Original Research

Mesenchymal stem cells alleviate hypoxia-induced oxidative stress and enhance the pro-survival pathways in porcine islets

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Impact statement

The utilization of mesenchymal stem cells (MSCs) is a promising approach to serve as adjuvant therapy for islet transplantation. But the inability to translate promising preclinical results into sound therapeutic effects in human subjects indicates a lack of key knowledge of MSC-islet interactions that warrant further research. Hypoxia and oxidative stress are critical factors which lead to a tremendous loss of islet grafts. However, previous studies mainly focused on other aspects of MSC protection such as inducing revascularization, enhancing insulin secretion, and reducing islet apoptosis. In this study, we aim to investigate whether MSC can protect islet cells from hypoxic damage by inhibiting ROS production and the potential underlying pathways involved. We also explore the effects of MSC-derived exosomes and IL-6 on hypoxia-injured islets. Our data provide new molecular targets for developing MSC applications, and this may ultimately promote the efficiency of clinical islet transplantation.

Abstract

Islet transplantation is a promising treatment for selected patients with type 1 diabetes mellitus (T1DM). Hypoxia and oxidative stress are major causes of damage to transplanted islets. Mesenchymal stem cells (MSCs) have been shown to enhance cell survival mainly through paracrine secretion. However, mechanisms of action underlying the protective effects of MSCs on islets have not been fully elucidated. In this study, we investigated whether human umbilical cord-derived MSCs (huc-MSCs) could inhibit hypoxia and ROS-related cell death of neonatal porcine islet cell clusters (NICCs) and further determined the underlying molecular mechanisms. NICCs were cultured *in vitro* under normoxic and hypoxic (1% O_2) conditions with or without MSC-conditioned medium (MSC-CM). Apoptosis of NICCs was evaluated by the AO/EB staining and Annexin V/PI flow cytometry analysis. Total and mitochondrial ROS production was detected by fluorometric assays. Western blot and the ERK pathway inhibitor, PD98059, were used to assess the possible pathways involved. The results showed that MSC-CM suppressed hypoxia-induced oxidative stress and cell death of NICCs. MSC-CM also activated several pro-survival pathways in NICCs under hypoxic conditions. Furthermore, MSC-secreted exosomes and IL-6 partially recapitulated the multifunctional benefits of MSC-CM. This study showed that huc-MSCs protected NICCs from hypoxia-induced cell death by regulating the cell redox state and cell signaling pathways. This increased understanding may enable MSCs to become a more promising adjuvant cell therapy for islet transplantation.

Keywords: Islet transplantation, mesenchymal stem cells, hypoxia, oxidative stress, the ERK pathway, exosome

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Introduction

Pancreatic islet transplantation has emerged as well-recognized clinical practice for selected patients with type 1 diabetes mellitus (T1DM) who suffer from repeated and severe hypoglycemia.[1] However, many obstacles including donor shortage and graft loss enormously limit the clinical application of this procedure.

Hypoxia is the primary initiator of islet injury and the leading cause of graft loss.[2] During islet isolation, culturing, and the early period after transplantation, islet cells face hypoxic stress due to the lack of vascularization.

Oxidative stress is a distinguishing characteristic associating with the β-cell injury. Due to the low expression of antioxidant enzymes, islets are considered to be particularly vulnerable to ROS attacks. Redox imbalance has a bad influence on both islet grafts and host immune cells which lead to inflammation.[3] In addition, oxidative stress interrupts the process of insulin secretion and insulin

action and causes defective angiogenesis.[4] Collectively, these changes may compromise islet viability and functionality *in vivo*.

On the other hand, activating pro-survival pathways restricts oxidative stress and cell death. However, the signaling events associated with islet survival have not been fully explored. The ERK and AKT pathways are involved in controlling cell proliferation and maintaining cell viability. Moreover, they are both in association with oxidative stress.

This study aims to identify the precise role of oxidative stress and pro-survival pathways in the huc-MSCs' protection against the hypoxia-related death of neonatal porcine islets (NICCs). Moreover, we investigate the possible roles of exosomes and interleukin 6 (IL-6) in MSCs' beneficial effects.

Materials and methods

MSC isolation, culture, and characterization

All clinical procedures followed the protocols approved by the Human Research and Ethics Committee of the Third Xiangya Hospital. Huc-MSCs were isolated from fresh umbilical cord samples as previously described.[5] Huc-MSCs were cultured in alpha-MEM medium (GE Healthcare, USA) supplemented with 2 mM L-Glutamine (GlutaMAXI, Thermo Fisher, USA) and 5% (v/v) UltraGROTM-Advanced cell culture supplement (Helios BioScience, USA). MSCs from passages 3–5 were used for downstream applications.

We evaluated the phenotype of huc-MSCs by a flow cytometer (Beckman, USA). OriCell™ human umbilical cord mesenchymal stem cell differentiation kits (Cyagen Biosciences, China) were used to detect the multilineage differentiation potential of huc-MSCs.

Generation of huc-MSCs-conditioned medium

When the huc-MSCs reached 90% confluence, they were washed with PBS and changed into serum-free medium to stimulate MSC secretion. After 48 h incubation at 37°C and 5% CO_2 in normoxic conditions, the medium was collected as the conditioned medium (CM). The CM was used immediately or stored at −80°C for later use.

Exosome isolation and characterization

Exosomes were isolated from the MSC-CM using the Minute™ Hi-Efficiency Exosome Precipitation Reagent (Invent Biotechnologies, USA). Exosomes were resuspended in phosphate-buffered saline (PBS) and stored at −80°C. Western blot was performed to detect the protein markers of exosomes. The size of the exosomes was measured by nanoparticle tracking analysis (NTA) technology using a Zetasizer Nano instrument (Malvern Panalytical, UK). Tecnai G2 Spirit transmission electron microscopy (Thermo, USA) was used to observe the morphology of exosomes and Orius CCD camera (Gatan, CA, USA) was used for image capture.

Porcine islets isolation and culture

Animal studies were approved by the Ethics Committee of the Third Xiangya Hospital of Central South University. Newborn (three to five-day-old) pigs (Hunan Xeno Life Science, China) were used to isolate NICCs. We performed the isolation protocol as previously described[6] and isolated NICCs were cultured in RPMI-1640 medium supplemented with 10% porcine serum (Invitrogen, USA), 10 mmol/L nicotinamide (Sigma, USA), 2 mmol/L L-glutamine (Invitrogen, USA), 50 mmol/L isobutylmethylxanthine (Sigma, USA), and 100 U/mL penicillin and streptomycin (Invitrogen). NICCs were cultured in both normoxic (20% O_2) and hypoxic (1% O_2) conditions. We added MSC-CM at the beginning of the assay and validated cell death after 72 h.

AO/EB staining

Acridine orange/ethidium bromide (AO/EB) fluorescence staining was used for estimating the viability of islets. Acridine orange (green fluorescence) stains live cells, while ethidium bromide (red fluorescence) stains for dead cells. Islets were stained with dithizone and counted under the microscope. The number of islets was converted into islet equivalents (IEQ) as previously described.[7] We calculated islet death by Image J software (National Institutes of Health, USA) as follows: the proportion of EB positive dead cells (%) = (red area/(red area + green area)) × 100. At least five images for each group, accounting for more than 50 NICCs, were analyzed under a fluorescence microscope (EVOS, Life technologies, USA).

Annexin V/PI apoptosis assay

We used the FITC Annexin V Apoptosis Detection Kit I (BD, USA) to measure the death of NICCs. The NICCs were first digested into a single cell solution by TrypLE Express (Gibco, 12604–013). The staining was performed according to the manufacturer's instruction. A flow cytometer (Beckman, USA) was used for sample acquisition, and the Beckman software was used for data analysis.

ROS measurement

The total ROS accumulation was measured with a fluorescent dye H2DCFDA (Invitrogen, USA). MitoSOX™ Red Mitochondrial Superoxide Indicator (Invitrogen, USA) was used to detect mitochondrial ROS production of NICCs. For both ROS detection kits, the fluorescence intensity was detected by an EnVision Multimode Plate Reader (PerkinElmer, USA) as well as a fluorescence microscope (EVOS, Life technologies, USA). N-acetylcysteine (NAC) (Sigma, USA) was used as a ROS scavenger. NAC was added to the NICCs culture after 24-h exposure to hypoxia and apoptosis was analyzed at 72 h. GSH/GSSG ratio detection assay kit (Abcam, UK) and glutathione peroxidase assay kit (Abcam, UK) were used to examine antioxidants in NICCs.

Western blot

NICCs were lysed in RIPA lysis and extraction buffer (Thermo, USA). Protein concentration was determined

using the PierceTM BCA protein assay kit (Thermo, USA). Lysates were separated in SDS-polyacrylamide gels and then transferred to nitrocellulose (NC) membranes. Membranes were blocked with 5% BSA and incubated with primary antibody overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using the Super ECL Plus chemiluminescent substrate (Thermo Pierce, USA) and recorded on X-ray films.

IL-6 and HGF detection

Human IL6 ELISA Kit (Proteintech, USA) and human hepatocyte growth factor (HGF) ELISA kit (CUSABIO, China) were used to detect the concentration of IL-6 and HGF in MSC-CM, respectively. Recombinant Human IL-6 (PeproTech, USA) was added to NICC culture as an exogenous source of IL-6.

Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis was performed using Prism 7 software (GraphPad Software, USA). The unpaired Student's *t*-test and ANOVA with Tukey's post-test were used to compare the results in each experiment. $P < 0.05$ was regarded as a statistical significance.

Results

Huc-MSC-CM abrogates hypoxia-induced death of NICCs

We characterized huc-MSCs from three to five passages, and these cells were highly positive for CD90, CD73, CD105 (>95%) but were negative for CD34, CD45, CD11b, and HLA-DR (<1%) (Figure S1(a)). Huc-MSCs could differentiate into osteoblasts, adipocytes, and chondroblasts after *in vitro* induction (Figure S1(b)).

We evaluated whether huc-MSC-conditioned medium (huc-MSC-CM) could reduce hypoxia-related death. AO/EB staining showed that hypoxia significantly raised the proportion of EB-positive dead islets compared with the normoxia group. MSC-CM markedly attenuated cytotoxicity under hypoxic conditions (the hypoxia group vs. the hypoxia+CM group, $P < 0.001$, Figure 1(a)). We then use the Annexin V/PI method to more precisely detect islet injury. A higher proportion of NICCs appeared as Annexin V+ in the hypoxia group compared with the normoxia group, and MSC-CM significantly reduced the level of Annexin V+ cells under hypoxia ($P < 0.001$) (Figure 1(b)).

We next assessed the effect of N-acetylcysteine (NAC), a well-known ROS scavenger, on hypoxia-associated NICCs death. We documented that 1 mM NAC significantly decreased the Annexin V+ cells in hypoxic conditions ($P < 0.01$, Figure 1(c)). Then the protective role of MSC-CM against exogenous ROS molecules was analyzed. We found that MSC-CM potently suppressed cytotoxicity caused by 2.5 mM H_2O_2 at 24 h and 48 h (H_2O_2 vs. H_2O_2+CM, $P < 0.01$, Figure 1(d)).

Huc-MSC-CM depresses oxidative stress of hypoxia-injured NICCs

To determine the effect of MSC-CM on the hypoxia-associated oxidative stress of NICCs, we used the H2DCFDA to detect the generation of total ROS by islet cells. The results obtained

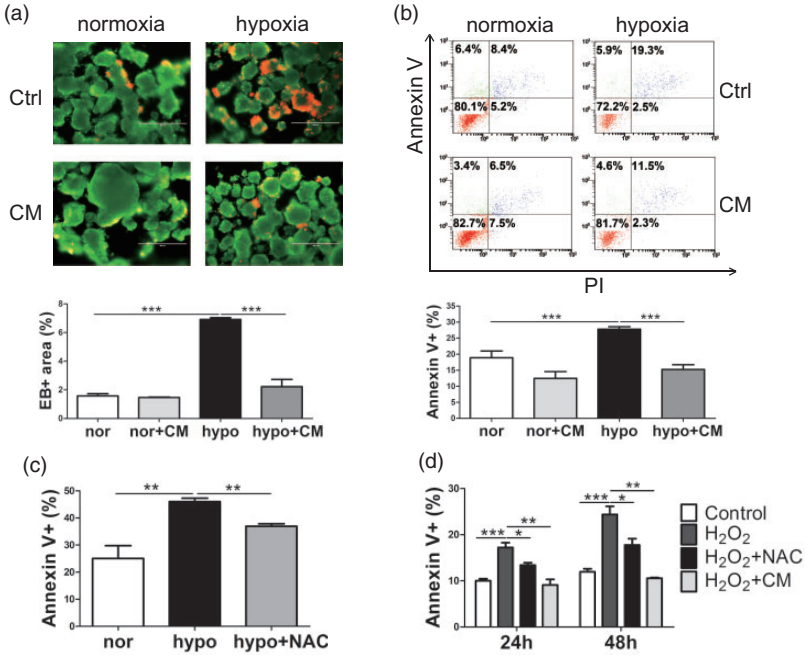

Figure 1. Huc-MSC-CM inhibits hypoxia-induced cell death of NICCs. (a) Representative overlay images of NICCs, stained with acridine orange/ethidium bromide (AO/EB) under normoxic and hypoxic (1% O_2) conditions with or without the MSC-CM. Scale bar = 400 μm. The percentages of EB positive area were analyzed by Image J software and shown as a bar chart. (b) Effects of huc-MSC-CM on the death of NICCs under hypoxia evaluated by Annexin V/PI apoptosis assay. Representative flow cytometry images were selected from five independent experiments. (c) 1 mM NAC inhibited the hypoxia-induced death of NICCs. (d) Inhibition of 2.5 mM H_2O_2-induced death of NICCs by MSC-CM. NAC (1 mM) is used as a positive control. $n = 3$. Ctrl: control group; CM: conditioned medium; nor: normoxia; hypo: hypoxia. Data are presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (A color version of this figure is available in the online journal.)

by a fluorescence microplate reader showed that hypoxia raised the fluorescence intensity (the normoxia group vs. the hypoxia group, 24 h: $P < 0.001$, 72 h: $P < 0.01$) and the MSC-CM significantly suppressed the total ROS level (the hypoxia group vs. the hypoxia+CM group, 24 h: $P < 0.001$, 72 h: $P < 0.01$) of NICCs (Figure 2(a)). Observation by the fluorescence microscope confirmed that MSC-CM blocked ROS production induced by hypoxia which was shown by green fluorescence (Figure 2(b)).

Another fluorometric assay was used to measure mitochondrial ROS (mROS). Similarly, the fluorescence microplate reader demonstrated that MSC-CM decreased the hypoxia-induced mROS generation (the hypoxia group vs. the hypoxia+CM group, 24 h: $P < 0.01$, 48 h: $P < 0.01$) (Figure 2(c)). The images from the fluorescence microscope showed induction of mROS in the hypoxia group and reduction of mROS by MSC-CM or NAC treatment at 24 h and 48 h (Figure S2(a)).

Next, we identified that 48-h exposure to hypoxia markedly reduced the concentration of reduced glutathione (GSH) ($^{**}P < 0.01$) and the activity of glutathione peroxidase (Gpx) ($^{***}P < 0.001$) of NICCs, while MSC-CM partially maintained the levels of these two antioxidants during hypoxia (Figure 2(d)).

Huc-MSC-CM protects NICCs by activating the ERK pathway

Then we further explored the molecular basis of MSCs' protection. Western blot analysis showed that hypoxia inhibited the phosphorylation levels of several marker proteins in the ERK pathway (including ERK1/2, MEK, and Raf), and MSC-CM enhanced the phosphorylation of these proteins under hypoxic conditions (Figure 3(a)). Moreover, MSC-CM also increased the phosphorylation of Akt protein and reduced the level of NLRP3 in the hypoxic environment.

To confirm the involvement of the ERK pathway in the beneficial effects of MSC-CM, we performed the Annexin V/PI assay. The results showed that PD98059, an ERK

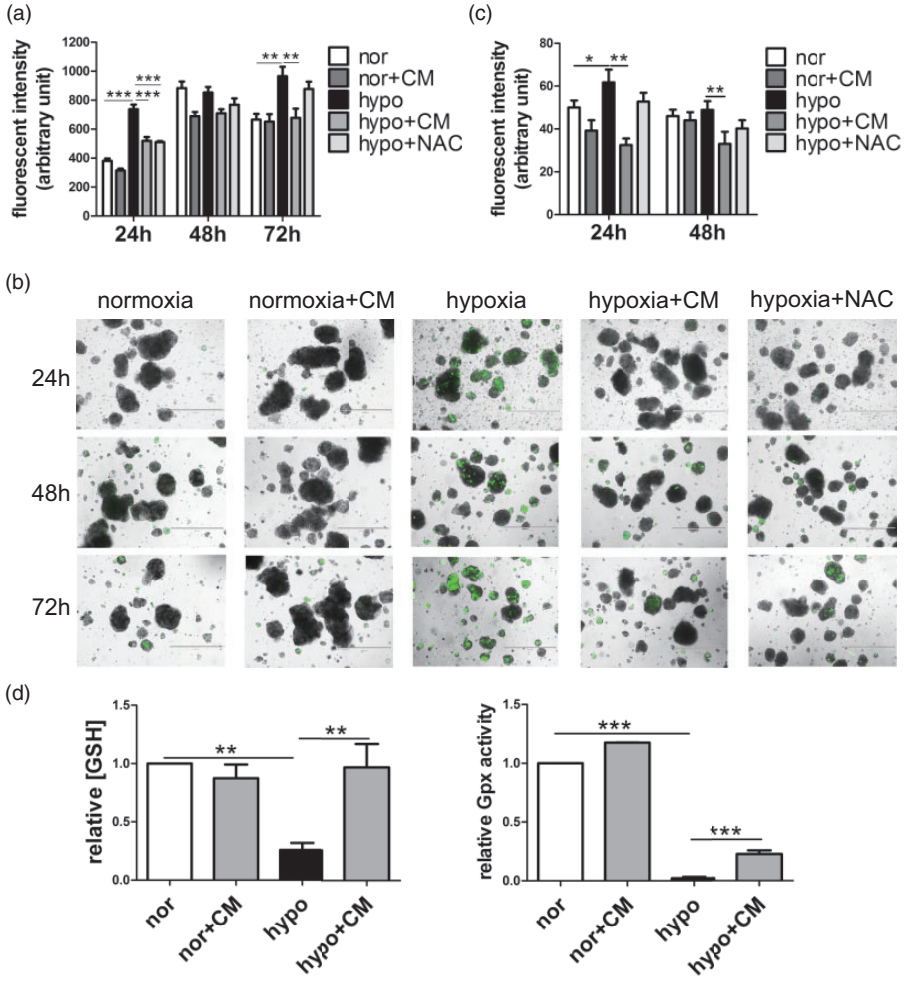

Figure 2. Huc-MSC-CM suppresses ROS generation of NICCs under hypoxic conditions. The total ROS was measured using H2DCFDA dye, and the fluorescence intensity was measured by a microplate reader (a, $n = 3$) and a fluorescence microscope (b, scale bar = 400 μm.). Mitochondrial ROS was detected by a microplate reader (c, $n = 3$). (d) MSC-CM maintained the concentration of reduced glutathione (GSH) and the activity of glutathione peroxidase (Gpx) of NICCs under hypoxic conditions. nor: normoxia; hypo: hypoxia; CM: conditioned medium. Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

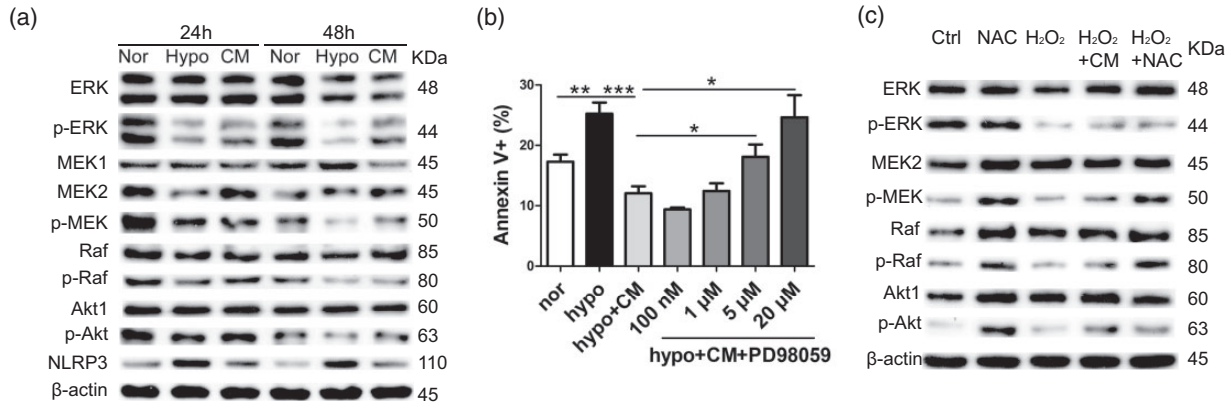

Figure 3. The effects of huc-MSC-CM on NICC pathways. (a) Representative Western blot images of marker proteins in several pathways. Nor: normoxia; Hypo: hypoxia; CM: hypoxia+MSC-conditioned medium. (b) The ERK pathway inhibitor, PD98059 was added at different concentrations to the hypoxia+CM groups to reverse the protection of MSC-CM. (c) MSC-CM alleviated the suppression of protein phosphorylation by exogenous H_2O_2. nor: normoxia; hypo: hypoxia; CM: conditioned medium; Ctrl: control group. Values are means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

inhibitor, dose-dependently rescued the anti-apoptotic effect of MSC-CM during hypoxia (Figure 3(b)).

To clarify the possible link between ROS and pro-survival pathways, we showed that exogenous H_2O_2 suppressed the expression of p-ERK and p-Raf in the ERK pathway, while MSC-CM activated the proteins of the pro-survival pathways (p-ERK, p-MEK, p-Raf, and p-Akt) under H_2O_2 exposure (Figure 3(c)).

Exosomes mediate the protective effects of huc-MSCs

For exosome identification, the nanoparticle tracking analysis (NTA) showed that the size distribution of exosomes was within 50–150 nm and had peaks of 118.0 ± 15.50 nm and 113.5 ± 19.36 nm when measured by intensity or volume, respectively (Figure S1(c)). Exosomes expressed protein markers including CD9, CD63, and tsg101 (Figure S1(d)). Transmission electron microscopy (TEM) demonstrated that exosomes had typical saucer-like or cup-shaped morphology (Figure S1(e)).

To determine whether exosomes involve in the positive effect of MSCs against the hypoxic injury of NICCs, we observed that 5 μg/mL huc-MSC-derived exosomes significantly decreased Annexin V+ NICCs (the hypoxia group vs. the hypoxia+exosome group, $P < 0.001$, Figure 4(a)). Similar to the effect of MSC-CM, exosomes inhibited the total ROS production of NICCs in hypoxic condition as demonstrated by both fluorescence microplate reader (Figure 4(b)) and fluorescence microscope (Figure S2(b)). Additionally, huc-MSC-derived exosomes antagonized the downregulation of several phosphorylated ERK signaling proteins after 24-h hypoxic culture. However, huc-MSC exosomes failed to increase the Akt phosphorylation (Figure 4(c)).

IL-6 involves in MSCs' protection

Besides exosomes, we detect some soluble proteins including interleukin 6 (IL-6) (778 ± 1.7 pg/mL) and hepatocyte growth factor (HGF) (593 ± 32 pg/mL) in MSC-CM (Figure 5(a)). In line with the results obtained from MSC-CM, our experiments further verified that 200 ng/mL recombinant human IL-6 (rIL-6) potently alleviated Annexin V+ cells (Figure 5(b)) and total ROS generation (Figure 5(c)) under hypoxic conditions. Furthermore, rIL-6 increased the level of p-ERK, p-MEK, p-Raf, and p-Akt under hypoxic conditions. rIL-6 also upregulated the expression of Nrf2, an antioxidative transcription factor (Figure 5(d)).

Discussion

Previous studies have established that MSCs protect transplanted islets mainly by inducing revascularization,[8] modulating the immune responses,[9] enhancing insulin secretion,[10] and reducing islet apoptosis. However, evidence suggested that hypoxia induced ROS production in islet cells[11] and MSCs suppressed ROS generation in many cell types other than islets by secreting antioxidants such as superoxide dismutase (SOD)[12] and glutathione (GSH).[13] Our previous observation using seahorse assay demonstrated that hypoxia significantly reduced the oxygen consumption rate (OCR) of NICCs and MSC-CM partially reversed the mitochondrial respiration efficiency of islet cells.[14] The evidence reveals that inhibition of oxidative stress may be a critical path through which MSCs protect hypoxia-exposed NICCs.

In the present study, we started by confirming that MSC-CM inhibited hypoxia-induced islet death. By using the ROS scavenger, NAC, we unveiled that oxidative stress is involved in the hypoxia-induced death of NICCs. Further results showed that MSC-CM downregulated total as well as mitochondrial ROS production in NICC under hypoxic conditions. Also, MSC-CM could suppress islet injury induced by exogenous H_2O_2. In addition, huc-MSCs might restrict ROS-induced islet damage by maintaining important antioxidants in NICCs. Critically, oxidative stress has an intricate relationship with many other cellular events such as ER stress and inflammation[15] and may influence multiple downstream cellular responses.

We next attempted to search for signaling molecules related to oxidative stress and islet death. We identified that hypoxia reduced the phosphorylation of several

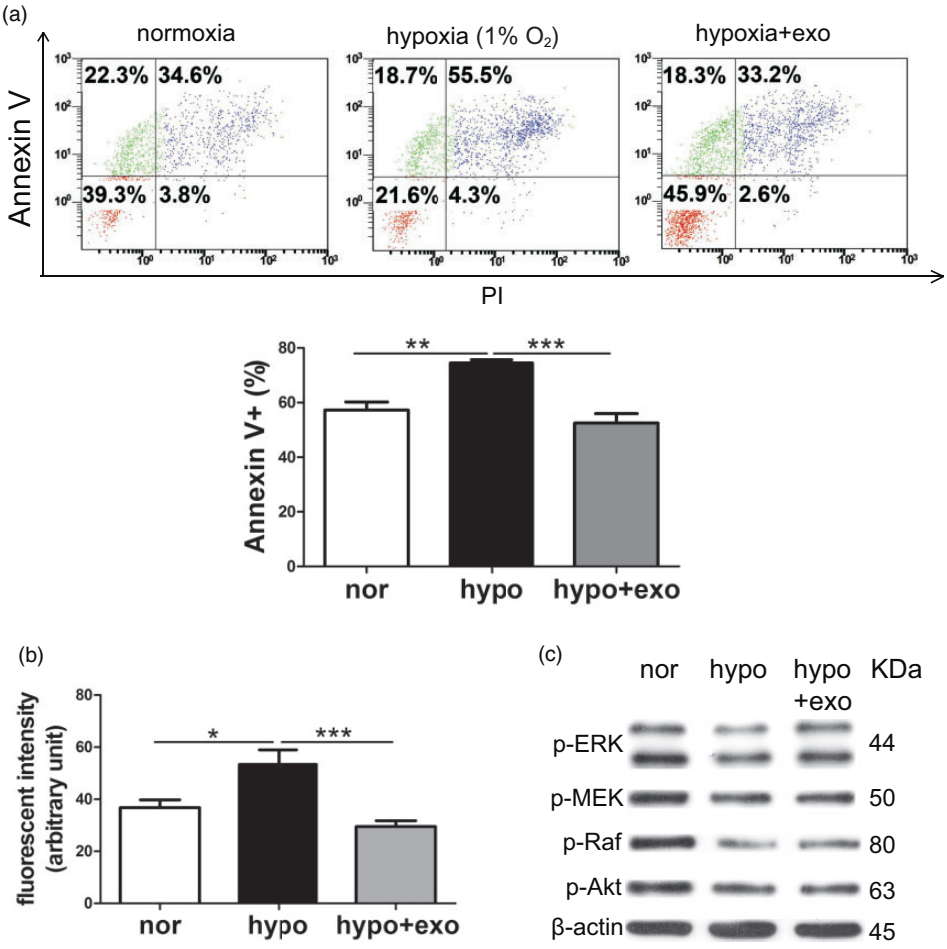

Figure 4. Exosomes mediate the protection of huc-MSCs on hypoxia-induced islet injuries. (a) MSC exosomes (5 μg/mL) inhibited the islet death under hypoxia. $n = 3$. (b) Measurement of the total ROS level of NICCs by a microplate reader with or without huc-MSC-derived exosomes (5 μg/mL). $n = 3$. (c) The MSC exosomes upregulated the protein phosphorylation in the ERK pathway at 24 h after hypoxia exposure shown by Western blot. nor: normoxia; hypo: hypoxia; exo: exosomes. Values are means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

protein markers in the ERK and AKT pathways and MSC-CM could reactivate these proteins in hypoxic conditions. The experiment using ERK inhibitor (PD98059) indicated that ERK, the downstream effector of the cascade, might be a crucial factor in promoting the survival capacity of islets. The extracellular signal-regulated kinase (ERK) pathway belongs to the MAPK pathways which also includes the p38 and JNK pathways. The ERK pathway is often shown to maintain cell survival, while p38 and JNK pathways are generally associated with cell death, and this is also true with islets.[16,17] Indeed, sustained p38 activities and declined ERK activation are a predictor of cell death after islet isolation.[16] Ghrelin prevented dexamethasone-induced INS-1 cell apoptosis via activating ERK and suppressing p38MAPK signaling.[18] Notably, both ERK and AKT pathways could act as a defender of oxidative stress. Activated ERK signal promoted cell survival under ROS microenvironment[19] and inactivated ERK pathway induced the ROS-mediated mitochondrial apoptotic pathway.[20] Similarly, AKT activation maintained the cell redox homeostasis, while AKT suppression weakened the antioxidant capacity of cells.[21]

Both proteins and extracellular vesicles, especially exosomes, have been reported to mediate pivotal paracrine functions of MSCs.[22] In the context of islet transplantation, one study showed that genetically modified exosome infusion after MSC transplantation improved islet graft efficacy in diabetic mice with decreased T cell infiltration and enhanced glucose tolerance results.[23] Moreover, MSCs are vital sources of anti-apoptosis exosomes.[24] Besides, MSC exosomes were shown to suppress oxidative stress in some cell types other than islets.[25] On the other hand, IL-6 is a multifunctional cytokine involved in a broad range of (patho)physiological conditions. IL-6 was recently proved to promote revascularization of islet grafts in a pig to non-human primate model.[26] IL-6 could suppress oxidative stress via activating the Nrf2-antioxidant pathway.[27] Many signaling pathways including MAPK, PI3K/AKT and AMPK can be activated by IL-6. But the involvement of these pathways seems to be context- and cell type-dependent.[28] In line with these findings, we observed that adding huc-MSC-derived exosomes as well as rIL-6 inhibited the death and total ROS production and activated the ERK pathway of NICCs under hypoxic conditions. The results

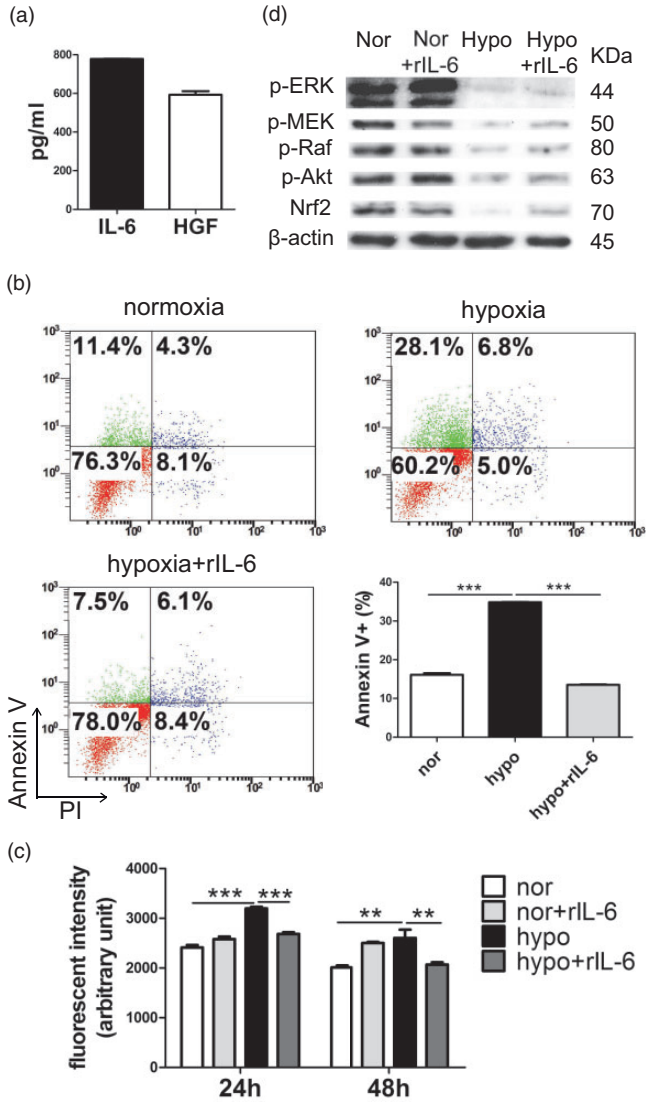

Figure 5. IL-6 plays a role in MSCs' protection. (a) ELISA quantification of IL-6 and HGF in MSC-CM. (b) The recombinant human IL-6 (rIL-6) decreased the hypoxia-induced death of NICCs. rIL-6 suppressed total ROS accumulation (c) and augmented protein phosphorylation (d) under hypoxic conditions. nor: normoxia; hypo: hypoxia; CM: conditioned medium. Values are means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

implied that exosomes or IL-6 involved in the beneficial paracrine mechanisms of huc-MSCs.

In conclusion, this study not only yields novel insights into how MSCs support the survival of NICCs under hypoxia conditions but also may provide new clues to develop MSC therapies that target oxidative and pro-survival signalings. The information gained moves us closer to improving functional graft survival and extending the usability of clinical islet transplantation.

Authors' contributions: MT, PR, and WW supervised the research. YT designed the study and wrote the manuscript. YT, XH, and YX conducted the experiments and analyzed data. WN, CC, JZ, and XM participated in critical review and discussion of the article. WW gave the final approval of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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