

Angiopoietin-Like 4 And A Method Of Its Use In Wound Healing

Cross-Reference to Related Application

[0001] This application claims the benefit of priority of United States of America Provisional Patent Application No. 61/673,463 filed July 19, 2012, the contents of which being hereby incorporated by reference in its entirety for all purposes.

Field

[0002] The invention relates to compositions for use in wound healing and methods of wound healing treatment.

Background

[0003] Type II diabetes is a medical menace that affects ~200 million people and continues to be an increasing burden on healthcare resources worldwide with its morbidities of retinopathy, cardiovascular diseases and diabetic nephropathy ¹. Impaired wound repair represents one of the most significant unmet medical needs in the world today and is a major complication of diabetes, resulting in significant morbidity, lost productivity, and healthcare expenditures ². Furthermore, poor healing diabetic wound is an open portal for infections, often resulting in chronic inflammation, sepsis, dehiscence and death. Despite the enormous impact these chronic wounds have, effective therapies have been lacking. To effectively manage these problems one must understand the healing process and to create a salubrious physical and biochemical environment conducive for healing.

[0004] Normal wound healing proceeds via a continuum of events that includes the acute inflammatory, proliferative and maturation phases ^{3,4}. These events entail a complex interplay between connective tissue formation, cellular activity, and growth factor

activation. All three of these physiologic processes is altered in the diabetic state ^{5,6}.

Extracellular matrix (ECM) components are integral to each phase of wound healing, interacting with cells and growth factors in a dynamic reciprocal manner that eventually results in wound closure.

[0005] Chronic wounds, such as venous, diabetic or pressure ulcers, represent one of the most significant unmet medical needs in the world today and are a major complication of diabetes, resulting in significant morbidity, lost productivity, and healthcare expenditures. Diabetic foot ulceration is a significant cause of morbidity and is the most common reason for hospital admission in diabetic patients. Approximately 15 % of diabetic patients will develop chronic ulcers during their lifetimes. In those who require lower-limb amputation, 70-90 % will be preceded by foot ulceration².

[0006] Diabetic wounds are characterized by an accumulation of devitalized tissue, increased/prolonged inflammation, poor wound-related angiogenesis and deficiencies in the ECM components ^{6,7}. Diabetic wounds show elevated levels of matrix metalloproteinases (MMPs), increased proteolytic degradation of ECM components, inactivation of growth factors that culminate in a corrupt ECM that cannot support healing ^{5,8}. Abnormal nitric oxide (NO) production also contributes to the pathogenesis of impaired healing. Cells such as keratinocytes, fibroblasts and macrophages display both dysfunctional expression and responses to many growth factors and cytokines. Thus, these wounds typically are non-responsive to most treatments. For these reasons, it may be most advantageous to intervene with aggressive strategies that could restore corrupt extracellular microenvironment in a diabetic wound. Wound healing strategies that replace the missing or dysfunctional ECM components may be beneficial. Ideally, such replacement should be multifaceted and interactive in nature, and closely approximate the

components of normal ECM. In this aspect, the role of matricellular proteins in wound healing is of interest. Matricellular proteins can associate with the diverse protein in extracellular matrix reservoir and bridged them with their cognate cell surface receptors⁹⁻¹¹. They are expressed temporally and spatially during wound healing and resided at the crossroads of cell-matrix communication serving as a modulator for several regulatory networks. Presumably, the regulatory pathways consist of complex networks making it difficult to design for compensatory adjustments required for wound repair. It may be most advantageous to intervene with aggressive healing strategies that replace the missing or dysfunctional extracellular matrix (ECM) components. Ideally, such replacement should be multifaceted and interactive in nature, and closely approximate the components of the normal ECM, leading to accelerated wound closure with minimal scar formation. Hence, while targeting or replacing the necessary matricellular proteins may be more efficacious than individual cytokine-mediated candidates it is difficult to know where to begin or what strategy may be successful.

[0007] To effectively manage these problems one must understand the healing process and to create a salubrious physical and biochemical environment conducive for healing. These non-healing wounds have been the subject of intensive investigation throughout the past 15 years. Much effort has focussed on recombinant growth factors. Given that the targets of members of the epidermal growth factor, fibroblast growth factor, platelet-derived growth factor (PDGF), and transforming growth factor- β families were cells that participated in the dermal wound repair process, it was logical to use this model as the first foray into clinical studies with these growth factors. With one notable exception (PDGF-BB or becaplermin), this drug development effort may be considered a failure for several reasons (Pierce & Mustoe 1995), among the most significant reason

was that these growth factors typically target a single biological process essential for wound healing. To date, the only growth factor approved by the US Food and Drug Administration for the treatment of diabetic foot ulcers is recombinant PDGF-BB (becaplermin), which comes in as a topical cream. PDGF-B is known to be a potent mitogen and chemotactic agent for stromal cells and may act to increase the wound vascularization by stimulating angiogenesis. Thus there is an urgent need for better, new or adjunctive treatments.

[0008] Angiopoietin-like protein 4 (ANGPTL4) are secreted proteins mainly expressed in liver that have been demonstrated to regulate triglyceride metabolism by inhibiting the lipolysis of triglyceride-rich lipoproteins. Experimental results show that ANGPTL4 function to regulate circulating triglyceride levels during different nutritional states and therefore play a role in lipid metabolism during feeding/fasting through differential inhibition of Lipoprotein lipase (LPL). The N-terminal domain of Angiopoietin-like proteins has been shown to play an active role in lipid metabolism. Using deletion mutants, it was demonstrated that the N-terminal domain containing fragment - (17-207) and not the C-terminal fibrinogen-like domain containing fragment - (207—460) increased the plasma triglyceride levels in mice: ANGPTL4 has been identified as a novel paracrine and, possibly, endocrine regulator of lipid metabolism and a target of peroxisome proliferators-activated receptors (PPARs). It is expressed in numerous cell types, such as adipocytes and hepatocytes, and is upregulated after fasting and hypoxia. Importantly, ANGPTL4 undergoes proteolytic processing to release its C-terminal fibrinogen-like domain (cANGPTL4), which circulates as a monomer yet whose function remains unclear. The N-terminal coiled-coil domain of ANGPTL4 (nANGPTL4) mediates the oligomerization of ANGPTL4 and binds to lipoprotein lipase to modulate

lipoprotein metabolism mediating oligomerization and lipoprotein metabolism. In contrast, cANGPTL4 exists as a monomer, and its function still remains unknown. ANGPTL4 has been showed to play a context-dependent role in angiogenesis and vascular permeability¹³⁻¹⁵. ANGPTL4, was a recently identified to be a matricellular protein implicated in regulation of energy metabolism and wound healing¹². The deficiency in ANGPTL4 in mice (ANGPTL4^{-/-}) resulted in delayed wound re-epithelialization, reduced matrix proteins expression, an increased inflammation and an impaired wound-related angiogenesis^{16,17}. However, the expression of ANGPTL4 and role in chronic wound repair, such as diabetic wound repair remains unclear.

Summary

[0009] Accordingly, a first aspect of the invention includes a method for increasing wound healing in an individual in need thereof, the method comprising administering an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof.

[0010] Another aspect of the invention includes a pharmaceutical composition for increasing wound healing in an individual, comprising an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof; and a pharmaceutically acceptable carrier.

[0011] Another aspect of the invention includes a method of determining whether a wound site will become a chronic slow-healing wound, comprising the steps of (a) determining the level of the angiopoietin like 4 protein (ANGPTL4) present in a sample taken from the wound site (b) comparing the level of the angiopoietin like 4 protein (ANGPTL4) from the sample with a control from a healthy individual (with normal wound healing), wherein a reduced level of ANGPTL4 in the sample compared with the control is indicative that the wound site will become a chronic slow-healing wound.

[0012] Other aspects of the invention would be apparent to a person skilled in the art with reference to the following drawings and discription of various non-limiting embodiments.

Brief description of the drawings

[0013] The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

Figure 1. Wound healing was retarded in diabetic (ob/ob) mice.

[0014] (A) Representative images of normal (ob/+) and diabetic (ob/ob) wound biopsies taken at day 1, 3, 5, 7 and 10 post wounding. Scale bar: 5 mm.

[0015] (B) Wound closure kinetics of ob/+ and ob/ob mice. Wound surface areas are plotted as percentage of day 0 (=100%) wound surface area. Data are mean $\pm$ SEM, n=10.

[0016] (C) Relative mRNA expression of ANGPTL4 in ob/+ and ob/ob wound biopsies on indicated day post-injury as determined by qPCR. The ribosomal protein L27 serves as reference housekeeping gene. Data are mean $\pm$ SEM from 3 independent studies performed in triplicates.

[0017] (D) Immunoblot analysis of cANGPTL4 from ob/+ and ob/ob wound biopsies. β -tubulin served as loading and transfer controls. The graph shows the relative protein expression level of cANGPTL4 from ob/+ and ob/ob wound biopsies on indicated days post-wounding. Densitometry values of protein bands were determined using imageJ software. Data are mean $\pm$ SEM from three independent wound studies.

[0018] (E) Immunofluorescence staining of ANGPTL4 (red) in ob/+ and ob/ob wound biopsies. Sections were counterstained with DAPI (blue). Dotted line delineates

the epidermis and dermis interface, scale bar: 40 μ m

Using Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Figure 2. Topical application of ANGPTL4 improved diabetic wound healing.

[0019] (A) Wound closure kinetics of diabetic ob/ob wounds treated with a single dose of either saline or cANGPTL4 (50 μ g) in 1% carboxyl methylcellulose. Wound surface areas are plotted as percentage of day 0 (=100%) wound surface area. Data are mean $\pm$ SEM, n=10. Using Mann-Whitney test: ** $p < 0.01$ and *** $p < 0.001$.

[0020] (B) Heatmaps showing gene expression profiles of ob/+, saline- and cANGPTL4-treated ob/ob wounds. Genes were sorted and clustered according to their biological gene functions: proliferation, angiogenesis, migration, ECM, apoptosis and inflammation. Color spectrum from blue to red depicts Log-fold change from -1.0 to 1.0.

[0021] (C) Venn diagram comparing total number of genes from ob/+ (blue), saline- (red) and cANGPTL4-treated ob/ob (black).

[0022] (D) Representative immunoblot of indicated proteins from ob/+, saline- and cANGPTL4-treated ob/ob wound biopsies on various days post-wounding. β -tubulin served as loading and transfer controls.

[0023] (E) The graph shows the relative protein expression level of indicated proteins from ob/+, saline- and cANGPTL4-treated ob/ob wound biopsies on indicated days post-wounding. Densitometry values of protein bands were determined using imageJ software. Data are mean $\pm$ SEM from three independent studies. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

[0024] (F) Immunohistochemistry and immunofluorescence staining of CD31 (brown) and Ki67 (green) of ob/+, saline- and cANGPTL4-treated ob/ob wound biopsies.

Dotted line delineates the epidermis and dermis interface, scale bar: 40 μ m.

Figure 3. ANGPTL4 regulate the NO production in ob/ob mice.

[0025] (A) Nitric oxide production in ob/+, saline- and cANGPTL4 treated ob/ob at indicated days post-wounding as determined by DAF-FM diacetate fluorescence (arbitrary unit, AU). Values were normalized with total protein concentration measured by UV 280nm spectrophotometry. Data are mean $\pm$ SEM from three independent experiments, n=10.

[0026] (B-C) Graph showing the relative mRNA expression level of eNOS (B, left panel) and iNOS (C, left panel) from ob/+, saline- and cANGPTL4-treated wound biopsies on indicated days post-wounding as determined by qPCR. The ribosomal protein L27 serves as reference housekeeping gene. Graphs show the relative mRNA level of iNOS in primary human fibroblasts (B, right panel) and eNOS in primary human dermal microvascular endothelial cells (C, right panel) upon treatment with cANGPTL4 compared with cognate untreated control. Data are mean $\pm$ SEM from 3 independent studies performed in triplicates **p < 0.01

[0027] (D) Immunofluorescence staining of iNOS (red) on saline- and cANGPTL4-treated ob/ob wound biopsies on day 7 post-injury wound biopsies. Nuclei (blue) are counterstained with DAPI. Dotted line delineates the epidermis and dermis interface, scale bar: 50 μ m. WB: wound bed.

Figure 4. ANGPTL4 regulates iNOS expression.

[0028] (A) Representative immunofluorescence staining of pSTAT3(Y705) (green), pSTAT1(Y701) (red) and pNF κ B(S276) (green) on ob/+, saline- and cANGPTL4-treated ob/ob Day

7 post-wounding biopsies. Nuclei (white) are counterstained with DAPI. Dotted line delineates the epidermis and dermis interface, scale bar: 40 μ m. WB: wound bed.

[0029] (B) ChIP assays were conducted using pre-immune IgG or antibodies against pSTAT3(Y705), pSTAT1(Y701) and pNFkB(S276) in saline- (U) and cANGPTL4-treated ob/ob (T) Day 7 wound biopsies. The regions spanning promoter binding sites of the mouse *iNOS* gene were amplified using appropriate primers (Table S1). A control region upstream of the binding sites served as negative control.

[0030] (C) Relative mRNA expression of ID3 in human dermal fibroblasts at indicated time post cANGPTL4 treatment. The ribosomal protein L27 serves as reference housekeeping gene. Data are mean $\pm$ SEM from three independent experiments.

[0031] (D) Relative stability of ID3 mRNA in human dermal fibroblasts treated with cANGPTL4 treated or saline (vehicle) in the presence of actinomycin D (10 μ g/ μ l). The ribosomal protein 18S serves as reference housekeeping gene. The relative mRNA expression level of ID3 was determined by qPCR, normalized to 18S values, and plotted as a percentage of value at time zero. The half-lives of each mRNA were calculated by linear regression analysis using Origin Pro 8.1. Data are mean $\pm$ SEM from three independent experiments.

Figure 5. ANGPTL4 reduced collagen scar tissue in ob/ob wounds.

[0032] (A) Hydroxyproline content of indicated wound tissues from saline- and cANGPTL4-treated ob/ob. Total amount of hydroxyproline (mg) were determined from a hydroxyproline standard curve and normalized with the total protein concentration measured by UV 280nm spectrophotometry. Data are mean $\pm$ SEM, $n=3$ *** $p<0.001$.

[0033] (B) Representative Van Gieson's staining of day 10 wound sections from

saline- and cANGPTL4-treated ob/ob wounds. Collagen is stained red, muscle or fibrin stained yellow and nuclei were stained black.

[0034] (C) Masson's trichrome staining of day 10 wound sections from saline-, cANGPTL4-treated and cANGPTL4 with aminoguanidine -treated ob/ob wounds. Collagen fibers were stained blue, cytoplasm stained red and nuclei were stained black.

[0035] (D) Representative scanning electron (SEM, top and middle panels) and transmission electron microscopy (TEM, bottom panel) images of connective tissue near the wound bed region of saline- and cANGPTL4-treated ob/ob wound from day 10 post wounding. Cross sectional image of collagen fibrils size from corresponding wound biopsies were imaged TEM. Scale bar: top panel, 50 μm ; middle panel, 5 μm and bottom panel, 50 nm.

Figure 6. Hematoxylin and Eosin (H&E) staining of wound sections.

[0036] (A) Representative Hematoxylin and Eosin (H&E) staining of wound sections from ob/+ and ob/ob mice at indicated days post wounding. WB, wound bed. Arrows indicate the wound edge. Dotted line delineates the epidermis and dermis interface. Scale bar: 100 μm .

[0037] (B) Schematic representation (right panel) of the H&E stained wound section (left panel). Epithelial gap (a) was measured as the distance of re-epithelization gap between the two margins of the inward growing epithelium. Epidermal wound area (b) was measured by the area of the epithelium within the epithelial tongue.

[0038] (C) Epidermal wound area of ob/+ and ob/ob wound biopsies sections. All the measurements are performed three times from three random sections for each indicated time point using Adobe Photoshop CS5.1. Image pixel was calibrated to μm using the scale bar. Data are mean $\pm$ SEM, $n=9$ (* $p<0.05$).

Figure 7. Topical application of recombinant ANGPTL4 on full-thickness splint wounds in diabetic ob/ob mice.

[0039] (A) A schematic diagram illustrating the location and dimensions of full-thickness excision splint wounds on the dorsal skin of diabetic mice. Topical application of cANGPTL4 and control saline were both performed on each mouse. Application sites were rotated to avoid site bias. Blue dotted lines indicate centrally dissected wound tissues.

[0040] (B) Photo images of saline- and cANGPTL4-treated ob/ob wound biopsies taken at day 3, 5, 7 and 10 post-wounding. Scale bar: 5 mm.

[0041] (C) Representative H&E staining of wound sections from saline- and cANGPTL4-treated ob/ob mice wound biopsies. Red arrows indicate the wound edge. Dotted line delineates the epidermis and dermis interface. Scale bar: 100 μ m. WB, wound bed.

Figure 8. Nitric oxide and iNOS levels in wounds treated or not with ANGPTL4.

[0042] (A) Representative fluorescence images of wound biopsies stained with DAF-FM diacetate (green) to detect nitric oxide. Wound sections were from day 7 post-injury biopsies of ob/+, saline- and cANGPTL4-treated ob/ob. Dotted line delineates the epidermis and dermis interface. Scale bar: 50 μ m. WB, wound bed.

[0043] (B) Mean fluorescence intensity of DAF-FM diacetate from wound sections stained as described in (A). Mean fluorescence intensity values (arbitrary unit, AU, $\pm$ SEM) were calculated from at least three biopsies and three microscopic fields of each tissue *p <0.05, ** p <0.01.

[0044] (C) Representative immunofluorescence staining of iNOS (green) in ob/+, saline- and cANGPTL4-treated ob/ob wound biopsies on day 7 post-injury. Nuclei were counterstained with DAPI (blue). Dotted line delineates the epidermis and dermis interface, scale bar: 50 μ m.

Figure 9. Effects of aminoguanidine of ANGPTL4 mediated wound healing.

[0045] (A) Photo images of saline-, cANGPTL4-treated ob/ob and cANGPTL4 with aminoguanidine (AG)-treated ob/ob wounds from two mice. Images were taken at day 7 post-wounding. Scale bar: 5 mm.

[0046] (B) Representative hematoxylin and eosin (H&E) images of wound sections from saline-, cANGPTL4- and cANGPTL4 with AG-treated ob/ob wounds at day 7 post-injury.

Scale bar: 500 μ m. Arrows point to the epithelial wound edge.

Detailed Description

[0047] We showed that topical application of ANGPTL4, specifically the C-terminal fibrinogen-like domain (cANGPTL4), accelerates wound closure of splint-wound model in diabetic mice and reduces collagen deposition i.e. scarring at the remodelling stage of wound healing. cANGPTL4 is a matricellular protein, thus it can modulate many critical regulatory networks during wound healing. Thus, matricellular protein ANGPTL4 is more efficacious than individual cytokine-mediated candidates.

[0048] Accordingly, a first aspect of the invention includes a method for increasing wound healing in an individual in need thereof, the method comprising administering an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof.

[0049] Polypeptides of the angiopoietin like 4 (ANGPTL4) polypeptide preferably

have about 406 amino acids, encoding an enzyme having many activities such as interacting with specific matrix proteins in the wound bed, delaying their proteolytic degradation by MMPs, and directly affects cell-matrix communication by altering the availability of intact matrix proteins. ANGPTL allows crosstalk of various cells such as wound keratinocytes, dermal fibroblasts, endothelial cells or inflammatory cells with the surrounding ECM. We also showed that ANGPTL4 reduced collagen deposition towards the later stage of wound healing.

[0050] Preferably, the angiopoietin like 4 (ANGPTL4) polypeptide or therapeutically active fragment thereof comprises ; (i) an amino acid sequence set forth in SEQ ID NO:1; or (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length; or (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 1 over its entire length; or (iv) a fragment of any one of (i) to (iii).

[0051] Preferably the therapeutically active fragment comprises the C terminal region of the angiopoietin like 4 (ANGPTL4) polypeptide or a functional fragment thereof. In various embodiments the fragment comprises: (i) the amino acid sequence set forth in SEQ ID NO:2; or (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:2 over its entire length; or (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 2 over its entire length.

[0052] The functional domain of the C terminal fibrinogen like domain of ANGPTL4

(cANGPTL4) comprises or consists of amino acids 186 to 406 of the amino acid sequence shown as SEQ ID NO: 1 or allelic variants, homologues or fragments, thereof. The C ANGPTL4 domain preferably comprises SEQ ID NO. 2 or a sequence having homology with the amino acid sequence set forth in SEQ ID NO: 2.

[0053] The term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. This term also does not refer to, or exclude modifications of the polypeptide, for example, glycosylates, acetylations, phosphorylations, and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, natural amino acids, etc.), polypeptides with substituted linkages as well as other modifications known in the art, both naturally and non-naturally occurring.

[0054] The term 'wound' refers to a type of injury where the dermis of the skin is disrupted forming a tear, cut, puncture, incision, laceration, abrasion, rip, slash, scratch, slit or rupture in the skin of an animal. Such wounds may include ulcers such as venous ulcers, pressure ulcers or diabetic foot. Diabetic foot refers to chronic slow healing wounds on the lower limbs of an individual who may have diabetes. In various embodiments the wound is selected from an ulcer, a chronic slow-healing wound and an open wound.

[0055] To increase wound healing refers to reducing the size of the wound surface area preferably until the dermis is mostly or completely covering the area, essentially, healing the wound. Wound healing may be measured as a percentage of the reduction of the wound surface area to the initial surface area at the time of the application of the composition or composition described herein. "Treatment" and "treat" and synonyms

thereof refer to therapeutic treatment wherein the object is to speed up (increase) wound healing by reducing the size of the wound surface area in the shortest amount of time.

Increasing wound healing may include reducing the amount of collagen in the wound site, reducing visible scarring or increased expression of nitric oxide synthase (iNOS).

Treatment may include prophylactic passive treatment of a patient at the time of surgery.

Those in need of such treatment include those with a wound such as a cut, an ulcer or a disruption of the skin or a chronic slow healing wound common in individuals with diabetes. An individual refers to an animal, such as a mammal, preferably a human. In various embodiments the individual has diabetes.

[0056] In various embodiments the methods of the invention may further comprise the step of performing an adjunct treatment. The adjunct treatment may include a debridement treatment such as the use of papain or other debridement agents known in the art. The adjunct treatment may include a cytokine treatment. The adjunct treatment may include a hyperbaric oxygen treatment. Similarly, the adjunct treatment may include dressing selection and diabetic shoes or any other treatments known in the art.

[0057] Preferably the angiopoietin like 4 (ANGPTL4) polypeptide comprises; (i) an amino acid sequence set forth in SEQ ID NO:1; or (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length; or (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 1 over its entire length; or (iv) a functional fragment of any one of (i) to (iii).

[0058] Preferably the polypeptide of the C terminal region of the angiopoietin like 4

(ANGPTL4) polypeptide or the functional fragment comprise; (i) the amino acid sequence set forth in SEQ ID NO:2; or (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:2 over its entire length; or (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 2 over its entire length.

[0059] In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 20, 50, 100, 200, 300 or 400 amino acids with the amino acid sequences set out in SEQ ID. NO 1 or the amino acid sequences set out in SEQ ID. NO 2. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for the function of the protein rather than non-essential neighbouring sequences. Preferred polypeptides of the invention comprise a contiguous sequence having greater than 50, 60 or 70% homology, more preferably greater than 80 or 90% homology, to one or more of amino acids of SEQ ID NO: 1 or to one or more of amino acids of SEQ ID NO: 2.

[0060] Other preferred polypeptides comprise a contiguous sequence having greater than 40, 50, 60, or 70% homology, of SEQ ID No: 1 and are capable of binding to SEQ ID No:1. Other preferred polypeptides comprise a contiguous sequence having greater than 50, 60, 70, or 80% homology, of SEQ ID No: 2 and are capable of binding to SEQ ID No:2 and having a similar effect on wound healing. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express

homology in terms of sequence identity. The terms "substantial homology" or "substantial identity", when referring to polypeptides, indicate that the polypeptide or protein in question exhibits at least about 70% identity with an entire naturally-occurring protein or a portion thereof, usually at least about 80% identity, and preferably at least about 90 or 95% identity.

[0061] Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

[0062] Percentage (%) homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

[0063] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

[0064] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a

sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps.

"Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

[0065] Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package and others known in the art. Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching.

[0066] Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in

the case of other software, the default matrix, such as BLOSUM62.

[0067] Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0068] The polypeptide homologues include those having the amino acid sequences, wherein one or more of the amino acids is substituted with another amino acid which substitutions do not substantially alter the biological activity of the molecule. A ANGPTL4 or cANGPTL4 homologue according to the invention preferably has 80 percent or greater amino acid sequence identity to the polypeptide amino acid sequence set out in SEQ ID NO: 1 or SEQ ID NO:2 and has a similar effect on wound healing. Examples of polypeptide homologues within the scope of the invention include the amino acid sequence of SEQ ID NOS: 1 or 2 wherein: (a) one or more aspartic acid residues is substituted with glutamic acid; (b) one or more isoleucine residues is substituted with leucine; (c) one or more glycine or valine residues is substituted with alanine; (d) one or more arginine residues is substituted with histidine; or (e) one or more tyrosine or phenylalanine residues is substituted with tryptophan.

[0069] Preferably "protein" or "polypeptide" refers to a protein or polypeptide encoded by a nucleic acid sequence expressing the amino acid sequences set forth in SEQ ID NO: 1 or SEQ ID NO.2, variants or fragments thereof. Also included are proteins encoded by DNA that hybridize under high or low stringency conditions, to the encoding nucleic acids. Closely related polypeptides or proteins retrieved by antisera to the polypeptide of SEQ ID NO. 1 or SEQ ID NO: 2 is also included.

[0070] "Protein modifications or fragments" are provided by the present invention for the polypeptides or fragments thereof which are substantially homologous to primary

structural sequences but which include, e.g., in vivo or in vitro chemical and biochemical modifications or which incorporate unusual amino acids. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, e.g., with radionuclides, and various enzymatic modifications, as will be readily appreciated by those well skilled in the art. A variety of methods for labeling polypeptides and of substituents or labels useful for such purposes are well known in the art, and include radioactive isotopes such as ^{32}P , ligands which bind to labeled antiligands (e.g., antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands which can serve as specific binding pair members for a labeled ligand. The choice of label depends on the sensitivity required, ease of conjugation with the primer, stability requirements, and available instrumentation. Methods of labeling polypeptides are well known in the art.

[0071] The 'polypeptide' may include any of the polypeptides described herein made according to methods known in the art such as recombinant production in prokaryotic or eukaryotic cells for example in CHO cells or via synthetic methods such as tBoc or Fmoc as is known in the art. Alternatively, the composition may include an agonist that increases the natural expression of ANGPTL4 in a wound site. Hypoxic conditions are known to induce ANGPTL4 expression. Hypoxia induction factor alpha (HIF- α) induces expression of ANGPTL4. Similarly, peroxisome proliferator - activated receptor (PPAR) proteins are transcription factors that activates ANGPTL4 expression. As such the composition may include a PPAR protein or a HIF- α protein.

[0072] Another aspect of the invention includes a pharmaceutical composition for increasing wound healing in an individual, comprising an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof; and a pharmaceutically

acceptable carrier.

Compositions of the Invention

[0073] Polypeptidess produced according to the invention, can be administered for the treatment of wounds in the form of pharmaceutical compositions.

[0074] Thus, the present invention also relates to compositions including pharmaceutical compositions comprising a therapeutically effective amount of (a) an agonist to angiopoietin like 4 protein (ANGPTL4) and, or (b) an agonist of the C terminal region of angiopoietin like 4 protein (ANGPTL4) and a carrier. As used herein a composition will be therapeutically effective if it is able to affect wound healing.

[0075] Pharmaceutical forms of the invention suitable for topical application include sterile aqueous solutions such as sterile phosphate-buffered saline (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of topical solutions and or one or more carrier. Alternatively, topical solutions may be delivered encapsulated in liposomes to assist their transport across cell membrane. Alternatively or in addition such preparations may contain constituents of self-assembling pore structures to facilitate transport across the cellular membrane. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating/destructive action of microorganisms such as, for example, bacteria and fungi.

[0076] The compositions of the present invention, in neat form, may be directly topically applied to an area of skin in need of treatment. In various embodiments, the resulting active compound is applied directly without dilution, or alternatively, the composition can be slightly diluted with water before being applied topically.

Alternatively, the active composition of the present invention can be formulated and

topically applied as a spray, soap, gel, cream, lotion, ointment or the like by the addition of pharmaceutically acceptable carriers or excipients. Preferred carriers include deionised water, vegetable or mineral oils, white petrolatum, branched chain fats or oils, animal fats and high molecular weight alcohol. Emulsifiers, stabilisers and antioxidants may also be included as well as colouring agents and essential oils to impart fragrance.

[0077] It is typical that the compositions of the present invention can be formulated as a lotion or tonic, where they are either applied directly, or diluted with water and then applied. The compositions can also be formulated as creams or ointments. In such formulations the active polypeptides may be added in an amount of 10% to 60% w/w of base moisturiser cream and mixed in with the base cream. For example, sorbolene cream or other moisturisers can have compositions of the present invention added to them in an amount of 10% to 60% w/w. Alternatively, macadamia oil, jojoba oil, almond oil or other nut and seed oils may have the active polypeptide of the present invention added to them in an amount of 10% to 60% w/w.

[0078] Other topical products in which the composition of the present invention can be formulated include skin products such as creams, gels, pastes, emulsions, salves, sprays, masques and peels, and the like.

[0079] Suitable topical vehicles for use with the compositions of the present invention are well known in the pharmaceutical areas and include water, lipid bases materials including oils and fats, soaps, surfactants, emollients, skin conditioning agents and emulsifying agents. Examples of these vehicles are described Martindale-The Extra Pharmacopoeia (Pharmaceutical Press). Clearly, the choice of a suitable vehicle depends on the mode of delivery of the formulation. The active composition is generally incorporated in the dermatologically acceptable vehicle/carrier in a conventional manner

well known in the pharmaceutical arts.

[0080] Topical application of an efficacious amount of the composition of the present invention to an area of skin in need of treatment affords fast and effective healing from the symptoms of various wounds such as ulcers, chronic non-healing wounds and acne. The area of treated skin takes on an improvement in that the surface area of the wound reduces in size as a percentage of the first application of the composition and preferably the area where the dermis has re-grown may have skin tone that appears smooth and taut.

[0081] Typically, a composition of the present invention is topically applied to an animal, preferably a human, for the treatment or prophylaxis of all epidermal wounds including ulcers, insect bites, first, second and third degree burns, healing of sores, wounds and skin infections. Typically, a composition of the present invention reduces scarring and reduces effects of prolific fibrosis on the epidermis. Preferably, a general improvement in clarity, skin texture and appearance of a final scar may be observed after application of a composition of the present invention compared to a similar wound where the composition is not applied.

[0082] Typically, a formulation containing a composition of the present invention, such as a composition and a carrier and/or dilutant is topically applied to the appropriate area and allowed to remain. In various embodiments several applications may be made consequently.

Accordingly, it is typical that the topical compositions of the present invention can be in the form of a masque or lotion or gel, or a cream or ointment.

[0083] The carrier can be a solvent or dispersion medium containing, for example, water, ethariol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The carrier may be

carboxyl methylcellulose. The proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Preventing the action of microorganisms in the compositions of the invention is achieved by adding antibacterial and/or antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0084] Sterile injectable solutions for local application may be prepared by incorporating the active compositions in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above.

[0085] The active ingredient may be held within a matrix which controls the release of the active agent. Preferably, the matrix comprises a substance selected from the group consisting of lipid, polyvinyl alcohol, polyvinyl acetate, polycaprolactone, poly(glycolic)acid, poly(lactic)acid, polycaprolactone, polylactic acid, polyanhydrides, polylactide-co-glycolides, polyamino acids, polyethylene oxide, acrylic terminated polyethylene oxide, polyamides, polyethylenes, polyacrylonitriles, polyphosphazenes, poly(ortho esters), sucrose acetate isobutyrate (S AIB), and combinations thereof and other polymers. Preferably, the matrix sustainedly releases the agonist.

[0086] Pharmaceutically acceptable carriers and/or diluents may also include any and all solvents, dispersion media, coatings, antibacterials and/or antifungals, isotonic and

absorption delaying agents and the like. The carrier and/or diluents may be carboxyl methylcellulose. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated.

[0087] The composition may further comprising an adjunct treatment agent. In various embodiments the adjunct treatment agent may comprise a debridement agent such as papain. Preferably the polypeptide or the composition is suitable for use in the treatment of the wound site.

[0088] "Treatment" and "treat" and synonyms thereof refer to therapeutic treatment wherein the object is to speed up (increase) wound healing by reducing the size of the wound surface area in the shortest amount of time, reducing the amount of collagen in the wound site, reducing visible scarring or increased expression of nitric oxide synthase (iNOS). Treatment may include prophylactic passive treatment of a patient at the time of surgery. Those in need of such treatment include those with a wound such as a cut, an ulcer or a disruption of the skin or a chronic slow healing wound common in individuals with diabetes. An individual refers to an animal, preferably a human.

[0089] To close a wound site refers to reducing the size of the wound surface area preferably until the dermis is mostly or completely covering the area, essentially, healing the wound. Wound healing may be measured as a percentage of the reduction of the wound surface area to the initial surface area at the time of the application of the polypeptide or composition described herein.

[0090] A therapeutically effective amount would be able to increase an angiopoietin like 4 protein (ANGPTL4) polypeptide in a wound site. As used herein a "therapeutically

effective amount" of a composition will be an amount of active agent that is capable of speeding up (increasing) wound healing. Dosages and administration of a composition of the invention in a pharmaceutical composition may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics. An effective amount of the composition to be employed therapeutically, for example a polypeptide, will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the mammal. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 10 ng to up to 100 mg per wound or more per day, preferably about 1 μ g to 10 mg per wound. Doses may include protein amount any where in the range of 10 to 100 μ g or more preferably 25, 50, or 75 μ g per wound.

[0091] Preferably, the angiopoietin like 4 (ANGPTL4) polypeptide or therapeutically active fragment thereof is as described above.

[0092] Wound site, refers to a type of injury where the dermis of the skin is disrupted forming a tear, cut, puncture, incision, laceration, abrasion, rip, slash, scratch, slit or rupture in the skin of an animal. Such wounds may include ulcers such as venous ulcers, pressure ulcers or diabetic foot. Diabetic foot refers to chronic slow healing wounds on the lower limbs of an individual who may have diabetes. Preferably, the wound site is selected from an ulcer, a chronic slow-healing wound and an open wound.

[0093] Another aspect of the invention includes a method of determining whether a wound site will become a chronic slow-healing wound, comprising the steps of (a) determining the level of the angiopoietin like 4 protein (ANGPTL4) present in a sample taken from the wound site (b) comparing the level of the angiopoietin like 4 protein (ANGPTL4) from the sample with a control from a healthy individual (with normal

wound healing) , wherein a reduced level of ANGPTL4 in the sample compared with the control is indicative that the wound site will become a chronic slow-healing wound.

[0094] A chronic slow-healing wound refers to impaired wound healing or wounds that do not appear to follow the normal healing process in less than 4 weeks.

[0095] The method may further comprising the step of determining the blood sugar level of the individual suspected of having the chronic slow-healing wound wherein a high blood sugar level compared to the blood sugar level of a healthy individual is further indicative that the wound site will become a chronic slow-healing wound.

[0096] ANGPTL4 produced by wound keratinocytes interacts with specific matrix proteins in the wound bed, delaying their proteolytic degradation by MMPs, and directly affects cell-matrix communication by altering the availability of intact matrix proteins. Thus, the multifaceted roles of ANGPTL4 link inflammation, diabetes and wound healing.

[0097] The management of the diabetic wound is complex requiring a multidisciplinary approach. Diabetic wounds are characterized by an accumulation of devitalized tissue, chronic inflammation, poor wound-related angiogenesis and deficiencies in several ECM components. Despite the enormous medical burden from poor diabetic wound healing, effective therapies that can regenerate this corrupted ECM have been lacking.

[0098] We have showed that ANGPTL allows crosstalk of various cells such as wound keratinocytes, dermal fibroblasts, endothelial cells or inflammatory cells with the surrounding ECM thus rendering it to be more efficacious than individual cytokine-mediated candidates. We also showed that ANGPTL4 reduced collagen deposition towards the later stage of in our diabetic wound healing. Unlike PDGF, ANGPTL4 can be administered as a topical cream or via biocompatible scaffolds. ANGPTL4 can also be

used as an anti-scarring agent.

[0099] Our study suggested that manipulation of ANGPTL4 may provide adjunctive or new therapeutics avenues in diabetes-associated complications, such as diabetic foot ulcers. It's anti-scarring properties could revolutionize wound healing strategy, not only by improving the healing rate but the aesthetic appearance of the skin. It can also be incorporated into over-the-counter creams, wound plasters, dressings, etc for improved healing and reduced scar formation.

Examples of preferred embodiments

[00100] We showed that the topical application of recombinant ANGPTL4 facilitates wound healing in diabetic mice. ANGPTL4 increases the production of NO level in wounded keratinocytes via integrin $\beta 1$ signaling/pSTAT3 induction of iNOS expression. The elevated NO in the wound microenvironment reduces collagen scar tissue via the stabilization of the ID3 mRNA, which inhibits the expression of COL1A2 in dermal fibroblasts. Our focused gene expression profiling also revealed that the majority of the dysregulated temporal and expression levels of cytokines, growth factors and transcription factors observed in diabetic mice wounds (ob/ob) when compared to wild type counterpart (ob/+), were restored upon treatment with ANGPTL4. Our study suggested that the replacement of ANGPTL4 provides an adjunctive or new therapeutic avenue in diabetics-associated complications, such as diabetic foot ulcers.

Example 1

[00101] Animals. Heterozygous BTBR.V (B6)-Lep^{ob}/WiscJ mice (+/ob) were purchased from The Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched homozygous ob/ob and wildtype (+/+) were obtained by interbreeding the +/ob

littermates. At 3-wk old, offsprings were separated from their parents and genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Male C57BL/6J mice were obtained from Laboratory Animal Centre, National University of Singapore. All mice used in this study were individually caged housed in a temperature-controlled room (23 °C) on a 12 h light-dark cycle, and allowed *ad libitum* access to standard mouse chow diet and water. The mutant mice exhibit severe diabetic conditions from 8 weeks of age and blood glucose level of diabetic mice was 473 ± 14.6 mg/dL and non-diabetic mice was 122.5 ± 5.21 mg/dL as determined by Accu-Chek Advantage glucometer (Roche Diagnostic).

Example 2

[00102] Genotyping of Crossbred mice with PCR-RFLP. A small tail biopsy was collected from each mouse for genotyping and ear tagged after anesthetics. Genomic DNA was extracted using a modified Proteinase K protocol as previously described ²⁵. Genotyping was performed on extracted DNA using PCR-RFLP assay. Primer sequences were (5'-TGTCCAAGATGGACCAGACTC-3') and (5'-ACTGGTCTGAGGCAGGGAGCA-3'). PCR product was digested with DdeI restriction enzyme. Digested fragments were resolved by 2 % agarose gel electrophoresis. Using PCR-RFLP, ++ mice show a single 155-bp band; heterozygous (+/ob) and homozygous mutant (ob/ob) yield 3 bands (155, 100 and 55-bp bands) and 2 bands (100 and 55-bp bands), respectively.

Example 3

[00103] Reduced ANGPTL4 expression in impaired diabetic wound healing. To demonstrate an impaired wound healing of a diabetic (ob/ob) mice compared with normal (ob/+) mice, we evaluated the progress and dynamics of wound healing of full-thickness

excisional splint wounds between ob/+ and ob/ob mice.

[00104] Mice were anesthetized prior to surgery by a single intraperitoneal anaesthetized with of ketamine/xylazine (80 mg/kg + 10mg/kg). Full-thickness excisional wounds (0.5 x0.5 cm²) were created on the dorso-medial back of each mouse. Mice were euthanized by CO₂ inhalation at Day 0, 3, 5, 7 and 10 post wounding. At the indicated time, photo imaging, histomorphometric and other biological analysis of wound biopsies was done.

[00105] Macroscopic observation revealed that ob/ob mice closed 40% of the wound when compared to the ob/+ mice which had complete wound closure by day 10 post-injury (Figure 1A). Haematoxylin and eosin (H&E) staining of day 3, 5, 7 and 10 post-injury biopsies showed impaired epithelial regeneration and granulation tissue formation in ob/ob when compared to ob/+ mice. Complete re-epithelialization of ob/+ wounds was observed on day 7 post-injury (Figure 6A).

[00106] Wound biopsies were fixed in 4% paraformaldehyde-PBS overnight at 4 °C, embedded in OCT tissue freezing medium (Leica) and frozen immediately with liquid nitrogen. Cryosections of 8 µm thickness were used for histological staining.

Histomorphometric analysis of centrally dissected ob/ob wound sections revealed significantly delayed re-epithelialization between day 3-10 post-wounding (ob/+ versus ob/ob: Day 3, 52.1% vs. 79.6%, $p<0.01$; Day 5, 13.3% vs. 35.4%, $p<0.01$; Day 7, 3.0% vs. 28.6%, $p<0.01$; Day 10, 0% vs. 23.6%, $p<0.01$; Figures 1B and Figure 6B). The epidermal wound area above the wound bed of the ob/ob mice remained larger compared with ob/+ mice on day 10 post-injury (11.8×10^5 vs. $2 \times 10^5 \mu\text{m}^2$, $p<0.01$; Figure 6C), suggesting delayed resolution of the wound re-epithelialization in ob/ob mice.

[00107] ANGPTL4 is important for the healing of normal full-thickness excision

wound. ANGPTL4-deficient mice showed a delay in wound healing associated with perturbed keratinocyte migration, poor wound-related angiogenesis that is highly reminiscent of diabetic wound healing. To investigate the role of ANGPTL4 in a diabetic wound, we first compared the spatiotemporal expression profile of ANGPTL4 mRNA and protein between wounded ob/ob mice and ob/+ mice.

[00108] RNA extraction and reverse transcription. At indicated time, skin wound biopsies were excised as previously described ²⁶ and total RNA was extracted using Illustra RNAspin Mini (GE Healthcare) following the supplier's protocol. Five µg of total RNA was reverse transcribed with oligo-dT primers using RevertAid™ H Minus M-MuLV. The RNAs were removed by RNase H digestion prior quantitative real-time PCR (qPCR). Total RNA was isolated from archival paraformaldehyde-fixed, paraffin embedded sections (FFPE) of human diabetic wounds using RecoverAll™ total nucleic acid isolation (Ambion). Fifty ng of RNA was subjected to Full Spectrum™ Complete Transcriptome RNA Amplification (System Biosciences) according to manufacturer's recommendation prior qPCR. The quantitative real-time PCR (qPCR) analyses revealed ~2-folds reduction in ANGPTL4 expression from day 3 post wounding in ob/ob mice compared with ob/+ mice (ob/ob vs ob/+: Day 3, 0.790 vs. 0.511, $p<0.01$; Day 5, 0.735 vs. 0.222, $p<0.01$; Day 7, 0.359 vs. 0.164, $p<0.01$; Figure 1C).

[00109] Wound biopsies were homogenized with ice-cold protein lysis buffer pH 8.0 (20 mM Na₂H₂PO₄, 250 mM NaCl, 1% Triton X-100, 0.1% SDS and 1 mM PMSF). Total protein lysates were precipitated by a chloroform/methanol solvent method prior been resolved on 10% SDS-PAGE. The proteins were electrotransferred onto a onto polyvinylidene fluoride membrane (Millipore). The membranes were blocked with 5% skim milk in TBS (0.25 M Tris.HCl, pH 7.6, 1.5 M NaCl) containing 0.05% Tween-20.

The membrane was incubated with indicated primary antibodies overnight at 4 °C and appropriate anti IgG-HRP secondary antibodies (1:10000) for 1 h at room temperature. The protein bands were revealed using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and signals were quantified by densitometry with the aid of ImageJ version 1.38x (NIH). Consistent with qPCR data, the western blot analysis showed a ~5-fold reduction in the level of ANGPTL4 at day 5 post wounding in ob/ob mice as compared with ob/+ (ob/ob vs ob/+; Day 3, 2.36 vs. 1.43, $p < 0.05$; Day 5, 1.90 vs. 0.38, $p < 0.01$; Day 7, 1.21 vs. 0.93, $p < 0.01$; Day 10, 1.25 vs. 0.62, $p < 0.01$; Figure 1D).

[00110] Similarly, the immunofluorescence staining of day 3-10 wound biopsies of ob/ob mice revealed a lower expression of ANGPTL4 in the wound epithelia and wound bed when compared with ob/+ (Figure 1E). Wound biopsies were fixed in 4% paraformaldehyde-PBS overnight at 4 °C, embedded in OCT tissue freezing medium (Leica) and frozen immediately with liquid nitrogen. Cryosections of 8 μ m thickness were used for immunofluorescence staining as previously described²⁶ except that Alexa488- or Alexa594-conjugated secondary antibodies were used. Apoptotic cells were detected using the TUNEL assay according to the manufacturer's recommendation (Roche). As positive control for TUNEL assay, the section was pre-treated with DNase I. The slides were counterstained with DAPI (Vectashield) and images capture using Eclipse TE2000-U microscope (Nikon).

Example 4

[00111] Topical application of cANGPTL4 improves the healing rate of diabetic wounds. Next, we examined the effect of topical application of recombinant ANGPTL4 on the rate of diabetic wound healing. We inflicted two full excisional splint wounds on the dorsal surface of DB mice, and treated one wound with cANGPTL4 and the other

with saline in 4% carboxymethylcellulose (Figure 7A).

[00112] Two circular 5mm diameter full excisional wounds were created on the dorso-media back of each mouse and silicon donut-shaped splints of 10mm diameter were centered over the wounds. The silicon splint was adhered to the skin with cyanoacrylate glue. On the day of surgery (day 0), 50 μ L of recombinant human ANGPTL4 (rhANGPTL4) protein of 1 mg/mL or 2 mg/mL or PBS mixed with 1% carboxyl methylcellulose (CMC) was applied topically to their respective cutaneous wound and protected with an occlusive dressing (Tegaderm, 3M) throughout the duration of the study.

[00113] The wound images revealed a distinct improvement in the wound closure of cANGPTL4-treated ob/ob mice compared to saline-treated ob/ob mice on day 7 and 10 post-injury (Figure 7B). Histomorphological examination of sections obtained as described in Example 3, from wounds harvested on indicated days showed that cANGPTL4 significantly accelerated re-epithelialization compared with saline control, as indicated by the reduced epithelial gap (saline- vs. cANGPTL4-treated; Day 3, 76.2% vs. 60.1%, $p < 0.05$; Day 5, 34.3% vs. 28.1%, $p < 0.05$; Day 7, 25.6% vs. 4.6%, $p < 0.01$; Day 10, 20.3% vs 0%, $p < 0.01$; Figure 2A and Figure 7C). ANGPTL4-treated wounds showed a significant reduction in the overall epidermal wound area on day 7-10 compared with saline control (3.6×10^5 vs. $6.0 \times 10^5 \mu\text{m}^2$, $p < 0.01$; Figure 7D).

[00114] The dysregulation of growth factors and cytokines production, and subsequent aberrant activation of signaling cascades contribute to poor diabetic wound healing. These factors and signaling mediators are known to be integral in the chemotaxis, migration, stimulation, and proliferation of cells and matrix substances necessary for wound healing. To investigate if treatment of diabetic wound with ANGPTL4 can affect the expression profiles of various genes involved several biological aspect of wound healing, focused

qPCR arrays were performed on wound biopsies derived from ob/+ , ob/ob and ob/ob mice treated with ANGPTL4 at various days post-wounding.

[00115] We analysed a total of 79 genes, whose temporal expression profile during wound healing can be clustered into their biological function such as proliferation, angiogenesis, cell migration, extracellular matrix (ECM), cell apoptosis and inflammation (Figure 2B). Real-time PCR arrays were used to analyze the expression of a focused panel of genes. qPCR was performed with KAPATM SYBR qPCR Universal Master Mix (KAPABiosystems). Melt curve analysis was included to assure that only one PCR product was formed. Primers were designed to generate a PCR amplification product of 100 to 250 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays. Expression was related to the control gene ribosomal protein P0 (RPLP0), which did not change under any of the experimental conditions studied. The sequences of qPCR primers are available in Table 1.

table 1. List of primer pairs sequences.

Genes	Sequence (5' to 3')	Sequence (5' to 3')
ADAM metallopeptidase domain 9 (ADAM9)	F: GGACGGAACCAGACTGCTG	R: CCACTGAACAAAGTTGCCCA
Adiponectin (ADIPOQ)	F: AGCCGCTTATATGTATCGCTCA	R: TGCCGTCATAATGATTCTGTTGG
AKT1	F: CCAAGGCCCAACACCTTTATC	R: TTCCTGCCTCTTGAGTCCATC
ANGPT1	F: TGCACTAAAGAAGGTGTTTTGCT	R: CCTCCCCATTACATCCATATT
ANGPT2	F: CGAGGCGCATTGCTGTAT	R: GGCTGATGCTACTTATTTTGCCC
ANGPTL4	F: TCCAACGCCACCCACTTAC	R: TGAAGTCATCTCACAGTTGACCA
CCL2	F: TTAAAAACCTGGATCGGAACCAA	R: GCATTAGCTTCAGATTACGGGT
CCL11	F: GAATCACCAACAACAGATGCAC	R: ATCCTGGACCCACTTCTTCTT
Cyclin-dependent kinase 4 (CDK4)	F: CCAATGTTGTACGGCTGATGG	R: TGTCCAGGTATGTCCTCAGGT
Cyclin-dependent kinase inhibitor 2B (CDKN2B)	F: CCCTGCCACCCTTACCAGA	R: CAGATACCTCGCAATGTCACG

cystatin A (CSTA)	F: TACGGAGGTGTTTCAGAGGC	R: CAGCGACGGCTTGAGTTTT
CXCL1	F: CTGGGATTACCTCAAGAACATC	R: CAGGGTCAAGGCAAGCCTC
CXCL5	F: TCGGTTGTGTTTGCTTAACCG	R: AGCTATGACTTCCACCGTAGG
CXCL9	F: GAACGGAGATCAAACCTGCCT	R: TG TAGTCTTCCTTGAACGACGA
CXCL10	F: CCAAGTGCTGCCGTCATTTTC	R: GGCTCGCAGGGATGATTTCAA
Epidermal growth factor receptor (EGFR)	F: GGGAGCATTTGGCACAGTGTA	R: GCCATCACATAGGCTTCGTCAA
EPHA3	F: TTCTGGTCGGGAGGTTTTGTG	R: ACTGCTTGAGTAGGGTCTTCA
EPHB3	F: ACCGTAAGAGACTGTAACAGCA	R: GTCCACTTTCACGTAGGGGTT
Fatty acid synthase (FAS)	F: AGAGATCCCCGAGACGCTTCT	R: GCCTGGTAGGCATTCTGTAGT
Fibroblast growth factor 1 (FGF1)	F: CAGCTCAGTGCGGAAAGTG	R: TGTCTGCGAGCCGTATAAAAG
FGF2	F: GCGACCCACACGTCAAACCTA	R: TCCATCTTCCTTCATAGCAAGGT
FBJ osteosarcoma oncogene B (FOSB)	F: GCCACTGCCGACCACAATTC	R: TTATTGGCGACAGTGCAGAACC
Fos related antigen 2 (FRA2)	F: AGCCTCCCGAAGAGGACAG	R: AGGACATTGGGGTAGGTGAA
Growth differentiation factor 3 (GDF3)	F: TAAGGTGGGCAGATTGCTTTTT	R: CTGGACAGTTACCCTGGAGTA
GLI-Kruppel family member GLI1 (GLI1)	F: GAGCCCTTCTTTAGGATTCCCA	R: ACCCCGAGTAGAGTCATGTGG
GM-CSF	F: TCGTCTCTAACGAGTTCTCCTT	R: GCAGTATGTCTGGTAGTAGCTGG
Hepatocyte growth factor (HGF)	F: CTGCTTCATGTCGCCATCC	R: TGGGTCTTCCTTGGTAAGAGTAG
HIF1 α	F: GGTTCATCGCAGTTGGAACCTCC	R: CGCTTGTGTCTTGGAAAGGCTTG
Baculoviral IAP repeat-containing 2 (IAP2)	F: AGGGACCATCAAGGGCACAG	R: TTTGTGTGTTTGGCGGTGTCTC
Insulin-like growth factor binding protein 4 (IGFBP4)	F: AGAAGCCCCTGCGTACATTG	R: TGTCCCCACGATCTTCATCTT
IL1R1	F: GCCAAGGTGGAGGACTCAG	R: CCAGGGTCATTCTCTAACACAGT
IL-6	F: TAGTCCTTCCTACCCCAATTTC	R: TTGGTCCTTAGCCACTCCTTC
IL-10	F: AGAAGCATGGCCCAGAAATCA	R: GGCCTTGTAGACACCTTGGT
IL-18	F: GTGAACCCAGACCAGACTG	R: CCTGGAACACGTTTCTGAAAGA
Inhibin, beta A (INHBA)	F: ATAGAGGACGACATTGGCAGG	R: ATAGAGGACGACATTGGCAGG
Integrin, alpha V (ITGAV)	F: CCTGTGCTCCATTGTACCACT	R: AGCATACTCAACGGTCTTTGTG
Jun B proto-oncogene (JUN-B)	F: GACCTGCACAAGATGAACCAC	R: AGGCTGGAGAGTAAGTCTGA
Keratinocyte growth factor (KGF)	F: CCGTGGCAGTTGGAATTGTG	R: CCTCCGCTGTGTGTCCATT
KI67	F: CTGCCTCAGATGGCTCAAAGA	R: GAAGACTTCGGTTCCTGTAAAC
Kruppel-like factor 9 (KLF9)	F: GCCGCCTACATGGACTTCG	R: GCCGTTACCTGTATGCAC

Mitogen-activated protein kinase 3 (MAPK3 (1B))	F: ACCACATTCTAGGTATCTTGGGT	R: GATGCGCTTGTGTTGGGTGAA
Mitogen-activated protein kinase kinase kinase 1 (MAP4K1)	F: CTCACAGCTCGCTCAGATCC	R: GAGGGGACAGCCGTTGAAT
Melan-a (MLANA)	F: TGGATACAGAACCTTGATGGACA	R: GGGCTGATGGGATTTCTCTTG
MMP9	F: AAACCACCTCTCCCGACTCCAG	R: AGCTCGGTGGTGTCTCCAATG
MMP13	F: ACCTCCACAGTTGACAGGCT	R: AGGCACTCCACATCTTGGTTT
OPN	F: ATCTCACCATTTCGGATGAGTCT	R: TCAGTCCATAAGCCAAGCTATCA
PAI1	F: GTGCATCACTCCACAAACCTGC	R: TAACGTGGGTGGCCAAGCATC
PDGFA	F: CGCTGCACTGGCTGTTGTA	R: TTCCCTACGCCTTCCTGTCTC
PDGFB	F: CGAGCCAAGACGCCTCAAG	R: CATGGGTGTGCTTAAACTTTCCG
Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM1)	F: TGCACCCCATCACTTACCACC	R: TAAAACGCGGTCTGTTCCTC
PPAR α	F: TCGGCGAACTATTCGGCTG	R: GCACTTGTGAAAACGGCAGT
PPAR β/δ	F: TTGAGCCCAAGTTCGAGTTTG	R: CGGTCTCCACACAGAATGATG
PPAR γ	F: TGTGGGGATAAAGCATCAGGC	R: CCGGCAGTTAAGATCACACCTAT
Ras homolog gene family, member A (RHOA)	F: AGCCTTTCTCACCTGGACTGC	R: CACCCACTGCCACCCATAAG
Rpl27	F: CAAGGGGATATCCACAGAGTACCTT	R: CTGGTGGCTGGAATTGACCGCTA
SKI-like oncogene (SKIL)	F: AGGCAGAGACAAGTAAGTCCA	R: CGTCTGGGTAAGACACTGTTTTT
SMAD3	F: CCCCCACTGGATGACTACAG	R: TCCATCTTCACTCAGGTAGCC
SOCS1	F: CTGCGGCTTCTATTGGGGAC	R: AAAAGGCAGTCGAAGGTCTCG
SOCS3	F: CAAGAACCTACGCATCCAGTG	R: CCAGCTTGAGTACACAGTCGAA
SPARC	F: ACTACATCGGACCATGCAAATAC	R: GTACAAGGTGACCAGGACATTTT
STAT1	F: GGAGCACGCTGCCTATGATG	R: CTCCAGAGAAAAGCGGCTGTA
STAT3	F: CAATACCATTGACCTGCCGAT	R: GAGCGACTCAAACCTGCCCT
STAT5A	F: AGTGGTTCGACGGGGTGAT	R: ATGGCTTCAGATTCCAGAGGT
TGF α	F: CACTCTGGGTACGTGGGTG	R: CACAGGTGATAATGAGGACAGC
TGF β 1	F: CCGCAACAACGCCATCTATG	R: CTCTGCACGGGACAGCAAT
TGF β 2	F: TCGACATGGATCAGTTTATGCG	R: CCCTGGTACTGTTGTAGATGGA
TGF β receptor 1 (TGF β R1)	F: TCCCAACTACAGGACCTTTTTCA	R: GCAGTGGTAAACCTGATCCAGA
TIMP1	F: CTTGGTTCCCTGGCGTACTC	R: ACCTGATCCGTCCACAAACAG
TIMP2	F: CTGGACGTTGGAGGAAAGAAG	R: GGTGATGCTAAGCGTGTCCC

TIMP3	F: GCGCAAGGGCCTCAATTAC	R: AGAGACACTCATTCTTGGAGGT
TLR2	F: CCAGACACTGGGGGTAACATC	R: CGGATCGACTTTAGACTTTGGG
TLR4	F: AAAGTGGCCCTACCAAGTCTC	R: TCAGGCTGTTTGTCCCAAATC
TNC	F: GCTACCGACGGGATCTTCG	R: TAGCCGTGGTACTGATGGTTT
TNF α	F: GGCTTTCCGAATTCACCTGGAG	R: CCCCAGGCCTTCCAAATAAA
TNF α interacting protein 2 (TNF α IP2)	F: AAAGGGATACCTACTTGCTGCT	R: CAAGCCCGACACCTTGAAG
TSP-1	F: GAAGCAACAAGTGGTGTCACT	R: ACAGTCTATGTAGAGTTGAGCCC
VEGFA	F: GCACATAGAGAGAATGAGCTTCC	R: CTCCGCTCTGAACAAGGCT
VG1 related sequence (VGR)	F: TCCTTGAACCGCAAGAGTCTC	R: CTCACCCTCAGGAATCTGGG
VHL	F: AAAGAGCGGTGCCTTCAGG	R: CACTTGGGTAGTCCTCCAAATC
X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1)	F: TCTTCAGTCGTATCAACAAGACG	R: GTTTGCTGGGAGGTTTCTCTG

[00116] Heatmaps were constructed from the qPCR focused array data (table 2, 3 and 4) for comparison (Figure 2B). Our analysis revealed that 18 genes out of the possible 79 (~ 22.7%), among them were mostly genes associated with angiogenesis and inflammation, had reverted back to ob/+ gene profiling in the ob/ob treated with cANGPTL4 (Figure 2C).

Table 2. Gene expression in ob/+ wound

GenBank Accession No.	Symbo l	Gene Description	Day 0	Day 3	Day 5	Day 7
NM_021274.1	CXCL10	chemokine (C-X-C motif) ligand 10	0.091558	2.265794	0.299061	0.121755
NM_009283	STAT 1	signal transducer and activator of transcription 1	0.762617	1.325453	1.484704	0.605908
NM_008808.3	PDGFA	platelet derived growth factor, alpha	1.034881	1.46755	2.094767	0.580995
BC109135	IL1R1	interleukin 1 receptor, type I	0.496046	1.924439	1.098497	0.982171
NM_011577.1	TGFB1	transforming growth factor, beta 1	0.208501	2.552748	0.930714	0.555158
NM_008402	ITGAV	integrin alpha V	0.846653	1.623685	1.197204	1.11023
NM_011905	TLR2	toll-like receptor 2	0.503839	2.670658	0.809051	0.342864
NM_011333.3	CCL2	chemokine (C-C motif) ligand 2	0.086708	1.764824	1.228643	0.195532
NM_008279.2	MAP4K1	mitogen-activated protein kinase kinase kinase 1	0.520385	0.820073	0.924272	0.432847
NM_009532	XRCC1	x-ray repair complementing defective repair in Chinese hamster cells 1	0.409888	4.198295	1.129176	1.564693
NM_011593	TIMP1	tissue inhibitor of metalloproteinase 1	0.097499	4.77994	0.615597	0.657043

NM_010548.2	IL-10	interleukin 10	0.020473	1.212068	1.264306	0.534042
NM_010431	HIF1A	hypoxia inducible factor 1, alpha subunit	0.7528	2.806556	1.038764	0.718689
NM_008599.4	CXCL9	chemokine (C-C motif) ligand 9	0.345309	0.727598	1.372522	0.509822
NM_009141.2	CXCL5	chemokine (C-X-C motif) ligand 5	0.149996	3.936088	1.464851	0.594453
NM_007707	SOCS3	Suppressor of cytokine signaling 3	0.526876	4.127815	1.742194	0.582746
NM_009507.3	VHL	von Hippel-Lindau tumor suppressor	1.001643	1.742515	1.298497	0.697237
NM_009263	OPN (Spp1)	secreted phosphoprotein 1	0.159337	3.38626	4.407381	0.200014
NM_013693	TNFA	Tumor necrosis factor, alpha	0.037312	10.2316	0.468858	0.201943
NM_008176	CXCL1	chemokine (C-X-C motif) ligand 1	0.083437	11.05786	0.793785	0.412259
NM_009652.3	AKT1	thymoma viral proto-oncogene 1	0.594666	3.446702	1.482059	1.029804
NM_020581.2	ANGPTL4	angiopoietin-like 4	0.03546	0.790093	0.735691	0.349754
NM_011057.3	PDGFB	platelet derived growth factor, B polypeptide	0.938039	1.825587	1.79318	0.734739
NM_001081117.2	Ki67	antigen identified by monoclonal antibody Ki 67	0.588614	5.438939	2.334188	3.044264
NM_010427.4	HGF	hepatocyte growth factor	0.291696	1.172812	1.299929	0.511323
AF016189.1	Smad3	MAD homolog 3	0.661817	1.268314	1.040053	0.561323
NM_010143	EPHB3	Eph receptor B3	0.529828	0.904733	1.098044	1.303584
NM_008037	FOSL2 (FRA2)	fos-like antigen 2	0.304307	2.714392	0.578645	1.117775
NM_010140	EPHA3	Eph receptor A3	0.171504	1.410735	1.281664	0.694695
NM_207655	EGFR	epidermal growth factor receptor	0.664681	1.006642	0.765933	0.826478
NM_009505	VEGFA	vascular endothelial growth factor A	1.256536	4.690086	2.205454	0.986008
NM_009870	CDK4	cyclin-dependent kinase 4	0.63752	1.941299	1.289818	1.160359
NM_007556	BMP6	bone morphogenetic protein 6	1.221781	2.120903	0.357451	0.136682
NM_001033239	CSTA	Cystatin A	0.188826	4.410111	19.62685	6.133511
NM_008108	GDF3	growth differentiation factor 3	0.229432	6.458987	2.496683	1.439967
NM_011386.2	SKIL (SnoN)	Ski like	0.933885	6.077622	5.291099	2.646154
NM_008416	JUN-B	Jun-B oncogene	0.156725	6.554231	0.686169	0.660104
NM_008380	INHBA	Inhibin, beta A	0.182848	3.492547	1.07148	0.774788
NM_009896	SOCS1	suppressor of cytokine signaling 1	0.75182	2.01507	2.614084	0.980315
NM_021297	TLR4	toll-like receptor 4	0.503916	1.382422	1.054945	0.449261
NM_007404	ADAM9	a disintegrin and metallopeptidase domain 9	0.395403	1.213515	0.922171	0.414878
NM_008607	MMP13	matrix metallopeptidase 13	0.23244	1.656137	0.666829	0.152405
NM_008871	PAI1 (Serpine 1)	serine (or cysteine) peptidase inhibitor, clade E, member 1	1.71784	0.88462	0.889975	0.345461
NM_013599	MMP9	matrix metallopeptidase 9	0.531998	4.645245	6.016101	1.260028
NM_007426.3	ANGPT2	angiopoietin 2	0.110251	0.61129	1.77125	0.371325
NM_011607	TNC	tenascin C	0.338232	4.641325	7.72361	5.58079
NM_031168.1	IL-6	interleukin 6	0.366964	1.705026	2.175877	0.588519
NM_011580	TSP-1	thrombospondin 1	0.476082	2.211606	1.336181	1.212617
NM_011145.3	PPAR β/δ	peroxisome proliferator activated receptor beta/delta	0.858071	0.998435	1.025421	1.068006

NM_009242	SPARC	secreted acidic cysteine rich glycoprotein	0.480204	0.790912	1.80714	0.794548
NM_031199.3	TGFA	transforming growth factor, alpha	1.10904	2.309913	2.735879	1.88485
NM_007670.4	p15 (CDKN2 B)	cyclin-dependent kinase inhibitor 2B	0.86872	1.674337	1.06653	1.406527
NM_009969.4	GM-CSF	Colony stimulating factor 2	1.305664	0.628033	1.980158	0.103102
NM_009370.2	TGFBR1 (ALK5)	transforming growth factor, beta receptor I	0.968227	0.932627	0.91463	1.035229
NM_010296.2	GLI1	GLI-Kruppel family member GLI1	1.502637	0.463211	0.562287	0.518731
NM_029993.1	MlanA	melan-A	0.366231	0.608786	0.160862	0.869004
NM_009640.3	ANGPT1	angiopoietin 1	0.312981	0.223352	0.185313	0.194421
NM_008816.2	PECAM1	platelet/endothelial cell adhesion molecule 1	1.588275	0.80874	0.777321	0.542357
NM_010638	KLF-9	Kruppel-like factor 9	0.68505	0.86225	0.386243	0.307509
NM_011330.3	CCL11	chemokine (C-C motif) ligand 11	0.529826	0.320064	0.593788	0.211562
NM_008008.4	KGF (FGF7)	fibroblast growth factor 7	0.514706	1.121522	0.805334	0.499904
NM_007987.2	FAS	Fas (TNF receptor superfamily member 6)	0.208006	0.226172	0.034424	0.357528
NM_011488	STAT 5A	signal transducer and activator of transcription 5A	0.453144	0.918977	0.756884	0.516671
NM_008036	FOSB	FBJ osteosarcoma oncogene B	0.844083	1.618836	0.867265	0.398194
BC003806	STAT 3	signal transducer and activator of transcription 3	0.704101	1.526852	1.145983	0.598204
NM_011952	MAPK3 (1b)	mitogen-activated protein kinase 3	0.3517	1.10514	0.604718	0.454422
NM_007464	IAP2	intracisternal A particle 2	0.665846	1.51123	1.13886	0.551961
NM_008360.1	IL-18	interleukin 18	0.366857	0.606514	0.960225	0.558545
NM_010517	IGFBP4	insulin-like growth factor binding protein 4	0.614106	0.888844	0.794956	0.476489
NM_009396.2	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	0.906535	0.835809	0.654888	0.250271
NM_016802	RHOA	ras homolog gene family, member A	0.981261	0.993775	0.959051	0.495424
NM_008006.2	FGF2	fibroblast growth factor 2	0.424226	0.758342	0.404547	0.155131
NM_009367.3	TGFB2	transforming growth factor, beta 2	1.28443	0.576548	0.736159	0.443463
NM_011594	TIMP2	tissue inhibitor of metalloproteinase 2	0.497669	0.489931	1.041379	0.386715
NM_010197.3	FGF1	fibroblast growth factor 1	1.506516	0.845095	0.630055	0.504144
NM_011595.2	TIMP3	tissue inhibitor of metalloproteinase 3	0.896732	0.560416	0.344548	0.387725
NM_011144.6	PPAR α	peroxisome proliferator activated receptor alpha	1.376648	0.632694	0.50422	0.457501
NM_00112733 0.1	PPAR γ	peroxisome proliferator activated receptor gamma	1.202532	0.414603	0.380897	0.311466
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing	0.257089	0.063623	0.0252	0.106677

Table 3. Gene expression in ob/ob wound

GenBank Accession No.	Symbo l	Gene Description	Day 0	Day 3	Day 5	Day 7
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NM_021274.1	CXCL10	chemokine (C-X-C motif) ligand 10	0.250498	1.126345	1.284465	0.778731
NM_009283	STAT 1	signal transducer and activator of transcription 1	1.007855	1.307954	2.155304	0.929762
NM_008808.3	PDGFA	platelet derived growth factor, alpha	0.300793	0.745053	1.267399	0.900313
BC109135	IL1R1	interleukin 1 receptor, type I	0.921124	1.726157	3.633038	0.723038
NM_011577.1	TGFB1	transforming growth factor, beta 1	0.549678	1.355657	3.658518	1.551075
NM_008402	ITGAV	integrin alpha V	1.486966	2.218466	3.554344	1.225875
NM_011905	TLR2	toll-like receptor 2	0.489705	2.922228	2.985972	0.923871
NM_011333.3	CCL2	chemokine (C-C motif) ligand 2	0.214683	8.392496	5.935198	0.475682
NM_008279.2	MAP4K1	mitogen-activated protein kinase kinase kinase 1	0.278249	0.832453	0.819214	0.485642
NM_009532	XRCC1	x-ray repair complementing defective repair in Chinese hamster cells 1	3.86801	0.530499	1.031126	1.463049
NM_011593	TIMP1	tissue inhibitor of metalloproteinase 1	0.092007	0.675698	0.641478	0.402068
NM_010548.2	IL-10	interleukin 10	0.297165	1.451524	1.288575	0.369205
NM_010431	HIF1A	hypoxia inducible factor 1, alpha subunit	0.895448	1.387657	0.906589	0.679313
NM_008599.4	CXCL9	chemokine (C-C motif) ligand 9	1.157953	2.190138	2.601004	2.928437
NM_009141.2	CXCL5	chemokine (C-X-C motif) ligand 5	0.501839	40.88581	49.65191	1.426044
NM_007707	SOCS3	Suppressor of cytokine signaling 3	1.077089	5.065714	2.008733	0.98434
NM_009507.3	VHL	von Hippel-Lindau tumor suppressor	1.440808	2.150269	2.576122	1.480475
NM_009263	OPN (Spp1)	secreted phosphoprotein 1	0.321897	11.29194	9.79651	2.078165
NM_013693	TNFA	Tumor necrosis factor, alpha	0.058466	1.534815	0.864856	0.825961
NM_008176	CXCL1	chemokine (C-X-C motif) ligand 1	0.103358	7.129337	4.410788	0.470175
NM_009652.3	AKT1	thymoma viral proto-oncogene 1	1.134636	1.321611	2.016966	0.934964
NM_020581.2	ANGPTL 4	angiopoietin-like 4	0.052951	0.511414	0.222601	0.264857
NM_011057.3	PDGFB	platelet derived growth factor, B polypeptide	0.669607	1.105467	1.523259	1.420103
NM_00108111 7.2	Ki67	antigen identified by monoclonal antibody Ki 67	1.853563	3.629809	4.337301	2.799198
NM_010427.4	HGF	hepatocyte growth factor	0.160628	1.743398	2.166984	0.765072
AF016189.1	Smad3	MAD homolog 3	0.742435	0.750201	0.441932	0.334552
NM_010143	EPHB3	Eph receptor B3	0.389309	1.173355	0.966125	0.874948
NM_008037	FOSL2 (FRA2)	fos-like antigen 2	0.389147	1.190892	0.566979	0.473821
NM_010140	EPHA3	Eph receptor A3	0.856996	0.32359	2.581308	0.473749
NM_207655	EGFR	epidermal growth factor receptor	1.023921	0.713711	1.00082	0.563818
NM_009505	VEGFA	vascular endothelial growth factor A	1.317539	1.37243	1.666211	0.653953
NM_009870	CDK4	cyclin-dependent kinase 4	0.813738	1.337735	1.360731	1.260869
NM_007556	BMP6	bone morphogenetic protein 6	4.061546	1.445786	2.047303	0.103315
NM_00103323 9	CSTA	Cystatin A	0.508856	27.13025	12.53943	3.082821
NM_008108	GDF3	growth differentiation factor 3	0.136515	4.965691	6.24347	2.480356
NM_011386.2	SKIL (SnoN)	Ski like	2.759996	8.323164	7.297358	4.831205
NM_008416	JUN-B	Jun-B oncogene	0.152556	3.172094	1.526778	1.134647
NM_008380	INHBA	Inhibin, beta A	0.919534	2.784224	5.537288	1.897602
NM_009896	SOCS1	suppressor of cytokine signaling 1	1.497767	4.148567	3.142511	1.11581

NM_021297	TLR4	toll-like receptor 4	0.713236	2.706313	2.601621	0.831974
NM_007404	ADAM9	a disintegrin and metallopeptidase domain 9	1.481524	1.773434	1.590105	0.674012
NM_008607	MMP13	matrix metallopeptidase 13	0.196111	1.485535	1.072455	1.113487
NM_008871	PAI1 (Serpine 1)	serine (or cysteine) peptidase inhibitor, clade E, member 1	0.82794	0.50429	0.910365	1.288952
NM_013599	MMP9	matrix metallopeptidase 9	0.48696	9.389738	5.523574	1.92419
NM_007426.3	ANGPT2	angiopoietin 2	2.597985	0.522414	1.412809	0.544707
NM_011607	TNC	tenascin C	0.196003	9.033474	22.59936	7.270111
NM_031168.1	IL-6	interleukin 6	0.195309	8.340513	5.607855	1.92322
NM_011580	TSP-1	thrombospondin 1	0.178282	2.934585	1.655054	1.467634
NM_011145.3	PPAR β/δ	peroxisome proliferator activated receptor beta/delta	0.514494	2.530547	2.382542	1.719608
NM_009242	SPARC	secreted acidic cysteine rich glycoprotein	0.766017	0.643615	1.251748	0.803739
NM_031199.3	TGFA	transforming growth factor, alpha	2.332045	5.16867	4.421783	2.799128
NM_007670.4	p15 (CDKN2 B)	cyclin-dependent kinase inhibitor 2B	1.499122	0.705773	1.415514	1.639463
NM_009969.4	GM-CSF	Colony stimulating factor 2	0.532663	1.575585	0.216542	3.897695
NM_009370.2	TGFBR1 (ALK5)	transforming growth factor, beta receptor I	2.250052	2.189751	1.554899	1.632469
NM_010296.2	GLI1	GLI-Kruppel family member GLI1	0.777933	0.523369	0.833232	0.629003
NM_029993.1	Mlana	melan-A	0.163832	0.130891	0.13634	1.018998
NM_009640.3	ANGPT1	angiopoietin 1	0.271623	0.541881	0.486119	0.274159
NM_008816.2	PECAM1	platelet/endothelial cell adhesion molecule 1	1.026536	1.012497	1.271402	0.708473
NM_010638	KLF-9	Kruppel-like factor 9	1.401337	0.431655	0.642859	0.400448
NM_011330.3	CCL11	chemokine (C-C motif) ligand 11	0.969593	0.610924	0.957601	0.415014
NM_008008.4	KGF (FGF7)	fibroblast growth factor 7	0.595196	1.110988	0.99184	0.680341
NM_007987.2	FAS	Fas (TNF receptor superfamily member 6)	0.439816	0.099373	0.224529	0.189167
NM_011488	STAT 5A	signal transducer and activator of transcription 5A	0.852498	0.754894	1.008012	0.39186
NM_008036	FOSB	FBJ osteosarcoma oncogene B	0.474459	2.473119	0.902717	0.39987
BC003806	STAT 3	signal transducer and activator of transcription 3	0.838114	1.418559	0.857724	0.241829
NM_011952	MAPK3 (1b)	mitogen-activated protein kinase 3	0.597765	0.716862	0.944315	0.538358
NM_007464	IAP2	intracisternal A particle 2	0.881733	0.995298	0.952775	0.67237
NM_008360.1	IL-18	interleukin 18	0.599095	0.486806	0.888455	0.438703
NM_010517	IGFBP4	insulin-like growth factor binding protein 4	0.847077	0.400526	1.231736	0.448635
NM_009396.2	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.334465	0.900658	0.394656	0.630798
NM_016802	RHOA	ras homolog gene family, member A	0.656541	1.501634	1.183005	0.931674
NM_008006.2	FGF2	fibroblast growth factor 2	1.317701	0.766727	0.71361	0.258482
NM_009367.3	TGFB2	transforming growth factor, beta 2	1.267494	1.106269	0.693061	0.685656
NM_011594	TIMP2	tissue inhibitor of metalloproteinase 2	0.615494	0.690985	1.270724	0.564646
NM_010197.3	FGF1	fibroblast growth factor 1	1.809058	1.298661	1.220293	0.831763
NM_011595.2	TIMP3	tissue inhibitor of metalloproteinase 3	1.082271	0.327056	0.547626	0.453588

NM_011144.6	PPAR α	peroxisome proliferator activated receptor alpha	1.358423	0.585544	0.716725	0.619864
NM_00112733 0.1	PPAR γ	peroxisome proliferator activated receptor gamma	1.476165	0.92328	0.820091	0.4815
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing	0.525416	0.118814	0.081237	0.050903

Table 4. Gene expression of ANGPTL4 treated ob/ob wound

GenBank Accession No.	Symbo l	Gene Description	Day 0	Day 3	Day 5	Day 7
NM_021274.1	CXCL10	chemokine (C-X-C motif) ligand 10	0.304805	0.72315	0.61108	0.735198
NM_009283	STAT 1	signal transducer and activator of transcription 1	0.874987	1.040915	1.396994	1.175607
NM_008808.3	PDGFA	platelet derived growth factor, alpha	0.621069	0.776066	0.783335	1.075653
BC109135	IL1R1	interleukin 1 receptor, type I	0.733946	0.774809	1.310378	0.999163
NM_011577.1	TGFB1	transforming growth factor, beta 1	0.369609	1.017253	0.795357	0.789618
NM_008402	ITGAV	integrin alpha V	0.911172	0.888832	1.301828	1.306485
NM_011905	TLR2	toll-like receptor 2	0.586327	0.942771	0.810887	0.921544
NM_011333.3	CCL2	chemokine (C-C motif) ligand 2	0.17887	0.676531	0.822543	0.350045
NM_008279.2	MAP4K1	mitogen-activated protein kinase kinase kinase 1	0.206677	0.70255	0.531403	0.559055
NM_009532	XRCC1	x-ray repair complementing defective repair in Chinese hamster cells 1	0.778193	0.964759	0.794793	0.816166
NM_011593	TIMP1	tissue inhibitor of metalloproteinase 1	0.12779	0.481093	0.376945	0.322528
NM_010548.2	IL-10	interleukin 10	0.361614	0.923416	0.915123	0.573573
NM_010431	HIF1A	hypoxia inducible factor 1, alpha subunit	1.107043	0.888913	1.23845	1.028589
NM_008599.4	CXCL9	chemokine (C-C motif) ligand 9	0.337254	0.683759	0.951498	1.139836
NM_009141.2	CXCL5	chemokine (C-X-C motif) ligand 5	0.506209	0.506209	0.588989	0.520007
NM_007707	SOCS3	Suppressor of cytokine signaling 3	0.586651	0.546553	0.535127	0.743986
NM_009507.3	VHL	von Hippel-Lindau tumor suppressor	0.730746	0.713839	1.030148	1.036704
NM_009263	OPN (Spp1)	secreted phosphoprotein 1	0.231809	0.246915	0.584065	0.477398
NM_013693	TNFA	Tumor necrosis factor, alpha	0.073604	0.323993	0.097211	0.288435
NM_008176	CXCL1	chemokine (C-X-C motif) ligand 1	0.142404	0.409071	0.213149	0.343446
NM_009652.3	AKT1	thymoma viral proto-oncogene 1	1.016809	1.022835	1.141897	1.084718
NM_020581.2	ANGPTL 4	angiopoietin-like 4	0.118174	0.551698	0.776718	0.951234
NM_011057.3	PDGFB	platelet derived growth factor, B polypeptide	0.838878	0.937201	0.865232	1.155474
NM_00108111 7.2	Ki67	antigen identified by monoclonal antibody Ki 67	0.80973	0.731895	0.984001	0.912941
NM_010427.4	HGF	hepatocyte growth factor	0.529111	0.883645	1.340757	1.247646
AF016189.1	Smad3	MAD homolog 3	0.954324	0.885912	0.521233	0.896738
NM_010143	EPHB3	Eph receptor B3	0.638463	0.648164	0.967867	1.213741
NM_008037	FOSL2 (FRA2)	fos-like antigen 2	0.517498	0.504218	0.424239	0.81525
NM_010140	EPHA3	Eph receptor A3	0.8117	0.984998	0.709375	0.800224

NM_207655	EGFR	epidermal growth factor receptor	0.99315	0.900182	1.408017	1.057561
NM_009505	VEGFA	vascular endothelial growth factor A	1.205035	0.925818	1.175286	0.980901
NM_009870	CDK4	cyclin-dependent kinase 4	0.796204	1.070902	0.861631	0.894941
NM_007556	BMP6	bone morphogenetic protein 6	0.997043	0.875784	1.168805	0.787381
NM_001033239	CSTA	Cystatin A	0.344823	0.271149	0.836511	1.096416
NM_008108	GDF3	growth differentiation factor 3	0.441969	0.933849	0.607563	0.893294
NM_011386.2	SKIL (SnoN)	Ski like	0.714242	0.601057	1.014233	1.240276
NM_008416	JUN-B	Jun-B oncogene	0.259351	0.412261	0.324982	0.478694
NM_008380	INHBA	Inhibin, beta A	0.437202	0.407295	0.243947	0.607651
NM_009896	SOCS1	suppressor of cytokine signaling 1	1.154803	0.952236	0.621538	1.058868
NM_021297	TLR4	toll-like receptor 4	0.439488	0.821909	1.18585	1.06559
NM_007404	ADAM9	a disintegrin and metallopeptidase domain 9	0.908046	0.828969	1.205345	0.672844
NM_008607	MMP13	matrix metallopeptidase 13	0.37852	0.430121	0.446309	0.750303
NM_008871	PAI1 (Serpine 1)	serine (or cysteine) peptidase inhibitor, clade E, member 1	0.693599	0.265534	0.833235	0.82168
NM_013599	MMP9	matrix metallopeptidase 9	0.417162	0.256081	0.727659	0.86045
NM_007426.3	ANGPT2	angiopoietin 2	1.188643	0.909358	1.040691	0.437665
NM_011607	TNC	tenascin C	0.195806	0.688135	0.605638	1.082937
NM_031168.1	IL-6	interleukin 6	0.370696	0.403678	0.662347	0.550118
NM_011580	TSP-1	thrombospondin 1	0.417082	0.823719	0.698756	0.862384
NM_011145.3	PPAR β/δ	peroxisome proliferator activated receptor beta/delta	0.88324	0.938287	1.672059	0.818705
NM_009242	SPARC	secreted acidic cysteine rich glycoprotein	0.702669	0.944087	0.739896	0.682164
NM_031199.3	TGFA	transforming growth factor, alpha	0.894606	0.816341	0.857122	1.168371
NM_007670.4	p15 (CDKN2 B)	cyclin-dependent kinase inhibitor 2B	0.644314	0.440738	0.691463	1.036704
NM_009969.4	GM-CSF	Colony stimulating factor 2	0.578629	0.584404	0.182076	1.03733
NM_009370.2	TGFBR1 (ALK5)	transforming growth factor, beta receptor I	1.091881	1.005272	1.024559	1.24505
NM_010296.2	GLI1	GLI-Kruppel family member GLI1	1.202765	0.587583	1.879333	1.169478
NM_029993.1	Mlana	melan-A	0.363229	0.317839	0.34455	0.61259
NM_009640.3	ANGPT1	angiopoietin 1	0.309335	0.558083	0.808304	0.402088
NM_008816.2	PECAM1	platelet/endothelial cell adhesion molecule 1	1.000219	1.019195	1.595884	1.206357
NM_010638	KLF-9	Kruppel-like factor 9	1.108882	1.096597	0.619882	0.588176
NM_011330.3	CCL11	chemokine (C-C motif) ligand 11	0.74805	1.011156	1.428584	0.610392
NM_008008.4	KGF (FGF7)	fibroblast growth factor 7	0.66413	0.814812	1.356676	1.011362
NM_007987.2	FAS	Fas (TNF receptor superfamily member 6)	0.482059	0.886083	0.957834	0.456364
NM_011488	STAT 5A	signal transducer and activator of transcription 5A	0.781767	1.043411	0.924494	0.786155
NM_008036	FOSB	FBJ osteosarcoma oncogene B	0.52427	0.49588	0.562765	0.468561
BC003806	STAT 3	signal transducer and activator of transcription 3	0.903481	1.014231	0.678205	0.801492
NM_011952	MAPK3 (1b)	mitogen-activated protein kinase 3	0.669488	0.992064	0.553663	0.610035

NM_007464	IAP2	intracisternal A particle 2	0.897358	0.903178	1.243896	0.923667
NM_008360.1	IL-18	interleukin 18	0.629251	0.846648	1.027115	0.944054
NM_010517	IGFBP4	insulin-like growth factor binding protein 4	0.811006	1.044133	0.837465	0.835327
NM_009396.2	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.362517	0.820266	0.973071	1.117801
NM_016802	RHOA	ras homolog gene family, member A	0.897189	0.894252	1.133156	1.211557
NM_008006.2	FGF2	fibroblast growth factor 2	1.11818	0.719789	1.409345	0.467684
NM_009367.3	TGFB2	transforming growth factor, beta 2	1.141916	1.096597	0.866636	0.981726
NM_011594	TIMP2	tissue inhibitor of metalloproteinase 2	0.656663	0.976303	1.102946	0.68267
NM_010197.3	FGF1	fibroblast growth factor 1	1.271444	0.853071	0.962985	1.246809
NM_011595.2	TIMP3	tissue inhibitor of metalloproteinase 3	0.953652	1.081293	1.029595	0.691683
NM_011144.6	PPAR α	peroxisome proliferator activated receptor alpha	1.227201	0.859424	1.228189	1.207679
NM_00112733 0.1	PPAR γ	peroxisome proliferator activated receptor gamma	0.951977	0.809746	1.381111	1.150956
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing	0.420466	0.858938	1.396699	0.220694
NM_021274.1	CXCL10	chemokine (C-X-C motif) ligand 10	0.304805	0.72315	0.61108	0.735198

[00117] To further understand how ANGPTL4 improves the healing rate of diabetic wounds, we examined the time course expression of specific protein markers such as endothelial cell marker (CD31), alpha smooth muscle actin (α SMA), cell proliferating marker (PCNA), and macrophage marker (F4/80) in ob/ob treated with ANGPTL4 compared with ob/+ and ob/ob using western blotting as described in Example 3. As expected, saline-treated ob/ob wounds displayed a reduced expression of CD31 and α SMA since day 3 post wounding when compared with the normal ob/+ wounds (Figure 2D, E). Notably, the expression profiles of CD31 and α SMA of cANGPTL4-treated ob/ob wounds were similar to ob/+ (Figure 2D, E).

Consistent with the western blot data, immunofluorescence staining of CD31, as described in Example 3, on day 5 post wounded biopsies showed increase in endothelial vasculature in cANGPTL4-treated ob/ob wounds compared with saline-treated ob/ob wounds (Figure 2F). In contrast to ob/+ wounds, the expression of F4/80 and PCNA in ob/ob wounds was remained elevated until day 10 post wounding, suggesting a persistent

infiltration and activation of macrophages (Figure 2D, E). Although the expression pattern of F4/80 and PCNA in ANGPTL4-treated wounds remain unchanged compared to saline control, their overall expression level was slightly reduced (Figure 2D). Taken together, the observations suggest that ANGPTL4 improves angiogenesis of diabetic wounds.

Example 5

[00118] ANGPTL4 regulate the NO production profile in ob/ob wounds. To understand how ANGPTL4 modulate angiogenesis, we examined the level of NO, which is a potent mediator of angiogenesis. Our earlier focused gene array analysis also revealed that the expression of iNOS was dramatically increased in ANGPTL4-treated ob/ob wounds compared with saline treatment (see Figure 2B). Nitric oxide has been shown to improve tissue repair by promoting endothelial proliferation, keratinocytes migration, and indirectly reducing inflammation.

[00119] Intracellular level of NO from wound biopsies were measured using cell-permeable 4,5-diamino-fluorescein (DAF-FM diacetate) (Invitrogen, USA). Wound biopsies were lysed in Krebs buffer, and incubated with 10 μ M DAF-FM diacetate for 30 min at 37 °C in darkness. The fluorophore signal was recorded immediately at 495 nm excitation and 515 nm emission wavelengths using a GloMax 20/20 Luminometer (Promega, USA). Fluorescence was expressed as arbitrary fluorescence units (AU), and was measured with the same instrument settings for all experiments.

[00120] Using DAF-FM diacetate, we compared the NO level in post-wound biopsies from saline- and ANGPTL4-treated ob/ob. We observed overall reduced NO level in ob/ob wounds when compared with ob/+ wounds (Figure 3A). ANGPTL4-treated ob/ob wounds showed significant increase in NO production from day 3 post-wounding

onwards when compared with saline control, suggesting that ANGPTL4 may mediate NO production (Figure 3A). Using DAF-FM diacetate fluorescence staining on wound biopsies, we observed that NO level was elevated in ANGPTL4-treated ob/ob wound tissue, particularly at the wound epithelia and in the dermal wound bed (Figure 8A). To further understand the underlying mechanism, we examined the mRNA expression of iNOS, eNOS and NO level at indicated days post wounding. In contrast to ob/+ wounds whose eNOS expression peaked at day 7 post wounding, the expression of eNOS in ob/ob wounds peaked earlier at day 3 post-wounding (Figure 3B, left panel). The treatment with ANGPTL4 shifted the peak expression of eNOS to day 5 post wounding, but has no impact on the expression level, suggesting that this may be a secondary effect. We further confirm that ANGPTL4 does not modulate the expression of eNOS using primary human dermal microvascular endothelial cells (Figure 3B, right panel). Our result showed diabetic ob/ob mice expressed little iNOS mRNA when compared to normal ob/+ mice whose iNOS expression peaked transiently at day 3 post-injury. The ANGPTL4 treatment of diabetic wounds increased iNOS expression, albeit peaking at day 7 post-injury (Figure 3C, left panel). We further confirmed that ANGPTL4 increases the expression of iNOS in fibroblasts (Figure 3C, right panel) and immunofluorescence staining, as described in Example 3, of day 7 wound biopsies (Figure 3D). Altogether, the observations suggest that the treatment of ANGPTL4 modulates the expression of iNOS, which increases NO generation at the wound site.

Example 6

[00121] ANGPTL4 regulates iNOS expression. ANGPTL4^{-/-} mice showed impaired angiogenesis during wound healing. Diabetic wounds are characterized by poor wound-related angiogenesis¹, which similarly expressed low level of ANGPTL4 protein (Figure

1D). The pro-angiogenic role of NO is known¹⁸⁻²⁰. However, how ANGPTL4 modulates iNOS and thus NO generation remains unclear. ANGPTL4 binds to integrin, activates focal adhesion kinase and subsequently downstream mediators such as ERK1 and STATs to modulate gene expression and cell behavior^{17,21}. The transcription factors STAT and NF- κ B have been shown to transcriptionally regulate the expression of iNOS²². Thus, we first examined the expression level of phosphorylated STAT1, 3 and NF- κ B in wound biopsies by immunofluorescence staining as described in Example 3. We observed elevated levels of phosphorylated STAT1, 3 and NF- κ B in ob/ob wounds treated with cANGPTL4 compared with saline control (Figure 4A). Next, we performed *in vivo* chromatin immunoprecipitation (ChIP) to determine these transcription factors were bound to the regulatory region of mouse *iNOS* gene. Our ChIP showed that phospho-STAT1, STAT3 and NF- κ B specifically bound to their cognate responsive elements in the promoter of mouse *iNOS* gene in ANGPTL4-treated but not in saline-treated ob/ob wounds (Figure 4B). No immunoprecipitation and amplification were seen with pre-immune IgG and with a control sequence upstream of the responsive elements in the promoter *iNOS* gene (Figure 4B). Nitric oxide has been shown to regulate gene expression and protein activities by transcriptional-independent stabilization of mRNA and modifications of cellular protein targets, respectively^{23,24}. Of particular interest, we observed that the mRNA expression of transcriptional factor Inhibitor of DNA binding 3 (ID3) was elevated in primary fibroblasts treated with ANGPTL4 (Figure 4C). Nitric oxide has been shown to stabilize ID3 mRNA²³. Thus, we examined the mRNA level in fibroblasts treated with either ANGPTL4 or saline in the presence of actinomycin D. Our data revealed a slower decrease in ID3 mRNA level in ANGPTL4-treated fibroblasts

compared to saline treatment (Figure 4D). Altogether, these observations indicate that ANGPTL4 stimulates the level of NO generation, at least through a mechanism that involves the transcriptional regulation of *iNOS* gene via the direct binding of STAT1, 3 and NF- κ B onto the promoter. In addition, we further showed that ANGPTL4-induced NO production stabilizes ID3 mRNA.

Example 7

[00122] ANGPTL4 reduce collagen deposition in ob/ob wounds. Delayed diabetic wound healing lead to excessive production and deposition of collagen at the wound bed, which at least in part may be attributed to reduce NO level. Studies have shown that expression of NOS and NO production are decreased in human hypertrophic scar and diabetic wounds^{27,28}. To address whether ANGPTL4 may influence the level of scarring, we measure collagen deposition in the saline- and cANGPTL4-treated ob/ob wounds. First, we measured the amount of hydroxyproline, a major component of collagen, from each wound biopsies of saline-treated and ANGPTL4-treated ob/ob wounds.

[00123] Hydroxyproline Assay. Wound biopsies were frozen in liquid nitrogen, and then homogenized thoroughly in distilled water. The net weight of the wound biopsies was predetermined for normalization. In addition, trans-4-hydroxy-L-proline (0-300 μ g/mL) was included as standards. Aliquots of samples (50 μ L) were hydrolyzed in 2 N NaOH at 120°C for 2 hours, and then oxidized with chloramine-T reagent (0.0127 g/mL) for 25 min at room temperature. The chromophore was then developed with the addition of p-dimethylaminobenzaldehyde (DMBA) reagent (0.3 g/mL dissolved in methanol/hydrochloric acid solution (2:1 v/v)). The absorbance of reddish hue complex formed was measured at 550 nm using SpectraMax® M2e Multi-Mode Microplate Reader and SoftMax® Pro Microplate Data Acquisition & Analysis Software (Molecular

Devices, USA). Absorbance values were plotted against the concentration of standard hydroxyproline, and the value of unknown hydroxyproline were then determined from the standard curve.

[00124] We detected a reduced level of hydroxyproline only with wound biopsies at day 10 post-injury, where complete wound closure was observed in ANGPTL4-treated wounds (Figure 5A and 2A). Van Gieson staining of wound Tissue sections from mice wound biopsies were deparaffinized and rehydrated in PBS. These sections were stained in Weigert's iron hematoxylin for 8 min at room temperature followed by staining in picric-fuchsin solution for 1 min at room. The Van Gieson staining showed a reduction in collagen deposition at the wound bed in cANGPTL4-treated ob/ob wounds when compared to saline-treated ob/ob wounds (Figure 5B).

[00125] Next, we co-treated ob/ob wounds with ANGPTL4 and aminoguanidine, a selective inhibitor of iNOS. Consistent with our above findings, wound re-epithelialisation was delayed (Figure 9) and the wound site had higher level of collagen deposition, as evidenced by Masson trichrome stain (Figure 5C). To further confirm our observation that ANGPTL4 reduced collagen scar tissue, we used scanning and transmission electron microscopy to characterize the architectural arrangement of collagen fibers in saline- and ANGPTL4-treated ob/ob wounds. As expected, collagen fibrils of saline-treated wounds were thicker and in a pronounced alignment in a single direction (Figure 5D), pointing to scar tissue formation. In contrast, collagen fibrils in ANGPTL4-treated wounds were thinner and in a randomised alignment (Figure 5D). Taken together, we showed that ANGPTL4 accelerated re-epithelialization and reduced collagen scar tissue in ob/ob diabetic wounds.

Example 8

[00126] Reagents. Antibodies used: Ki67 and keratin 6 (NovoCastra); PCNA (PC10) and α SMA (alpha-SM1) (Santa Cruz Biotechnology); CD31 (BD Pharmingen); CD68 (FA-11) (Biolegend); F4/80 (AbD Serotec); cANGPTL4: monoclonal antibodies against the C-terminal mouse (190-410 amino acids) of ANGPTL4 were produced by ProSci, respectively; goat anti-rabbit and anti-mouse IgG-HRP (Santa Cruz Biotechnology); Alexa Fluor 488 or 594 goat anti-mouse IgG, anti-rat IgG and anti-rabbit IgG (Molecular probes). DAB peroxidase substrate kit (Vector Laboratories). Unless mentioned otherwise, all chemicals were from Sigma-Aldrich and molecular biology enzymes from Fermentas. All oligonucleotides were synthesized by Sigma-Proligo.

Example 9

[00127] Assessment of wound healing. Images of wounds were captured at Day 1, 3, 5, 7 and 10 post-wounding using a Canon G12 digital camera. A ruler was included in each image to allow standard calibration of measurements. Surface wound area was quantified using Image-Pro® Plus version 5.1.0.20 software (Media Cybernetics, USA). Surface wound area at each time point were standardized and expressed as a percentage of initial wound area at Day 1 (100%). Histomorphometric measurement was made from sections through the center of the wound to obtain the actual wound representation. Sections of wound biopsies over the indicated time were stained by haematoxylin-eosin staining. Histological images were visualized with Nikon Eclipse 90i brightfield microscope using a Plan Fluor, 10x/0.30 objective and taken with QCapture Pro version 5.0.1.26 software (QImaging). The measurements were performed three times from random sections using Adobe Photoshop CS5.1 and image pixel was calibrated to μ m using the scale bar.

[00128] By "comprising" it is meant including, but not limited to, whatever follows

the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[00129] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[00130] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00131] By "about" in relation to a given numerical value, such as for temperature and period of time, it is meant to include numerical values within 10% of the specified value.

[00132] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a

proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00133] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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SEQUENCE LISTING

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<120> Angiopoietin-like 4 and a method of its use in wound healing

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Gly Asp Pro His Gly Glu Phe Trp Leu Gly Leu Glu Lys Val His Ser
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Ile Thr Gly Asp Arg Asn Ser Arg Leu Ala Val Gln Leu Arg Asp Trp
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Asp Gly Asn Ala Glu Leu Leu Gln Phe Ser Val His Leu Gly Gly Glu
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Asp Thr Ala Tyr Ser Leu Gln Leu Thr Ala Pro Val Ala Gly Gln Leu
 115 120 125

Gly Ala Thr Thr Val Pro Pro Ser Gly Leu Ser Val Pro Phe Ser Thr
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Leu Ser Gly Gly Trp Trp Phe Gly Thr Cys Ser His Ser Asn Leu Asn
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Gly Gln Tyr Phe Arg Ser Ile Pro Gln Gln Arg Gln Lys Leu Lys Lys
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Claims

1. A method for increasing wound healing in an individual in need thereof, the method comprising administering an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof.
2. The method of claim 1 wherein the angiopoietin like 4 (ANGPTL4) polypeptide or therapeutically active fragment thereof comprises ;
 - (i) an amino acid sequence set forth in SEQ ID NO:1; or
 - (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length; or
 - (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 1 over its entire length; or
 - (iv) a fragment of any one of (i) to (iii).
3. The method of claim 1 or 2 wherein the therapeutically active fragment comprises the C terminal region of the angiopoietin like 4 (ANGPTL4) polypeptide or a functional fragment thereof.
4. The method of claim 3, wherein the fragment comprises:
 - (i) the amino acid sequence set forth in SEQ ID NO:2; or
 - (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:2 over its entire length; or
 - (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 2 over its entire length.
5. The method of any one of claims 1 to 4 wherein the wound is selected from an ulcer, a chronic slow-healing wound and an open wound.
6. The method of any one of claims 1 to 5, wherein the individual is a mammal, preferably a human.
7. The method of any one of claims 1 to 6, wherein the individual has diabetes.
8. A pharmaceutical composition for increasing wound healing in an individual, comprising an angiopoietin like 4 (ANGPTL4) polypeptide or a therapeutically active fragment thereof; and a pharmaceutically acceptable carrier.
9. The pharmaceutical composition of claim 8 wherein the carrier is carboxyl

methylcellulose.

10. The pharmaceutical composition of claim 8 or 9 further comprising a further wound treatment agent.

11. The pharmaceutical composition of any one of claims 8 to 10 for use in the treatment of a wound site.

12. The pharmaceutical composition of any one of claims 8 to 11 wherein the angiopoietin like 4 (ANGPTL4) polypeptide of therapeutically active fragment thereof comprises;

- (i) the amino acid sequence set forth in SEQ ID NO:1; or
- (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:1 over its entire length; or
- (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 1 over its entire length; or
- (iv) a fragment of any one of (i) to (iii).

13. The pharmaceutical composition of claim 12, wherein the fragment comprises:

- (i) the amino acid sequence set forth in SEQ ID NO:2; or
- (ii) an amino acid sequence having at least 60, at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 % sequence identity with the amino acid sequence set forth in SEQ ID NO:2 over its entire length; or
- (iii) an amino acid sequence having at least 70, at least 80, at least 85, at least 90, at least 95, at least 97, at least 98 or at least 99 sequence homology with the amino acid sequence set forth in SEQ ID NO: 2 over its entire length.

14. A method of determining whether a wound site will become a chronic slow-healing wound, comprising the steps of (a) determining the level of the angiopoietin like 4 protein (ANGPTL4) present in a sample taken from the wound site (b) comparing the level of the angiopoietin like 4 protein (ANGPTL4) from the sample with a control from a healthy individual (with normal wound healing), wherein a reduced level of ANGPTL4 in the sample compared with the control is indicative that the wound site will become a chronic slow-healing wound.

15. The method of claim 14 further comprising the step of determining the blood sugar level of the individual suspected of having the chronic slow-healing wound wherein a high blood sugar level compared to the blood sugar level of a healthy individual is further indicative that the wound site will become a chronic slow-healing wound.