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# Human mesenchymal stem cells–derived conditioned medium inhibits hypoxia-induced death of neonatal porcine islets by inducing autophagy

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## Abstract

**Background:** The dysfunction of islet grafts is generally attributed to hypoxia-induced damage. Mesenchymal stem cells (MSCs) are currently thought to effectively protect cells from various risk factors via regulating autophagy. In our study, we investigated if human umbilical cord-derived MSCs could ameliorate hypoxia-induced apoptosis in porcine islets by modulating autophagy, and we explored the underlying mechanisms.

**Methods:** Neonatal porcine islet cell clusters (NICCs) were cultured with human umbilical cord-derived MSC conditioned medium (huc-MSC-CM) and RPMI-1640 medium (control) under hypoxic conditions (1% $O_2$) in vitro. NICCs were treated with 3-methyladenine (3-MA) and chloroquine (CQ) to examine the role of huc-MSC-CM in regulating autophagy. Finally, the levels of several cytokines secreted by huc-MSCs were detected by ELISAs, and the corresponding inhibitors were applied to investigate which cytokine mediates the protective effects of huc-MSC-CM. The effects of huc-MSC-CM on NICCs viability and autophagy were examined using AO/PI staining, flow cytometry analysis, transmission electron microscopy (TEM) and confocal fluorescence microscopy analysis. The insulin secretion of NICCs was tested with an insulin immunoradiometric assay kit.

**Results:** Compared to the control, the huc-MSC-CM treatment improved the viability of NICCs, inhibited apoptosis, increased autophagic activity and the levels of PI3K class III and phosphorylated Akt, while the ratio of phosphorylated mTOR/mTOR was reduced. These changes were reversed by CQ and 3-MA treatments. High concentrations of IL-6 were detected in hu-MSC-CM. Furthermore, recombinant IL-6 pre-treatment exerted similar effects as huc-MSC-CM, and these effects were reversed by a specific inhibitor of IL-6 (Sarilumab).

**Conclusions:** Our results demonstrated that huc-MSC-CM improved islet viability and function by increasing autophagy through the PI3K/Akt/mTOR pathway

**Abbreviations:** huc-MSC-CM, human umbilical cord derived mesenchymal stromal cells conditioned medium; MSCs, mesenchymal stromal cells; NICCs, neonatal porcine islet cell clusters.

under hypoxic conditions. Additionally, IL-6 plays an important role in the function of huc-MSC-CM.

KEYWORDS
apoptosis, autophagy, hypoxia, mesenchymal stem cells, neonatal porcine islet cell clusters

## 1 | INTRODUCTION

Xenotransplantation of porcine islets is currently a promising treatment option for type 1 diabetes and an alternative to mainstream procedures.[1-3] However, present clinical porcine islet transplantation protocols are inefficient. Currently, up to 70% of functional islets are destroyed due to hypoxia-induced damage during the transplantation process.[4,5] It is well established that pancreatic islets are sensitive to hypoxia and easily become apoptotic and necrotic under hypoxic conditions.[6,7] Autophagy, a highly conserved catabolic process that captures, degrades, and recycles excess or damaged intracellular contents, promotes cellular homoeostasis and survival.[8,9] A previous study indicated that hypoxia is a strong stimulus that induces autophagy.[10] Researchers have not yet determined whether the survival of islets after exposure to hypoxia is regulated by autophagy, or the relationship between autophagy and apoptosis in islets exposed to hypoxia.

Mesenchymal stem cells (MSCs), a class of pluripotent cells, have been proved to exert anti-apoptosis, anti-inflammatory and immunoregulatory effects, and they are an emerging and promising resource that may protect islets from hypoxia or other damage, thereby promoting graft survival.[11-14] Interestingly, a growing body of research has indicated there may be a potential link between the protective role of MSCs and autophagy.[15-20] However, it is currently unknown whether the favourable effect of MSCs on protecting islets subjected to hypoxia is related to the regulation of autophagy. Additionally, the underlying mechanism involved in this process remains to be elucidated.

Previous reports have illustrated that one of the potential mechanisms that contribute to the protective effect of MSCs is paracrine effects. As shown in our previous studies, exosomes derived from human MSCs protect NICCs from hypoxia-induced apoptosis,[21] but the mechanism mediating this process remains unclear. MSCs have been reported to secrete various cytokines, including interleukin-6 (IL-6), hepatocyte growth factor (HGF) and others.[11,22,23] Among other cytokines, IL-6 is a trophic factor that is secreted at high levels by MSCs.[22] Therefore, we hypothesized that the protective effect of MSCs on islet cells partially depends on IL-6. In support of this hypothesis, recombinant IL-6 has been reported to prevent INS-1 cells from undergoing apoptosis by activating autophagy.[24] However, it remains unknown whether IL-6 secreted by MSCs exerts anti-apoptotic effects under hypoxic conditions, and whether it plays a primary role in MSCs for the regulation of autophagy to alleviate the hypoxia-induced damage of islet cells.

To address these questions, we examined autophagy and apoptosis in NICCs cultured under hypoxic conditions. Moreover, we investigated the protective role of huc-MSC-CM in attenuating the hypoxia-induced death of NICCs and explored the underlying mechanisms related to autophagy. Additionally, we investigated the potential role of IL-6 in modulating the beneficial effect of huc-MSC-CM. Our results have improved our understanding of the mechanisms by which huc-MSCs protect islet cells suffering from hypoxia, and we provide new insights into the development of novel cell-free therapeutic methods using MSCs in islet transplantation.

## 2 | MATERIALS AND METHODS

### 2.1 | Preparation of huc-MSC-CM

The collection of umbilical cords was approved by the Human Research and Ethics Committee of the Third Xiangya Hospital. Umbilical cords were donated by healthy volunteer mothers. MSCs were then isolated from umbilical cords, using a recently published protocol,[25] and cultured with alpha-MEM medium (GE Healthcare) supplemented with 5% (v/v) UltraGROTM-Advanced cell culture supplement (Helios BioScience) and 2 mmol/L L-glutamine (GlutaMAXI, Thermo Fisher) at 37°C and 5% $CO_2$. The supernatant of passage 3-5 huc-MSCs was collected as conditioned medium (huc-MSC-CM) as previously described.[21] Briefly, the human umbilical cord-derived MSCs, at passage 3-5, were seeded into 100-mm tissue culture dishes and cultured with complete medium containing serum in incubators under normoxic conditions (20% $O_2$, 5% $CO_2$ and 37°C). Once cells reached 90% confluence, medium was replaced with serum-free RPMI-1640 (Biological Industries), and cells were cultured for 48 hours under normoxic conditions. Human umbilical cord-derived MSC medium fractions were then collected and centrifuged at 3000 × *g* at 4°C for 10 minutes. The cell-free supernatant was collected, concentrated with a 0.22-μm Millipore filter (Millipore), and stored at −80°C.

### 2.2 | Preparation of porcine islets

This animal study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University. Three- to five-day-old pigs, designated pathogen-free (DPF), of either sex were obtained from Hunan Xeno Life Science. NICCs were isolated from a donor pancreas as described previously[26] and cultured with the previously described medium.[21] After one day of incubation (on day 0), NICCs were rinsed twice with phosphate-buffered saline (PBS). The medium was then changed according to the experimental design. In the huc-MSC-CM group, the medium was changed to

RPMI-1640 medium supplemented with 30% huc-MSC-CM. In the control group, islets were still cultured with RPMI-1640 medium. The culture medium of the two groups was changed every other day thereafter. After one day of culture at 37°C, 20% $O_2$ and 5% $CO_2$, NICCs were incubated for 1-2 days under normoxic (20% $O_2$) and hypoxic conditions (1% $O_2$), respectively, in the presence or absence of 30% huc-MSC-CM.

## 2.3 | Acridine orange (AO)/propidium iodide (PI) staining

AO/PI staining was used to detect the viability of NICCs after the indicated treatment. NICCs were simultaneously stained with acridine orange (AO: 0.67 mol/L) and propidium iodide (PI: 0.75 mol/L) (AO/PI) and viewed under a fluorescence microscope (Olympus). Red indicated dead cells, while green indicated living cells.

## 2.4 | Flow cytometry analysis

Islets (approximately 6000 IEQs) were seeded into six-well plates and were subjected to hypoxia with or without a pre-treatment. NICCs were collected in a 5-mL centrifuge tube. An appropriate amount (1 mL/1000 IEQs) of trypsin was added to NICCs in a water bath at constant temperature (37°C), and cells were gently pipetted for about approximately 3 minutes until the NICCs had been digested into a single-cell suspension. The Annexin V-FTIC/PI apoptosis detection kit (BD Bioscience) and a flow cytometry analysis were used to detect NICCs apoptosis. Staining was performed according to the manufacturer's instructions. The apoptotic rates were analysed using FCM (Accuri C6; BD Biosciences).

## 2.5 | Confocal fluorescence microscopy analysis

Porcine islet cells were collected after digestion with the TrypLE™ Express enzyme (Thermo Fisher) and filtered with a Millipore filter (Millipore). Cells were then labelled with the CYTO-ID® Autophagy Detection Kit (Enzo, Switzerland, ENZ-51031) according to the manufacturer's protocol. Stained cells were viewed with an Olympus FV1000 confocal laser-scanning microscope with excitation filters of 530 nm (blue) and 488 nm (green). Blue fluorescence indicated nuclear staining with Hoechst 33 342, while green fluorescence indicated the production of autophagic vesicles.

## 2.6 | Transmission electron microscopy analysis

Islets in the experimental group (three batches, 6000 IEQs/batch) and the control group (three batches, 6000 IEQs/batch) were collected and washed twice; cell aggregates were pre-fixed with 2.5% glutaraldehyde at 4°C overnight. Cell aggregates were then post-fixed with 1% buffered osmium tetroxide, dehydrated with an ethanol gradient and embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were examined under a transmission electron microscope (TEM; Hitachi, H-7700). The results were analysed by TEM experts who were blinded to the groups.

## 2.7 | Test of NICCs function

A total of 6000 NICC, IEQs were extracted from each group after treatment and cultured according to our experimental design. NICCs were divided into two groups, each of which consisted of three wells, and cultured with medium containing 2.5 mmol/L glucose for 1 hour. Then the medium was changed to medium containing 25.0 mmol/L glucose, and NICCs were cultured for 2 hours. Finally, supernatants were collected to measure the insulin level with an insulin immunoradiometric assay kit (Biosource). Glucose-stimulated insulin release was measured and reported as the stimulation index, which was calculated as the ratio of insulin release in high (25.0 mmol/L) glucose to low (2.5 mmol/L) glucose.[27]

## 2.8 | Western blotting

For the Western blot analysis, total proteins were extracted with lysis buffer containing protease inhibitors (Roche, Sigma). Equal amounts of protein (50 μg) were separated using 8%-10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (PVDF, Roche). Different membranes were prepared for each Western blot. The transferring time is depended on the molecular weight of detected protein. Membranes were blocked with non-fat milk for 90 minutes and incubated overnight with specific primary antibodies at 4°C. The following primary antibodies were used: rabbit anti-cleaved-caspase3 (Cell Signaling Technology), rabbit anti-LC3B(Abcam), rabbit anti-mTOR(Cell Signaling Technology), rabbit anti-Bad (Cell Signaling Technology), rabbit anti-pBcl2 (Cell Signaling Technology), rabbit anti-pmTOR (Abcam), rabbit anti-Beclin1 (Cell Signaling Technology), rabbit anti-p62 (Cell Signaling Technology,), rabbit anti-PI3K class III (Abcam), rabbit anti-pAkt (Thr308) (Cell Signaling Technology), rabbit anti-pAkt (Ser473) (Cell Signaling Technology), rabbit anti-Akt (Cell Signaling Technology) and rabbit anti-β-actin (Proteintech). On the next day, membranes were washed and incubated with HRP-conjugated goat anti-mouse and goat anti-rabbit IgG secondary antibodies for 90 minutes. Blots were visualized using Super Signal enhanced chemiluminescence development reagent (ECL, Thermo Scientific Pierce). Band intensities were analysed using Quantity One software.

## 2.9 | Enzyme-linked immuno-sorbent assay

The huc-MSC-CM from 3-5 passages MSCs was collected. Islet cell culture supernatants were also collected from the different groups. ELISA kits (PeproTech) were used to measure the levels of cytokines such as IL-6, VEGF-A, HGF, and TGF-β according to the manufacturer's instructions. All of the ELISA kits used in the present study detected human cytokines. All of the measurements were performed in triplicate.

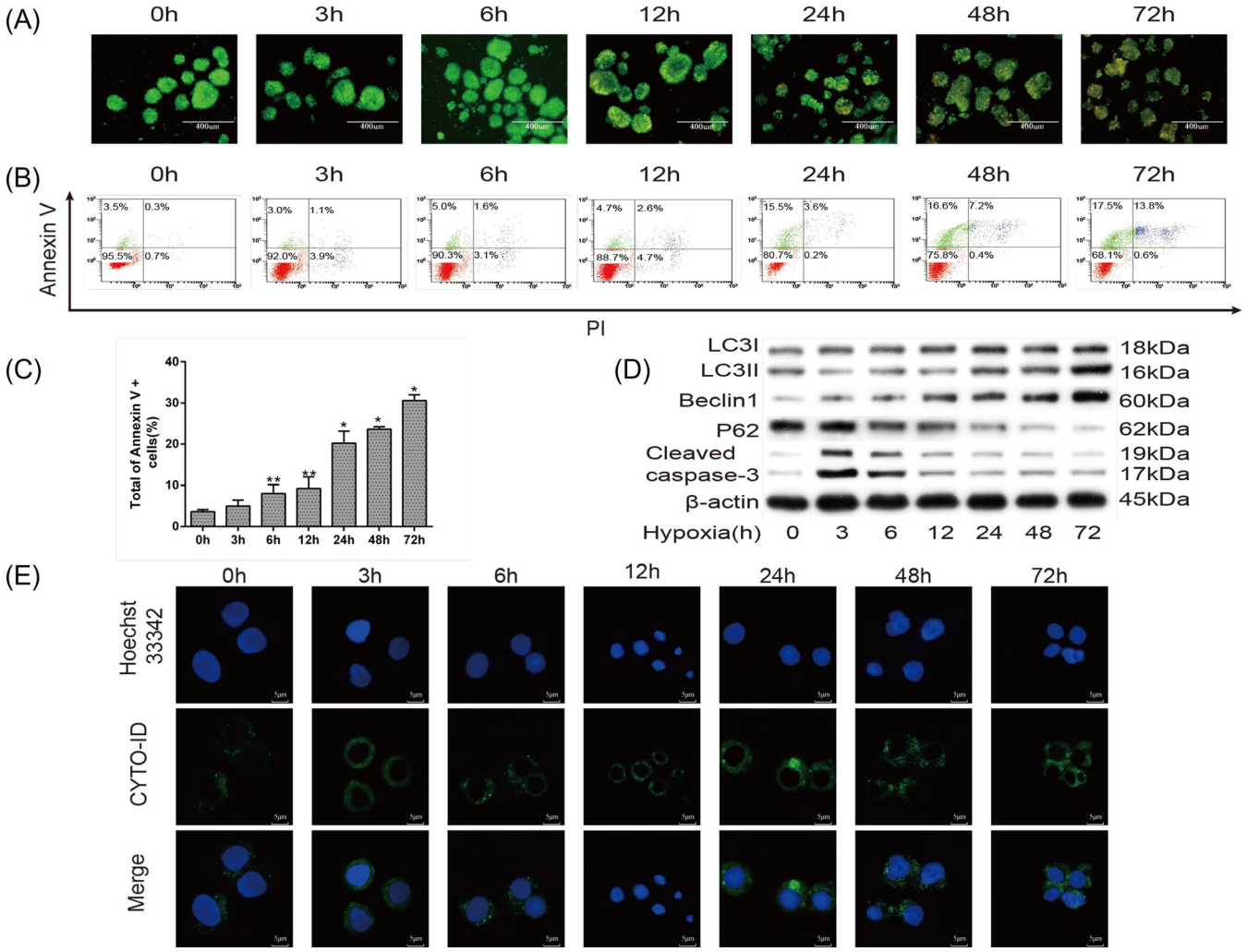


**FIGURE 1** Hypoxia induces autophagy and apoptosis but inhibits the viability of NICCs. Viability was measured using A, AO/PI staining (red indicated dead cells, while green indicated living cells) and B, flow cytometry analysis. C, Quantification of the percentage of apoptotic cells (*$P$ < .01 and *$P$ < .05 compared with the 0-hour group). D, Proteins related to autophagy and apoptosis were visualized and analysed (*$P$ < .01 and **$P$ < .05). E, Autophagy flux was analysed using a CYTO-ID® Autophagy Detection Kit and confocal fluorescence microscopy. Nuclei were labelled with Hoechst 33 342 (blue), and CYTO-ID®-stained autophagic cells were stained with green. Magnification, ×500

### 2.10 | Reagents and inhibitors

To confirm the protective role of huc-MSC-CM in inducing autophagy, NICCs were pre-treated with two commonly used inhibitors of autophagy, namely, CQ (50 μmol/L, Sigma) and 3-MA (100 μmol/L, Sigma) for 24 hours and then cultured with huc-MSC-CM for another 24 hours under hypoxic conditions. To assess the signalling pathway, LY294002 (50 μmol/L, Abcam), an inhibitor of PI3K class III, and Akti1/2(10 μmol/L, Selleck) were used to pre-treat NICCs for 2 hours. To determine the role of IL-6 in huc-MSC-CM, NICCs were pre-treated with Sarilumab (400 μg/mL, Selleck), a specific inhibitor of IL-6, and recombinant human IL-6 (200 ng/mL, PeproTech) for 6 hours. Cells were then cultured with huc-MSC-CM for 48 hours under hypoxic conditions. Cells were collected at pre-specified time points.

### 2.11 | Statistical analysis

Results are presented as the mean ± SD from at least three independent experiments. All data were analysed by SPSS version 22.0. Comparisons between each group were performed using unpaired Student's *t* tests and ANOVAs. *P* values <.05 were accepted as statistically significant.

## 3 | RESULTS

### 3.1 | Characteristics of huc-MSCs

Cells expressed CD73, CD90, and CD105 (>95%) and presented very low levels of CD11b, CD34, HLA-DR and CD45 staining (<1%; Figure. S1A). The trilineage differentiation of MSCs was induced to

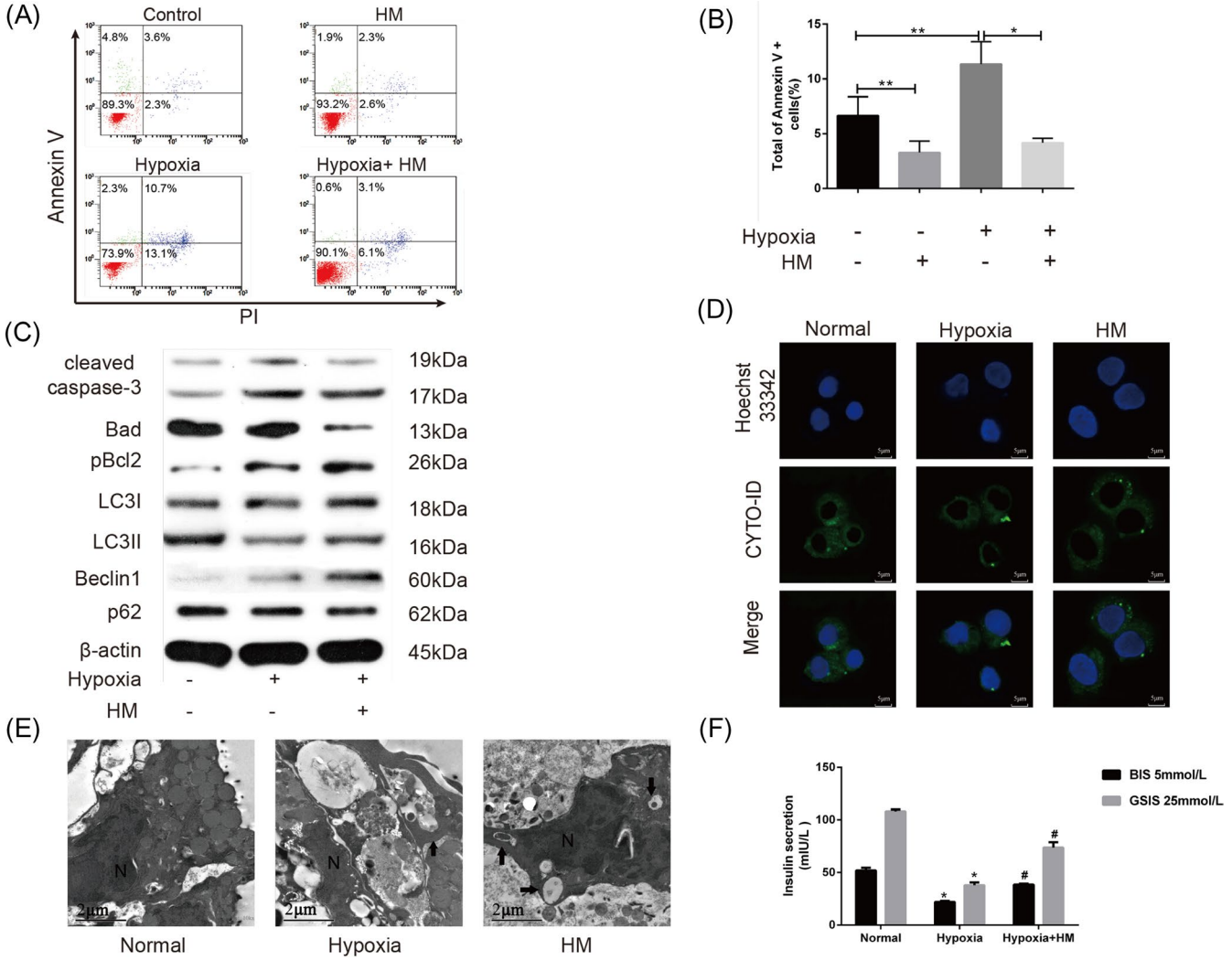


**FIGURE 2** A treatment with huc-MSC-CM inhibits apoptosis and increases autophagy in islet cells exposed to hypoxia. (A and B) The huc-MSC-CM treatment remarkably reduced the percentage of dead cells, (C) decreased the levels of cleaved caspase-3 and Bad, increased autophagic activity, as confirmed using D, confocal fluorescence microscopy, and E, TEM analysis and C, Western blotting, and F, alleviated the impaired insulin secretion of NICCs exposed to hypoxia (*$P$ < .01 compared with the control group and #$P$ < .01 compared with the hypoxia group). In D, nuclei are stained with Hoechst 33 342 (blue), and autophagic cells are stained with CYTO-ID® (green). Magnification, ×500. In (E), N indicates the nucleus, and black arrows highlight autophagosomes. Scale bar, 2 μm. Magnification, ×10,000 (*$P$ < .01 and **$P$ < .05; HM, huc-MSC-CM)

confirm their properties. After induction, cells differentiated into adipocytes, osteoblasts and chondrocytes (Figure S1B). Cells were positive for Oil O red (adipocytes), Alizarin Red (osteocytes) and Alcian blue staining (chondrocytes).

## 3.2 | The viability of NICCs is affected by autophagy and apoptosis

To investigate, whether the viability of NICCs was regulated by autophagy and apoptosis under hypoxic conditions, NICCs were cultured with RPMI-1640 medium under normoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions, and assessed for islet viability and changes in autophagy and apoptosis after hypoxia at different time points. The number of viable islets was reduced when NICCs were exposed to hypoxia for 24 hours and decreased after 72 hours (Figure 1A). Hypoxia stimulation significantly increased the death rate of NICCs in a time-dependent manner (Figure 1B).

The percentage of Annexin V-positive cells (3.63 ± 0.39% under normoxic conditions) gradually increased beginning at 3 hours and was remarkably increased at 24 hours (4.97 ± 1.16%, 8 ± 1.77%, 9.23 ± 2.32%, 20.23 ± 2.35%, 23.63 ± 0.46%, and 30.6 ± 1.13% at 3, 6, 12,24, 48 and 72 hours, respectively; Figure 1C). The level of cleaved caspase-3, a commonly used apoptosis marker, was increased after hypoxia treatment for 3 hours (the ratio of cleaved caspase-3/β-actin was 0.027 and 0.31 at 0 and 3 hours, respectively), but this increase was gradually reduced after 6 hours and substantially decreased at 24 hours (the ratio of cleaved caspase-3/β-actin was 0.19 at 6 hours vs 0.07 at 24 hours) (Figure 1D and S3, $P$ < .05). Interestingly, the levels of

autophagy protein markers, such as LC3II and Beclin1, were increased directly proportional to time. Moreover, the level of p62 was down-regulated after hypoxia treatment ($P < .05$). As shown in Figure 1E, the number of CYTO-ID® positive cells increased and the intensity of green fluorescence gradually increased over time, which correlated with the changes in the levels of autophagy-related proteins. These results suggest that apoptosis was significantly increased at the early stages of hypoxia, while autophagy was induced later in NICCs.

### 3.3 | Huc-MSC-conditioned medium attenuates apoptosis and enhances autophagy in NICCs exposed to hypoxia

Compared to the control group, the ratio of alive cells was significantly reduced when NICCs were exposed to hypoxia for 24 hours, and the huc-MSC-CM group significantly increased the percentage of viable cells (Figure 2A) and strikingly reduced the percentage of dead cells (Annexin V-positive), particularly cells cultured under hypoxic conditions (Figure 2B). When compared to the hypoxia treatment group, the levels of the pro-apoptotic proteins cleaved caspase-3 (the ratio of cleaved caspase-3/β-actin was 0.31 and 0.41 in the huc-MSC-CM group and the hypoxia group, respectively) and Bad (the ratio of Bad/β-actin was 0.13 and 0.49 in the huc-MSC-CM group and the hypoxia group, respectively) were decreased, while the level of the anti-apoptotic protein p-Bcl2 was significantly upregulated in the huc-MSC-CM culture group (the ratio of p-Bcl2/β-actin was 0.35 and 0.16 in the huc-MSC-CM group and the hypoxia group, respectively; Figure 2C and Figure S4A, $P < .05$). Thus, huc-MSC-CM inhibited hypoxia-induced NICC apoptosis. Furthermore, we investigated the function of NICCs cultured under hypoxic conditions. The

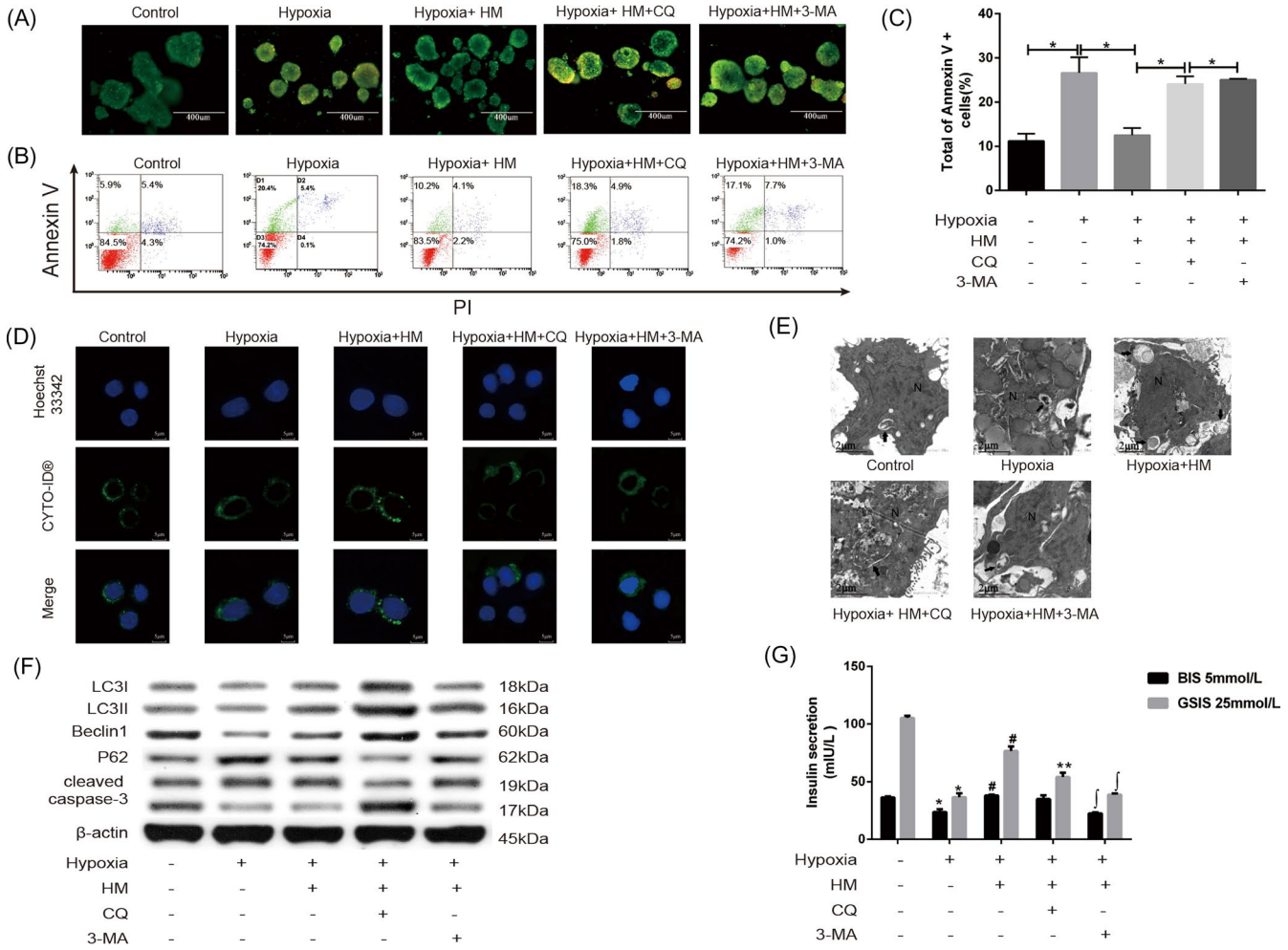


**FIGURE 3** Inhibition of autophagy accelerates hypoxia-induced NICC death. After pre-treatment with CQ or 3-MA, NICCs were exposed to hypoxia in the presence or absence of huc-MSC-CM. A, The relative viability of NICCs treated with CQ (50 μmol/L) or 3-MA (100 μmol/L) was determined using AO/PI staining (red indicated dead cells, while green indicated living cells). B, The survival of NICCs exposed to hypoxia was detected using Annexin V/PI staining and a flow cytometry analysis. C, Quantification of the Annexin V-positive cells in B. D, Confocal fluorescence microscopy and E, TEM analyses were performed to assess autophagy in NICCs. Autophagic cells are labelled with CYTO-ID® (green), while nuclei are labelled with Hoechst 33 342 (blue). Black arrows indicate autophagosomes. F, Key protein markers related to autophagy and apoptosis were detected using Western blotting. G Insulin secretion from NICCs in different groups (*$P < .01$ compared with the control group, #$P < .01$ compared with the hypoxia group, **$P < .01$ compared with the HM group, and $^{\int}P < .01$ compared with the HM group). (*$P < .01$ and **$P < .05$, HM, huc-MSC-CM)

results showed significantly higher insulin secretion from cells cultured under hypoxic conditions with huc-MSC-CM in the presence of both low glucose (5 mmol/L) and high glucose (25 mmol/L) compared with NICCs cultured without huc-MSC conditioned medium (Figure 2F).

In addition, we measured the levels of three commonly used autophagy-related protein markers, that is microtubule-associated protein 1 light chain 3 (LC3), Beclin1 and p62. It can be observed from Figure 2C that the levels of LC3II and Beclin1 were significantly increased in huc-MSC-CM-treated NICCs, suggesting increased autophagosome formation. To confirm our Western blot results, a confocal fluorescence microscopy analysis and TEM analysis were performed to detect the formation of autophagosomes. Using the method described above, we determined that treatment with huc-MSC-CM increases autophagy activity in NICCs exposed to hypoxia (Figure 2D, 2). The numbers of autophagosomes and CYTO-ID® Green-stained cells were increased in NICCs cultured with huc-MSC-CM.

### 3.4 | The anti-apoptotic effect of huc-MSC-conditioned medium depends on enhancement of autophagy

Compared to the hypoxia group, the percentage of Annexin V-positive cells was significantly reduced in NICCs cultured with huc-MSC-CM (Figure 3A, 3). Pre-treatment with CQ or 3-MA noticeably increased the percentage of dead cells (24.13 ± 1.39% and 25.07 ± 0.19% for the CQ and 3-MA groups compared with 12.5 ± 1.34% for the huc-MSC-CM group; Figure 3C), as well as the level of cleaved caspase-3. Meanwhile, the addition of 3-MA or CQ also significantly impaired insulin secretion from NICCs cultured with huc-MSC-CM under low glucose (5 mmol/L) or high glucose (25 mmol/L) conditions (Figure 3G). We then investigated whether CQ and 3-MA altered the autophagy activity in NICCs. The number of autophagic cells (green-stained) and the intensity of autophagy were obviously increased in NICCs cultured with huc-MSC-CM, while pre-treatment with CQ and 3-MA reversed these changes

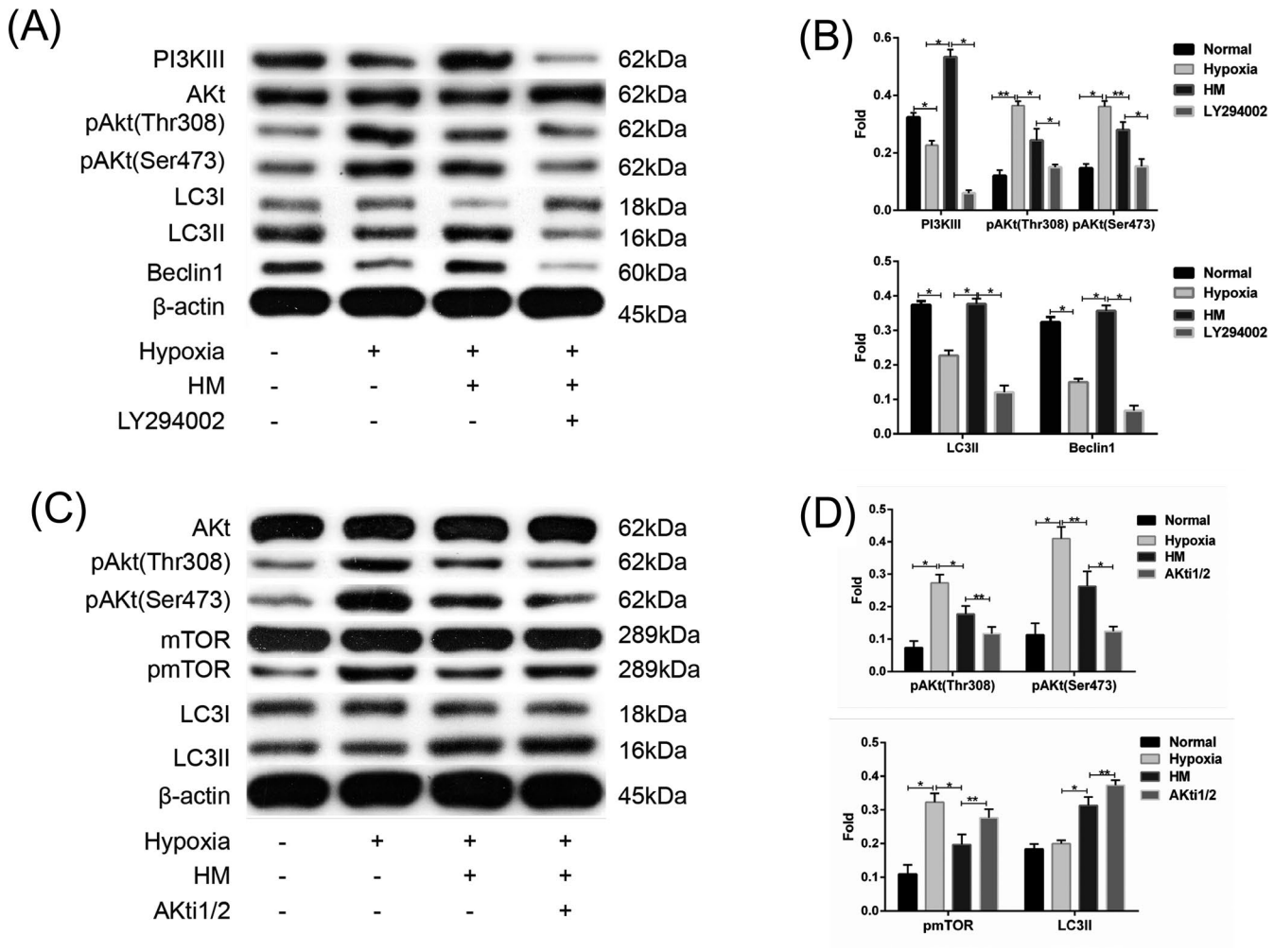


**FIGURE 4** The PI3K/Akt/mTOR signalling pathway mediates autophagy. A, Representative Western blot showing the levels of activated PI3K class III and p-Akt. LC3II levels were significantly increased in the huc-MSC-CM group. Pre-treatment with LY294002 prevented the activation of PI3K class III and p-Akt, and the increased levels of LC3II were decreased. B, Supplement to Figure 5A. Quantitative analysis of levels of the PI3KIII, p-Akt, LC3II, Beclin1. C, Pre-treatment with Akti-1/2 inhibited the activation of p-Akt and restored the activation of p-mTOR. D, Supplement to Figure 5C. Quantitative analysis of levels of the p-Akt, LC3II, p-mTOR (*$P$ < .01 and **$P$ < .05; HM, huc-MSC-CM)

(Figure 3D). Moreover, the TEM analysis (Figure 3E) revealed a noticeable reduction in the number of autophagosomes after the 3-MA or CQ pre-treatment. Western blotting results confirmed these observations (Figure 3F). The huc-MSC-CM treatment increased the LC3II and Beclin1 levels and decreased the level of the pro-apoptotic protein cleaved caspase-3. However, after in NICCs pre-treated with 3-MA, LC3II and Beclin1 levels were decreased, and p62 levels were slightly increased. CQ, a lysosomal protease inhibitor that blocks autophagy at the late stage, also induced LC3II accumulation (the ratio of LC3II/β-actin was 0.25 and 0.55 in the huc-MSC-CM group and the CQ group, respectively), and downregulated p62 (ratio of p62/β-actin was 0.25 and 0.12 in the huc-MSC-CM group and the CQ group, respectively) (Figure S4B). Cleaved caspase-3 levels were increased after pre-treatment with CQ and 3-MA. Collectively, the protective effect of huc-MSC-CM on islet cell survival under hypoxic conditions was diminished by an autophagy inhibitor, suggesting that huc-MSC-CM alleviated hypoxia-induced injury by modulating autophagy in NICCs.

### 3.5 | Huc-MSC-conditioned medium activates the PI3K/Akt/mTOR pathway to increase autophagy in NICCs exposed to hypoxia

Huc-MSC-CM co-culture increased the levels of PI3KIII and Beclin1, and significantly increased the LC3II/I ratio, while p-Akt levels were slightly decreased (Figure 4A). To further confirm the role of PI3K in activating autophagy, NICCs were pre-treated with LY294002, a specific PI3K inhibitor, and cultured under hypoxic conditions. The increased levels of PI3KIII, Beclin1, the LC3II/LC3I ratio, and the Akt phosphorylated at Ser473 and Thr308 were substantially decreased by LY294002. Akti-1/2 was used to determine whether the downstream factors, namely, Akt and mTOR, were involved in the activation of autophagy in NICCs. As shown in Figure 4B, cells cultured with huc-MSC-CM exhibited a significantly reduced p-mTOR/mTOR ratio and increased autophagy, as LC3II expression was significantly upregulated by the treatment. Compared to the hypoxia-only group and the huc-MSC-CM culture group, the Akti-1/2 pre-treatment blocked the phosphorylation of Akt, increased the ratio of p-mTOR/mTOR and cell autophagy. In general, huc-MSC-CM-induced autophagy in NICCs through the PI3K/Akt/mTOR pathway under hypoxic conditions.

### 3.6 | IL-6 mediates the protective effect of huc-MSC-conditioned medium on inhibiting NICCs death

The levels of trophic factors secreted by huc-MSCs, such as IL-6, VEGF-A, HGF and TGF-β were detected. Huc-MSC-CM contained high concentration of VEGF-A, IL-6 and HGF (662.55 ± 199.11, 575.85 ± 167.18 and 568.06 ± 53.58 pg/mL, respectively; Figure 5A (a). VEGF-A had the highest concentration, while TGF-β had the lowest concentration. As shown in Figure 5A (b), NICCs secreted small amounts of IL-6 under normoxic or hypoxic conditions, and we inferred that huc-MSC-CM promoted IL-6 secretion from NICCs cultured under hypoxic conditions. Furthermore, the optimal concentration of IL-6 was determined to be 200 ng/mL (Figure S2). As shown in Figure 5B-D, IL-6 increased the viability of NICCs, reduced the percentage of dead cells (11.53 ± 1.18% and 22.6 ± 0.98% in the IL-6 group and the hypoxia group, respectively), and restored the impaired insulin secretion of NICCs exposed to hypoxia (Figure 5G), similar to huc-MSC-CM (the percentage of dead cells was 10.77 ± 1.32%) under hypoxic conditions. Western blot results showed decreased levels of cleaved caspase-3 and increased levels of the anti-apoptotic protein Bcl-XL in the huc-MSC-CM and IL-6 groups. Additionally, autophagy activity was increased in the IL-6 pre-treated group, as confirmed by the increased levels of LC3II and Beclin1. In addition, the administration of Sarilumab significantly reduced cell viability and impaired insulin secretion (Figure 5G) in NICCs cultured with IL-6 or huc-MSC-CM under hypoxic conditions. As shown in Figure 5C, 5, the percentage of Annexin V-positive cells was significantly increased in the huc-MSC-CM and IL-6 groups, (23.7 ± 0.25% and 26.8 ± 0.79% in the huc-MSC-CM + Sarilumab group and the IL-6+ Sarilumab group respectively), and the level of the pro-apoptotic protein cleaved caspase-3 was increased, while the level of the anti-apoptotic protein Bcl-XL was decreased in the Sarilumab pre-treated group. As shown in Figure 5E, the levels of LC3II and Beclin1, as well as the ratio of LC3II/I, were decreased in NICCs pre-treated with Sarilumab (Figure S5A). Furthermore, as shown in Figure 5F, the protein involved in the signalling pathway, namely, PI3KIII, as well as the phosphorylation level of Akt Ser473 and Thr308 was significantly upregulated after IL-6 pre-treated group (Figure S5B), producing a similar effect as huc-MSC-CM on activating autophagy under hypoxic conditions. In summary, the protective effects of huc-MSC-CM and IL-6 on islet cell function and survival under hypoxic conditions were diminished after pre-treated with Sarilumab, a specific inhibitor of IL-6. The effect of huc-MSC-CM on alleviating the hypoxia-induced apoptosis of NICCs by increasing autophagy through the activation of the PI3K/Akt/mTOR pathway at least partially depends on IL-6.

## 4 | DISCUSSION

The current literature suggests that hypoxia of islet graft pre- and post-transplantation leads to apoptosis and graft failure.[28-30] Autophagy, the process by which cells self-regulate and dispose of abnormal components, plays a crucial role in β cell survival after exposed to various stressors, such as hypoxia and starvation.[31-33] Excessive autophagy, however, can lead to β cell death by interacting with apoptosis pathways.[33] Therefore, moderate regulation of autophagy is conducive to the survival of β cells under stress.

Autophagy and apoptosis have an interaction, as each process can affect the other.[34-37] In the current study, autophagy and apoptosis were simultaneously induced in NICCs exposed to hypoxia, which was consistent with a previous literature.[38] The level of apoptotic protein marker cleaved caspase-3 was significantly increased at 3 hours and markedly decreased at 24 hours, while the levels of the autophagy-related protein LC3II were significantly increased at

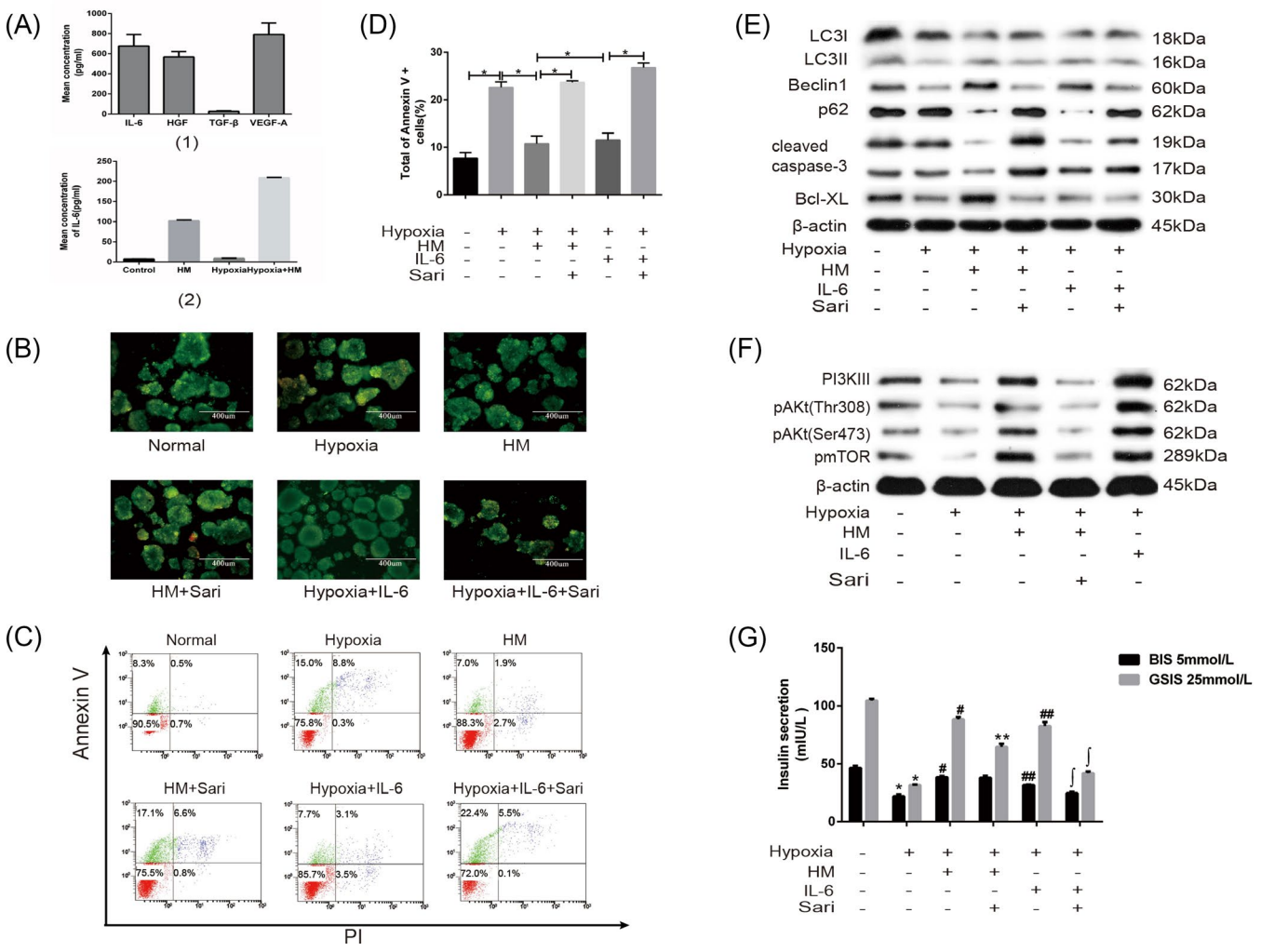


**FIGURE 5** IL-6 contributes to the anti-apoptotic effect of huc-MSC-CM on protecting NICCs from hypoxia-induced death. A, The concentrations of (a) common cytokines secreted by huc-MSC-CM and (b) IL-6 in the supernatant collected from different groups were measured using ELISAs. B, An IL-6 pre-treatment increased the number of viable cells, (C and D) reduced the percentage of dead cells and decreased the levels of cleaved capase-3, while increasing the levels of E, LC3II, Beclin1, (F) PI3KIII, and p-Akt and G, reversing the impaired insulin secretion from NICCs exposed to hypoxia. ($^{*}P < .01$ compared with the control group, $^{\#}P < .01$ versus hypoxia group; $^{**}P < .01$ compared with the HM group, $^{\#\#}P < .01$ compared with the HM group, $^{\int}P < .01$ compared with the IL-6 group). Sarilumab pre-treatment reversed these effects of IL-6 and huc-MSC-CM. (*$P < .01$ and $^{**}P < .05$; HM, huc-MSC-CM; Sari, Sarilumab)

24 hours. Thus, autophagy is likely the major cause of cell death induced by 24 hours of hypoxia. These results confirm the hypothesis from our previous study that apoptosis is not the main cause of cell death after 24 hours of hypoxia.

In the early stage of hypoxia, autophagy in NICCs is decreased, while apoptosis is substantially increased, thus decreasing the survival of NICCs. Based on accumulating evidence, the mechanism regulating autophagy represents a novel and effective approach to protect cells from apoptosis in many disease contexts.[33,39-42] Therefore, we hypothesized that interventions targeting autophagy, within certain limits, represent an alternative method to prevent NICCs from undergoing hypoxia-induced apoptosis.

Mesenchymal stem cells have displayed great promise as an efficient and effective therapeutic strategy due to their capacity to inhibit autoimmune reactions, regulate the microenvironment and secrete various trophic factors.[14,43,44] Recently, MSCs were reported to exert a protective effect on myocardial cells by regulating autophagy.[45-47] However, whether the role of MSCs in protecting porcine islets from hypoxia-induced damage is attributed to the activation of autophagy is still unknown. In addition, huc-MSC-derived exosomes increased the resistance of porcine islets to hypoxia in our previous study. Indeed, the huc-MSC-CM treatment inhibited hypoxia-induced apoptosis and enhanced the impaired function of insulin secretion in NICCs. This finding is consistent with the results of our previous study.[21] Notably, the results from our previous study revealed less autophagy in the huc-MSC-CM group, while in this study, autophagy activity was increased in NICCs treated with huc-MSC-CM after exposure to hypoxia, as evidenced by the increased numbers of CYTO-ID®-positive cells and autophagosomes detected using TEM, as well as the levels of LC3II and Beclin1, which seems to contradict our previous findings. In fact, NICCs were cultured for 6 days and then exposed to hypoxia for 24 hours or 48 hours with

or without huc-MSC-CM in our previous study[21] while NICCs were cultured for 1 day and then exposed to hypoxia with or without huc-MSC-CM for 24 hours or 48 hours in this study. We assumed that different durations of hypoxia lead to different autophagy activity. According to the report by Li and his/her colleagues, a certain level of autophagy activity may be beneficial for cell metabolism, while excessive activation of autophagy may further aggravate the ischaemia- and hypoxia-induced cellular injury.[35] Therefore, the opposite roles of autophagy at different time points of hypoxia exposure are responsible for the difference in autophagy activity of NICCs cultured with huc-MSC-CM.

Several pathways are involved in the regulation and control of autophagy signalling,[48,49] and therefore, the direct mechanism by which huc-MSC-CM activates autophagy remains unclear. The PI3K/Akt signalling pathway is involved in a wide range of intracellular metabolic processes, including autophagy.[50] The PI3K signalling pathway is upstream of mTOR,[51] which plays an important role in the induction of autophagy. PI3K class III signalling enhances autophagy, while PI3K class I/Akt inhibits autophagy in an IRI lung injury model.[42] Based on our results, we hypothesized that huc-MSC-CM activates PI3K class III signalling, as the ratio of PI3K III/β-actin in the huc-MSC-CM group was approximately three times higher than that of in the hypoxia group (Figure S4). Moreover, LY294002 and Akti-1/2 pre-treatments significantly decreased the levels of PI3KIII and p-Akt (Thr308 andSer473), respectively. PI3K/Akt/mTOR signalling plays a role in the huc-MSC-CM-induced activation of autophagy by, supporting the hypothesis described above. However, it should be noted that the changes in LC3II levels and the ratio of LC3II/I in the Akti-1/2 pre-treatment group did not completely correspond to the changes in the p-mTOR/mTOR ratio, implying that autophagy was partially activated by another signalling pathway. Exosomes derived from huc-MSC-CM were proven to exert an anti-apoptotic effect on NICCs exposed to hypoxia in our previous study.[21] As trophic factors such as IL-6, HGF and VEGF-A were detected in huc-MSC-CM, we aimed to determine which of these proteins contributed to the effect of huc-MSC-CM on activating autophagy to alleviate apoptosis in NICCs under hypoxic conditions.

IL-6 is a double-edged sword. On one hand, IL-6 plays an important role in regulating metabolism,[52] particularly glucose homoeostasis.[53] IL-6 has been identified as a cytoprotective molecule in inflammatory disorders, as shown in multiple pre-clinical trials of organ transplantation,[54-59] and therefore may have potential applications in clinical islet transplantation as a novel therapeutic molecule. On the other hand, IL-6 is regarded as an inflammatory factor and an important indicator in the field of xenotransplantation.[60] Furthermore, HGF is a key factor responsible for the cytoprotective and regenerative potential of hepatocytes,[61,62] while VEGF has long been thought to be a potent neurotrophic factor that promotes the survival of spinal cord neurons.[63] Hence, an exploration of whether IL-6 mediates the protective effect of huc-MSC-CM on inhibiting the hypoxia-induced death of NICCs is interesting. In the present study, the concentration of IL-6 was the second-highest measured, after VEGF-A, in huc-MSC-CM. However, this conclusion contradicts a previous study.[22] We speculate that detection methods and the source of the MSCs likely account for these differences. Pre-treatment with a moderate concentration (200 ng/mL) of recombinant IL-6 rendered NICCs resistant to hypoxia-induced death (Figure S2), supporting the hypothesis proposed in a previous study that IL-6 prevents cell death.[58] Interestingly, the concentrations of (a) IL-6 in huc-MSC-conditioned medium used to culture NICCs and (b) recombinant IL-6 varied considerably, but they exerted equivalent anti-apoptotic effects on cells undergoing hypoxic stress, suggesting that huc-MSC-CM is more efficient than IL-6 in suppressing NICCs apoptosis and impaired insulin secretion under hypoxic conditions in vitro. Consistent with our original hypothesis, the anti-apoptotic effects of huc-MSC-CM and IL-6 were abolished after pre-treatment with Sarilumab, and the increase in autophagy was prevented. Additionally, the higher levels of PI3KIII and Akt phosphorylated Ser473 and Thr308 were observed after the IL-6 pre-treatment, suggesting that IL-6 might be responsible for the anti-apoptotic effect of huc-MSC-CM on protecting NICCs by inducing autophagy through the activation of the PI3K/Akt signalling pathway. However, the roles of other factors secreted by MSCs cannot be excluded, since Sarilumab, a specific inhibitor of the IL-6 receptor, should not theoretically act on other factors, such as HGF and VEGF. While interpreting these promising findings, experimental limitations should also be considered. NICCs were cultured with huc-MSC-CM, and therefore the effects of huc-MSCs on NICCs should be noted.

## 5 | CONCLUSIONS

In the present study, the protective effect of huc-MSC-CM against the hypoxia-induced death of NICCs was related to the activation of autophagy through PI3K/Akt/mTOR signalling pathway. Our data support the hypothesis that IL-6 might be involved in huc-MSC-induced autophagy in NICCs, implying that IL-6 overexpressing MSCs are a prospective tool to protect islet grafts from hypoxia. This study demonstrates the protective effects of huc-MSC-CM on porcine islet grafts suffering from hypoxia-induced apoptosis and indicates that a cell-free treatment based on MSC paracrine factors potentially represents a novel therapeutic strategy for hypoxia-ischaemia-related diseases.

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### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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