● RESEARCH ARTICLE

Transfer of mitochondria from mesenchymal stem cells derived from induced pluripotent stem cells attenuates hypoxia-ischemia-induced mitochondrial dysfunction in PC12 cells

Yan Yang[1, 2, #], Gen Ye[1, 3, #], Yue-Lin Zhang[1], Hai-Wei He[1], Bao-Qi Yu[4], Yi-Mei Hong[1], Wei You[1], Xin Li[1, *]

1 Department of Emergency Medicine, Department of Emergency and Critical Care Medicine, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong Province, China
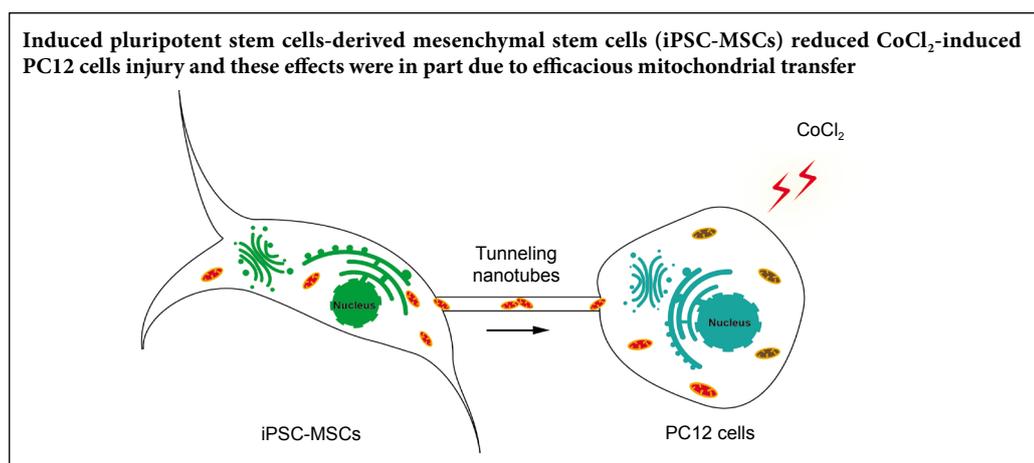2 Department of Emergency, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province, China
3 Shantou University Medical College, Shantou, Guangdong Province, China
4 Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Capital Medical University, Key Laboratory of Remodelling-related Cardiovascular Diseases, Ministry of Education, Beijing, China

Graphical Abstract

Induced pluripotent stem cells-derived mesenchymal stem cells (iPSC-MSCs) reduced CoCl$_2$-induced PC12 cells injury and these effects were in part due to efficacious mitochondrial transfer

*Correspondence to:
Xin Li, MD, PhD,
xlidoct@qq.com.

#These authors contributed equally to this study.

orcid:
0000-0003-0469-5121 (Xin Li)

Abstract

Mitochondrial dysfunction in neurons has been implicated in hypoxia-ischemia-induced brain injury. Although mesenchymal stem cell therapy has emerged as a novel treatment for this pathology, the mechanisms are not fully understood. To address this issue, we first co-cultured 1.5 × 10^5 PC12 cells with mesenchymal stem cells that were derived from induced pluripotent stem cells at a ratio of 1:1, and then intervened with cobalt chloride (CoCl$_2$) for 24 hours. Reactive oxygen species in PC12 cells was measured by Mito-sox. Mitochondrial membrane potential (ΔΨm) in PC12 cells was determined by JC-1 staining. Apoptosis of PC12 cells was detected by terminal deoxynucleotidal transferase-mediated dUTP nick end-labeling staining. Mitochondrial morphology in PC12 cells was examined by transmission electron microscopy. Transfer of mitochondria from the mesenchymal stem cells derived from induced pluripotent stem cells to damaged PC12 cells was measured by flow cytometry. Mesenchymal stem cells were induced from pluripotent stem cells by lentivirus infection containing green fluorescent protein in mitochondria. Then they were co-cultured with PC12 cells in Transwell chambers and treated with CoCl$_2$ for 24 hours to detect adenosine triphosphate level in PC12 cells. CoCl$_2$-induced PC12 cell damage was dose-dependent. Co-culture with mesenchymal stem cells significantly reduced apoptosis and restored ΔΨm in the injured PC12 cells under CoCl$_2$ challenge. Co-culture with mesenchymal stem cells ameliorated mitochondrial swelling, the disappearance of cristae, and chromatin margination in the injured PC12 cells. After direct co-culture, mitochondrial transfer from the mesenchymal stem cells stem cells to PC12 cells was detected via formed tunneling nanotubes between these two types of cells. The transfer efficiency was greatly enhanced in the presence of CoCl$_2$. More importantly, inhibition of tunneling nanotubes partially abrogated the beneficial effects of mesenchymal stem cells on CoCl$_2$-induced PC12 cell injury. Mesenchymal stem cells reduced CoCl$_2$-induced PC12 cell injury and these effects were in part due to efficacious mitochondrial transfer.

Key Words: apoptosis; brain injury; hypoxia-ischemia; induced pluripotent stem cells; mesenchymal stem cells; mitochondrial membrane potential; mitochondrial transfer; PC12 cells; tunneling nanotubes

Introduction

Hypoxia-ischemia-induced brain injury caused by inadequate blood supply is a major cause of morbidity and mortality in infants, especially in developing countries (Johnston et al., 2000; Liu et al., 2015; Rodriguez et al., 2018; Davies et al., 2019). This type of brain injury has a prevalence of ~1.5–4/1000 births, affecting around 4 million children per year (Mir and Chalak, 2014). Although the pathogenesis of this condition has been intensively studied, the underlying mechanisms remain largely unknown. Nevertheless, accumulating evidence indicates that mitochondrial dysfunction in neurons is a contributing factor (Arteaga et al., 2017; Leaw et al., 2017). Hypoxia-ischemia-induced mitochondrial dysfunction leads to excessive reactive oxidative species (ROS) generation, triggering cell apoptosis in the brain (Jiang et al., 2014; Arteaga et al., 2017; Thornton et al., 2017). Administration of dichloroacetate has been shown to markedly reduce hypoxia-ischemia-induced brain injury in neonatal mice by improving mitochondrial metabolism, indicating that mitochondrial injury participates in the condition's pathology (Sun et al., 2016). Despite advanced interventions, there is no definitive treatment for reducing brain injury except for induced hypothermia. Even so, application of this method is greatly limited due to clinical complications and decreased autoimmunity (Lu et al., 2015). Identification of an alternative strategy is urgently needed. Given that mitochondrial dysfunction is closely linked to hypoxia-ischemia-induced brain injury, an effective strategy that targets mitochondria injury likely offers great potential.

Over the past decade, stem cell-based therapy, including neural progenitor cells, neural stem cells, and mesenchymal stem cells (MSCs), has been proposed as a novel treatment for hypoxia-ischemia-induced brain injury, but the mechanisms through which this treatment operates are not fully understood (Ophelders et al., 2016; Archambault et al., 2017; Braccioli et al., 2017; Lee et al., 2017; Hawkins et al., 2018). Among the various types of stem cells, MSCs have attracted huge attention due to their ease of isolation and expansion, immune privilege, and capacity for multiple lineage differentiation (Zhang et al., 2015a, b; Song et al., 2018). Nonetheless, the functionality of MSCs that come from aged or diseased donors decline, thus limiting their beneficial effects (Block et al., 2017; Song et al., 2017; Liang et al., 2019). Compared with adult MSCs extracted from bone marrow, induced pluripotent stem cells-derived MSCs (iPSC-MSCs) not only share the same phenotype, but also exhibit a higher proliferative capacity-without loss of self-renewal capacity-and less batch-to-batch variation (Lian et al., 2010). In addition to direct cell differentiation and paracrine effects, MSCs have been reported to transfer their mitochondria to injured cells and rescue tissue damage (Jiang et al., 2016; Zhang et al., 2016; Leaw et al., 2017; Yao et al., 2018). Furthermore, iPSC-MSCs exhibited higher mitochondrial transfer efficiency to injured cells under stress conditions than did bone marrow MSCs (Li et al., 2014; Zhang et al., 2016). PC12 cells, a rat pheochromocytoma cell, are currently used as a model for the neural cell biology. Cobalt chloride ($CoCl_2$) can induce hypoxia-like

responses in several cell lines, including PC12 cells. Nonetheless, the therapeutic effects of iPSC-MSCs on $CoCl_2$-induced PC12 cell injury have not been determined. In this study, we investigated whether iPSC-MSCs could attenuate $CoCl_2$-induced mitochondrial damage in PC12 cells and thus implicate mitochondria transfer as one of the underlying mechanisms that regulates recovery.

Materials and Methods

Cell culture

PC12 cells (purchased from Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicilin/streptomycin. PC12 cells at passage 12–15 were used in this study. Human iPSC-MSCs (kind gifts from Dr. Qizhou Lian, University of Hong Kong, Hong Kong SAR, China) were cultured and characterized as previously reported (Lian et al., 2010; Lian et al., 2016). We used two iPSC-MSCs cell lines: Lee NL-iPSC-MSCs and IMR90-iPSC-MSCs.

Cell Counting Kit-8 assay

The viability of PC12 cells was evaluated using a Cell Counting Kit-8 (CCK-8) kit (Shanghai Tongren, CK04). Briefly, PC12 cells at 5×10^3 were plated in one well of 96-well plate and exposed to 200, 400, or 600 μM $CoCl_2$ for 24 hours and finally incubated with CCK-8 working solution. The optical density values were measured at 450 nm by automatic microplate reader (Thermo Fisher, Waltham, MA, USA).

Direct co-culture

PC12 cells at 1.5×10^5 were co-cultured with iPSC-MSCs in one well of a 6-well plate at a 1:1 ratio in MSC medium (Gibco) and PC12 cell medium (Sigma-Aldrich) (1:1) and then treated with 400 μM $CoCl_2$ (Sigma-Aldrich) for 24 hours.

Celltrace violet labeling

PC12 cells were labeled with 1 μM Celltrace violet (Molecular Probes, Eugene, OR, USA) for half an hour and washed with fresh medium. Cells at 1×10^6 were incubated with 1 μM 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester (Molecular Probes) for half an hour and washed with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum.

ROS and mitochondrial membrane potential measurement

The ROS and mitochondrial membrane potential ($\Delta\Psi m$) of treated PC12 cells were measured by Mito-sox and JC-1 dye staining (Invitrogen, Carlsbad, CA, USA), respectively. Briefly, Celltrace violet-labeled PC12 cells were co-cultured with iPSC-MSCs and then exposed to $CoCl_2$. After treatment, cells were incubated with 5 μM Mito-sox or 2 μM of JC-1 for half an hour at 37°C. Finally, the ROS and $\Delta\Psi m$ of sorted Celltrace violet-labeled PC12 cells were measured by fluorescence activated cell sorting flow cytometry (BD Biosciences, San Jose, CA, USA).

TUNEL staining

The apoptotic rate of PC12 cells was examined by terminal deoxynucleotidal transferase-mediated dUTP nick end-labeling (TUNEL) staining (11767291910, Roche, Basel, Switzerland) for each type of treatment. Briefly, after fixation with 4% paraformaldehyde, cells were treated with blocking solution. After incubation with permeabilization solution, cells were incubated with 50 μL TUNEL reaction mixture at 37°C for 1 hour. Meanwhile, 50 μL of Label Solution was used as the negative control. Finally, after washing with PBS three times, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI). Apoptosis of PC12 cells were assessed as previously described (Zhao et al., 2016). The apoptotic ratio of PC12 cells was calculated by dividing the number of positive TUNEL cells by the total number of cells per viewing area. The TUNEL staining images were captured by a motorized inverted fluorescence microscope (IX81-ZDC2; Olympus, Tokyo, Japan) at 100× magnification from five fields on each slide.

Transwell co-culture

iPSC-MSCs were seeded on the top and PC12 cells were plated on the bottom in a transwell (Corning, NY, USA) with 0.4-μm inserts. The cell number was 1.5×10^5. Finally, the co-cultured cells were treated with 400 μM CoCl$_2$ for 24 hours.

Mitochondrial transfer assessment

To examine mitochondrial transfer, iPSC-MSCs were infected with lentivirus-containing mitochondria with GFP (mito-GFP) (Cyto102-PA-1, System Biosciences, Palo Alto, CA, USA), as previously described (Zhang et al., 2016). Mito-GFP-iPSC-MSCs at 1×10^4 were then co-cultured with Celltrace-labeled PC12 cells (1:1) with or without 400 μM CoCl$_2$ treatment for 24 hours in a 24-well plate with a cover slide. Mito-GFP-iPSC-MSCs were also co-cultured with Celltrace-labeled PC12 cells (1:1) in a 10-cm culture plate for 24 hours with or without CoCl$_2$ treatment. The efficiency of mitochondrial transfer from iPSC-MSCs to PC12 cells was examined by fluorescence activated cell sorting (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest software (BD Biosciences). The mitochondrial transfer ratio was calculated as the number of PC12 cells with Mito-GFP divided by the total number of PC12 cells. Images showing mitochondrial transfer from MSCs to PC12 cells were captured using the same microscope at 400× magnification. Tunneling nanotubes were observed by an inverted microscope or fluorescence microscope, and the ratio of formed tunneling nanotubes was calculated as the total number of tunneling nanotubes divided by the total number of cells per viewing area.

Adenosine triphosphate (ATP) measurement

ATP was extracted from PC12 cells using boiling distilled water as previously reported (Yang et al., 2002). ATP content was examined by an ATP Determination Kit (Molecular Probes).

Statistical analysis

Data are shown as the mean ± SEM. Statistical analyses were performed using Prism 5.04 Software (GraphPad Software for Windows, San Diego, CA, USA). Unpaired Student's *t*-tests were used to compare two groups. One-way analysis of variance followed by the Bonferroni test was used to compare multiple groups. $P < 0.05$ was considered statistically significant.

Results

CoCl$_2$-induced PC12 cell injury is dose-dependent

Compared with control PC12 cells, the viability of PC12 cells was greatly reduced to 83%, 50% and 33% in the 200, 400, and 600 μM CoCl$_2$-treated groups, respectively, indicating that CoCl$_2$-induced PC12 cells injury was dose-dependent ($P < 0.05$ or $P < 0.001$; **Figure 1**). Based on the above results, 400 μM of CoCl$_2$ was chosen for further studies. CoCl$_2$ had no impact on MSC proliferation (data not shown).
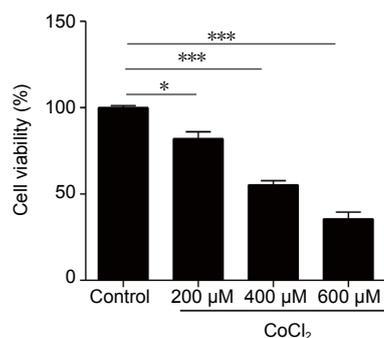

Figure 1 CoCl$_2$ induced PC12 cell injury is dose-dependent *in vivo*.
Cell viability of PC12 cells that were treated with different concentrations of CoCl$_2$ for 24 hours was examined by Cell Counting Kit-8 assay. Cell viability is expressed as the percentage relative to the control group. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by the Bonferroni test). All experiments were conducted in triplicate. ∗$P < 0.05$, ∗∗∗$P < 0.001$. CoCl$_2$: Cobalt chloride.

iPSC-MSCs ameliorate CoCl$_2$-induced mitochondrial damage in PC12 cells

To examine whether iPSC-MSCs protect against PC12 cell injury, PC12 cells were co-cultured with GFP-iPSC-MSCs and then treated with 400 μM CoCl$_2$ for 24 hours. Next, flow cytometry was used to identify iPSC-MSCs as GFP-positive cells and PC12 cells as GFP-negative cells. Mitochondria ROS levels were examined in sorted PC12 cells. Compared with normal PC12 cells, ROS was dramatically enhanced in CoCl$_2$-treated PC12 cells that were not co-cultured with iPSC-MSCs ($P < 0.01$; **Figure 2A**). However, co-culture with iPSC-MSCs greatly down-regulated the increased ROS induced by CoCl$_2$ ($P < 0.01$; **Figure 2A**). Moreover, while CoCl$_2$ significantly reduced ΔΨm in PC12 cells compared with the control group ($P < 0.01$), when co-cultured with iPSC-MSCs this effect was reversed ($P < 0.01$; **Figure 2B**). Transmission electron microscopy revealed that co-culture with iPSC-MSCs attenuated mitochondrial swelling, disappearance of cristae, and chromatin margination in the

injured PC12 cells that was induced by CoCl$_2$ (**Figure 2C**). We also examined ATP in PC12 cells from different groups. Compared with normal PC12 cells, ATP levels were greatly reduced in the CoCl$_2$-treated PC12 cells ($P < 0.001$; **Figure 2D**). However, iPSC-MSCs co-culture dramatically reversed this effect of CoCl$_2$ ($P < 0.01$; **Figure 2D**). Collectively, these data indicate that co-culture with iPSC-MSCs ameliorates CoCl$_2$-induced mitochondrial damage in PC12 cells.

iPSC-MSCs reduces CoCl$_2$-induced apoptosis of PC12 cells

Because mitochondrial dysfunction can lead to apoptosis, we examined whether iPSC-MSCs protected against CoCl$_2$-induced apoptosis in PC12 cells. PC12 cells were co-cultured with GFP-iPSC-MSCs and then incubated with 400 μM CoCl$_2$ for 24 hours. Next, iPSC-MSCs were identified as GFP-positive cells and PC12 cells as GFP-negative cells using flow cytometry. Apoptosis was measured in the sorted PC12 cells by TUNEL. Compared with the control PC12 cells, apoptosis of PC12 cells was significantly enhanced under 400 μM CoCl$_2$ challenge ($P < 0.001$; **Figure 3**). However, iPSC-MSCs co-culture reduced t his increased apoptosis caused by CoCl$_2$ ($P < 0.001$; **Figure 3**). Collectively, these results suggest that iPSC-MSCs co-culture inhibits CoCl$_2$-induced apoptosis in PC12 cells.

Paracrine action of iPSC-MSCs on CoCl$_2$-induced mitochondrial damage in PC12 cells

To examine whether the protective effects of iPSC-MSCs on CoCl$_2$-induced mitochondrial damage in PC12 cells was due to the paracrine action, the iPSC-MSCs were co-cultured with PC12 cells using a trans-well under CoCl$_2$ challenge. Although there was a trend towards downregulation of mitochondrial ROS in PC12 cells co-cultured with iPSC-MSCs in trans-well compared with PC12 cells that were not co-cultured with iPSC-MSCs, the difference was not significant ($P > 0.05$; **Figure 4A**). Furthermore, no differences in apoptosis or ΔΨm were observed between PC12 cells co-cultured with iPSC-MSCs in trans-well and those not co-cultured with iPSC-MSC ($P > 0.05$; **Figure 4B** and **C**). Collectively, these findings indicate that the beneficial effects of iPSC-MSCs on PC12 cell injury are not entirely attributable to paracrine effects.

Mitochondrial transfer from iPSC-MSCs to PC12 cells

Recent studies show that iPSC-MSCs can transfer mitochondria to damaged cells via tunneling nanotubes. We first examine tunneling nanotubes formation between PC12 cells and iPSC-MSCs. As shown in **Figure 5A**, tunneling nanotubes were clearly visible connecting CFSE-labeled PC12 cells and iPSC-MSCs (**Figure 5A**). Furthermore, CoCl$_2$ treatment stimulated an increase in tunneling nanotubes between the two cell types ($P < 0.01$; **Figure 5B**). Next, to detect whether iPSC-MSCs can transfer mitochondria to injured PC12 cells, we co-cultured the Mito-GFP-iPSC-MSCs and Celltrace-labeled PC12 cells in the presence of CoCl$_2$ and examined the mitochondrial transfer. As shown in **Fig-

ure 5C**, GFP-positive mitochondria from iPSC-MSCs were found in Celltrace-labeled PC12 cells, indicating that Mito-GFP-iPSC-MSCs transferred mitochondria to the PC12 cells (**Figure 5C**). Furthermore, tunneling-nanotube formation was clearly visible between Celltraced-labeled PC12 cells and iPSC-MSCs, suggesting that mitochondria may be transferred via tunneling nanotubes. Subsequently, mitochondrial transfer efficiency was examined by flow cytometry. The results revealed that CoCl$_2$ treatment increased the mitochondrial transfer ratio from iPSC-MSCs to PC12 cells ($P < 0.05$; **Figure 5D**). Next, we examined whether the mitochondria transferred from iPSC-MSCs rescue the injured PC12 cells. Celltrace-labeled PC12 cells were co-cultured with Mito-GFP-iPSC-MSCs under CoCl$_2$ treatment for 24 hours. Celltrace-labeled PC12 cells were separated by cell sorting into those that received mitochondrial transfer and those that did not, and the ATP content was measured and compared between groups. Compared with the PC12 cells that did not receive mitochondria, those that did receive mitochondria exhibited large increases in intracellular ATP levels, indicating that the mitochondria transferred from iPSC-MSCs to the injured PC12 cells were functional ($P < 0.01$; **Figure 5E**). Furthermore, PC12 cells that received mitochondria from MSCs exhibited less cell apoptosis than those that did not ($P < 0.01$; **Figure 5F**). Taken together, these results show that mitochondrial transfer from MSCs ameliorates CoCl$_2$-induced PC12 cell injury.

Inhibition of tunneling nanotubes reduces the protective effects of iPSC-MSCs on CoCl$_2$-induced PC12 cell injury

To examine whether tunneling nanotubes are essential for mitochondrial transfer of iPSC-MSCs to PC12 cells, we treated the co-cultured cells with Cytochalasin D (Cyto D), which inhibits tunneling nanotubes formation (Bittins and Wang, 2017). Administration of Cyto D had no impact on iPSC-MSC or PC12 cell viability (data not shown). Treatment with Cyto D greatly reduced the mitochondrial transfer efficiency from iPSC-MSCs to PC12 cells in the presence of CoCl$_2$, indicating that tunneling nanotubes play a critical role in mediating mitochondrial transfer ($P < 0.05$; **Figure 6A**). Next, to determine whether Cyto D treatment can reduce the protective effect of iPSC-MSCs, we examined mitochondrial ROS and apoptosis in PC12 cells after co-culture in the presence of CoCl$_2$ and Cyto D. As expected, co-culture with iPSC-MSCs greatly inhibited the increased ROS and apoptosis induced by CoCl$_2$ in PC12 cells ($P < 0.001$; **Figure 6B** and **C**). However, the beneficial effects of iPSC-MSCs on injured PC12 cells were partially blocked by Cyto D treatment ($P < 0.05$ or $P < 0.01$; **Figure 6B** and **C**). These findings demonstrate that inhibiting tunneling nanotubes reduces the protective effects of iPSC-MSCs on CoCl$_2$-induced PC12 cell injury.

Discussion

There are several major findings in this study. First, iPSC-MSCs protected against CoCl$_2$-induced PC12 cell injury by restoring mitochondrial function, and these beneficial effects were not totally attributed to paracrine action of
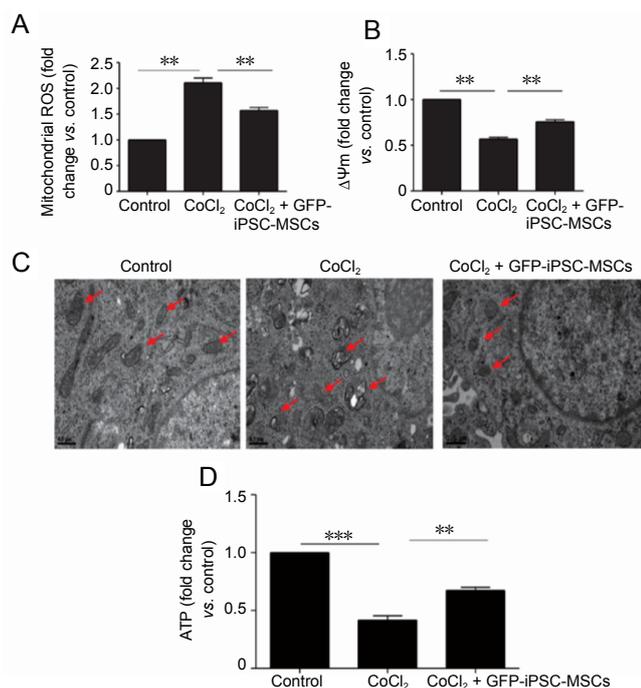
Figure 2 iPSC-MSCs co-culture alleviates CoCl$_2$-induced mitochondrial injury in PC12 cells.
(A) Mitochondrial ROS generation in PC12 cells for each treatment, as determined by Mito-sox staining. (B) The ΔΨm in PC12 cells for each treatment, as determined by JC-1 staining. (C) Representative transmission electron microscope images showing the morphology of mitochondria in PC12 cells for each treatment. Arrows point to mitochondria. Scale bars: 0.5 μm. (D) ATP levels in PC12 cells with varying treatments. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by the Bonferroni test). All experiments were conducted in triplicate. $**P < 0.01$, $***P < 0.001$. CoCl$_2$: Cobalt chloride; iPSC-MSCs: induced pluripotent stem cell-mesenchymal stem cells; ROS: reactive oxidative species; ΔΨm: mitochondrial membrane potential; ATP: adenosine triphosphate.
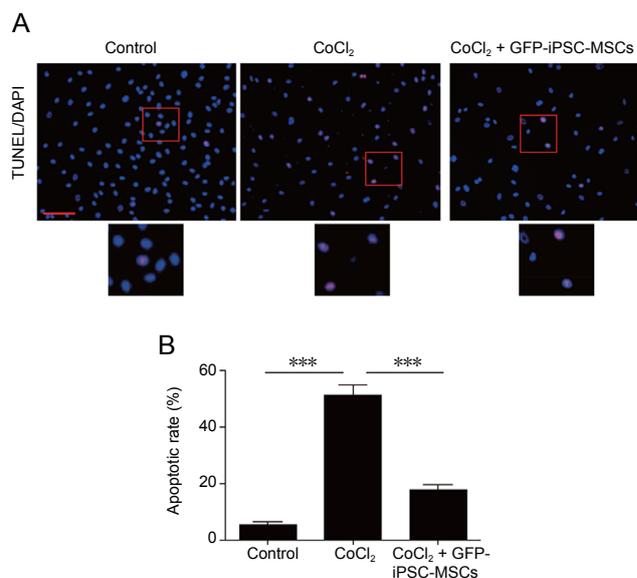
Figure 3 iPSC-MSCs co-culture reduces CoCl$_2$-induced apoptosis in PC12 cells.
(A) Representative images showing the effects of iPSC-MSC co-culture on CoCl$_2$-induced apoptosis in PC12 cells (in red) as determined by TUNEL staining. Scale bar: 100 μm. (B) The apoptotic rate for each PC12 cell treatment. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by the Bonferroni test). All experiments were conducted in triplicate. $***P < 0.001$. CoCl$_2$: Cobalt chloride; DAPI: 4′,6-diamidino-2-phenylindole; GFP: green fluorescence protein; iPSC-MSCs: induced pluripotent stem cell-mesenchymal stem cells; TUNEL: terminal deoxynucleotidal transferase mediated dUTP nick end-labeling.
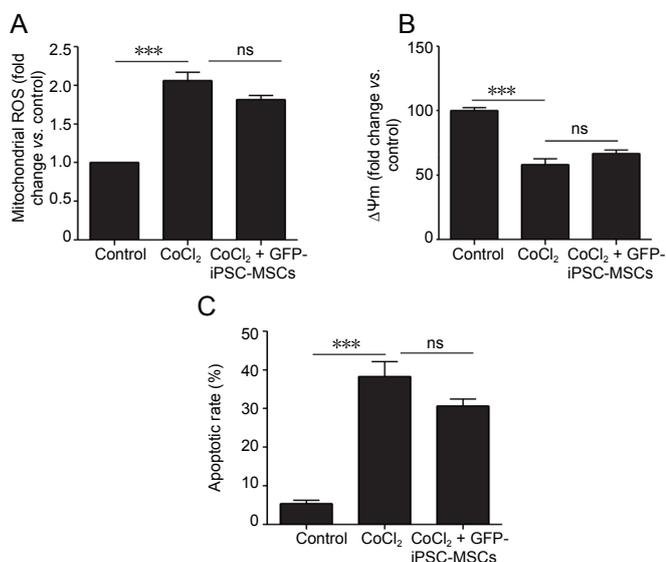

Figure 4 Effects of iPSC-MSC co-culture on CoCl$_2$-induced mitochondrial dysfunction and apoptosis in PC12 cells using a transwell culture.
(A) Effect of iPSC-MSC co-culture on ROS generation in PC12 cells induced by CoCl$_2$ in trans-well culture. (B) Effect of iPSC-MSC co-culture on the ΔΨm in PC12 cells induced by CoCl$_2$ in trans-well culture. (C) Effect of iPSC-MSC co-culture on apoptosis of PC12 cells induced by CoCl$_2$ in trans-well culture. Data are expressed as the mean ± SEM ($n = 3$; one-way analysis of variance followed by the Bonferroni test). All experiments were conducted in triplicate. $***P < 0.01$. CoCl$_2$: Cobalt chloride; DAPI: 4′,6-diamidino-2-phenylindole; GFP: green fluorescence protein; iPSC-MSCs: induced pluripotent stem cell-mesenchymal stem cells; ns: not significant; ROS: reactive oxidative species; ΔΨm: mitochondrial membrane potential.
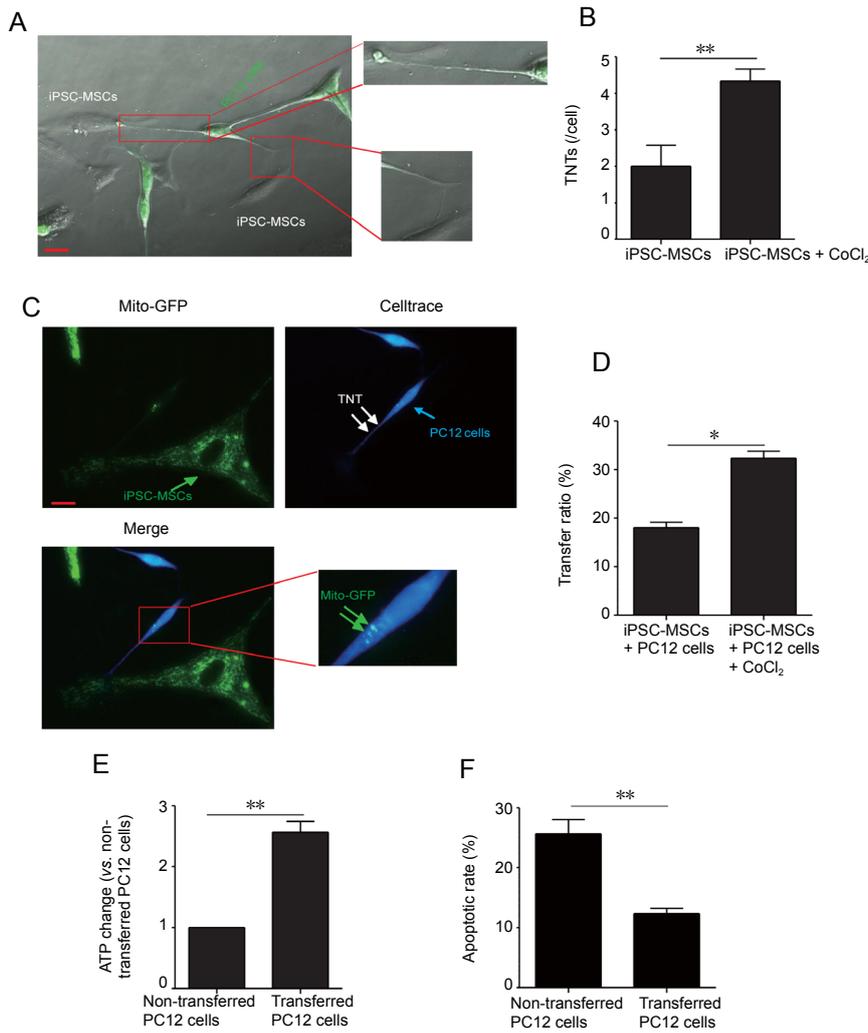
Figure 5 Mitochondria are transferred from iPSC-MSCs to injured PC12 cells under CoCl$_2$ challenge.
(A) TNTs formed between PC12 cells (in green) and iPSC-MSCs. Scale bar: 10 μm. (B) The number of iPSC-MSC TNTs was greatly enhanced under CoCl$_2$ challenge. (C) Mitochondria were transferred from Mito-GFP-iPSC-MSCs (in green) to Cell-trace-labelled-PC12 cells (in blue) via TNTs. Scale bar: 10 μm. (D) The efficiency of mitochondrial transfer from iPSC-MSCs to PC12 cells was dramatically enhanced under CoCl$_2$ treatment compared with no CoCl$_2$ treatment. (E) ATP levels were dramatically increased in the PC12 cells that received mitochondria from iPSC-MSCs compared with those that did not. (F) Apoptosis was dramatically reduced in the PC12 cells that received mitochondria from iPSC-MSCs compared with those that did not. Data are expressed as the mean ± SEM (n = 3; unpaired Student's t-test). All experiments were conducted in triplicate. $*P < 0.05$, $**P < 0.01$. ATP: Adenosine triphosphate; GFP: green fluorescence protein; iPSC-MSCs: induced pluripotent stem cell-mesenchymal stem cells; TNTs: tunneling nanotubes.
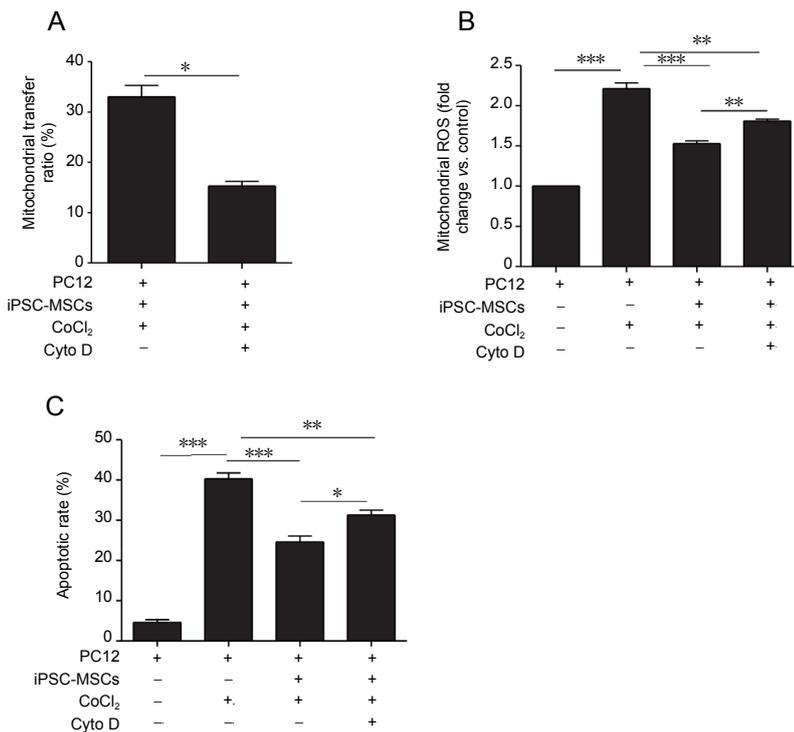

Figure 6 Inhibition of tunneling nanotubes disrupts the beneficial effects of iPSC-MSCs on CoCl$_2$-induced PC12 cell injury.
(A) Cyto D treatment greatly reduced the efficiency of mitochondrial transfer from iPSC-MSCs to PC12 cells in the presence of CoCl$_2$. (B) Cyto D treatment partially abrogated the beneficial effects of iPSC-MSCs on inhibiting ROS generation in the injured PC12 cells. (C) Cyto D treatment reduced the beneficial effects of iPSC-MSCs on inhibiting apoptosis of injured PC12 cells. Data are expressed as the mean ± SEM (n = 3, one-way analysis of variance followed by the Bonferroni test). All experiments were conducted in triplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. CoCl$_2$: Cobalt chloride; Cyto D: Cytochalasin D; iPSC-MSCs: induced pluripotent stem cell-mesenchymal stem cells; ROS: reactive oxidative species.

iPSC-MSCs. Second, iPSC-MSCs transferred their own mitochondria to the injured PC12 cells via the formation of tunneling nanotubes between the two cell types, which rescued the injuries in the PC12 cells. Third, inhibiting tunneling nanotubes decreased mitochondrial transfer from iPSC-MSCs to PC12 cells, which reduced the beneficial effects of iPSC-MSCs. Our results suggest that iPSC-MSCs provide a novel therapeutic strategy for hypoxia-ischemia-induced brain injury.

Accumulating evidence has demonstrated that although the underlying mechanisms are unclear, MSC-based therapy offers obvious therapeutic promise for hypoxia-ischemia-induced brain injury (Fleiss et al., 2014; Cho et al., 2016; Oppliger et al., 2016; Zheng et al., 2018). MSCs derived from Wharton's Jelly prevented the apoptosis of neuronal cells induced by hypoxia-ischemia through secretion of extracellular vesicles that contained a high level of let-7a and let-7e miRNAs, which regulate caspase-3 (Joerger-Messerli et al., 2018). Transplantation of MSCs attenuated the hypoxia-ischemia-induced brain injury-as determined by improved behavioral outcomes in mice-by activating astrocytes to release angiogenic factors (Cho et al., 2016). Transplantation of MSCs that overexpress brain-derived neurotrophic factor has been shown to alleviate hypoxia-ischemia-induced brain damage by stimulating the proliferation and differentiation of neural stem cells in the injured brain (van Velthoven et al., 2014; Gong and Liu, 2019; Zhou et al., 2019). Nonetheless, the functionality and number of MSCs derived from adult tissue decline with age, thus reducing their capacity for repair and regeneration (Lian et al., 2016; Li et al., 2017b; Ma et al., 2018). Therefore, exploring an alternative source of MSCs is of great importance. MSCs derived from embryonic stem cells or iPSCs provide a novel source of MSCs (Lian et al., 2010; Zhang et al., 2012). iPSC-MSCs exhibit higher proliferative and differentiation capacity than bone marrow mesenchymal stem cells (Lian et al., 2010). More importantly, iPSC-MSCs exhibit better therapeutic efficacy than bone marrow-derived mesenchymal stem cells in attenuating cigarette smoke-induced damage and hind limb ischemia. This is because of their higher differentiation potential and paracrine effects (Zhang et al., 2016; Li et al., 2017a). In the current study, iPSC-MSCs greatly reduced $CoCl_2$-induced apoptosis of PC12 cells. However, iPSC-MSCs only slightly alleviated the apoptosis of PC12 cells in a trans-well co-culture, suggesting that the protective effects of iPSC-MSCs are not entirely attributed to their paracrine effects. Thus, there is an urgent need to identify the mechanisms underlying iPSC-MSC rescue of $CoCl_2$-induced PC12 cell injury.

Mitochondrial dysfunction in neurons is the major cause of hypoxia-ischemia-induced brain damage (Leaw et al., 2017; Sun et al., 2017; Silachev et al., 2018). Hypoxia-ischemia markedly reduces the mitochondrial transmembrane potential and elevates ROS generation, leading to brain injury (Ten and Starkov, 2012; Bratek et al., 2018; Odorcyk et al., 2018). Treatment with resveratrol can alleviate brain damage by its ability to scavenge the ROS, indicating that the neuroprotective effects are mainly due to its antioxidant activity

(Arteaga et al., 2015). Damage to mitochondrial membrane integrity induced by hypoxia-ischemia has been shown to activate autophagy, resulting in apoptosis of neurons (Luo et al., 2015). These studies suggest that mitoprotective-based therapy can provide a novel intervention strategy for hypoxia-ischemia-induced brain injury. Here, we found that $CoCl_2$ enhanced ROS generation and reduced the $\Delta\Psi m$ in PC12 cells, indicating that $CoCl_2$ induced mitochondrial damage. However, co-culture with iPSC-MSCs obviously reversed the mitochondrial damage in PC12 cells induced by $CoCl_2$. Indeed, MSC-based therapy has shown promising results in rescuing mitochondrial damage. Recent studies have demonstrated that these cells can deliver new mitochondria to the injured cell to alleviate tissue damage. iPSC-MSCs have been reported to transfer mitochondria to cardiomyocytes injured by doxorubicin, which then attenuates the cardiomyopathy (Zhang et al., 2016). Furthermore, mitochondria transferred from iPSC-MSCs have also been shown to have beneficial effects on mitochondrial injury in airway smooth muscle cells that was induced by oxidative stress (Li et al., 2018). In our study, iPSC-MSCs delivered their own mitochondria to the injured PC12 cells and thus inhibited cell injury. Further, we observed that mitochondria were delivered to the injured PC12 cells via tunneling nanotubes that formed between the two types of cells. Accumulating evidence indicates that tunneling nanotubes are involved in regulating the transport of cellular contents and pathogens between cells (de Rooij et al., 2017; Sanchez et al., 2017). Previous studies have shown that iPSC-MSCs can transfer their mitochondria to injured cells via tunneling nanotubes (Li et al., 2014; Jiang et al., 2016). More importantly, we found that formation of tunneling nanotubes was dramatically enhanced in the presence of $CoCl_2$. This may explain why $CoCl_2$ challenge increased the mitochondrial transfer efficiency from iPSC-MSCs to PC12 cells. Indeed, doxorubicin can activate TNF-α/NF-κB signaling and thus stimulate formation of tunneling nanotubes that extend from iPSC-MSCs. Additionally, inhibition of NF-κB activity can reduce tunneling-nanotube formation (Zhang et al., 2016; Omsland et al., 2017). Whether $CoCl_2$ stimulated tunneling nanotubes formation and whether it is involved in regulating the NF-κB signaling pathway requires further investigation.

There are some limitations to the current study. First, it has not been determined whether iPSC-MSCs can transfer mitochondria to injured neuron cells in a rodent model of hypoxia-ischemia-induced brain injury. Future study is required to illustrate whether MSCs can transfer mitochondria to the injured neurons in an animal model. Second, in addition to mitochondria, whether iPSC-MSCs can transfer other cellular contents to injured PC12 cells should be carefully examined. A recent study demonstrated that autophagosomes can be transferred from MSCs to leukemic cells via tunneling nanotubes (de Rooij et al., 2017). Third, it is still unclear how $CoCl_2$ stimulates tunneling-nanotube formation in this study. Fourth, in addition to tunneling nanotubes, the possibility that other mechanisms can mediate mitochondrial transfer from iPSC-MSCs was not evaluated in this study.

Taken together, iPSC-MSCs protected against CoCl$_2$-induced PC12 cell injury, partially due to their paracrine effects, but also due to mitochondrial transfer via tunneling nanotubes. iPSC-MSC-based therapy is thus a novel strategy for hypoxia-ischemia-induced brain injury.

References

Archambault J, Moreira A, McDaniel D, Winter L, Sun L, Hornsby P (2017) Therapeutic potential of mesenchymal stromal cells for hypoxic ischemic encephalopathy: A systematic review and meta-analysis of preclinical studies. PLoS One 12:e0189895.

Arteaga O, Revuelta M, Uriguen L, Alvarez A, Montalvo H, Hilario E (2015) Pretreatment with resveratrol prevents neuronal injury and cognitive deficits induced by perinatal hypoxia-ischemia in rats. PLoS One 10:e0142424.

Arteaga O, Alvarez A, Revuelta M, Santaolalla F, Urtasun A, Hilario E (2017) Role of antioxidants in neonatal hypoxic-ischemic brain injury: new therapeutic approaches. Int J Mol Sci 18:E265.

Bittins M, Wang X (2017) TNT-induced phagocytosis: tunneling nanotubes mediate the transfer of pro-phagocytic signals from apoptotic to viable cells. J Cell Physiol 232:2271-2279.

Block TJ, Marinkovic M, Tran ON, Gonzalez AO, Marshall A, Dean DD, Chen XD (2017) Restoring the quantity and quality of elderly human mesenchymal stem cells for autologous cell-based therapies. Stem Cell Res Ther 8:239.

Braccioli L, Heijnen CJ, Coffer PJ, Nijboer CH (2017) Delayed administration of neural stem cells after hypoxia-ischemia reduces sensorimotor deficits, cerebral lesion size, and neuroinflammation in neonatal mice. Pediatr Res 81:127-135.

Bratek E, Ziembowicz A, Bronisz A, Salinska E (2018) The activation of group II metabotropic glutamate receptors protects neonatal rat brains from oxidative stress injury after hypoxia-ischemia. PLoS One 13:e0200933.

Cho SR, Suh H, Yu JH, Kim HH, Seo JH, Seo CH (2016) Astroglial activation by an enriched environment after transplantation of mesenchymal stem cells enhances angiogenesis after hypoxic-ischemic brain injury. Int J Mol Sci 17:E1550.

Davies A, Wassink G, Bennet L, Gunn AJ, Davidson JO (2019) Can we further optimize therapeutic hypothermia for hypoxic-ischemic encephalopathy? Neural Regen Res 2019;14:1678-1683.

de Rooij B, Polak R, Stalpers F, Pieters R, den Boer ML (2017) Tunneling nanotubes facilitate autophagosome transfer in the leukemic niche. Leukemia 31:1651-1654.

Fleiss B, Guillot PV, Titomanlio L, Baud O, Hagberg H, Gressens P (2014) Stem cell therapy for neonatal brain injury. Clin Perinatol 41:133-148.

Gong J, Liu M (2019) Human induced pluripotent stem cell transplantation for hypoxic-ischemic encephalopathy in neonatal mice. Zhongguo Zuzhi Gongcheng Yanjiu 23:5322-5327.

Hawkins KE, Corcelli M, Dowding K, Ranzoni AM, Vlahova F, Hau KL, Hunjan A, Peebles D, Gressens P, Hagberg H, de Coppi P, Hristova M, Guillot PV (2018) Embryonic stem cell-derived mesenchymal stem cells (MSCs) have a superior neuroprotective capacity over fetal MSCs in the hypoxic-ischemic mouse brain. Stem Cells Transl Med 7:439-449.

Jiang D, Gao F, Zhang Y, Wong DS, Li Q, Tse HF, Xu G, Yu Z, Lian Q (2016) Mitochondrial transfer of mesenchymal stem cells effectively protects corneal epithelial cells from mitochondrial damage. Cell Death Dis 7:e2467.

Jiang W, Chen L, Zhang XJ, Chen J, Li XC, Hou WS, Xiao N (2014) Red photon treatment inhibits apoptosis via regulation of bcl-2 proteins and ROS levels, alleviating hypoxic-ischemic brain damage. Neuroscience 268:66-74.

Joerger-Messerli MS, Oppliger B, Spinelli M, Thomi G, di Salvo I, Schneider P, Schoeberlein A (2018) Extracellular vesicles derived from Wharton's Jelly mesenchymal stem cells prevent and resolve programmed cell death mediated by perinatal hypoxia-ischemia in neuronal cells. Cell Transplant 27:168-180.

Johnston MV, Trescher WH, Ishida A, Nakajima W (2000) Novel treatments after experimental brain injury. Semin Neonatol 5:75-86.

Leaw B, Nair S, Lim R, Thornton C, Mallard C, Hagberg H (2017) Mitochondria, bioenergetics and excitotoxicity: new therapeutic targets in perinatal brain injury. Front Cell Neurosci 11:199.

Lee IS, Koo KY, Jung K, Kim M, Kim IS, Hwang K, Yun S, Lee H, Shin JE, Park KI (2017) Neurogenin-2-transduced human neural progenitor cells attenuate neonatal hypoxic-ischemic brain injury. Transl Res 183:121-136.e9.

Li X, Zhang Y, Liang Y, Cui Y, Yeung SC, Ip MS, Tse HF, Lian Q, Mak JC (2017a) iPSC-derived mesenchymal stem cells exert SCF-dependent recovery of cigarette smoke-induced apoptosis/proliferation imbalance in airway cells. J Cell Mol Med 21:265-277.

Li X, Michaeloudes C, Zhang Y, Wiegman CH, Adcock IM, Lian Q, Mak JCW, Bhavsar PK, Chung KF (2018) Mesenchymal stem cells alleviate oxidative stress-induced mitochondrial dysfunction in the airways. J Allergy Clin Immunol 141:1634-1645.e5.

Li X, Zhang Y, Yeung SC, Liang Y, Liang X, Ding Y, Ip MS, Tse HF, Mak JC, Lian Q (2014) Mitochondrial transfer of induced pluripotent stem cell-derived mesenchymal stem cells to airway epithelial cells attenuates cigarette smoke-induced damage. Am J Respir Cell Mol Biol 51:455-465.

Li X, Song Y, Liu D, Zhao J, Xu J, Ren J, Hu Y, Wang Z, Hou Y, Zhao G (2017b) MiR-495 promotes senescence of mesenchymal stem cells by targeting Bmi-1. Cell Physiol Biochem 42:780-796.

Lian Q, Zhang Y, Liang X, Gao F, Tse HF (2016) Directed differentiation of human-induced pluripotent stem cells to mesenchymal stem cells. Methods Mol Biol 1416:289-298.

Lian Q, Zhang Y, Zhang J, Zhang HK, Wu X, Zhang Y, Lam FF, Kang S, Xia JC, Lai WH, Au KW, Chow YY, Siu CW, Lee CN, Tse HF (2010) Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. Circulation 121:1113-1123.

Liang X, Ding Y, Lin F, Zhang Y, Zhou X, Meng Q, Lu X, Jiang G, Zhu H, Chen Y, Lian Q, Fan H, Liu Z (2019) Overexpression of ERBB4 rejuvenates aged mesenchymal stem cells and enhances angiogenesis via PI3K/AKT and MAPK/ERK pathways. FASEB J 33:4559-4570.

Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE (2015) Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet 385:430-440.

Lu Y, Tucker D, Dong Y, Zhao N, Zhuo X, Zhang Q (2015) Role of mitochondria in neonatal hypoxic-ischemic brain injury. J Neurosci Rehabil 2:1-14.

Luo C, Li Q, Gao Y, Shen X, Ma L, Wu Q, Wang Z, Zhang M, Zhao Z, Chen X, Tao L (2015) Poloxamer 188 attenuates cerebral hypoxia/ischemia injury in parallel with preventing mitochondrial membrane permeabilization and autophagic activation. J Mol Neurosci 56:988-998.

Ma Y, Qi M, An Y, Zhang L, Yang R, Doro DH, Liu W, Jin Y (2018) Autophagy controls mesenchymal stem cell properties and senescence during bone aging. Aging Cell 17:e12709.

Mir IN, Chalak LF (2014) Serum biomarkers to evaluate the integrity of the neurovascular unit. Early Hum Dev 90:707-711.

Odorcyk FK, Kolling J, Sanches EF, Wyse ATS, Netto CA (2018) Experimental neonatal hypoxia ischemia causes long lasting changes of oxidative stress parameters in the hippocampus and the spleen. J Perinat Med 46:433-439.

Omsland M, Bruserud O, Gjertsen BT, Andresen V (2017) Tunneling nanotube (TNT) formation is downregulated by cytarabine and NF-κB inhibition in acute myeloid leukemia (AML). Oncotarget 8:7946-7963.

Ophelders DR, Wolfs TG, Jellema RK, Zwanenburg A, Andriessen P, Delhaas T, Ludwig AK, Radtke S, Peters V, Janssen L, Giebel B, Kramer BW (2016) Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. Stem Cells Transl Med 5:754-763.

Oppliger B, Joerger-Messerli M, Mueller M, Reinhart U, Schneider P, Surbek DV, Schoeberlein A (2016) Intranasal delivery of umbilical cord-derived mesenchymal stem cells preserves myelination in perinatal brain damage. Stem Cells Dev 25:1234-1242.

Rodriguez J, Zhang Y, Li T, Xie C, Sun Y, Xu Y, Zhou K, Huo K, Wang Y, Wang X, Andersson D, Ståhlberg A, Xing Q, Mallard C, Hagberg H, Modjtahedi N, Kroemer G, Blomgren K, Zhu C (2018) Lack of the brain-specific isoform of apoptosis-inducing factor aggravates cerebral damage in a model of neonatal hypoxia-ischemia. Cell Death Dis 10:3.

Sanchez V, Villalba N, Fiore L, Luzzani C, Miriuka S, Boveris A, Gelpi RJ, Brusco A, Poderoso JJ (2017) Characterization of tunneling nanotubes in Wharton's jelly mesenchymal stem cells. an intercellular exchange of components between neighboring cells. Stem Cell Rev 13:491-498.

Silachev DN, Plotnikov EY, Pevzner IB, Zorova LD, Balakireva AV, Gulyaev MV, Pirogov YA, Skulachev VP, Zorov DB (2018) Neuroprotective effects of mitochondria-targeted plastoquinone in a rat model of neonatal hypoxic-ischemic brain injury. Molecules 23:E1871.

Song CG, Zhang YZ, Wu HN, Cao XL, Guo CJ, Li YQ, Zheng MH, Han H (2018) Stem cells: a promising candidate to treat neurological disorders. Neural Regen Res 2018;13:1294-1304.

Song HF, He S, Li SH, Yin WJ, Wu J, Guo J, Shao ZB, Zhai XY, Gong H, Lu L, Wei F, Weisel RD, Xie J, Li RK (2017) Aged human multipotent mesenchymal stromal cells can be rejuvenated by neuron-derived neurotrophic factor and improve heart function after injury. JACC Basic Transl Sci 2:702-716.

Sun Y, Li T, Xie C, Zhang Y, Zhou K, Wang X, Blomgren K, Zhu C (2016) Dichloroacetate treatment improves mitochondrial metabolism and reduces brain injury in neonatal mice. Oncotarget 7:31708-31722.

Sun Y, Li T, Xie C, Xu Y, Zhou K, Rodriguez J, Han W, Wang X, Kroemer G, Modjtahedi N, Blomgren K, Zhu C (2017) Haploinsufficiency in the mitochondrial protein CHCHD4 reduces brain injury in a mouse model of neonatal hypoxia-ischemia. Cell Death Dis 8:e2781.

Ten VS, Starkov A (2012) Hypoxic-ischemic injury in the developing brain: the role of reactive oxygen species originating in mitochondria. Neurol Res Int 2012:542976.

Thornton C, Leaw B, Mallard C, Nair S, Jinnai M, Hagberg H (2017) Cell death in the developing brain after hypoxia-ischemia. Front Cell Neurosci 11:248.

van Velthoven CT, Braccioli L, Willemen HL, Kavelaars A, Heijnen CJ (2014) Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. Mol Ther 22:645-654.

Yang NC, Ho WM, Chen YH, Hu ML (2002) A convenient one-step extraction of cellular ATP using boiling water for the luciferin-luciferase assay of ATP. Anal Biochem 306:323-327.

Yao Y, Fan XL, Jiang D, Zhang Y, Li X, Xu ZB, Fang SB, Chiu S, Tse HF, Lian Q, Fu QL (2018) Connexin 43-mediated mitochondrial transfer of iPSC-MSCs alleviates asthma inflammation. Stem Cell Rep 11:1120-1135.

Zhang Y, Liao S, Yang M, Liang X, Poon MW, Wong CY, Wang J, Zhou Z, Cheong SK, Lee CN, Tse HF, Lian Q (2012) Improved cell survival and paracrine capacity of human embryonic stem cell-derived mesenchymal stem cells promote therapeutic potential for pulmonary arterial hypertension. Cell Transplant 21:2225-2239.

Zhang Y, Chiu S, Liang X, Gao F, Zhang Z, Liao S, Liang Y, Chai YH, Low DJ, Tse HF, Tergaonkar V, Lian Q (2015a) Rap1-mediated nuclear factor-kappaB (NF-κB) activity regulates the paracrine capacity of mesenchymal stem cells in heart repair following infarction. Cell Death Discov 1:15007.

Zhang Y, Liang X, Liao S, Wang W, Wang J, Li X, Ding Y, Liang Y, Gao F, Yang M, Fu Q, Xu A, Chai YH, He J, Tse HF, Lian Q (2015b) Potent paracrine effects of human induced pluripotent stem cell-derived mesenchymal stem cells attenuate doxorubicin-induced cardiomyopathy. Sci Rep 5:11235.

Zhang Y, Yu Z, Jiang D, Liang X, Liao S, Zhang Z, Yue W, Li X, Chiu SM, Chai YH, Liang Y, Chow Y, Han S, Xu A, Tse HF, Lian Q (2016) iPSC-MSCs with high intrinsic MIRO1 and sensitivity to TNF-alpha yield efficacious mitochondrial transfer to rescue anthracycline-induced cardiomyopathy. Stem Cell Reports 7:749-763.

Zhao L, Liu X, Zhang Y, Liang X, Ding Y, Xu Y, Fang Z, Zhang F (2016) Enhanced cell survival and paracrine effects of mesenchymal stem cells overexpressing hepatocyte growth factor promote cardioprotection in myocardial infarction. Exp Cell Res 344:30-39.

Zheng Z, Zhang L, Qu Y, Xiao G, Li S, Bao S, Lu QR, Mu D (2018) Mesenchymal stem cells protect against hypoxia-ischemia brain damage by enhancing autophagy through brain derived neurotrophic factor/mammalin target of rapamycin signaling pathway. Stem Cells 36:1109-1121.

Zhou Y, Mo GL, Zhao J, Fang P (2019) Neuroprotective effect and mechanism of human umbilical cord blood mesenchymal stem cells on hypoxic-ischemic brain injury in neonatal rats. Zhongguo Zuzhi Gongcheng Yanjiu 23:2035-2041.