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Canadian Intellectual Property Office

CA 2658972 C 2019/11/26

(11)(21) 2 658 972

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2007/07/31
(87) Date publication PCT/PCT Publication Date: 2008/02/07
(45) Date de délivrance/Issue Date: 2019/11/26
(85) Entrée phase nationale/National Entry: 2009/01/26
(86) N° demande PCT/PCT Application No.: IL 2007/000959
(87) N° publication PCT/PCT Publication No.: 2008/015675
(30) Priorité/Priority: 2006/07/31 (US60/834,157)

(51) Cl.Int./Int.Cl. *C12N 15/12* (2006.01), *A61K 38/17* (2006.01), *A61K 48/00* (2006.01), *C07K 14/47* (2006.01), *C12N 15/63* (2006.01), *C12N 5/10* (2006.01)

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(54) Titre : POLYPEPTIDES ET POLYNUCLEOTIDES LES CODANT ET LEUR UTILISATION DANS LE TRAITEMENT D'AFFECTIONS MEDICALES ASSOCIEES A L'ISCHEMIE

(54) Title: POLYPEPTIDES AND POLYNUCLEOTIDES ENCODING SAME AND USE THEREOF IN THE TREATMENT OF MEDICAL CONDITIONS ASSOCIATED WITH ISCHEMIA

(57) Abrégé/Abstract:
An isolated polynucleotide is disclosed comprising a nucleic acid sequence encoding a polypeptide having an amino acid sequence of HIF-1alpha, the polypeptide being stably expressed and constitutively active. Isolated polypeptides encoded by same are also disclosed and uses thereof.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 February 2008 (07.02.2008)

PCT

(10) International Publication Number
WO 2008/015675 A3

(51) International Patent Classification:
C07H 21/04 (2006.01) ***C12N 5/10*** (2006.01)
C07K 14/47 (2006.01) ***A61K 48/00*** (2006.01)
C12N 15/63 (2006.01) ***A61K 38/17*** (2006.01)

(21) International Application Number:
PCT/IL2007/000959

(22) International Filing Date: 31 July 2007 (31.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/834,157 31 July 2006 (31.07.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
12 March 2009

WO 2008/015675 A3

(54) Title: POLYPEPTIDES AND POLYNUCLEOTIDES ENCODING SAME AND USE THEREOF IN THE TREATMENT OF MEDICAL CONDITIONS ASSOCIATED WITH ISCHEMIA

(57) Abstract: An isolated polynucleotide is disclosed comprising a nucleic acid sequence encoding a polypeptide having an amino acid sequence of HIF-1alpha, the polypeptide being stably expressed and constitutively active. Isolated polypeptides encoded by same are also disclosed and uses thereof.

POLYPEPTIDES AND POLYNUCLEOTIDES ENCODING SAME AND USE THEREOF IN THE TREATMENT OF MEDICAL CONDITIONS ASSOCIATED WITH ISCHEMIA.

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to polypeptides and polynucleotides encoding same and use thereof in the treatment of medical conditions associated with ischemia.

Angiogenesis is the budding of new blood vessels from pre-existing ones. It occurs in various physiological conditions such as the female reproductive cycle, as well as pathological conditions which include tumors, tissue ischemia and wound healing.

Ischemic heart disease is the leading cause of mortality in many industrialized countries and is responsible for over 500,000 deaths in the US alone each year. Current treatment options include drug therapy, coronary angioplasty and the more invasive coronary artery bypass grafting (CABG).

However, in all these cases, technical issues including the size of the artery involved, lack of appropriate distal vasculature, the complexity of the arterial lesions that cause the occlusion, and the general clinical conditions of the patient frequently prevent revascularization of the ischemic tissues.

A less invasive approach which has recently been developed is therapeutic angiogenesis. This term refers to the introduction of proangiogenic factors aimed at enhancing neovascularization of the ischemic tissue, thus alleviating the ischemia. Two main methods have been utilized in the field of therapeutic angiogenesis; the first is angiogenic gene therapy either in the form of naked DNA or with viral vehicles to deliver various cytokines, the most commonly used being Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF). A more recent method is cell therapy with fully differentiated cells, endothelial progenitor cells or mesenchymal stem cells. Both methods have shown success in animal models and early phase clinical trials.

However, many obstacles still have to be overcome before therapeutic angiogenesis becomes a real clinical alternative for patients suffering from ischemic diseases.

For angiogenic gene therapy, these obstacles include the need for tissue specificity of transgene expression, choice of delivery vehicle, optimization of dose

and timing, optimization of route of administration and the potential of adverse events such as edema and tumor development.

Another limitation to the success of angiogenic gene therapy is the lack of maturation of the newly formed vessels and their subsequent regression, thus preventing a significant long-lasting therapeutic effect. This may be because the delivered angiogenic genes are expressed only for a relatively short period of time which does not allow for vessel maturation and recruitment of smooth muscle cells to take place or because multiple angiogenic factors are required for vessel maturation to occur. A possible solution is to use an upstream angiogenic regulator which would activate multiple angiogenic factors simultaneously, thus more closely resembling physiological angiogenesis. Such a factor is hypoxia-inducible factor 1 (HIF-1).

Hypoxia Inducible Factor-1 (HIF-1) is a transcription factor and a master regulator of the response to oxygen deprivation, activating over 40 genes during hypoxia. It is a heterodimeric transcription factor consisting of two subunits; the subunit α is subject to tight regulation by the level of oxygen and is induced during hypoxia, whereas subunit β is constitutively expressed regardless of oxygen tension.

HIF-1α contains two transactivation domains (N-TAD and C-TAD) and an oxygen-dependent degradation domain (ODDD). The protein von-Hippel-Lindau (VHL) interacts with the ODDD of HIF-1α under normoxic conditions and acts as part of an E3-ubiquitin ligase complex, thus sending HIF-1α to proteosomal degradation. In hypoxia, HIF-1α dimerizes with HIF-1β and activates the transcription of its target genes in the nucleus.

Recently, it was shown that the interaction of VHL with HIF-1α is enabled by prolyl hydroxylation of two specific residues within the HIF-1α protein (Epstein, A.C. et al..2001, *Cell* 107, 43-54. Masson, N.,et al. 2001, *Embo J* 20, 5197-206).

Three prolyl hydroxylases (PHD 1-3) were found to hydroxylate HIF-1α during normoxia. PHD 1 and 2 hydroxylate at residues 402 and 564 whereas PHD 3 hydroxylates at residue 564 only.

A second mechanism of regulation of HIF-1α was uncovered in 2002 (Lando, D et al , 2002, *Science* 295, 858-61) and includes the asparaginyl hydroxylation of HIF-1α at residue 803, effected by an asparaginyl hydroxylase, also termed Factor Inhibiting HIF-1 (FIH-1). This hydroxylation prevents the interaction of HIF-1α with the co-factor p300, thus hindering the transcriptional activity of HIF-1α.

Hence, during normoxia two mechanisms of regulation are responsible for the decrease in HIF-1α activity, one concerning its stability via prolyl hydroxylations and the other concerning its transcriptional activation via asparaginyl hydroxylation.

These prolyl and asparaginyl hydroxylases are all dioxygenases which are 2-oxoglutarate and iron dependent and their requirement for cellular oxygen could provide the basis for their activity as oxygen sensors.

Two specific point mutations at residues 402 and 564 were demonstrated to abolish the interaction of HIF-1α with VHL, thus rendering HIF-1α constitutively stable (Masson et al., 2001, Embo J 20, 5197-206). The resultant mutant HIF-1α was as active as hypoxia treatment in driving the expression of an HRE-Luciferase construct. Two mutations at residues 564 and 803 were shown to give HIF-1α full transcriptional activity, similar to that obtained by treatment with the hypoxia mimetic iron chelator 2,2'-Dipyridyl (Lando et al., 2002, Science 295, 858-61).

Transgenic mice overexpressing a mutant hHIF-1α in which residues 401 to 602 are deleted, under the regulation of Keratin 14 promoter showed increased vascularization in the skin (Elson, D.A. et al.2001, Genes Dev 15, 2520-32). These mice showed up-regulation of mRNA of Glut-1 and VEGF, known targets of HIF-1α. In comparison with VEGF overexpressing mice, these mice had blood vessels which were less leaky and showed greater maturity. This may be explained by the fact that HIF-1α induces the activation of multiple angiogenic factors (i.e. erythropoietin), similar to the physiological angiogenic response, in contrast to VEGF alone. In addition, HIF-1α induces the activation of many isoforms of VEGF and other genes, again more closely resembling the physiological response, which could not be achieved by the administration of a single isoform.

A constitutively active form of HIF-1α was first tested in angiogenic gene therapy in 2000 (Vincent, K.A. et al. 2000, *Circulation* 102, 2255-61). It contained the DNA binding domain and dimerization domains of HIF-1α attached to the transactivation domain VP16 of the Herpes Simplex Virus (HSV) under the regulation of CMV promoter. The resultant mutant was able to induce HIF target genes in-vitro. When administered locally as naked DNA it caused increased capillary density and blood perfusion in a mouse hindlimb ischemia model. The same construct also showed therapeutic effect when injected IM in a rat MI model (Shyu, K.G. et al, 2002, *Cardiovasc Res* 54, 576-83).

An adenovirus expressing a constitutively active mutant HIF-1α, with a deletion of residues 401-602, under the regulation of CMV promoter was able to induce angiogenesis in the non-ischemic tissue of the retina (Kelly, B.D. et al, 2003 *Circ Res* 93, 1074-81).

The two constructs mentioned above bear large deletions of the HIF-1α molecule, and lack one or both of the native HIF-1α transactivation domains (N-TAD and C-TAD), which may result in reduced activation potential and specificity of HIF-1α. In addition, HIF-1α is expressed in these constructs under the regulation of CMV, a versatile and non-specific promoter, which may limit its application due to non-specific expression and potential side-effects.

Various routes of administration have been used to deliver the therapeutic gene to the ischemic region, including intravascular and intramuscular in the case of peripheral ischemia, and intramyocardial, intrapericardial and intracoronary in the case of myocardial ischemia. The intravenous route confers advantages which include easy access without the need for an invasive procedure, technical safety and low cost as well as accessibility to a large patient population. However, despite its obvious clinical appeal, the use of this administration route is uncommon due to systemic distribution of the vector leading to low transgene expression in the target organ along with unwanted expression in non-target organs resulting in systemic side-effects, which limit the dose that may be administered. This limitation, in turn, tends to restrict the efficacy of the treatment. Unwanted expression of a pro-angiogeneic gene could induce pathological angiogenesis, possibly leading to tumor development and retinopathy, and may therefore be unacceptable. Thus, the ability to direct transgene expression specifically to the ischemic target organ is of outmost importance for systemic administration to be efficacious and safe.

There is thus a widely recognized need for, and it would be highly advantageous to have pro-angiogenic factors together with safe and effective methods of delivering same.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding a polypeptide having an

amino acid sequence of HIF-1α, the polypeptide being stably expressed and constitutively active.

According to further features in preferred embodiments of the invention described below, the nucleic acid sequence is as set forth in SEQ ID NO:1.

According to still further features in the described preferred embodiments the amino acid sequence is as set forth in SEQ ID NO: 2.

According to still further features in the described preferred embodiments the amino acid sequence being at least 90 % homologous to SEQ ID NO: 3 and comprising a mutation at a position corresponding to proline 402 of SEQ ID NO: 3, a mutation at a position corresponding to proline at 564 of SEQ ID NO: 3 and a mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3.

According to another aspect of the present invention there is provided an isolated polypeptide comprising an amino acid sequence encoding a polypeptide having a HIF-1a amino acid sequence, the polypeptide being stably expressed and constitutively active.

According to still further features in the described preferred embodiments the amino acid sequence is as set forth in SEQ ID NO: 2.

According to still further features in the described preferred embodiments the amino acid sequence being at least 90 % homologous to SEQ ID NO: 3 and comprising a mutation at a position corresponding to proline 402 of SEQ ID NO: 3, a mutation at a position corresponding to proline at 564 of SEQ ID NO: 3 and a mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3.

According to another aspect of the present invention there is provided a nucleic acid construct comprising the polynucleotide of the present invention.

According to still further features in the described preferred embodiments the nucleic acid construct further comprising a cis-regulatory element.

According to still further features in the described preferred embodiments the cis-regulatory element comprises a promoter element.

According to still further features in the described preferred embodiments the promoter element is an endothelial specific promoter element.

According to still further features in the described preferred embodiments the endothelial specific promoter element comprises at least one copy of the PPE-1 promoter.

According to still further features in the described preferred embodiments the PPE-1 promoter is as set forth in SEQ ID NO: 4.

According to still further features in the described preferred embodiments the cis-regulatory element further comprises a hypoxia response element.

According to yet another aspect of the present invention there is provided a cell comprising the nucleic acid construct.

According to still another aspect of the present invention there is provided a pharmaceutical composition comprising as an active agent the nucleic acid construct and a pharmaceutically acceptable carrier.

According to an additional aspect of the present invention there is provided a method of treating a medical condition associated with hypoxia or ischemia, reduced tissue perfusion inclusion or 'low flow', the method comprising administering to a subject in need thereof a therapeutically effective amount of an agent capable of upregulating the polypeptide of the present invention in cells of the subject, thereby treating an angiogenesis-related disease.

According to still further features in the described preferred embodiments the agent is the polypeptide.

According to still further features in the described preferred embodiments the agent is the nucleic acid construct.

According to still further features in the described preferred embodiments the administering is effected systemically.

According to still further features in the described preferred embodiments the disease or condition associated with ischemia is selected from the group consisting of wound healing, ischemic stroke, ischemic heart disease, peripheral vascular disease, renal artery disease, gastrointestinal lesions, burns, skin transplantation, vascular grafts, organ repair, bone reparative disorders, liver disorders, uterine disorders, ocular angiogenesis disorders, bone regeneration disorders, cartilage repair disorders and smooth muscle cell disorders.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel treatments for medical conditions associated with ischemia.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent

to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-E are bar graphs and photographs illustrating the transcriptional activity of the triple mutant HIF-1α as compared to the P402A P564G and P564A N803A double mutants. HRE-mediated transcription was measured in HEK293 (Figure 1A) and BAEC (Figure 1B) following co-transfection of p2.1 HRE-luc with different HIF-1α plasmids or pcDNA3 control. Data are expressed as luciferase ratios and are normalized to β-gal (mean ± S.D). *$p<0.001$ compared with double mutants. Figure 1C is an RT-PCR analysis of mRNA isolated from HeLa cells 48 hours post-transfection with HIF-1α plasmids or pcDNA3 control. Treatment with 2,2'-dipyridyl (2,2'-DP) is illustrated in Figure 1D. HRE-mediated transcription was measured in VHL-deficient renal cell carcinoma cells following co-transfection of p2.1 HRE-luc with different HIF-1α plasmids or pcDNA3 control. Data are expressed as luciferase ratios and are normalized to β-gal (mean ± S.D). *$p< 0.001$ and +$p = 0.003$ vs. P402A P564G mutant. HEK293 cells were co-transfected with p2.1 HRE-luciferase, HIF-1a plasmids and the indicated amount of FIH-1 antisense (Figure 1E). Data are expressed as luciferase ratios and are normalized to β -gal (mean ± S.D).

FIGs. 2A-H are photographs and bar graphs illustrating that the triple mutant HIF-1α shows greater angiogenic potency than P402A P564G and P564A N803A double mutants. Figures 2A-F are representative photographs of an in-vitro angiogenesis assay in HUVECs transfected with pcDNA3 (Figure 2A), wt-HIF-1α (Figure 2B), P402A P564G (Figure 2C), P564A N803A (Figure 2D), Triple mutant (Figure 2E) or VEGF (Figure 2F). Figure 2G is a bar graph illustrating tube formation of transfected HUVECs, which was quantified by counting the number of capillary branches per high power microscopic field. Results are expressed as mean ± S.D of 5 random microscopic fields. Figure 2H is a bar graph illustrating VEGF protein concentration of transfected HUVECs, as determined by ELISA and results are expressed as mean ± S.D. *p = 0.029 vs. P402A P564G.

FIGs. 3A-H are photographs of fluorescent microscope sections illustrating the endothelial and ischemia specificity of PPE1-3x promoter. Figures 3A-F illustrate fluoresent microscopic sections of gastrocnemius muscles of mice subjected to either hindlimb ischemia or sham procedure, which were injected systemically via the tail vein 7 days later with either saline (Figures 3A and 3D), Ad-CMV-GFP (Figures 3B and 3E), or Ad-PPE1-3x-GFP (Figures 3C and 3F). Figures 3G-H illustrate sections of gastrocnemius muscle of mouse treated with Ad-PPE-1-3x-GFP as seen in fluorescent microscopy (Figure 3G) or phase microscopy (Figure 3H) showing erythrocytes within a capillary (arrows) made of GFP expressing endothelial cells (arrowhead).

FIGs. 4A-F illustrate Post-ischemic angiogenesis is augmented by local Ad-CMV-Triple and Ad-PPE-Triple treatments. Figure 4A is a line graph of Blood flow in the ischemic limb measured immediately following and at 7, 14, 21 and 28 days after left femoral artery ligation. Data are expressed as the ratio of the left (ischemic) to right (non-ischemic) limb perfusion. Saline, n = 8; Ad-CMV-Luc, n = 11; Ad-CMV-wt, n = 11; Ad-CMV-Triple, n = 12; Ad-PPE-Triple, n = 11. Figure 4B are representative laser Doppler blood flow images of the ischemic (left) and non-ischemic (right) limbs at day 28 post-surgery. In color-coded images, normal perfusion is depicted in red while low and/or no perfusion in blue. Figure 4C are representative photos of mouse hindlimbs 21 days post-surgery. Figure 4D is a bar graph illustrating the percentage of mice showing toe necrosis by day 21 post-surgery. Figures 4E-F illustrate representative CD31 staining (Figure 4E) and quantitation

(Figure 4F) of capillaries from sections of gastrocnemius muscles 28 days following femoral artery ligation. *p<0.01.

FIGs. 5A-N illustrate that Ad-PPE-Triple is less toxic than Ad-CMV-Triple following systemic administration. Figure 5A is a point graph illustrating the changes in mice body weights over time following systemic injection of saline or adenovirus. Figures 5B-D are bar graphs illustrating Serum bilirubin (Figure 5B), AST (Figure 5C) and ALT (Figure 5D) levels at 5 and 21 days following systemic injection of saline or adenovirus. Figures 5E-N are histological sections of mice livers 5 (Figures 5E, G, I, K and M) and 21 days (Figures 5F, H, J, L and N) following systemic injection of saline (Figures 5E-F), AdCMV-Luc (Figures 5G-H), AdCMV-wt (Figures 5I-J), AdCMV-triple (Figures 5K-L), AdPPE-triple (Figures 5M-N).

FIGs. 6A-F illustrate that post-ischemic angiogenesis is augmented by systemic Ad-PPE-Triple but not by Ad-CMV-Triple treatment. Figure 6A is a point graph of blood flow in the ischemic limb measured immediately following and at 7, 14, 21 and 28 days after left femoral artery resection. Data are expressed as the ratio of the left (ischemic) to right (non-ischemic) limb perfusion. Saline, n = 9; Ad-CMV-Luc, n = 8; Ad-CMV-wt, n = 9; Ad-CMV-Triple, n = 7; Ad-PPE-Triple, n = 8. Figure 6B is a bar graph illustrating the percentage of mice showing toe necrosis by day 21 post-surgery. Figure 6C illustrate representative photos of mice hindlimbs 21 days post-surgery. Figure 6D are representative laser Doppler blood flow images of the ischemic (left) and non-ischemic (right) limbs at day 28 post-surgery. In color-coded images, normal perfusion is depicted in red while low and/or no perfusion in blue. Figures 6E-F illustrate representative CD31 staining (Figure 6E) and quantitation (Figure 6F) of capillaries from sections of gastrocnemius muscles 28 days after femoral artery ligation. *p<0.01.

FIG. 7A is a comparison of HRE-mediated transcription of HIF-1α expressing adenoviruses in BAEC. BAEC were transfected with p2.1 HRE-Luc, followed 24h later by infection with adenoviruses at MOI 20 or MOI 200. Data are expressed as luciferase light units (mean ± S.D).

FIG. 7B is a Western blot of HIF-1α in HeLa cells. Infection of HeLa cells with Ad-CMV-GFP, AD-CMV-wtHIF-1α, Ad-CMV-Triple or Ad-PPE1-3x-Triple, or mock infection were carried out. Western blot was performed 48h following infection.

FIGs. 7C-G: In-vitro angiogenesis assay for HIF-1α expressing adenoviruses in HUVEC. HUVEC were either mock infected (C), infected with Ad-CMV-GFP (D),

Ad-CMV-wtHIF-1α (E), Ad-CMV-Triple (F) or Ad-PPE1-3x-Triple (G). Forty eight hours later, in-vitro angiogenesis assay was performed. Figures C-G are representative photographs of tube formation.

FIG. 7H: is a bar graph illustrating VEGF protein concentration of infected HUVECs, as determined by ELISA and results are expressed as mean ± S.D. *p = 0.01 vs. Ad-CMV-wtHIF-1α.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of HIF-1α polypeptides and polynucleotides encoding same, pharmaceutical compositions which comprise the same and methods of producing and using same.

Specifically, the present invention can be used in the treatment of diseases associated with ischemia.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Hypoxia-inducible factor 1α (HIF-1α) is a heterodimeric transcription factor and a key regulator of the response to low oxygen levels, and has been suggested as a potential candidate for therapeutic angiogenesis. Stabilization of HIF-1α may be achieved by point mutations P402A and P564G [Masson et al., 2001, Embo J 20, 5197-206] while constitutive activity of its C-transactivation domain (C-TAD) is achieved by point mutation N803A [Lando et al., 2002, Science 295, 858-61].

While reducing the present invention to practice the present inventors genetically engineered a mutant form of HIF-1α combining the three point mutations P402A P564G N803A (triple mutant), rendering HIF-1α both stable and constitutively active. Surprisingly, the triple mutant showed a synergistic effect on the expression of a reporter gene linked to a hypoxia response element as compared to the two known double mutant forms of HIF-1α, as illustrated in Figures 1A-B. Thus, the present inventors showed that constitutive activation of the HIF-1α C-transactivation domain, and not merely stabilization of the HIF-1α molecule, is essential for optimal HIF-mediated transcription and proangiogenic effects.

Accordingly, the triple mutant HIF-1α, or polynucleotide encoding same, of the present invention can be used to treat diseases or conditions associated with angiogenesis.

As illustrated in Figures 2G-H, the triple mutant of the present invention shows a greater angiogenic potency than P402A P564G and P564A N803A double mutants as demonstrated in in-vitro angiogenesis assays. Furthermore, as illustrated in Figures 4A-F, an adenovirus expressing the triple mutant HIF-1α of the present invention showed enhanced blood perfusion and increased capillary density compared with an adenovirus expressing wild-type HIF-1α and controls in a mouse hindlimb ischemic model. A modified preproendothelin-1 promoter was shown to allow specific expression in ischaemic endothelial cells (Figures 3C and 3F). Expression of the triple mutant HIF-1α under the regulation of such a promoter reduced ectopic expression and systemic side-effects as compared to expression of the triple mutant under the control of a constitutively active promoter (Figures 5A-N and 6A-D).

Thus, according to one aspect of the present invention, there is provided an isolated polypeptide comprising an amino acid sequence encoding a polypeptide having a HIF-1α amino acid sequence, the polypeptide being stably expressed and constitutively active.

As used herein, the phrase "HIF-1α" refers to at least an active portion of HIF-1α (i.e., a portion having HIF-1α activity). Preferably the HIF-1α of the present invention is human HIF-1α, e.g. GenBank Accession No: NM001530.

As used herein the phrase "HIF-1α activity" refers to at least the transcription factor activity of HIF-1α i.e., the ability of HIF-1α to trancriptionally up-regulate target angiogenic genes. In order to function as a transcription factor, HIF-1α, dimerizes with HIF-1β and binds co-factors such as P300. Accordingly the HIF-1α of the present invention preferably comprises a functional DNA binding domain and functional co-factor and HIF-1β binding domains. The HIF-1α of the present invention does not have to comprise for example amino or carboxy terminal amino acids since these terminal sequences are not required for HIF-1α activity.

As used herein, the phrase "constitutively active" refers to HIF-1α which comprises transcriptional activity which is not regulated by the hypoxic state of the cell.

As used herein, the phrase "stably expressed" refers to a polypeptide which is not subject to an increase in proteosomal degradation in response to hypoxia.

Accordingly the half life of the polypeptide is not altered by the presence of Von-Hippel-Lindau (VHL). Preferably, the half life of the polypeptides of the present invention are at least about 2 times greater, and even more preferably 5 times the half life of the wild type polypeptides (i.e. not comprising a mutation).

According to a preferred embodiment of this aspect of the present invention, the polypeptide of the present invention is at least 50 % homologous, more preferably at least 60 % homologous, more preferably at least 70 % homologous, more preferably at least 80 % homologous, and most preferably at least 90 % homologous to SEQ ID NO: 3 and comprises a mutation at a position corresponding to proline 402 of SEQ ID NO: 3, a mutation at a position corresponding to proline at 564 of SEQ ID NO: 3 and a mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3.

As used herein, the term "mutation" refers to an alteration in an amino acid sequence compared to the wild type sequence (GenBank Accession No: NM001530 (GI:31077212)

The mutation may comprise a deletion or a substitution. Exemplary mutations include an alanine corresponding to proline at position 402, a glycine corresponding to proline at position 564 and an alanine corresponding to asparagine at position 803.

Thus, according to a preferred embodiment the polypeptide of the present invention is as set forth in SEQ ID NO: 2.

In addition, the polypepide of the present invention may comprise other conservative variations of SEQ ID NO: 3.

The phrase "conservative variation" as used herein refers to the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, or methionine for another, or the substitution of one solar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Methods of protein engineering e.g. display techniques may be used to uncover other mutations which impart stability and constitutive activity to the HIF-1α of the present invention as well as active portions of HIF-1α.

Methods of constructing display libraries are well known in the art. Such methods are described in, for example, Young AC, *et al.*, "The three-dimensional structures of a polysaccharide binding antibody to Cryptococcus neoformans and its complex with a peptide from a phage display library: implications for the identification of peptide mimotopes" J Mol Biol 1997 Dec 12;274(4):622-34; Giebel LB *et al.* "Screening of cyclic peptide phage libraries identifies ligands that bind streptavidin with high affinities" Biochemistry 1995 Nov 28;34(47):15430-5; Davies EL *et al.*, "Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes" J Immunol Methods 1995 Oct 12;186(1):125-35; Jones C RT al. "Current trends in molecular recognition and bioseparation" J Chromatogr A 1995 Jul 14;707(1):3-22; Deng SJ *et al.* "Basis for selection of improved carbohydrate-binding single-chain antibodies from synthetic gene libraries" Proc Natl Acad Sci U S A 1995 May 23;92(11):4992-6; and Deng SJ *et al.* "Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display" J Biol Chem 1994 Apr 1;269(13):9533-8.

Other mutations which impart stability and constitutive activity to the HIF-1α of the present invention can also be uncovered using computational biology. For example, various mutated HIF-1α peptide sequences can be computationally analyzed for an ability to impart stability and constitutive activity using a variety of three dimensional computational tools. Software programs useful for displaying three-dimensional structural models, such as RIBBONS (Carson, M., 1997. Methods in Enzymology 277, 25), O (Jones, TA. *et al.*, 1991. Acta Crystallogr. A47, 110), DINO (DINO: Visualizing Structural Biology (2001)); and QUANTA, INSIGHT, SYBYL, MACROMODE, ICM, MOLMOL, RASMOL and GRASP (reviewed in Kraulis, J., 1991. Appl Crystallogr. 24, 946) can be utilized to model prospective mutant peptide sequences to identify useful mutations.

The term "polypeptide" as used herein encompasses native polypeptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides) and peptidomimetics (typically, synthetically synthesized polypeptides), as well as as peptoids and semipeptoids which are polypeptide analogs, which may have, for example, modifications rendering the polypeptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, polypeptide

bond modification, including, but not limited to, CH2-NH, CH2-S, CH2-S=O, O=C-NH, CH2-O, CH2-CH2, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992). Further details in this respect are provided hereinunder.

Polypeptide bonds (-CO-NH-) within the polypeptide may be substituted, for example, by N-methylated bonds (-N(CH3)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH2-), α-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH2-NH-), hydroxyethylene bonds (-CH(OH)-CH2-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), polypeptide derivatives (-N(R)-CH2-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the polypeptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the polypeptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2) which can be used with the present invention.

Table 1

Amino Acid	Three-Letter Abbreviation	One-letter Symbol
alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
glycine	Gly	G
Histidine	His	H
isoleucine	Iie	I
leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

Table 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgin
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
D-valine	Dval	α -methyl-γ-aminobutyrate	Mgabu
D- α -methylalanine	Dmala	α ethylcyclohexylalanine	Mchexa
D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
D- α -methylasparagine	Dmas	α -methyl- α -napthylalanine	Manap

	n		
D- α -methylaspartate	Dmasp	α - methylpenicillamine	Mpen
D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- α -methylisoleucine	Dmile	N- amino- α -methylbutyrate	Nmaabu
D- α -methylleucine	Dmleu	α -napthylalanine	Anap
D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D- α -methylvaline	Dmval	N-cyclododeclglycine	Ncdod
D- α -methylalnine	Dnmala	N-cyclooctylglycine	Ncoct
D- α -methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D- α -methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D- α -methylasparatate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D- α -methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl-*t*-butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α thylglutamine	Mgln	L- α -methylglutamate	Mglu

L- α -methylhistidine	Mhis	L- α -methylhomo phenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl-*t*-butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α ethylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
L- α thylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	mser	L- α -methylthreonine	Mthr
L- α ethylvaline	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylleucine	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl-glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl(1)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl ethylamino)cyclopropane	Nnbm Nmbc		

Table 2 Cont.

The isolated polypeptides of present invention can be biochemically synthesized such as by using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. These methods are preferably used when it

cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence) and therefore involves different chemistry or when short peptides are synthesized.

Solid phase polypeptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Polypeptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic polypeptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

Recombinant techniques are preferably used to generate the isolated polypeptides of the present invention since these techniques are better suited for generation of relatively long polypeptides (e.g., longer than 20 amino acids) and large amounts thereof. Such recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

These techniques may be used to generate the polypeptide of the present invention in vitro, ex vivo and in vivo (the latter two are further described hereinbelow).

To produce an isolated HIF-1α polypeptide of the present invention using recombinant technology, an isolated polynucleotide comprising a nucleic acid sequence encoding such a polypeptide may be used. An exemplary nucleic acid sequence is set forth in SEQ ID NO: 1.

The term "nucleic acid sequence" refers to a deoxyribonucleic acid sequence composed of naturally-occurring bases, sugars and covalent internucleoside linkages (e.g., backbone) as well as oligonucleotides having non-naturally-occurring portions which function similarly to respective naturally-occurring portions. Such modifications are enabled by the present invention provided that recombinant expression is still allowed.

A nucleic acid sequence of HIF-1α according to this aspect of the present invention can be a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

In order to generate the HIF-1α polypeptides of the present invention using recombinant techniques, the polynucleotides encoding same are ligated into nucleic acid expression vectors, such that the polynucleotide sequence is under the transcriptional control of a cis-regulatory sequence (e.g., promoter sequence).

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the polypeptides of the present invention. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the polypeptide coding sequence; yeast transformed with recombinant yeast expression vectors containing the polypeptide coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the polypeptide coding sequence.

Constitutive promoters suitable for use with this embodiment of the present invention include sequences which are functional (i.e., capable of directing

transcription) under most environmental conditions and most types of cells such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV).

Inducible promoters suitable for use with this embodiment of the present invention include for example the tetracycline-inducible promoter (Srour, M.A., et al., 2003. Thromb. Haemost. 90: 398-405).

The expression vector according to this embodiment of the present invention may include additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). Typical cloning vectors contain transcription and translation initiation sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals).

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983

Polyadenylation sequences can also be added to the expression vector in order to increase the translation efficiency of a polypeptide expressed from the expression vector of the present invention. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40.

In addition to the elements already described, the expression vector of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

The expression vector of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

Examples for mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can also be used by the present invention. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Various methods can be used to introduce the expression vector of the present invention into cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant polypeptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the polypeptide), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed polypeptide.

Depending on the vector and host system used for production, resultant polypeptides of the present invention may either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

Following a predetermined time in culture, recovery of the recombinant polypeptide is effected.

The phrase "recovering the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

Thus, polypeptides of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

To facilitate recovery, the expressed coding sequence can be engineered to encode the polypeptide of the present invention and fused cleavable moiety. Such a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. Where a cleavage site is engineered between the polypeptide and the cleavable moiety, the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth *et al.*, Immunol. Lett. 19:65-70 (1988); and Gardella *et al.*, J. Biol. Chem. 265:15854-15859 (1990)].

Following production of the polypeptides of the present invention, antibodies may be generated that specifically recognize the mutant forms and not the native forms. Such antibodies may be of use for verifying expression thereof.

Since the polypeptides of the present invention comprise angiogenic effects, they may be used in the treatment a disease or condition associated with ischemia.

Thus, according to another aspect of the present invention, there is provided a method of treating a disease or condition associated with ischemia, the method comprising administering to a subject in need thereof a therapeutically effective amount of an agent capable of upregulating the polypeptides of the present invention.

As used herein the term "treating" refers to preventing, alleviating or diminishing a symptom associated with an angiogenesis-related disease. Preferably, treating cures, e.g., substantially eliminates, the symptoms associated with the angiogenesis-related disease.

As used herein the term "subject" refers to any (e.g., mammalian) subject, preferably a human subject.

As used herein, the term "hypoxia" refers to a state of reduced oxygen. This can occur when the lungs are compromised or blood flow is reduced.

As used herein, the term "ischemia" refers to a reduction in blood flow, which can be caused by the obstruction of an artery or vein by a blood clot (thrombus) or by any foreign circulating matter (embolus), or by a vascular disorder such as atherosclerosis. Alternatively, ischemia can be caused when the artery is not obstructed but target tissue demand for oxygen is increased relative to supply. Reduction in blood flow can have a sudden onset and short duration (acute ischemia), or can have a slow onset with long duration or frequent recurrence (chronic ischemia). Acute ischemia is often associated with regional, irreversible tissue necrosis (an infarct), whereas chronic ischemia is usually associated with transient hypoxic tissue injury. If the decrease in perfusion is prolonged or severe, however, chronic ischemia can also be associated with an infarct. Infarctions commonly occur in the spleen, kidney, lungs, brain, and heart, producing disorders such as intestinal infarction, pulmonary infarction, ischemic stroke, and myocardial infarction.

The present invention thus clearly contemplates methods that can be applied to the treatment of medical conditions associated with any ischemic event, whether acute, transient or chronic. Acute ischemic events can include those associated with surgery, organ transplantation, infarction (e.g., cerebral, intestinal, myocardial, pulmonary, etc.), trauma, insult, or injury, etc. Chronic events associated with ischemia can include hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, cirrhosis, congestive heart failure, systemic sclerosis, etc.

Other diseases or disorders associated with ischemia include but are not limited to wound healing, gastrointestinal lesions, autoimmune diseases and neurodegenerative disorders.

Examples of specific conditions include, but are not limited to, wound healing, ischemic stroke, ischemic heart disease, peripheral vascular disease, renal artery disease, gastrointestinal lesions, burns, skin transplantation, vascular grafts, organ repair (e.g., ex vivo and in vivo), bone reparative disorders, liver disorders, uterine disorders, ocular angiogenesis disorders, (i.e. retinal detachment, age related macular

degeneration), bone regeneration disorders, cartilage repair disorders and smooth muscle cell disorders.

According to one embodiment, agents capable of upregulating the polypeptides of the present invention are the HIF-1α polypeptides of the present invention i.e. the polypeptides are generated and subsequently administered to a subject. Methods of generating the isolated polypeptides of the present invention are described hereinabove.

Since it is preferable that the HIF-1α polypeptides of the present invention are provided at the site of the angiogenesis (i.e. proliferating endothelial cells), preferably the polypeptides are delivered locally or comprise a targeted delivery system (e.g. targeted liposomes).

A particularly preferred method of targeting the HIF-1α polypeptides of the present invention to the site of angiogenesis is by targeted gene therapy.

Gene therapy as used herein refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition or phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. For review see, in general, the text "Gene Therapy" (Advanced in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) ex vivo and (2) *in vivo* gene therapy. In ex vivo gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material in situ. The cells may be autologous or non-autologous to the subject. Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism in situ, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired in situ (Culver, 1998. (Abstract) Antisense DNA & RNA based therapeutics, February 1998, Coronado, CA).

These genetically altered cells have been shown to express the transfected genetic material in situ.

To confer specificity, preferably the nucleic acid constructs used to express the polypeptides of the present invention comprise endothelial cell-specific promoter sequence elements. The endothelial specific promoter element may include, for example, at least one copy of the PPE-1 promoter as set forth in SEQ ID NO: 4. Examples of suitable promoters/enhancers which can be utilized by the nucleic acid construct of the present invention include the endothelial-specific promoters: preproendothelin-1, PPE-1 promoter (Harats D, J Clin Invest. 1995 Mar;95(3):1335-44)., the PPE-1-3x promoter [PCT/IL01/01059; Varda-Bloom N, Gene Ther 2001 Jun;8(11):819-27], the TIE-1 (S79347, S79346) and the TIE-2 (U53603) promoters [Sato TN, Proc Natl Acad Sci U S A 1993 Oct 15;90(20):9355-8], the Endoglin promoter [Y11653; Rius C, Blood 1998 Dec 15;92(12):4677-90], the von Willebrand factor [AF152417; Collins CJ Proc Natl Acad Sci U S A 1987 Jul;84(13):4393-7], the KDR/flk-1 promoter [X89777, X89776; Ronicke V, Circ Res 1996 Aug;79(2):277-85],The FLT-1 promoter [D64016 AJ224863; Morishita K, : J Biol Chem 1995 Nov 17;270(46):27948-53], the Egr-1 promoter [AJ245926; Sukhatme VP, Oncogene Res 1987 Sep-Oct;1(4):343-55], the E-selectin promoter [Y12462;Collins T J Biol Chem 1991 Feb 5;266(4):2466-73], The endothelial adhesion molecules promoters: ICAM-1 [X84737; Horley KJ EMBO J 1989 Oct;8(10):2889-96], VCAM-1 [M92431; Iademarco MF , J Biol Chem 1992 Aug 15;267(23):16323-9], PECAM-1 [AJ313330 X96849; CD31, Newman PJ, Science 1990 Mar 9;247(4947):1219-22], the vascular smooth-muscle-specific elements: CArG box X53154 and aortic carboxypeptidase-like protein (*ACLP*) promoter [AF332596;Layne MD, *Circ Res.* 2002; 90: 728–736] and Aortic Preferentially Expressed Gene-1 [Yen-Hsu Chen J. Biol. Chem., Vol. 276, Issue 50, 47658-47663, December 14, 2001]. Other suitable endothelial specific promoters are well known in the art, such as, for example, the EPCR promoter (US Patent Nos. 6,200,751 to Gu et al) and the VEGF promoter (US Patent Application 5,916,763 to Williams et al).

Preferably, the nucleic acid constructs of the present invention further include a hypoxia response element, for example at least one copy of the sequence set forth in SEQ ID NO:5. Since HIF-1 binds to such a response element, the HIF-1α transgene expressed under the regulation of such a response element should, in turn, activate the promoter further, thus creating a positive feedback loop, enhancing the transcriptional response.

Introduction of nucleic acids by infection in both in vivo and ex vivo gene therapy offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral utilizes its natural specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art.

It will be appreciated that although adenoviruses are employed in the experiments described in examples presented hereinbelow, the constructs of the present invention could be easily adapted by those of ordinary skill in the art to other viral delivery systems.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

Since transduction of cells with conditionally replicating adenoviral vectors is significantly more effective in target cell lysis and spread of viral infection, the nucleic acid construct can include a conditionally replicating adenovirus.

The viral vectors, containing the endothelial cell specific promoters, can also be used in combination with other approaches to enhance targeting of the viral vectors. Such approaches include short peptide ligands and /or bispecific or bifunctional molecule or diabodies (Nettelbeck et al. Molecular Therapy 3:882;2001).

The polypeptides and polynucleotides of the present invention can be provided to the individual *per se*, or as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the polypeptide or polynucleotide preparation, which is accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does

not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition

Suitable routes of administration may, for example, include oral, rectal, transmucosal, transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the preparation in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

A recombinant vector can be administered in several ways. If vectors are used which comprise endothelial cell specific promoters, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. Thus, according to a preferred embodiment of the present invention, the nucleic acid constructs are administered systemically (e.g. intravenously or subcutaneously). As illustrated in Example 5, systemic administration of a polynucleotide of the present invention linked to an endothelial cell specific promoter showed a much higher safety profile than systemic administration of the same polynucleotide linked to a constitutively active promoter as well as being more effective at the disease site at promoting angiogenesis.

Injection of viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with appropriate target specificity for infection.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or

pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran.

Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro assays. For example, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

It will be appreciated that the polypeptides and polynucleotides of the present invention can be provided to the individual with additional active agents to achieve an improved therapeutic effect as compared to treatment with each agent by itself. In such therapy, measures (e.g., dosing and selection of the complementary agent) are taken to adverse side effects which may be associated with combination therapies.

Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996)

. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

EXAMPLE 1

Triple mutant HIF-1α shows greater transcriptional activity than P402A P564G and P564A N803A double mutants

Point mutations P402A and P564G within HIF-1α were previously shown to abrogate its interaction with the tumor suppressor VHL, thus preventing its subsequent proteosomal degradation. This resulted in stabilized HIF-1α during normoxia, reaching activity levels comparable to hypoxic condition. Similarly, HIF-1α with point mutations P564A N803A was demonstrated to be as active in normoxia as wild-type HIF-1α following treatment with the hypoxia mimetic iron chelator 2,2'-dipyridyl. The present inventors hypothesized that a combination of all three mutations may result in a mutated HIF-1α, termed 'Triple mutant', which is more active than the P402A P564G mutant and more stable than the P564A N803A mutant, thus making it more potent for therapeutic angiogenesis purposes.

MATERIALS AND METHODS

Cell culture: The following cell lines were used: Bovine aortic endothelial cells (BAECs) were a kind gift from N. Savion (Goldschleger Eye Institute, Sheba Medical Center). Human embryonic kidney cells (HEK293 cells) were purchased from American Type Culture Collection (Rockville, Maryland, USA). Passages 20–26 of 293 cells were used. HeLa cells were a kind gift from Y. Keisary (Tel Aviv University). Human umbilical vein ECs (HUVECs) were produced as previously described [Jaffe, E.A.. et al, J Clin Invest 52, 2745-56 (1973)]. Renal cell carcinoma (RCC) cells were a kind gift from G. Lavi. (Angiogenesis Institute, Sheba Medical Center). BAEC, HEK293 cells and RCC were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine and 100 U/ml penicillin/streptomycin. HeLa cells were cultured in MEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. HUVECs were maintained in EGM-2 and 2 % FCS (Clonetics, San-Diego, California, USA). All cells were maintained at 37 °C in a 5 % CO_2-humidified incubator.

Expression construct: Wild-type HIF-1α and P564A N803A HIF-1α mutant in pEF-bos plasmids were kind gifts from M. Whitelaw (Adelaide University). P402A P564G HIF-1α mutant in pcDNA3 plasmid was a kind gift from PJ. Ratcliffe (Oxford University). pCR3.1-HA-FIH-1 antisense plasmid was a kind gift from G. Semenza (Johns Hopkins University). p2.1 HRE-luciferase was purchased from American Type Culture Collection (Rockville, Maryland, USA) and was previously described [Mahon, P.C., et al., Genes Dev 15, 2675-86 (2001)].

Plasmid SV40-β-gal was purchased from Promega (Madison WI, USA). Triple mutant HIF-1α was constructed by insertion of Bsu26I/NotI digested fragment of P564A N803A HIF-1α mutant into Bsu26I/NotI digested P402A P564G HIF-1α vector. The integrity of the plasmid was confirmed by DNA sequencing. For transfection experiments, wild-type and mutant HIF-1a cDNAs were subcloned into pcDNA3 vector (Invitrogen Corp., Carlsbad, California, USA).

Transient Transfection Experiments: HEK 293, BAEC and RCC cells were co-transfected in triplicates in 24-well plates with 200 ng p2.1 HRE-luciferase, 100 ng pSV40-β-gal and 100 ng of either pcDNA3 empty vector, wild-type HIF-1α, P402A P564G, P564A N803A or Triple mutant using Lipofectamine Plus reagent (Invitrogen Corp., Carlsbad, California, USA) according to the manufacturer's instructions. For the FIH-1 antisense experiment, transfection was carried out in triplicates using Lipofectamine Plus reagent with 100 ng p2.1 HRE-luciferase, 100 ng pSV40-β-gal, 100 ng of either pcDNA3 empty vector, wild-type HIF-1α, P402A P564G, P564A N803A or Triple mutant and 0-400 ng of pcDNA3-HA-FIH-1 antisense or pcDNA3. Forty eight hours later, luciferase and β-gal activities were determined using the β-gal assay system (Promega, Madison WI, USA) according to the manufacturer's instructions. Subsequently, luciferase was measured using a Turner Luminometer model 20e (Turner Designs, Sunnyvale, CA, USA). Luciferase activity results were normalized for transfection efficiency using β-gal levels. For the RT-PCR experiment in HeLa, transfection of 300 ng of the various plasmids was carried out in 24-well plates with Lipofectamine Plus reagent. Magnofection of HUVEC, grown in 60 mm dishes, with plasmids was performed using Polymag (Chemicell, GmbH, Berlin, Germany) according to the manufacturer's instructions. Where indicated, treatment with 150 μM 2,2'-dipyridyl was given 16 hours prior to cell lysis.

Luciferase and β-galactosidase assays: Luciferase and β-gal activities were determined using the β-gal assay system (Promega, Madison WI, USA) according to the manufacturer's instructions. Luciferase activity results were normalized for transfection efficiency using β-gal levels.

RT-PCR assay: Forty eight hours post-transfection, cells were lysed and total RNA was isolated with RNeasy miniprep kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was then reverse transcribed to cDNA using the FastStart PCR Master kit (Roche, Mannheim, Germany). Semi-quantitative RT-PCR was performed for the following genes: HIF-1a, GAPDH, VEGF, carbonic anhydrase

9 (CA-9) and PDGF-B. Primers, annealing temperatures and cycle numbers are detailed in Table 3 hereinbelow.

Table 3

Gene	*Primers and SEQ ID NOs*	*Annealing Temp. (°C)*	*No. of Cycles*
HIF-1α	Forward:GTCGGACAGCCTCACCAAACAGAG SEQ ID NO: 6 Reverse: GTTAACTTGATCCAAAGCTCTGAG SEQ ID NO: 7	60	24
CA-9	Forward: CACGTGGTTCACCTCAGCAC SEQ ID NO: 8 Reverse: CAGCGATTTCTTCCAAGCG SEQ ID NO: 9	60	30
VEGF	Forward: CAGCGCAGCTACTGCCATCCAATCGAGA SEQ ID NO: 10 Reverse: GCTTGTCACATCTGCAAGTACGTTCGTTTA SEQ ID NO: 11	59	34
PDGF-B	Forward: ATCGCCGAGTGCAAGACGCG SEQ ID NO: 12 Reverse: AAGCACCATTGGCCGTCCGA SEQ ID NO: 13	60	34
GAPDH	Forward: ACCACAGTCCATGCCATCAC SEQ ID NO: 14 Reverse: TCCACCACCCTGTTGCTGTA SEQ ID NO: 15	60	24

RESULTS

In both human embryonic kidney 293 (HEK293) and bovine aortic endothelial cells (BAEC), transiently transfected triple mutant HIF-1α showed a 2 to 2.5-fold increase in transcriptional activity in normoxia compared with P402A P564G and P564A N803A double mutants (Figures 1A-B).

The triple mutant also led to a greater mRNA induction of HIF-1α target genes. For this, transient transfection of HeLa cells with the different mutants was followed by mRNA extraction and RT-PCR analysis. As shown in Figure 1C, mRNA of HIF-1α in all cells transfected with HIF-1α constructs was comparable and much higher than empty vector control, as expected following transfection. In agreement with the previous results, the triple mutant HIF-1α caused greater mRNA induction of the HIF-1α target genes carbonic anhydrase 9 (CA-9), VEGF and PDGF-B than the double mutants and wild-type HIF-1a. Protein degradation of P564A N803A mutant mediated by VHL could theoretically still take place by virtue of its P402 residue which is exposed to prolyl hydroxylation. In order to prove that the observed difference in transcription induction between the triple mutant and P564A N803A mutant is due to VHL-mediated degradation, a VHL-deficient renal cell carcinoma

(RCC) line was used. Assessment of HRE-mediated transcription of luciferase following transient transfection of the HIF-1α mutants in RCC in normoxia revealed an increase in activity of wild-type HIF-1α to levels of the stabilized P402A P564G mutant, indicating inhibition of proteosomal degradation (Figure 1D). As expected, an in increase in activity of the P564A N803A mutant to the levels of the triple mutant was observed.

The P402A P5645G mutant, though stabilized, could theoretically still be hydroxylated at residue N803 by FIH-1, leading to its inactivation, which is the most likely explanation for the observed difference in transcription between it and the triple mutant. Inhibition of FIH-1 by antisense knockout treatment in HEK293 resulted in a dose-dependent increase of HRE-mediated transcription by wild-type HIF-1α in normoxia [Figure 1E]. As expected, FIH-1 inhibition caused a greater fold-induction of P402A P564G mutant activity than of the Triple mutant, indicating that FIH-1 is indeed at least partly responsible for the previously observed higher activity of the triple mutant.

EXAMPLE 2

Activation of C-transactivation domain is essential for optimal HIF-1α -mediated angiogenesis.

In order to assess the significance of the triple mutant's increased transcription ability in terms of therapeutic angiogenesis, a comparison of the different mutants was carried out using an in-vitro angiogenesis assay following transfection of the HIF-1α constructs in human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

In-vitro angiogenesis assay: Transfected or infected HUVECs, grown in 60 mm dishes, were maintained for 24 hours in EGM-2 medium followed by medium replacement with EBM-2. Cells were maintained in EBM-2 for further 24 hours, prior to assay. Cells were then trypsinized and seeded at concentration of 50,000 cells per well on 24-well plate pre-coated with growth factor reduced matrigel (BD Biosciences, USA). Capillary and tube formation was tracked for 8 hours. Quantitation of capillary formation was performed by counting the number of capillary branches per high power microscopic field in 5 random microscopic fields.

VEGF ELISA: Transfected or infected HUVECs, grown in 60 mm dishes, were maintained for 24 hours in EGM-2 medium followed by medium replacement with EBM-2. Cells were maintained in EBM-2 for a further 24 hours, prior to addition

of lysis buffer (20 mM Tris pH 7.5 in PBS, 1 % Triton with complete mini protease inhibitor cocktail tablet, Roche Diagnostics, GmBH, Germany). Cell lysates were centrifuged at 10,000 rpm for 15 minutes. ELISA for human VEGF protein was performed according to the manufacturer's protocol (R& D Systems).

Western blot analysis: HUVECs were cultured in 60-mm dishes and grown to subconfluence. Cells were infected with Ad-PPE-Triple, Ad-CMV-Triple, Ad-CMV-wt or Ad-CMV-GFP as described above. After lysis and evaluation of protein content (Micro BCA; Pierce Biotechnology Inc., Rockford, Illinois, USA), samples containing equal amounts of protein were separated by SDS-PAGE and transferred to Optitran BA-S83 reinforced nitrocellulose membranes (Schleicher & Schuell BioScience Inc., Keene, New Hampshire, USA). The membranes were probed with mouse anti-human HIF-1α polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), followed by HRP-conjugated secondary antibodies and ECL reaction.

RESULTS

While the empty vector caused no tube formation, all HIF-1α constructs, VEGF and FCS positive controls stimulated angiogenesis (Figures 2A-F). Wild-type HIF-1α and P402A P564G stimulated tube formation to a similar extent but both had a significantly reduced angiogenic effect compared to P564A N803A and the triple mutant ($p < 0.001$) (Figure 2G). In agreement with these findings, the VEGF protein concentration in HUVECs following treatment with wt- HIF1α or P402A P564G was very similar and significantly lower than treatments with P564A N803A or Triple mutant ($p = 0.029$) (Figure 2H). Treatment with the P564A N803A or Triple mutant resulted in a 6-fold and 10-fold increase in VEGF protein respectively, compared with wt-HIF1α and P402A P564G (Figure 2H). These results indicate that stability of HIF-1α is insufficient and that a constitutively active C-TAD is essential for maximal angiogenic effect of HIF-1α. No difference was observed between P564A N803A and the triple mutant. This may be due to high HIF-1α overexpression which had saturated the hydroxylase enzymes, thus masking any differences which are due to stability, similar to the state found within the RCC in Figure 1D.

EXAMPLE 3

Expression and specificity of the PPE-1-3x promoter in ischemia

A prerequisite for the feasibility and safety of systemic pro-angiogenic gene therapy is the sufficiently robust and specific expression within the target ischemic

tissue. In order to characterize the expression and specificity of a modified PPE-1 promoter in the setting of ischemia, an adenovirus expressing GFP under the regulation of PPE-1-3x was used (Ad-PPE-1-3x-GFP).

MATERIALS AND METHODS

Construction of adenoviral vectors: Ad-PPE-Triple, Ad-CMV-Triple, and Ad-CMV-wt vectors were generated by homologous recombination by Vector Biolabs (Philadelphia, USA). Successful creation of viral constructs was verified by PCR, following which two rounds of plaque purification were performed. Viral plaques subsequently underwent large-scale amplification in HEK293, followed by CsCl purification.

***Animals*:** Twelve-week old female C57BL/6J mice (Harlan Laboratories Ltd., Jerusalem, Israel) were used. All animal procedures were approved by the Animal Care and Use Committee of Sheba Medical Center.

Hindlimb ischemia model and adenovirus injections: Twelve-week old C57BL/6J female mice were anesthetized using a mixture of ketamine and xylazine. They were subjected to hindlimb ischemia by a modification to the procedure described by Couffinhal et al. [15]. The skin overlying the left femoral artery was incised, followed by ligations of the femoral artery proximal to the bifurcation of the deep and superficial femoral arteries, and proximal to the popliteal artery. The portion of the artery between the ligatures was then removed and all side branches were excised. For local intramuscular injections, 20µl of 2×10^{10}VP or saline were injected at time of surgery at each of three sites: quadriceps femoris, adductor and gastrocnemius muscles. For systemic intravenous injections, 100µl of 1×10^{11}VP or saline were injected via the tail vein 7 days post-surgery.

GFP analysis: Mice were sacrificed 7 days after virus injection, and gastrocnemius muscle tissues were collected and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer at 4 ° C for 24 hours, soaked in 30 % sucrose solution at 4 ° C for 48 hours and then frozen in OCT compound (Sakura, CA, USA) . Tissue blocks were sliced by a cryomicrotome into 5 µm-thick transverse sections and observed directly under a fluorescent microscope (FITC filter). All tissue processing was performed under dim light to prevent GFP bleaching.

RESULTS

Mice were subjected to hindlimb ischemia by ligation of the femoral artery or to sham procedure, followed by intamuscular injection of Ad-PPE-1-3x-GFP. Control

animals were injected with Ad-CMV-GFP or saline. Expression of GFP following Ad-CMV-GFP injection was detected in muscle histological sections in both myocytes and endothelial cells of ischemic and non-ischemic muscles. In contrast, GFP expression following Ad-PPE-1-3x-GFP injection was detected in endothelial cells of ischemic muscles while no expression was identified in non-ischemic muscle. These results demonstrate the specificity to endothelium and to ischemic condition of the modified PPE-1 promoter.

Intravenous delivery of Ad-PPE-1-3x-GFP was effected in order to determine whether this promoter could also lead to expression following intravenous systemic injection, where the virus is disseminated in the body and only a small fraction of it reaches the target organ. For this end, mice subjected to hindlimb ischemia or to sham procedure were injected 7 days post-surgery via the tail vein with Ad-PPE-1-3x-GFP and Ad-CMV-GFP or saline as controls. Since immediately following femoral artery ligation the blood flow in the ischemic hindlimb falls to less than 10 % of the normal limb and is almost absent in our model, there was a waiting interval of 7 days prior to injection, with the aim of enabling sufficient vascularisation to develop, thus allowing the virus to reach its ischemic target. As shown in Figures 3A-F, whereas non-specific expression of GFP was detected in histological sections of both ischemic and non-ischemic muscles following systemic injection of Ad-CMV-GFP (Figures 3B and 3E), specific expression of GFP only in endothelial cells of ischemic muscle was noted following injection of Ad-PPE-1-3x-GFP (Figures 3C and 3F).

EXAMPLE 4

Local injection of Ad-PPE-Triple HIF-1a augments ischemic neovascularization

The following experiments were performed in order to determine whether intramuscular treatment with adenoviral-mediated triple mutant HIF-1α under the expression of PPE-1-3x promoter could promote angiogenesis in-vivo.

MATERIALS AND METHODS

Monitoring of hindlimb blood flow: Mice were anesthetized and placed for 5 minutes on a heating plate at 37 °C in order to minimize temperature variation. Hindlimb blood flow was measured with a laser Doppler perfusion imager system (Moor Instruments Ltd., UK) immediately after surgery and at days 7, 14, 21 and 28 post-surgery. Low and/or no perfusion signal was displayed in blue, whereas the highest perfusion signal was displayed in red. Color photographs were recorded and

analyses were performed by calculating the average perfusion of the ischemic and non-ischemic hindlimb. To account for variables such as ambient light and temperature, the results are expressed as ratio of perfusion in the left (ischemic) vs. right (non-ischemic) hindlimb.

Immunohistochemistry and quantification of capillary density: Upon sacrifice at day 28, hindlimb muscle tissues were excised and frozen in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, California, USA) and cryosectioned transversely. ECs were immunostained using rat monoclonal anti-CD31 antibodies (Pharmingen, San Diego, California, USA). The background was stained with hematoxylin.

RESULTS

Hindlimb ischemia was induced in mice followed by intramuscular injection of Ad-PPE-1-3x-Triple, Ad-CMV-Triple or Ad-CMV-wt. Control animals were injected with Ad-CMV-Luc or saline. Blood flow, clinical outcome and indices of angiogenesis were examined over a period of 28 days. Immediately following surgery, blood flow in the ischemic limb fell to less than 10 % of the normal limb in all mice groups. By day 21, mice treated with Ad-CMV-Triple or Ad-PPE-1-3x-Triple showed significantly increased hindlimb blood flow relative to mice treated with Ad-CMV-wt, Ad-CMV-Luc or saline controls (Figure 4A). By day 28, both Ad-CMV-Triple and Ad-PPE1-3x-Triple improved perfusion in the ischemic limb to approximately 50 % of the normal limb, significantly more than Ad-CMV-wt or control treatments (Figures 4A-B). Moreover, treatment with Ad-CMV-Triple or Ad-PPE-Triple managed to profoundly reduce ischemia-induced toe necrosis and autoamputation, which were prominent in the mice treated with Ad-CMV-wt or controls, consistent with improved blood flow to the ischemic limb (Figures 4C- D). In agreement with the blood flow findings, quantification of muscle capillary density following CD31 staining revealed higher capillary to muscle fiber ratio in the animals treated with Ad-CMV-Triple and Ad-PPE-1-3x-Triple than Ad-CMV-wt and control-treated animals (Figures 4E and 4F).

EXAMPLE 5

Systemic administration of Ad-PPE-1-3x-Triple is safer and shows no side-effects compared with Ad-CMV-Triple

The following experiments were performed to compare the safety profile of systemic administration of Ad-PPE-1-3x-Triple to that of Ad-CMV-Triple. In contrast to local intramuscular injection where most of the adenovirus is concentrated at the site of injection, following intravenous administration via the tail vein most of the adenovirus reaches and transduces the liver. Therefore, with regards to safety of the treatment, it was especially important to monitor any changes in liver function and other systemic manifestations occurring in the mice.

MATERIALS AND METHODS

Measurement of serum bilirubin, AST and ALT: Mice sera were obtained at day 5 and 21 post-injection and analyzed using an automatic analyzer.

Immunohistochemistry and quantification of capillary density: as described in Example 4.

Monitoring of hindlimb blood flow: as described in Example 4.

Statistical methods: SigmaStat (SPSS Science, Chicago, Illinois, USA) was used for statistical analysis. One-way repeated-measures ANOVA was performed in the in vitro studies, and Student's *t* test was used for the hindlimb ischemia model; *P* less than 0.05 was considered significant. Data are presented as mean ± SEM and were graphed using SigmaPlot or Microsoft Excel (Microsoft Corp., Redmond, Washington, USA).

RESULTS

Mice subjected to hindlimb ischemia were injected systemically via the tail vein 7 days post-surgery with Ad-PPE-1-3x-Triple, Ad-CMV-Triple and Ad-CMV-wt, while Ad-CMV-Luc and saline served as controls. One day following injection, all groups of mice but the saline-treated showed a marked reduction in body weight. However, whereas all other adenovirus- treated mice regained their original body weight by day 3 post-injection, the Ad-CMV-Triple treated mice continued to decrease in weight reaching a peak 10 % reduction by day 4, indicating systemic illness (Figure 5A). Liver function tests of the mice were performed 5 days post-injection and revealed a marked 25-fold serum bilirubin increase in the Ad-CMV-Triple treated mice over saline control, demonstrating severe jaundice. Ad-CMV-wt caused a milder serum bilirubin increase (Figure 5C). Furthermore, serum AST and

ALT levels were profoundly elevated by day 5 in mice treated with Ad-CMV-Triple, Ad-CMV-wt and also Ad-CMV-Luc (Figures 5B and 5D). In contrast to the overt systemic manifestations of hepatic illness exhibited by the Ad-CMV-Triple treated mice, the mice treated with Ad-PPE-Triple showed no signs of illness and had their serum bilirubin, AST and ALT levels comparable to saline-treated mice. In accordance with these findings, liver histological sections of mice sacrificed at day 5 revealed remarkable inflammation and lymphocytic infiltration in the Ad-CMV-Triple treated mice while Ad-PPE-1-3x-Triple, Ad-CMV-Luc and saline controls showed no pathology (Figures 5E-N). Mild lymphocytic infiltration was also noted in the Ad-CMV-wt treated group. Following this acute phase of hepatic pathology, the Ad-CMV-Triple treated mice showed gradual partial recovery. They regained their weight, and by day 28 post-injection their serum bilirubin had returned to normal. However, their serum AST and ALT were still elevated compared with Ad-PPE-1-3x-Triple and saline control treated mice, similarly to Ad-CMV-wt and Ad-CMV-Luc groups. This partial liver recovery was seen also in liver histological sections from day 21 post-injection (Figure 5E-N), showing mild residual lymphocytic infiltration in the animals treated with Ad-CMV-triple.

Following treatment, mice treated with Ad-PPE-Triple demonstrated a significantly greater improvement in blood perfusion in the ischemic hindlimb compared with Ad-CMV-Triple and all other treatment groups, which was first noted on day 21 post-surgery, reaching approximately 45 % perfusion of the normal limb by day 28 post-surgery vs. 30 % in the Ad-CMV-Triple group (Figures 6A-B). The clinical outcome of the ischemic hindlimb was consistent with the increased blood flow, demonstrating a marked reduction in toe necrosis and autoamputation in the Ad-PPE-Triple mice vs. all other groups (Figures 6C-D). In addition, quantification of muscle capillary density following CD31 staining showed higher capillary to muscle fiber ratio in the group treated with Ad-PPE-1-3x-Triple than the Ad-CMV-Triple and all other groups (Figures 6E-F).

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A vector comprising an endothelial cell-specific promoter which comprises at least one copy of SEQ ID NO: 4.

2. The vector of claim 1, which is a prokaryotic vector or a eukaryotic vector.

3. The vector of claim 1, which is an adenovirus.

4. The vector of claim 1, wherein the endothelial cell-specific promoter is operably linked to a nucleotide sequence encoding a polypeptide.

5. The vector of claim 4, wherein the polypeptide has a Hypoxia-Inducible Factor 1-α activity.

6. The vector of claim 5, wherein the polypeptide comprises an amino acid sequence at least 90% identical to the full length sequence of SEQ ID NO: 3.

7. The vector of claim 6, wherein the polypeptide comprises SEQ ID NO: 3 except a mutation at a position corresponding to proline 402 of SEQ ID NO: 3, a mutation at a position corresponding to proline at 564 of SEQ ID NO: 3, or a mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3.

8. The vector of claim 7, wherein the polypeptide comprises SEQ ID NO: 3 except a mutation at a position corresponding to proline 402 of SEQ ID NO: 3, a mutation at a position corresponding to proline at 564 of SEQ ID NO: 3, and a mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3.

9. The vector of claim 7, wherein the mutation at a position corresponding to proline 402 of SEQ ID NO: 3 is to alanine, the mutation at a position corresponding to proline at 564 of SEQ ID NO: 3 is to glycine, or the mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3 is to alanine.

10. The vector of claim 9, wherein the mutation at a position corresponding to proline 402 of SEQ ID NO: 3 is to alanine, the mutation at a position corresponding to proline at 564 of

SEQ ID NO: 3 is to glycine, and the mutation at a position corresponding to asparagine 803 of SEQ ID NO: 3 is to alanine.

11. The vector of claim 4, wherein the polypeptide comprises SEQ ID NO: 2.

12. The vector of claim 11, wherein the nucleotide sequence comprises SEQ ID NO: 1.

13. The vector of any one of claims 4 to 12, which is a prokaryotic vector or a eukaryotic vector.

14. The vector of any one of claims 4 to 12, which is an adenovirus.

15. A recombinant protein comprising the polypeptide encoded by the vector of any one of claims 4 to 14.

16. A pharmaceutical composition comprising the vector of any one of claims 4 to 14 and a pharmaceutically acceptable carrier.

17. The pharmaceutical composition of claim 16, wherein the composition is formulated for systemic administration or local administration.

18. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is wound healing.

19. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is ischemic stroke.

20. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is ischemic heart disease.

21. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is peripheral vascular disease.

22. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is renal artery disease.

23. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is gastrointestinal lesions.

24. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is burns.

25. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is skin transplantation.

26. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is vascular grafts.

27. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the

composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is organ repair.

28. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is bone reparative disorders.

29. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is liver disorders.

30. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is uterine disorders.

31. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is ocular angiogenesis disorders.

32. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is bone regeneration disorders.

33. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is cartilage repair disorders.

34. Use of a pharmaceutical composition in the manufacture of a medicament for treating a medical condition associated with hypoxia or ischemia in a subject in need thereof, the composition comprising the vector of any one of claims 6 to 14, and wherein the medical condition associated with hypoxia or ischemia is smooth muscle cell disorders.

35. The use of any one of claims 18 to 34, wherein the composition is formulated for systemic administration or local administration.

36. The use of claim 35, wherein the composition is formulated for oral administration.

37. The use of claim 35, wherein the composition is formulated for rectal administration.

38. The use of claim 35, wherein the composition is formulated for transmucosal administration.

39. The use of claim 35, wherein the composition is formulated for transnasal administration.

40. The use of claim 35, wherein the composition is formulated for intestinal administration.

41. The use of claim 35, wherein the composition is formulated for parenteral administration.

42. The use of claim 35, wherein the composition is formulated for intramuscular administration.

43. The use of claim 35, wherein the composition is formulated for subcutaneous administration.

44. The use of claim 35, wherein the composition is formulated for intramedullary administration.

45. The use of claim 35, wherein the composition is formulated for intrathecal administration.

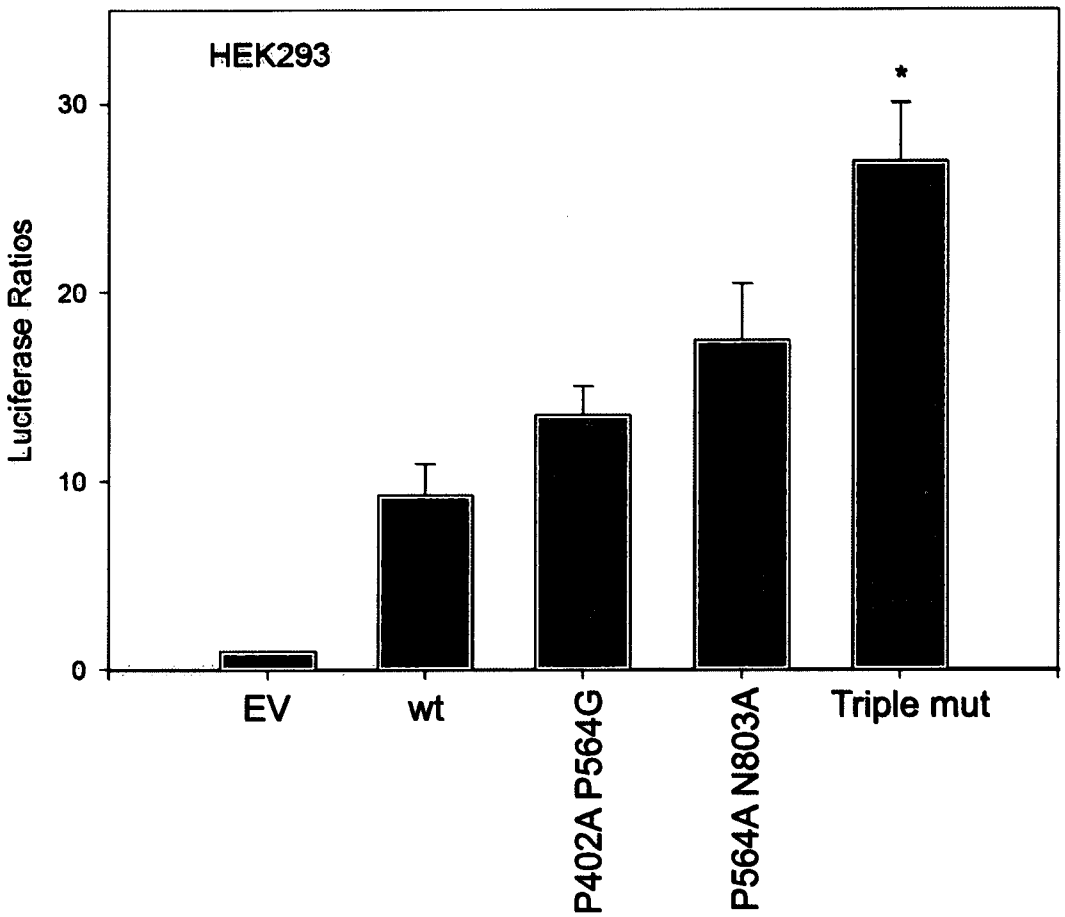
46. The use of claim 35, wherein the composition is formulated for direct intraventricular administration.

47. The use of claim 35, wherein the composition is formulated for intravenous administration.

48. The use of claim 35, wherein the composition is formulated for intraperitoneal administration.

49. The use of claim 35, wherein the composition is formulated for intranasal administration.

50. The use of claim 35, wherein the composition is formulated for intraocular administration.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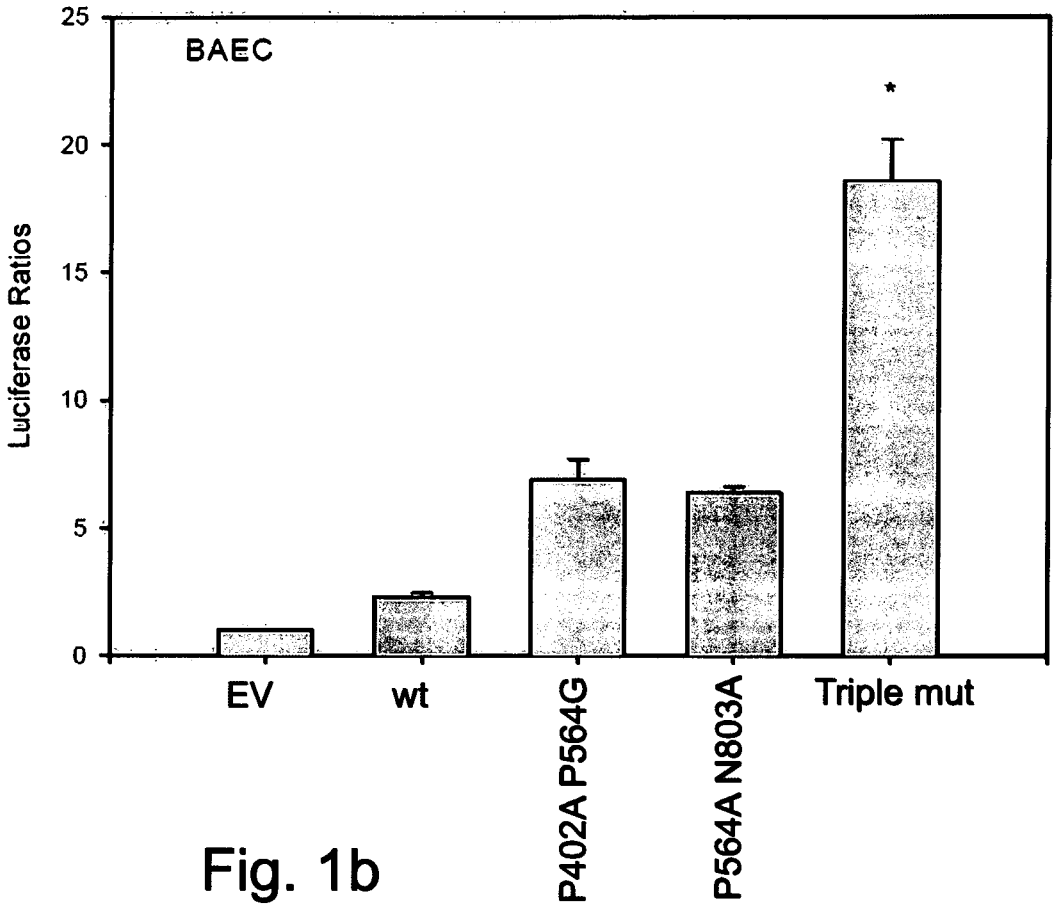
20
10
0
*
EV
wt
P402A P564G
P564A N803A
Triple mut

Fig. 1a


BAEC
Luciferase Ratios
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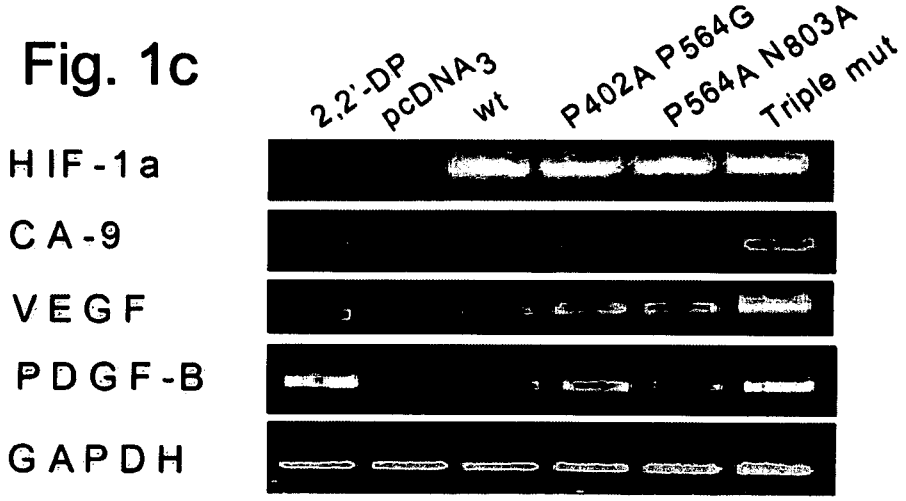
5
0
*
EV
wt
P402A P564G
P564A N803A
Triple mut

Fig. 1b

Fig. 1c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wt
P402A P564G
P564A N803A
Triple mut
HIF-1a
CA-9
VEGF
PDGF-B
GAPDH

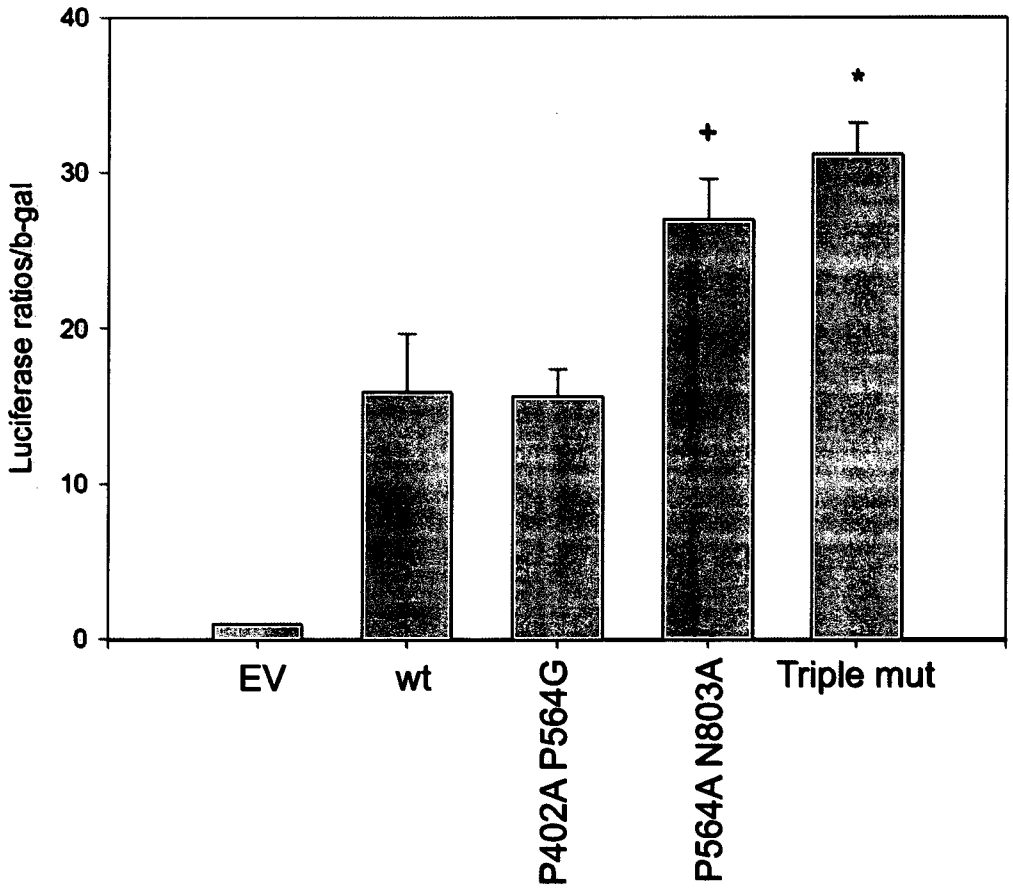

40
30
20
10
0
Luciferase ratios/b-gal
+
*
EV
wt
P402A P564G
P564A N803A
Triple mut

Fig. 1d

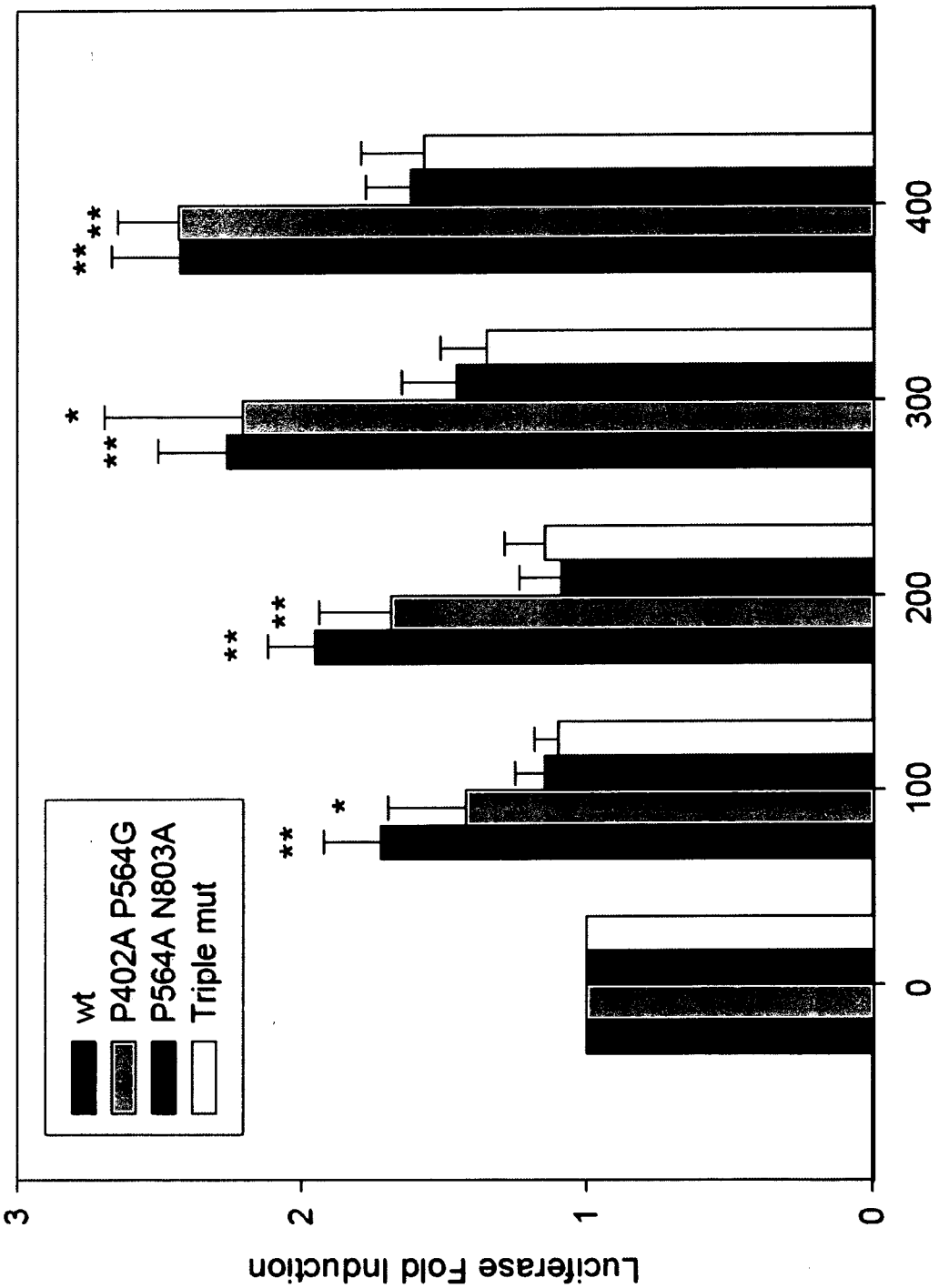

Luciferase Fold Induction
0
1
2
3
AS-FIH-1 amount (ng)
0
100
200
300
400
wt
P402A P564G
P564A N803A
Triple mut
*
**
**
**
*
**
**
***

Fig. 1e

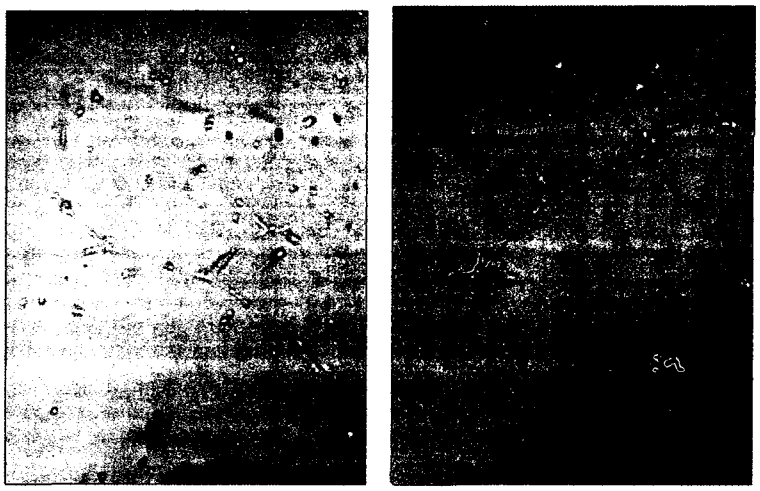


Fig. 2c

Fig. 2f

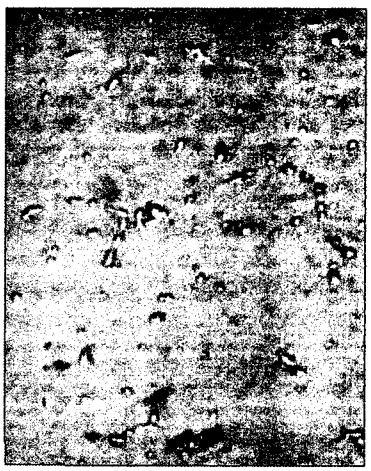


Fig. 2b

Fig. 2e

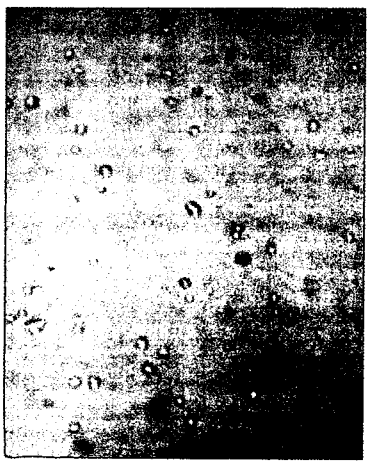


Fig. 2a

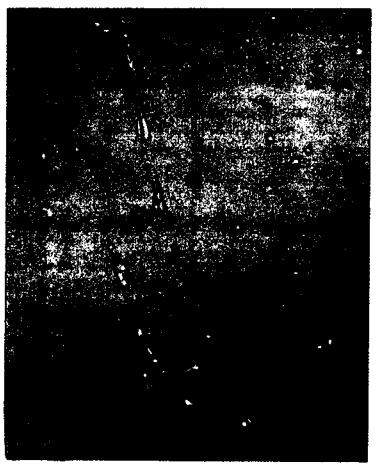


Fig. 2d

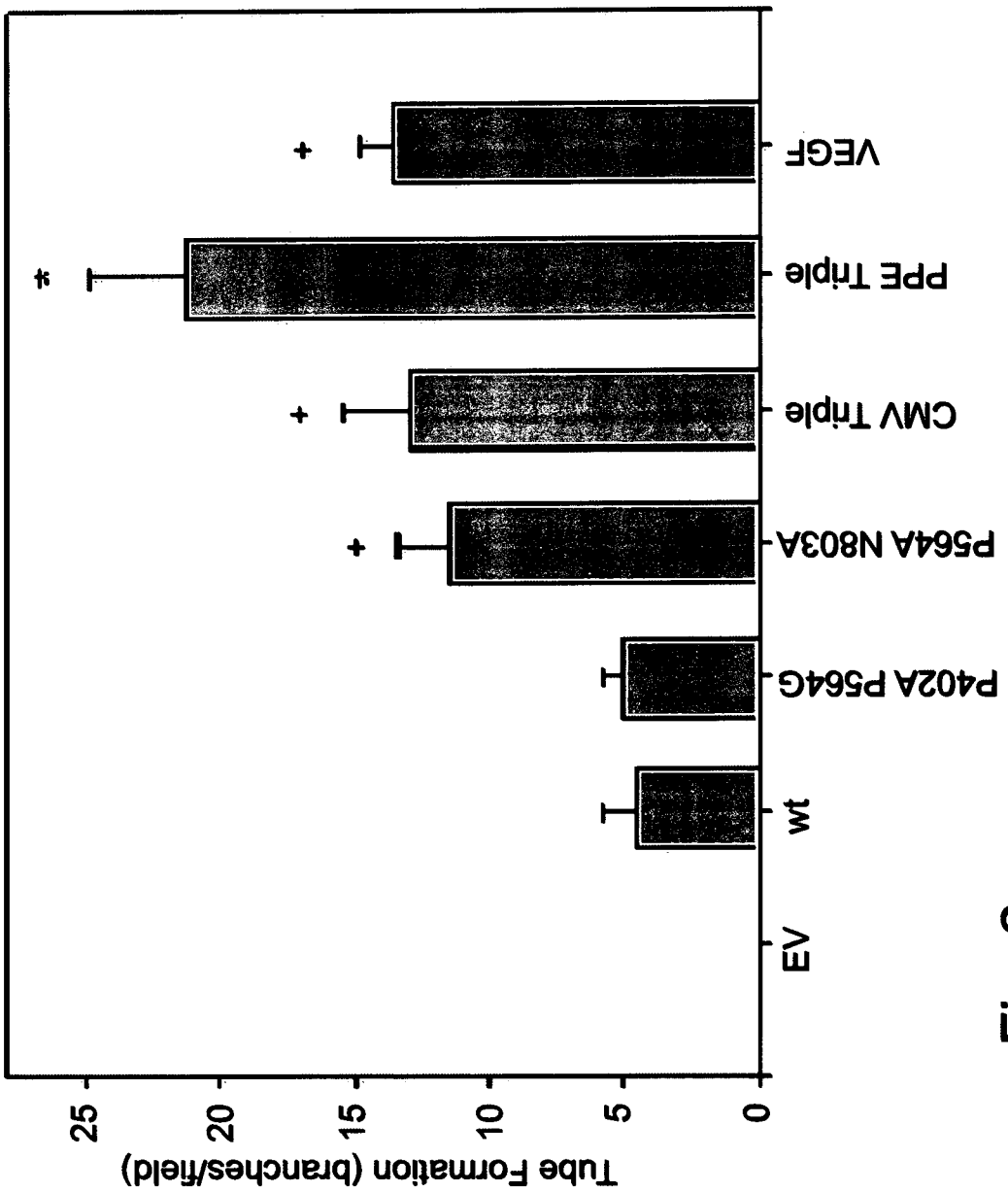

Tube Formation (branches/field)
0
5
10
15
20
25
EV
wt
P402A P564G
P564A N803A
CMV Triple
PPE Triple
VEGF
+
+
+
*

Fig. 2g

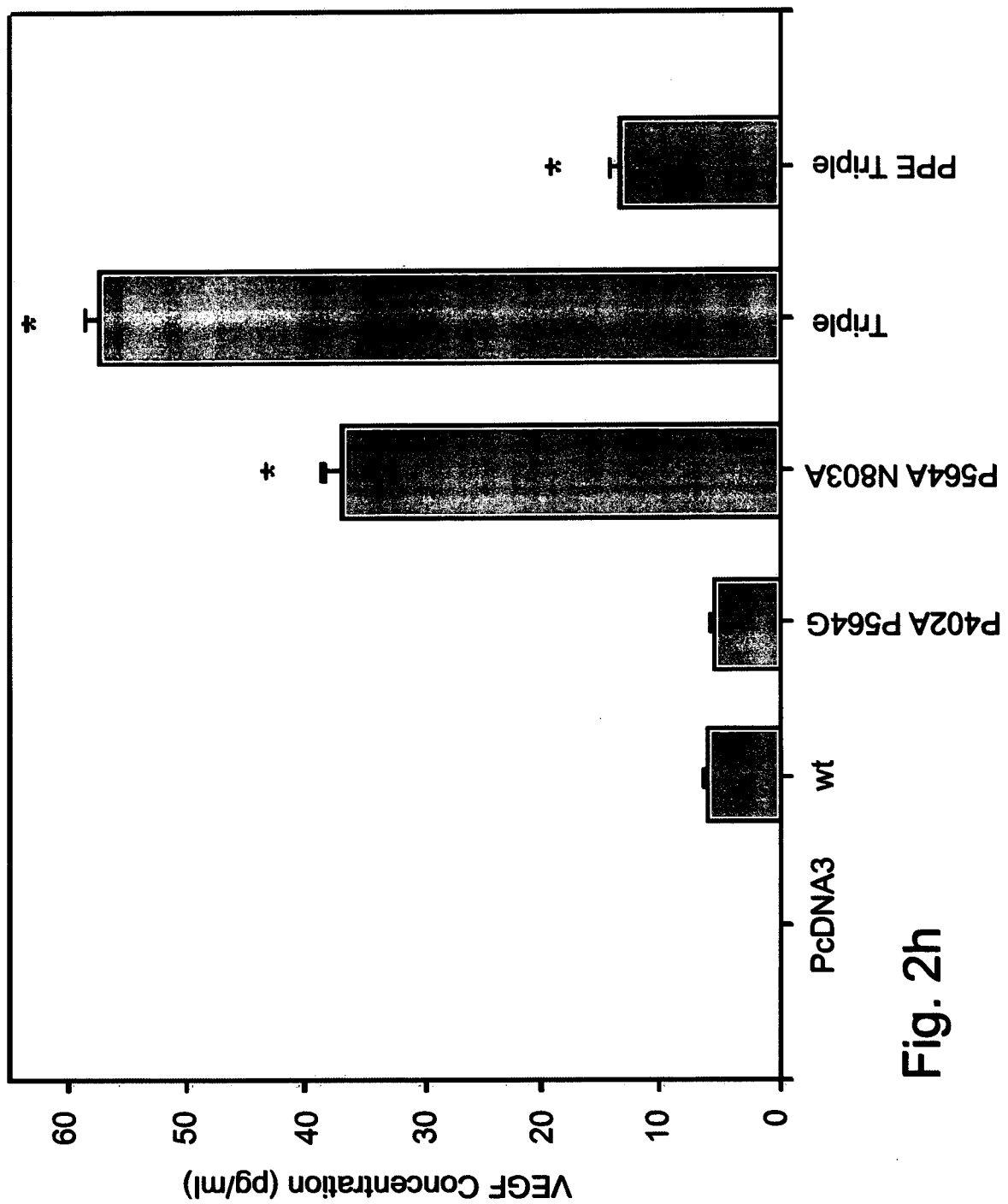

VEGF Concentration (pg/ml)
0
10
20
30
40
50
60
PcDNA3
wt
P402A P564G
P564A N803A
Triple
PPE Triple
*
*
*

Fig. 2h

Fig. 3a

Fig. 3b

Fig. 3c

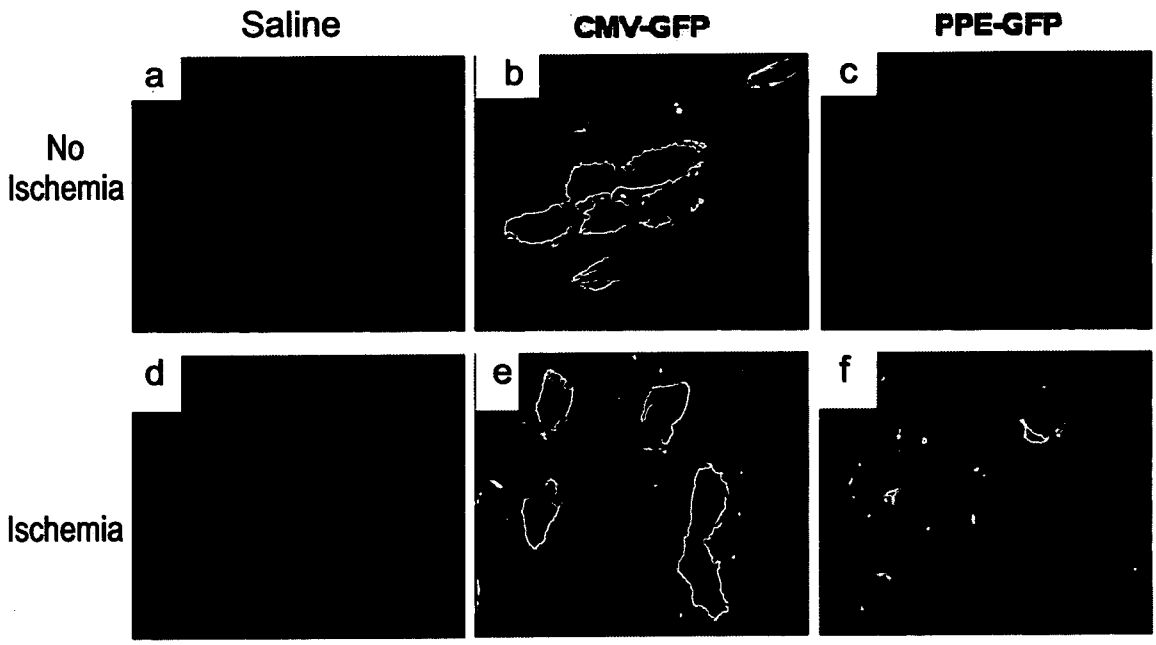


Fig. 3d

Fig. 3e

Fig. 3f

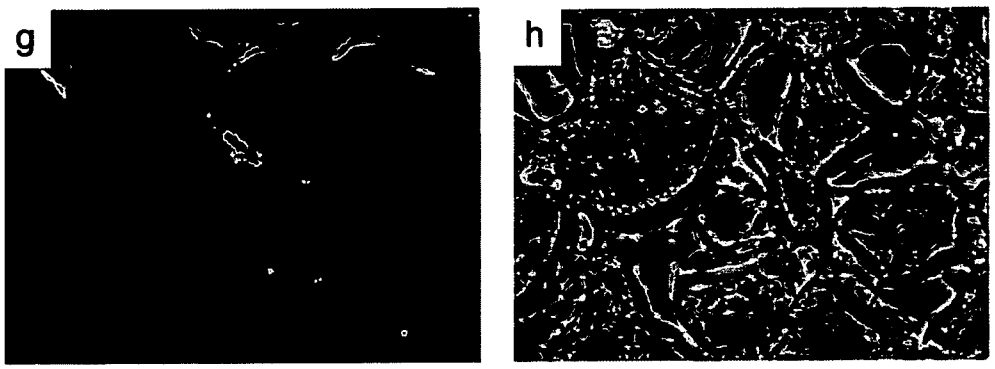


Fig. 3g

Fig. 3h

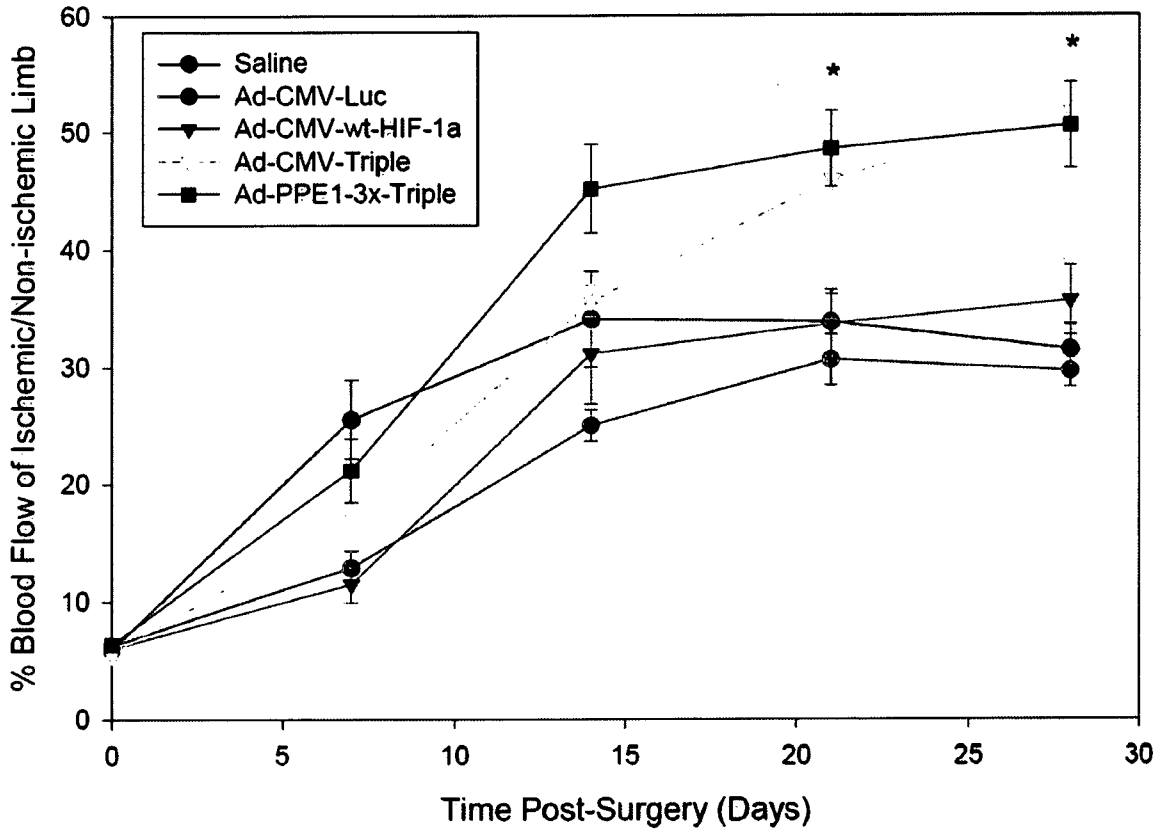

Saline
Ad-CMV-Luc
Ad-CMV-wt-HIF-1a
Ad-CMV-Triple
Ad-PPE1-3x-Triple
% Blood Flow of Ischemic/Non-ischemic Limb
Time Post-Surgery (Days)
0
10
20
30
40
50
60
0
5
10
15
20
25
30
*
*

Fig. 4a

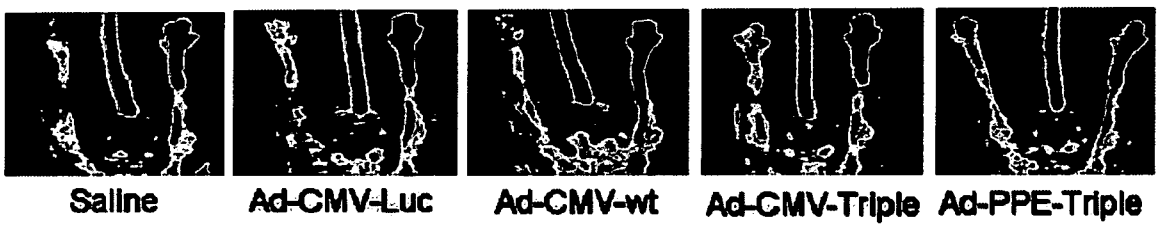

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 4b

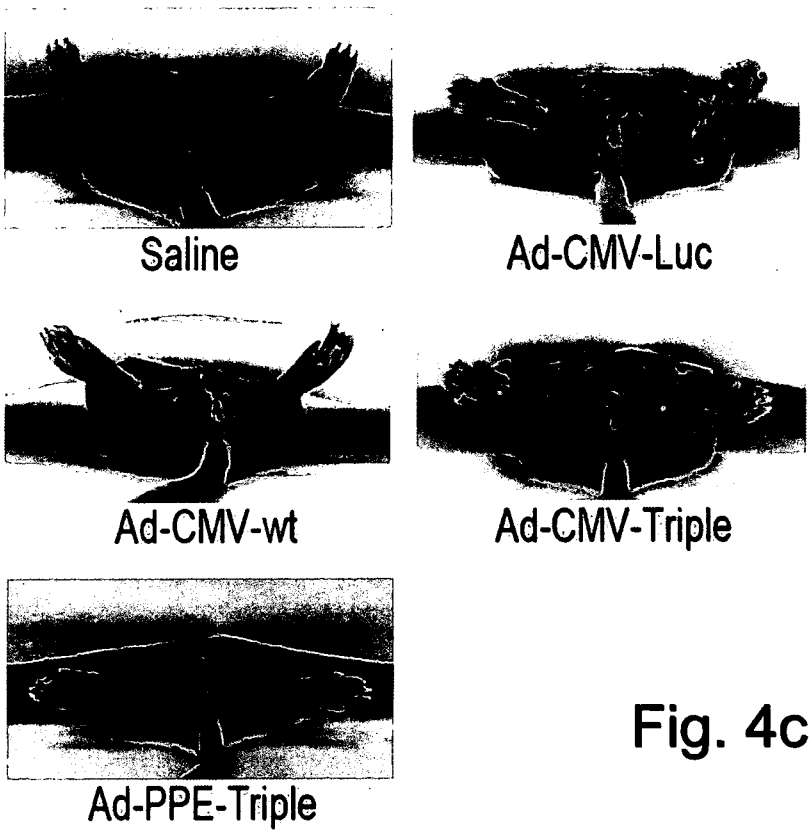

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 4c

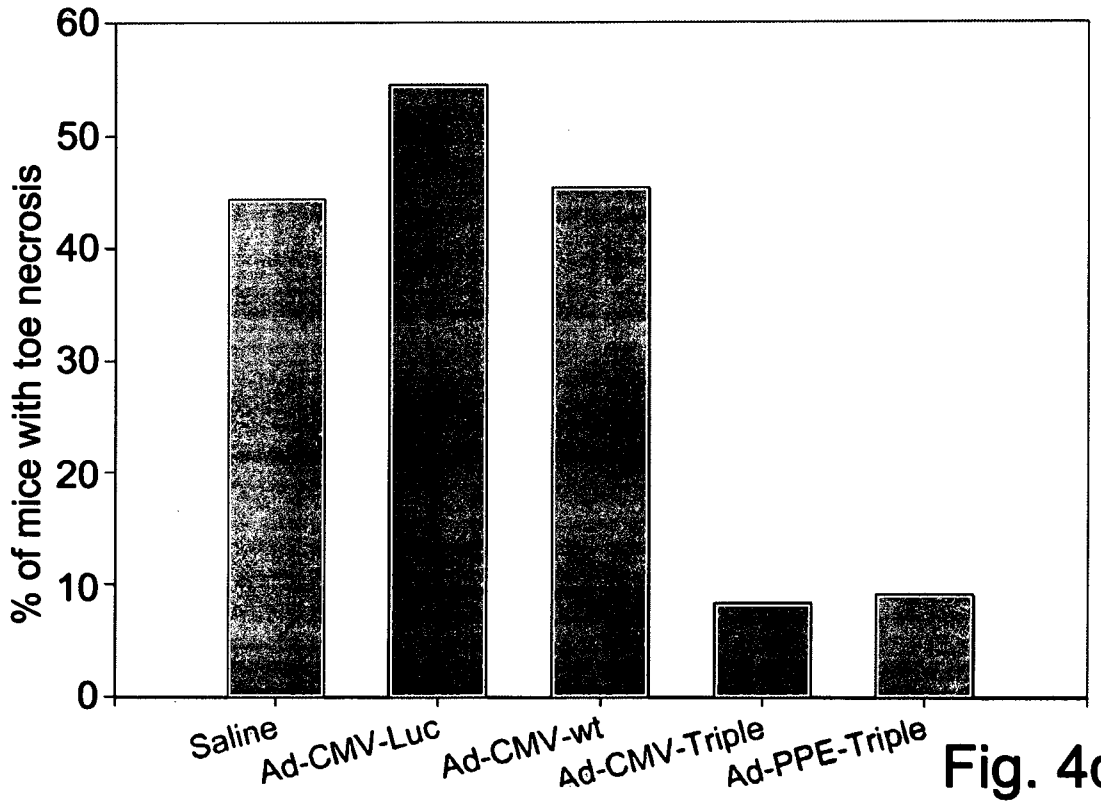

% of mice with toe necrosis
0
10
20
30
40
50
60
Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 4d

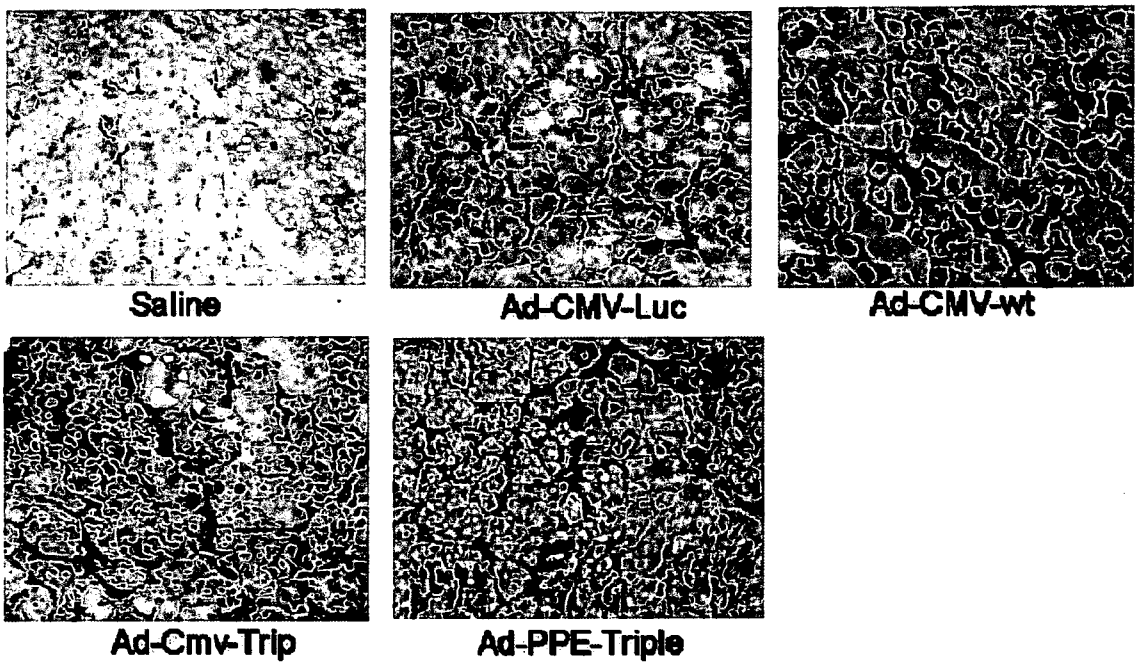

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-Cmv-Trip
Ad-PPE-Triple

Fig. 4e

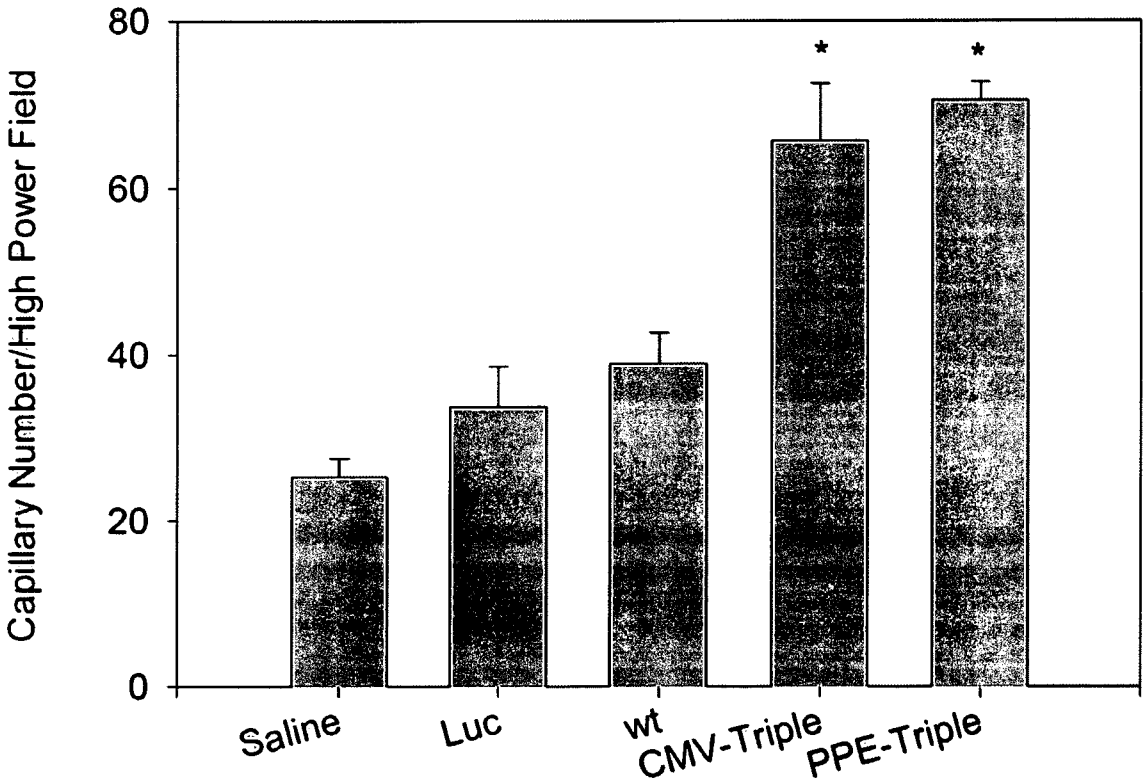

Capillary Number/High Power Field
80
60
40
20
0
*
*
Saline
Luc
wt
CMV-Triple
PPE-Triple

Fig. 4f

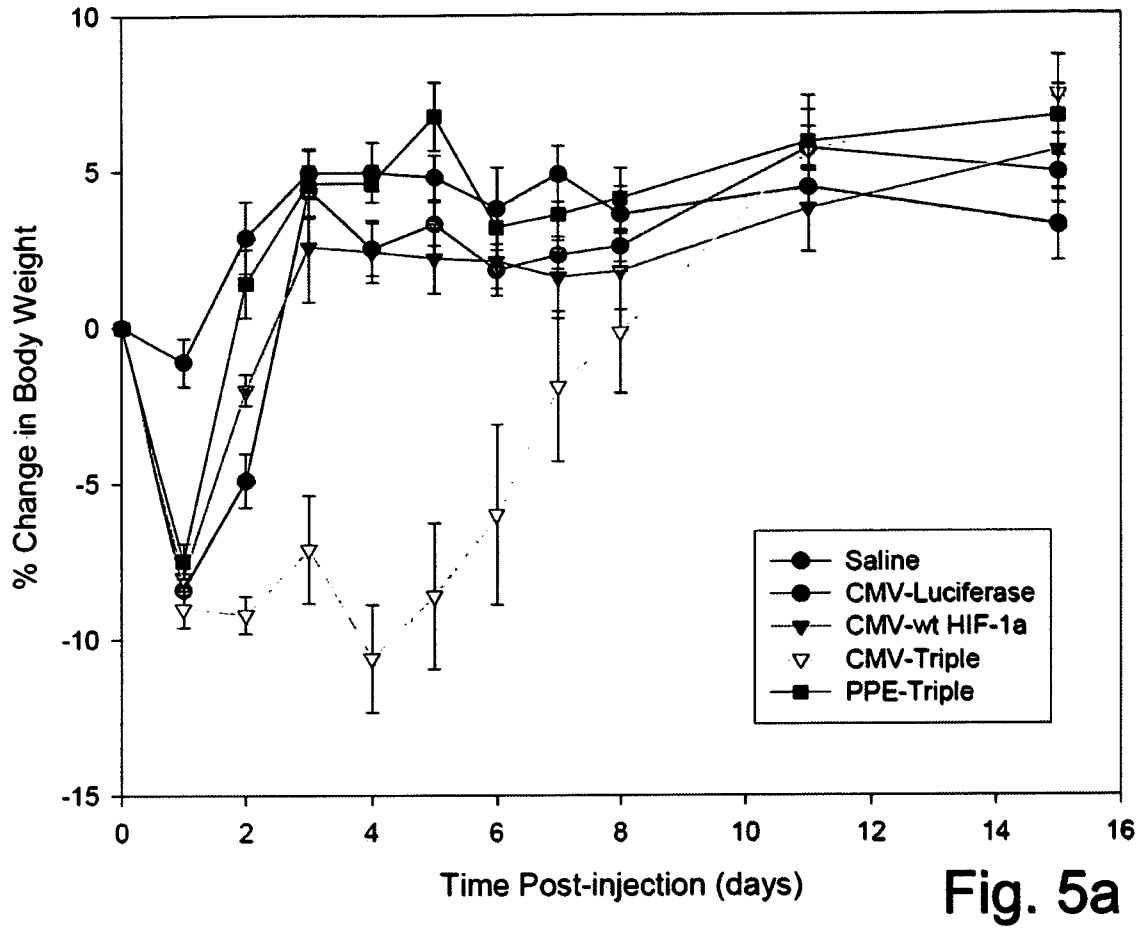

% Change in Body Weight
Time Post-injection (days)
Saline
CMV-Luciferase
CMV-wt HIF-1a
CMV-Triple
PPE-Triple

Fig. 5a

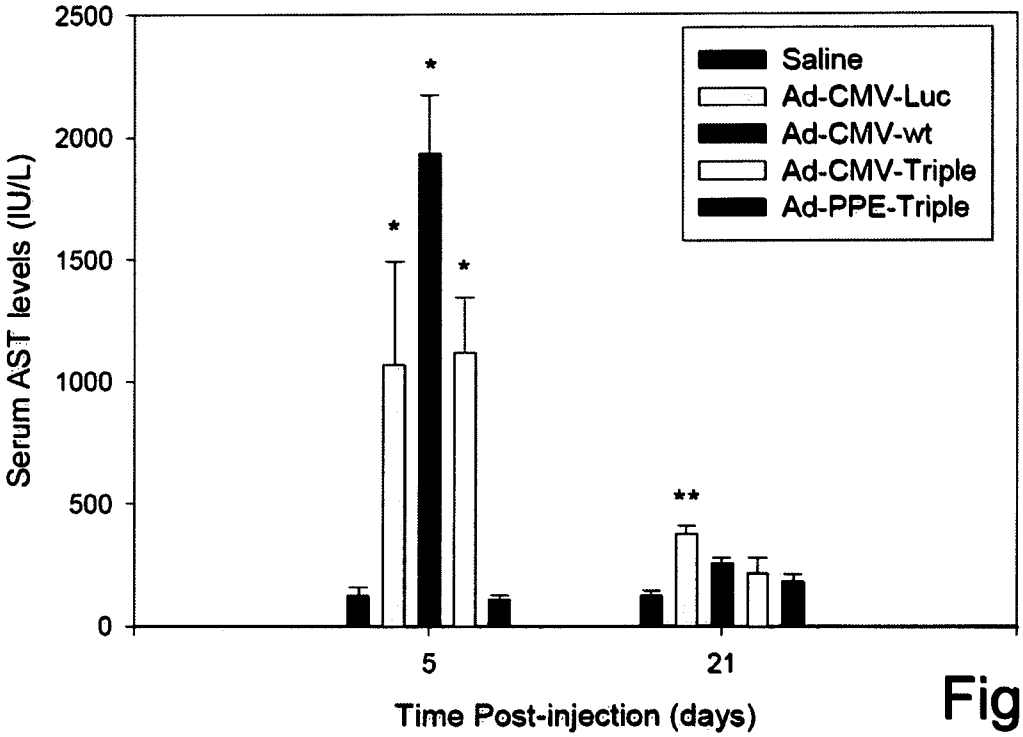

Serum AST levels (IU/L)
Time Post-injection (days)
Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 5b

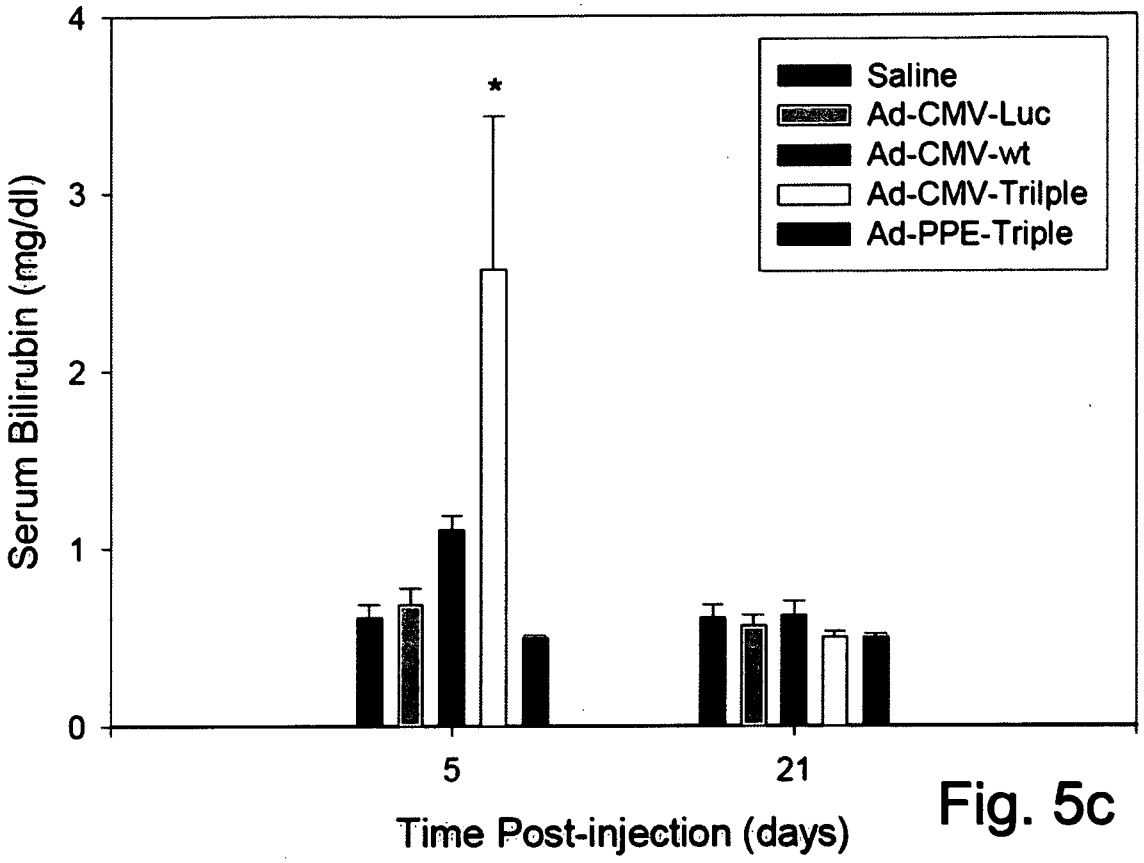

Serum Bilirubin (mg/dl)
4
3
2
1
0
*
Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Trilple
Ad-PPE-Triple
5
21
Time Post-injection (days)

Fig. 5c

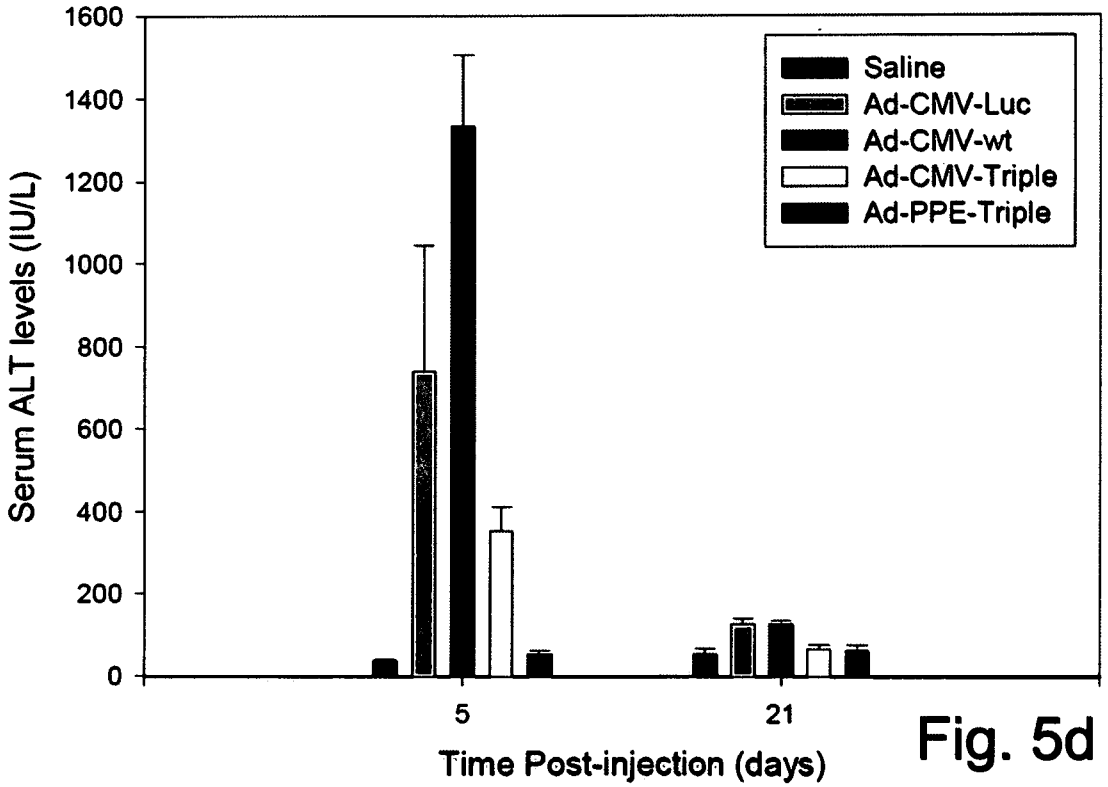
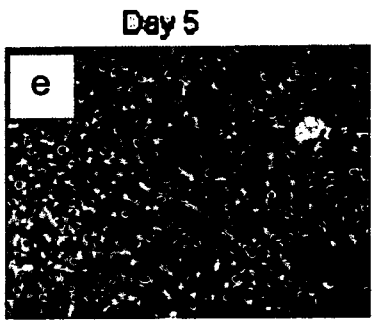

Serum ALT levels (IU/L)
1600
1400
1200
1000
800
600
400
200
0
Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple
5
21
Time Post-injection (days)

Fig. 5d



Saline

Fig. 5e

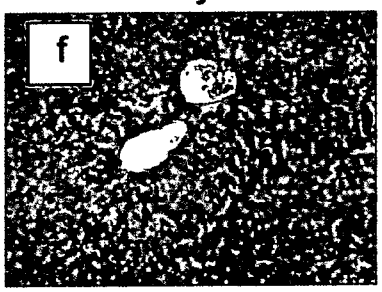
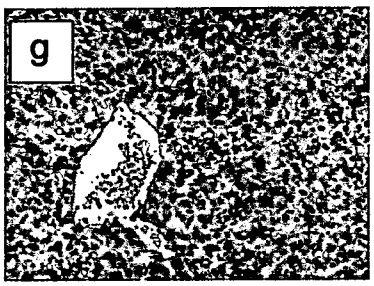


Fig. 5f



Ad-CMV-Luc

Fig. 5g

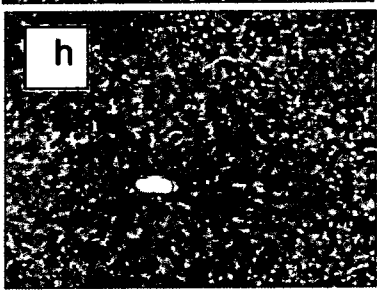
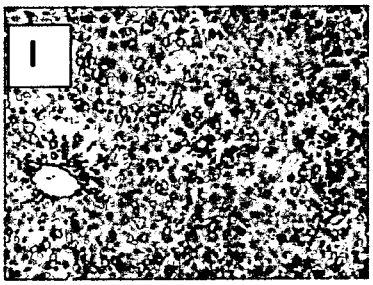


Fig. 5h



Ad-CMV-wt

Fig. 5i

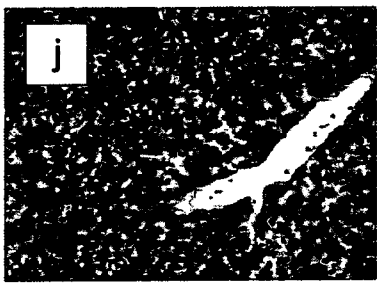
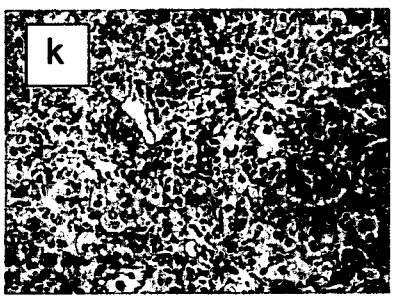


Fig. 5j



Ad-CMV-Triple

Fig. 5k

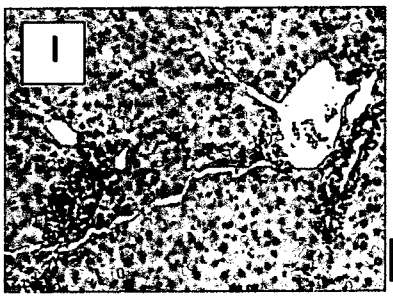
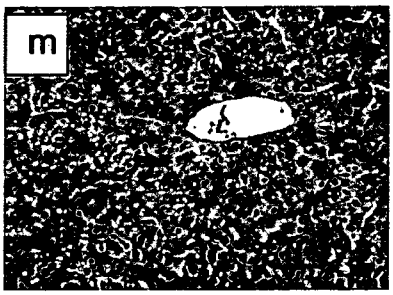


Fig. 5l



Ad-PPE-Triple

Fig. 5m

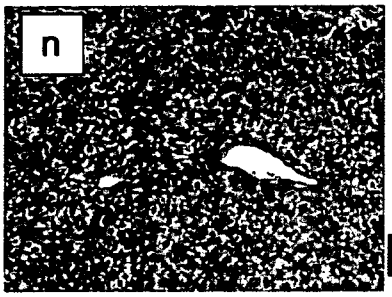


Fig. 5n

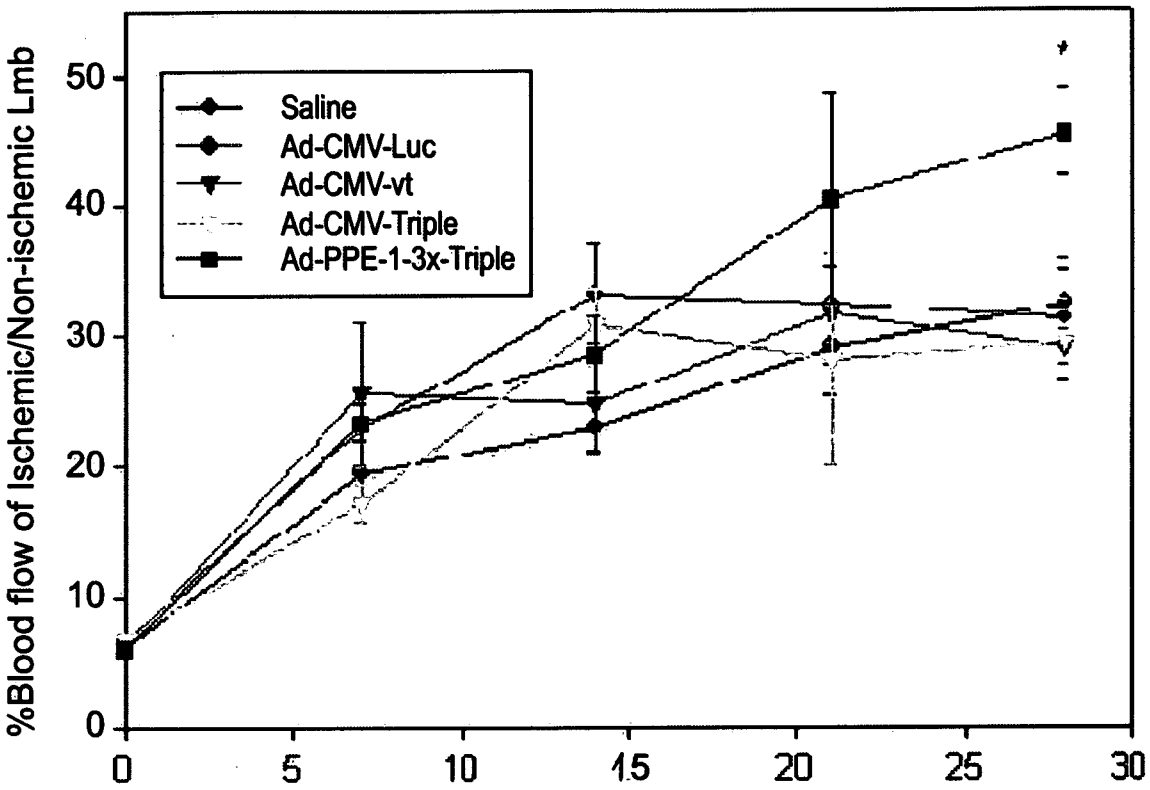

%Blood flow of Ischemic/Non-ischemic Lmb
Saline
Ad-CMV-Luc
Ad-CMV-vt
Ad-CMV-Triple
Ad-PPE-1-3x-Triple
0
10
20
30
40
50
0
5
10
15
20
25
30

Fig. 6a

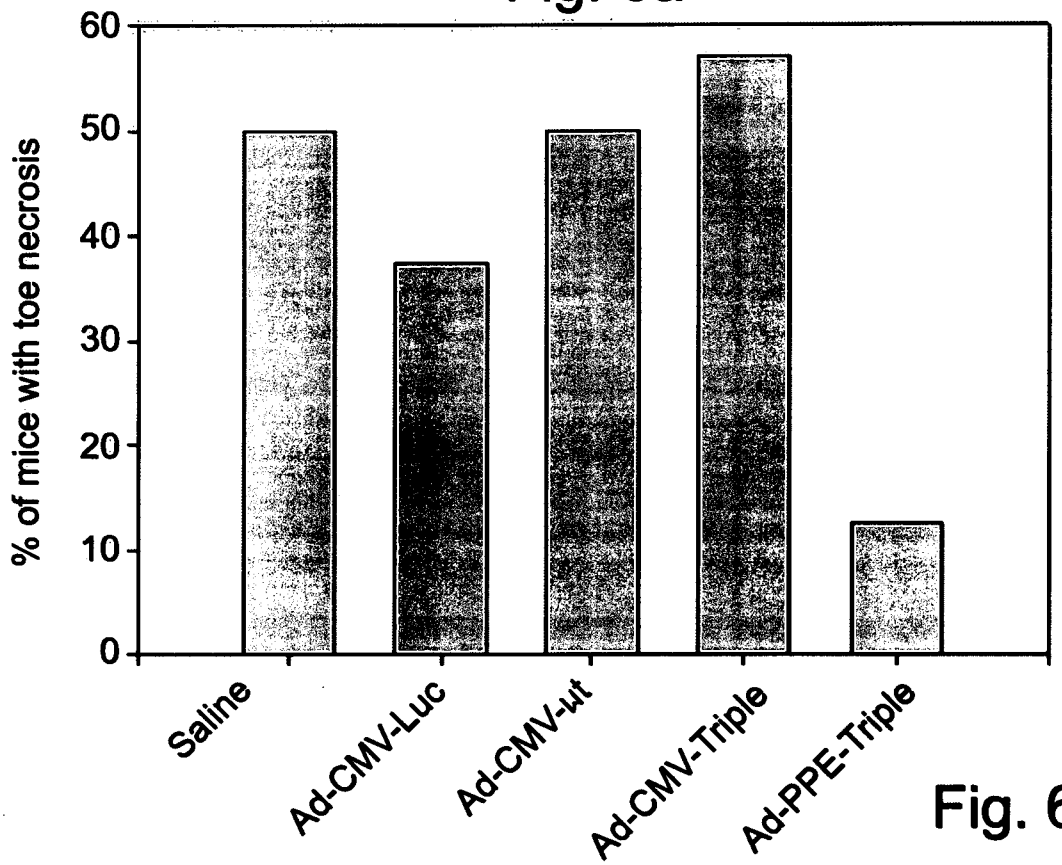

% of mice with toe necrosis
0
10
20
30
40
50
60
Saline
Ad-CMV-Luc
Ad-CMV-vt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 6b

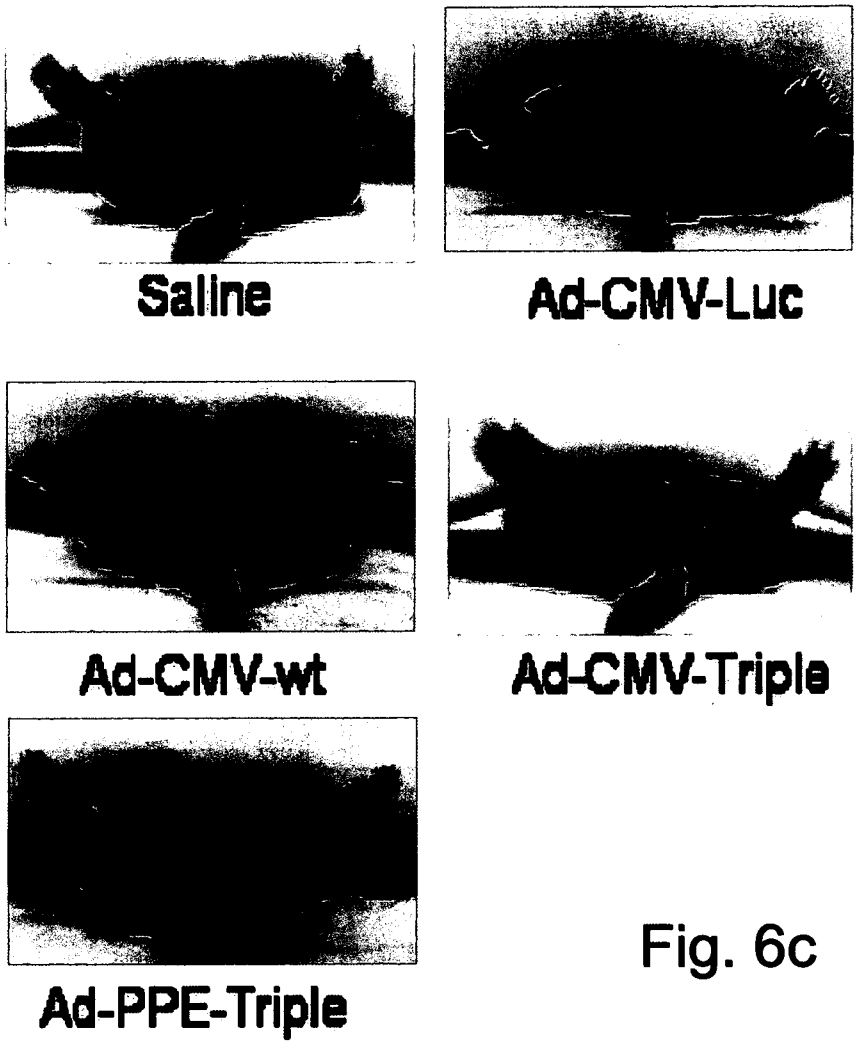

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 6c

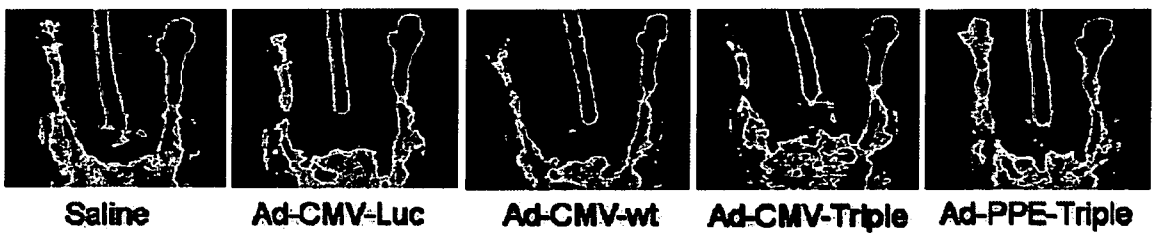

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-CMV-Triple
Ad-PPE-Triple

Fig. 6d

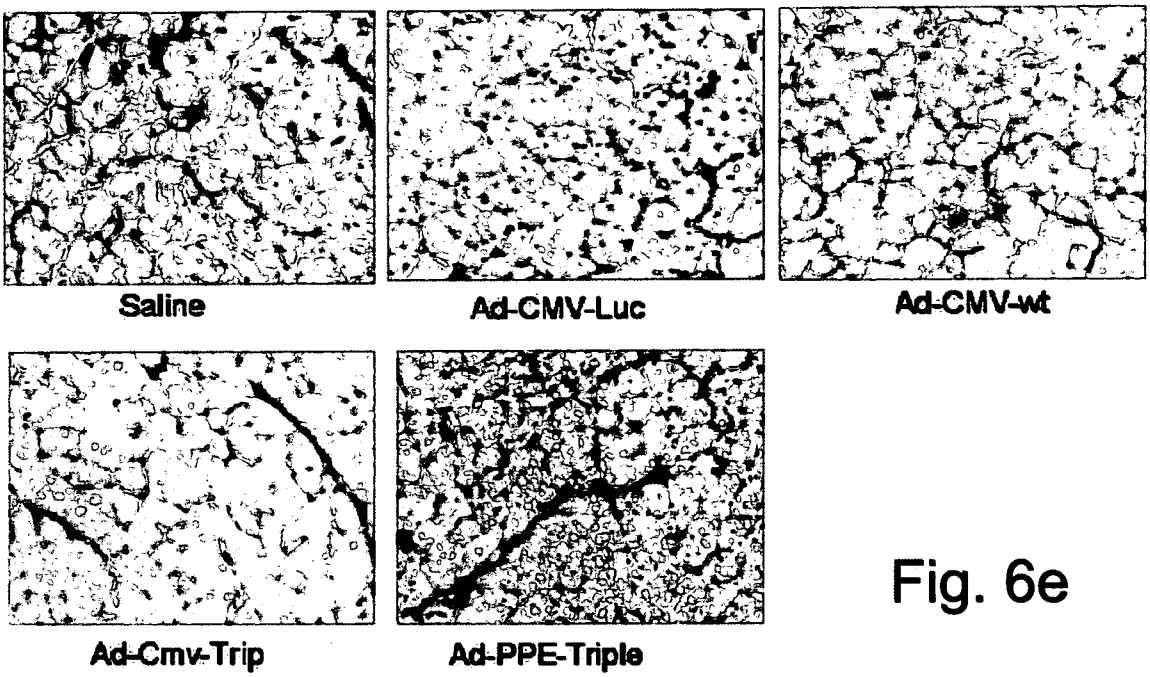

Saline
Ad-CMV-Luc
Ad-CMV-wt
Ad-Cmv-Trip
Ad-PPE-Triple

Fig. 6e

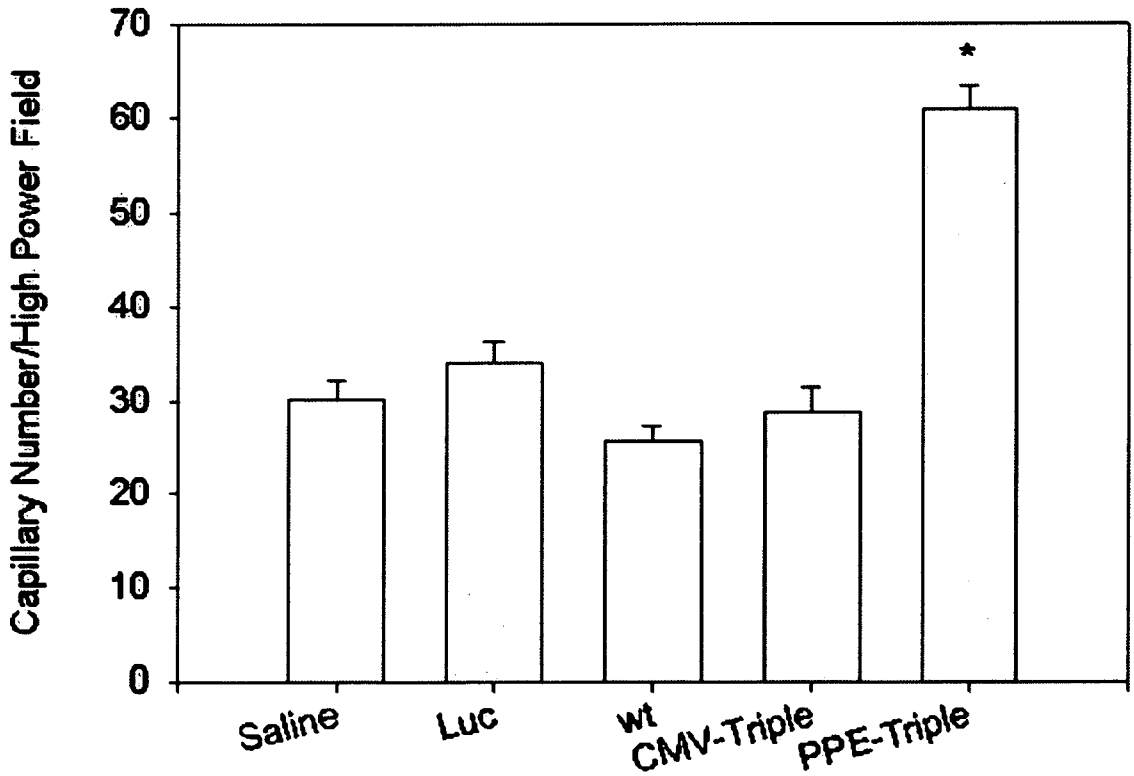

Capillary Number/High Power Field
70
60
50
40
30
20
10
0
*
Saline
Luc
wt
CMV-Triple
PPE-Triple

Fig. 6f

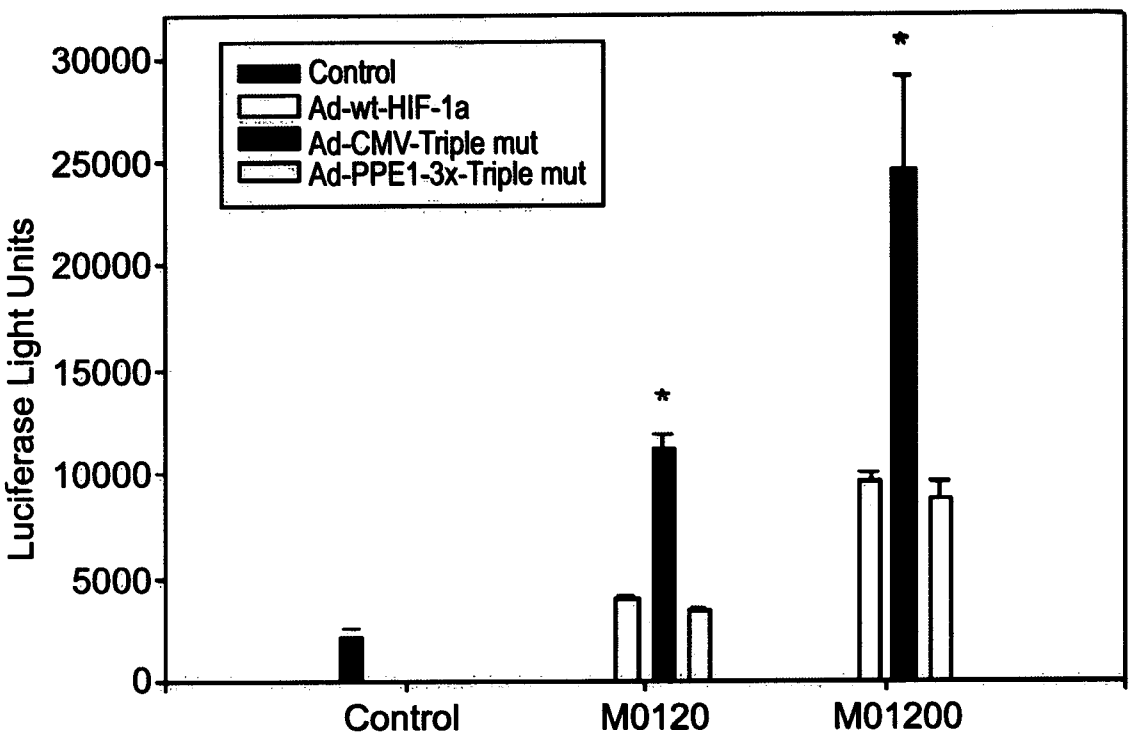

Luciferase Light Units
30000
25000
20000
15000
10000
5000
0
Control
Ad-wt-HIF-1a
Ad-CMV-Triple mut
Ad-PPE1-3x-Triple mut
*
*
Control
M0120
M01200

Fig. 7a

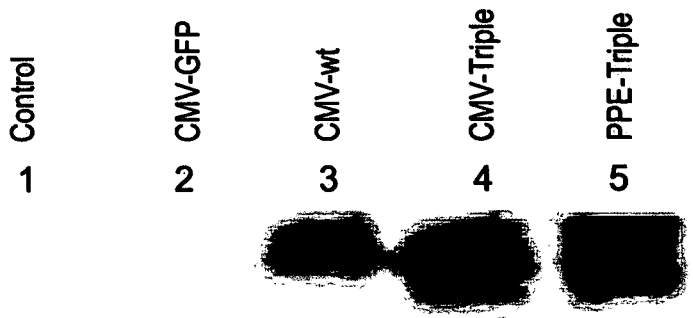
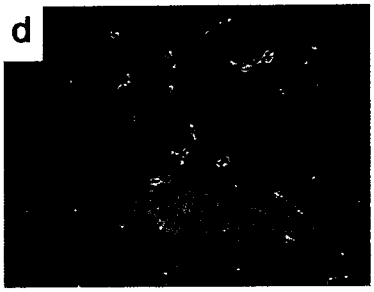

Control
CMV-GFP
CMV-wt
CMV-Triple
PPE-Triple
1
2
3
4
5

Fig. 7b

Fig. 7c

Fig. 7d



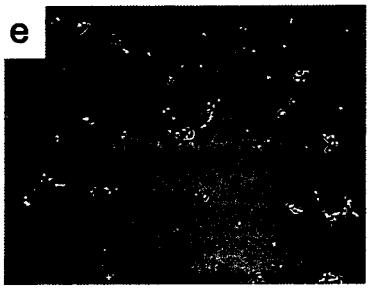


Fig. 7e

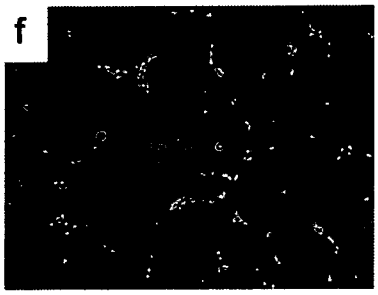


Fig. 7f

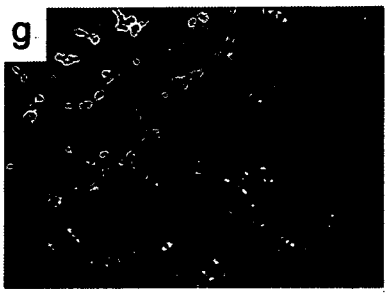


Fig. 7g

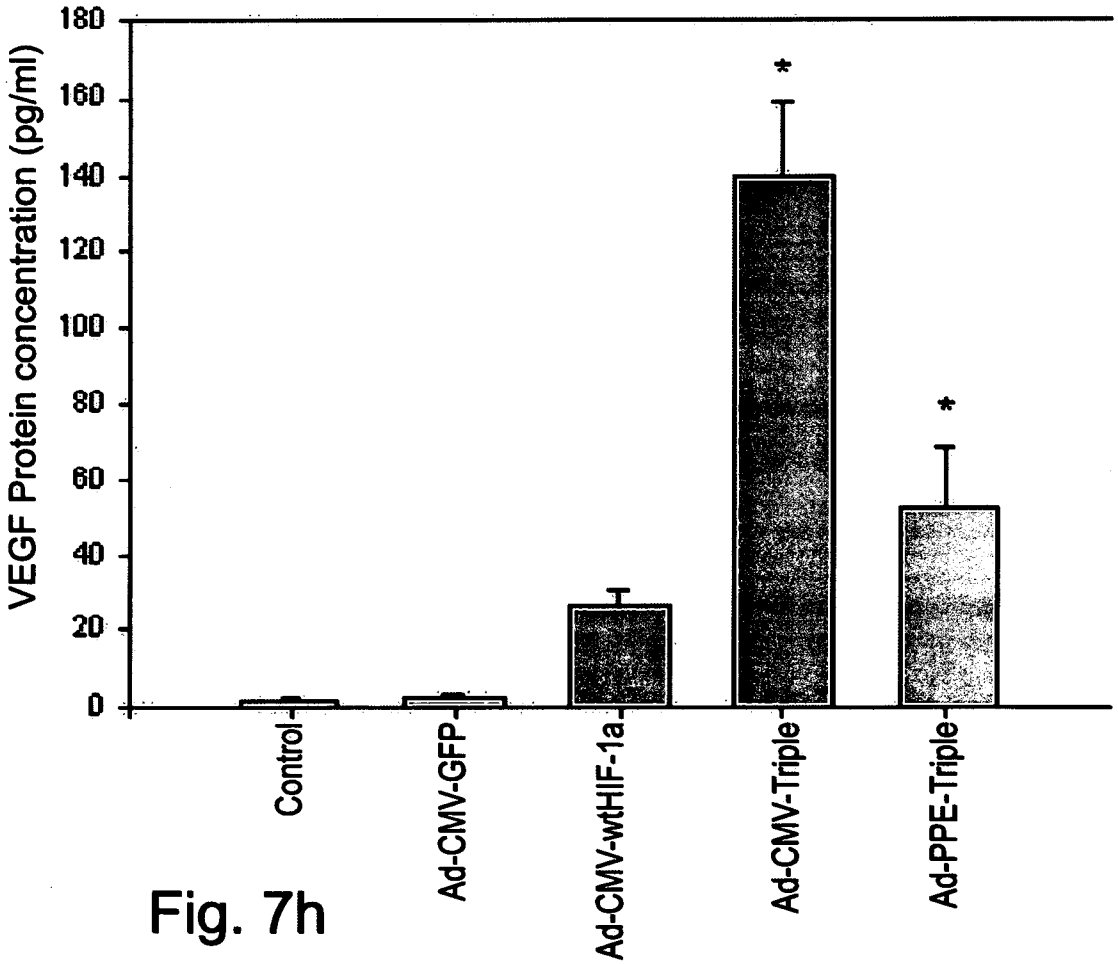


Fig. 7h