Netrin-1 restores cell injury and impaired angiogenesis in vascular endothelial cells upon high glucose by PI3K/AKT-eNOS

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Abstract

Diabetic foot ulceration (DFU) represents a common vascular complication of diabetes mellitus (DM) with high morbidity and disability resulting from amputation. Netrin-1 level was decreased in type 2 DM patients and has been identified as a protective regulator against diabetes-triggered myocardial infarction and nephropathy. Unfortunately, its role and molecular mechanism in DFU remain poorly elucidated. Here, netrin-1 levels were reduced in DM and DFU patients relative to healthy controls, with netrin-1 levels being the lowest in DFU patients. Moreover, exposure to high glucose (HG) also suppressed netrin-1 expression in human umbilical vein endothelial cells (HUVECs). Elevated netrin-1 expression by infection with Ad-netrin-1 adenovirus vector protected against HUVEC injury in response to HG by ameliorating the inhibitory effects on cell viability, lactate dehydrogenase (LDH) and malondialdehyde (MDA) levels, cell apoptotic rate and caspase-3 activity. Importantly, HG-impaired angiogenesis was improved after netrin-1 overexpression by elevating cell migration, capillary-like tube formation and VEGF production. Mechanism assay substantiated that netrin-1 elevation increased the phosphorylation levels of AKT and eNOS, and NO production, which was notably suppressed by HG, indicating that netrin-1 overexpression restored HG-triggered impairment of the PI3K/AKT-eNOS pathway. More intriguingly, preconditioning with LY294002 (PI3K/AKT antagonist) or NG-monomethyl-L-arginine (eNOS inhibitor) antagonized netrin-1-induced activation of the PI3K/AKT-eNOS pathway. Concomitantly, treatment with these antagonists also attenuated the protective role of netrin-1 in endothelial dysfunction upon HG stimulation. These results suggest that elevation of netrin-1 may restore HG-triggered impairment of HUVEC and angiogenesis by activating the PI3K/AKT-eNOS pathway, indicating a potential agent for wound healing in DFU patients.

Key Words

- ▶ diabetic foot ulcer
- ▶ netrin-1
- ▶ endothelial dysfunction
- ▶ high glucose
- ▶ PI3K/AKT
- ▶ eNOS

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Introduction

Diabetes mellitus (DM) ranks as a complicated and chronic health disease and is commonly accepted to be the fifth common cause of death with the escalating incidence worldwide (Roglic *et al.* 2005). It is estimated that 366million people suffered from diabetes in 2011, which is predicted to reach 522million by 2030. DM is recognized as a major contributor to high mortality and morbidity through a series of diabetic complications such as nephropathy and stroke. Among these, diabetic foot ulceration (DFU) is a proverbial consequence of DM, having 15% prevalence among hospitalized DM patients (Woo *et al.* 2013). Patients with DFU suffer higher mortality and disability than patients without foot ulcers. DFU is characterized by lessened vascularity and angiogenesis and leads to 84% of diabetes-related lower leg amputations (Liu *et al.* 2014). Importantly, DFU represents a main contributor to non-traumatic lower limb amputation in developed countries, resulting in an annual cost of 300million pounds (Posnett & Franks 2008). Consequently, there is an urgent need to elucidate the mechanism underlying DFU and to develop efficient therapeutic strategies against DFU.

Hyperglycemia, a known characteristic of diabetes, usually results in refractory wound lesions and endothelial dysfunction, which, in turn, contributes to diabetic vascular complications including DFU (Marston & Dermagraft 2006, Wu & Chen 2016). It is generally believed that DFU is caused by neuropathy, infection and peripheral vascular disorders, which results in poor arterial inflow within limbs and consequently worse outcomes (Woo *et al.* 2013, Volmer-Thole & Lobmann 2016). Impairment of angiogenesis induced by chronic hyperglycemia is a pivotal cause of delayed healing in DFU due to insufficient blood supply (Brem & Tomic-Canic 2007, Carmeliet & Jain 2011, Costa & Soares 2013, Ruzehaji *et al.* 2014). The healing of diabetic wounds is a complex process, involving in various cellular processes and cytokines including NO (Brem & Tomic-Canic 2007). Enhanced synthesis of NO and VEGF, both of which are critical regulators for vasculogenesis, can improve blood flow and, consequently, contribute to wound healing of diabetic foot ulcers (Mohajeri-Tehrani *et al.* 2014). Recent study confirmed that elevating the pro-angiogenic response by attenuating flightless I obviously accelerated skin wound healing, implying a promising therapeutic strategy against DFU (Ruzehaji *et al.* 2014).

Netrin-1 is initially recognized to be an axon guidance molecule, and it has an analogous structure to laminin. Netrin-1 possesses a prominent role in a variety of physiological processes, including cell injury, proliferation and migration (Son *et al.* 2013, Yang *et al.* 2017). In umbilical cord blood-derived mesenchymal stem cells (UCB-MSC), netrin-1 protected against hypoxic injury-induced cell apoptosis via the expression of heat shock protein (HSP) 27 (Son *et al.* 2013). The growing body of evidence has corroborated a vital function of netrin-1 in injury repair of multiple diseases (Wang *et al.* 2008, Ke *et al.* 2014). Administration of netrin-1 antagonizes both ischemia-reperfusion (I/R) injury of kidney and kidney function repair by suppressing endothelial cell activation and leukocyte infiltration (Wang *et al.* 2008). Emerging report demonstrated the reduction of plasma netrin-1 levels in patients diagnosed with type 2 DM, and netrin-1 levels was found to be negatively related to insulin resistance and glucose homeostasis (Liu *et al.* 2016). Importantly, administration with netrin-1-expressing mesenchymal stem cells facilitated the repair of myocardial infarction-triggered ischemic injury in diabetic mice by promoting NO production and subsequent neovessel formation (Ke *et al.* 2014). Netrin-1 also exerts the beneficial efficacy in the treatment of diabetic nephropathy (Mohamed *et al.* 2012, Tak *et al.* 2013). Unfortunately, its role and molecular mechanism in DFU are poorly elucidated. In this study, we aimed to investigate the levels of netrin-1 in DFU patients and hyperglycemia conditions *in vitro*. Furthermore, the role of netrin-1 and its involvement in a potential mechanism of HUVEC dysfunction under high glucose were also investigated in the present research.

Materials and methods

Patients

Type 2 diabetic patients with DFU (DFU group, $n=35$) or not (DM group, $n=35$) and healthy individuals (control group, $n=30$) were recruited from the Department of Endocrinology, Xijing Hospital, Forth Military Medical University, during the period of August 2013–December 2014. Patients with DFU were enrolled into this research with Wagner classification DFU stages (2–4) and wound surface area over 1.5cm². All patients were matched for age and gender. Exclusion criteria for DFU patients included severe infection, hyperthyroidism, active

osteomyelitis, skin diseases, pregnancy and kidney failure. Patients receiving any pharmaceutical treatment to enhance wound healing were also excluded. Individuals without a history of diabetes and with a normal fasting blood glucose levels were defined as the control group. All biochemical parameters were determined by routine techniques using a Hitachi-912 Autoanalyzer (Hitachi). This research was approved by the Ethics Committee of Xijing Hospital, Forth Military Medical University and conducted according to the Helsinki Declaration. All participants gave written informed consent.

Detection of netrin-1 levels in plasma

Participant blood samples were collected in EDTA containers and centrifuged at 503g for 10 min. Then, plasma specimens were stored at -80°C for subsequent experiments. The concentration of netrin-1 in plasma specimens was measured according to the instruction of a commercial human netrin-1 ELISA kit (ToYong Bio, Shanghai, China).

Cell culture and treatment

HUVECs were purchased from ATCC. Cells were seeded in 75-cm² flasks and incubated with M199 medium (Grand Island, NY, USA) supplement with 10% FBS. For glucose exposure, cells were cultured with M199 medium containing 5.5 mmol/L glucose (normal glucose group) or 33 mmol/L glucose (high glucose group) for 48 h. All cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Assessment of netrin-1 mRNA levels by quantitative real-time PCR (qRT-PCR)

Total RNA from cells was extracted using RNAiso Plus (TaKaRa Bio). Then, 1.5 µg of extracted RNA was reverse transcribed to synthesize the cDNA with an ImProm II reverse-transcriptase kit (Promega). Afterward, 1 µL of cDNA was used as a template for qRT-PCR assay using a SYBR Premix Ex TaqTM II Kit (TaKaRa). The specific primers for netrin-1 and β-actin were synthesized by Sangon (Shanghai, China) and used as follows: netrin-1, sense: 5'-TGGGGCGGTTGCTCCTATTTC-3', anti-sense: 5'-GCCTTGTTGCTTGGCGTGT-3'; β-actin, sense: 5'-CACGATGGAGGGGCCGACTCATC-3', anti-sense: 5'-TAAAGACCTCTATGCCAACACA GT-3'. The reaction was carried out using ABI PRISM Sequence Detector System 7500 (Perkin-Elmer, Applied Biosystems). The mRNA

levels of netrin-1 were normalized to β-actin. Relative mRNA levels were calculated using 2^{-ΔΔCT} method.

Immunoblotting

After lysis in RIPA lysis buffer, the extracted protein was separated by 12% SDS-PAGE and then electroblotted onto PVDF membranes (Millipore). For Western blotting assay, the membrane was blocked with 5% skim milk for 2 h. After rinsing, primary antibodies were added and incubated at 4°C overnight, including antibodies against netrin-1 (1:3000) (Abcam), phosphorylated (p)-AKT and AKT (1:1000) (Cell Signaling Technology), p-eNOS (1:600, Abcam), eNOS (1:600, Abcam), VEGF (1:100) (Invitrogen) and β-actin (1:500, Invitrogen). Then, the goat anti-rabbit (1:500) and anti-mouse (1:10,000) secondary antibodies conjugated with HRP (Invitrogen) were added and incubated at room temperature for 1 h. To visualize the immunoreactive proteins, ECL reagent (Invitrogen) was added. The binding bands were analyzed using a Gel DocTM XR imaging system (Bio-Rad) and quantified via Quantity One software (Bio-Rad). The relative protein expression was normalized to β-actin.

Recombinant adenovirus vector construction and transduction

To construct a recombinant adenovirus vector expressing netrin-1, full-length cDNA of human netrin-1 was inserted into pAdTrack-CMV (Agilent) plasmid-containing green fluorescent protein (GFP). After homologous recombination with pAdEasy-1 in BJ5183, *E. coli* strain, DNA sequencing was carried out to evaluate the insert identity and orientation by Sangon Company. The Ad-netrin-1 vectors were then transfected into HEK293T cells (ATCC). After propagation in 293T cells, the amplified virus was purified using CsCl₂ gradient centrifugation. Then, the viral titer was assessed using the Adeno-X Rapid Titer kit (BD Biosciences, San Jose, CA, USA). For transduction, HUVECs were infected with the recombinant virus suspension of Ad-Srx-1, or Ad-GFP, at the titer of 1 × 10⁹ TU/mL for 48 h at 37°C.

Cell proliferation assay by MTT

Cells were seeded into 96-well plates at a density of 5000 cells/well. Then, cells were treated with either eNOS inhibitor N^G-monomethyl-L-arginine (NMA, 100 µM) or PI3K/AKT inhibitor LY294002 (20 µM) (Sigma) prior to infection with Ad-netrin-1 under high glucose. After that,

20 μ L of MTT (5 mg/mL, Sigma) was added to each well for a further 4-h incubation. Subsequently, formazan crystals were dissolved by DMSO for 10 min. Cell viability was then assessed by detecting the absorbance at 570 nm (OD570) and expressed by calculating the absorbance percentage of the treated group vs control group.

Detection of lactate dehydrogenase (LDH) and malondialdehyde (MDA) levels

After the collection of culture medium, the activities of LDH and MDA were measured as per the manufacturer's protocols of LDH and MDA assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Caspase-3 assay

The activity of caspase-3 was detected for different groups using the caspase-3 Fluorescent Assay Kits (Clontech). Cells under the indicated treatment were lysed and centrifuged. Then, the harvested supernatants were incubated for 1 h with 5 μ L of DEVD-AFC, a specific substrate for caspase-3. Finally, caspase-3 activity was evaluated by measuring the fluorescence intensities using a FL600 fluorescent reader (BioTek Instruments).

Apoptosis analysis

Cell apoptotic rates were measured by flow cytometric analysis using Annexin V-FITC/PI staining. Briefly, cells in different groups were trypsinized. After rinsing with PBS, cells were suspended in binding buffer, and then incubated with 10 μ L Annexin V-FITC (Beyotime, Shanghai, China) for 15 min under darkness. Then, 5 μ L of PI was also added for an additional 5 min. The specimens were subjected into FACSCalibur flow cytometer (BECTON, Franklin Lakes, NJ, USA) to determine the apoptotic rates.

Detection of cell migration by Transwell assay

The migration of HUVECs under various treatment specimens was evaluated using a Transwell system (Millipore) with 8 μ m pore size. For migration, cells (2×10^4) were seeded into the upper chamber with serum-free M199 medium, and chemotaxis was performed by loading 10% FBS to the lower chamber. After approximately 24 h, cells that had migrated to the bottom surface of membrane

were fixed with 4% paraformaldehyde, followed by staining with 0.1% crystal violet. The mean number of migrated cells was calculated from five random fields per specimen using a BX51 microscope (Olympus).

Tube formation *in vitro*

The formation of capillary tube was assessed by Matrigel analysis. After seeding in 96-well plates pre-coated with Matrigel (BD Biosciences), cells (1×10^4 /well) were treated with the indicated conditions. Twenty-four hours later, the formation of capillary-like tubular structures were captured under an inverted microscope. The number of tubes was quantified by assessing five randomly selected fields and presented as the percentage of tubes formed in control group.

ELISA assay of VEGF levels

After treatment with indicated conditions, the culture medium was harvested and centrifuged. Then, VEGF levels released into supernatants were determined using a Human VEGF Quantikine ELISA Kit (R&D Systems) in accordance with the manufacturer's instructions.

Measurement of NO production

NO production in different culture medium was evaluated by determining the stable metabolite of NO, nitrite. After 10-min incubation with Griess reagent containing 1% sulfanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride, samples were then analyzed by a SpectraMax Plus 384 spectrophotometer (Molecular Device, Sunnyvale, CA, USA) at 540 nm. Nitrite contents were assessed by reference to standard curves of nitrite.

Statistical analysis

All data were obtained from at least three independent trials and analyzed by SPSS 19.0 software. Results were exhibited as mean $\pm$ standard deviation (s.d.). Statistical significances ($P < 0.05$ and $P < 0.01$) were evaluated by Student's *t*-test for two groups and one-way ANOVA for three or more groups, followed by a *post hoc* least significant difference (LSD) test.

Table 1 Characteristics of patients.

	Healthy control (n=30)	DM (n=35)	DFU (n=35)
Age (years)	52.4±2.1	54.3±2.6	55.2±3.1
Gender			
Male	17	16	18
Female	13	19	17
Weight (kg)	62.18±5.79	64.26±6.17	65.11±5.87
Fasting plasma glucose (mmol/L)	5.14±0.64	9.87±2.89*	10.02±2.51*
HbA1c (%)	4.06±0.38	8.76±0.41*	8.25±0.53*
Serum triglyceride (mmol/L)	7.96±3.11	8.43±3.32	8.22±3.54
Total cholesterol (mmol/L)	9.53±2.14	8.76±2.87	8.84±3.06

Data were exhibited by mean ± S.D.

*P < 0.05 vs healthy control groups.

Results

Decreased expression of netrin-1 in patients with DFU and HUVECs under HG

As shown in Table 1, no differences were observed in the clinical and biochemical characterizations of patient subjects, excepting the higher concentrations of fasting plasma glucose and HbA1c in the DM and DFU groups. Further analysis corroborated an obvious downregulation of netrin-1 levels in plasma specimens taken from DM and DFU patients, compared to that in control group (Fig. 1A). Importantly, netrin-1 was presented at the lower content in DFU group relative to DM group. Moreover, the dramatic decreases in netrin-1 mRNA (Fig. 1B) and protein levels (Fig. 1C) were also demonstrated in HUVECs

upon HG exposure in contrast to normal glucose-treated group. Together, these results suggested a potential role of netrin-1 in the progression of DFU.

Elevation of netrin-1 antagonizes HUVEC injury triggered by HG exposure

To clarify the effect of netrin-1 on vascular endothelial cell injury under HG condition, cells were infected with Ad-netrin-1. The upregulation of netrin-1 in Ad-netrin-1 group was validated by qRT-PCR and Western assay (Fig. 2A and B). Functional analysis manifested that HG exposure reduced HUVEC viability to 40.11%, whereas this inhibitory function was notably attenuated after netrin-1 upregulation (Fig. 2C). Additionally, administration with HG augmented LDH release (Fig. 2D) and MDA levels (Fig. 2E), both the indices of cell injury. Intriguingly, ectopic expression of netrin-1 restrained the increases in LDH and MDA levels. Simultaneously, elevation of netrin-1 further attenuated the apoptotic rates of HUVECs upon HG from 32.67% to 12.78% (Fig. 2F). Furthermore, the high activity of caspase-3, a widely used indicator of cell apoptosis, in HUVECs under HG was also dramatically suppressed by netrin-1 overexpression (Fig. 2G). These data indicated that netrin-1 could protect against high glucose-induced HUVEC injury.

Ectopic expression of netrin-1 restores HG-impaired angiogenesis in HUVECs

The numbers of migrated cells were significantly reduced in response to HG stimulation (Fig. 3A). However,

Figure 1

Downregulation of netrin-1 in patients with DFU and HUVECs upon HG exposure. (A) Plasma samples were collected from health control, DM and DFU patients. The levels of netrin-1 in plasma were determined by ELISA assay. **P < 0.01 vs control groups. *P < 0.05 vs DM groups. (B) HUVECs were exposed to normal glucose, or HG, for 48 h, the mRNA levels of netrin-1 were evaluated using qRT-PCR. (C) The protein levels of netrin-1 in normal and HG groups. *P < 0.05 vs normal glucose group.

Figure 2

Upregulation of netrin-1 protected against HUVEC injury by HG. HUVECs were infected with Ad-netrin-1, or Ad-GFP, prior to subject to HG. (A and B) Expression levels of netrin-1 mRNA (A) and protein (B) were detected independently by qRT-PCR and Western blotting. (C) MTT assay was performed to assess the cell viability. (D and E) Effect of netrin-1 overexpression on LDH leakage (D) and MDA concentration (E) triggered by HG. (F) After dual-staining with Annexin V and PI, cell apoptotic rate was measured by flow cytometry. (G) Caspase-3 activity was measured by fluorometric assay. * $P < 0.05$.

the inhibitory role of HG was reversed after netrin-1 overexpression. Importantly, upregulation of netrin-1 attenuated the inhibitory effect of HG on capillary-like tube formation by HUVECs (Fig. 3B). Moreover, exposure of HUVECs to HG dampened the VEGF expression, a critical factor in angiogenesis, which was noticeably ameliorated after netrin-1 elevation (Fig. 3C). Concomitantly, the decreased concentrations of VEGF in medium under HG stimulation were alleviated when cells were infected with Ad-netrin-1 vectors (Fig. 3D). These observations manifested that elevation of netrin-1

could execute the protective response to HG-injured angiogenesis in HUVECs.

Netrin-1 upregulation restores the injury in the activation of PI3K/AKT-eNOS pathway upon HG stimulation

To elucidate the underlying mechanism for the beneficial role of netrin-1 upregulation in endothelial dysfunction under HG, the activation of PI3K/AKT-eNOS signaling was explored. Here, exposure to HG reduced the phosphorylation level of AKT, but not in the

Figure 3

Effect of netrin-1 elevation on the impairment of angiogenesis in response to HG. After infection with Ad-netrin-1, HUVECs were incubated with HG. (A) Cell migration was analyzed by Transwell chamber. (B) Effect on capillary-like tube formation was evaluated by culturing on Matrigel (magnification $\times 100$). (C) The expression of VEGF protein was determined by Western blotting assay. (D) Upregulation of netrin-1 ameliorated the inhibitory role of HG in VEGF concentration in culture medium. * $P < 0.05$ vs control groups. # $P < 0.05$ vs HG groups.

Figure 4

Netrin-1 restored the impairment of the PI3K/AKT-eNOS pathway in HG-stimulated HUVECs. (A) Phosphorylation of AKT and eNOS was determined in HG-treated cells that were infected with Ad-netrin-1, or not, by Western blotting. (B) Quantification analysis of p-AKT/AKT protein. (C) Quantification analysis of p-eNOS/eNOS expression. (D) Effects of netrin-1 on NO concentration in the indicated groups. * $P < 0.05$ vs control groups. # $P < 0.05$ vs HG groups.

Figure 5

The PI3K/AKT-eNOS signaling was involved in the protective role of netrin-1 in endothelial dysfunction. (A) After preconditioning with LY294002, or NMA, the activation of PI3K/AKT-eNOS pathway in netrin-1-overexpressed cells upon HG was analyzed by Western blotting. (B) The corresponding quantified assay of protein expression. (C) Pretreatment with LY294002, or NMA, attenuated the production of NO induced by netrin-1. (D, E, F, G, H and I) The corresponding effects on cell viability (D), LDH leakage (E), cell apoptosis (F), cell migration (G), tube formation (H) (magnification $\times 100$) and VEGF production (I). * $P < 0.05$ vs control groups. # $P < 0.05$ vs netrin-1-treated groups.

expression of AKT protein (Fig. 4A). Simultaneously, the protein expression of p-eNOS was also suppressed under HG, whereas without the change in eNOS expression. However, upregulation of netrin-1 dramatically increased the phosphorylation levels of AKT and eNOS, but had little effects on the total AKT and eNOS expression (Fig. 4B

and C), indicating that netrin-1 elevation restored the impairment of PI3K/AKT-eNOS signaling induced by HG. Additionally, HG exposure decreased the concentration of NO production from $24.56 \mu\text{mol/L}$ to $10.23 \mu\text{mol/L}$, which was increased into $18.74 \mu\text{mol/L}$ when netrin-1 was overexpressed in HUVECs (Fig. 4D).

Netrin-1 improves HUVEC dysfunction in response to HG through the PI3K/AKT-eNOS pathway

We further investigated whether the PI3K/AKT-eNOS pathway was responsible for the protective effects of netrin-1 on HG-triggered cell and angiogenesis impairment. As shown in Fig. 5A and B, prior administration with LY294002 mitigated the augment in the expression of p-AKT and p-eNOS induced by netrin-1 overexpression. Concomitantly, pretreatment with NMA, an antagonist of the eNOS pathway, also attenuated netrin-1-increased phosphorylation levels of eNOS. The augmented production of NO in netrin-1-overexpressed groups was consistently decreased in HG-treated cells after pretreatment with both LY294002 and NMA (Fig. 5C). Moreover, ectopic expression of netrin-1 elevated cell viability upon HG, which was decreased when cells were preconditioned with LY294002 and NMA (Fig. 5D). At the same time, blocking the PI3K/AKT and eNOS pathways with their respective antagonists also potentiated the release of LDH (Fig. 5E) and cell apoptosis (Fig. 5F), which was attenuated by netrin-1 overexpression. Further, functional assays documented that netrin-1-induced increases in migrated cell number (Fig. 5G) and tube formation (Fig. 5H) were both inhibited in LY294002- and NMA-pretreated groups. Additionally, preconditioning with LY294002 and NMA also antagonized the production of VEGF triggered by netrin-1 elevation (Fig. 5I).

Discussion

Hyperglycemia is a predominantly metabolic feature of diabetes and can trigger various macrovascular and microvascular-related complications. As a common and serious chronic complication of diabetes, DFU remains an unsolved health problem due to the high mortality and disability rates, with a poignant amputation being performed approximately every 30s worldwide (Boulton *et al.* 2005). To develop a new and effective strategy to treat DFU is urgently needed. Decreased levels of netrin-1 had been observed in diabetic patients (Liu *et al.* 2016). Importantly, increasing evidences have corroborated the protective role of netrin-1 expression against diabetes-induced complications such as diabetic nephropathy and myocardial infarction (Tak *et al.* 2013, Ke *et al.* 2014). To date, no data are available on its function in diabetes-induced DFU. Coincident with a previous research (Liu *et al.* 2016), an obvious downregulation of netrin-1 in plasma was observed in diabetic patients in contrast to healthy controls. More intriguingly, lower

levels of netrin-1 were detected in patients with DFU relative to those without DFU, indicating a potential critical role of netrin-1 for wound healing in DFU.

Endothelial dysfunction in diabetes is a pivotal characteristic of diabetes-mediated vascular complications including DFU (Gerasimchuk *et al.* 2014, Dong *et al.* 2016, Suganya *et al.* 2016). Once impaired by hyperglycemia, vascular endothelial cells will lose their ability to regulate blood flow, nutrient delivery and angiogenesis, and this, in turn, can affect wound healing in diabetic patients (Marston & Dermagraft 2006). Netrin-1 has recently attracted increasing interests in wound repair progression in diabetic complications. Previous researches have revealed a protective role of netrin-1 against ischemia/reperfusion kidney injury and cardiac injury (Tak *et al.* 2013, Siu *et al.* 2015). Importantly, ectopic expression of netrin-1 exerts a benefit effect on repair impairment in diabetes-induced myocardial infarction and diabetic nephropathy, making it as a potentially promising agent against diabetic complication (Tak *et al.* 2013, Ke *et al.* 2014). To further elucidate the role of netrin-1 in DFU, we investigated its function in vascular endothelial cell dysfunction under high glucose exposure. As expected, high glucose treatment inhibited netrin-1 expression in HUVECs. Importantly, upregulation of netrin-1 ameliorated high glucose-inhibited cell viability and suppressed high glucose-increased cell apoptosis. Simultaneously, the release of LDH induced by high glucose, a marker for cell injury, was also reduced after netrin-1 overexpression concomitant with MDA decrease, an indicator to estimate reactive oxygen species (ROS)-induced cell injury upon high glucose. These results suggest that netrin-1 elevation may protect against HUVEC injury in response to high glucose.

Impaired vasculogenesis is widely recognized to be a key contributor to delayed wound healing (Ruzehaji *et al.* 2014, Zhou *et al.* 2017). We, therefore, next investigated the angiogenic function of netrin-1 in HUVECs stimulated with high glucose. Intriguingly, ectopic expression of netrin-1 restored the inhibitory effects of high glucose on cell migration and tube formation. Concomitantly, netrin-1 also elevated the production of VEGF, a pro-angiogenic cytokine, under high glucose. A previous study also proved an essential role of netrin-1 in angiogenesis of rat placenta (Xie *et al.* 2011). Analogously, netrin-1 expression can improve capillary formation and blood vessel density in mice with diabetic myocardial infarction (Ke *et al.* 2014). Hence, these observations provoke us to conclude that overexpression of netrin-1 may facilitate wound healing of DFU by ameliorating endothelial

dysfunction by restoring the impairment of cell growth and vascularization.

Cumulatively, abundant researchers agree that the PI3K/AKT pathway possesses a critical role in wound healing under diverse pathological conditions including hyperglycemia (Liu *et al.* 2014, Zhu *et al.* 2014). Once activated, the phosphorylation of AKT can directly phosphorylate eNOS and induce the subsequent production of NO (Yao *et al.* 2016). eNOS convincingly acts as a pivotal regulator of vascularization by mediating NO generation. To clarify the molecular mechanism involved in the protective role of netrin-1 against high glucose-induced endothelial dysfunction, we explored the activation of PI3K/AKT-eNOS signaling. Within agreement with this hypothesis, elevation of netrin-1 attenuated the decrease in the phosphorylation of AKT and eNOS when exposed to high glucose, as well as increased NO levels. As a critical and potent vasodilator, NO released by endothelia can maintain vascular integrity of normal blood vessels and its decrease results in vascular malfunction during the progression of insulin resistance (Zhu *et al.* 2014). Moreover, the deficiency of NO accounts for the damaged vascular regeneration in diabetic ischemic injury animal model (Yan *et al.* 2009). Further analysis confirmed that blocking the PI3K/AKT or eNOS pathway attenuated the restored potential of netrin-1 elevation in cell growth and pro-angiogenic impairment under conditions of high glucose exposure.

Collectively, this study demonstrated the decrease of netrin-1 levels in patients with DFU. More importantly, overexpression of netrin-1 could antagonize endothelial dysfunction by restoring cell injury and impaired angiogenesis upon high glucose exposure by regulating the PI3K/AKT-eNOS signaling. Therefore, this study highlights netrin-1 as a potentially promising agent for wound healing of diabetic vascular complications, including DFU. Emerging studies have verified the critical role of augmented inflammatory response in the pathological process of diabetic vascular complications (Tuttolomondo *et al.* 2015, Afarideh *et al.* 2016, Wu & Chen 2016). Recently, several studies have corroborated that netrin-1 exerts the critical roles in the progression of inflammatory diseases (Podjaski *et al.* 2015, Mediero *et al.* 2016). Accordingly, further studies will focus on the effect of netrin-1 on the inflammatory response to hyperglycemia *in vitro* and DFU model.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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