High Levels of Pigment Epithelium-derived Factor in Diabetes Impair Wound Healing through Suppression of Wnt Signaling

Running Title: PEDF and wound healing

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Diabetic foot ulcer (DFU), caused by impaired wound healing, is a common vascular complication of diabetes. The present study revealed that plasma levels of pigment epithelium-derived factor (PEDF) were elevated in Type 2 diabetic patients with DFU and in db/db mice. To test whether elevated PEDF levels contributes to skin wound healing delay in diabetes, endogenous PEDF was neutralized with an anti-PEDF antibody in db/db mice. Our results showed that neutralization of PEDF accelerated wound healing, increased angiogenesis in the wound skin, and improved functions and numbers of endothelial progenitor cells (EPCs) in the diabetic mice. Further, PEDF deficient mice showed higher baseline blood flow in the skin, higher density of cutaneous micro-vessels, increased skin thickness, improved circulating numbers and functions of EPCs, and accelerated wound healing, compared to the Wt mice. Over-expression of PEDF suppressed the Wnt signaling pathway in the wound skin. Lithium chloride-induced Wnt signaling activation at downstream of the PEDF-interaction site attenuated the inhibitory effect of PEDF on EPCs and rescued the wound healing deficiency in diabetic mice. Taken together, these results suggest that elevated circulating PEDF levels contribute to impaired
wound healing on the process of angiogenesis and vasculogenesis through inhibition of Wnt/β-catenin signaling.

Non-standard Abbreviations and Acronyms

PEDF pigment epithelium-derived factor
DFU diabetic foot ulcer
EPCs endothelial progenitor cells
ECs endothelial cells
SDF-1 stromal cell-derived factor-1
SCF stem cell factor
Fz frizzled
LRP6 low-density lipoprotein receptor-related protein 6
TCF T cell factor
LiCl lithium chloride

Diabetes affects approximately 170 million patients worldwide(1), which is associated with multiple vascular complications. Diabetic foot ulcer (DFU), affecting 15% of diabetic patients, is a common vascular complication, which might leads to amputation(2).

The pathological impairments of wound healing are the main reason of amputation. The dynamic process of wound healing has been divided into three phases—reperfusion, reepithelization and neovascularization, and remodeling (3; 4). Neovascularization, including angiogenesis and vasculogenesis, plays a significant role in skin maintenance and repair, relying on various cell participation and coordination, especially endothelial progenitor cells (EPCs) and endothelial cells (ECs) (5).
EPCs are a subpopulation of bone marrow cells which are mobilized into peripheral blood and recruited to ischemic sites where they differentiate \textit{in situ} into mature ECs, participating in the process of neovascularization\cite{6; 7}. A previous study identified that specific cell surface markers of EPCs such as CD34, CD133, Flk-1 are present on the cells participating in vasculogenesis\cite{8; 9}. The mobilization of EPCs is initiated by the key regulator VEGF, which is secreted by multiple cell types including macrophages, fibroblasts and epithelial cells during wound-induced hypoxia\cite{1}. VEGF induces the activation of eNOS, subsequently leads to the production of NO\cite{10; 11}, administrates stem cell factor (SCF) binding to c-kit positive progenitor cells\cite{12; 13}, orchestrating the mobilization of EPCs into the bloodstream. Furthermore, the recruitment of EPCs to the wound site depends on ischemia-induced up-regulation of stromal cell-derived factor-1 (SDF-1), which is secreted by epithelial cells and myofibroblasts\cite{1}.

Previous studies have shown the down-regulation of VEGF results in impaired EPCs mobilization and functions \cite{14; 15}. In addition, the decreased expression of SDF-1 has also been suggested to contribute to deficient EPCs recruitment \cite{14}.

PEDF is a 50-kDa secreted glycoprotein in the serine proteinase inhibitor (Serpins) family \cite{16}. PEDF has a broad spectrum of activities including neuroprotection, regulation of lipid metabolism, and up-regulated in insulin resistant and obese human subjects \cite{17; 18}. In addition, PEDF is known to have a potent anti-angiogenic activity\cite{19}, counterbalancing the pro-angiogenic activity of VEGF\cite{20}. Our previous studies have shown that PEDF levels are increased in the circulation of type 2 diabetic patients and type 1 diabetic patients associated with microvascular complications\cite{21; 22}. These observations suggest that the elevated circulating PEDF levels in diabetes are probably compensatory responses to diabetic retinopathy and
nephropathy. Although the effect of PEDF on corneal epithelial wound healing has been report(23), the role of PEDF on diabetic skin wound healing has not been documented. Our recent study showed that PEDF functions as an endogenous inhibitor of the Wnt pathway (24). Wnts are a family of evolutionarily conserved, secreted glycoproteins modulating wound healing (24; 25). In the canonical Wnt signaling pathway, Wnt ligands bind with their receptors frizzled (Fz) and the co-receptor low-density lipoprotein receptor-related protein 6 (LPR6), resulting in phosphorylation of LRP6, and preventing phosphorylation and degradation of the transcriptional factor β-catenin. The stabilized β-catenin translocates into the nucleus, and associates with T cell factor (TCF) to activate Wnt target genes(26). Wnt signaling plays a pivotal role in numerous processes including inflammatory responses(27), carcinogenesis, fibrosis and angiogenesis(28-32). Furthermore, our previous study firstly verified that the Wnt signaling pathway plays the regulatory role in EPCs release during retinal neovascularization in the oxygen-induced retinopathy model(33). Nevertheless, the role of PEDF in regulating EPCs through the Wnt signaling pathway has not been studied. Kallistatin, another member of serpin family, with potent anti-angiogenic, impairs skin function and repair through suppression of the Wnt pathway (34). However, the modulation of PEDF in Wnt signaling during wound healing has not been verified.

In the present study, we investigate the role of PEDF in the regulation of wound healing through the Wnt signaling pathway. We also tested that hypothesis that PEDF impairs the numbers and functions of circulating EPCs by suppressing Wnt signaling, consequently delays the process of wound healing.

**RESEARCH DESIGN AND METHODS**
Human subjects

The collection of the human samples was in strict agreement with the institutionally approved guidelines by the Second Affiliated Hospital of Sun Yat-Sen University, and each participant gave written informed consent. All the patients with and without DFU were diagnosed and confirmed type 2 diabetes clinically. Blood was collected from 30 diabetic patients with DFU, 30 diabetic patients without DFU and 25 non-diabetic subjects. Clinical data of the subjects are presented in Table S1.

Enzyme-linked immunosorbent assay (ELISA) specific for PEDF/VEGF/SCF/NO

PEDF levels in human plasma were quantified by ELISA (Millipore, Billerica, MA). The factors of mice plasma were determined using commercial ELISA kits for PEDF (CusaBio Biotech, China), VEGF (ExCell Bio, China), SCF (RayBiotech, Norcross, GA), total NO and Nitrate/Nitrite (R&D Systems, Minneapolis, MN).

Experimental animals and protocols

All of the animal experiment procedures were approved by The Institutional Animal Care and Use Committee in the Sun Yat-Sen University and University of Oklahoma Health Sciences Center. The strain of mouse used for the type 1 diabetes model, the db/db mouse, the PEDF knockout mouse and the Wnt reporter mouse are all B6. For the diabetic mouse model, mice (6 weeks old) were fed high fat food (HFD) for 4 weeks (D12492:60% calories from fat; animal center of Guangdong Province, Guangzhou, China). Then, the mice received daily intraperitoneal injections of streptozotocin (STZ, 40 mg/kg/day) for 7 days to induce diabetes. Db/db mice were purchased from Model Animal Research Center of Nanjing University (China). The PEDF knockout (PEDF KO) mice were provided as a generous gift by Dr. S.J. Wiegand from Regeneron Pharmaceuticals, Inc. (Tarrytown, NY)(35), and Wnt reporter mice were
obtained from Jackson Lab and were bred in the barrier of University of Oklahoma Health Sciences Center. Recombinant GST-PEDF (PEDF) and a PEDF-neutralizing antibody (PEDF Ab) were obtained as previously described (18). Mice were injected intraperitoneally with recombinant PEDF (2 mg/kg) daily for 7 days, and the control group was injected with the same concentration of GST. For neutralizing PEDF in diabetic mice, mice were injected intraperitoneally with the PEDF Ab (Genscript, China) 0.4 mg/kg/day, for 15 days, and the control group was injected with the same concentration of non-specific IgG (Beyotime, China). Adenovirus expressing PEDF (Ad-PEDF) was generated from the full-length human PEDF cDNA as previously described (35), and Ad-GFP was used as the control virus. The viruses were injected into the wound bed (5×10^7 PFU/wound) at day 1 after wound was generated. Lithium chloride (LiCl, Sigma-Aldrish, St. Louis, MO) was injected intraperitoneally (100 mg/kg) to 3 month-old diabetic mice daily for 7 days, with the same concentration of NaCl as control as our described previously(33). The metabolic phenotype of PEDF KO mice and type 2 diabetes with PEDF Ab are presented in table S2.

**Skin wound healing and microvessel density assay**

Dorsum was clipped in anesthetized mice, and then standardized circular wounds were made with a full-thickness 6-mm skin punch (Acuderm, Fort Lauderdale, FL). Wound closure rates were measured by tracing the wound area every day through taking photos and quantified by Image J software (NIH). The frozen slides of wound tissue are stained with CD31 antibody (BD Biosciences, Franklin Lakes, NJ). The slides were examined under 100X magnification to identify the highest vascular density area, and five randomized high power field (hpf) areas of highest MVD were selected for counting under 200X magnication. The average of the five areas was recorded as the MVD level of this case.
Laser Doppler flowmetry

Anesthetized mice were fixed in modified centrifuge tube using mild-adhesive tape, keeping the temperature of mice around 32°C, and the laser Doppler probe was fixed firmly to skin to measure perfusion units (PU) using the PerimedPeriFlux System 5000 (Perimed, Stockholm, Sweden).

EPC isolation and culture

Bone marrow cells were isolated from the femur and tibia of mice individually. Briefly, the bone was carefully crushed in M199 medium (GIBCO, Gaithersburg, MD) after removal of the muscle around the bone followed by removing the red blood cells and washing. Then the cells were grown in fibronectin-coated dish in the presence of 10% fetal bovine serum (FBS, GIBCO, Gaithersburg, MD) in EGM-2 medium (VEGF, hydrocortisone, hFGF, IGF, ascorbic acid, hEGF, heparin, Lonza)(36).

EPC function assays

EPCs were cultured in EGM-2 medium and the culture medium was replaced at day 4 in culture to remove non-adherent cells. EPCs were used for function assays at day 7. For the migration assay, 10% FBS in 600 μl EGM-2 medium was placed in the lower chamber of 12-well plates, and then the cells (1×10^4) were plated on the upper chamber with 200 μl EGM-2 medium including 1% FBS for 12 hr. The cells were fixed with 4% paraformaldehyde and stained with crystal violet (Sigma-Aldrich, St. Louis, MO). For the adhesion assay, 96-well plates were coated with fibronectin (Sigma-Aldrich, St. Louis, MO) for 45 min at 37°C with 5% CO2. Then the plates were washed for 3 times with PBS to remove the non-adherent cells. The adherent cells were fixed with 4% paraformaldehyde and stained with crystal violet. For tube formation assay, 48-well plates were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ).
Bone marrow-derived EPCs (2×10⁴/well) were plated in 150 µl EGM-2 medium and incubated at 37°C with 5% CO₂ for 12 hr.

**Flow cytometry analysis**

To quantify circulation and bone marrow EPCs by fluorescence-activated cell sorter analysis (FACS), cells were acquired from either 100 µl of peripheral blood or bone marrow. The cells were incubated for 1 hr on ice and stained with a FITC-conjugated anti-mouse CD34 antibody, PE-conjugated anti-mouse CD133 antibody, PerCP(cy5.5)-conjugated anti-mouse Flk-1 antibody. All of the antibodies was purchased from BD (BD Biosciences, Franklin Lakes, NJ).

**Western blot analysis**

Wound skin tissue samples of approximate 5-mm circle around the wound were isolated from six mice and were lysed with 500 µl 1×SDS buffer for the total protein extraction. Western blot analysis was performed as described previously (37; 38). Antibodies for phosphorylated LRP6 (p-LPR6) and non-phosphorylated β-catenin (non-p-β-catenin) were purchased from Cell Signaling (Danvers, MA). Antibodies for VEGF and SDF-1 were purchased from Santa Cruz Biotechnology (Dallas, TX). The same blot was stripped and re-blotted for an antibody for β-actin (Sigma-Aldrich, St. Louis, MO).

**X-Gal staining**

Skin and wounds from BAT-gal transgenic mice were fixed for 2 hr in 4% paraformaldehyde, stained with 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal, Sigma-Aldrich, St. Louis, MO), and incubated at 37°C for 12 hr according to manufacturer’s instruction (Sigma-Aldrich, St. Louis, MO).

**Statistics**
For all studies comparing more than two groups, one-way ANOVA of SPSS 13.0 software was used, followed by LSD-t test for multiple comparisons amongst groups. Student’s t-test was used for statistical analysis between two groups. Statistical significance was considered at P-value less than 0.05. All data were expressed as mean±standard deviation.

RESULTS

Elevation of circulating PEDF in humans with diabetic foot ulcers

As showed in Figure 1A, circulating PEDF levels were significantly higher in diabetic patients with DFU (DM+DFU, 7.0±0.6 µg/mL, p<0.01) compared to diabetic patients without DFU (DM, 6.1±0.7 µg/mL) and non-diabetic controls (NDM, 4.0±0.5 µg/mL). Circulating EPCs, identified by CD34 and Flk-1 co-staining, were counted by FACS. The numbers of circulating CD34+/Flk-1+ cells were significantly lower in the DM+DFU group (0.03±0.02%, p<0.01) compared to DM (0.05±0.01%) and NDM (0.09±0.02%) groups (Fig. 1B). Furthermore, correlation analysis showed that circulating PEDF levels were negatively associated with numbers of CD34+/Flk-1+ cells (Fig. 1C, r= -0.76, p<0.01) in DFU patients (Fig. 1C). HbA1c levels were significantly higher in diabetic patients than non-diabetic individuals; but there was no difference between DM+DFU and DM (Fig. 1D). Collectively, circulating PEDF levels were elevated in diabetic patients with DFU, associated with the decreased circulating CD34+/Flk-1+ cells.

Plasma PEDF levels were up-regulated in diabetic mice

Plasma PEDF levels were significantly higher in 3 month-old db/db mice (7.85±2.41 ng/mL, p<0.01) compared with their NDM littermates (3.31±1.11 ng/mL) (Fig. 2A). HFD and STZ-induced DM mice also showed increased PEDF levels compared to age-matched NDM mice (p<0.01) (Fig. S1A). Plasma PEDF levels were up-regulated in diabetic mice, which is consistent with the clinical data in diabetic humans.
PEDF neutralization improved wound healing in diabetic mice

The PEDF Ab accelerated wound closure in db/db mice (Fig. 2B, C) and DM mice (Fig. S1B, C) compared with IgG. To further verify the role of PEDF in wound healing, diabetes was induced in PEDF KO and WT littermates by HFD in combination with STZ injection. The wound healing delay was ameliorated in PEDF KO (DM) mice compared to WT (DM) mice (Fig. 2D). Taken together, our results showed that wound healing in diabetic mice was accelerated via blocking PEDF.

PEDF delays skin wound repair

Wound closure in NDM mice treated with recombinant PEDF lagged behind control group with protein GST (Fig. 2E). Moreover, we measured the rate of wound closure in non-diabetic PEDF KO and WT mice. The result showed that the wound healing of PEDF KO mice was faster compared with WT littermates (Fig. 2F). Our observations suggested that PEDF administration alone impairs wound healing in non-diabetic mice.

PEDF inhibits angiogenesis in wound bed

Skin micro-vessel density (MVD) is measured, as PEDF plays a significant role as an angiogenesis inhibitor (39). Compared to IgG control group, the PEDF Ab significantly increased microvascular density in db/db mice skin (Fig. 3A, B). In contrast, recombinant PEDF administration significantly reduced vascular density in NDM mice, compared with GST-treated control (Fig. 3B). In NDM or DM conditions, PEDF KO mice had a higher vascular density in the skin compared with the WT littermates (Fig. 3B). Further, PEDF KO mice developed significantly increased dorsal skin thickness (Fig. 3C, D) and higher skin blood flow rate (Fig. 3E, F), compared to WT mice. Moreover, PEDF Ab increased doral skin thickness in db/db mice.
compared with IgG (Fig. S2A). Taken together, our results showed that PEDF inhibited angiogenesis, decreased the dorsal skin thickness and impeded skin blood flow.

**High levels of PEDF contribute to the decreased circulating EPC in diabetic mice**

Although PEDF is a well-known anti-angiogenic factor, its effect on EPC mobilization has not been documented. The PEDF Ab significantly increased the number of CD34\(^+\)/Flk-1\(^+\) and CD133\(^+\)/Flk-1\(^+\) cells in the peripheral blood of db/db mice, compared to that in the IgG-treated group (Fig. 4A-E). In contrast, recombinant PEDF decreased the numbers of CD34\(^+\)/Flk-1\(^+\) and CD133\(^+\)/Flk-1\(^+\) cells in NDM mice compared with GST-treated control (Fig. 4F). PEDF KO mice had more CD34\(^+\)/Flk-1\(^+\) and CD133\(^+\)/Flk-1\(^+\) cells in the circulation than the WT littermates (Fig. 4G-H). However, the injection of PEDF and the PEDF Ab had no effect on numbers of CD34\(^+\)/Flk-1\(^+\) and CD133\(^+\)/Flk-1\(^+\) cells in the bone marrow (Fig. S3A-B). Our observation suggested that PEDF decreased CD34\(^+\)/Flk-1\(^+\) and CD133\(^+\)/Flk-1\(^+\) cells in the peripheral blood of mice.

**PEDF impairs the function of EPCs**

To determine the effect of the PEDF Ab on the function of EPCs, we measured the migration, adhesion and tube formation of bone marrow-derived EPCs *ex vivo* (Fig. 4I, M, Q). In a transwell migration assay, the cells isolated from the db/db mice with the PEDF Ab treatment showed substantially higher migration than those from IgG-injected mice (Fig. 4J). On the other hand, recombinant PEDF injection slowed the migration of isolated EPCs, compared to GST injection in NDM mice (Fig. 4K). The EPCs from PEDF KO mice showed a higher migration activity than WT EPCs (Fig. 4L).

In an adhesion assay, the PEDF Ab improved the adhesion function of the EPCs from db/db mice (Fig. 4N). Recombinant PEDF reduced the adhesion of EPCs, compared to GST control in
NDM mice (Fig. 4O). The EPCs from PEDF KO mice had more adherent cells to the plate vs WT control (Fig. 4P).

In a tube formation assay, the PEDF Ab increased tube and branch formation of EPCs from db/db mice (Fig. 4R). Recombinant PEDF reduced the tube formation of EPCs, compared to the GST control (Fig. 4S). The EPCs from PEDF KO mice formed more tubes and branches than the WT mice (Fig. 4T). Moreover, PEDF Ab increased circulating EPC and improved the functions of EPCs in DM mice compared with IgG (Fig. S4A-D). Our results showed that the function of EPCs was improved by neutralizing PEDF.

**PEDF decreases the plasma VEGF levels, and expression of VEGF and SDF-1 in the wound tissue**

The expression of VEGF and SDF-1 was significantly higher at protein levels in db/db mice treated with the PEDF Ab compared with IgG in the wound tissue at day 15 after wounding (Fig. 5A-C). In contrast, PEDF down regulated the VEGF and SDF-1 expression in the wound tissue at day 7 after wounding (Fig. 5D-F). PEDF KO mice had higher VEGF and SDF-1 expression in the wound tissue than WT littermates (Fig. 5G-I). VEGF levels were significantly higher in db/db mice treated with the PEDF Ab (152.8±17.5 pg/mL) compared to diabetic mice with IgG (102.1±17.3 pg/mL, Fig. 5J). Plasma VEGF levels were significantly lower in NDM with the PEDF treatment (82.0±11.7 pg/mL) compared to NDM with GST (138.1±15.3 pg/mL, Fig. 5J).

Similar to the change of VEGF levels, plasma SCF and NO levels were increased after the treatment of the PEDF Ab in db/db mice, and decreased after the injection of recombinant PEDF (Fig. 5K, L). Moreover, PEDF Ab increased the plasma VEGF, NO, SCF levels (Fig. S6A-C) and the wound tissue VEGF, SDF-1 expression (Fig. S6D) in DM mice compared with IgG. We also measured the NO content in the wound tissue which functions to induce endothelial cell
migration and proliferation (40). The PEDF Ab increased the local NO level compared with diabetic mice treated with control IgG (Fig. S7A, B). The NO content in the wound tissue was decreased by injection of recombinant PEDF compared to the GST-injected control group (Fig. S7C). Our observation suggested that PEDF decreases the expression of VEGF, SDF-1 in the wound, and down-regulates the plasma VEGF, NO and SCF levels.

**PEDF inhibits Wnt/β-catenin signaling in the wound tissue**

Wound closure was lagged after Ad-PEDF injection compared with that in the control virus Ad-GFP injection (Fig. S5A). X-gal staining showed that Wnt signaling was activated in the periphery of wounded skin (Fig. 6A, B), and the Ad-PEDF treatment reduced X-gal staining in the periphery of the wounded skin, compared to the Ad-GFP (Fig. 6C). Western blot analysis showed that PEDF reduced levels of phosphorylated LRP6 (p-LPR6) and levels of non-phosphorylated β-catenin (non-p-β-catenin) in the wound tissue at days 4 and 7 after wounding, suggesting an inhibitory effect on Wnt signaling (Fig. 6D-I). Furthermore, p-LPR6 and non-p-β-catenin levels were increased in the wounded skin of PEDF KO mice compared to that in the WT mice (Fig. 6J-L). Our results showed that PEDF inhibits Wnt/β-catenin signaling in wound healing.

**Activation of the Wnt signaling at downstream of LRP6 attenuates the effect of PEDF on wound healing in diabetic mice.**

To further confirm that PEDF has a regulatory effect on wound healing through Wnt signaling, LiCl was injected to the 3 month-old diabetic mice. LiCl injection enhanced the wound healing of diabetic mice, compared with that in the NaCl group (Fig. S5B). The plasma VEGF levels and CD34+/Flk-1+ and CD133+/Flk-1+ cells were increased by LiCl injection compared with the NaCl controls (Fig. 7A-F). Western blot analysis showed that non-p-β-catenin, VEGF and SDF-1
levels were also increased in the LiCl injection group compared to the NaCl control (Fig. 7G-J). These findings demonstrated that LiCl reached the wound bed and induced Wnt activation at downstream of LRP6, the target of PEDF. Consequently, LiCl offset the PEDF-induced wound healing delay. Taken together, these results suggest that the effect of PEDF on wound healing is, at least in part, through blockade of Wnt signaling.

**DISCUSSION**

Impaired wound healing is a common cause of amputation in diabetic patients (1). The molecular basis underlying the pathogenesis of diabetes-induced wound healing deficiency is not completely understood. The present study, for the first time, demonstrated that elevated PEDF levels in the plasma are associated with DFU. Consistent with that in human subjects, plasma PEDF levels are up-regulated in diabetic mice. Using a PEDF neutralizing Ab in diabetic mice, PEDF KO mice and injection of recombinant PEDF, we have demonstrated that high levels of PEDF contribute to wound healing delay in diabetes through its anti-angiogenic activity. Using Wnt reporter mice and activation of Wnt signaling downstream of LRP6, our studies suggest that PEDF’s effect on wound healing is through blockade of the Wnt pathway. These observations for the first time established the association of elevated PEDF levels in diabetes with impaired wound healing and DFU, suggesting that PEDF is a promising drug target for the treatment of DFU.

PEDF is a multifunctional protein, which is characterized by its broad functions including anti-tumorigenicity, anti-metastaticity (41; 42), anti-inflammation (43) and anti-angiogenicity (16) activities. Our previous study reported that circulation PEDF levels are elevated in Type 2 diabetic patients (21) and Type 1 diabetic patients with microvascular complications (22), while
PEDF levels were decreased in renal (44) and retinal tissues (45). Here we showed that elevated circulating PEDF levels in patients with DFU compared with diabetic patients without DFU and with non-diabetic individuals. Furthermore, our result showed that the plasma PEDF levels were increased in diabetic mice. This is the first evidence suggesting that high levels of PEDF may play a role in regulation of wound healing in diabetes. To establish the role of PEDF in wound healing, we used loss-of-function approach by a PEDF Ab to block PEDF activity in diabetic mice and PEDF KO mice. Our results showed that the PEDF Ab not only improved delayed skin wound closure, but also increased micro-vessel density in the wound tissue. Diabetic patients have the thinner skin and reduced density of capillaries in the dermal layer which result in higher morbidity of poorer blood flow response, neuropathy, ulceration and gangrene, that can eventually develop into lower limb amputation (46) (47; 48). The relationship between PEDF levels and these clinical characters are not yet entirely clear. Thus, PEDF’s effect on the skin, particularly on the reduction of vascular density in the panniculus adiposus layer, provide a possible explanation how high levels of PEDF may contribute to impaired skin function and repair in patients with or at a high risk of peripheral vascular disease and amputations. In addition, which patients with diabetic peripheral vascular disease often have impaired skin function and blood flow in lower limb skin. Our results showed that PEDF KO mice had thicker resting skin from the top of epidermis to the bottom of the dermis, an increased skin microvascular density and higher blood flow rate than WT mice, In the gain-of-function approach, we injected recombinant PEDF into mice during wound healing. PEDF protein alone is sufficient to result in wound healing delay in mice. Taken together, these approaches all suggest that elevated PEDF levels in the circulation indeed contribute to wound healing delay in diabetes.
Both angiogenesis and vasculogenesis contribute to the process of wound healing. Recent evidence indicated that vasculogenesis occurs during both of physiological development of vasculature and pathological neovascularization with postnatal vasculogenesis(49). Thus, it is a new target for pathological neovascularization through improvement of the decreased numbers and impaired function of EPCs. Our study showed that an intraperitoneal injection of the PEDF Ab in diabetic mice increased skin microvascular density, improved numbers and function of EPCs mobilization and recruitment. The results of unchanged EPC abundance in bone marrow in diabetic mice suggest that PEDF might not affect the generation of EPCs.

Wnt signaling is known to play a key role in modulating angiogenesis(27; 28), and is crucial for the regulation of EPC mobilization (33). However the process of vasculogenesis has not been well understood. The numbers and functions of EPCs are crucial in the process of mobilization and recruitment. Our results demonstrated that decreased circulation numbers and dysfunction of EPCs correlate with Wnt signaling inhibition in the wound bed after injections of recombinant PEDF. Our previous study showed that binding of PEDF to LRP6 blocked the activation of the Wnt pathway through hindering the Wnt ligand-induced dimerization of LRP6 and Fz receptor(24). To further confirm that Wnt signaling contributes to the EPCs mobilization, lithium chloride (LiCl), a potent activator of canonical Wnt signaling by inhibiting GSK-3β and stabilizing β-catenin, and subsequently activating VEGF expression(50; 51), was employed as an agent to bypass the effects of PEDF on Wnt signaling. The results demonstrated that LiCl inhibited the effects of PEDF on the EPCs mobilization and the β-catenin activation in wound healing. Taken together, our data suggests that PEDF is a Wnt/β-catenin inhibitor in postnatal murine skin. In addition, this study provides strong evidence that the process of mobilization and recruitment of bone marrow-derived EPCs contributes to the skin neovascularization in the
wound healing model and PEDF regulates the EPC mobilization through the Wnt signaling pathway.

This EPC mobilization cascade starts with peripheral hypoxia-induced tissue release of VEGF(10; 11). In this process, the increase of NO and SCF levels in the peripheral blood results in the mobilization of EPCs from BM niches to circulation, ultimately allowing for their participation in vasculogenesis in wound tissue and wound healing(15). In the wounded tissue, EPC recruitment depends on ischemia-induced up-regulation of stromal cell-derived factor-1 (SDF-1) (52). Since the mobilization and function of EPCs are regulated by VEGF, NO and SCF, and the recruitment of EPCs are modulated by SDF-1, we measured and found the increased expression of VEGF, NO, SCF and SDF-1 following the treatment with the PEDF Ab. Therefore, these observations strongly suggest that the PEDF Ab improved vascularization in the wound tissue by targeting EPCs. Although representative endogenous anti-angiogenic proteins such as PEDF play an undoubted role in angiogenesis in vitro and in vivo(16), the PEDF’s regulatory role on vasculogenesis has not been well established. Thus, our data demonstrates that PEDF blockage increases numbers and functions of EPCs during the pathogenesis of wound neovascularization in diabetic model.

Based on our results, we propose the following model: High levels of PEDF in diabetes inhibit Wnt/β-catenin signaling, leading to suppressed mobilization and function of EPCs and consequently, angiogenesis and vasculogenesis during wound healing. These activities contribute to wound healing delay in diabetic patients. Hence, blockade of PEDF may benefit the treatment of DFU and prevention of amputation.
AUTHORSHIP CONTRIBUTIONS

This work presented here was carried out in collaboration between all authors. Guoquan Gao, Jianxing Ma, Xia Yang and Li Yan defined the study concepts. Weiwei Qi and Chuan Yang researched literature, designed methods and experiments, carried out the laboratory experiments, acquired and analyzed the data, did the statistical analysis, interpreted the results, and wrote the paper. Zhiyu Dai, Di Che, Juan Feng, Yuling Mao and Xiaoqiong Gu co-designed the animal experiments. Zhongxiao Wang and Xuemin He co-worked on the EPCs isolation, culture and associated assessment. Rui Cheng and Ti Zhou co-worked on associated data collection and their interpretation. Guoquan Gao, Jianxing Ma, Xia Yang and Li Yan co-designed experiments, discussed analyses, interpretation, manuscript editing & revision and presentation. All authors have contributed to, seen and approved the manuscript.

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Diabetes 2005;54:243-250

Figure Legends

Figure 1. Elevated plasma PEDF levels in Type 2 diabetic patients with DFU. (A) Plasma PEDF levels of NDM (n=25), DM (n=30) and DM+DFU (n=30) groups (mean±SD, **p<0.01). (B) Circulating EPCs numbers (mean±SD, **p<0.01). (C) Correlation analysis of plasma PEDF levels with circulating EPCs numbers. (D) Hemoglobin A1C (HbA1C, mean±SD, **p<0.01). (Statistical significance was considered at P-value less than 0.05. All data were expressed as mean±standard deviation).

Figure 2. PEDF blockage improved wound closure of diabetic mice. (A) Plasma PEDF levels (mean±SD, n=10 animals/group, **p<0.01). (B) Image of representative wound. (C-F) Rate of
wound closure (3 month-old male mice, n=10 animals/group, *p<0.05, **p<0.01). (C)
db/db+IgG vs db/db+PEDF Ab, (D) PEDF KO (DM) vs WT(DM), (E) NDM+GST vs NDM+PEDF, (F) PEDF KO vs WT. (Statistical significance was considered at P-value less than 0.05. All data were expressed as mean±standard deviation).

**Figure 3.** Higher levels of PEDF impaired skin structure and function. (A) CD31 and DAPI double staining in wound bed. Scale bar=100 µm. (B) Vessel numbers (vessel numbers measured in each high power field (hpf) (representative cohort of n=10 animals/group; 2 hpfs per mice sample, **p<0.01). (C) H&E staining of dorsal skin section, WT vs PEDF KO mice. Scale bar=500 µm. (D) Skin thickness from the top of epidermis (EPI) to the bottom of the dermis measured at thickest areas in each hpf, 2 hpfs per mice sample (representative cohort of n=5 animals/group). (E) Laser Doppler flowmetry in back skin: representative traces are shown for 3 month-old mice. (F) Baseline perfusion on back skin of WT vs PEDF KO mice (n=5 animals/group, **p<0.01). (Statistical significance was considered at P-value less than 0.05).

**Figure 4.** PEDF blockage improved reduced circulating CD34+/CD133+/Flk-1+ cells and impaired EPC functions including migration, adhesion and tube formation of diabetic mice. (A-D) Representative FACs results of peripheral blood are shown. (E-H) Quantification of CD34+/Flk-1+ and CD133+/Flk-1+ cells in peripheral blood (mean±SD, n=10 animals/group, **p<0.01): (E) db/db+IgG vs db/db+PEDF Ab, (F) NDM+GST vs NDM+PEDF, (G) PEDF KO vs WT, (H) PEDF KO (DM) vs WT (DM). (I) Representative migration results of *in vitro* are shown. (J-L) Migration: cell numbers (representative cohort of n=5 animals/group). **p<0.01. (M) Representative adhesion results of *in vitro* are shown. (N-P) Adhesion assay: cell numbers
(representative cohort of n=5 animals/group), *p<0.05; **p<0.01. (Q) Representative tube
formation results of in vitro are shown. (R-T) Tube formation: branch point counting
(representative cohort of n=5 animals/group), **p<0.01. (Statistical significance was considered
at P-value less than 0.05. All data were expressed as mean±standard deviation).

**Figure 5.** PEDF decreased the plasma levels of VEGF, and expression of VEGF and SDF-1 in
the wound skin. (A) Western blot for VEGF and SDF-1 in wound tissue after injecting PEDF Ab
for 15 days. (D) Western blot for VEGF and SDF-1 in wound tissue after injecting PEDF for 7
days. (G) Western blot for VEGF and SDF-1 in wound tissue of WT vs PEDF KO mice at day 7
after wounded. (B, C, E, F, H, I) The Western blotting results were semi-quantified by
densitometry, normalized by β-actin levels, averaged in three independent experiments, and
expressed as percentage of the respective control. All values are mean±SD (n=4), **p<0.01. (J,
K, L) Plasma VEGF, SCF and NO content of db/db+PEDF Ab vs db/db+IgG and NDM+PEDF
vs NDM+GST (representative cohort of n=10 in each group, mean±SD ,*p<0.05, **p<0.01).
(Statistical significance was considered at P-value less than 0.05. All data were expressed as
mean±standard deviation).

**Figure 6.** PEDF inhibits Wnt/TCF/β-catenin signaling in the resting and wounded skin. (A-C)
Day 7 wound tissue. Representative skin sections of Wnt-reporter Bat-gal mice were stained with
X-gal after the Ad-PEDF injection or Ad-GFP injection: (A) resting skin stained with X-gal; (B)
wounded skin stained with X-gal after the Ad-GFP injection; (C) wound skin stained with X-gal
after the Ad-PEDF injection. Scale bar=200µm. (D) Western blot analysis of p-LRP6 and non-p-
β-catenin in wounded skin after injecting Ad-PEDF or Ad-GFP in the wound bed at day 4. (G)
Western blot analysis of p-LRP6 and non-p-β-catenin in wound tissue 7 days after injection of Ad-GFP and Ad-PEDF in the wound bed. (H) Western blot analysis of p-LRP6 and non-p-β-catenin in wound tissue of WT vs PEDF KO at day 7 after wounded. (E, F, H, I, K, L) The Western blotting results were semi-quantified by densitometry, normalized by β-actin levels, averaged in three independent experiments, and expressed as percentage of the respective control. All values are mean±SD (n=9), **p<0.01. (Statistical significance was considered at P-value less than 0.05. All data were expressed as mean±standard deviation).

**Figure 7. Lithium attenuates the effects of PEDF.** (A) Plasma VEGF content was increased in diabetic mice injected with LiCl compared to those with NaCl injection (representative cohort of n=5 in each group, mean±SD, *p<0.05, **p<0.01). (B-E) representative FACs results of peripheral blood are shown. (F) Quantification of CD34+/Flk-1+, CD133+/Flk-1+ cells of peripheral blood (mean±SD, **p<0.01). (G) Western blot for non-p-β-catenin, VEGF and SDF-1 in wound tissue after injecting LiCl and NaCl for 7 days. (H-J) The Western blotting results were semi-quantified by densitometry, normalized by β-actin levels, averaged in three independent experiments, and expressed as percentage of the respective control. All values are mean±SD (n=3), **p<0.01. (Statistical significance was considered at P-value less than 0.05. All data were expressed as mean±standard deviation).
SUPPLEMENTAL FIGURE LEGEND

Figure S1. PEDF blockage improved delayed wound closure of diabetic mice. (A) Plasma PEDF level (n=10 animals/group, mean±SD, **p<0.01). (B) Image of representative wound. (C) Rate of wound closure (3 month-old male mice), n=10 in diabetic mice with PBS (DM), n=10 in diabetic mice with PEDF Ab (DM+PEDF Ab).

Figure S2. PEDF blockage increased skin thickness of db/db mice. (A) Skin thickness from the top of epidermis (EPI) to the bottom of the dermis measured at thickest areas in each hpf, 2 hpfs per mice sample (representative cohort of n=5 animals/group, *p<0.05).

Figure S3. PEDF had no effect on numbers of the CD34+/CD133+/Flk-1+ cells in the bone marrow. (A) Quantification of CD34+/Flk-1+ cells in the bone marrow (not significant). (B) Quantification of CD133+/Flk-1+ cells in the bone marrow (not significant).

Figure S4. PEDF blockage increased circulating CD34+/CD133+/Flk-1+ cells and impaired EPCs function of diabetic mice. (A) Quantification of CD34+/Flk-1+ and CD133+/Flk-1+ cells in peripheral blood (mean±SD, **p<0.01), n=10 in DM, n=10 in DM+PEDF Ab. (B) Migration: cell numbers (representative cohort of n=5 animals/group), **p<0.01. (C) Adhesion: cell numbers (representative cohort of n=5 animals/group, **p<0.01 (D) Tube formation: branch point counting (representative cohort of n=5 animals/group), **p<0.01.

Figure S5. PEDF delayed wound healing through the Wnt pathway. (A) Rate of wound closure of Wnt-reporter mice at day 4 and day 7 (representative cohort of n=5 animals/group), **p<0.01 (B) Rate of wound closure of LiCl and NaCl injection of diabetic mice at day 7 (representative cohort of n=5 animals/group), **p<0.01

Figure S6. PEDF decreased the plasma levels of VEGF, and expression of VEGF and SDF-1 in the wound. (A-C) Plasma VEGF, SCF and NO content of DM+PEDF Ab vs DM+PBS (representative cohort of n=10 in each group, mean±SD, *p<0.05, **p<0.01). (D) Western blot analysis of VEGF and SDF-1 in wound tissue 10 days after injection of the PEDF Ab; the western blotting results were semi-quantified by densitometry, normalized by β-actin levels, averaged in three independent
experiments, and expressed as percentage of the respective control. All values are mean±SD (n=4), **p<0.01.

Figure S7. PEDF antibody increased NO content in wound tissue of diabetic mice. (A) NO content of db/db+PEDF Ab vs db/db+IgG (Representative cohort of n=10 in each group, mean±SD, *p<0.05, **p<0.01). (B) NO content of DM+PEDF Ab vs DM+PBS (representative cohort of n=10 in each group, mean±SD, **p<0.01). (C) NO content of NDM+PEDF vs NDM+ GST (Representative cohort of n=10 in each group, mean±SD,*p<0.05, **p<0.01).
Table S1
Clinical characteristics in non-diabetic (NDM) control subjects, patients with Type 2 diabetes (DM) and diabetic patients with diabetic foot ulcer (DM+DFU).

<table>
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<tr>
<th></th>
<th>NDM</th>
<th>DM</th>
<th>DM+DFU</th>
<th>p value (NDMvsDM)</th>
<th>p value (DMvsDM+DFU)</th>
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</thead>
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<tr>
<td>n</td>
<td>25</td>
<td>30</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>50.3±4.1</td>
<td>54.7±2.9</td>
<td>55.6±3.8</td>
<td>0.08</td>
<td>0.69</td>
</tr>
<tr>
<td>TC(mg/dl)</td>
<td>4.9±0.4</td>
<td>4.4±1</td>
<td>4.7±0.6</td>
<td>0.78</td>
<td>0.61</td>
</tr>
<tr>
<td>LDL(mg/dl)</td>
<td>2.8±0.3</td>
<td>2.8±0.7</td>
<td>3±0.6</td>
<td>0.82</td>
<td>0.85</td>
</tr>
<tr>
<td>HDL(mg/dl)</td>
<td>1.5±0.4</td>
<td>1.3±0.4</td>
<td>1±0.2</td>
<td>0.81</td>
<td>0.24</td>
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<tr>
<td>TG(mg/dl)</td>
<td>1.1±0.7</td>
<td>1.2±0.6</td>
<td>2±0.8</td>
<td>0.83</td>
<td>0.03</td>
</tr>
<tr>
<td>PLT(×10^9 /L)</td>
<td>225±44</td>
<td>220±60</td>
<td>287±96</td>
<td>0.72</td>
<td>0.04</td>
</tr>
<tr>
<td>WBC(×10^9 /L)</td>
<td>5.9±0.9</td>
<td>6.6±2.6</td>
<td>8.2±4.2</td>
<td>0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>RBC(×10^9 /L)</td>
<td>4.3±0.2</td>
<td>4.7±0.6</td>
<td>3.8±0.8</td>
<td>0.62</td>
<td>0.07</td>
</tr>
<tr>
<td>Hb(g/L)</td>
<td>133±6</td>
<td>129±13</td>
<td>115±23</td>
<td>0.73</td>
<td>0.08</td>
</tr>
<tr>
<td>UA(umol/L)</td>
<td>314±96</td>
<td>323±66</td>
<td>360±109</td>
<td>0.85</td>
<td>0.67</td>
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Table S2
The metabolic phenotype of PEDF KO mice and type 2 diabetes with PEDF Ab.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PEDF KO</th>
<th>p value</th>
<th>DM</th>
<th>DM+PEDF Ab</th>
<th>p value</th>
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<tr>
<td>Body weight</td>
<td>26.4±1.48</td>
<td>25.7±0.78</td>
<td>0.61</td>
<td>32.4±4.17</td>
<td>31.7±4.88</td>
<td>0.89</td>
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<tr>
<td>GLU(mmol/L)</td>
<td>6.45±0.48</td>
<td>6.52±0.35</td>
<td>0.89</td>
<td>22.0±2.18</td>
<td>25.3±0.87</td>
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<tr>
<td>Urea(mmol/L)</td>
<td>52.5±8.57</td>
<td>49.0±7.07</td>
<td>0.88</td>
<td>34.5±4.95</td>
<td>30.5±17.7</td>
<td>0.78</td>
</tr>
<tr>
<td>B-HB(mmol/L)</td>
<td>1.01±0.23</td>
<td>1.14±0.31</td>
<td>0.67</td>
<td>1.92±1.39</td>
<td>1.68±0.31</td>
<td>0.83</td>
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<tr>
<td>TC(mmol/L)</td>
<td>2.84±0.03</td>
<td>2.68±0.10</td>
<td>0.85</td>
<td>6.62±0.06</td>
<td>7.95±0.31</td>
<td>0.03</td>
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<tr>
<td>HDL-C(mmol/L)</td>
<td>2.15±0.13</td>
<td>1.99±0.72</td>
<td>0.79</td>
<td>3.84±0.14</td>
<td>4.61±0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>LDL-C(mmol/L)</td>
<td>0.29±0.03</td>
<td>0.35±0.05</td>
<td>0.31</td>
<td>0.87±0.25</td>
<td>0.83±0.17</td>
<td>0.87</td>
</tr>
<tr>
<td>TG(mmol/L)</td>
<td>0.81±0.12</td>
<td>1.17±0.01</td>
<td>0.05</td>
<td>1.25±0.14</td>
<td>1.20±0.22</td>
<td>0.80</td>
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<tr>
<td>Cr(umol/L)</td>
<td>16.5±2.12</td>
<td>16.0±1.41</td>
<td>0.81</td>
<td>18.5±0.71</td>
<td>18.5±2.12</td>
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</tr>
<tr>
<td>UA(umol/L)</td>
<td>35.5±3.53</td>
<td>49.0±7.07</td>
<td>0.14</td>
<td>34.5±4.95</td>
<td>30.5±17.7</td>
<td>0.78</td>
</tr>
<tr>
<td>GSP(mmol/L)</td>
<td>3.57±0.13</td>
<td>3.56±0.24</td>
<td>0.96</td>
<td>4.91±0.30</td>
<td>5.71±0.18</td>
<td>0.08</td>
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</table>