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A method for stimulating angiogenesis in a tissue of a mammal comprising contacting tissue with a protein substantially similar to pyruvate kinase M2.

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PROTEIN TO PROMOTE BLOOD VESSEL GROWTH AND USES THEREOF

BACKGROUND

10 The disclosure relates to methods for promoting angiogenesis, including vascularizing an avascular site, or extending or enhancing existing vasculature.

BRIEF DESCRIPTION OF THE DRAWINGS

15 [0001] The detailed description particularly refers to the accompanying figures in which:

 [0002] Fig. 1A shows graphically that endothelial HUVEC cells treated with a pyruvate kinase M2 had increased formation of endothelial tubes, more branch points, and longer tubes compared with the cells treated with controls;

20 [0003] Fig. 1B shows graphically endothelial cells treated with a pyruvate kinase M2 had increased branch points in the tube formation assays compared with cells treated with controls;

 [0004] Fig. 2 shows that the area of wounds treated with a pyruvate kinase M2 protein reduces more effectively and had earlier granulation formation compared with wounds treated
25 with controls;

 [0005] Fig. 3A shows graphically that the wound treated with a pyruvate kinase M2 protein reduces more effectively compared with wounds treated with controls;

30 [0006] Fig. 3B shows graphically (from inside the wound) that the wound areas treated with a pyruvate kinase M2 protein reduces more effectively compared with wounds treated with controls; and

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[0007] Fig. 4 shows that wounds treated with a pyruvate kinase M2 protein have increased angiogenesis compared with wounds treated with controls.

[0008] Fig. 5A shows a representative pictures of the retrieved matrix-gel plaques.

[0009] Fig. 5B shows that the hemoglobin contents in the matrix-gel plaques having
5 PKM2 gel and rPKM1 gel.

[0010] Fig. 6 shows that wounds treated with pyruvate kinase M2 protein show significantly greater healing percentages than those treated with controls.

[0011]

DEFINITIONS

10 [0012] The following definitions are provided to facilitate understanding of certain terms used throughout this specification.

[0013] The term "amino acid" refers to naturally occurring and non-natural amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally encoded amino acids are the 20
15 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrrolysine and selenocysteine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, by way of example only, an alpha.-carbon that
20 is bound to a hydrogen, a carboxyl group, an amino group, and an R group. Such analogs may have modified R groups (by way of example, norleucine) or may have modified peptide backbones, while still retaining the same basic chemical structure as a naturally occurring amino acid. Non-limiting examples of amino acid analogs include homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium.

25 [0014] The term "effective amount," as used herein, refers to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. By way of example, an agent or a compound being administered includes,
30 but is not limited to, a natural amino acid polypeptide, non-natural amino acid polypeptide, modified natural amino acid polypeptide, or modified non-amino acid polypeptide.

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[0015] Compositions containing such natural amino acid polypeptides, non-natural amino acid polypeptides, modified natural amino acid polypeptides, or modified non-natural amino acid polypeptides can be administered for prophylactic, enhancing, and/or therapeutic treatments.

5 **[0016]** An appropriate "effective" amount in any individual case may be determined using techniques, such as a dose escalation study.

[0017] The term "nucleic acid sequence" as used herein, refers to the order and identity of the nucleotides comprising a nucleic acid.

10 **[0018]** Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. The term nucleic acid is used interchangeably with gene, cDNA,
15 mRNA, oligonucleotide, and polynucleotide.

[0019] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and
20 non-naturally occurring amino acid polymer.

[0020] The term "pharmaceutically acceptable", as used herein, refers to a material, including but not limited, to a salt, carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in
25 a deleterious manner with any of the components of the composition in which it is contained.

[0021] The term "prophylactically effective amount," as used herein, refers that amount of a composition containing at least one non-natural amino acid polypeptide or at least one modified non-natural amino acid polypeptide prophylactically applied to a patient which will relieve to some extent one or more of the symptoms of a disease, condition or
30 disorder being treated. In such prophylactic applications, such amounts may depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art

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for one to determine such prophylactically effective amounts by routine experimentation, including, but not limited to, a dose escalation clinical trial.

[0022] The phrase "substantially similar," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 75%, or at least 85%, or at least 90%, 95% or higher or any integral value therebetween nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm such as those described below for example, or by visual inspection. A substantial identity exists over a region of the sequences that is at least about 10, or about 20, or about 40-60 residues in length or any integral value therebetween, preferably over a longer region than 60-80 residues, more preferably at least about 90-100 residues, and most preferably the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a nucleotide sequence for example.

[0023] The term "therapeutically effective amount," as used herein, refers to the amount of a composition containing at least one non-natural amino acid polypeptide and/or at least one modified non-natural amino acid polypeptide administered to a patient already suffering from a disease, condition or disorder, sufficient to cure or at least partially arrest, or relieve to some extent one or more of the symptoms of the disease, disorder or condition being treated. The effectiveness of such compositions depend conditions including, but not limited to, the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician. By way of example only, therapeutically effective amounts may be determined by routine experimentation, including but not limited to a dose escalation clinical trial. A therapeutically effective amount of protein kinase M2 or variant thereof would include an amount that can stimulate angiogenesis.

[0024] The term "subject" as used herein includes mammals and humans.

[0025] The term "effective amount" is meant to include any amount of pyruvate kinase M2 or variant thereof that is sufficient to bring about a desired therapeutic result, especially upon administration to an animal or human subject.

[0026] The term "dosage" as used herein refers to the amount of pyruvate kinase M2 or variant thereof administered to an animal or human. Suitable dosage units for use in the

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methods of the present invention range from mg/kg body weight of the recipient subject to mg/kg. The therapeutic agent may be delivered to the recipient as a bolus or by a sustained (continuous or intermittent) delivery.

5 **[0027]** DETAILED DESCRIPTION

10 **[0028]** The angiogenic activity and/or endothelial cell proliferative or migration potential of a pyruvate kinase M2, or a protein substantially similar to pyruvate kinase, can be assessed by assays and methodology. The pyruvate kinase protein can be any vertebrate or mammalian pyruvate kinase, and may be a native pyruvate kinase, or a recombinant or other synthetic protein. The amino acid sequence for human pyruvate kinase is for instance provided by GenBank Accession No. MP0011193727 pyruvate kinase. The amino acid sequence identity of pyruvate kinase for example is highly conserved between species with human having 98% amino acid sequence identity with mouse, hamster and rat. The amino acid sequence for human pyruvate kinase is disclosed herein. In one specific embodiment, angiogenic active pyruvate kinase protein is a dimer, that is, the subtype M2. A broad range of proteins substantially similar to pyruvate kinase M2 are useful with specific embodiments.

15 **[0029]** An animal from which native pyruvate kinase protein is purified can for instance be a member of the bovine, ovine, porcine, equine, canine, feline, primate, rodent or other mammalian family. In at least some forms, the pyruvate kinase protein will be a human pyruvate kinase protein purified from bacterial production.

20 **[0030]** A recombinant pyruvate kinase protein can have an identical amino acid sequence to the native pyruvate kinase or one or more amino acid differences compared to the native protein. The amino acid changes can comprise the addition, deletion and/or substitution of one or more amino acids. Inversion of amino acids and other mutational changes that result in modification of the native pyruvate kinase protein sequence are also encompassed. Moreover, a recombinant protein can comprise an amino acid or amino acids not encoded by the genetic code.

25 **[0031]** The substitution of an amino acid can be a conservative or non-conservative substitution. The term conservative amino acid substitution is to be taken in the normally accepted sense of replacing an amino acid residue with another amino acid having similar

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properties which substantially does not adversely affect the angiogenic and/or wound healing activity of the pyruvate kinase protein. For example, a conservative amino acid substitution can involve substitution of a basic amino acid such as arginine with another basic amino acid such as lysine. Likewise, for instance a cysteine residue can be replaced with serine, or a non-polar amino acid may be substituted with another non-polar amino acid such as alanine. Amino acids amenable to substitution or deletion in a pyruvate kinase protein amino acid sequence may be determined by comparison of the sequence with closely related pyruvate kinase proteins to identify non-conserved amino acids and by routine trial and experimentation well within the skill of the addressee. A modified recombinant pyruvate kinase protein can be provided by introducing nucleotide change(s) in nucleic acid sequence encoding the native protein such that the desired amino acid changes are achieved upon expression of the nucleic acid in a host cell.

[0032] A recombinant or other synthetic pyruvate kinase protein useful in a method embodied by the invention will have amino acid sequence identity with the native pyruvate kinase of about 60% or greater, and more usually at least about 70%, 80%, 90%, 95%, 98% or greater, or 100%, and all sequence homologies and ranges thereof within those enumerated above are expressly encompassed. Sequence identity between amino acid sequences is determined by comparing amino acids at each position in the sequences when optimally aligned for the purpose of comparison. The sequences are considered identical at a position if the amino acids at that position are the same. A gap, that is a position in an alignment where an amino acid residue is present in one sequence but not the other is regarded as a position with non-identical residues. Alignment of sequences may be performed using any suitable program or algorithm. Computer assisted sequence alignment can be conveniently performed using standard software programs.

[0033] The pyruvate kinase protein can also be chemically synthesized. The provision and use of fusion proteins incorporating a pyruvate kinase protein as described herein is also expressly encompassed by the invention. Nucleic acid encoding a fusion protein can be provided by joining separate DNA fragments encoding the pyruvate kinase protein and, for example, a lipophilic amino acid sequence for enhancing the lipophilic characteristics of the protein by employing blunt-ended termini and oligonucleotide linkers, digestion to provide staggered termini and ligation of cohesive ends as required.

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[0034] Host cells that can be transfected for expression of recombinant pyruvate kinase proteins and fusion proteins as described herein include bacteria such as *E. coli*, *Bacillus* strains (eg., *B. subtilis*), *Streptomyces* and *Pseudomonas* bacterial strains, yeast such as *Sacchromyces* and *Pichia*, insect cells, avian cells and mammalian cells such as Chinese Hamster Ovary cells (CHO), COS, HeLa, HaRas, WI38, SW480, and NIH3T3 cells. The host cells are cultured in a suitable culture medium under conditions for expression of the introduced nucleic acid (typically in an appropriate expression vector) prior to purification of the expressed product from the host cells, and/or supernatants as required using standard purification techniques.

[0035] Pyruvate kinase proteins as described herein can also be modified by coupling one or more proteinaceous or non-proteinaceous moieties to the protein to improve solubility, lipophilic characteristics, stability, biological half-life, or for instance to act as a label for subsequent detection or the like. Modifications can also result from post-translational or post-synthesis modification such as by the attachment of carbohydrate moieties, or chemical reaction(s) resulting in structural modification(s) (e.g., the alkylation or acetylation of one or more amino acid residues or other changes involving the formation of chemical bonds). For example, the pyruvate kinase protein can have one or more modifications selected from the group consisting of methylation, phosphorylation, oxidation of tyrosine and/or tryptophan residues, glycosylation, and S-methylcysteine covalent attachment.

[0036] The pyruvate kinase protein can be of a size with a range of deviating from the complete protein. However, the pyruvate kinase should have a length sufficient to enable a dimer formation.

[0037] C-terminal and N-terminal extensions of native pyruvate kinase proteins are involved in stabilization of quaternary structure and the generation of aggregates of the protein. Thus, pyruvate kinases lacking such C-terminal and N-terminal extensions forms aggregates poorly. Pyruvate kinases form large aggregates. Electrostatic interactions between pyruvate kinase proteins are also involved in pyruvate kinase aggregate formation, and ionization of histidine residues below a pH of 7 can disrupt the aggregates. Typically, the pyruvate kinase protein used in a method embodied by the invention will be in dimeric form.

[0038] The intact and truncated forms of a pyruvate kinase protein useful in embodiments of the invention may be subjected to post translational modifications not

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limited to acetylation, methylation, ethylation, phosphorylation, oxidation and glycosylation modifications in the native pyruvate kinase protein. Suitable conditions for alkaline phosphatase activity include suitable zinc, magnesium or calcium containing buffers.

[0039] Partially hydrolysed forms of pyruvate kinase proteins can be purified for use in embodiments of the invention by any suitable purification technique including, e.g., filtration and chromatography protocols.

[0040] Tissue sites and wounds that may be treated in accordance with the invention include acute and chronic wounds, burns including burns arising from exposure to ionizing radiation, chemical wounds, surgical wounds, oral wounds, skin and muscle trauma, open skin wounds, diabetic skin sores including diabetic foot ulcers, diabetic neuropathic foot ulcers, ischemic tissue including ischemic neuropathic foot ulcers, venous stasis ulcers, pressure sores, and hypoxic tissue. Examples of ischemic and hypoxic tissues include ischemic heart tissue and hypoxic tissues associated with stroke. Conditions in which the wound healing process may be promoted by the administration of the pyruvate kinase protein include in circumstances of delayed wound healing in which healing is impaired or prevented by for example, tissue hypoxia, repeated trauma, or systemic causes such as diabetes and vascular disease. Examples of endothelial cell types that may be induced to proliferate and/or migrate by pyruvate kinase proteins in accordance with the invention include human umbilical vein endothelial cells, human microvascular endothelial cells, and aorta endothelial cells.

[0041] The pyruvate kinase protein can be administered to a subject so to stabilize stem cells and tissue cultures within the subject. By administering the protein, the culture or cells may undergo angiogenesis.

[0042] In one specific embodiment, stem cells can be treated with a pyruvate kinase protein so to facilitate blood vessel to the transplanted stem cells, so to promote survival of the stem cells. The source of the stem cells may be adipose stromal cells, embryonic stem cells, and/or stem cells derived from bone marrow aspirate. The stem cells can be mammalian stem cells, and in various embodiments, are human stem cells. Stem cells can be applied to the site just prior to the administration of the pyruvate kinase protein, and can be mixed with stem cells for co-transplantation.

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[0043] In another embodiment, tissue transplantation may be facilitated by the treatment of the transplanted tissue with protein substantially similar to pyruvate kinase or pyruvate kinase. The transplanted tissue may be treated with pyruvate kinase protein. A scaffold may be included to contain, support, or retain the pyruvate kinase protein, along with other materials, at the tissue defect site. In one example, a scaffold may contain or support stem cells to enabling growth and/or retention of the stem cells at the site of implantation. In addition, the scaffold may facilitate migration of endogenous cells into the administration site. The scaffold may be implanted or applied at the tissue defect site, followed by the administration of the pyruvate kinase protein. One example includes a method for facilitating tissue transplantation and cell transplantation contacting a site of transplantation with a protein substantially similar to pyruvate kinase M2. In some examples, the cells to be transplanted can be mixed with a protein substantially similar to pyruvate kinase.

[0044] The pyruvate kinase protein can be administered to a subject in need of such treatment alone or be co-administered with one or more other therapeutic agents. For example, the pyruvate kinase protein can be co-administered in combination with therapeutic agents conventionally used for promoting angiogenesis and/or wound healing. By "co-administered" is meant simultaneous administration in the same formulation or in two different formulations by the same or different routes, or sequential administration by the same or different routes, whereby the pyruvate kinase protein and other therapeutic agent(s) exhibit overlapping therapeutic windows. By "sequential" administration is meant one is administered after the other. Such further agents that may be co-administered with the pyruvate kinase protein include platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), platelet-derived wound healing factor, insulin growth factor (IGF), keratinocyte growth factor (KGF), anti-inflammatory agents and anti-microbial agents. Further examples of other therapeutic agents used for promoting angiogenesis and/or wound healing that may be co-administered with the pyruvate kinase protein include indoleamine 2,3-dioxygenase (IDO), tryptophan dioxygenase (TDO), sphingosine-1-phosphate (SIP), N-acylethanolamines, grapefruit extract and other plant phytochemicals including ascein, green tea catechins, melatonin, arginine and other amino acids for support of vessel growth.

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[0045] The pyruvate kinase protein will generally be formulated into a pharmaceutical composition comprising the protein and a pharmaceutically acceptable carrier. Suitable pharmaceutical compositions include topically acceptable formulations such as creams, lotions, ointments and gels, and biodegradable gel or matrix for internal or external application. Topically acceptable compositions can be applied directly to the site of treatment including by way of dressings and the like impregnated with the preparation. Gels comprising a pyruvate kinase protein precipitate are particularly suitable. Gels and other pharmaceutical compositions as described herein may also include calcium ions and/or salts. The presence of calcium in the composition can enhance wound healing.

[0046] A pharmaceutical composition as described herein can also incorporate one or more preservatives such as parabens, chlorobutanol, and sorbic acid, binders such as corn starch or gelatin, thickening agents, emulsifiers, surfactants, gelling agents, and other components typically used in such compositions. Pharmaceutically acceptable carriers include any suitable conventionally known topically and physiologically acceptable solvents, dispersion media, isotonic preparations and solutions. Use of such ingredients and media for pharmaceutically active substances is well known. Except insofar as any conventional media or agent is incompatible with the pyruvate kinase protein, use thereof is expressly encompassed.

[0047] Pharmaceutical compositions embodied by the invention include therapeutic compositions for human or veterinary use. Typically, the pharmaceutical composition will have a pH suitable for application of the composition directly to a wound. Generally, the pH will be above 5 and usually, about 7 or higher. Pyruvate kinase protein precipitate gels as described herein can be precipitated from a solution containing the protein and a physiologically acceptable buffer system such that the precipitate has the desired pH. Alternatively, the pH of the gel can be altered to the desired pH by the addition of any suitable pH modifier(s) to the gel.

[0048] A pharmaceutical composition embodied by the invention will generally contain at least about 0.001% by weight of the pyruvate kinase protein up to about 80% w/w of the composition. For example, the pharmaceutical composition can contain about 0.05%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80% by weight of the pyruvate kinase protein or substantially similar protein. The amount of the protein in the composition

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will be such that a suitable effective dosage will be delivered to the subject taking into account the proposed mode of administration.

[0049] The dosage of the pyruvate kinase protein administered in accordance with an embodiment of the invention will depend on a number of factors including whether the protein is to be administered for prophylactic or therapeutic use, the disease or condition for which the protein is intended to be administered, the severity of the condition, the sex and age of the subject, and related factors including weight and general health of the subject, and can be determined in accordance with accepted medical principles. For instance, a low dosage can initially be given which is subsequently increased at each administration following evaluation of the subject's response. Similarly, the frequency of administration can be determined in the same way that is, by continuously monitoring the subject's response between each dosage and if desirable, increasing the frequency of administration or alternatively, reducing the frequency of administration.

[0050] Typically, the pyruvate kinase protein will be administered in accordance with a method embodied by this disclosure. For topical application, the pyruvate kinase protein will be administered to a tissue or wound site by covering the site with the pharmaceutical composition containing 0.5%–0.01% by weight PKM2

[0051] Routes of administration include but are not limited to topically, respiratorially, intravenously, orally, intraperitoneally, subcutaneously, intramuscularly, rectally, topically and by implant. With respect to intravenous routes, particularly suitable routes are via injection into blood vessels which supply the target tissue to be treated. The pyruvate kinase protein can also be delivered into cavities such for example the pleural or peritoneal cavity, cranial or be injected directly into the tissues to be treated. For oral administration, the pyruvate kinase protein can be encapsulated or otherwise provided in an enteric for passage through the stomach and release in the small intestine. Any suitable such enteric formulation or coating can be utilized.

[0052] Moreover, a pyruvate kinase protein can also be coated onto the surface of a stent or balloon of a catheter such as an angioplasty catheter, or other surgical instrument for application to the interior wall of a blood vessel during angioplasty or other surgical procedure. The pyruvate kinase can for instance be applied to the wall of the blood vessel in

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this manner in the form of a gel or any other appropriate formulation to promote wound healing and/or angiogenesis or epithelial cell migration to the site of treatment.

[0053] Suitable pharmaceutically acceptable carriers and formulations useful in compositions embodied by the invention can for instance be found in handbooks and texts.

5 [0054] Examples

[0055] Example 1

[0056] The following is an exemplary pyruvate kinase M2 sequence.

PKM2 Accession No. NP 002645

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1  mskphseagt afiqtqqlha amadtflehm crldidsppi tarntgiict igpasrsvet
61 lkemiksgmn varlnfshgt heyhaetikn vrtatesfas dpilyrpvav aldtkgpeir
121 tgliksgta evelkkgatl kitldnayme kcdenilwld yknickvvev gskiyvddgl
181 islqvkkqga dflvteveng gslgskkgvn lpgaavdlpa vsekdiqdlk fgveqdvdmv
15 241 fasfirkasd vhevrkvlge kgknikiisk ienhegvrrf deileasdgi mvargdlgie
301 ipaekvflaq kmmigr-cnra gkpvicatqm lesmikkprp traegsdvan avldgadcim
361 lsgetakgdy pleavrmqhl iareaeaaay hlqlfeelrr lapitsdpte atavgaveas
421 fkccsgaiiv ltksgrsahq varyrprapi iavtrnpqta rqahlyrgif pvlckdpvqe
481 awaedvdrlv nfamnv-gkar gffkkgdvvi vltgwrpgsg ftntmrvvvpv p

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[0057] Example 2

[0058] *In vitro* tests supported that PKM2 promoted angiogenesis. PKM2 expressed
25 as recombinant PKM2 (ref to as rPKM2) and its isoenzyme PKM1 (ref to as rPKM1) as a control. The rPKM2, the rPKM2+ fructose bisphosphate (FBP), the rPKM1, and a buffered solution alone were added to the culture medium of the cells. Formation of the endothelial tubes was analyzed. As shown in Fig. 1A and Fig. 1B, rPKM2 strongly promoted
30 endothelial tube formation both in tube density and the sprouts of the formed tubes. The time required for formation of the tubes was also substantially shortened, and the formed tubes were maintained much longer time. The rPKM2 + FBP had less effects compared to that of the rPKM2. The rPKM1 had only marginal effects, while buffered saline had no effects.

[0059] Example 3

[0060] *In vivo* tests supported that PKM2 promoted wound healing by facilitating
35 angiogenesis. A wound was induced in mice. A cream without PKM2 was administered on the wound to a sample of the mice, and a cream with PKM2 was administered on the wound to another sample of the mice. The wounds were then covered and dressed. The topical

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applications of the agents were applied again three days and six days after wound inductions. The experiments were stopped at nine days. After 3 days, there was a difference in wound healing and closure. Figures 2 through 4 show this data.

[0061] Example 4

- 5 **[0062]** The following are the amino acid sequences of modified pyruvate kinase proteins in which at least arginine has been substituted with a glutamate.

R399E Mutant

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1   mskphseagt afigtqqqlha amadtflehm crldidsppi tarntgiict igpasrsvet
10  61 lkemiksgmn varlnfshgt heyhaetikn vrtatesfas dpilyrpvav aldtkgpeir
    121 tglksgsgta evelkkgatl kitldnayme kcdenilwld yknickvvev gskiyvddgl
    181 islqvkkqka dflvtevang gslgskkgvn lpgaavdlpa vsekdiqdlk fgveqdvdmv
    241 fasfirkasd vhevrkvlge kgknikiisk ienhegvrrf deileasdgi mvargdlgie
    301 ipaekvflaq kmmigrcnra gkpvicatqm lesmikkprp traegsdvan avldgadcim
15  361 lsgetakgdy pleavrmqhl iareaeaaay hlqlfeeler lapitsdpte atavgaveas
    421 fkccsgaiiv ltksgrsahq varyrprapi iavtrnpqta rqahlyrgif pvlckdpvqe
    481 awaedvdrlv nfamnvskar gffkkgdvvi vltgwrpgsg ftntmrsvpv p

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Y105E Mutant

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20  1   mskphseagt afigtqqqlha amadtflehm crldidsppi tarntgiict igpasrsvet
    61 lkemiksgmn varlnfshgt heyhaetikn vrtatesfas dpilyrpvav aldtkgpeir
    121 tglksgsgta evelkkgatl kitldnayme kcdenilwld yknickvvev gskiyvddgl
    181 islqvkkqka dflvtevang gslgskkgvn lpgaavdlpa vsekdiqdlk fgveqdvdmv
    241 fasfirkasd vhevrkvlge kgknikiisk ienhegvrrf deileasdgi mvargdlgie
25  301 ipaekvflaq kmmigrcnra gkpvicatqm lesmikkprp traegsdvan avldgadcim
    361 lsgetakgdy pleavrmqhl iareaeaaay hlqlfeelrr lapitsdpte atavgaveas
    421 fkccsgaiiv ltksgrsahq varyrprapi iavtrnpqta rqahlyrgif pvlckdpvqe
    481 awaedvdrlv nfamnvskar gffkkgdvvi vltgwrpgsg ftntmrsvpv p

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- 30 **[0063]** Example 5

[0064] A gel plaque assay, a matrix-gel having rPKM2 (3 mM) or rPKM1 (3 mM), respectively, was implanted into the right flank of Balb/c mice. The matrix-gel was pre-mixed with other materials suggested by vendor before addition of rPKM1 and rPKM2 gels. After seven days, the matrix-gel plaques were retrieved from mouse. Fig. 5A shows a

35 representative pictures of the retrieved matrix-gel plaques. Fig. 5B shows that the hemoglobin contents in the matrix-gel plaques having PKM2 gel was larger relative to the hemoglobin content in the matrix-gel plaques having rPKM1 gel. The assay indicates the activity of rPKM2 in promoting angiogenesis.

[0065] Example 6

-14-

In vivo tests supported that PKM2 promoted wound healing by facilitating angiogenesis with diabetic db/db mice. A wound was induced in mice. A cream without PKM2 was administered on the wound to a group of the mice, and a cream with PKM2 was administered on the wound to another sample of the mice. The wounds were then covered and dressed.

- 5 FIG. 6 shows a wound that was induced in diabetic db/db mice (13 weeks old, six mice per group). A cream without PKM2 was topically administered on the wound, and a cream with PKM2/Buffer (0.004% w/w) was topically administered on the wound, and a cream with PKM1 (0.004% w/w)(Blue) was topically administered on the wound. The degree or percentage of healing was plainly higher in mice where PKM2 was applied thereto. The
- 10 percent (%) of healing was determined by measuring the initial wound areas and then subtracting measured wound areas at a given day/initial wound areas. The error bars are the standard deviations among the five mice.

CLAIMS

What is claimed is:

1. Use of a protein substantially similar to pyruvate kinase M2 to stimulate angiogenesis in a group of cells, tissue or organism, wherein the protein shares at least 60% amino acid sequence identity with pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
2. Use of a protein substantially similar to pyruvate kinase M2 to promote endothelial cell migration in mammalian tissue, wherein the protein shares at least 60% amino acid sequence identity with pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
3. Use of a protein substantially similar to pyruvate kinase M2 to facilitate wound healing, tissue transplantation, tissue engineering and cell transplantation, wherein the protein shares at least 60% amino acid sequence identity pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
4. Use of a pyruvate kinase M2 to stimulate angiogenesis in a group of cells, tissue or organism, wherein the protein is a dimer.
5. Use of a pyruvate kinase M2 to promote endothelial cell migration in mammalian tissue, wherein the pyruvate kinase M2 is a dimer.

6. Use of a pyruvate kinase M2 to facilitate wound healing, tissue transplantation, tissue engineering and cell transplantation, wherein the pyruvate kinase M2 is a dimer.
7. The use of any of claims 1, 2 and 3 wherein the protein substantially similar to pyruvate kinase M2 is recombinant pyruvate kinase M2.
8. The use of any of claims 1, 2, 3 and 7 wherein the protein shares at least 60% amino acid sequence identity with human pyruvate kinase M2 and exhibits angiogenic activity.
9. The use of any one of claims 1, 2, 3, 7 and 8 wherein the protein is incorporated into a pharmaceutically acceptable carrier.
10. The use of claim 9 wherein the protein and pharmaceutically acceptable carrier are formulated into any one of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.
11. The use of any of claims 4, 5 and 6 wherein the pyruvate kinase M2 is recombinant pyruvate kinase M2.
12. The use of any of claims 4, 5, 6 and 11 wherein the pyruvate kinase M2 is human pyruvate kinase M2.
13. The use of any one of claims 4, 5, 6, 11 and 12 wherein the pyruvate kinase M2 is incorporated into a pharmaceutically acceptable carrier.

14. The use of claim 13 wherein the pyruvate kinase M2 and pharmaceutically acceptable carrier are formulated into any one of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.
15. The use of any of claims 1 and 4 wherein the organism has an angiogenesis-related disorder.
16. The use of claim 15 wherein the angiogenesis-related disorder is one of macular degeneration, corneal ulceration, stroke, ischemic heart disease, infertility, ulcers, scleroderma, ischemia, myocardial infarction, myocardosis, angina pectoris and unstable angina.
17. The use of claim 3 wherein the protein substantially similar to pyruvate kinase M2 is mixed with stem cells.
18. The use of claim 6 wherein the pyruvate kinase M2 is mixed with stem cells.
19. A pharmaceutical composition for use in stimulating angiogenesis, comprising pyruvate kinase M2 and a pharmaceutically acceptable carrier, wherein the pyruvate kinase M2 is a dimer.
20. The pharmaceutical composition of claim 19, wherein the concentration of pyruvate kinase M2 is greater than 3 μ M.

21. The pharmaceutical composition of claim 19, wherein the pyruvate kinase M2 is recombinant pyruvate kinase M2.
22. The pharmaceutical composition of any of claims 19 to 21, wherein the pyruvate kinase M2 is human pyruvate kinase M2.
23. The pharmaceutical composition of claim 19 wherein the pyruvate kinase M2 is mixed with stem cells.
24. The pharmaceutical composition of any of claims 19 to 23, wherein the pyruvate kinase M2 and pharmaceutically acceptable carrier are formulated into any of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.
25. A pharmaceutical composition for use in stimulating angiogenesis, comprising a protein substantially similar to pyruvate kinase M2 and a pharmaceutically acceptable carrier, wherein the protein shares at least 60 % amino acid sequence identity with pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
26. The pharmaceutical composition of claim 25, wherein the concentration of the protein is greater than 3 μ M.
27. The pharmaceutical composition of claim 25, wherein the protein is recombinant pyruvate kinase M2.

28. The pharmaceutical composition of claim 25, wherein protein is substantially similar to human pyruvate kinase M2.
29. The pharmaceutical composition of claim 25 wherein the protein is mixed with stem cells.
30. The pharmaceutical composition of any of claims 25 to 29, wherein the protein and pharmaceutically acceptable carrier are formulated into any of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.
31. Use of a protein substantially similar to pyruvate kinase M2 for preparing a medicament to stimulate angiogenesis in a group of cells, tissue or organism, wherein the protein shares at least 60% amino acid sequence identity with pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
32. Use of a protein substantially similar to pyruvate kinase M2 for preparing a medicament to promote endothelial cell migration in mammalian tissue, wherein the protein shares at least 60% amino acid sequence identity pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.
33. Use of a protein substantially similar to pyruvate kinase M2 for preparing a medicament to facilitate wound healing, tissue transplantation, tissue engineering and cell transplantation, wherein the protein shares at least 60% amino acid sequence identity with pyruvate kinase M2 and exhibits angiogenic activity, and wherein the protein is a dimer.

34. Use of a pyruvate kinase M2 for preparing a medicament to stimulate angiogenesis in a group of cells, tissue or organism, wherein the pyruvate kinase M2 is a dimer.
35. Use of a pyruvate kinase M2 for preparing a medicament to promote endothelial cell migration in mammalian tissue, wherein the pyruvate kinase M2 is a dimer.
36. Use of a pyruvate kinase M2 for preparing a medicament to facilitate wound healing, tissue transplantation, tissue engineering and cell transplantation, wherein the pyruvate kinase M2 is a dimer.
37. The use of any of claims 31 to 33 wherein the protein substantially similar to pyruvate kinase M2 is recombinant pyruvate kinase M2.
38. The use of any of claims 31 to 33 and 37 wherein the protein shares at least 60% amino acid sequence identity with human pyruvate kinase M2 and exhibits angiogenic activity.
39. The use of any one of claims 31 to 33, 37 and 38 wherein the protein is incorporated into a pharmaceutically acceptable carrier.
40. The use of claim 39 wherein the protein and pharmaceutically acceptable carrier are formulated into any one of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.

41. The use of any of claims 34 to 36 wherein the pyruvate kinase M2 is recombinant pyruvate kinase M2.
42. The use of any of claims 34 to 36 and 41 wherein the pyruvate kinase M2 is human pyruvate kinase M2.
43. The use of any one of claims 34 to 36, 41 and 42 wherein the pyruvate kinase M2 is incorporated into a pharmaceutically acceptable carrier.
44. The use of claim 43 wherein the pyruvate kinase M2 and pharmaceutically acceptable carrier are formulated into any one of a cream, lotion, ointment, gel, biodegradable gel and biodegradable matrix.
45. The use of any of claims 31 and 34 wherein the organism has an angiogenesis-related disorder.
46. The use of claim 45 wherein the angiogenesis-related disorder is one of macular degeneration, corneal ulceration, stroke, ischemic heart disease, infertility, ulcers, scleroderma, ischemia, myocardial infarction, myocardosis, angina pectoris and unstable angina.
47. The use of claim 33 wherein the protein substantially similar to pyruvate kinase M2 is mixed with stem cells.
48. The use of claim 36 wherein the pyruvate kinase M2 is mixed with stem cells.

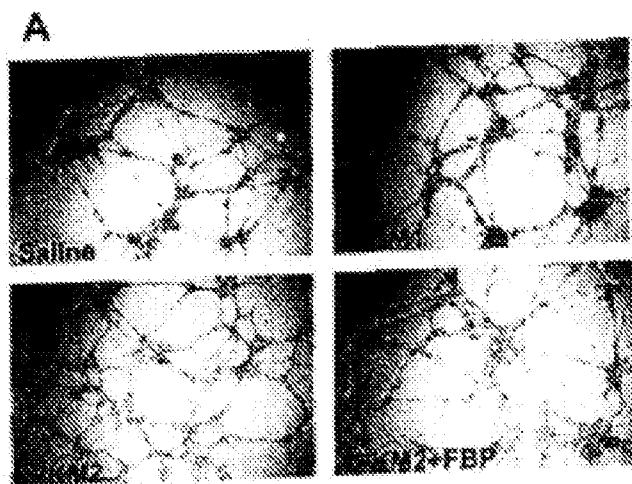


FIG. 1A

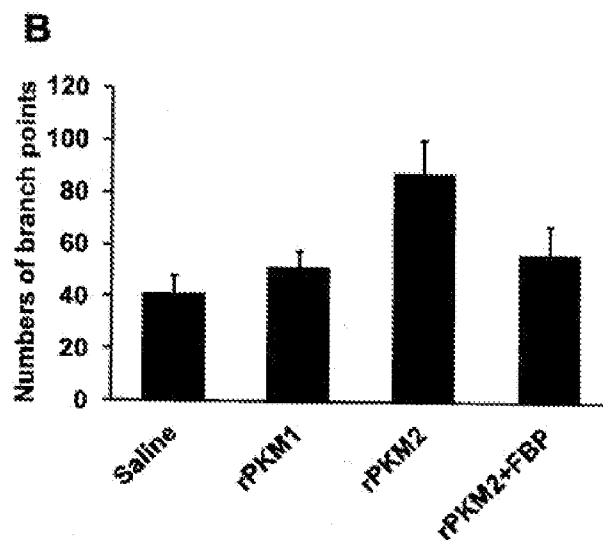


FIG. 1B

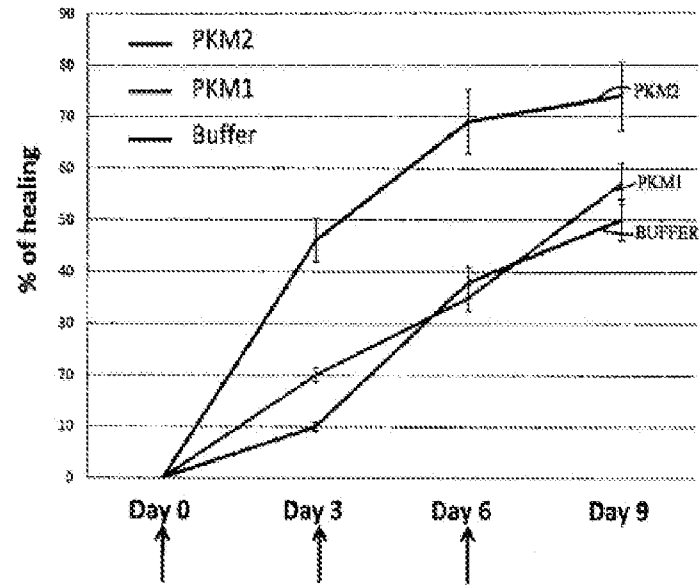


Fig. 2

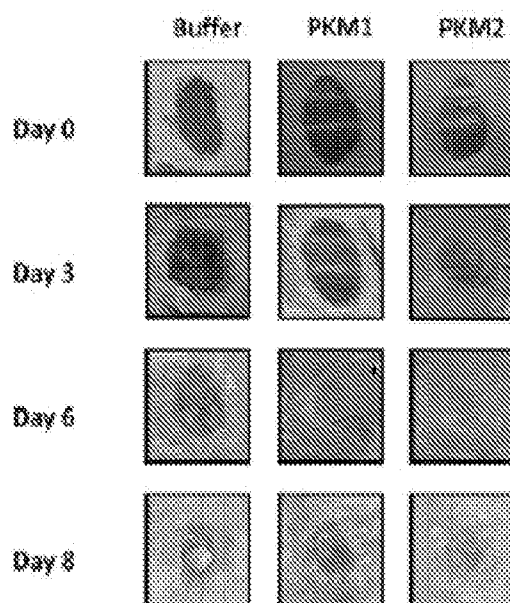


Fig. 3A

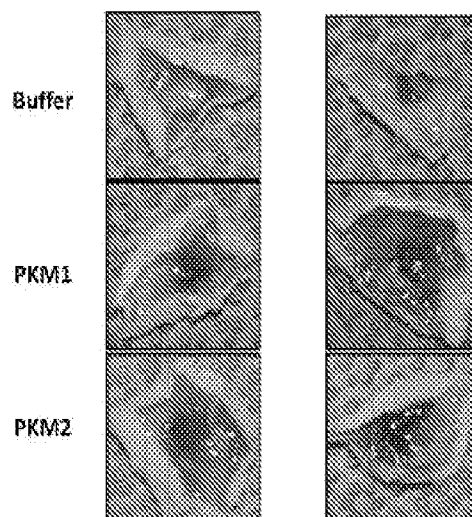
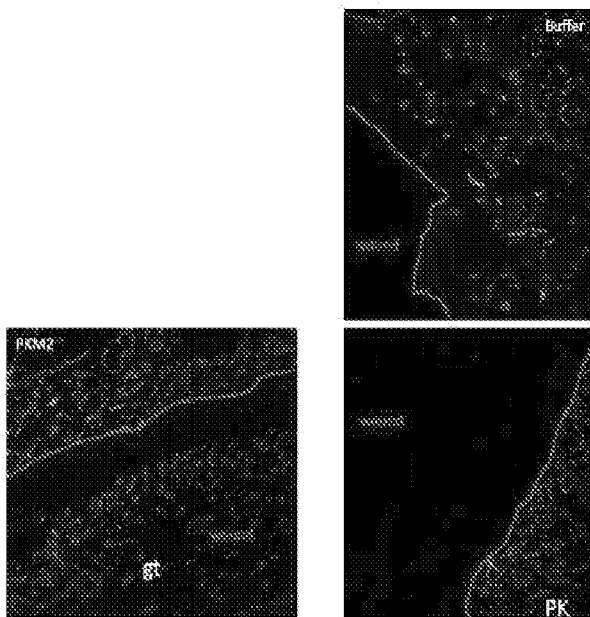


Fig. 3B

FIG. 4



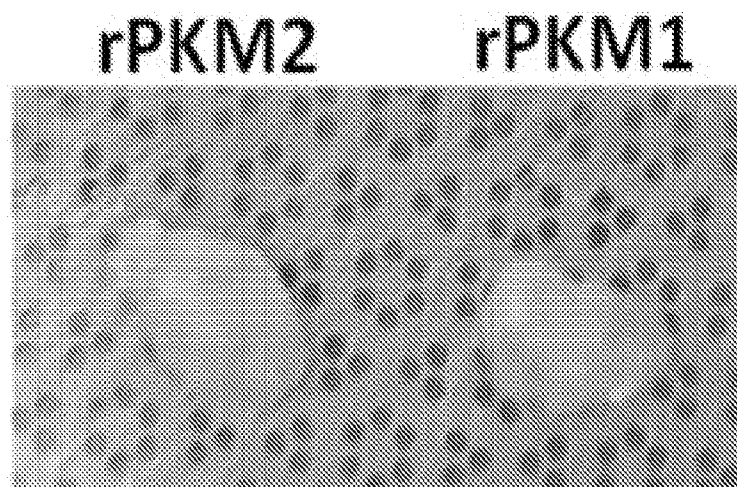


FIG. 5A

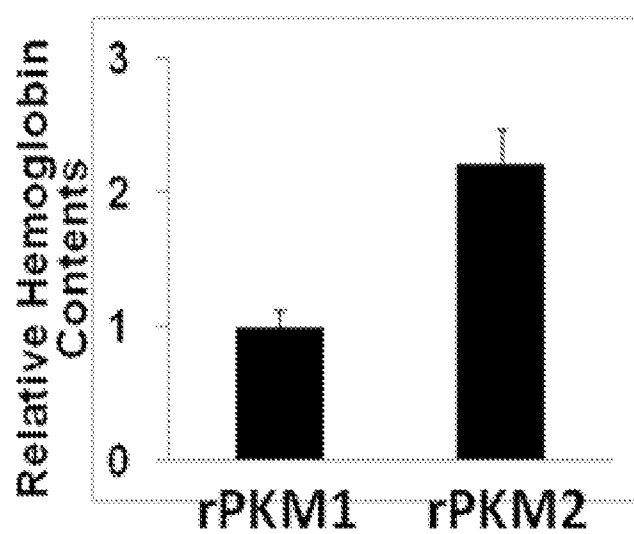


FIG. 5B

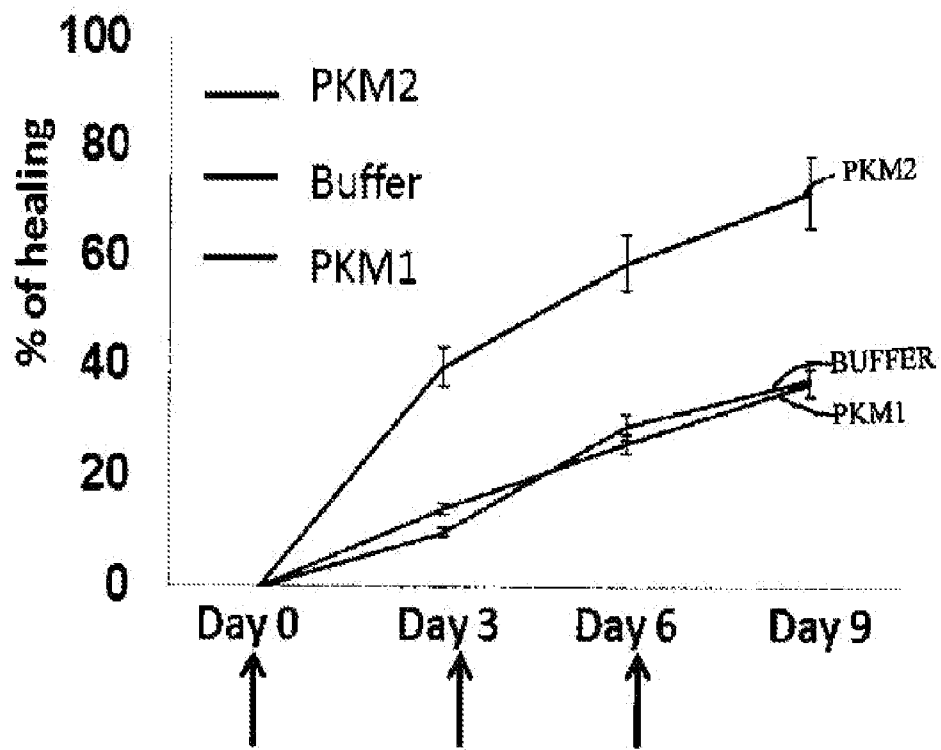


FIG. 6