatin, such as Kringle 1, Kringle 2, Kringle 3, and combinations thereof, and endostatin which exhibit physiologically similar behavior to naturally occurring full-length angiostatin and endostatin.

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Another embodiment of the present invention provides for bifunctional hybrid constructs of angiogenesis inhibitors. As used herein, a bifunctional hybrid molecule or construct means a protein produced by combining two protein subunits, where the two subunits can be derived from different proteins. Each protein subunit has its own independent function so that in the hybrid molecule, the functions of the two subunits may be additive or synergistic. Such functional hybrid proteins would allow the synergistic effect of angiostatin and endostatin to be explored in animal models. A preferred bifunctional hybrid may comprise at least two different angiogenesis inhibitors linked in tandem, either directly or by means of a polypeptide linker. For example, in a preferred embodiment, the target sequence encodes at least a portion of angiostatin linked in frame with at least a portion of endostatin and both the angiostatin and endostatin domains exhibit anti angiogenesis activity or angiogenesis inhibition. The two units may be linked by a polypeptide linker.

As used herein the term "polypeptide linker is understood to mean an peptide sequence that can link two proteins together or a protein and an Fc region. The polypeptide linker preferably comprises a plurality of amino acids such as glycine and/or serine. Preferably, the polypeptide linker comprises a series of glycine and serine peptides about 10-15 residues in length. See, for example, U.S. Patent No. 5,258,698. It is contemplated however, that the optimal linker length and amino acid composition may be determined by routine experimentation.

It is found that when different parts of the angiostatin are expressed as Fc fusion molecules, high levels of expression are obtained, presumably because the Fc portion acts as a carrier, helping the polypeptide at the C-terminus to fold correctly. In addition, the Fc region can be glycosylated and highly charged at physiological pH, thus the Fc region can help to solubilize hydrophobic proteins.

The present invention also provides methods for the production of angiostatin and endostatin of non-human species as Fc fusion proteins. Non-human angiogenesis inhibitor fusion proteins are useful for preclinical studies of angiogenesis inhibitors because efficacy and toxicity studies of a protein drug must be performed in animal model systems before testing in humans. A human protein may not work in a mouse

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model because the protein may elicit an immune response, and/or exhibit different pharmacokinetics skewing the test results. Therefore, the equivalent mouse protein is the best surrogate for the human protein for testing in a mouse model.

The standard Lewis lung carcinoma model in mice (O'Reilly et al. (1997) Cell
5 88: 277) was used to compare soluble huFc-huAngiostatin, huFc-huEndostatin, muFc-
muAngiostatin, muFc-muEndostatin with the insoluble proteins produced in an *E. coli*
expression system. The soluble Fc fusion proteins were more efficacious in suppressing
tumor growth in the Lewis lung model than the corresponding proteins produced in *E.*
coli. Furthermore, laboratory mice are inbred and their tumors are induced and not
10 spontaneous. Therefore, efficacy in a mouse model

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may not correlate to probable efficacy against human tumors. Preclinical studies in dogs will provide more precise information about the efficacy of these angiogenesis inhibitors on spontaneous tumors because there are numerous naturally occurring, spontaneous canine tumors. The methods of producing murine (mu) Fc-mu angiostatin, muFc-mu endostatin, and canine (ca) Fc-ca angiostatin, caFc-ca endostatin of the present invention will facilitate preclinical studies of angiogenesis inhibitors in both murine and canine systems.

The present invention provides methods of treating a condition mediated by angiogenesis by administering the DNA, RNA or proteins of the invention. Conditions mediated by angiogenesis include, for example: solid tumors; blood born tumors, tumor metastasis, benign tumors including hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyrogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases (diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma) retrolental fibroplasia, rubeosis, Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints' angiofibroma; and wound granulation; and excessive or abnormal stimulation of endothelial cells, intestinal adhesions, arteriosclerosis, sclerodermal and hypertrophic scars, i.e., keloids.

The DNA constructs disclosed herein can be useful in gene therapy procedures in which the endostatin or angiostatin gene is delivered into a cell by one of various means e.g., native DNA associated with a promoter or DNA within a viral vector. Once inside a cell, the angiostatin and/or endostatin gene or gene fragment is expressed and the protein is produced *in vivo* to carry out its normal biological function. The DNA construct of the present invention results in high levels of expression of the fusion protein. The fusion proteins of the present invention may also be useful in treating conditions mediated by angiogenesis and may have greater clinical efficacy than native angiogenesis inhibitors and other recombinant angiogenesis inhibitors because the angiogenesis inhibitor immunofusins of the present invention have a longer serum half-life than the other recombinant angiogenesis inhibitors or native angiogenesis inhibitors alone. The bivalent and dimeric forms of the present invention should have higher binding affinity due to the bivalent and dimeric structure. The bifunctional hybrid molecules of the present invention may have a higher clinical efficacy due to possible synergistic effects of two different angiogenesis inhibitors connected by the fused Fc region or a flexible polypeptide linker.

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The compositions of the present invention may be provided to an animal by any suitable means, directly (e.g., locally, as by injection, implantation or topical administration to a tissue locus) or systemically (e.g., parenterally or orally). Where the composition is to be provided parenterally, such as by intravenous, subcutaneous, ophthalmic, intraperitoneal, intramuscular, 5 buccal, rectal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal or by aerosol administration, the composition preferably comprises part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the carrier or vehicle is physiologically acceptable so that in addition to delivery of the desired composition to the patient, it does not otherwise adversely affect the patient's 10 electrolyte and/or volume balance. The fluid medium for the agent thus can comprise normal physiologic saline (e.g., 9.85% aqueous NaCl, 0.15 M, pH 7-7.4).

Preferred dosages of the immunofusins per administration are within the range of 50 ng/m² to 1 g/m², more preferably 5 µg/m² to 200 mg/m², and most preferably 0.1 mg/m² to 50 mg/m².

Preferred dosages of nucleic acids encoding the immunofusins per administration are within the 15 range of 1 µg/m² to 100 mg/m², more preferably 20 µg/m² to 10 mg/m², and most preferably 400 µg/m² to 4 mg/m². It is contemplated, however, that the optimal modes of administration, and dosages may be determined by routine experimentation well within the level of skill in the art.

The invention is illustrated further by the following non-limiting examples.

EXAMPLES

20 Example 1. Expression of huFc-huEndostatin

Human endostatin was expressed as a human Fc-human endostatin (huFc-huEndo) fusion protein according to the teachings of Lo et al. (1998) Protein Engineering 11:495. Fc refers to the Fc fragment of the human immunoglobulin gamma (DNA sequence set forth in SEQ ID NO:1; amino acid sequence set forth in SEQ ID NO:2). (Polymerase chain reactions PCR) was 25 used to adapt the endostatin cDNA (SEQ ID NO:3; whose amino acid sequence is disclosed in SEQ ID NO:4), for expression in an Fc-Endo fusion protein. The forward primer was either 5'-CC CCG GGT AAA CAC AGC CAC CGC GAC TTC C (SEQ ID NO:5; encoded amino acids disclosed in SEQ ID NO:6) or 5'-C AAG CTT CAC AGC CAC CGC GAC TTC C (SEQ ID NO:7; encoded amino acids disclosed in SEQ ID NO:8), where the XmaI site or the HindIII site

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was followed by sequence encoding the N-terminus of endostatin. The primer with the XmaI site adapted the endostatin cDNA for ligation to the XmaI site at the end of the CH₃ domain of the IgGFc region. The primer with the HindIII site adapted the endostatin cDNA for ligation to the HindIII site of the pdCs-Fc (D₄K) vector, which contains the enterokinase recognition site Asp₄-Lys (LaVallie et al. (1993) J. Biol. Chem. 268: 23311-23317) at the junction of the fusion protein. The reverse primer was 5'-C CTC GAG CTA CTT GGA GGC AGT CAT G (SEQ ID NO: 9), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of endostatin, and this was followed by an XhoI site. The PCR products were cloned and sequenced, and the XmaI-XhoI fragment was ligated to the resulting XmaI and XhoI digested pdCs-Fc vector. Similarly, the HindIII-XhoI fragment encoding endostatin was ligated into appropriately digested pdCs-huFc (D₄K) vector. Stable clones expressing Fc-endo or Fc (D₄K)-endostatin were obtained by electroporation of NS/0 cells followed by selection in growth medium containing 100 nM methotrexate. Protein expression level was assayed by anti-human Fc ELISA (Example 3) and confirmed by SDS-PAGE, which showed a protein product of ~52 kD. The best producing clones were subcloned by limiting dilutions.

Example 2. Cell culture and transfection

For transient transfection, the plasmid was introduced into human kidney 293 cells by co-precipitation of plasmid DNA with calcium phosphate (Sambrook et al. (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, NY) or by lipofection using LipofectAMINE Plus™ (Life Technologies, Gaithersburg, MD) according to supplier's protocol.

In order to obtain stably transfected clones, plasmid DNA was introduced into the mouse myeloma NS/0 cells by electroporation. NS/0 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. About 5 x 10⁶ cells were washed once with PBS and resuspended in 0.5 ml PBS. Ten µg of linearized plasmid DNA then was incubated with the cells in a Gene Pulser™ Cuvette (0.4 cm electrode gap, BioRad, Hercules, CA) on ice for 10 min. Electroporation was performed using a Gene Pulser (BioRad, Hercules, CA) with

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settings at 0.25 V and 500 μ F. Cells were allowed to recover for 10 min. on ice, after which they were resuspended in growth medium and then plated onto two 96 well plates. Stably transfected clones were selected by growth in the presence of 100 nM methotrexate (MTX), which was introduced two days post-transfection. The cells were fed every 3 days for three more times, and MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-Fc ELISA to identify high producers. High producing clones were isolated and propagated in growth medium containing 100 nM MTX.

Example 3. ELISA Procedures

10 Three different ELISAs were used to determine the concentrations of protein products in the supernatants of MTX-resistant clones and other test samples. The anti-human Fc (huFc) ELISA was used to measure the amount of human Fc-containing proteins. The anti-murine Fc (muFc) and anti-canine Fc (caFc) antibodies were used in ELISAs to measure the amount of murine Fc- and canineFc-containing proteins,
15 respectively. The procedure for the anti-huFc ELISA is described in detail herein below.

A. Coating plates

ELISA plates were coated with AffiniPure Goat anti-Human IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) at 5 μ g/ml in PBS, and 100 μ l/well in 96-well plates (Nunc-Immuno plate MaxiSorp™, Nalge Nunc International,
20 Rochester, NY). Coated plates were covered and incubated at 4°C overnight. Plates then were washed 4 times with 0.05% Tween 20™ in PBS, and blocked with 1% BSA/1% Goat Serum in PBS, 200 μ l/well. After incubation with the blocking buffer at 37°C for 2 hours, the plates were washed 4 times with 0.05% Tween™ in PBS and tapped dry on paper towels.

B. Incubation with test samples and secondary antibody

Test samples were diluted to the proper concentrations in a sample buffer, containing 1% BSA/1% Goat Serum/0.05% Tween™ in PBS. A standard curve was prepared with a chimeric antibody (with a human Fc), the concentration of which was known. To prepare a standard curve, serial dilutions were made in the sample buffer to

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give a standard curve ranging from 125 ng/ml to 3.9 ng/ml. The diluted samples and standards were added to the plate, 100 μ l/well and the plate was then incubated at 37°C for 2 hr. After incubation, the plate was washed 8 times with 0.05% Tween™ in PBS. To each well was then added 100 μ l of secondary antibody, the horse radish peroxidase (HRP)-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), diluted about 1:120,000 in sample buffer. The exact dilution of the secondary antibody had to be determined for each lot of the HRP-conjugated Anti-Human IgG. After incubation at 37°C for 2 hr, the plate was washed 8 times with 0.05% Tween™ in PBS.

10 C. Development

A substrate solution was prepared by dissolving 30 mg (1 tablet) of o-phenylenediamine dihydrochloride (OPD) into 15 ml of 0.025 M citric acid/0.05 M Na₂HPO₄ buffer, pH 5, containing 0.03% of freshly added H₂O₂. The substrate solution was added to the plate at 100 μ l/well. The color was allowed to develop for 30 min. at room temperature in the dark. The developing time can be subject to change, depending on lot to lot variability of the coated plates, the secondary antibody, etc. The reaction was stopped by adding 4N H₂SO₄, 100 μ l/well. The plate was read by a plate reader, which was set at both 490 and 650 nm, and programmed to subtract the background OD at 650 nm from the OD at 490 nm.

20 The procedure for the anti-muFc ELISA was similar, except that ELISA plate was coated with AffiniPure Goat anti-murine IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) at 5 μ g/ml in PBS, and 100 μ l/well; and the secondary antibody was horse radish peroxidase-conjugated goat anti-muIgG, Fc γ (Jackson ImmunoResearch West Grove, PA), used at 1 in 5000 dilution. Similarly, for the anti-caFc ELISA, the ELISA plate was coated with AffiniPure Rabbit anti-dog IgG, Fc Fragment specific (Jackson ImmunoResearch, West Grove, PA) at 5 μ g/ml in PBS, and 100 μ l/well; and the secondary antibody was horse radish peroxidase-conjugated AffiniPure rabbit anti-dog IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA), used at 1 in 5000 dilution.

30 Example 4. Expression of huFc-huAngiostatin

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Human angiostatin (DNA sequence set forth in SEQ ID NO: 10; amino acid sequence set forth in SEQ ID NO: 11) was expressed as a human Fc-human angiostatin (huFc-huAngio) fusion protein essentially as described in Example 1. PCR was used to adapt the angiostatin cDNA (SEQ ID NO: 3), for expression in the pdCs-huFc or pdCs-huFc (D₄K) vectors. The respective forward primers were 5'-CC CCG GG T AAG AAA GTG TAT CTC TCA GAG (SEQ ID NO 12; encoded amino acids disclosed in SEQ ID NO: 13), and 5'-C CCC AAG CTT AAA GTG TAT CTC TCA GAG (SEQ ID NO: 14; encoded amino acids disclosed in SEQ ID NO: 15), where the XmaI site or the HindIII site was followed by sequence encoding the N-terminus of

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angiostatin. The reverse primer was 5'-CCC CTC GAG CTA CGC TTC TGT TCC TGA GCA (SEQ ID NO:16), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of angiostatin, and this was followed by an XhoI site. The PCR products were cloned and sequenced, and the resulting XmaI-XhoI fragment and the HindIII-XhoI fragment encoding angiostatin were ligated to the pdCs-huFc and the pdCs-huFc(D₄K) vectors, respectively. Stable NS/0 clones expressing huFc-huAngio and huFc(D₄K)-huAngio were selected and assayed as described in Examples 2 and 3.

Example 5. Expression of muFc-mu-Endostatin

Murine endostatin (DNA sequence set forth in SEQ ID NO:17; amino acid sequence set forth in SEQ ID NO:18) and murine Fc (DNA sequence set forth in SEQ ID NO:19; encoded amino acids set forth in SEQ ID NO:20) were expressed as a murine Fc-murine endostatin (muFc-muEndo) fusion protein essentially as described in Example 1. PCR was used to adapt the endostatin cDNA (SEQ ID NO:4), for expression in the pdCs-muFc(D₄K) vector. The forward primer was 5'-C CCC AAG CTT CAT ACT CAT CAG GAC TTT C (SEQ ID NO:21; encoded amino acids disclosed in SEQ ID NO:22), where the HindIII site was followed by sequence encoding the N-terminus of endostatin. The reverse primer was 5'-CCC CTC GAG CTA TTT GGA GAA AGA GGT C (SEQ ID NO:23), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of endostatin, and this was followed by an XhoI site. The PCR product was cloned and sequenced, and the resulting HindIII-XhoI fragment encoding endostatin was ligated into the pdCs-muFc(D₄K) vector. Stable NS/0 clones expressing muFc(D₄K)-muEndo were selected and assayed (anti-muFc ELISA) as described in Examples 2 and 3.

Example 6. Expression of muFc-muAngiostatin

Murine angiostatin (DNA sequence set forth in SEQ ID NO:24; amino acid sequence set forth in SEQ ID NO:25) was expressed as a murine Fc-murine angiostatin (muFc-muAngio) fusion protein essentially as described in Example 1. PCR was used to adapt the angiostatin cDNA (SEQ ID NO:6) for expression in the pdCs-Fc(D₄K) vector. The forward primer was 5'-C CCC AAG CTT GTG TAT CTG TCA GAA TGT AAG CCC TCC TGT CTC TGA GCA (SEQ ID NO: 26; encoded amino acids disclosed in SEQ ID NO:27), where the HindIII site was

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followed by sequence encoding the N-terminus of angiostatin. The reverse primer was 5'-CCC CTC GAG CTA CCC TCC TGT CTC TGA GCA (SEQ ID NO:28), which was designed to put a translation STOP codon (anticodon, CTA) immediately after the C-terminus of angiostatin, and this was followed by an XhoI site (CTCGAG). The PCR product was cloned and sequenced, and the HindIII-XhoI fragment encoding angiostatin was ligated to the pdCs-muFc(D₄K) vector. Stable NS/0 clones expressing muFc(D₄K)-muAngio were selected and assayed (anti-muFc ELISA) as described in Examples 2 and 3.

Example 7. Expression of canine Fc (caFc)

Canine peripheral blood monocytic cells (PBMCs) isolated from dog's blood were used to prepare mRNA. After synthesis of the first strand cDNA with reverse transcriptase and oligo(dT), PCR was performed to amplify the canine Fc (Kazuhiko et al., (1992) JP 1992040894-A1) using the forward primer 5'-CC TTA AGC GAA AAT GGA AGA GTT CCT CGC (SEQ ID NO:29; encoded amino acids disclosed in SEQ ID NO:30), in which an AflII site was introduced immediately upstream of the sequence encoding the hinge region of the canine Fc, and the reverse primer 5'-C CTC GAG TCA TTT ACC CGG GGA ATG GGA GAG GGA TTT CTG (SEQ ID NO:31), in which an XhoI site was introduced after the translation STOP codon (anticodon, TCA) of the canine Fc. The reverse primer also introduced a silent mutation to create a XmaI restriction site, which facilitates the construction of the pdCs-caFc(D₄K) vector through a linker-adaptor and ligation to DNA constructs encoding canine endostatin or angiostatin. Similar to the construction of pdCs-huFc, which was described in detail in Lo et al. (Lo et al., Protein Engineering (1998) 11:495), the expression vector for the pdCs-caFc was constructed as follows. The AflII-XhoI fragment encoding the canine Fc was ligated to the XbaI-AflII fragment encoding the light chain signal peptide and the XbaI-XhoI digested pdCs vector. The resulting pdCs-caFc expression vector then was used to transfect 293 cells. About 3 days post-transfection, the supernatant was purified by Protein A chromatography. Expression of dog Fc (DNA sequence set forth in SEQ ID NO:32; amino acid sequence set forth in SEQ ID NO:33) was confirmed by SDS-PAGE followed by Western blot analysis using a peroxidase-conjugated Rabbit anti-Dog IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA).

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Example 8. Expression of caFc-caEndostatin.

The coding sequence for canine endostatin (DNA sequence set forth in SEQ ID NO:34; amino acid sequence set forth in SEQ ID NO:35) was adapted to a HindIII-XhoI fragment for expression as a Fc fusion protein, essentially as described in Example 5. At the 3' end, a STOP codon was introduced, for example, by PCR, immediately after the codon encoding the C-terminal lysine residue, and this was followed by the NotI restriction site. At the 5' end, however, there was a DraIII restriction site convenient for reconstruction. An oligonucleotide duplex consisting of a HindIII and a DraIII sticky ends was chemically synthesized and used to ligate to the DraIII-XhoI restriction fragment which encodes the rest of the canine endostatin cDNA. The duplex used is shown below:

HindIII

5'-AGCTT CAC ACC CAC CAG GAC TTC CAG CCG GTG CTG CAC CTG (SEQ ID NO:36)

A GTG TGG GTG GTC CTG AAG GTC GGC CAC GAC GTG-5' (SEQ ID NO:38)

DraIII

15

The first CAC in the duplex encodes the N-terminal histidine residue of the canine endostatin. The HindIII-XhoI fragment encoding the full-length canine endostatin thus could be ligated to the HindIII-XhoI digested pdCs-caFc vector (see Example 7) for expression. Stable NS/0 clones expressing caFc-caEndo were selected and assayed by anti-caFc ELISA, as described in Examples 2 and 3. The protein product was analyzed on SDS-PAGE and confirmed by Western blot analysis.

20

Example 9. Expression of caFc-caAngiostatin

The cDNA encoding the full length canine angiostatin (DNA sequence set forth in SEQ ID NO:39; amino acid sequence set forth in SEQ ID NO:40) was adapted for expression as a caFc fusion protein essentially as in the aforementioned examples. Briefly, at the 3' end, a STOP codon was introduced, for example, by PCR, immediately after the codon encoding the C-terminal lysine residue and this was followed by a NotI restriction site instead of an XhoI site, since there was an internal XhoI restriction site in the cDNA of the canine angiostatin. At the 5' end, a HindIII site was introduced in-frame immediately upstream of the N-terminus of angiostatin. The HindIII-NotI fragment encoding the full length canine angiostatin then was ligated to the HindIII-NotI digested pdCs-caFc vector (where the NotI site was introduced at the

30

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XhoI site through linker ligation) for expression. Stable NS/0 clones expressing caFc-caAngio were selected and assayed by anti-caFc ELISA, as described in Examples 2 and 3. The protein product was analyzed on SDS-PAGE and confirmed by Western blot analysis.

Example 10. Expression of muFc-K1 of muAngio

5 Angiostatin comprises the first four of the five Kringle domains of plasminogen. To determine if any one or several Kringle domains are responsible for the observed anti-angiogenic activity of angiostatin, it is possible to produce single Kringle domains by themselves or combination thereof for testing. To demonstrate the utility of Fc as a fusion protein partner, the expression of the first Kringle domain of murine angiostatin (K1) was achieved in the following way. The first Kringle domain ends at Glu-87 of murine angiostatin (SEQ ID NO:25). There was a convenient NsiI restriction site in the cDNA at this position so that after digestion by NsiI, the four-base 3'-overhang was removed by T4 polymerase to create a blunt end. A translation STOP codon was introduced immediately downstream of the GAA encoding Glu-87 via ligation to the palindromic linker TGA CTC GAG TCA (SEQ ID NO: 41), where the STOP codon TGA was followed by an XhoI site. The HindIII-XhoI fragment encoding this truncated angiostatin, i.e., first Kringle only, then was ligated into the pdCs-muFc(D₄K) vector for expression. High levels of expression were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 11. Expression of muFc-innerK1 of muAngio

20 A Kringle domain consists of multiple loops, including an outer loop and an inner loop. In the first Kringle of murine angiostatin, the inner loop is defined by Cys 55 and Cys 79, which together form a disulfide bond at the base of the loop. The Cys-67 of the inner loop forms another disulfide bond with a Cys residue of the outer loop to give the Kringle structure. To test if the inner loop has any anti-angiogenic activity, it was expressed as a muFc-inner K1 (Kringle 1) as follows. With a DNA fragment encoding the first Kringle as template, a mutagenic primer having the sequence 5'GGG CCT TGG AGC TAC ACT ACA (SEQ ID NO: 42; encoded amino acids disclosed in SEQ ID NO:43) was used to mutagenize TGC (Cys-67) to AGC (Ser), by PCR. This ensures that the Cys-67 does not form a disulfide bond when the inner loop of Kringle 1 is expressed without the outer loop. An upstream primer having the sequence

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5'GCGGATCCAAGCTT AGT ACA CAT CCC AAT GAG GG (SEQ ID NO:44; encoded amino acids disclosed in SEQ ID NO:45) was used to introduce a HindIII site in frame immediately 5' to the codon for Ser-43 (AGT). A BamHI site was also introduced immediately upstream of the HindIII site. The BamHI site is useful for ligating to the BamHI site at the end of the flexible Gly-Ser linker shown in Example 12 below. Thus a HindIII-XhoI DNA fragment encoding Ser-43 through Glu-87 of murine angiostatin was ligated to the pdCs-muFc(D₄K) vector for expression. High levels of expression of muFc-innerK1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 12. Expression of muFc-muEndo-GlySer linker-innerK1 of muAngio

10 The hybrid molecule muFc-muEndo-innerK1 comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to the inner loop of the first Kringle of murine angiostatin. The DNA construct was assembled as follows.

15 There is a BspHI site at the 3' end of the murine endostatin cDNA. To introduce a flexible linker of glycine and serine residues at the C-terminus of murine endostatin, a 540-bp HindIII-BspHI fragment encoding endostatin was ligated to an overlapping oligonucleotide duplex formed by the oligonucleotides disclosed in SEQ ID NO:46 and SEQ ID NO:48. The amino acid linker encoded by SEQ ID NO:46 is disclosed in SEQ ID NO:47.

20 The HindIII-BamHI fragment encoding murine endostatin and the Gly-Ser linker was subcloned into a standard cloning vector. The BamHI site was then used to introduce the BamHI-XhoI fragment encoding the innerK1 in Example 11. The resulting HindIII-XhoI fragment encoding muEndo-GlySer linker-innerK1, was ligated to the pdCs-muFc(D₄K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-innerK1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

25 Example 13. Expression of muFc-muEndo-GlySer linker-K1 of muAngio

The hybrid molecule muFc-muEndo-K1 comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to the first Kringle of murine angiostatin. The DNA construct was assembled as follows.

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The BamHI end of the HindIII-BamHI fragment encoding the muEndo-GlySer linker (Example 12) was ligated to the HindIII-XhoI fragment encoding the Kringle 1 of murine angiostatin (Example 10) via the following adaptor:

BamHI
 5' GA TCC TCA GGC C (SEQ ID NO:49)
 G AGT CCG GTCGA (SEQ ID NO:50)
 HindIII

The adaptor has a HindIII' sticky end, which upon ligation, would not regenerate the HindIII site. Thus, the resulting HindIII-XhoI fragment, which encodes the muEndo-GlySer linker-Kringle 1, was ligated to the pdCs-muFc(D₄K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-K1 were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 14 Expression of muFc-muEndo-GlySer linker-muAngio

The hybrid molecule muFc-muEndo-GlySer linker-muAngio comprises muFc-muEndo joined by a polypeptide linker containing glycine and serine residues, to murine angiostatin. The DNA construct was assembled essentially as follows. The BamHI end of the HindIII-BamHI fragment encoding the muEndo-GlySer linker (Example 12) was ligated to the HindIII-XhoI fragment encoding murine angiostatin via the adaptor described in Example 13. The resulting HindIII-XhoI fragment, which encodes the muEndo-GlySer linker-muAngio, was ligated to the pdCs-muFc(D₄K) vector for expression. High levels of expression of muFc-muEndo-GlySer linker-muAngio were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

Example 15. Expression of huAngio-huFc-huEndo

The hybrid molecule huAngio-huFc-huEndo comprises human angiostatin joined by a peptide bond to huFc-huEndo. The DNA construct was assembled as follows. A HindIII-XhoI fragment which encodes human angiostatin without a STOP codon was first generated by PCR, so that the codon for the last amino acid residue of angiostatin was followed immediately by CTCGAG of the XhoI site. The HindIII at the 5' end was ligated to an XbaI-AflIII fragment of

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the light chain signal peptide (Lo et al., Protein Engineering (1998) 11:495) via a AflIII-HindIII' adaptor:

```

      AflIII
5' TTA AGC GGC C      (SEQ ID NO:51)
      CG CGG GTCGA    (SEQ ID NO:52)
      HindIII'
  
```

The HindIII' sticky end of the adaptor, upon ligation, would not regenerate a HindIII site. At the 3' end, the XhoI site was ligated to the AflIII site of the AflIII-XhoI fragment encoding the huFc-hu-Endo via the following XhoI'-AflIII adaptor:

```

      XhoI'
5' TC GAC TCC GGC    (SEQ ID NO:53)
      G AGG CCG AATT  (SEQ ID NO:54)
      AflIII
  
```

The XhoI sticky end of the adaptor, upon ligation, would not regenerate a XhoI site. The resulting XbaI-XhoI fragment encoding the signal peptide-human angiostatin-huFc-human endostatin was cloned into the pdCs vector for expression. High levels of expression of were obtained in both transient and stable expression, as analyzed by anti-muFc ELISA and SDS-PAGE.

20 Example 16 Pharmacokinetics

In one set of pharmacokinetic studies, C57/BL6 mice with implanted Lewis lung tumors at 100-200 mm³ were injected in the tail vein with 720 µg huFc-huAngio per mouse. The size of the tumors and the dosage of huFc-huAngio used in this study were chosen to simulate the actual treatment protocol described by O'Reilly (O'Reilly et al., (1996) Nature Medicine 2:689). Blood was harvested by retro-orbital bleeding at 1/2, 1, 2, 4, 8, 24, and 48 hr. post injection. The blood samples were analyzed by anti-huFc ELISA followed by Western analysis. HuFc-huAngio was found to have a circulating half-life of about 32 hr. in mouse and Western analysis showed that over 90% of the hu-Fc-huAngio remained as an intact molecule in circulation.

The pharmacokinetic studies was also repeated in Swiss mice without tumors at a dosage of 200 µg/mouse. In this case huFc-huAngio was found to have a circulating half-life of about 33 hr.

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Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the
5 invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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SEQUENCE LISTING

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			260					265					270						
Glu	Leu	Thr	Pro	Val	Val	Gln	Asp	Cys	Tyr	His	Gly	Asp	Gly	Gln	Ser				
		275					280					285							
Tyr	Arg	Gly	Thr	Ser	Ser	Thr	Thr	Thr	Thr	Gly	Lys	Lys	Cys	Gln	Ser				
	290					295					300								
Trp	Ser	Ser	Met	Thr	Pro	His	Arg	His	Gln	Lys	Thr	Pro	Glu	Asn	Tyr				
305					310					315					320				
Pro	Asn	Ala	Gly	Leu	Thr	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Asp				
				325					330					335					
Lys	Gly	Pro	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Ser	Val	Arg	Trp	Glu	Tyr				
			340					345					350						
Cys	Asn	Leu	Lys	Lys	Cys	Ser	Gly	Thr	Glu	Ala									
	355						360												

<210> 12
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Forward primer
 for human Fc-Angio

<220>
 <221> CDS
 <222> (3)..(29)

<400> 12
 cc ccg ggt aag aaa gtg tat ctc tca gag
 Pro Gly Lys Lys Val Tyr Leu Ser Glu
 1 5

29

<210> 13
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<400> 13

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Pro Gly Lys Lys Val Tyr Leu Ser Glu
 1 5

<210> 14
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Forward primer
 for human Fc-Angio

<220>
 <221> CDS
 <222> (2)..(28)

<400> 14
 c ccc aag ctt aaa gtg tat ctc tca gag 28
 Pro Lys Leu Lys Val Tyr Leu Ser Glu
 1 5

<210> 15
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<400> 15
 Pro Lys Leu Lys Val Tyr Leu Ser Glu
 1 5

<210> 16
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Reverse primer
 for human Fc-Angio

<400> 16
 cccctcgagc tacgcttctg ttcttgagca 30

<210> 17
 <211> 552
 <212> DNA
 <213> Mus musculus

<220>
 <221> CDS
 <222> (1)..(552)
 <223> endostatin

<400> 17
 cat act cat cag gac ttt cag cca gtg ctc cac ctg gtg gca ctg aac 48

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His	Thr	His	Gln	Asp	Phe	Gln	Pro	Val	Leu	His	Leu	Val	Ala	Leu	Asn	
1				5					10					15		
acc	ccc	ctg	tct	gga	ggc	atg	cgt	ggt	atc	cgt	gga	gca	gat	ttc	cag	96
Thr	Pro	Leu	Ser	Gly	Gly	Met	Arg	Gly	Ile	Arg	Gly	Ala	Asp	Phe	Gln	
			20					25					30			
tgc	ttc	cag	caa	gcc	cga	gcc	gtg	ggg	ctg	tcg	ggc	acc	ttc	cgg	gct	144
Cys	Phe	Gln	Gln	Ala	Arg	Ala	Val	Gly	Leu	Ser	Gly	Thr	Phe	Arg	Ala	
		35					40					45				
ttc	ctg	tcc	tct	agg	ctg	cag	gat	ctc	tat	agc	atc	gtg	cgc	cgt	gct	192
Phe	Leu	Ser	Ser	Arg	Leu	Gln	Asp	Leu	Tyr	Ser	Ile	Val	Arg	Arg	Ala	
	50					55					60					
gac	cgg	ggg	tct	gtg	ccc	atc	gtc	aac	ctg	aag	gac	gag	gtg	cta	tct	240
Asp	Arg	Gly	Ser	Val	Pro	Ile	Val	Asn	Leu	Lys	Asp	Glu	Val	Leu	Ser	
65					70					75					80	
ccc	agc	tgg	gac	tcc	ctg	ttt	tct	ggc	tcc	cag	ggt	caa	gtg	caa	ccc	288
Pro	Ser	Trp	Asp	Ser	Leu	Phe	Ser	Gly	Ser	Gln	Gly	Gln	Val	Gln	Pro	
				85					90					95		
ggg	gcc	cgc	atc	ttt	tct	ttt	gac	ggc	aga	gat	gtc	ctg	aga	cac	cca	336
Gly	Ala	Arg	Ile	Phe	Ser	Phe	Asp	Gly	Arg	Asp	Val	Leu	Arg	His	Pro	
			100				105						110			
gcc	tgg	ccg	cag	aag	agc	gta	tgg	cac	ggc	tcg	gac	ccc	agt	ggg	cgg	384
Ala	Trp	Pro	Gln	Lys	Ser	Val	Trp	His	Gly	Ser	Asp	Pro	Ser	Gly	Arg	
		115					120					125				
agg	ctg	atg	gag	agt	tac	tgt	gag	aca	tgg	cga	act	gaa	act	act	ggg	432
Arg	Leu	Met	Glu	Ser	Tyr	Cys	Glu	Thr	Trp	Arg	Thr	Glu	Thr	Thr	Gly	
	130					135					140					
gct	aca	ggt	cag	gcc	tcc	tcc	ctg	ctg	tca	ggc	agg	ctc	ctg	gaa	cag	480
Ala	Thr	Gly	Gln	Ala	Ser	Ser	Leu	Leu	Ser	Gly	Arg	Leu	Leu	Glu	Gln	
145					150					155					160	
aaa	gct	gcg	agc	tgc	cac	aac	agc	tac	atc	gtc	ctg	tgc	att	gag	aat	528
Lys	Ala	Ala	Ser	Cys	His	Asn	Ser	Tyr	Ile	Val	Leu	Cys	Ile	Glu	Asn	
				165					170					175		
agc	ttc	atg	acc	tct	ttc	tcc	aaa									552
Ser	Phe	Met	Thr	Ser	Phe	Ser	Lys									
			180													
<210>	18															
<211>	184															
<212>	PRT															
<213>	Mus musculus															
<400>	18															
His	Thr	His	Gln	Asp	Phe	Gln	Pro	Val	Leu	His	Leu	Val	Ala	Leu	Asn	
1				5					10					15		
Thr	Pro	Leu	Ser	Gly	Gly	Met	Arg	Gly	Ile	Arg	Gly	Ala	Asp	Phe	Gln	

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	20		25		30										
Cys	Phe	Gln	Gln	Ala	Arg	Ala	Val	Gly	Leu	Ser	Gly	Thr	Phe	Arg	Ala
		35					40					45			
Phe	Leu	Ser	Ser	Arg	Leu	Gln	Asp	Leu	Tyr	Ser	Ile	Val	Arg	Arg	Ala
	50					55					60				
Asp	Arg	Gly	Ser	Val	Pro	Ile	Val	Asn	Leu	Lys	Asp	Glu	Val	Leu	Ser
65					70					75				80	
Pro	Ser	Trp	Asp	Ser	Leu	Phe	Ser	Gly	Ser	Gln	Gly	Gln	Val	Gln	Pro
				85					90					95	
Gly	Ala	Arg	Ile	Phe	Ser	Phe	Asp	Gly	Arg	Asp	Val	Leu	Arg	His	Pro
			100					105					110		
Ala	Trp	Pro	Gln	Lys	Ser	Val	Trp	His	Gly	Ser	Asp	Pro	Ser	Gly	Arg
		115					120						125		
Arg	Leu	Met	Glu	Ser	Tyr	Cys	Glu	Thr	Trp	Arg	Thr	Glu	Thr	Thr	Gly
	130					135					140				
Ala	Thr	Gly	Gln	Ala	Ser	Ser	Leu	Leu	Ser	Gly	Arg	Leu	Leu	Glu	Gln
145					150					155					160
Lys	Ala	Ala	Ser	Cys	His	Asn	Ser	Tyr	Ile	Val	Leu	Cys	Ile	Glu	Asn
				165					170					175	
Ser	Phe	Met	Thr	Ser	Phe	Ser	Lys								
			180												

<210> 19
 <211> 699
 <212> DNA
 <213> Mus musculus

<220>
 <221> CDS
 <222> (1)..(699)
 <223> Fc

<400> 19																	
gag	ccc	aga	ggg	ccc	aca	atc	aag	ccc	tgt	cct	cca	tgc	aaa	tgc	cca		48
Glu	Pro	Arg	Gly	Pro	Thr	Ile	Lys	Pro	Cys	Pro	Pro	Cys	Lys	Cys	Pro		
1				5				10						15			
gca	cct	aac	ctc	ttg	ggt	gga	cca	tcc	gtc	ttc	atc	ttc	cct	cca	aag		96
Ala	Pro	Asn	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Lys		
			20					25					30				
atc	aag	gat	gta	ctc	atg	atc	tcc	ctg	agc	ccc	ata	gtc	aca	tgt	gtg		144
Ile	Lys	Asp	Val	Leu	Met	Ile	Ser	Leu	Ser	Pro	Ile	Val	Thr	Cys	Val		
		35				40						45					
gtg	gtg	gat	gtg	agc	gag	gat	gac	cca	gat	gtc	cag	atc	agc	tgg	ttt		192
Val	Val	Asp	Val	Ser	Glu	Asp	Asp	Pro	Asp	Val	Gln	Ile	Ser	Trp	Phe		

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50	55	60	
gtg aac aac gtg gaa gta cac aca gct cag aca caa acc cat aga gag Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu 65 70 75 80			240
gat tac aac agt act ctc cgg gtg gtc agt gcc ctc ccc atc cag cac Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His 85 90 95			288
cag gac tgg atg agt ggc aag gag ttc aaa tgc aag gtc aac aac aaa Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys 100 105 110			336
gac ctc cca gcg ccc atc gag aga acc atc tca aaa ccc aaa ggg tca Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser 115 120 125			384
gta aga gct cca cag gta tat gtc ttg cct cca cca gaa gaa gag atg Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met 130 135 140			432
act aag aaa cag gtc act ctg acc tgc atg gtc aca gac ttc atg cct Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro 145 150 155 160			480
gaa gac att tac gtg gag tgg acc aac aac ggg aaa aca gag cta aac Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn 165 170 175			528
tac aag aac act gaa cca gtc ctg gac tct gat ggt tct tac ttc atg Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met 180 185 190			576
tac agc aag ctg aga gtg gaa aag aag aac tgg gtg gaa aga aat agc Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser 195 200 205			624
tac tcc tgt tca gtg gtc cac gag ggt ctg cac aat cac cac acg act Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr 210 215 220			672
aag agc ttc tcc cgg acc ccg ggt aaa Lys Ser Phe Ser Arg Thr Pro Gly Lys 225 230			699
 <210> 20 <211> 233 <212> PRT <213> Mus musculus			
 <400> 20 Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Lys Cys Pro 1 5 10 15 Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys 20 25 30			

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Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
35 40 45

Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
50 55 60

Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
65 70 75 80

Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
85 90 95

Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
100 105 110

Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys Gly Ser
115 120 125

Val Arg Ala Pro Gln Val Tyr Val Leu Pro Pro Pro Glu Glu Glu Met
130 135 140

Thr Lys Lys Gln Val Thr Leu Thr Cys Met Val Thr Asp Phe Met Pro
145 150 155 160

Glu Asp Ile Tyr Val Glu Trp Thr Asn Asn Gly Lys Thr Glu Leu Asn
165 170 175

Tyr Lys Asn Thr Glu Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Met
180 185 190

Tyr Ser Lys Leu Arg Val Glu Lys Lys Asn Trp Val Glu Arg Asn Ser
195 200 205

Tyr Ser Cys Ser Val Val His Glu Gly Leu His Asn His His Thr Thr
210 215 220

Lys Ser Phe Ser Arg Thr Pro Gly Lys
225 230

<210> 21

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Forward primer
for mouse Fc-Endo

<220>

<221> CDS

<222> (2)..(28)

<400> 21

c ccc aag ctt cat act cat cag gac ttt c
Pro Lys Leu His Thr His Gln Asp Phe
1 5

29

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<210> 22
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<400> 22
 Pro Lys Leu His Thr His Gln Asp Phe
 1 5

<210> 23
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Reverse primer
 for mouse Fc-Endo

<400> 23
 cccctcgagc tatttgagaga aagaggtc 28

<210> 24
 <211> 1086
 <212> DNA
 <213> Mus musculus

<220>
 <221> CDS
 <222> (1)..(1086)
 <223> Angiostatin

<400> 24
 gtg tat ctg tca gaa tgt aag acc ggc atc ggc aac ggc tac aga gga 48
 Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly
 1 5 10 15
 acc atg tcc agg aca aag agt ggt gtt gcc tgt caa aag tgg ggt gcc 96
 Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly Ala
 20 25 30
 acg ttc ccc cac gta ccc aac tac tct ccc agt aca cat ccc aat gag 144
 Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn Glu
 35 40 45
 gga cta gaa gag aac tac tgt agg aac cca gac aat gat gaa caa ggg 192
 Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly
 50 55 60
 cct tgg tgc tac act aca gat ccg gac aag aga tat gac tac tgc aac 240
 Pro Trp Cys Tyr Thr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn
 65 70 75 80
 att cct gaa tgt gaa gag gaa tgc atg tac tgc agt gga gaa aag tat 288
 Ile Pro Glu Cys Glu Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys Tyr

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					85						90						95	
gag	ggc	aaa	atc	tcc	aag	acc	atg	tct	gga	ctt	gac	tgc	cag	gcc	tgg	336		
Glu	Gly	Lys	Ile	Ser	Lys	Thr	Met	Ser	Gly	Leu	Asp	Cys	Gln	Ala	Trp			
					100						105						110	
gat	tct	cag	agc	cca	cat	gct	cat	gga	tac	atc	cct	gcc	aaa	ttt	cca	384		
Asp	Ser	Gln	Ser	Pro	His	Ala	His	Gly	Tyr	Ile	Pro	Ala	Lys	Phe	Pro			
					115						120						125	
agc	aag	aac	ctg	aag	atg	aat	tat	tgc	cac	aac	cct	gac	ggg	gag	cca	432		
Ser	Lys	Asn	Leu	Lys	Met	Asn	Tyr	Cys	His	Asn	Pro	Asp	Gly	Glu	Pro			
					130						135						140	
agg	ccc	tgg	tgc	ttc	aca	aca	gac	ccc	acc	aaa	cgc	tgg	gaa	tac	tgt	480		
Arg	Pro	Trp	Cys	Phe	Thr	Thr	Asp	Pro	Thr	Lys	Arg	Trp	Glu	Tyr	Cys			
					145						150						155	160
gac	atc	ccc	cgc	tgc	aca	aca	ccc	ccg	ccc	cca	ccc	agc	cca	acc	tac	528		
Asp	Ile	Pro	Arg	Cys	Thr	Thr	Pro	Pro	Pro	Pro	Pro	Ser	Pro	Thr	Tyr			
					165						170						175	
caa	tgt	ctg	aaa	gga	aga	ggt	gaa	aat	tac	cga	ggg	acc	gtg	tct	gtc	576		
Gln	Cys	Leu	Lys	Gly	Arg	Gly	Glu	Asn	Tyr	Arg	Gly	Thr	Val	Ser	Val			
					180						185						190	
acc	gtg	tct	ggg	aaa	acc	tgt	cag	cgc	tgg	agt	gag	caa	acc	cct	cat	624		
Thr	Val	Ser	Gly	Lys	Thr	Cys	Gln	Arg	Trp	Ser	Glu	Gln	Thr	Pro	His			
					195						200						205	
agg	cac	aac	agg	aca	cca	gaa	aat	ttc	ccc	tgc	aaa	aat	ctg	gaa	gag	672		
Arg	His	Asn	Arg	Thr	Pro	Glu	Asn	Phe	Pro	Cys	Lys	Asn	Leu	Glu	Glu			
					210						215						220	
aac	tac	tgc	cgg	aac	cca	gat	gga	gaa	act	gct	ccc	tgg	tgc	tat	acc	720		
Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Gly	Glu	Thr	Ala	Pro	Trp	Cys	Tyr	Thr			
					225						230						235	240
act	gac	agc	cag	ctg	agg	tgg	gag	tac	tgt	gag	att	cca	tcc	tgc	gag	768		
Thr	Asp	Ser	Gln	Leu	Arg	Trp	Glu	Tyr	Cys	Glu	Ile	Pro	Ser	Cys	Glu			
					245						250						255	
tcc	tca	gca	tca	cca	gac	cag	tca	gat	tcc	tca	gtt	cca	cca	gag	gag	816		
Ser	Ser	Ala	Ser	Pro	Asp	Gln	Ser	Asp	Ser	Ser	Val	Pro	Pro	Glu	Glu			
					260						265						270	
caa	aca	cct	gtg	gtc	cag	gaa	tgc	tac	cag	agc	gat	ggg	cag	agc	tat	864		
Gln	Thr	Pro	Val	Val	Gln	Glu	Cys	Tyr	Gln	Ser	Asp	Gly	Gln	Ser	Tyr			
					275						280						285	
cgg	ggt	aca	tcg	tcc	act	acc	atc	aca	ggg	aag	aag	tgc	cag	tcc	tgg	912		
Arg	Gly	Thr	Ser	Ser	Thr	Thr	Ile	Thr	Gly	Lys	Lys	Cys	Gln	Ser	Trp			
					290						295						300	
gca	gct	atg	ttt	cca	cac	agg	cat	tcg	aag	acc	cca	gag	aac	ttc	cca	960		
Ala	Ala	Met	Phe	Pro	His	Arg	His	Ser	Lys	Thr	Pro	Glu	Asn	Phe	Pro			
					305						310						315	320

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gat gct ggc ttg gag atg aac tac tgc agg aac ccg gat ggt gac aag 1008
 Asp Ala Gly Leu Glu Met Asn Tyr Cys Arg Asn Pro Asp Gly Asp Lys
 325 330 335

ggc cct tgg tgc tac acc act gac ccg agc gtc agg tgg gaa tac tgc 1056
 Gly Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys
 340 345 350

aac ctg aag cgg tgc tca gag aca gga ggg 1086
 Asn Leu Lys Arg Cys Ser Glu Thr Gly Gly
 355 360

<210> 25
 <211> 362
 <212> PRT
 <213> Mus musculus

<400> 25
 Val Tyr Leu Ser Glu Cys Lys Thr Gly Ile Gly Asn Gly Tyr Arg Gly
 1 5 10 15

Thr Met Ser Arg Thr Lys Ser Gly Val Ala Cys Gln Lys Trp Gly Ala
 20 25 30

Thr Phe Pro His Val Pro Asn Tyr Ser Pro Ser Thr His Pro Asn Glu
 35 40 45

Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Gln Gly
 50 55 60

Pro Trp Cys Tyr Thr Thr Asp Pro Asp Lys Arg Tyr Asp Tyr Cys Asn
 65 70 75 80

Ile Pro Glu Cys Glu Glu Glu Cys Met Tyr Cys Ser Gly Glu Lys Tyr
 85 90 95

Glu Gly Lys Ile Ser Lys Thr Met Ser Gly Leu Asp Cys Gln Ala Trp
 100 105 110

Asp Ser Gln Ser Pro His Ala His Gly Tyr Ile Pro Ala Lys Phe Pro
 115 120 125

Ser Lys Asn Leu Lys Met Asn Tyr Cys His Asn Pro Asp Gly Glu Pro
 130 135 140

Arg Pro Trp Cys Phe Thr Thr Asp Pro Thr Lys Arg Trp Glu Tyr Cys
 145 150 155 160

Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Pro Thr Tyr
 165 170 175

Gln Cys Leu Lys Gly Arg Gly Glu Asn Tyr Arg Gly Thr Val Ser Val
 180 185 190

Thr Val Ser Gly Lys Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro His
 195 200 205

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Arg His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Glu Glu
 210 215 220

Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
 225 230 235 240

Thr Asp Ser Gln Leu Arg Trp Glu Tyr Cys Glu Ile Pro Ser Cys Glu
 245 250 255

Ser Ser Ala Ser Pro Asp Gln Ser Asp Ser Ser Val Pro Pro Glu Glu
 260 265 270

Gln Thr Pro Val Val Gln Glu Cys Tyr Gln Ser Asp Gly Gln Ser Tyr
 275 280 285

Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln Ser Trp
 290 295 300

Ala Ala Met Phe Pro His Arg His Ser Lys Thr Pro Glu Asn Phe Pro
 305 310 315 320

Asp Ala Gly Leu Glu Met Asn Tyr Cys Arg Asn Pro Asp Gly Asp Lys
 325 330 335

Gly Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys
 340 345 350

Asn Leu Lys Arg Cys Ser Glu Thr Gly Gly
 355 360

<210> 26

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Forward primer
for mouse Fc-Angio

<220>

<221> CDS

<222> (2)..(49)

<400> 26

c ccc aag ctt gtg tat ctg tca gaa tgt aag
 Pro Lys Leu Val Tyr Leu Ser Glu Cys Lys
 1 5 10

31

<210> 27

<211> 10

<212> PRT

<213> Artificial Sequence

<400> 27

Pro Lys Leu Val Tyr Leu Ser Glu Cys Lys
 1 5 10

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<210> 28
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Reverse primer
 for mouse Fc-Angio

<400> 28
 cccctcgagc taccctcctg tctctgagca

30

<210> 29
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Forward primer
 for canine Fc

<220>
 <221> CDS
 <222> (3)..(29)

<400> 29
 cc tta agc gaa aat gga aga gtt cct cgc
 Leu Ser Glu Asn Gly Arg Val Pro Arg
 1 5

29

<210> 30
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<400> 30
 Leu Ser Glu Asn Gly Arg Val Pro Arg
 1 5

<210> 31
 <211> 40
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Reverse primer
 for canine Fc

<400> 31
 cctcgagtca ttaccocggg gaatgggaga gggatttctg

40

<210> 32

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<211> 702
 <212> DNA
 <213> Canis familiaris

<220>
 <221> CDS
 <222> (1)..(702)
 <223> Fc

<400> 32

gaa aat gga aga gtt cct cgc cca cct gat tgt ccc aaa tgc cca gcc	48
Glu Asn Gly Arg Val Pro Arg Pro Pro Asp Cys Pro Lys Cys Pro Ala	
1 5 10 15	
cct gaa atg ctg gga ggg cct tgc gtc ttc atc ttt ccc ccg aaa ccc	96
Pro Glu Met Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys Pro	
20 25 30	
aag gac acc ctc ttg att gcc cga aca cct gag gtc aca tgt gtg gtg	144
Lys Asp Thr Leu Leu Ile Ala Arg Thr Pro Glu Val Thr Cys Val Val	
35 40 45	
gtg gat ctg gga cca gaa gac cct gag gtg cag atc agc tgg ttc gtg	192
Val Asp Leu Gly Pro Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val	
50 55 60	
gac ggt aag cag atg caa aca gcc aag act cag cct cgt gag gag cag	240
Asp Gly Lys Gln Met Gln Thr Ala Lys Thr Gln Pro Arg Glu Glu Gln	
65 70 75 80	
ttc aat ggc acc tac cgt gtg gtc agt gtc ctc ccc att ggg cac cag	288
Phe Asn Gly Thr Tyr Arg Val Val Ser Val Leu Pro Ile Gly His Gln	
85 90 95	
gac tgg ctc aag ggg aag cag ttc acg tgc aaa gtc aac aac aaa gcc	336
Asp Trp Leu Lys Gly Lys Gln Phe Thr Cys Lys Val Asn Asn Lys Ala	
100 105 110	
ctc cca tcc ccg atc gag agg acc atc tcc aag gcc aga ggg cag gcc	384
Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Gln Ala	
115 120 125	
cat cag ccc agt gtg tat gtc ctg ccg cca tcc cgg gag gag ttg agc	432
His Gln Pro Ser Val Tyr Val Leu Pro Pro Ser Arg Glu Glu Leu Ser	
130 135 140	
aag aac aca gtc agc ttg aca tgc ctg atc aaa gac ttc ttc cca cct	480
Lys Asn Thr Val Ser Leu Thr Cys Leu Ile Lys Asp Phe Phe Pro Pro	
145 150 155 160	
gac att gat gtg gag tgg cag agc aat gga cag cag gag cct gag agc	528
Asp Ile Asp Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Ser	
165 170 175	
aag tac cgc acg acc ccg ccc cag ctg gac gag gac ggg tcc tac ttc	576
Lys Tyr Arg Thr Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe	
180 185 190	

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Thr Phe Ile Cys Ala Val Met His Glu Ala Leu His Asn His Tyr Thr
 210 215 220

Gln Lys Ser Leu Ser His Ser Pro Gly Lys
 225 230

<210> 34
 <211> 552
 <212> DNA
 <213> Canis familiaris

<220>
 <221> CDS
 <222> (1)..(552)
 <223> Endostatin

<400> 34
 cac acc cac cag gac ttc cag ccg gtg ctg cac ctg gtg gcc ctg aac 48
 His Thr His Gln Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn
 1 5 10 15
 agc ccg cag ccg ggc ggc atg cga ggc atc cgg gga gcg gac ttc cag 96
 Ser Pro Gln Pro Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
 20 25 30
 tgc ttc cag cag gcg cgc gcc gcg ggg ctg gcc ggc acc ttc cgg gcc 144
 Cys Phe Gln Gln Ala Arg Ala Ala Gly Leu Ala Gly Thr Phe Arg Ala
 35 40 45
 ttc ctg tcg tcg cgg ctg cag gac ctc tac agc atc gtg cgc cgc gcc 192
 Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
 50 55 60
 gac cgc acc ggg gtg ccc gtc gtc aac ctc agg gac gag gtg ctc ttc 240
 Asp Arg Thr Gly Val Pro Val Val Asn Leu Arg Asp Glu Val Leu Phe
 65 70 75 80
 ccc agc tgg gag gcc tta ttc tcg ggc tcc gag ggc cag ctg aag ccc 288
 Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Gln Leu Lys Pro
 85 90 95
 ggg gcc cgc atc ttc tct ttc gac ggc aga gat gtc ctg cag cac ccc 336
 Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Gln His Pro
 100 105 110
 gcc tgg ccc cgg aag agc gtg tgg cac ggc tcc gac ccc agc ggg cgc 384
 Ala Trp Pro Arg Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
 115 120 125
 cgc ctg acc gac agc tac tgc gag acg tgg cgg acg gag gcc ccg gcg 432
 Arg Leu Thr Asp Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ala
 130 135 140
 gcc acc ggg cag gcg tcg tcg ctg ctg gcg ggc agg ctg ctg gag cag 480
 Ala Thr Gly Gln Ala Ser Ser Leu Leu Ala Gly Arg Leu Leu Glu Gln
 145 150 155 160

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gag gcc gcg agc tgc cgc cac gcc ttc gtg gtg ctc tgc atc gag aac 528
 Glu Ala Ala Ser Cys Arg His Ala Phe Val Val Leu Cys Ile Glu Asn
 165 170 175

agc gtc atg acc tcc ttc tcc aag 552
 Ser Val Met Thr Ser Phe Ser Lys
 180

<210> 35
 <211> 184
 <212> PRT
 <213> Canis familiaris

<400> 35
 His Thr His Gln Asp Phe Gln Pro Val Leu His Leu Val Ala Leu Asn
 1 5 10 15
 Ser Pro Gln Pro Gly Gly Met Arg Gly Ile Arg Gly Ala Asp Phe Gln
 20 25 30
 Cys Phe Gln Gln Ala Arg Ala Ala Gly Leu Ala Gly Thr Phe Arg Ala
 35 40 45
 Phe Leu Ser Ser Arg Leu Gln Asp Leu Tyr Ser Ile Val Arg Arg Ala
 50 55 60
 Asp Arg Thr Gly Val Pro Val Val Asn Leu Arg Asp Glu Val Leu Phe
 65 70 75 80
 Pro Ser Trp Glu Ala Leu Phe Ser Gly Ser Glu Gly Gln Leu Lys Pro
 85 90 95
 Gly Ala Arg Ile Phe Ser Phe Asp Gly Arg Asp Val Leu Gln His Pro
 100 105 110
 Ala Trp Pro Arg Lys Ser Val Trp His Gly Ser Asp Pro Ser Gly Arg
 115 120 125
 Arg Leu Thr Asp Ser Tyr Cys Glu Thr Trp Arg Thr Glu Ala Pro Ala
 130 135 140
 Ala Thr Gly Gln Ala Ser Ser Leu Leu Ala Gly Arg Leu Leu Glu Gln
 145 150 155 160
 Glu Ala Ala Ser Cys Arg His Ala Phe Val Val Leu Cys Ile Glu Asn
 165 170 175
 Ser Val Met Thr Ser Phe Ser Lys
 180

<210> 36
 <211> 41
 <212> DNA
 <213> Artificial Sequence

<220>

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<223> Description of Artificial Sequence:HindIII/DraIII
linker: top strand

<220>

<221> CDS

<222> (3)..(41)

<400> 36

ag ctt cac acc cac cag gac ttc cag ccg gtg ctg cac ctg 41
Leu His Thr His Gln Asp Phe Gln Pro Val Leu His Leu
1 5 10

<210> 37

<211> 13

<212> PRT

<213> Artificial Sequence

<400> 37

Leu His Thr His Gln Asp Phe Gln Pro Val Leu His Leu
1 5 10

<210> 38

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:HindIII/DraIII
linker: bottom strand

<400> 38

gtgcagcacc ggctggaagt cctggtgggt gtga 34

<210> 39

<211> 1077

<212> DNA

<213> Canis familiaris

<220>

<221> CDS

<222> (1)..(1077)

<223> angiostatin

<400> 39

ata tat ctt tca gag tgc aag act gga aat ggg aaa acc tac agg ggg 48
Ile Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Thr Tyr Arg Gly
1 5 10 15

acc atg gcc aaa acg aag aat gat gtt gcc tgt caa aaa tgg agt gac 96
Thr Met Ala Lys Thr Lys Asn Asp Val Ala Cys Gln Lys Trp Ser Asp
20 25 30

aat tct ccg cac aaa cct aac tat acg cct gag aag cac ccc ttg gag 144
Asn Ser Pro His Lys Pro Asn Tyr Thr Pro Glu Lys His Pro Leu Glu
35 40 45

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ggg ctg gag gag aac tat tgc agg aac cct gac aac gac gag aac ggg	192
Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Asn Gly	
50 55 60	
ccc tgg tgc tac acc aca aac cca gac gtg agg ttc gac tac tgc aac	240
Pro Trp Cys Tyr Thr Thr Asn Pro Asp Val Arg Phe Asp Tyr Cys Asn	
65 70 75 80	
att cca gaa tgt gaa gag gaa tgt atg cat tgc agt ggg gaa aat tat	288
Ile Pro Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr	
85 90 95	
gag ggc aaa att tcc aag aca aag tct gga ctc gag tgc caa gcc tgg	336
Glu Gly Lys Ile Ser Lys Thr Lys Ser Gly Leu Glu Cys Gln Ala Trp	
100 105 110	
aac tct caa acc cca cat gct cat gga tat att cct tcc aaa ttt cca	384
Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro	
115 120 125	
agc aag aac ttg aag atg aat tac tgc cgt aac cct gat ggg gag ccc	432
Ser Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn Pro Asp Gly Glu Pro	
130 135 140	
cgc cca tgg tgt ttc acc atg gat ccc aac aaa cgc tgg gaa ttc tgt	480
Arg Pro Trp Cys Phe Thr Met Asp Pro Asn Lys Arg Trp Glu Phe Cys	
145 150 155 160	
gac att ccc cgc tgt aca aca cca cca ccc cct tcg ggc cca acg tac	528
Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Gly Pro Thr Tyr	
165 170 175	
cag tgt ctg aag ggc aga ggg gag agc tac cga ggg aag gtg tcc gtc	576
Gln Cys Leu Lys Gly Arg Gly Glu Ser Tyr Arg Gly Lys Val Ser Val	
180 185 190	
act gtc tct gga cat aca tgt cag cac tgg agt gaa cag acc cct cac	624
Thr Val Ser Gly His Thr Cys Gln His Trp Ser Glu Gln Thr Pro His	
195 200 205	
aag cac aac agg acc cca gaa aac ttc cct tgc aaa aat ttg gat gaa	672
Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu	
210 215 220	
aac tac tgt cgc aac cct gat gga gaa aca gct cca tgg tgc tac aca	720
Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr	
225 230 235 240	
acc aac agt gag gtg agg tgg gaa cac tgc cag att ccg tcc tgt gag	768
Thr Asn Ser Glu Val Arg Trp Glu His Cys Gln Ile Pro Ser Cys Glu	
245 250 255	
tcc tct cca ata acc aca gaa tat ttg gat gcc cca gct tca gtg cca	816
Ser Ser Pro Ile Thr Thr Glu Tyr Leu Asp Ala Pro Ala Ser Val Pro	
260 265 270	
cct gaa caa act cct gtg gtc cag gag tgc tac cac ggc aat ggg cag	864

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Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr His Gly Asn Gly Gln
 275 280 285

agt tat cga ggc aca tca tcc act act atc aca gga aga aaa tgt cag 912
 Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Arg Lys Cys Gln
 290 295 300

tct tgg tca tct atg aca cca cac cga cat gag aag acc cca gaa cac 960
 Ser Trp Ser Ser Met Thr Pro His Arg His Glu Lys Thr Pro Glu His
 305 310 315 320

ttc ccg gag gct ggc ctg aca atg aac tac tgc agg aat ccc gac gcc 1008
 Phe Pro Glu Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala
 325 330 335

gac aaa agc cct tgg tgt tac acc acc gac ccc tct gtg cgc tgg gag 1056
 Asp Lys Ser Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu
 340 345 350

ttc tgt aac ttg aga aaa tgc 1077
 Phe Cys Asn Leu Arg Lys Cys
 355

<210> 40
 <211> 359
 <212> PRT
 <213> Canis familiaris

<400> 40
 Ile Tyr Leu Ser Glu Cys Lys Thr Gly Asn Gly Lys Thr Tyr Arg Gly
 1 5 10 15

Thr Met Ala Lys Thr Lys Asn Asp Val Ala Cys Gln Lys Trp Ser Asp
 20 25 30

Asn Ser Pro His Lys Pro Asn Tyr Thr Pro Glu Lys His Pro Leu Glu
 35 40 45

Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Asn Asp Glu Asn Gly
 50 55 60

Pro Trp Cys Tyr Thr Thr Asn Pro Asp Val Arg Phe Asp Tyr Cys Asn
 65 70 75 80

Ile Pro Glu Cys Glu Glu Glu Cys Met His Cys Ser Gly Glu Asn Tyr
 85 90 95

Glu Gly Lys Ile Ser Lys Thr Lys Ser Gly Leu Glu Cys Gln Ala Trp
 100 105 110

Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro
 115 120 125

Ser Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn Pro Asp Gly Glu Pro
 130 135 140

Arg Pro Trp Cys Phe Thr Met Asp Pro Asn Lys Arg Trp Glu Phe Cys

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145 150 155 160
 Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Pro Ser Gly Pro Thr Tyr
 165 170 175
 Gln Cys Leu Lys Gly Arg Gly Glu Ser Tyr Arg Gly Lys Val Ser Val
 180 185 190
 Thr Val Ser Gly His Thr Cys Gln His Trp Ser Glu Gln Thr Pro His
 195 200 205
 Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu
 210 215 220
 Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Ala Pro Trp Cys Tyr Thr
 225 230 235 240
 Thr Asn Ser Glu Val Arg Trp Glu His Cys Gln Ile Pro Ser Cys Glu
 245 250 255
 Ser Ser Pro Ile Thr Thr Glu Tyr Leu Asp Ala Pro Ala Ser Val Pro
 260 265 270
 Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr His Gly Asn Gly Gln
 275 280 285
 Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Arg Lys Cys Gln
 290 295 300
 Ser Trp Ser Ser Met Thr Pro His Arg His Glu Lys Thr Pro Glu His
 305 310 315 320
 Phe Pro Glu Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala
 325 330 335
 Asp Lys Ser Pro Trp Cys Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu
 340 345 350
 Phe Cys Asn Leu Arg Lys Cys
 355

<210> 41
 <211> 12
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:palindromic
 linker where the STOP codon TGA is followed by an
 XhoI site

<400> 41
 tgactcgagt ca

12

<210> 42
 <211> 21

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<212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Mutagenic
 primer for murine angiostatin

<220>
 <221> CDS
 <222> (1)..(21)

<400> 42
 ggg cct tgg agc tac act aca
 Gly Pro Trp Ser Tyr Thr Thr
 1 5

21

<210> 43
 <211> 7
 <212> PRT
 <213> Artificial Sequence

<400> 43
 Gly Pro Trp Ser Tyr Thr Thr
 1 5

<210> 44
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer used to
 introduce HindIII into murine angiostatin

<220>
 <221> CDS
 <222> (9)..(32)

<400> 44
 gcggatcc aag ctt agt aca cat ccc aat gag gg
 Lys Leu Ser Thr His Pro Asn Glu
 1 5

34

<210> 45
 <211> 8
 <212> PRT
 <213> Artificial Sequence

<400> 45
 Lys Leu Ser Thr His Pro Asn Glu
 1 5

<210> 46
 <211> 59

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<212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:BspHI/BamHI
 linker: top strand

<220>
 <221> CDS
 <222> (2)..(58)

<400> 46
 c atg acc tct ttc tcc aaa tcg agc ggg ggc agc ggg ggc gga ggc agc 49
 Met Thr Ser Phe Ser Lys Ser Ser Gly Gly Ser Gly Gly Gly Gly Ser
 1 5 10 15

ggc ggc ggc g 59
 Gly Gly Gly

<210> 47
 <211> 19
 <212> PRT
 <213> Artificial Sequence

<400> 47
 Met Thr Ser Phe Ser Lys Ser Ser Gly Gly Ser Gly Gly Gly Gly Ser
 1 5 10 15

Gly Gly Gly

<210> 48
 <211> 59
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:BspHI/BamHI
 linker: bottom strand

<400> 48
 gatccgcccc cgccgctgcc tccgcccccg ctgccccgc tcgatttgga gaaagaggt 59

<210> 49
 <211> 12
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:BamHI/HindIII
 linker: top strand

<220>
 <221> CDS
 <222> (3)..(11)

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<400> 49
ga tcc tca ggc c 12
Ser Ser Gly
1

<210> 50
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:BamHI/HindIII
linker: bottom strand

<400> 50
agctggcctg ag 12

<210> 51
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:AflIII/HindIII
linker: top strand

<220>
<221> CDS
<222> (1)..(9)

<400> 51
tta agc ggc c 10
Leu Ser Gly
1

<210> 52
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:AflIII/HindIII
linker: bottom strand

<400> 52
agctgggcgc 10

<210> 53
<211> 11
<212> DNA
<213> Artificial Sequence

<220>

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<223> Description of Artificial Sequence:XhoI/AflII
linker: top strand

<220>

<221> CDS

<222> (3)..(11)

<400> 53

tc gac tcc ggc

Asp Ser Gly

1

11

<210> 54

<211> 11

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:XhoI/AflII
linker: bottom strand

<400> 54

ttaagccgga g

11

What is claimed is:

1. A homodimeric fusion protein having angiogenesis inhibitor activity comprising an immunoglobulin Fc-region and a target protein, wherein the Fc region comprises a hinge region, a CH2 domain and a CH3 domain, and the target protein has the angiogenesis inhibitor activity of endostatin and is endostatin or a biologically active fragment thereof, and is linked to the N-terminal ends or the C-terminal ends of said immunoglobulin Fc region.
2. The homodimeric fusion protein according to claim 1, wherein the target protein comprises the amino acid sequence set forth in SEQ ID No. 4.
3. The homodimeric fusion protein according to claim 1 or claim 2, wherein the target protein is connected to the C-terminal end of the Fc region.
4. The homodimeric fusion protein according to any one of claims 1 to 3, wherein the immunoglobulin is IgG1.
5. The DNA molecule coding for a signal sequence and the fusion protein as specified in any one of claims 1 to 4.
6. A replicable expression vector for transfecting a mammalian cell, the vector comprising the DNA molecule of claim 5.
7. A method for producing the homodimeric fusion protein as specified in any one of claims 1 to 4 comprising the steps:
 - (a) transfecting a mammalian cell with the vector of claim 6,
 - (b) culturing the mammalian cell to produce said fusion protein, and
 - (c) isolating said fusion protein.

