

(51) International Patent Classification:  
C12N 5/071 (2010.01)(21) International Application Number:  
PCT/US2015/040795(22) International Filing Date:  
16 July 2015 (16.07.2015)

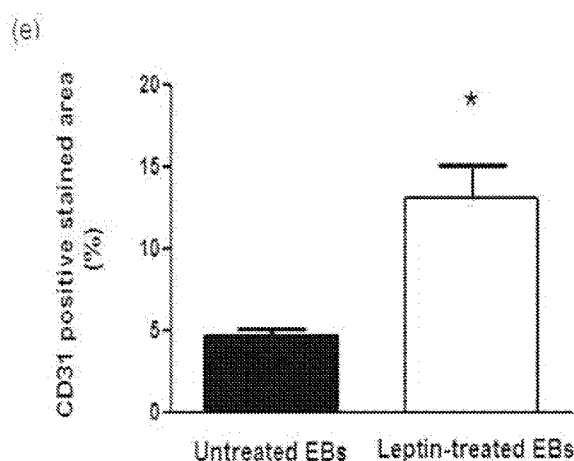
(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
62/025,908 17 July 2014 (17.07.2014) US(71) Applicant: CEDARS-SINAI MEDICAL CENTER  
[US/US]; 8700 Beverly Boulevard, Los Angeles, California 90048 (US).(72) Inventors: TALAVERA-ADAME, Dodanim; 18034  
Anne's Circle Apt. 102, Canyon Country, California 91387  
(US). DAFOE, Donald C.; 24798 Brown Latigo St.,  
Malibu, California 90265 (US).(74) Agents: CHEN, Stephen W. et al.; Nixon Peabody LLP,  
Gas Company Tower, 555 West Fifth Street, 46th Floor,  
Los Angeles, California 90013 (US).(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,  
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,  
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,  
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,  
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,  
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,  
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,  
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,  
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,  
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,  
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,  
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,  
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,  
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, KM, ML, MR, NE, SN, TD, TG).**Published:**

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: BLOOD-VESSEL THERAPEUTIC PATCH COMPOSED OF ENDOTHELIAL CELLS DERIVED FROM IPSCS PRE-TREATED WITH LEPTIN



(57) **Abstract:** Described herein are compositions and techniques related to generation of endothelial cells from pluripotent stem cells. Via leptin addition and activation of the canonical JAK/STAT pathway, vascular density improves in leptin-treated EBs compared to untreated controls in murine and human cells. Endothelial cell and angiogenesis marker expression analyzed via qRT-PCR demonstrated elevated CD31 and phosphoSTAT3 (pSTAT3) expression. Such results allow for generation of therapeutic microvessels derived from pluripotent stem cells with enhanced capacity for angiogenesis and vasculogenesis, in addition to in vivo capillary network formation, thereby finding regenerative medicine applications for diseases such as diabetes and conditions such as burn victims.

5        **BLOOD-VESSEL THERAPEUTIC PATCH COMPOSED OF ENDOTHELIAL  
         CELLS DERIVED FROM iPSCs PRE-TREATED WITH LEPTIN**

**FIELD OF THE INVENTION**

                 Described are methods and compositions for generation of endothelial cells from  
10 pluripotent stem cells, which provide therapies for vascular related conditions.

**BACKGROUND**

                 Vascular biology, mediated by endothelial cells (ECs) in the body, plays an important  
role in normal and disease-relevant physiology, including establishing barrier function,  
15 mediating inflammation, modulating cell migration, angiogenesis and vasculogenesis, among  
many others. Differentiation of pluripotent stem cells, such as human embryonic stem cells  
(hESCs) and induced pluripotent stem cells (iPSCs), into ECs has the potential to provide an  
unlimited source of cells for novel transplantation therapies by supporting angiogenesis and  
vasculogenesis, while further establishing informative disease models for drug and  
20 therapeutic research.

                 Endothelial differentiation studies relying on hESC and iPSC sources have been  
hampered by poor efficiency of the so-called “3-dimensional” embryoid body (EB) method.  
Other “2-dimensional” culturing methods frequently require co-culturing with feeder layers  
such as on bone marrow stromal OP9 cells, or mouse embryonic fibroblasts (MEFs), thereby  
25 requiring use of animal products limit future clinical application of hESC-ECs. Thus, there is  
a critical need in the art is for development of reliable vascular biology compositions and  
methods, such as efficient production of functional pSC-derived endothelial cells.

                 The metabolic regulation of leptin and its angiogenic effects have been well  
characterized in adult mammals. However, the role of leptin in the differentiation of  
30 endothelium and angiogenesis during embryo development has not been completely  
characterized. Furthermore, leptin effects on the differentiation of ESCs to ECs and  
angiogenesis has not been fully explored. In addition, it is unknown if JAK/STAT pathway  
activation plays a pivotal role in this differentiation. Described herein is the discovery that  
leptin promotes differentiation of embryonic stem cells (ESCs) to endothelial cells (ECs)  
35 through activation of the canonical JAK/STAT pathway and stimulates angiogenesis. Using  
an *in vitro* model consisting of murine ESCs (mESCs) aggregated into EBs, significant  
upregulation of endothelial markers (CD31, eNOS, vWF, CD34) and angiogenic markers  
(FLK1, TIE1, TIE2, ANG1, and ANG2) was observed, and FLT-1 was found specifically in

leptin-treated EBs compared to controls. In addition, significant increase in vessel density and increased CD31 (65% vs. 17%) expression was observed in leptin-treated EBs compared to controls.

Described herein are compositions and techniques related to generation of large amounts of iPSC-derived endothelial cells following embryoid body (EB) formation, culture on ECM proteins (collagen I, IV and laminin I) deposited on a scaffold, in combination with leptin addition. The iPSC-derived endothelial cells are described as functional, mature cells possessing microvessels capable of forming an *in vivo* network. The combination of scaffold and iPSC-derived endothelial cells can constitute a “patch” that has wide applications ranging from cardiac and wound repair to diabetes treatment. The discovery of a specific role for leptin in EC differentiation and angiogenesis through JAK/STAT pathway activation provides compositions of pSC-derived ECs and techniques for producing significant quantities of these cells.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Fig. 1a to Fig. 1e.** Quantification of vascular density in embryoid bodies (EBs) treated or untreated with leptin. (a) EBs treated with 1 nM leptin and stained with anti-CD31. (b) Binarized image of “a” using the image tools of imageJ (see methods). (c) Untreated EBs stained with anti-CD31. (d) Binarized image of “c”. (e) Percentage of the area occupied by CD3-positive cells in ten random fields of EBs treated or untreated with leptin. Bar = 50  $\mu$ m. \*P < 0.05.

**Fig. 2a to Fig. 2f.** Angiogenic effects of leptin in mouse EBs stained with anti-CD31. (a) EBs treated with 1 nM leptin and stained with rat anti-mouse CD31 as primary antibody and Alexa 555 as secondary antibody. (b) EBs treated with 10 nM leptin. (c) EBs treated with 0.5 U/mL erythropoietin (EPO). (d) EBs treated with a combination of 1 nM leptin and 0.5 U/mL EPO. (e) Untreated EBs. (f) Isotype controls. Bar = 50  $\mu$ m.

**Fig. 3a to Fig. 3e.** Endothelial-cell marker expression in mouse embryoid bodies (EBs) measured by quantitative RTPCR. These EBs were treated or untreated with two different leptin concentrations (1 nM or 10 nM) at 10, 20, and 30 days of EB age. (a) CD31. (b) eNOS. (c) von Willebrand Factor (vWF). (d) CD34. \* P < 0.05. \*\* P < 0.005 (e) Long form of mouse leptin receptor as function of EB age in presence or absence of leptin (C = control, L = leptin). \* P<0.05. \*\*P<0.005.

**Fig. 4a to Fig. 4f.** Angiogenesis marker expression in mouse embryoid bodies (EBs) measured by quantitative RTPCR. The EBs were treated with leptin at 1 or 10 nM and

5 harvested for analysis at 10, 20, and 30 days of EB age. (a) FLK1 (VEGFR2). (b) Flt-1 (VEGFR1). (c) Tie1. (d) Tie2. (e) Ang1. (f) Ang2. \*  $P < 0.05$ . \*\*  $P < 0.005$

**Fig. 5a to Fig. 5h.** FACS and magnetic sorting isolation of endothelial cells (ECs) from mouse EBs at day 10. (a) Leptin-treated EBs that show higher amounts of CD31 positive cells (green). (b) Untreated EBs with scarce CD31 positive cells also stained to CD31 (green). (c) Quantification of CD31 positive cells by FACS in EBs treated with leptin (1 nM). (d) Quantification of CD31 positive cells in untreated EBs. (e) ECs isolated from leptin-treated EBs. (f) ECs isolated from untreated EBs. (g) Higher magnification of the cell clusters shown in “e” composed of cells that express CD31(green) or cardiotin (red) (yellow arrows). (h) Tube-like structures formed by CD31 positive cells isolated from leptin-treated EBs. (a, b) Bar = 50  $\mu\text{m}$ . (e, f) Bar = 100  $\mu\text{m}$ . (g, h) Bar = 25  $\mu\text{m}$ .

**Fig. 6a to Fig. 6f.** Co-expression of CD31 and pSTAT3 in cells derived from leptin-treated or untreated EBs. (a) Endothelial cell clusters derived from leptin-treated EBs at day 10 stained with CD31 (green) and (b) phosphoSTAT3 (red). (c) Merged image and an inset that shows these cells at higher magnification (asterisk). (d) Untreated EBs stained to CD31 and (e) phosphoSTAT3. (f) Merged image. (a to f) Bar = 100  $\mu\text{m}$ . Inset in “c” bar = 10  $\mu\text{m}$ .

**Fig. 7a to Fig. 7f.** Embryoid body (EBs) formation *in vitro* and blood vessel development. (a) EB day 3 in suspension. (b) EB day 6 cultured alone attached to a coverslip. (c) EB day 10 treated with leptin (1 nM) observed with phase contrast microscope. (d) EB day 10 treated with leptin (1 nM) stained with anti-CD31 antibody observed with fluorescent microscope. (e) Untreated EB day 10 observed with phase contrast microscopy. (f) Untreated EB day 10 observed with fluorescent microscope. (a, b, c, e) Bars = 100  $\mu\text{m}$ . (d, f) Bar = 50  $\mu\text{m}$ .

**Fig. 8a to Fig. 8d.** EBs developed in hanging drops for two days and then (a) after suspension for three more days. (b) EBs were cultured in dishes with coverslips. After attachment, EB cells spread out at the periphery. (c, d) After ten days in culture, enhancement in the number of blood vessels was observed in leptin-treated EBs (e,f) in contrast to few primitive vessels observed in controls. The pattern observed resembled intussusceptive angiogenesis instead of branching (d).

**Fig. 9.** Formation of tube-like structures in human induced pluripotent stem cell-derived endothelial cells treated or untreated with leptin in collagen-laminin gels.

**Fig. 10a to Fig. 10u.** Co-expression of CD31/PECAM1 with angiogenesis markers in EBs treated (1 nM leptin) for ten days or untreated EBs. EBs treated with leptin that co-expressed (a) DAPI, (b) CD31/PECAM1, and (c) TIE2. Untreated EBs that co-expressed (d)

5 DAPI, (e) CD31/PECAM, and (f) TIE2. EBs treated with leptin that co-expressed (g) DAPI, (h) CD31/PECAM1, and (i) FLK-1. Untreated EBs stained to (j) DAPI, (k) CD31, and (l) FLK-1. EBs treated with leptin that co-expressed (m) DAPI, (n) CD31/PECAM1, and (o) Ang2. Untreated EBs that co-expressed (p) DAPI, (q) CD31/PECAM1, and (r) Ang2. Isotype controls that express (s) DAPI and no mouse marker expression (t, u). Bar = 100  $\mu$ m.

## SUMMARY OF THE INVENTION

Described herein are a method of differentiating human pluripotent stem cells into microvessels including endothelial cells, including: (a) providing a quantity of human pluripotent stem cells (pSCs), (b) inducing formation of embryoid bodies (EBs), (c) 15 generating endothelial cells by culturing the EBs in the presence of leptin, an (d) further culturing the endothelial cells on a scaffold including at least one extracellular matrix (ECM) component, wherein the leptin and at least one ECM component form microvessels including endothelial cells. In other embodiments, wherein inducing generating endothelial cells comprises culturing EBs for 7 to 23 days in the presence of leptin. In other embodiments, the 20 endothelial cells in the microvessel express one or more of CD31+, VE-CAM+, VWF+, KLF-2+, and KLF-4+. In other embodiments, the endothelial cells are isolated before placing onto the scaffold including at least one ECM component. In other embodiments, the scaffold including at least one extracellular matrix ECM component includes at least one collagen and at least one laminin. In other embodiments, the at least one extracellular matrix ECM 25 component includes collagen I, IV, and laminin I. In other embodiments, the scaffold including at least one extracellular matrix ECM component includes a biocompatible material suitable for transplantation. In other embodiments, the method includes further culturing the endothelial cells on a scaffold including at least one extracellular matrix (ECM) component includes leptin addition.

Also described herein is a composition produced by a method of differentiating human pluripotent stem cells into microvessels including endothelial cells, including: (a) providing a quantity of human pluripotent stem cells (pSCs), (b) inducing formation of embryoid bodies (EBs), (c) generating endothelial cells by culturing the EBs in the presence of leptin, an (d) further culturing the endothelial cells on a scaffold including at least one 35 extracellular matrix (ECM) component, wherein the leptin and at least one ECM component form microvessels including endothelial cells.

Further described herein is a composition, including: a quantity of microvessels including endothelial cells, wherein the endothelial cells are capable of angiogenesis and/or

5    vasculogenesis. In other embodiments, the endothelial cells synthesize and/or release nitric  
oxide (NO) and/or prostacyclin. In other embodiments, the composition includes at least one  
extracellular matrix (ECM) component. In other embodiments, the at least one ECM  
component includes at least one collagen and at least one laminin. In other embodiments, the  
at least one ECM component includes collagen I, IV, and laminin I. In other embodiments,  
10   the composition includes a biocompatible material suitable for transplantation. In other  
embodiments, the endothelial cells express one or more of CD31+, VE-CAM+, VWF+, KLF-  
2+, and KLF-4+.

Also described herein is a method of treatment, including: providing a composition  
including microvessels including endothelial cells, administering to a subject in need of  
15   treatment for wound repair, the composition including microvessels including endothelial  
cells, wherein in vivo formation of a capillary network treats the subject. In other  
embodiments, the endothelial cells synthesize and/or release nitric oxide (NO) and/or  
prostacyclin. In other embodiments, the composition includes at least one extracellular  
matrix (ECM) component. In other embodiments, the at least one ECM component includes  
20   collagen I, IV, and laminin I. In other embodiments, the method includes a biocompatible  
material suitable for transplantation. In other embodiments, the endothelial cells express one  
or more of CD31+, VE-CAM+, VWF+, KLF-2+, and KLF-4+. In other embodiments, the  
subject in need of treatment for wound repair is afflicted with diabetes, obesity with wound  
healing impairment, peripheral vascular problems, physical trauma requiring wound healing,  
25   or burns.

### DETAILED DESCRIPTION

All references cited herein are incorporated by reference in their entirety as though  
fully set forth. Unless defined otherwise, technical and scientific terms used herein have the  
30   same meaning as commonly understood by one of ordinary skill in the art to which this  
invention belongs. Allen *et al.*, *Remington: The Science and Practice of Pharmacy* 22<sup>nd</sup> ed.,  
Pharmaceutical Press (September 15, 2012); Hornyak *et al.*, *Introduction to Nanoscience and  
Nanotechnology*, CRC Press (2008); Singleton and Sainsbury, *Dictionary of Microbiology  
and Molecular Biology* 3<sup>rd</sup> ed., revised ed., J. Wiley & Sons (New York, NY 2006); Smith,  
35   *March's Advanced Organic Chemistry Reactions, Mechanisms and Structure* 7<sup>th</sup> ed., J. Wiley  
& Sons (New York, NY 2013); Singleton, *Dictionary of DNA and Genome Technology* 3<sup>rd</sup>  
ed., Wiley-Blackwell (November 28, 2012); and Green and Sambrook, *Molecular Cloning: A  
Laboratory Manual* 4<sup>th</sup> ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY

5 2012), provide one skilled in the art with a general guide to many of the terms used in the present application. For references on how to prepare antibodies, see Greenfield, *Antibodies A Laboratory Manual 2<sup>nd</sup> ed.*, Cold Spring Harbor Press (Cold Spring Harbor NY, 2013); Köhler and Milstein, *Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion*, Eur. J. Immunol. 1976 Jul, 6(7):511-9; Queen and Selick, *Humanized immunoglobulins*, U. S. Patent No. 5,585,089 (1996 Dec); and Riechmann *et al.*, *Reshaping human antibodies for therapy*, Nature 1988 Mar 24, 332(6162):323-7.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described.

15 For purposes of the present invention, the following terms are defined below.

As used in the description herein and throughout the claims that follow, the meaning of “a,” “an,” and “the” includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein, the meaning of “in” includes “in” and “on” unless the context clearly dictates otherwise.

20

As described, prior attempts to generate endothelial cells (ECs) from pluripotent stem cells (pSCs) have relied on embryoid body (EB) formation or co-culture techniques. Two common methods can be described as “2-D” culturing on a flat culture surface consisting of extracellular matrix (ECM) proteins or co-culture with other cells (*e.g.*, OP9 or fibroblasts) or “3-D” culturing involving formation of spherical embryoid bodies (EBs) or placement on scaffolds. Examples of 2-D culture including growth and differentiation of human embryonic stem cells (hESCs) collagen IV- coated plates, or 2-D methods involving co-culture includes hESC grown on bone marrow stromal OP9 cells. Examples of 3-D culture include induced human EB formation on gelatin-coated plates or human EBs grown on methylcellulose. In some instances, there is reported use of scaffolds for differentiation of endothelial cells, this includes 3-D culturing via ECM coated polyethylene glycol (PEG) scaffolds. While each of the above methods in the first category purports to establish a platform for generating PSC-derived endothelial cells, as characterized by phenotypic properties (*e.g.*, expression of CD31<sup>+</sup>, vascular endothelial-cadherin (VE-CAM<sup>+</sup>), and von Willebrand Factor (vWF<sup>+</sup>), cobblestone morphology), some reports have observed that many alleged pSC-derived cells may lack full functional, mature characteristics when compared to endogenous endothelial cells.

25

30

35

5 Further variations attempt to generate functionalized, mature endothelial cells. As a result, various additives or adjustments have been undertaken to coax cells with some, all, or more than all of core features of endothelial cells for the sake of further differentiating them. These additives or adjustments can vary widely, and include examples such as application of biomechanical shear forces, addition of exogenous factors such as combinations of BMP4, bFGF, VEGF and DKK1 or co-culture of endothelial cells with mesenchymal cells or human umbilical cord vein endothelial cells (HUVECs) to develop endothelial cells with vasculature. What can be appreciated from this myriad of reports is that a variety of methods have yet to establish an efficient, xeno-free platform for generation of significant quantities of pSC-derived endothelial cells. Moreover, it is not clear if any the alleged pSC-derived ECs possess the requisite characteristics of functional, mature ECs that would provide for their clinical use.

Leptin is a 16-kDa product of the obese (*ob*) gene with multiple biological effects. Expressed in adipocytes, leptin plays an important role in the regulation of food intake and body weight in humans. Leptin has also been shown to have a potent angiogenic effect. Through the use of computer-assisted image-analysis software, the Inventors previously characterized the angiogenic effects of leptin in quail chorioallantoic membranes (CAMs) and concluded that leptin promotes blood vessel growth in CAMs through a mechanism known as intussusception or splitting angiogenesis. In addition, it has been reported that leptin-treated human endothelial progenitor cells form tubelike structures *in vitro* via the long form of leptin receptor. Therefore, leptin not only promotes angiogenesis but also blood vessel remodeling.

Endothelial cells and other cells also respond to leptin via leptin membrane receptors. Leptin receptor (*Ob-R*) is encoded by the diabetes gene (*db*) and six isoforms of this receptor have been identified. These isoforms are generated by alternate splicing (*OB-Ra-f*) in mice. Most of these isoforms are membrane-spanning proteins with extracellular and intracellular domains. The extracellular domains are identical for all leptin isoforms. In contrast, the intracellular domain can be short and define the short forms of leptin receptor (*OB-Ra,c,d,f*) while another isoform has a long intracellular domain and it is known as the long form of leptin receptor (*OB-R<sub>b</sub>*). Another form of leptin receptor named *OB-Re* has no transmembrane domain and circulates as soluble receptor. Apparently, *OB-R<sub>b</sub>* leptin receptor has a major role in food intake in mammals since its mutation may result in the obese phenotype in mice. In general, leptin receptor has homologies with the class I cytokine receptor superfamily including interleukin-6 receptors (*IL-6R*), leukemia inhibitor factor

5 (LIF), and granulocyte-colony stimulating factor (G-CSF). Activation of leptin receptor causes phosphorylation of janus tyrosine kinase 2 (JAK2) which in turn activates signal transducers and activators of transcription (STAT) proteins. JAK2 phosphorylates leptin receptor and activates STAT3 that translocates to the nucleus and function as transcription factor that targets multiple genes. Most of the leptin effects are exerted through this  
10 JAK/STAT pathway and have been evaluated mainly in adult tissues and organs.

With the emergence of embryonic stem cells (ESCs) that can be maintained undifferentiated in culture, the role of hormones such as leptin in EC differentiation and blood vessel development can be explored. When these ESCs are plated in suspension, they form structures composed with the all three germ layers: ectoderm, mesoderm, and endoderm  
15 and are called embryoid bodies (EBs). Mesoderm gives rise to several structures including the cardiovascular system. Then, ECs can be derived from ESCs. The function of these derived ECs has been also evaluated *in vivo*. In addition, the effects of angiogenic molecule such as erythropoietin in cardiovascular enhancement in embryoid bodies have been also described. It has been reported that leptin is crucial for embryo pre-implantation as well as  
20 the epithelial to mesenchymal cellular transition necessary for cardiac valve development, and adipocyte differentiation. Furthermore, the role of leptin in early hematopoietic differentiation has also been described. Apparently, endothelium interactions with different tissues during development is crucial for adequate organogenesis. In this work we used ESCs as an *in vitro* model to characterize leptin inductive effects in EC differentiation and  
25 embryonic angiogenesis.

Such understanding allows one to adopt therapeutic avenues otherwise unavailable. For example, according to the American Diabetes Association, in 2012, 29.1 million Americans, or 9.3% of the population, had diabetes. Approximately 1.25 million American  
30 children and adults have type 1 diabetes. Of the 29.1 million, 21 .0 million were diagnosed, and 8.1 million were undiagnosed. Diabetic foot ulcers (DFUs) are responsible for more hospitalizations than any other complication of diabetes. Diabetes is the leading cause of non-traumatic lower extremity amputations in the United States, with approximately 5% of diabetics developing foot ulcers each year. DFUs typically result from peripheral neuropathy and/or vascular disease. Approximately 15% of DFUs result in lower-extremity amputation.  
35 More than 85% of lower-extremity amputations in patients with diabetes occur in people who have had an antecedent foot ulcer. The estimated cost to the US healthcare system of diabetic foot ulceration and related amputations is more than \$10.9 billion. Mainstays of treatment

5 include debridement, infection control, wound care and offloading. Bioengineered skin substitute to stimulate healing in nonresponding wounds after 4 weeks' treatment is the optimal care. Only a small number of wound-care products have proven their value in accelerating DFU healing in prospective, randomized registration trials. These include becaplermin, a topical gel containing recombinant human platelet-derived growth factor, and  
10 2 living skin equivalents: a bilayered skin substitute and a human fibroblast-derived dermal substitute.

As it is known that leptin is also involved in embryo development and its effects are crucial for embryo pre-implantation as well as the epithelial to mesenchymal cellular  
15 transition necessary for cardiac valve development, and adipocyte differentiation. Nevertheless, leptin effects on the differentiation of ESCs to ECs and angiogenesis has not been fully explored. In addition, it is unknown if JAK/STAT pathway activation plays a pivotal role in this differentiation. The study of these effects on blood vessel development are relevant since endothelial cells exert essential signaling for adequate organogenesis. Without  
20 being bound by any particular theory, the Inventors hypothesized that leptin promotes differentiation of ECs and angiogenesis in ESCs and that these effects are mediated by JAK/STAT pathway activation.

Described herein are the effects of leptin on the differentiation of ECs and  
25 angiogenesis using murine and human EBs *in vitro*. The Inventors generated EBs composed of the three germ layers and demonstrated growth and differentiation effects on mesoderm that are essential to blood vessel formation. The Inventors further found that leptin enhances the differentiation of ECs and promotes intussusceptive angiogenesis. Leptin also induces proliferation and survival of ECs derived from pSCs. Finally, ECs obtained from leptin-  
30 treated EBs co-expressed CD31 and pSTAT3 suggesting that canonical JAK/STAT pathway is involved in the differentiation process. These results improve the understanding leptin effects on vascular differentiation that may be relevant to understand leptin effects in organogenesis as well as enable the derivation of functional ECs for regenerative medicine applications. For instance, endothelial cells derived from leptin-treated ESCs or human  
35 pluripotent stemcells (hPSCs) can be used to promote vascularization of chronic wounds and those tissues damaged by hypoxia or trauma and in this way accelerate wound healing.

5 Described herein is a method of differentiating human pluripotent stem cells into microvessels composed of endothelial cells, including (a) providing a quantity of human pluripotent stem cells (pSCs), (b) inducing formation of embryoid bodies (EBs), (c) generating endothelial cells by culturing the EBs in the presence of leptin, (d) further culturing the endothelial cells on a scaffold composed of at least one extracellular matrix  
10 (ECM) component, wherein the leptin and at least one ECM component form microvessels composed of endothelial cells. In certain embodiments, endothelial cells express CD31<sup>+</sup>. In other embodiments, the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>. In various embodiments, the endothelial cells are functionally mature, and capable of *in vivo* network formation. In other embodiments, the endothelial cells are  
15 capable of angiogenesis and/or vasculogenesis. In other embodiments, the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin. In other embodiments, endothelial cells are isolated via marker expression (e.g., magnetic bead sorting or flow cytometry) before placing onto the scaffold composed of at least one ECM component. In other embodiments, additional quantities of leptin are added to the endothelial cells cultured  
20 on the at least one ECM component. In various embodiments, the leptin is provided to EBs or endothelial cells at a concentration of 0.1-0.5 nM, 0.5-1 nM, 1-5 nM, 5-10 nM or 10 nM or more.

In another embodiment, inducing the formation of embryoid bodies (EBs) can be for multiple days, optionally including the addition of growth factors, exogenous factors or small  
25 molecules. In various embodiments, culturing the pSCs includes inducing the formation of EBs, which can be cultured up to 1, 2, 3, 4, 5, 6, 7 days, 1 week or more, 2 weeks or more, 3 weeks or more, to promote various states of differentiation. For example, EBs can be cultured for 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 days in the presence of leptin to promote endothelial cell formation. In certain embodiments, the EBs can be  
30 cultured in the presence of ROCK inhibitor, or in a high density plate or other apparatus to promote uniformity in shape, size, and consistency in differentiation state of the cells of the EB.

In other embodiments, placing the endothelial cells on a scaffold composed of at least one extracellular matrix (ECM) component includes a scaffold with at least one collagen and  
35 at least one laminin. This includes, for example, a gel mixture containing collagen I, IV, and laminin I. In another embodiment, the ECM component includes at least one ECM component selected from the following: a collagen, a laminin, an integrin, a fibronectin, a proteoglycan, and an elastin. In another embodiment, the ECM component includes collagen

5 I, IV, and laminin I. In other embodiments, the at least one ECM component is deposited on the surface of a biocompatible material suitable for transplantation. Examples of such materials including polyethylene glycol hydrogel, Dacron mesh, Transwell filters and other materials readily known to one of ordinary skill.

For example, EBs can be generated using AggreWell system  
 10 (STEMCELL Technologies, Vancouver, Canada) and according to manufacturer instructions. EBs can be were maintained in AggreWell medium (STEMCELL Technologies, Vancouver, Canada), optionally supplemented with 10  $\mu$ M ROCK inhibitor (Sigma-Aldrich, St. Louis, MO), at 37°C in humidified incubator at 5% CO<sub>2</sub>. Alternatively, EB formation media include IMDM is first prepared in a 15 mL falcon tube on ice (17% KO Serum Replacer, 1% MEM-  
 15 NEAA, 1% L-alanyl-L-glutamine, 110 $\mu$ M Beta-mercaptoethanol, 10 $\mu$ M ROCK inhibitor, remainder up to 100% volume IMDM). Cells are harvested with Accutase, and placed in suspension. For example, from 2-3 confluent (70-80%) wells of a 6-well plate, these cells can make EBs in one 384-well plate. The cells can be counted to ensure that the number of cell plated is in a range of 5000 cells per well of a 384-well plate. It means that the total  
 20 should be approximately  $2 \times 10^6$  cells/plate. The cell suspension is transferred to a tube and spin down at 1100 RPM for 5 min. Supernatant is aspirated and re-suspend in 10 mL of IMDM differentiation media, optionally including 10  $\mu$ M ROCK inhibitor cold matrigel (0.5 mg/384well plate) in the pre-chilled tube in ice. Use 25  $\mu$ L volume cell suspension to plate cells per well, which should be 10 ml/384-well plate. Seeding in the wells constitutes day 0  
 25 for EBs (EBd0). A sterile lid is placed on the plate, spun at 1,400 rpm at 4°C for 10 min, and placed in incubator overnight at 37°C. Optionally, EBs are transferred from the 384-well plate to Petri dishes, with further culturing in the presence of leptin for a period of 10-20 days before being placed and cultured on a scaffold composed of an ECM component. For example, cells can be added collagen I, IV, and laminin I containing 1X MEM, 1 M HEPES  
 30 buffer, 7.5% Bicarbonate solution 0.1 N NaOH, sterile water, 1 mg/mL laminin and 1 mg/mL collagen IV placed in ice. After mixing the cells gently in this solution, endothelial cells-gel mixture solution can be placed in Petri dishes and incubated for gel solidification at 37°C during 10-20 min.

35 Further described herein is a composition including microvessels composed of endothelial cells. In certain embodiments, endothelial cells express CD31<sup>+</sup>. In other embodiments, the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>. In various embodiments, the endothelial cells are functionally mature, and

5 capable of *in vivo* network formation. In other embodiments, the endothelial cells are capable of angiogenesis and/or vasculogenesis. In other embodiments, the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin. In another embodiment, the composition is composed of at least one extracellular matrix (ECM) component includes a scaffold with at least one collagen and at least one laminin. This includes, for example, a gel  
10 mixture containing collagen I, IV, and laminin I. In other embodiments, the at least one ECM component is deposited on the surface of a biocompatible material suitable for transplantation. Examples of such materials including polyethylene glycol hydrogel, Dacron mesh, Transwell filters and other materials readily known to one of ordinary skill.

15 Also described herein is a composition including microvessels composed of endothelial cells produced by method of differentiating human pluripotent stem cells. In various embodiments, the method includes (a) providing a quantity of human pluripotent stem cells (pSCs), (b) inducing formation of embryoid bodies (EBs), (c) generating endothelial cells by culturing the EBs in the presence of leptin, (d) further culturing the  
20 endothelial cells on a scaffold composed of at least one extracellular matrix (ECM) component, wherein the leptin and at least one ECM component form microvessels composed of endothelial cells. In certain embodiments, endothelial cells express CD31<sup>+</sup>. In other embodiments, the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>. In various embodiments, the endothelial cells are functionally mature,  
25 and capable of *in vivo* network formation. In other embodiments, the endothelial cells are capable of angiogenesis and/or vasculogenesis. In other embodiments, the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin. In other embodiments, endothelial cells are isolated via marker expression (e.g., magnetic bead sorting or flow cytometry) before placing onto the scaffold composed of at least one ECM component. In  
30 other embodiments, additional quantities of leptin are added to the endothelial cells cultured on the at least one ECM component. In various embodiments, the leptin is provided to EBs or endothelial cells at a concentration of 0.1-0.5 nM, 0.5-1 nM, 1-5 nM, 5-10 nM or 10 nM or more. In other embodiments, the at least one ECM component is deposited on the surface of a biocompatible material suitable for transplantation. Examples of such materials including  
35 polyethylene glycol hydrogel, Dacron mesh, Transwell filters and other materials readily known to one of ordinary skill.

In another embodiment, inducing the formation of embryoid bodies (EBs) can be for multiple days, optionally including the addition of growth factors, exogenous factors or small

5 molecules. In various embodiments, culturing the pSCs includes inducing the formation of EBs, which can be cultured up to 1, 2, 3, 4, 5, 6, 7 days, 1 week or more, 2 weeks or more, 3 weeks or more, to promote various states of differentiation. For example, EBs can be cultured for 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 days in the presence of leptin to promote endothelial cell formation. In certain embodiments, the EBs can be  
10 cultured in the presence of ROCK inhibitor, or in a high density plate or other apparatus to promote uniformity in shape, size, and consistency in differentiation state of the cells of the EB.

In other embodiments, placing the endothelial cells on a scaffold composed of at least one extracellular matrix (ECM) component includes a scaffold with at least one collagen and  
15 at least one laminin. This includes, for example, a gel mixture containing collagen I, IV, and laminin I. In another embodiment, the ECM component includes at least one ECM component selected from the following: a collagen, a laminin, an integrin, a fibronectin, a proteoglycan, and an elastin. In another embodiment, the ECM component includes collagen I, IV, and laminin I. In other embodiments, the at least one ECM component is deposited on  
20 the surface of a biocompatible material suitable for transplantation. Examples of such materials including polyethylene glycol hydrogel, Dacron mesh, Transwell filters and other materials readily known to one of ordinary skill.

Further described herein is a method of treatment using a composition including  
25 microvessels composed of endothelial cells. In certain embodiments, endothelial cells express CD31<sup>+</sup>. In other embodiments, the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>. In various embodiments, the endothelial cells are functionally mature, and capable of *in vivo* network formation. In other embodiments, the endothelial cells are capable of angiogenesis and/or vasculogenesis. In other embodiments,  
30 the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin. In another embodiment, the composition is composed of at least one extracellular matrix (ECM) component includes a scaffold with at least one collagen and at least one laminin. This includes, for example, a gel mixture containing collagen I, IV, and laminin I. In other embodiments, the composition including microvessels composed of endothelial cells is  
35 produced by method of differentiating human pluripotent stem cells. In various embodiments, the method includes (a) providing a quantity of human pluripotent stem cells (pSCs), (b) inducing formation of embryoid bodies (EBs), (c) generating endothelial cells by culturing the EBs in the presence of leptin, (d) further culturing the endothelial cells on a

5 scaffold composed of at least one extracellular matrix (ECM) component, wherein the leptin and at least one ECM component form microvessels composed of endothelial cells. In different embodiments, the method of treatment is for diabetes, obesity with wound healing impairment, peripheral vascular problems, physical trauma requiring wound healing, burns or similar disease and conditions requiring treatment.

10 In different embodiments, a population of endothelial cells are produced by any of the methods described herein. In another embodiment, the present invention includes a cell line including endothelial cells as produced by any of the methods described herein. In certain embodiments, endothelial cells express CD31<sup>+</sup>. In other embodiments, the endothelial cells  
15 express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>. In various embodiments, the endothelial cells are functionally mature, and capable of *in vivo* network formation. In various embodiments, cells or cell lines produced by the described methods can be expanded for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 10 or more passages without a loss of karyotype stability. In various embodiments, the methods described herein are able to  
20 convert 10, 20, 30, 40, 50, 60, 70, 80, 90, 90% or more of a quantity of pSCs into endothelial cells. In various embodiments, the pSCs are induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs).

25 *Example 1*  
*Cells and reagents*

Mouse ESC line R1 (from [strains 129 /Sv x 129/Sv-CP] F1 3.5-day blastocyst) (Samuel Lunenfeld Research Institute, ON, Canada) passage 20-25 were plated on Mitomycin C (Sigma, St. Louis, MO) -inactivated mouse embryonic fibroblasts (MEFs)  
30 (ATCC, Manassas, VA) as feeder layers. The culture medium for cell maintenance consisted of high glucose Dulbecco Modified Eagle Medium (DMEM-H) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Omega Scientific Inc., Tarzana), 1 mM Sodium Pyruvate, 0.1 mM non-essential amino-acids, 200  $\mu$ M L-glutamine (Invitrogen, Grand Island, NY), 1000 U/mL leukemia inhibitor factor (LIF) (Chemicon, Temecula, CA) and 100  $\mu$ M -  
35 mercaptoethanol (Sigma, St. Louis, MO). MEFs were grown at 37°C under 5% CO<sub>2</sub> in DMEM-H (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (Omega Scientific, Tarzana, CA). To induce formation of embryoid bodies (EBs), mouse ESCs (R1) were cultured in hanging drops after disaggregating with accutase (Innovative Cell Technologies,

5 San Diego, CA). Six hundred cells were plated in each drop of 20  $\mu$ L hanging on the lid of a Petri dish for two days in DMEM-H supplemented with 20% FBS (Omega Scientific Inc., Tarzana, CA). After this time, complete media was added to the cells to keep them in suspension for an additional three days for EB formation.

10 *Example 2*  
*Immunocytochemistry and FACS*

For immunocytochemistry, the EBs plated on coverslips treated or untreated with leptin were fixed with paraformaldehyde 4% (Polysciences, Inc., Warrington, PA) at different  
15 time points and permeabilized with 0.3% triton X-100 in PBS for 5 minutes. After rinsing with PBS, cells were blocked with PBS/5% BSA for 1 hour and exposed overnight using primary antibodies to cardiotin, PARP (AB3565), Ki-67 (Millipore, Billerica, MA), CD31 (BD Biosciences Pharmingen, San Diego, CA), phospho-STAT3 (Cell Signaling Technologies, Danvers, MA), mouse IgG1, and rat IgG2a (isotype controls; Santa Cruz,  
20 Biotechnology, Inc., Santa Cruz, CA). The secondary antibodies used were as follows: Alexa Fluor 555 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rat IgG, and Alexa Fluor 555 goat anti-rat IgG (Molecular Probes, Eugene, OR). All the secondary antibodies were diluted 1:1000 in blocking solution (BSA 5% in 1X PBS). Images were acquired with a multi-purpose zoom microscope (Nikon AZ 100, USA; <http://www.nikon.com/>) attached to a DS-  
25 Qi1 High-sensitivity CCD Camera and analyzed using an imaging software NIS-Elements AR 3.10 (Nikon Instruments, Melville, N.Y.) and the image tools of ImageJ 1.30v software (Wayne Rasband National Institutes of Health; USA). Another group of images were acquired with a TCS SP5 X confocal microscope (Leica Microsystems, Mannheim, Germany).

30 For FACS analysis, about  $1 \times 10^6$  cells were obtained from EBs either untreated or treated with leptin (1 nM) for 10 days. The cell suspension was incubated for 30 min with rat anti mouseCD31 in ice. After this time, the cells were washed several times and treated with the secondary antibody Alexa Fluor 488 goat anti rat for 30 min in ice. After several washes the cells were resuspended in 500  $\mu$ L and analyzed by flow cytometry using BD LSRFortessa  
35 (BD Biosciences, San Jose, CA).

5

*Example 3*  
*Image Analysis*

For image binarization, the images of the EB blood vessels were captured using a Spot camera RT-KE Slider 7.4.2 attached to a Nikon Eclipse TE2000-S fluorescent microscope (Diagnostic Instruments, Inc., Sterling Heights, MI). The SPOT v4.6 software (Diagnostic Instruments, Inc., Sterling Heights, MI) was used to obtain the higher resolution images that were analyzed using the image tools of imageJ software 1.37v (Wayne Rasband National Institutes of Health; USA). The photos were binarized using the image tools of imageJ software 1.37v (Wayne Rasband National Institutes of Health; USA).

15

*Example 4*  
*Leptin and EPO treatment*

Recombinant mouse leptin (Prospec, East Brunswick, NJ) was added directly at 1 nM or 10 nM concentration to growing EBs for 10, 20, and 30 days. The media was replaced with fresh media with leptin every three days. 0.5 U/mL recombinant erythropoietin (EPO) (Stem Cell Technologies, Vancouver, BC) was added directly the media of growing EBs for 10, 20, and 30 days alone or in combination with 1 nM recombinant mouse leptin. EPO was used as positive control of angiogenesis in healthy EBs.

25

*Example 5*  
*Magnetic-activated cell sorting (MACS)*

Magnetic sorting was performed according to manufacturer instructions (Miltenyi Biotec, Auburn, CA). Briefly, after 10 days in culture, EBs treated and untreated with leptin were harvested. One million cells were treated with rat anti mouse anti-CD31 (BD Biosciences Pharmingen, San Diego, CA) as primary antibody for 5 min in ice. After several washes, goat anti-rat IgG conjugated to magnetic beads was added to the cell suspension as secondary antibody and incubated for 15 min in ice. The labeled cells were washed and transfer to a MACS MS column separator (positive selection) and put into a magnetic field. The column was then removed from the separator and placed on a collection tube. Magnetically labeled cells were then collected and plated on NUNC 4-well plates (Thermo scientific, Logan, UT) and transferred at 1:2 split ratio. For cell maintenance, 1 nM recombinant mouse leptin was added to the media consisted in MCDB131 supplemented with 10% FBS (Omega Scientific Inc., Tarzana, CA), 200  $\mu$ M L-glutamine (Invitrogen, Grand

5 Island, NY), and ECs growth supplement (ECGS) (BD Biosciences, San Jose, CA, USA). Cells obtained either from leptin-treated EBs or untreated EBs were cultured in the same medium.

### Example 6

#### Quantitative real time RT-PCR (qRT-PCR)

10 Total RNA was isolated from 100 EBs cultured alone treated or untreated with leptin using RNAeasy mini kit (Qiagen, Valencia, CA). After cDNA synthesis, using a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA), quantitative real-time PCR analysis was performed using SYBR Green RT-PCR kit (Qiagen, Valencia, CA) and the LightCycler instrument (AB Applied Biosystems, Foster City, CA). PCR cycle conditions included a first step for initial polymerase activation for 10 minutes at 95°C and 45 cycles of denaturation at 15 94°C for 30 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds. The forward and reverse primers used (all sequences are 5'-3') were as follows:

#### *CD31:*

GCTTGGCAGCGAAACACT (SEQ ID NO: 1) and  
TGGGAGGTGATGAATGGG (SEQ ID NO: 2)

#### *CD34:*

TAGCACAGAACTTCCCAGCAAAC (SEQ ID NO: 3) and  
CTCAGATCACAGTTCTGTGTCAGC (SEQ ID NO: 4)

#### *Endothelial nitric oxide synthase (eNOS):*

CCCCACAGCTCTGCATTCA (SEQ ID NO: 5) and  
CACCCAGTCAATCCCTTTGG (SEQ ID NO: 6)

#### *von Willebrand factor (vWF):*

GTCATTGTGATGGTGTCAACTT (SEQ ID NO: 7) and  
35 AGCACCTCTGTAGCACCA (SEQ ID NO: 8)

#### *fms-like tyrosine kinase 1 (FLT-1):*

GAGGAGGATGAGGGTGTCTATAGGT (SEQ ID NO: 9) and  
GTGATCAGCTCCAGGTTTGAATT (SEQ ID NO: 10)

#### *Fetal liver kinase 1 (FLK-1):*

GCCCTGCTGTGGTCTCACTAC (SEQ ID NO: 11) and  
CAAAGCATTGCCCATTCGAT (SEQ ID NO: 12)

#### *Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1):*

CAAGGTCACACACCGGTGAA (SEQ ID NO: 13) and  
GCCAGTCTAGGGTATTGAAGTAGGA (SEQ ID NO: 14)

*TEK tyrosine kinase (Tie-2):*

ATGTGGAAGTCGAGAGGCGAT (SEQ ID NO: 15) and  
CGAATAGCCATCCACTATTGTCC (SEQ ID NO: 16)

*Angiopoietin-1 (Ang1):*

CATTCTTCGCTGCCATTCTG (SEQ ID NO: 17) and  
GCACATTGCCCATGTTGAATC (SEQ ID NO: 18)

*Angiopoietin-2 (Ang2):*

TTAGCACAAAGGATTTCGGACAAT (SEQ ID NO: 19) and  
TTTTGTGGGTAGTACTGTCCATTCA (SEQ ID NO: 20)

*Glyceraldehyde-3-phosphate dehydrogenase:*

(GAPDH), ATTGACCACTACCTGGGCAA (SEQ ID NO: 21) and  
GAGATACACTTCAACACTTGACCT (SEQ ID NO: 22)

Negative controls were included in each analysis (No RT). All samples were run in triplicate and PCR products were observed by gel electrophoresis on 2% agarose ethidium bromide-stained gels. Analysis was performed using 7300 Sequence Detection Software (SDS) Version 1.3 After real time PCR, a dissociation curve was run to detect primer dimmers, contaminating DNA, and PCR products from misannealed primers. The Inventors used a standard curve obtained by running a GAPDH-plasmid with a known copy-number value based on its molecular weight. The standard curve was used as a reference for extrapolating quantitative information for mRNA targets of unknown concentrations. In this way the absolute number of copies was determined for each marker. The absolute number of copies of the specific marker was then divided by the absolute number of copies of GAPDH of the same sample for normalization (mouse housekeeping gene).

#### *Example 7* *Statistics*

Data are expressed as mean  $\pm$  standard error of absolute quantification of gene expression values from three independent experiments. To find significant differences in the tested EB groups, the values were assessed by Student's *t*-test using GraphPad Prism software (GraphPad Software v5.01, Inc. La Jolla, CA).

#### *Example 8* *Leptin promotes angiogenesis in mouse EBs*

EBs developed in hanging drops for two days and then, after suspension for three more days, EBs were cultured in dishes with coverslips. After attachment, EB cells spread

5 out at the periphery. After ten days in culture, enhancement in the number of blood vessels was observed in leptin-treated EBs (Fig. 1c, d) in contrast to few primitive vessels observed in controls. The pattern observed resembled intussusceptive angiogenesis instead of branching. EB cells stained positive for Ki-67 indicating cell proliferation and negative for cleaved poly ADP-ribose polymerase (PARP) (not shown) indicating a lack of apoptosis. EB  
10 blood vessel area was quantified using the image tools of ImageJ (see methods). Once the images were binarized in ten random fields of EBs center, the total area occupied by the blood vessels was quantified as a measurement of vascular density. Higher blood vessel density was observed in leptin-treated EBs (Figs. 1a, b, and e [white bar]) in comparison to untreated EBs (Figs. 1c, d, and e [black bar]). The angiogenic effects of leptin were dose-  
15 dependent. The blood vessel pattern in EBs treated with 1 nM leptin (Fig. 2a) (Fig. 2b) contrasted with 10 nM leptin where blood vessel remodeling, branching, and tortuosity was observed (Fig. 2b). As positive control of angiogenesis the Inventors used erythropoietin (EPO). EPO induces angiogenesis mainly by EC proliferation as depicted by thicker blood vessels with intussusceptive and branching angiogenesis (Fig. 2c). The Inventors reproduced  
20 these experiments in our model and compared these effects with angiogenesis induced by leptin treatment alone. Synergistic effects were observed using leptin and EPO together with abundant thick blood vessels in networks (Fig. 2d). Untreated controls showed scarce blood vessel formation (Fig. 2e) and no CD31 staining in isotype controls (Fig. 2f). These data indicate that leptin promotes angiogenesis by enhancement of EC proliferation,  
25 intussusception, and remodeling in mouse EB blood vessels.

#### *Example 9*

##### *Leptin induces upregulation of endothelial-cell genes and genes associated with angiogenesis*

30 In order to test the response to leptin as function of EB age, the Inventors evaluated the expression of endothelial genes by qRT-PCR in EBs treated with two leptin concentrations (1 nM and 10 nM) at 10 (EBd10), 20 (EBd20), and 30 (EBd30) days of age. The normal physiological concentration of leptin in the plasma is about 1 -10 ng/ml, a concentration that corresponds to 0.06 to 0.6 nM. As expected, higher expression of CD31  
35 was observed in EBd10 and EBd20 with 1 or 10 nM leptin (Fig. 3a). Similar effects were observed in the expression of eNOS (Fig. 3b), vWF (Fig. 3c) and CD34 (Fig. 3d). However, down-regulation of some markers such as CD31, eNOS, and CD34 (Figs. 3a, b, and c) was also observed at day 30 especially with 1 nM leptin. Low endothelial marker expression was observed at day 10, 20, and 30 in untreated EBs which confirms previous observations.

5 Furthermore, the long form of leptin receptor was upregulated at day 20 but apparently this upregulation is no-leptin dependent since leptin receptor expression is not affected by 1 nM of leptin treatment (Fig. 3e). Expression of angiogenesis markers was also regulated by leptin in these EBs. Expression of angiogenic markers was also regulated by leptin in these EBs. For instance, the expression of FLK1 was up-regulated by leptin in EBd10 and 20 and down-regulated in EBd30 (Fig. 4a) while FLT-1 was down-regulated in early EBs (EBd10 and 20) and up-regulated in late EBs (EBd30) (Fig. 4b). Tie1 was up-regulated in EBd10 and 20 while minimal expression was observed at EBd30; whereas, Tie2 was up-regulated only at EBd10 (Fig. 4d). Finally, Ang1 (Fig. 4e) and Ang2 (Fig. 4f) were up-regulated mainly in early EBs (EBd10 and 20) with no significant leptin effects at EBd30. These results indicate that leptin effects on EC differentiation and angiogenesis in mouse EBs depend on leptin concentration and EB age.

#### Example 10

##### *ECs derived from leptin-treated EBs can be isolated and expanded*

20 Leptin-treated EBs showed enhanced vasculature (Fig. 5a) in comparison to untreated EBs (Fig. 5b). Quantification of CD31 expression by FACS indicated higher expression of this EC marker in the cell population derived from leptin-treated EBs (Fig. 5c) compared to untreated controls (Fig. 5d). Isolation by magnetic sorting resulted in more cellular yield and enhanced survival of CD31 positive cells when derived from leptin-treated EBs (Fig. 5e ,f). Surprisingly, some clusters of cells derived from leptin-treated EBs that expressed CD31 were composed of cells that also expressed cardiotin (cardiomyocyte marker) (yellow arrows in Fig. 5g). This finding was not evident in controls (Fig. 5f). In addition, unlike controls, CD31 positive cells from leptin-treated EBs tended to form tube-like structures (Figs. 5h). Such sorted cells not only survived but could be expanded up to three passages. These data confirm previous observations and indicate that leptin promotes survival of isolated EC derived from mouse ESCs.

#### Example 11

##### *Endothelial cells derived from leptin-treated EBs co-express CD31 and phosphoSTAT3 (pSTAT3)*

One of the best characterized pathways in leptin signaling is the janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway. After activation of leptin receptor (OB-R), an Ob-R/JAK2 complex is formed that results in

5 receptor phosphorylation. This phosphorylation is crucial to STAT3 phosphorylation (pSTAT3) and nuclear translocation.

The Inventors studied the expression of pSTAT3 in isolated ECs from EBs treated and untreated with leptin. Cells derived from leptintreated EBs had co-expression of CD31 and pSTAT (Figs. 6a, b, c). The inset in Fig. 6c shows a higher magnification image in which  
10 expression of CD31 is more intense in the intercellular junction and nuclear expression of pSTAT. Many clusters with cells that co-expressed these proteins were identified in these cultures (yellow arrows in Fig. 6a, b, c). In contrast, no co-expression was found in CD31 positive cells derived from untreated EBs (Figs. 6d, e, f). These data suggest that leptin promotes the differentiation of ECs through STAT3 activation.

15  
*Example 12*  
*Formation of tube-like structures in iECs treated or untreated with leptin in collagen-laminin gels*

20 The Inventors have extended the above observations for generation of human induced pluripotent stem cell (hiPSC) derived endothelial cell (ECs). Specifically, hiPSC EBs are formed, pre-treated with leptin (10 nM) for approximately 10-20 days. Following recovery of CD31<sup>+</sup> EC candidate cells via magnetic bead sorting, these cells are and plated on scaffolds composed of collagen I, IV, and laminin I. Cells are fed with basal media for  
25 microvascular endothelial cells and additional leptin treatment (10 nM) for maintenance is provided at this time. Importantly, unlike other EC cell differentiation techniques, the described method allows for production of EC cells with microvessel network architecture. Fig. 9.

Functional properties of the resulting scaffold can be studied via transplantation.  
30 Digestion via collagenase allows for recovery of the EC cells. For a period of approximately 12 months, scaffold performance can be observed in normal or leptin receptor deficient mice (with impaired wound healing). Vascularization, as evaluated by Doppler ultrasound, can establish changes in blood flow quantification.

35  
*Example 13*  
*Leptin enhances expression of endothelial-cell and angiogenesis markers*

The Inventors evaluated co-expression of CD31 with the main angiogenesis markers by ICC. Higher number of cell clusters and networks that coexpressed CD31 and TIE2 was

5 observed in EBs treated with 1 nM leptin for ten days (Fig. 10a, b, c). In contrast, lower number of clusters that coexpressed these markers was found in untreated EBs (Fig. 10d, e, f). Another angiogenesis marker, FLK-1/VEGFR2, was also abundant in networks that expressed CD31 in leptin-treated EBs (Fig. 10g, h, i). In contrast, no co-expression was observed in untreated EBs (Fig. 10j, k, l). Higher co-expression of CD31 and angiopoietin-2 (Ang2) was also observed in several clusters of leptin-treated EBs (Fig. 10m, n, o) in comparison to untreated EBs with few clusters positive for these markers (Fig. 10p, q, r). The isotype controls indicated no unspecific staining (Fig. 10s, t, u).

#### Example 14 Discussion

15 Embryonic stem cells (ESCs) have the ability to self-renew and differentiate into all cell types of an organism. When these cells are plated in hanging drops, embryoid bodies (EBs) are formed and spontaneous differentiation gives rise to cells from three germ layers (endoderm, mesoderm, and ectoderm). Plated EBs attach to the substrate and spread out to form a monolayer. In this way differentiated cells can be analyzed by conventional methods. This *in vitro* EB model lends itself to the assessment of growth factors, drugs and other substances on cell differentiation of any germ layer. Leptin is a hormone with multiple biological effects discovered first as an adipokine with an important role in the regulation of food intake in mammals. Other important effects of this hormone have been recently discovered. However, leptin role in differentiation of embryonic stem cells toward endothelial cells as well as angiogenesis in embryonic vessels is still under investigation. To explore such leptin effects *in vitro*, the Inventors used EBs as model. The Inventors analyzed the expression of ECs and angiogenesis specific markers at 10, 20, and 30 days of EB age. In the present work, leptin treatment caused an increase in vascular density that was more evident during the first 10-20 days of EB development. Similar to leptin effects, other factors such as vascular endothelial growth factor (VEGF) have been tested in human EBs with formation of functional blood vessels. Effects of erythropoietin (EPO) have also been tested in EBs and this angiogenic effects were used as positive control of angiogenesis in our EB model. The Inventors confirmed the angiogenic effects of EPO in healthy EBs and compared them to leptin-treated EBs. While EPO induced endothelial cell (EC) proliferation, leptin effects were more apparent in vascular remodeling through intussusceptive angiogenesis. The Inventors have describe these effects previously using EBs cultured on quail chorioallantoic membranes. Leptin angiogenic effects on adult blood vessels can be mediated through

5 synergism with FGF-2 and VEGF. Other investigations indicate that leptin and interleukin-1 (IL-1) upregulate VEGF and, in this way, promote angiogenesis. In our model, VEGF receptor 1(Flt-1) was downregulated by leptin at early EB stages and upregulated at late EB stages. On the contrary, VEGF receptor 2 (Flk-1) was upregulated at early stages and downregulated at late stages. Then, leptin modulates the regulatory effects exerted by Flt-1 on Flk-1  
10 bioactivity. In addition, the Inventors observed increase of ANG1 and ANG2 in EBs at days 10 and 20. However, more production of ANG1 was observed. It is known that ANG1 is mainly produced by perivascular cells. This ligand interacts with Tie2 leading to recruitment of perivascular cells for vessel stabilization. Conversely, ANG2 acts as antagonist of Tie2 and it is produced by ECs. Since the Inventors did not detect upregulation of Tie2 in EBs at  
15 days 20 or 30, the Inventors assumed that most of the ANG1/2 effects observed were exerted at early ages of EBs. Taken together, our results suggest that stabilization driven by ANG1-Tie2 and vessel remodeling driven by ANG2-Tie2 is taking place at the same time. However, higher upregulation of ANG1 may explain the dominant increase and vessel stabilization at early EB stages. Some EC markers were downregulated in our *in vitro* system at 30 days of EB  
20 age. These EBs responded only at higher leptin concentrations suggesting that VEGF or leptin receptor were less sensitive at this EB age.

Additionally, leptin induced downregulation of FLK-1 and upregulation of FLT-1 at late EB stages. These modulatory effects may explain the diminished expression of some EC markers (CD31, eNOS, and CD34) in EBs at day 30. However, another explanation is that  
25 leptin may exert opposite effects at lower concentrations which has also been reported. Upregulation of eNOS, vWF, and CD34 was observed only at higher leptin concentrations suggesting changes in leptin sensitivity and that some angiogenic effects may not be mediated by VEGF at late EB stages. Therefore, some effects can be direct through activation of a leptin receptor. Several isoforms of this receptor have been identified. In our model, we  
30 observed that leptin increased the expression of EC markers during the first 20 days of EB development as confirmed by expression of the long form of leptin receptor in mouse EBs during this period. The Inventors observed higher levels of pSTAT3 in the nuclei of ECs derived from leptin-treated EBs suggesting that JACK/STAT signaling may be involved in leptin-mediated EC differentiation and angiogenesis. Sorted ECs survived up to five passages  
35 in culture suggesting that leptin may also enhance *in vitro* viability of ESC-derived ECs. A previous study reported that ESCs yielded low number of ECs that exhibited poor *in vitro* propagation characteristics. Another study suggested that leptin increased proliferation and survival of human ECs which is consistent with our results. Our results also indicated that

5 leptin increased the number of cardiotin-expressing cells. This effect could be due to leptin-mediated activation of VEGF receptors and is shown to be important in cardiomyocyte differentiation of ESCs.

It has been reported that leptin increases expression of VEGF and Flk-1 and, in this way, promotes angiogenesis. In addition to VEGF-mediated leptin effects, leptin angiogenic  
10 effects can be direct through activation of a leptin receptor. Several isoforms of this receptor have been identified. Leptin receptor (ObRb) is member of gp 130 family of cytokine receptors that stimulate gene transcription through STAT activation by phosphorylation and linkage to activation of VEGF receptor 2 (VEGFR2) with subsequent signaling by p38  
15 <sup>MAPK</sup>/Akt/COX-2. Once activated by leptin, the long form of leptin receptor becomes phosphorylated and controls STAT3 activation, pSTAT3 nuclear translocation and subsequent specific gene expression. In our current model, the Inventors observed that leptin increased the expression of EC markers during the first 20 days of EB development as confirmed by expression of the long form of leptin receptor in mouse EBs during this time period. At EBday30, these effects could only be achieved at higher leptin concentrations  
20 suggesting that leptin sensitivity decreases over time. The Inventors also observed the expression of nuclear pSTAT3 in ECs derived from leptin treated EBs suggesting that this phosphorylation is an important step in leptin-mediated EC differentiation and angiogenesis.

Blood vessels and cardiomyocytes have been found to develop “spontaneously” within EBs. Apparently, factors are released during the three germ layer structure formation  
25 that favor the development of endothelium. Regarding these factors, the Inventors recently identified the expression of bone morphogenetic protein 2 and 4 (BMP-2 and BMP-4), which are considered pro-angiogenic factors, in tumors, mouse EBs, and primary cultures of mouse dermal ECs. The downregulation of CD31, eNOS, and CD34 observed in our model, can be related to downregulation of Flk-1 at 1 nM leptin. Interestingly, at the same EBs age, Flt-1 is  
30 upregulated and may explain the diminished activity of Flk-1.

However, another explanation is that leptin may exert opposite effects at lower concentrations. For instance, paradoxical leptin effects have been reported in mouse embryos treated with different leptin concentrations. In fact the Inventors observed downregulation of Flk-1 at 1 nM leptin that apparently caused decreased expression of EC markers. At this  
35 leptin concentration, vWF expression was similar to controls suggesting more heterogeneity of ECs in which some of them do not produce vWF. According to this fact, it has been reported that vWF expression is regulated by specific microenvironment and therefore upregulation of these factor may not be seen in some ECs.

5           The Inventors obtained survival of approximately 65% of CD31 positive cells *in vitro* and transferred them up to three passages. Previous work described lower cell yields with and no *in vitro* propagation of ECs derived from human ESCs. Leptin has been shown to increase expression of the apoptosis inhibitor (bcl-2) and reduce the expression of cell cycle checkpoint genes p53 and p21 with subsequent hyper-phosphorylation of the Retinoblastoma protein (pRb) resulting in proliferation and survival in human ECs consistent with our results presented herein. Our results also determined that leptin increased the number of cells that expressed cardiotin - a cardiomyocyte marker. These cells when sorted together with ECs grew *in vitro* and formed small cell aggregates. The effects of leptin on cardiomyocyte differentiation have not been completely explored. VEGF activation has been reported to promote cardiomyocyte differentiation from ESCs. Leptin may have a similar effect. Furthermore, it has been reported that some cells define as cardiohemangioblasts co-express markers such as Flk-1, CD31, and VE-cadherin and are able to generate cardiac cells as well as endothelial and hematopoietic cells. After isolation of CD31 positive cells the Inventors observed the presence of cardiotin positive cells in some clusters. This finding suggests that some CD31 positive cells may give rise to cardiac cells and therefore its proliferation may be promoted by leptin. However, further studies should be done to this respect to elucidate the role of leptin in cardiohemangioblast generation from ESCs.

25           The various methods and techniques described above provide a number of ways to carry out the invention. Of course, it is to be understood that not necessarily all objectives or advantages described may be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as may be taught or suggested herein. A variety of advantageous and disadvantageous alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several advantageous features, while others specifically exclude one, another, or several disadvantageous features, while still others specifically mitigate a present disadvantageous feature by inclusion of one, another, or several advantageous features.

35           Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be

5 mixed and matched by one of ordinary skill in this art to perform methods in accordance with principles described herein. Among the various elements, features, and steps some will be specifically included and others specifically excluded in diverse embodiments.

Although the invention has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the  
10 invention extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

Many variations and alternative elements have been disclosed in embodiments of the present invention. Still further variations and alternate elements will be apparent to one of skill in the art. Among these variations, without limitation, are sources of pluripotent stem  
15 cell (pSC)-derived endothelial cells, compositions of such pSC-derived cells, methods of producing pSC-derived endothelial cells, and the particular use of the products created through the teachings of the invention. Various embodiments of the invention can specifically include or exclude any of these variations or elements.

In some embodiments, the numbers expressing quantities of ingredients, properties  
20 such as concentration, reaction conditions, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term “about.” Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some  
25 embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable. The numerical values presented in some  
30 embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

In some embodiments, the terms “a” and “an” and “the” and similar references used in the context of describing a particular embodiment of the invention (especially in the context of certain of the following claims) can be construed to cover both the singular and the  
35 plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any

5 suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language  
10 in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group can be included in, or deleted from, a group for  
15 reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations on those preferred  
20 embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the invention can be practiced otherwise than specifically described herein. Accordingly, many embodiments of this invention include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law.  
25 Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above cited references and printed publications are  
30 herein individually incorporated by reference in their entirety.

In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that can be employed can be within the scope of the invention. Thus, by way of example, but not of  
35 limitation, alternative configurations of the present invention can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present invention are not limited to that precisely as shown and described.

## THE CLAIMS

1. A method of differentiating human pluripotent stem cells into microvessels comprising endothelial cells, comprising:
  - (a) providing a quantity of human pluripotent stem cells (pSCs),
  - (b) inducing formation of embryoid bodies (EBs),
  - (c) generating endothelial cells by culturing the EBs in the presence of leptin, and
  - (d) further culturing the endothelial cells on a scaffold comprising at least one extracellular matrix (ECM) component, wherein the leptin and at least one ECM component form microvessels comprising endothelial cells.
2. The method of claim 1, wherein inducing generating endothelial cells comprises culturing EBs for 7 to 23 days in the presence of leptin
3. The method of claim 1, wherein the endothelial cells in the microvessel express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>.
4. The method of claim 1, wherein the endothelial cells are isolated before placing onto the scaffold comprising at least one ECM component.
5. The method of claim 1, wherein the scaffold comprising at least one extracellular matrix ECM component comprises at least one collagen and at least one laminin.
6. The method of claim 5, wherein the at least one extracellular matrix ECM component comprises collagen I, IV, and laminin I.
7. The method of claim 1, wherein the scaffold comprising at least one extracellular matrix ECM component comprises a biocompatible material suitable for transplantation.
8. The method of claim 1, wherein further culturing the endothelial cells on a scaffold comprising at least one extracellular matrix (ECM) component comprises leptin addition.
9. A composition produced by the method of claim 1.
10. A composition, comprising:

a quantity of microvessels comprising endothelial cells, wherein the endothelial cells are capable of angiogenesis and/or vasculogenesis.

11. The composition of claim 10, wherein the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin.

12. The composition of claim 10, comprising at least one extracellular matrix (ECM) component.

13. The composition of claim 12, wherein the at least one ECM component comprises at least one collagen and at least one laminin.

14. The composition of claim 13, wherein the at least one ECM component comprises collagen I, IV, and laminin I.

15. The composition of 10, comprising a biocompatible material suitable for transplantation.

16. The composition of claim 10, wherein the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>.

17. A method of treatment, comprising:

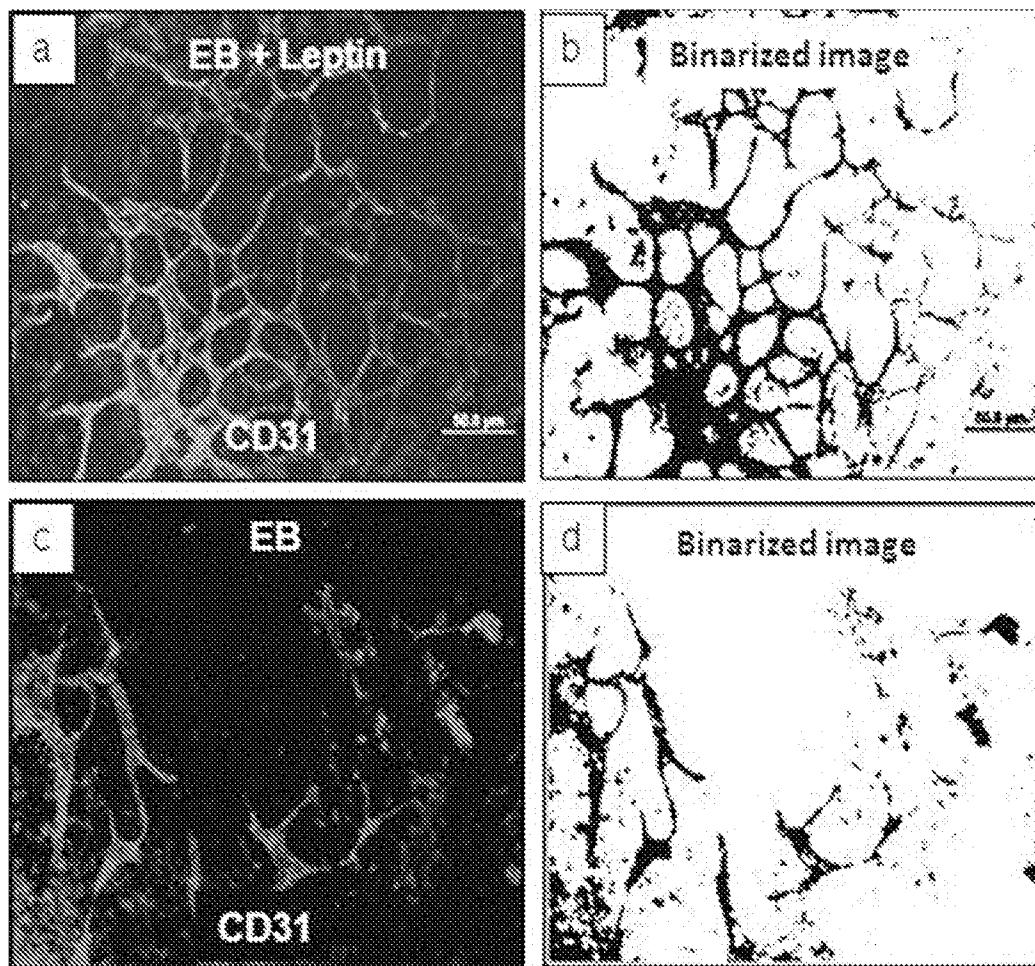
providing a composition comprising microvessels comprising endothelial cells,  
administering to a subject in need of treatment for wound repair, the composition comprising microvessels comprising endothelial cells, wherein *in vivo* formation of a capillary network treats the subject.

18. The method of claim 17, wherein the endothelial cells synthesize and/or release nitric oxide (NO) and/or prostacyclin.

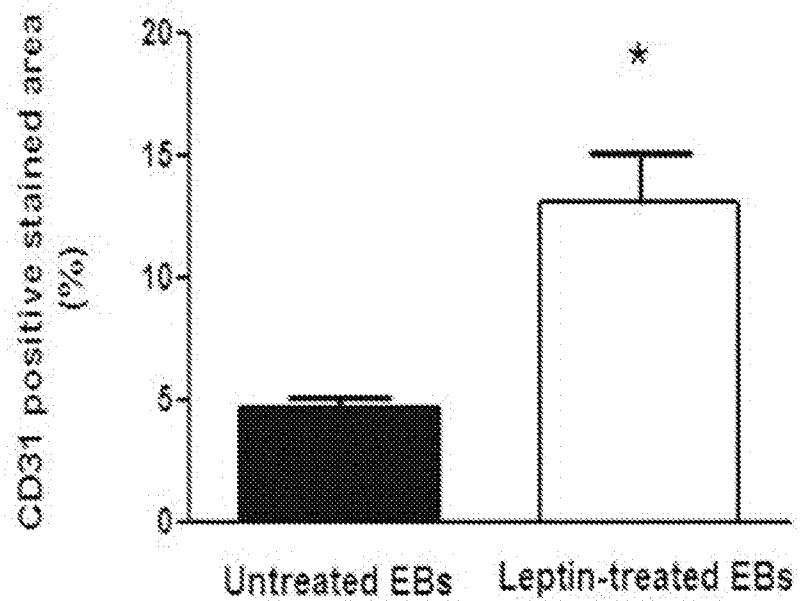
19. The method of claim 17, wherein the composition comprises at least one extracellular matrix (ECM) component.

20. The method of claim 19, wherein the at least one ECM component comprises collagen I, IV, and laminin I.

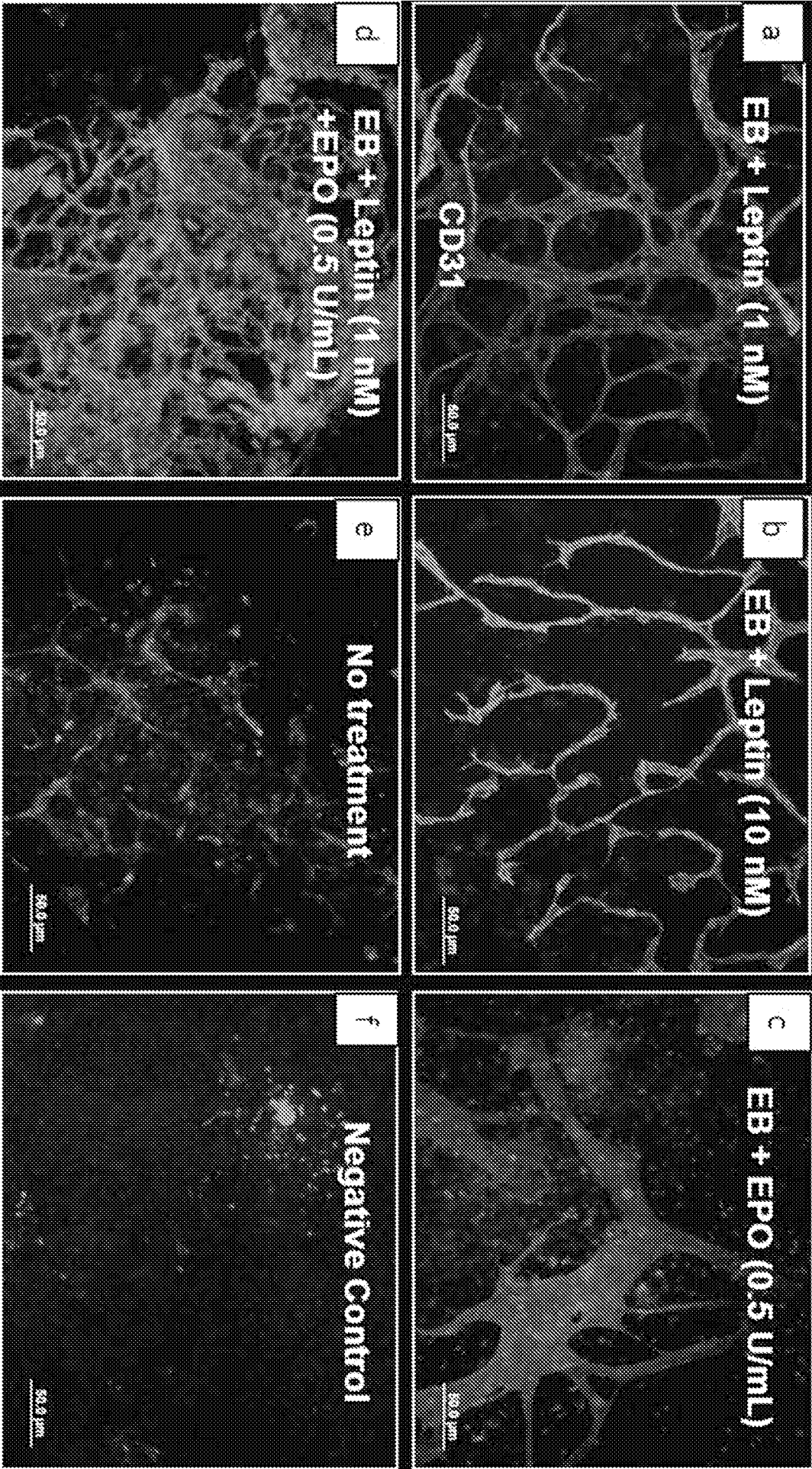
21. The method of 17, comprising a biocompatible material suitable for transplantation.
22. The method of claim 17, wherein the endothelial cells express one or more of CD31<sup>+</sup>, VE-CAM<sup>+</sup>, VWF<sup>+</sup>, KLF-2<sup>+</sup>, and KLF-4<sup>+</sup>.
23. The method of claim 17, wherein the subject in need of treatment for wound repair is afflicted with diabetes, obesity with wound healing impairment, peripheral vascular problems, physical trauma requiring wound healing, or burns.

**Figure 1.**

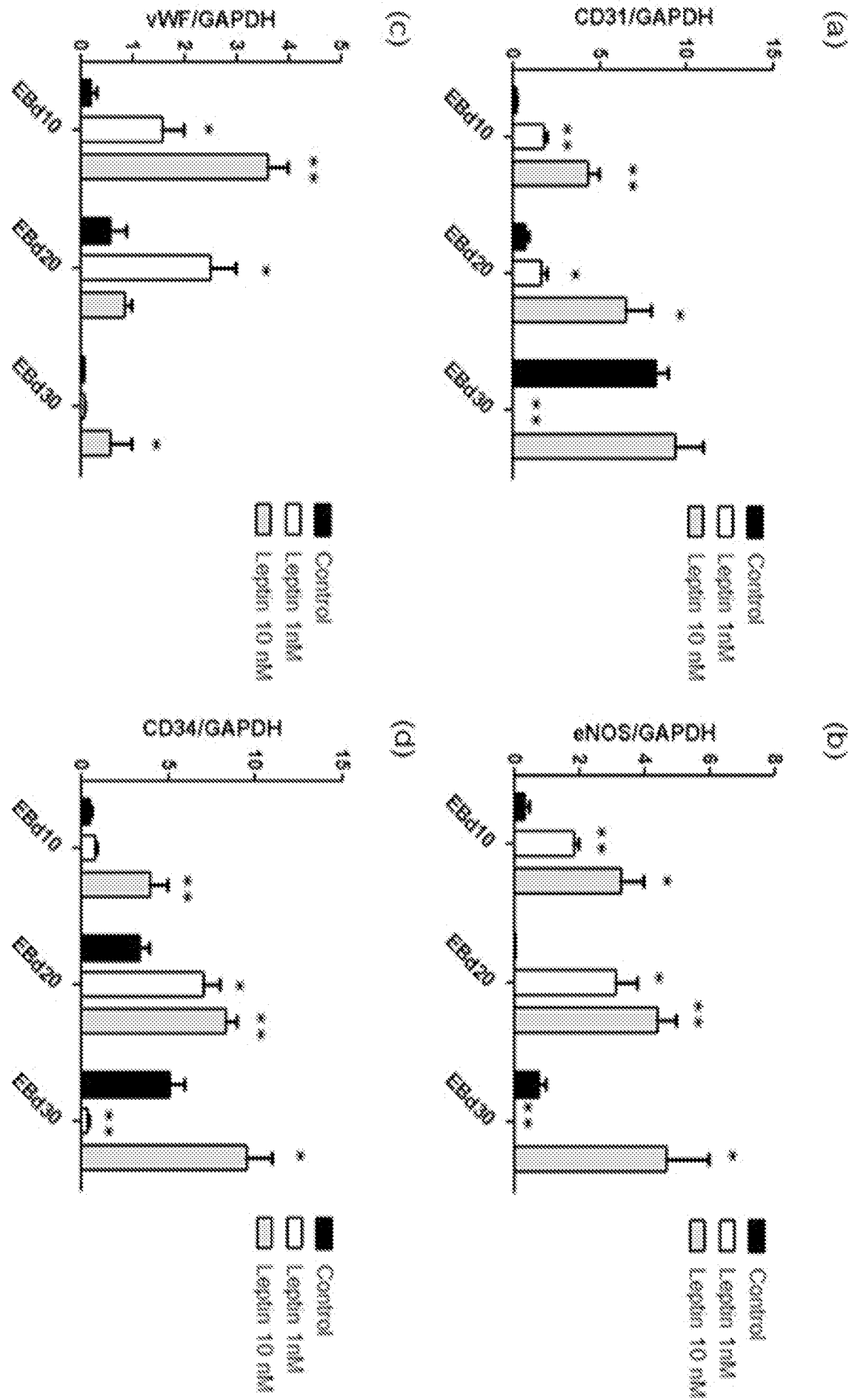
(e)



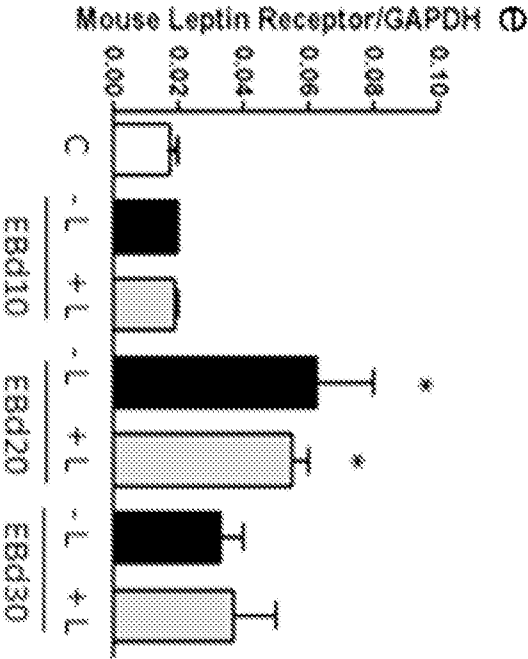
**Figure 2.**



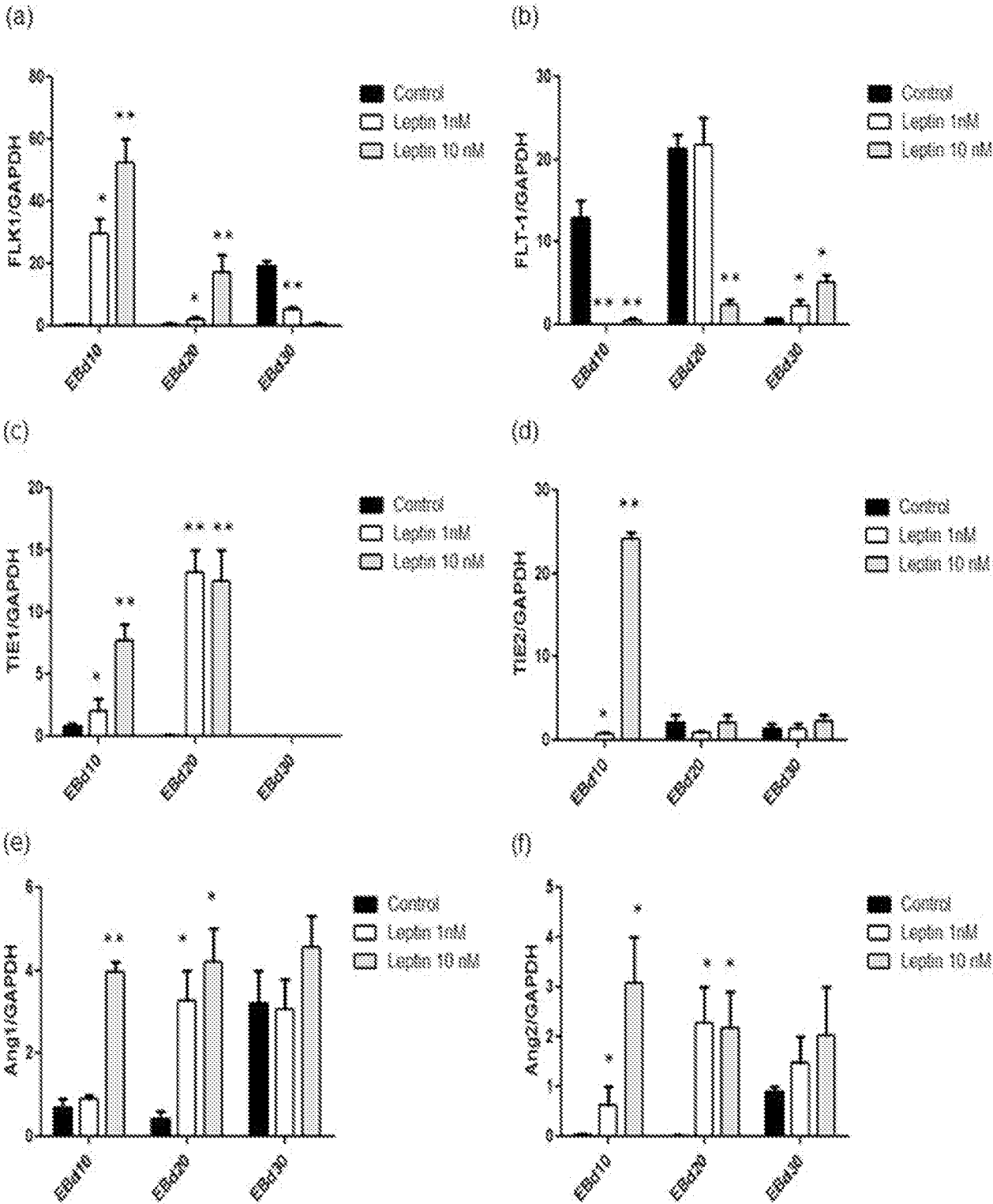
**Figure 3.**



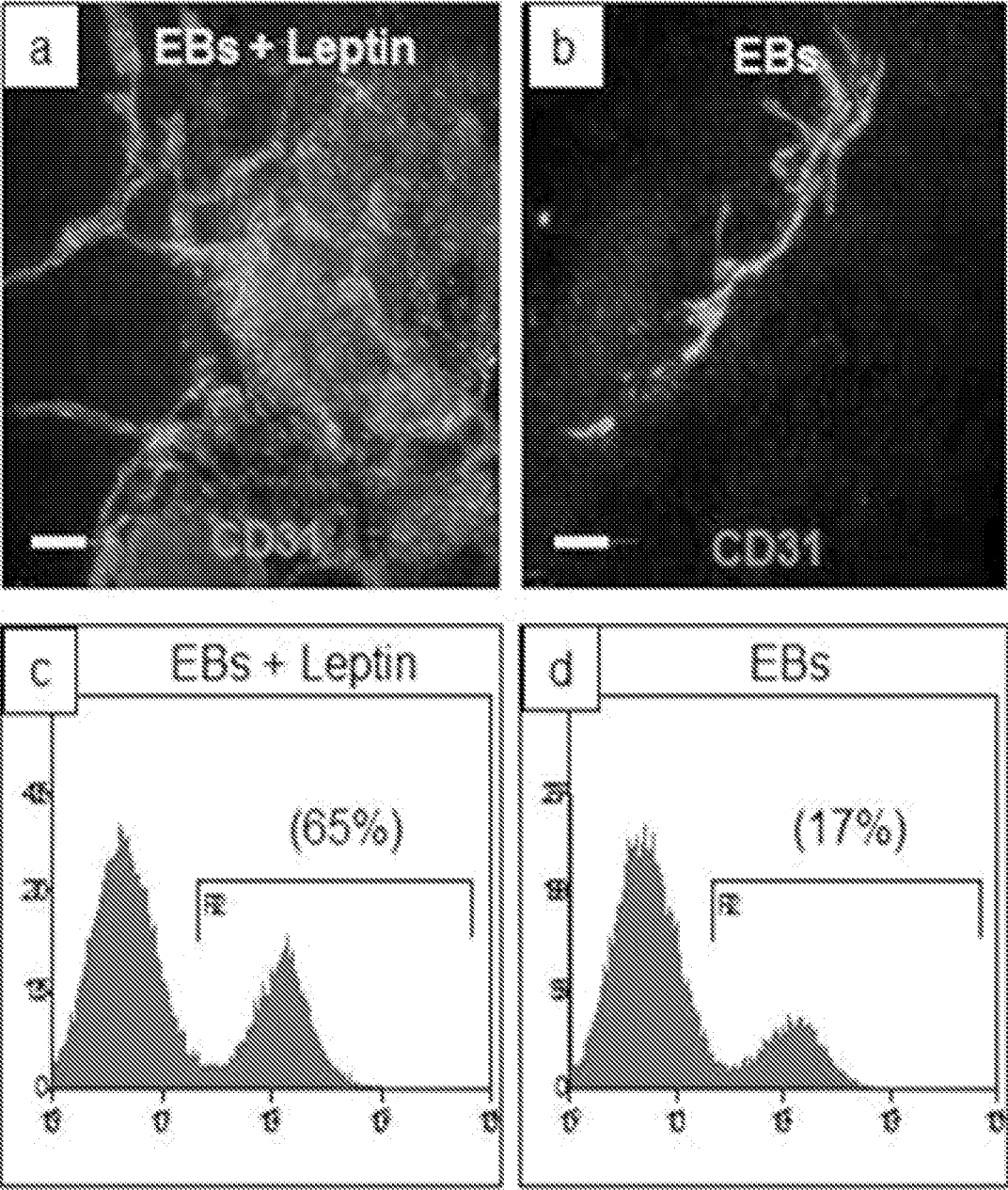
**Figure 3.**



**Figure 4.**



**Figure 5.**



**Figure 6.**

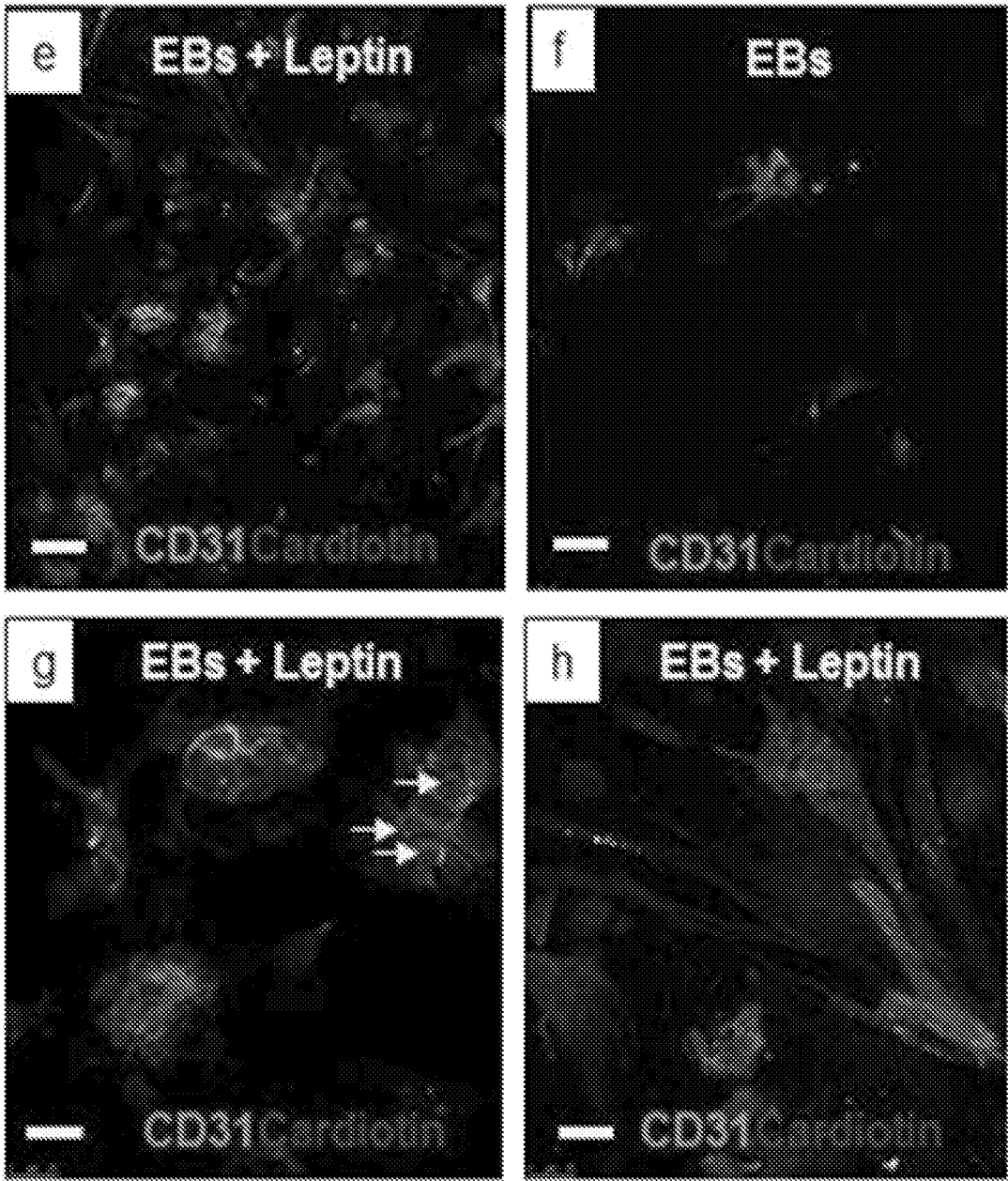
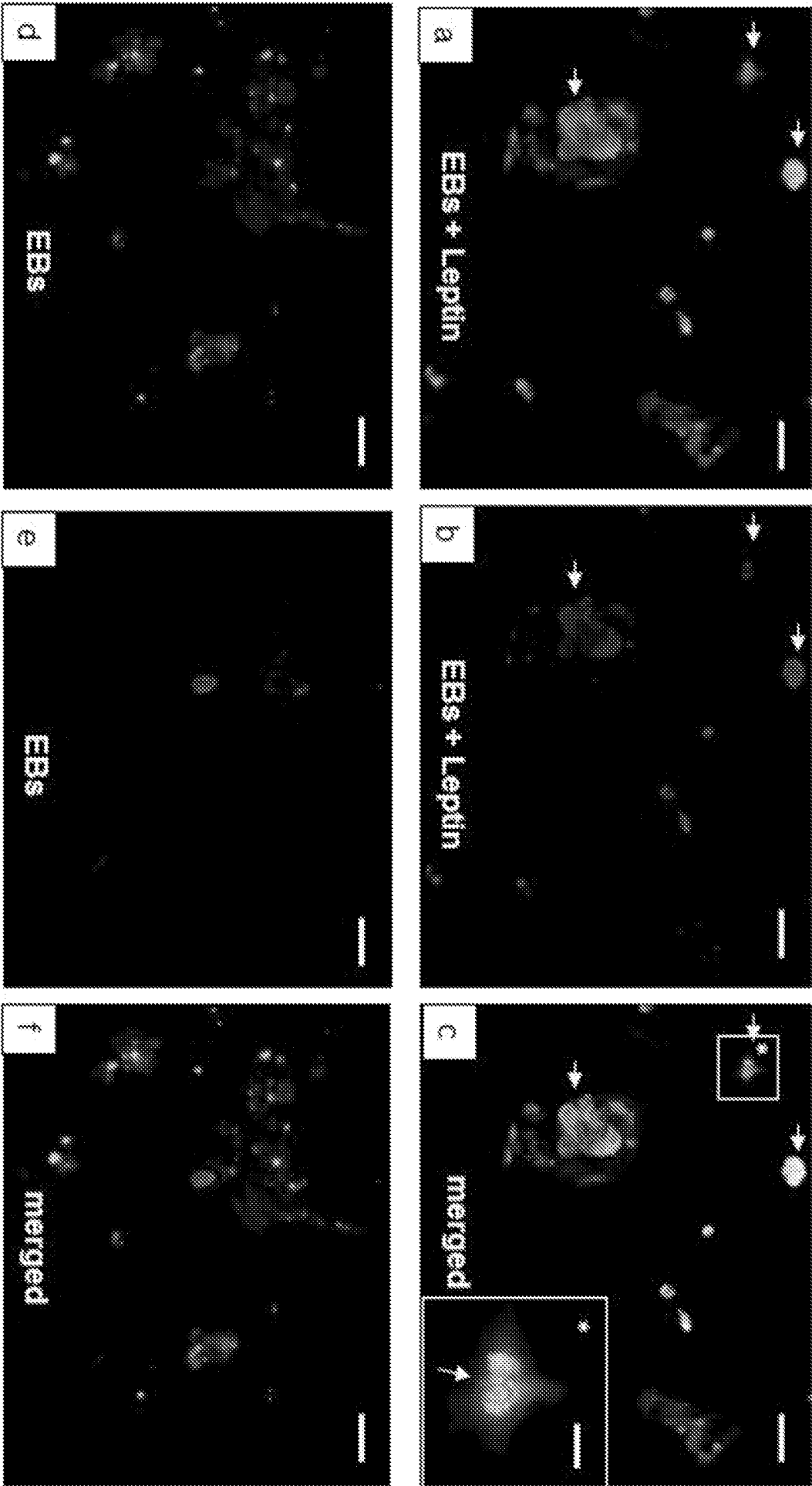
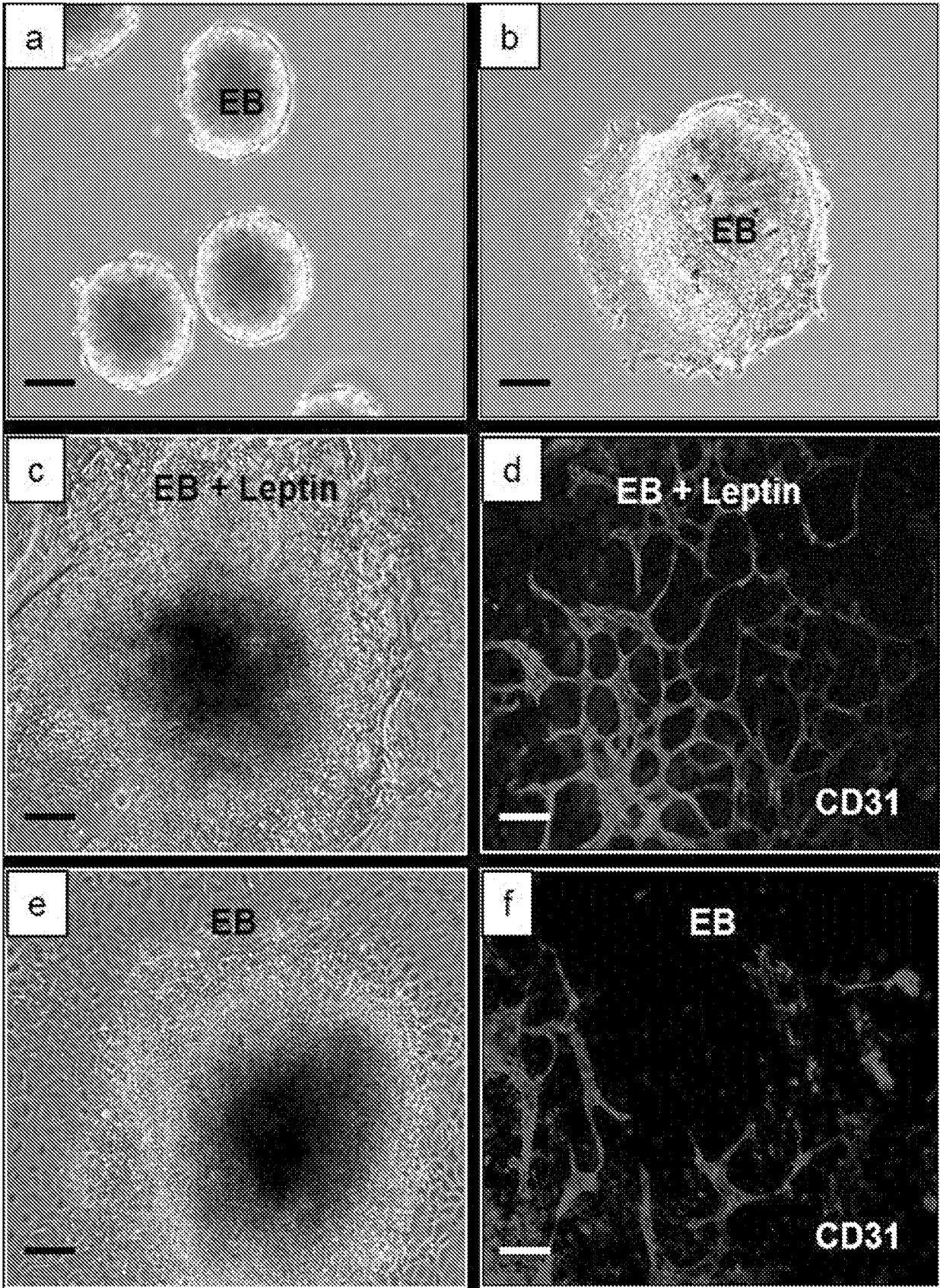


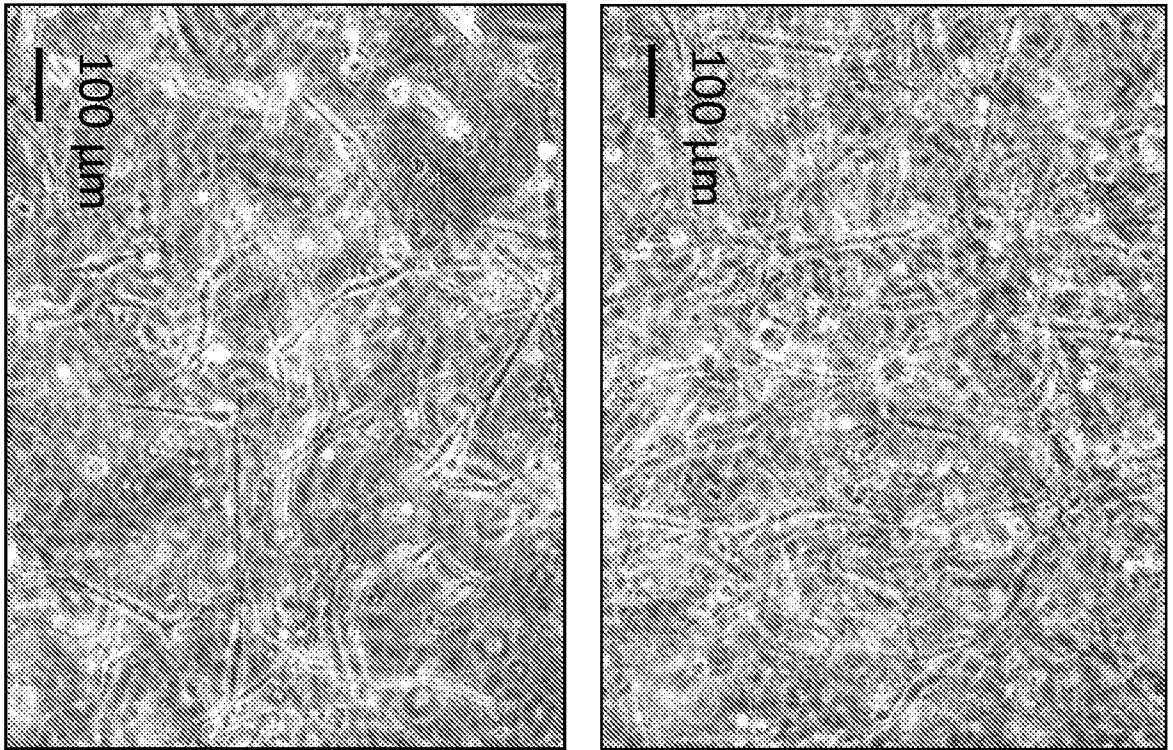
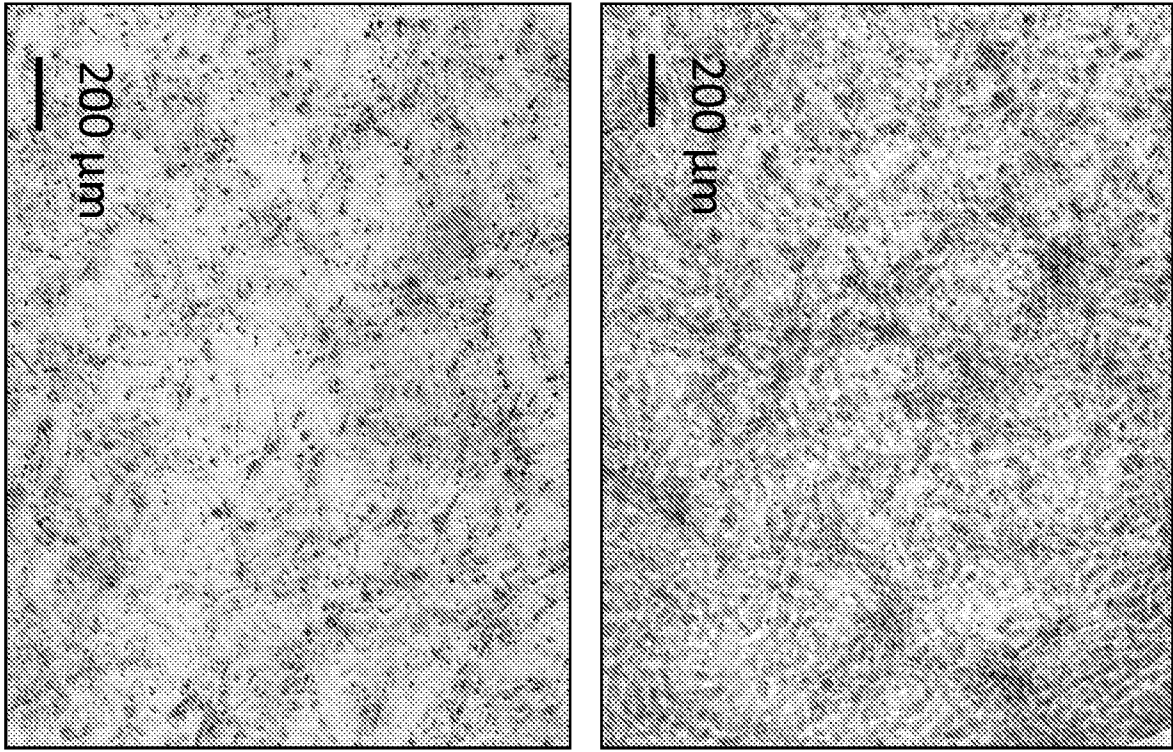
Figure 7.



**Figure 8.**



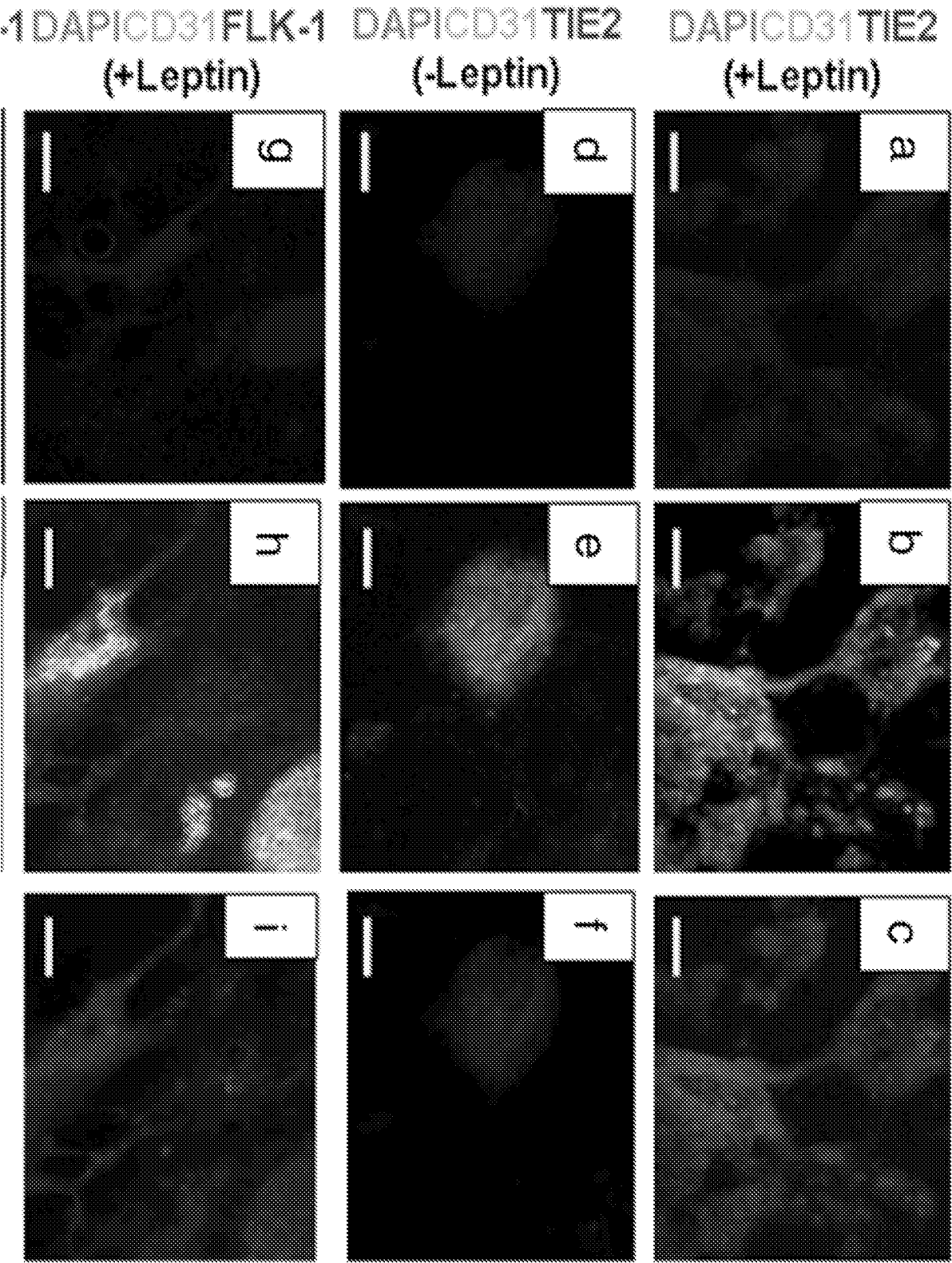
**Figure 9.**



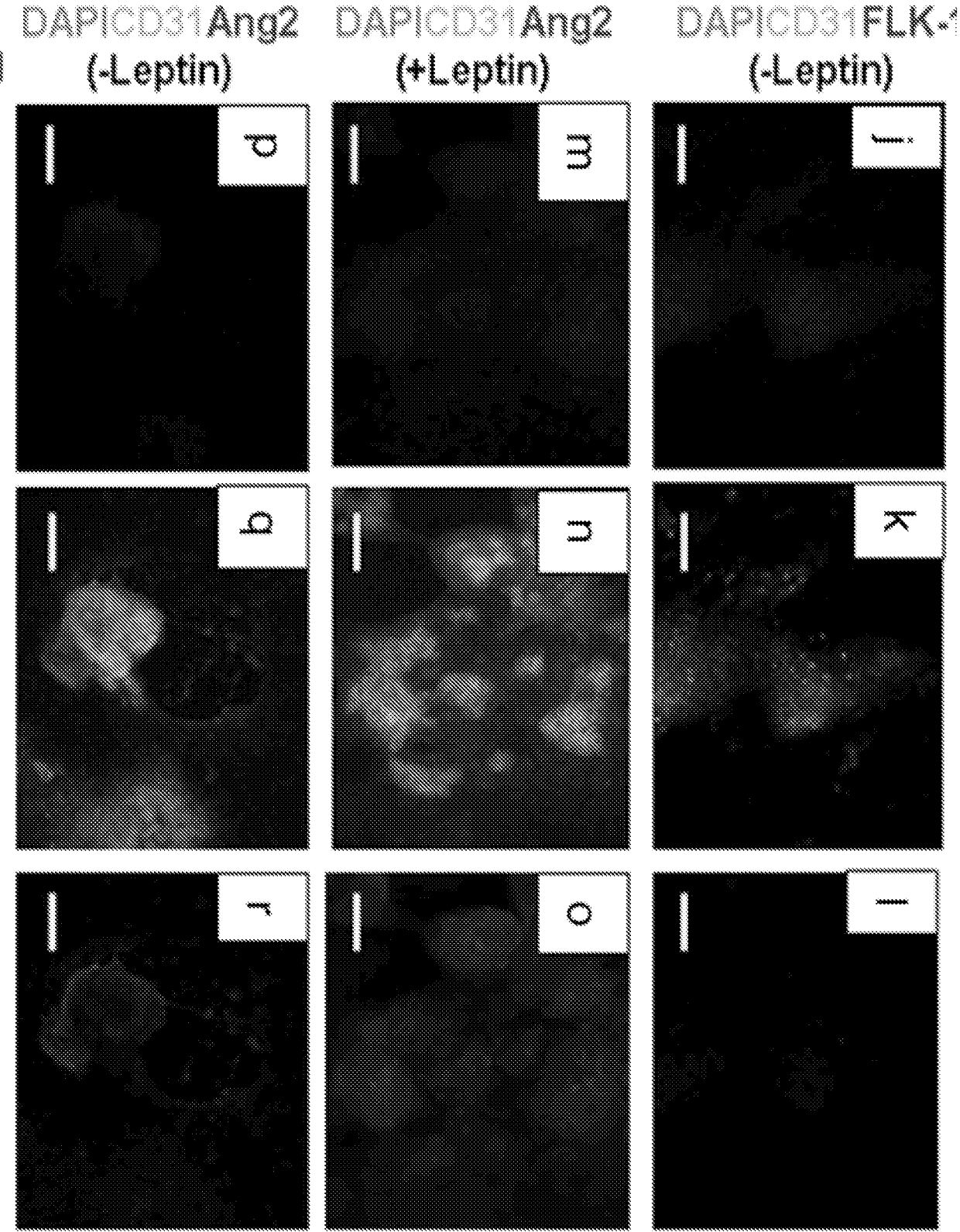
IECs + Leptin

IECs

**Figure 10.**



**Figure 10.**



DAPI  
Isotype Control

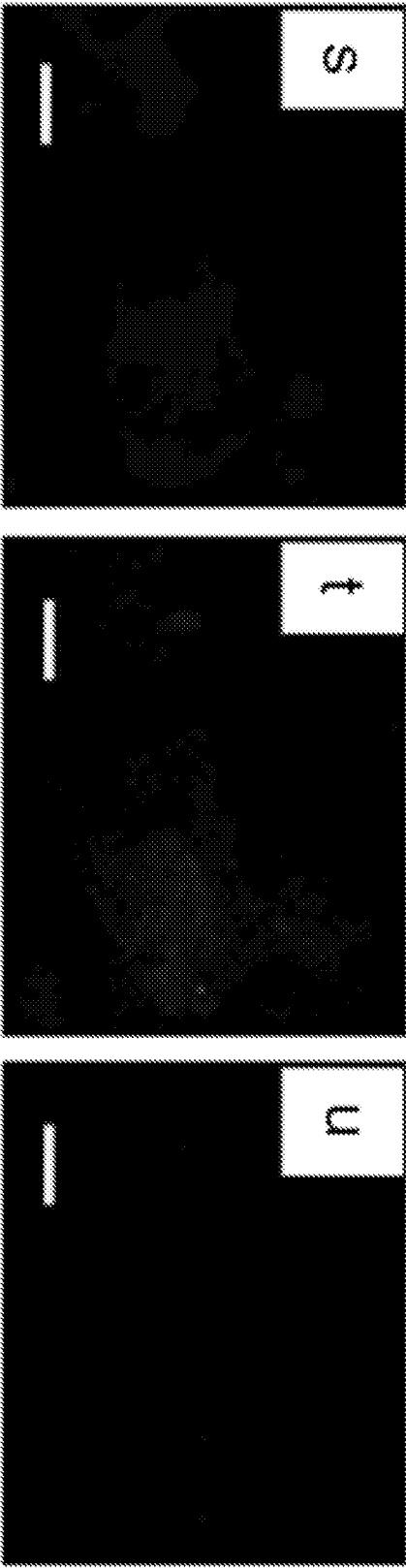


Figure 10.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/40795

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/071 (2015.01)

CPC - A61K 35/12; A61K 38/00; A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12N 5/071 (2015.01)

CPC - A61K 35/12; A61K 38/00; A61K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
UPC: 435/366,367Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Minesoft patbase, Google Scholar; keywords: lpsc, multipotent, pluripotent, setm cells, endothelial, angiogenesis, growth factor\*, differentiation factor\*, CD31+, matrix

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | US 2010/0233132 A1 (Ferreira et al.) 16 September 2010 (16.09.2010) abstract, para [0003], [0036], [0037], [0038], [0039], [0042], [0048], [0051], [0058], [0086], [0087], [0089] | 1-9                   |
| Y         | US 2011/0250182 A1 (Abbot et al.) 13 October 2011 (13.10.2011) para [0008], [0263]                                                                                                | 1-9                   |
| Y         | US 2012/0015395 A1 (Shusta et al.) 19 January 2012 (19.01.2012) para [0007], [0020], [0030]                                                                                       | 3                     |

☐ Further documents are listed in the continuation of Box C.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

05 November 2015 (05.11.2015)

Date of mailing of the international search report

08 DEC 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/40795

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:  
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-9, directed to a method of differentiating human pluripotent stem cells into microvessels comprising endothelial cells comprising generating endothelial cells by culturing the EBs in the presence of leptin

Group II: claims 10-16, directed to a composition, comprising microvessels

Group III: claims 17-23, directed to a method of treatment, comprising: providing a composition comprising microvessels comprising endothelial cells, administering to a subject in need of treatment for wound repair.

---please see supplemental box ---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-9

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

**Box III: lack of unity**

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

**Special technical features:**

The special technical feature of Group I is a method of differentiating human pluripotent stem cells into microvessels comprising endothelial cells comprising generating endothelial cells by culturing the EBs in the presence of leptin which is not required by groups II-III

The special technical feature of Group III is a method of treatment, comprising: providing a composition comprising microvessels comprising endothelial cells, administering to a subject in need of treatment for wound repair, which is not required by groups I and II

**Common technical features:**

Groups I-III share the technical feature of microvessels comprising endothelial cells.

Groups II-III share the technical feature of a composition, comprising microvessels endothelial cells

However, this shared technical feature does not provide a contribution over the prior art because these shared technical features as being anticipated by US 2004/0072342 A1 to Bothwell et al. (hereafter 'Bothwell'). Bothwell teaches composition, comprising microvessels endothelial cells (abstract; para [0001], .. remodeling into complex microvessels lined by the endothelial cells...; claim 13)

Groups I-III, therefore, lack unity under PCT Rule 13.2, because they do not share a same or corresponding special technical feature providing a contribution over the prior art.