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EPO protects mesenchymal stem cells from hyperglycaemic injury via activation of the Akt/FoxO3a pathway

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ABSTRACT

Introduction: Mesenchymal stem cell (MSC)-based therapies have demonstrated positive outcomes for treating cardiovascular disease. However, the proliferative ability of MSCs decreases during chronic exposure to hyperglycaemia; their ability to contribute to endogenous injury repair is thus reduced. Erythropoietin (EPO) was recently reported to protect against hyperglycaemia-related injury in various cells and may be a good candidate for enhancing MSC functions under hyperglycaemic conditions.

Methods: Bone marrow-derived MSCs were isolated from male donor rats weighing 60–80 g. The roles of EPO in regulating cell viability, senescence, angiogenesis and inflammation were investigated using the Cell Counting Kit-8 (CCK-8) assay and 5-ethynyl-2′-deoxyuridine (EdU) assays; senescence-associated β-galactosidase (SA-β-gal) staining; VEGF, HGF, IGF, bFGF ELISAs and TNF-α ELISA, respectively. ROS production was measured by flow cytometry. The expression levels of Akt, forkhead box class O3a (FoxO3a) and VEGF proteins in MSCs were analysed by western blotting. Matrigel was used for tube formation assays.

Results: The results of the current study showed that EPO has beneficial effects on MSCs exposed to hyperglycaemia by promoting proliferation, inhibiting senescence and the release of pro-inflammatory factors, increasing the secretion of proangiogenic cytokines, and enhancing the ability of MSCs to stimulate tube formation among human umbilical vein endothelial cells (HUVECs). In addition, the beneficial effects of EPO may result from the activation of the Akt/FoxO3a signalling pathway.

Conclusions: Our study demonstrates for the first time that EPO protects MSCs from hyperglycaemia-induced damage by targeting the Akt/FoxO3a signalling pathway.

1. Introduction

Coronary artery diseases (CADs), especially myocardial infarction (MI), remain the leading cause of mortality across the world. Diabetes mellitus (DM) is a risk factor for CAD, and CAD-associated mortality rates are 2–8 times higher in people with diabetes than in those without diabetes [1]. Chronic exposure to hyperglycaemia promotes the development of MI and inhibits the recovery process through various mechanisms, among which endothelial cell (EC) injury and decreased angiogenesis play vital roles. Additionally, hyperglycaemia induces the excessive production of ROS, which aggravates the inflammatory reaction and delays the cardiomyocyte recovery process [2].

To treat MI, bone marrow-derived mesenchymal stem cells (MSCs) function as one of the most suitable candidate seed cells compared with other kinds of stem cells for cardiomyocyte recovery. A recent study showed that bone marrow-derived MSCs (BMSCs) transplantation can increase the ability of ECs to form blood vessels, reduce the expression levels of collagen and matrix metalloproteinase-2 (MMP-2), and inhibit diabetes-induced ventricular remodelling [3]. However, stem cells from diabetic individuals could not repair the MI. When transplanted into rats to improve heart function, human MSCs (hMSCs) obtained from patients with both CAD and DM have significantly reduced abilities to proliferate and offer myocardial protection compared to MSCs from patients with CAD alone [4]. Further studies have shown that hyperglycaemia prevents MSCs from secreting pro-survival growth and anti-inflammation factors [5,6]; and promotes the secretion of large amounts of inflammatory mediators such as TNF-α, which leads to healing disorders and enhanced local inflammation [7]. Therefore, identifying how to enhance the biological activity of MSCs from DM patients to increase the autologous transplantation efficiency is of great importance.

Pretreating or modifying MSCs with some small molecules prior to

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transplantation may be a useful method for enhancing transplantation efficiency. A recent study showed that EPO can protect against hyperglycaemic injury by reducing apoptosis and promoting the anti-inflammatory factors released by ECs; additionally, EPO promotes endothelial progenitor cell mobilization and angiogenesis [8]. In our previous study, we showed that EPO treatment alleviates the inflammatory reaction and promotes the tube formation ability of ECs under hyperglycaemic conditions [9]. However, whether EPO can protect MSCs from hyperglycaemic injury and increase the angiogenic capability through communication with ECs in high glucose (HG) microenvironments are unclear, and the precise mechanism by which these functions are accomplished is unknown.

This study explored the effect of EPO on MSCs and ECs under hyperglycaemic conditions and investigated the communication between these two cell populations.

2. Materials and methods

2.1. Reagents

Recombinant human EPO (PeproTech, Rocky Hill, NJ, USA) was dissolved in 0.1% BSA and stored at −20 °C. Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12), RPMI 1640 and foetal bovine serum (FBS) were purchased from HyClone (Grand Island, NY, USA). A senescence cell histochemical staining kit, diluted 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluoroprobe and cell counting kit-8 (CCK-8) were obtained from Beyotime (Shanghai, China). A 5-ethynyl-2′-deoxyuridine (EdU) assay kit was purchased from RiboBio (Guangzhou, China). VEGF, HGF, IGF, bFGF and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from Sigma-Aldrich (R&D Systems Inc., Minneapolis, MN, USA). The Akt (cst-4691 s), phosphorylated Akt (p-Akt (Ser473); cst-4060 s), p-Akt (Thr308); cst-4056 s) and VEGF (cst-5874 sc) antibodies were obtained from Cell Signaling Technology; FoxO3a (AF609) and phosphorylated FoxO3a (phospho-FoxO3a (Thr32); AF605) antibodies were obtained from Beyotime Institute of Biotechnology; and HRP-conjugated goat anti-rabbit IgG (H + L) was obtained from Beyotime (Shanghai, China). Matrigel was obtained from BD Biosciences (Bedford, MA, USA). The commercial transfection reagent X-treme siRNA Transfection Reagent was from Roche Applied Science (Penzberg, Germany). siRNA was purchased from GenePharma (Shanghai, China). All other drugs and chemicals used in this study were purchased from Sigma Chemical Co., Ltd.

2.2. Cell culture and treatment

MSCs were isolated from the bone marrow of Sprague-Dawley (SD) rats (weighing 60–80 g) as previously described [10]. All SD rats were obtained from the Laboratory Animal Science Department of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Briefly, femurs and tibias were removed from SD rats; then, the bone marrow was removed from the bone with 10 ml of DMEM/F12 medium. The cells were centrifuged at 300 ×*g* for 5 min. The resulting cell pellets were harvested in 6 ml of DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. These cells were plated in a 25 cm^2 plastic flask and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every 3 days, and non-adherent cells were removed. Upon reaching 80–90% confluence, the cells were expanded at a dilution of 1:2 or 1:3 after being detached from the flasks using 0.25% trypsin. MSCs were characterized by flow cytometric analysis for the expression of the typical markers, CD90, CD29, and CD44 (All from BD Biosciences, Franklin Lakes, NJ, USA), and the absence of the hematopoietic markers CD45 (eBioscience, San Diego, CA, USA) and CD34 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), as previously reported [10]. The cells in the normal glucose (NG) group were cultured in medium containing 5.5 mM glucose, while those in the HG group were cultured in medium containing 25 mM glucose. Cells were cultured for as long as 28 d.

Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in 25 cm^2 plastic flasks in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. The medium was replaced every 3 d. Upon reaching 80–90% confluence, the cells were expanded at a dilution of 1:2 or 1:3 with the above method. Cells at passages 4–6 were used for experiments. To investigate the effect of EPO on the communication between MSCs and ECs, the MSCs were cultured on chamber slides with high (25 mM) levels of glucose and with or without EPO (50 IU/ml) for a designed time. The culture supernatant was then collected and used for culturing HUVECs for 24 h. After culture, the cells were collected for different assessments.

2.3. Cell transfection

Before transfection, MSCs were replated into six-well plates at a density of 2×10^5 cells/well and incubated overnight. For Akt inhibition, 100 nM Akt siRNA was transfected into the cells. As controls, cells were transfected with scrambled siRNA (siRNA-NT). siRNA-NT or siRNA-Akt was transfected into MSCs using a commercial transfection reagent (X-treme siRNA Transfection Reagent) according to the manufacturer's protocol. Cells were harvested 48 h after transfection for further analysis.

2.4. SA-β-gal staining

MSC senescence was determined by in situ staining for senescence-associated β-galactosidase (SA-β-gal) using the Senescence Cell Histochemical Staining Kit. Briefly after treatment MSCs were fixed in fixation buffer for 30 min at room temperature. After being washed with PBS cells were incubated with β-galactosidase staining solution for 16 h at 37 °C without CO_2. The reaction was stopped by the addition of PBS. Statistical analysis was performed by counting 600 cells for each sample.

2.5. ROS staining

Cells were left untreated or were treated with EPO, siRNA-Akt, and siRNA-NT separately or in combination and were then stimulated with the diluted fluoroprobe DCFH-DA for 20 min at 37 °C with slight shaking every 5 min. After washing with serum-free culture medium, the cells were collected and examined by flow cytometry.

2.6. Cell proliferation assay

Cell proliferation was assessed with a CCK-8 assay and an EdU proliferation assay according to the manufacturer's instruction. For the CCK-8 assay, cells were plated in 96-well plates (3×10^3 cells/well) with EPO (10–100 IU/ml) in triplicate. Assays were performed on days 1 to 4 after plating by adding 100 μl of fresh medium and 10 μl of CCK-8 solution to the wells and incubating the plates for an additional 2 h at 37 °C. The optical density (OD) was measured at 450 nm. The assay was repeated three times.

For the EdU assay, cells were incubated with EPO (50 IU/ml) for 3 d in 96-well plates and then were stained with Edu and Hoechst 33342 for 30 min. The EdU-positive cells were visualized with fluorescence microscopy (DMI4000B; Leica, Wetzlar, Germany), and the total number of EdU-positive cells were calculated by counting at least three random separate fields.

2.7. Western blot analysis

After the designated treatments, cells were lysed in RIPA buffer. The protein concentration was determined with a bicinchoninic acid (BCA)

kit according to the manufacturer's instructions. For western blot analysis, 60 μg protein was separated on SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in TBS containing 0.5% Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies diluted in TBST. The membranes were washed with TBST for 10 min 3 times and were then reprobed with HRP-conjugated secondary antibodies for 1 h. The membranes were then washed three times with TBST as described previously and visualized with Beyo ECL Plus reagent in an ECL chemiluminescence detection system. Antibodies that recognized the following proteins were used at the indicated dilutions: Akt (1:1000), p-Akt (Thr 308) (1:1000), p-Akt (Ser 473) (1:1000), FoxO3a (1:1000), p-FoxO3a (Thr32) (1:750), VEGF (1:1000), and HRP-conjugated secondary rabbit antibody (1:5000). Images were analysed using Image Lab software (version 4.1, Bio-Rad).

2.8. Cytokine measurement via ELISA

Using 500 μl of supernatant containing 5×10^5 cells, the VEGF, bFGF, HGF, IGF-1 and TNF-α concentrations were assessed with the manufacturer's instructions. The VEGF, bFGF, HGF, IGF-1 and TNF-α concentrations obtained from each assay were calculated based on calibration curves constructed from serial dilutions of human recombinant standards. The data are expressed in nanograms per millilitre, and the sensitivity of the VEGF, bFGF, HGF, IGF-1 and TNF-α assays was 2 pg/ml.

2.9. Tube formation assay

Matrigel tube formation assays were performed as previously described. Briefly, 48-well plates were coated with 60 μl of Matrigel per well and incubated for 30 min at 37 °C. HUVECs cultured with supernatant from the different treatment groups were then seeded at a density of 1.5×10^4 cells per well in RPMI 1640 medium supplemented with 0.2% FBS and incubated for 6 h at 37 °C. Subsequently, capillary-like structures were observed using an inverted microscope (Olympus IX73, Tokyo, Japan). The number of junctions per field was determined, and at least three different viewing fields were analysed.

2.10. Data analyses and statistics

The data are expressed as the mean ± SD of at least three independent experiments. Statistical analysis was performed with Student's *t*-test for comparisons between two sets of values. One-way ANOVA followed by Dunnett's test was used for comparisons involving three or more groups. $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered significant; these values are indicated by *, ** or ***, respectively.

3. Results

3.1. Hyperglycaemia affects the biological abilities of MSCs

To determine the effect of exposure to a hyperglycaemic environment on the biological properties of MSCs, we first examined the senescence and self-renewal potential of MSCs cultured under NG and HG conditions using β-gal staining and CCK-8 and EdU assays, respectively. We found that high glucose levels progressively increased the senescence (Fig. 1A and B) and decreased the viability of MSCs in a time-dependent manner (Fig. 1C–E); the difference became significant at 14 d and reached an even greater level of significance at 28 d. MSCs secrete a variety of cytokines and growth factors that can function in both paracrine and autocrine manners. Such trophic effects of MSCs are key proangiogenic factors that mediate the function of MSCs in tissue and vascular repair [11]. In our study, high glucose levels damaged the paracrine function of MSCs in relation to VEGF, bFGF, HGF and IGF expression (Fig. 1F–I). Taken together, the above results show that, under HG conditions, MSCs become senescent and that their proliferation rate and ability to release proangiogenic factors decreases. Previous studies focused on short-term culture under hyperglycaemia; in the clinical setting, however, chronic exposure to hyperglycaemia was more significant. To observe the effect of EPO in protecting cells against hyperglycaemia and mimic the environment of DM patients, the next experiment in our study was conducted over 28 d.

3.2. EPO restores the survival and function of MSCs exposed to a hyperglycaemic environment in a concentration-dependent manner

To determine whether EPO can restore the abilities damaged by hyperglycaemia and to assess the optimum concentration for treatment, we treated MSCs cultured under HG conditions with different concentrations of EPO (10–100 IU/ml). Since senescence reduces the regenerative potential of MSCs and is one of the main reasons for the increased susceptibility of MSCs to apoptotic cell death under ischaemic conditions [12], we examined the effect of EPO on regenerating the anti-senescence function of MSCs cultured under HG conditions and found that these cells were significantly less senescent than MSCs not exposed to EPO. The difference became significant at 50 IU/ml and reached an even greater level of significance at 100 IU/ml (Fig. 2A and B).

Next, we assayed cell proliferation using a CCK-8 assay and found that when we treated MSCs cultured under HG conditions with EPO, the proliferation rate increased significantly starting on day 3 of treatment and continuing until day 4 of treatment, at which point the proliferation rate started to resemble that of MSCs cultured under NG conditions (Fig. 2C).

Importantly, HG and anoxic microenvironments prevent MSCs from inhibiting tissue inflammation and promote the secretion of large amounts of inflammatory mediators, such as TNF-α. These effects enhance local inflammation and result in tissue and vessel repair-related disorders. Our results show that EPO can significantly decrease the production of TNF-α (Fig. 2D). In addition, ROS induced by HG microenvironments also damage the biological abilities and transplant efficacy of MSCs, and our findings suggest that EPO may exert its protective effect against oxidative stress-induced MSC apoptosis by reducing excessive amounts of ROS (Fig. 2E).

To determine whether EPO can restore the trophic activity in MSCs cultured under HG conditions, we performed ELISAs to quantify the levels of VEGF, bFGF, HGF and IGF in the culture medium of MSCs cultured under HG conditions with and without EPO treatment. We found that the paracrine ability of the HG + EPO group of MSCs was significantly better than that of the HG group of MSCs. In summary, compared with HG conditions alone, EPO treatment significantly enhanced the secretion of all four growth factors (Fig. 2F–I). These results suggest that EPO not only has a restorative function on MSCs cultured under HG conditions but might also exhibit anti-inflammatory properties. Since the difference can be observed at 50 IU/ml EPO, the cells in the subsequent experiment were treated with 50 IU/ml EPO.

3.3. EPO exposure leads to activation of the Akt/FoxO3a signalling pathway

After we validated the pro-survival effect of EPO on MSCs cultured under HG conditions, we further explored the underlying mechanism. Because Akt/FoxO3a signalling protects the heart against ischaemic injury and hyperglycaemia-induced damage, we examined the relationship between EPO and the Akt/FoxO3a pathway in MSCs cultured under HG conditions by treating MSCs with EPO (50 IU/ml) for 72 h. Compared with the HG group, the HG + EPO group exhibited Akt activation, as evidenced by the increased phosphorylation (p-Akt) at Ser473 and Thr308 observed in the HG + EPO group (Fig. 3A and B). Furthermore, FoxO3a activation (p-FoxO3a) was also observed (Fig. 3C

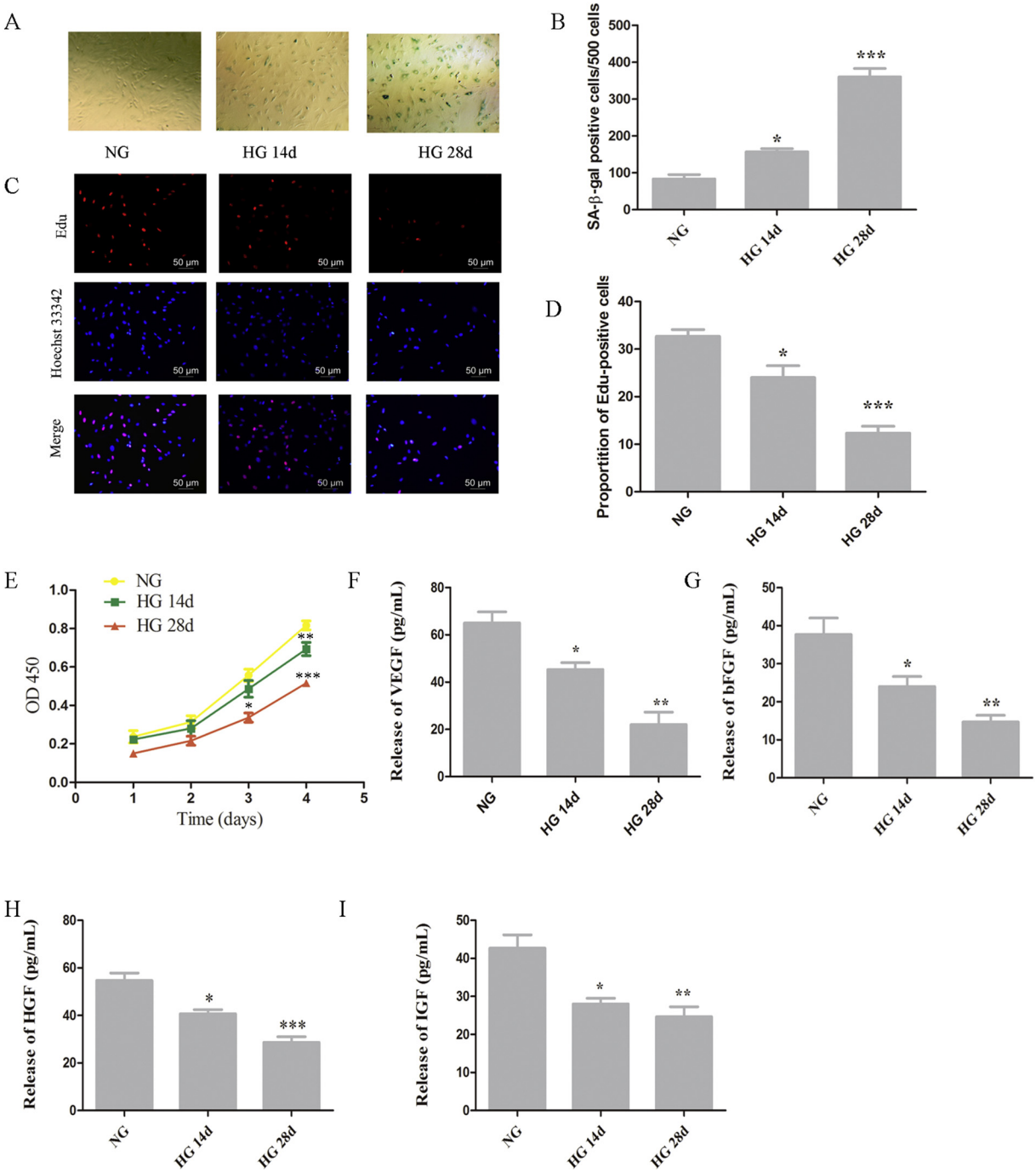

Fig. 1. Hyperglycaemia affects the biological abilities of MSCs.
MSCs were cultured in NG (5.5 mM) or HG (25 mM) culture medium for 14 d or 28 d. (A, B) Cellular senescence was analysed by SA-β-gal staining. Cell viability was determined by an EdU assay (C, D) and a CCK-8 assay (E). (F–I) The expression levels of VEGF, bFGF, HGF and IGF were measured by ELISAs. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$ compared with the NG group. SA-β-gal, senescence-associated β-galactosidase.

and D), which strongly suggests that the Akt/FoxO3a signalling pathway plays a pivotal role in the EPO-mediated effect on MSCs cultured under HG conditions.

3.4. The protective role EPO against hyperglycaemia is mediated by the Akt/FoxO3a signalling pathway

To further confirm a role for the Akt/FoxO3a pathway in mediating the protective effect of EPO against hyperglycaemia, Akt was blocked with the specific inhibitor siRNA-Akt. Western blotting revealed that siRNA-Akt inhibited Akt expression in the HG + EPO group (Fig. 4A and B). Moreover, co-incubation with siRNA-Akt partially abrogated the protective role of EPO on MSCs exposed to hyperglycaemia; compared with cells that were not treated with siRNA-Akt, the number of β-gal positive cells was increased (Fig. 4C and D) and the levels of VEGF, bFGF, HGF and IGF-1 in the conditioned medium were decreased in cells incubated with siRNA-Akt (Fig. 4G–J). Furthermore, pretreatment of MSCs with siRNA-Akt attenuated the anti-inflammatory effect of EPO and increased the TNF-α level and ROS production in the conditioned medium (Fig. 4E and F).

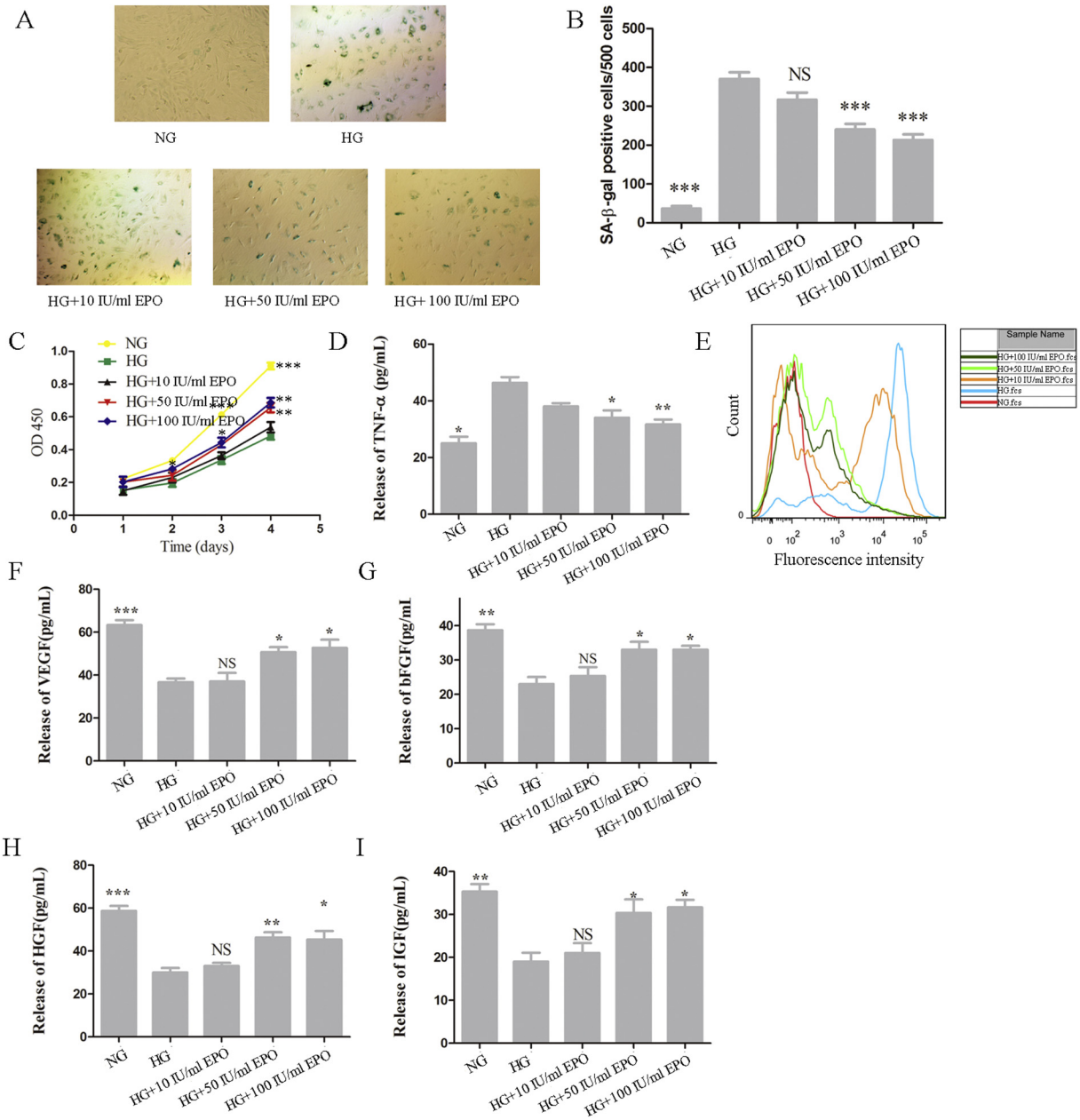

Fig. 2. EPO restores the survival and function of MSCs that have been exposed to a hyperglycaemic environment in a concentration-dependent manner
MSCs were cultured in NG (5.5 mM) or HG (25 mM) culture medium with or without exposure to different doses (10–100 IU/ml) of EPO for 72 h. (A, B) Cellular senescence was analysed by SA-β-gal staining. (C) Cell viability was determined by a CCK-8 assay. (E) Cellular ROS production was assessed by measuring the fluorescence intensity of DCFH-DA using flow cytometry. (D, F–I) The release of pro-inflammatory factor TNF-α and proangiogenic factors, namely VEGF, bFGF, HGF and IGF, was quantified by ELISA. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$ compared with the HG group. DCFH, 2–7′-dichlorodihydrofluorescein.

3.5. EPO increases the ability of MSCs to stimulate tube formation among HUVECs and enhances angiogenic growth factor secretion

A tube formation assay was used to test whether the conditioned medium from MSCs treated with EPO could stimulate angiogenesis in vitro. MSCs were cultured under HG conditions in the presence or absence of siRNA-Akt for 48 h. Then they were treated with EPO (50 IU/ml) for another 72 h and then with fresh medium for 24 h. The supernatant from the MSCs was then collected and used to stimulate HUVECs for 24 h. The results (Fig. 5A and B) indicate that compared with HG group, the conditioned medium from untreated MSCs and from MSCs preincubated with EPO (50 IU/ml) increased the capillary tube-forming capacity of ECs in vitro; the maximum proangiogenic effect was observed in the presence of the conditioned medium from MSCs that were preincubated with EPO, suggesting that a paracrine mechanism was involved in the proangiogenic effect of EPO on MSCs.

To further explore the relationship among EPO, MSCs cultured under HG conditions and the tube-forming ability of ECs, we detected the expression of VEGF with western blotting under different types of stimulation. The results showed that EPO can promote VEGF expression in ECs and that blocking the expression of Akt with siRNA-Akt abolishes the protective effect of EPO on MSCs (Fig. 5C and D).

Altogether, these data suggest that EPO can enhance the proangiogenic paracrine effect of MSCs under hyperglycaemic conditions and thereby augment the capillary tube-forming capacity of ECs in vitro.

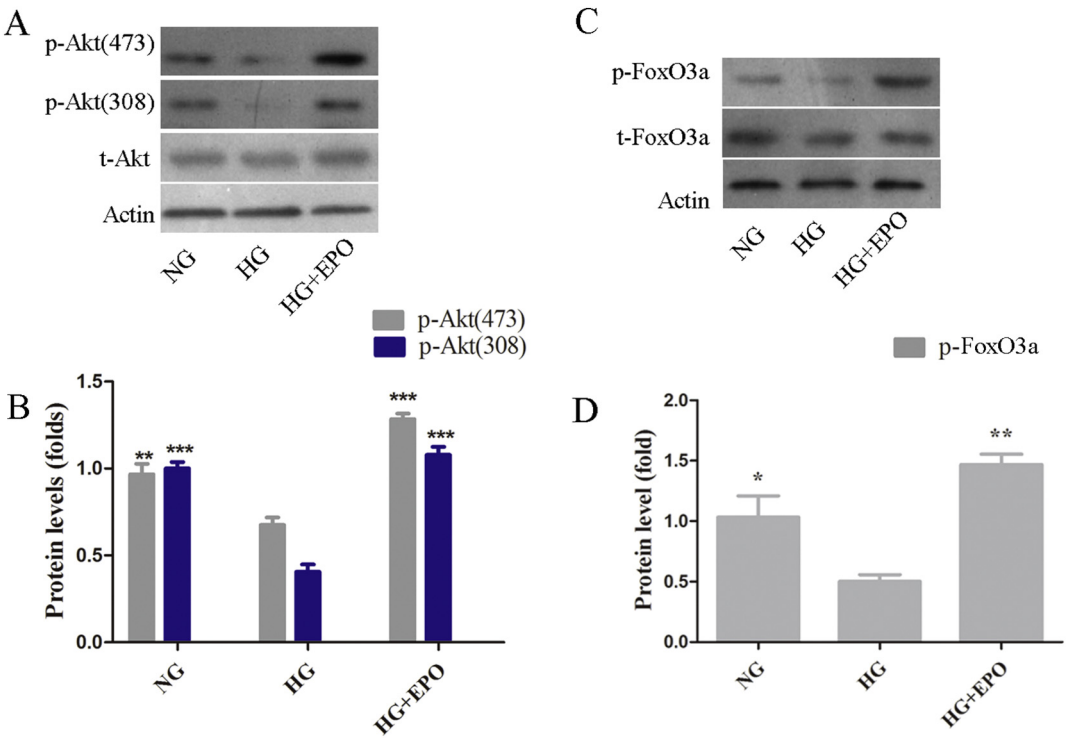

Fig. 3. EPO exposure leads to the activation of the Akt/FoxO3a signalling pathway.
MSCs were stimulated with EPO (50 IU/ml) for 72 h under hyperglycaemic conditions. Then, Akt/FoxO3a signalling pathway activation was detected in total cell lysates from the NG, HG and HG + EPO groups by western blot analysis. (A, B) p-Akt [(Ser473) or (Thr 308)] was upregulated in the HG + EPO group. (C, D) The expression levels of p-FoxO3a and t-FoxO3a were measured by western blot analysis. Data are presented as the means ± SDs of three separate experiments. $^{*}P < 0.05$ compared with the HG group. t-Akt, total Akt; p-Akt, phospho-Akt [(Ser473) or (Thr 308)]; p-FoxO3a, phospho-FoxO3a(Thr32); t-FoxO3a, total FoxO3a.

4. Discussion

In this study, we tested the hypothesis that EPO can ameliorate chronic hyperglycaemia-induced MSC injury, as observed by decreased senescence, increased proliferation and the promotion of angiogenic growth factor secretion. Furthermore, EPO can increase the ability of MSCs to stimulate the tube formation of ECs under hyperglycaemic conditions. Moreover, we showed that the effects of EPO were mediated at least partially through the Akt/FoxO3a signalling pathway.

Coronary artery disease remains the leading cause of morbidity and mortality in the Western world [13]. People with diabetes are two to four times more likely to develop cardiovascular disease than those without diabetes [14]. The population of DM patients is expected to increase to 592 million by 2035 [15]. Due to the large population and the worse outcome of CAD patients with DM relative to CAD patients without DM, we urgently need to explore what pathway DM mediates to affect the developments of MI.

Chronic hyperglycaemia exposure is an important promoter of MI. Hyperglycaemia extends the QT period and exacerbates the no reflow phenomenon, besides abnormal glycolysis increases the oxygen-consuming activity of myocardial cells and aggravates the ischaemic injury of myocardial cells [16]. Additionally, chronic hyperglycaemia exposure places the body in a chronic inflammation state, increases the release of pro-inflammation and vasoconstriction factors, and promotes the production of ROS; in turn, excessive ROS exposure increases the apoptosis of myocardial cells [17,18].

To treat MI, stem cell therapy is the most appealing treatment. In particular, BMSCs show promising results. Stem cell transplantation can increase the tube formation ability of ECs, reduce collagen and MMP-2 expression and inhibit ventricular remodelling in streptozotocin (STZ)-induced diabetic rats [3]. However, BMSCs from DM patients cannot repair a damaged myocardium. Govaert et al. transplanted autologous bone marrow mononuclear cells from normal or diabetic mice to the infarcted area and found that the latter ones cannot enhance the ejection fraction (EF) and left ventricular fractional shortening (LVFS) after 35 d [19]. An additional study showed that BMSCs from DM patients have reduced proliferation rates, are more likely to undergo apoptosis [4] and are more likely to enter senescence [20]. Culturing BMSCs with serum from DM patients reduced their angiogenic and restorative abilities [21]. All the above results show that hyperglycaemia attenuates the efficiency of stem cell transplantation and that MSCs from DM patients are not directly suitable for autologous transplantation. Thus, strategies to pretreat and modify the MSCs from DM patients to enhance MSC survival are pivotal for improving MSC transplantation efficiency. In the present study, we cultured the MSCs under high (25 mM) levels of glucose for as long as 28 d to mimic the chronic hyperglycaemia exposure conditions of MSCs from DM patients.

Numerous methods, such as pretreatment or modification with cytofactors, siRNA or drugs, to enhance MSC transplantation efficiency show certain effects [10,22,23]. In the present study, we focus on EPO. EPO ameliorated obesity and glucose homeostasis in diet-induced obese mice [24], and EPO pretreatment before I/R injury led to better myocardial protection under hyperglycaemic conditions than non-pretreatment group [25]. In our previous study, we showed that EPO treatment alleviated the inflammatory reaction, inhibited M1 polarization and promoted the tube forming ability of ECs under hyperglycaemic conditions. In this study, we explored whether EPO could reverse the damage that hyperglycaemia causes in MSCs. As shown in Fig. 1, we found that exposure to HG conditions for 28 d in vitro substantially increased the senescence of MSCs, as seen by the increase in β-gal-positive cells, and reduced their viability and proliferative ability. The reverse effect was seen in MSCs cocultured with EPO. In addition, EPO could inhibit the expression of hyperglycaemia-induced pro-inflammatory factors and abundant ROS production; both roles are important for restoring the viability and tissue repair ability of MSCs.

Various pathways are involved in the EPO-mediated protective

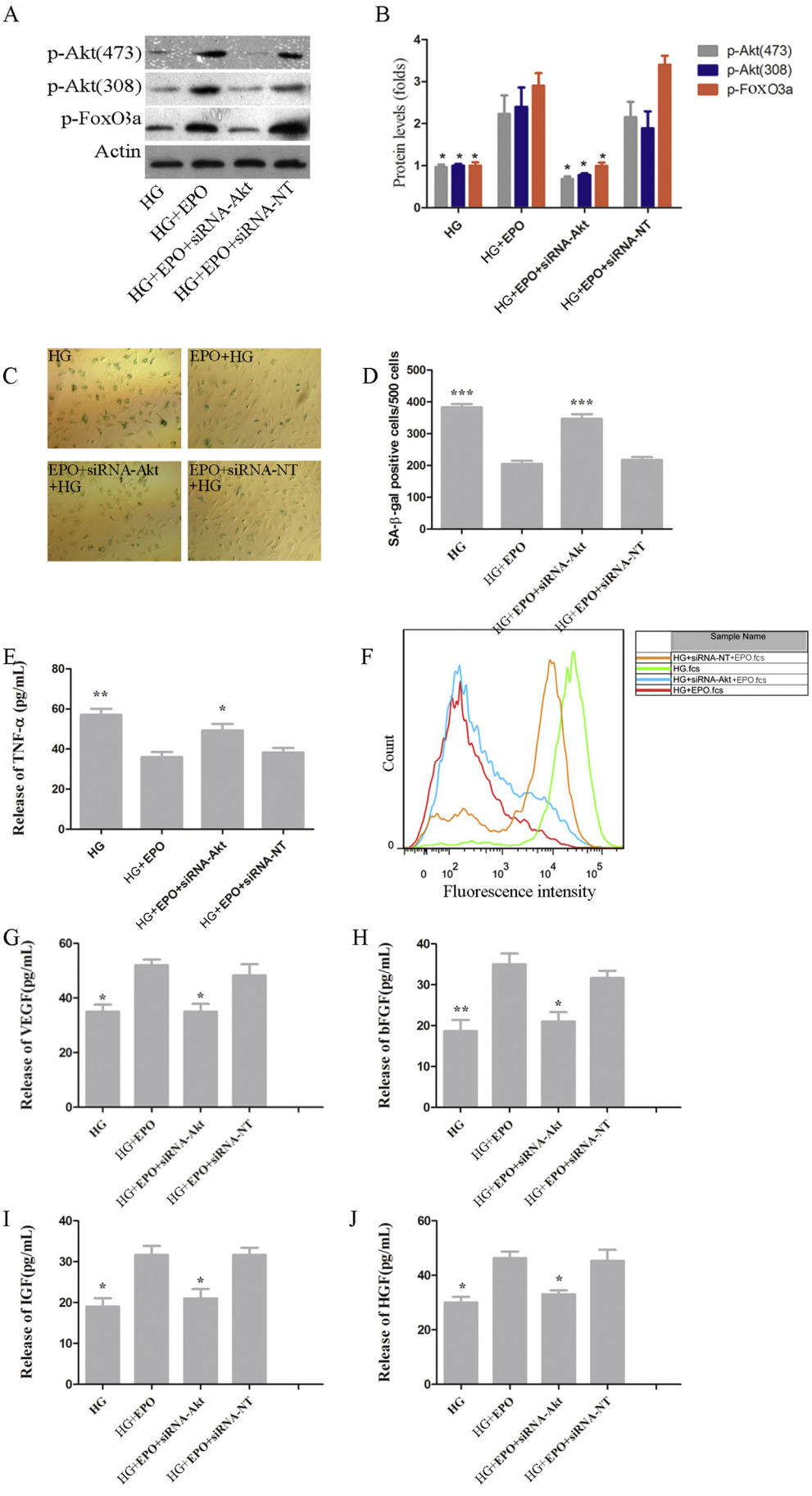

Fig. 4. The protective role of EPO against hyperglycaemia is mediated through the Akt/FoxO3a signalling pathway.
MSCs were cultured under HG conditions with or without the addition of 100 nM siRNA-Akt for 48 h and then stimulated with 50 IU/ml EPO for another 72 h. (A, B) The effect of siRNA-Akt on the Akt/FoxO3a signalling pathway was determined by western blot analysis. (C, D) Cellular senescence was analysed by SA-β-gal staining. (F) Cellular ROS production was assessed by measuring the fluorescence intensity of DCFH-DA using flow cytometry. (E, G–J) The release of pro-inflammatory factor TNF-α and proangiogenic factors, namely VEGF, bFGF, HGF and IGF, was determined by ELISA. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$ compared with the HG + EPO group.

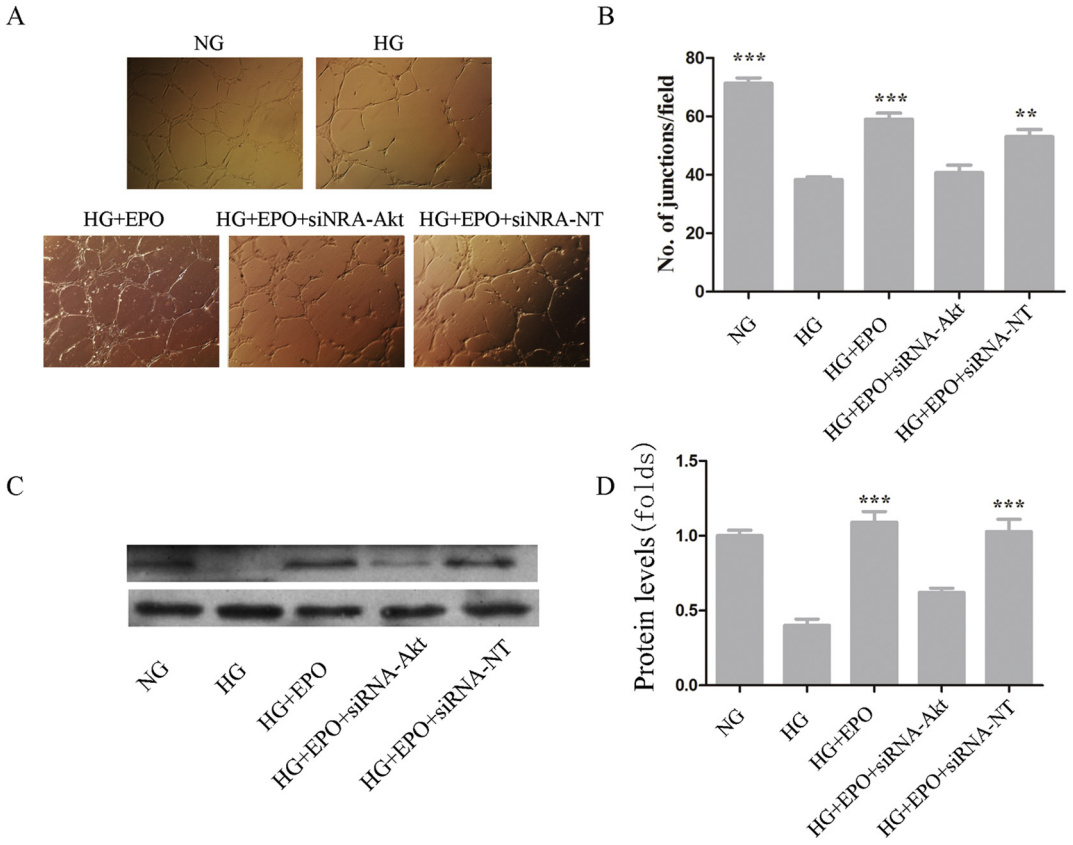

Fig. 5. EPO increased the ability of MSCs to stimulate tube formation among HUVECs and enhanced angiogenic growth factor secretion.
MSCs were cultured under HG conditions in the presence or absence of siRNA-Akt for 48 h. Then the medium were replaced with fresh ones containing EPO (50 IU/ml) for another 72 h and then fresh medium for 24 h. The supernatant from the MSCs was then collected and used to stimulate HUVECs for 24 h. (A, B) HUVECs were cultured under normal conditions, stimulated with high glucose levels or treated with different supernatants from MSCs in Matrigel-coated 48-well plates for 6 h. The number of tube junctions/field was counted. (C, D) HUVECs treated as above were cultured for 24 h, and the expression level of VEGF was determined by western blot analysis. Data are presented as the means ± SEMs of three separate experiments. $^{*}P < 0.05$ compared with the HG group.

effect against hyperglycaemia, of which Akt is a key factor for promoting cell survival and angiogenesis [26]. There are 3 Akt isoforms, each of which has different major functions. Akt1 regulates body size and adipogenesis [27], Akt2 controls insulin resistance and diabetes [28], and Akt3 affects the brain and neuronal cell function [29]. Akt2, rather than Akt1 or Akt3, knockout mice have severe insulin resistance following diabetes [30]. Akt phosphorylates its downstream targets to induce the above effect; among its targets, FoxO3a is a critical regulator of glucose homeostasis [31]. We further examined whether the Akt/FoxO3a signalling pathway is involved in the protective effect of EPO on MSCs under hyperglycaemic conditions. Our results showed that EPO increases Akt phosphorylation in response to hyperglycaemia and that these protective effects are effectively blocked by siRNA-Akt, as seen by the decrease in cell proliferation and the release of pro-angiogenic factors and by the increase in senescence, the production of ROS and the release of pro-inflammatory factors. These effects strongly suggest an essential role for the Akt/FoxO3a signalling pathway in the EPO-mediated protection of MSCs that are exposed to hyperglycaemic stress.

For MI patients, the formation of new blood vessels to offer adequate nutrient and oxygen is a key factor for recovery. Over the past decade, MSCs have been demonstrated to promote angiogenesis by secreting proangiogenic factors (e.g., VEGF, bFGF, HGF and IGF-1) that contribute to tissue repair and enhance the reparative process [32]. However, previous studies revealed a reduced expression of VEGF in the myocardial cells of diabetic patients [33]. In the present study, we observed that hyperglycaemia decreases the release of proangiogenic factors (VEGF, bFGF, HGF and IGF-1) from MSCs and damaged the tube-forming ability of ECs, while EPO reversed the negative effect of hyperglycaemia. To determine the role that EPO played in the communication between MSCs and ECs in angiogenesis under hyperglycaemic conditions, we used supernatant from EPO-cultured MSCs to test its effect on the tube-forming ability of ECs on Matrigel. As shown in Fig. 5, we found that the conditioned medium from EPO-pretreated MSCs significantly increased the number of junctions per field compared with those in the untreated group. This result suggests that EPO enhances the release of proangiogenic factors from MSCs and indirectly promotes tube-forming capacity by communicating with ECs, showing that pretreating MSCs with EPO under hyperglycaemic conditions may improve vascular recovery. Furthermore, blocking the Akt pathway in MSCs abolished the protective effect of EPO on their tube-forming capacity and their ability to release proangiogenic cytokines, suggesting that EPO exerts its protective effect against hyperglycaemia partly through the Akt pathway.

5. Conclusions

Overall, the results of the present study showed that EPO-pretreated MSCs showed better proliferation ability, less senescence and reduced release of pro-inflammatory factors compared with the untreated MSCs under hyperglycaemic conditions. Additionally, EPO can increase the secretion of proangiogenic cytokines and enhance the ability of MSCs to stimulate tube formation among ECs. The beneficial effects of EPO may result from Akt/FoxO3a signalling pathway activation. These data collectively demonstrate that EPO-pretreated MSCs may be used as a suitable candidate for stem cell-based therapy in DM patients who are

suffering from MI.

Abbreviations

CCK-8 cell counting kit-8
DCFH-DA 2′,7′-dichlorodihydrofluorescein diacetate
DMEM/F12 Dulbecco's modified Eagle's medium/nutrient mixture F-12
EdU 5-ethynyl-2′-deoxyuridine
ELISA enzyme-linked immunosorbent assay
FGF-2 fibroblast growth factor-2
FoxO3a Forkhead box O3a
HGF hepatocyte growth factor
HUVEC human umbilical vein endothelial cell
IGF-1 insulin-like growth factor-1
MSC mesenchymal stem cell
p-Akt phosphorylated Akt
PBS phosphate-buffered saline
PVDF polyvinylidene difluoride
ROS reactive oxygen species
SD Sprague-Dawley
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS Tris-buffered saline
TBST TBS with Tween-20
VEGF vascular endothelial growth factor

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Not applicable.

Authors' contributions

JJC contributed to the experimental design, performed the molecular biology experiments and the statistical analysis, and drafted the manuscript. XHL participated in designing the study and revised the manuscript. ZQZ performed the flow cytometric assay and revised the manuscript. YLX was responsible for MSC culture and statistical analysis and helped draft the manuscript. XXL participated in the EdU and Hoechst 33342 dye assays and helped draft the manuscript. FYZ conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

All study procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University (reference no. KY2016-180). This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Academy Press (National Institutes of Health, revised in 1996).

Consent for publication

Not applicable.

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