Lin28 Enhances Glucose Metabolism in Human Placental Mesenchymal Stem Cells during Hypoxia via the PI3K-Akt Pathway

Xi Zhou
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Junbo Li
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Jin Wang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Huifang Yang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Jingzeng Wang
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Xiaoyun Xie
Tongji University Tenth People's Hospital: Shanghai Tenth People's Hospital
Zhishui Chen
Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology
Bo Zhang (zhangbo@tjh.tjmu.edu.cn)
Huazhong University of Science and Technology

Research

Keywords: LIRI, PMSC, Glucose metabolism, PI3K-Akt Pathway, LDHA

Posted Date: September 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-892582/v1

Abstract

Mesenchymal stem cells (MSCs) are widely used to treat and prevent liver ischemia–reperfusion injury (LIRI), which commonly occurs after liver surgery. Lin28 is an RNA-binding protein crucial for early embryonic development, stem cell differentiation/reprogramming, tumorigenesis, and metabolism. However, whether Lin28 can enhance metabolism in human placental MSCs (PMSCs) during hypoxia to improve the protective effect against LIRI remains unclear. First, a Lin28 overexpression construct was introduced into PMSCs; glucose metabolism, the expression of glucose metabolism - and PI3K-AKT pathway-related proteins, and the levels of microRNA Let-7 family members were examined using a glucose metabolism kit, western blots, and real-time quantitative PCR, respectively. Next, treatment with an AKT inhibitor was performed to understand the association of Lin28 with the PI3K-Akt pathway. Subsequently, AML12 cells were co-cultured with PMSCs to construct an in vitro model of PMSC protecting liver cells from hypoxia injury. Finally, C57BL/6J mice were used to establish a partial warm hepatic ischemia–reperfusion model in vivo. Lin28 increased the glycolysis capacity of PMSCs, allowing these cells to produce more energy under hypoxic conditions. Lin28 also activated PI3K-Akt signaling under hypoxic conditions, and AKT inhibition attenuated the effects of Lin28. In addition, Lin28 overexpression was found to protect cells against LIRI-induced liver damage, inflammation, and apoptosis and attenuate hypoxia-induced hepatocyte injury. Inconclusion, Lin28 enhances glucose metabolism under hypoxic conditions in PMSCs, thereby providing protective effects against LIRI via the activation of the PI3K-Akt signaling pathway. Our study first reported the application of gene-modified mesenchymal stem cell-based therapy in LIRI.

1. Introduction

Liver ischemia–reperfusion injury (LIRI) is sometimes observed in cases of liver surgery (such as transplantation and hepatectomy) and shock and is an important type of clinical liver injury [1]. LIRI mainly leads to primary graft dysfunction after transplantation, liver dysfunction after hepatectomy, and biliary tract injury [2, 3]. During liver surgery, the blood vessels in the liver are often blocked, leading to ischemia in liver cells and inducing their death [4]. In addition, after blood supply is restored, free radicals attack the liver cells that have regained blood supply. Together, these conditions lead to necrosis and apoptosis, causing inflammatory cell infiltration and the aggregation of inflammatory mediators, which further damage liver cells [5, 6]. Studies have shown that the damage caused by reperfusion is far greater than that caused by ischemia [7]. With the progress in medicine, the mechanism underlying LIRI has been extensively studied. Surgical strategies that reduce the duration of liver ischemia, intraoperative and postoperative liver protection strategies, and immunosuppressing drugs that prevent liver damage have played an obvious role in reducing the incidence of LIRI [8–10]. Mesenchymal stem cells (MSCs) are present at multiple sites in the body, such as bone marrow, cord blood, and umbilical cord and placental tissue, and in adults, these act as non-hematopoietic stem cells with multidirectional potential. MSCs are mainly used during bone marrow transplantation for preventing graft versus host disease, improving tissue repair, and providing anti-inflammatory and immunomodulatory effects[11–14]. Placental MSCs

(PMSCs) show better immunogenicity and immune regulation than other types of MSCs owing to their negative immune -regulating function and inhibition of the body's immune response. These cells restore the immune balance and can inhibit the inflammation associated with injury in several organs, and they have therefore been used for kidney, brain, heart, and liver treatment [15–17]. Nevertheless, the use of MSCs remains associated with some problems, including the survival duration of MSCs, number of organs to be protected, and injury to MSCs during ischemia and reperfusion[18]. Further, the regulation of MSC survival under hypoxic conditions has not been examined.

Cancerous cells typically gain energy from glycolysis instead of aerobic respiration. Owing to this switch, called the Warburg effect, cancer cells show mitochondrial utilization that is different from that in normal cells [19]. Lin28 is an RNA-binding protein required for early embryonic development, stem cell differentiation/reprogramming, tumorigenesis, and metabolism [20–22]. Studies have shown that the Lin28/Let-7 pathway can regulate mammalian glucose metabolism, and that this pathway itself is intricately controlled [23]. Another important intracellular pathway is the PI3K-Akt signaling pathway, which regulates processes such as energy metabolism, growth and development, and autophagy [24, 25]. Studies have shown that this pathway is activated under anoxic conditions and enables the switch from aerobic respiration to glycolysis, allowing cells to produce enough energy [26]. However, whether Lin28 can enhance metabolism in human placental MSCs (PMSCs) during hypoxia to improve the protective effect against LIRI, and whether pi3K-Akt signaling pathway plays a role in it remains unclear. In the present study, a partial hepatic ischemia–reperfusion injury mouse model and hypoxia-treated hepatocyte model were used to examine whether Lin28 can promote glycolytic ability in PMSCs under hypoxic conditions, thereby protecting against LIRI. We also explored whether the effect of Lin28 on PMSC glycolysis was mediated by PI3K-Akt signaling.

2. Results

2.1. Lin28 Changes Glucose Metabolism Levels in PMSCs

A PMSC hypoxia model was used to examine the expression of Lin28 in PMSCs under hypoxic conditions. We found that hypoxia obviously increased Lin28 expression in PMSCs (Figure 1A). The Lin28 overexpression lentivirus showed a 76.36±2.002% transfection efficiency in PMSCs (Figure 1B,C). On examining several biochemical indicators related to cell glycolysis, we found that PMSCs transfected with Lin28 had a higher LA level, NADPH/NADP+ ratio, and ATP content and lower intracellular glucose levels than untransfected cells. Although these differences were observed in both normoxic and anoxic environments, they were starker in anoxic environments (Figure 1D,E). In line with this, we found that the basal glucose metabolism and glycolytic capacity of PMSCs increased after Lin28 overexpression, indicating that Lin28 promoted glycolysis in PMSCs (Figure 1F,G). A CCK-8 assay demonstrated that the proliferation of PMSCs was significantly reduced under hypoxia, and the proliferation of PMSCs overexpressing Lin28 was also significantly reduced (Figure 1H). These results indicated that the overexpression of Lin28 under hypoxic conditions could promote anaerobic glycolysis in PMSCs, allowing them to produce more energy under hypoxia and ensuring appropriate functioning.

2.2. Lin28 Can Regulate Glycometabolism-related Proteins in PMSCs

In order to understand the pathway via which Lin28 increases the glycolytic capacity of PMSCs, we examined the levels of glycolysis-related proteins in PMSCs under normoxic and hypoxic conditions. We found that lactate dehydrogenase A(LDHA), 3-phosphoinositide dependent kinase-1(PDK1), M2-pyruvate kinase (PKM2), Hexokinase I (HEX I), Hexokinase II (HEX II), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2(PFKFB2) — all enzymes involved in the regulation of glycolysis — showed elevated protein levels during hypoxia. However, in PMSCs overexpressing Lin28, these proteins — with the exception of LDHA — showed no significant difference in expression (Figure 2A). Meanwhile, the mitochondrial proteins UQCRC and COXII showed increased levels in PMSCs overexpressing Lin28, while ATP5A, SDHB, and NDUFB8 showed no significant difference (Figure 2B). Therefore, we speculated that Lin28 may regulate glucose metabolism in PMSCs by regulating LDHA.

2.3. Lin28 Regulates LDHA via the Let7-PI3K-Akt Pathway

Lin28 can downregulate the microRNA Let7 family. Using qRT-PCR, we found that the levels of microRNA Let7a, Let7b, and Let7c were dramatically lower in the PMSC-Lin28 group than in the PMSC group, whereas microRNA Let7d, Let7e, Let7f, and Let7g showed no significant difference (Figure 3A). Previous studies have confirmed that a reduction in Let7c leads to the activation of PI3K and the PI3K-Akt pathway, which leads to changes in the expression of LDHA. The transcription levels of PI3K and LDHA were quantified in PMSCs and PMSCs-Lin28 under normoxia and hypoxia using qRT-PCR, and the protein expression of AKT, pAKT, Lin28, and LDHA was detected using western blots. Transcription levels of PI3K and LDHA were higher in PMSCs-Lin28 group than in PMSCs and were also higher under hypoxia than under normoxia (Figure 3B). The protein levels of pAKT and LDHA showed similar trends. However, the protein expression of AKT was comparable among all groups (Figure 3C). Based on these results, we speculated that Lin28 reduces the levels of Let7c by interacting with the microRNA Let7c, thus promoting the expression of PI3K, activating the PI3K-Akt pathway, and promoting AKT phosphorylation. Such changes lead to an increase in LDHA expression, thereby promoting the switch from aerobic oxidation to anaerobic glycolysis in PMSCs.

2.4. Inhibitors of the PI3K-Akt Pathway Weaken the Effect of Lin28 on Glucose Metabolism

In order to verify that Lin28 regulates glucose metabolism via the PI3K-Akt pathway, we treated PMSCs with the AKT phosphorylation inhibitor MK2206 in vitro. After treatment with MK2206, the intracellular lactate level, NADPH/NADP+ ratio, and ATP content decreased in Lin28-overexpressing PMSCs, whereas the intracellular glucose content increased significantly; these effects were observed under both normoxic and anoxic conditions (Figure 4A,B). On examining protein expression, we found that cells from the PMSC+MK2206 and PMSC-Lin28+MK2206 groups showed lower levels of pAKT and LDHA than did those from the PMSC and PMSC-Lin28 groups. In contrast, the levels of AKT and Lin28 were similar between the PMSC-Lin28+MK2206 and PMSC-Lin28 groups and the PMSC+MK2206 and PMSC groups (Figure 4C). Furthermore, ECAR analysis showed that basal glucose metabolism and glycolysis were

reduced in PMSCs overexpressing Lin28 after MK2206 treatment (Figure 4D,E). Hence, we concluded that Lin28 enhances the viability of PMSCs in anoxic environments by activating the PI3K-Akt pathway.

2.5. Lin28 Overexpression in PMSCs Enhances its Protective Effects against Ischemia–Reperfusion-induced Apoptosis in AML12 Cells

After 24 hours of anoxic culture as mentioned above, the cells were cultured in a normoxic incubator containing 5%CO2 for 1 hour and then harvested and analyzed using flow cytometry. The apoptosis rate was found to be markedly lower in the AML12+PMSC and AML12+PMSC-Lin28 groups than in the AML12 group. Furthermore, this value was much lower in the AML12+PMSC-Lin28 group than in the AML12+PMSC group (Figure 5A,B). This indicated that Lin28 promotes the protective effect of PMSCs against apoptosis in an AML12 hypoxia model.

2.6. Lin28 Overexpression in PMSCs Enhances its Protective Effects against LIRI in Mice

We examined the effects of treatment with Lin28-overexpressing PMSCs in a mouse model of LIRI using biochemical and histological examinations. Serum ALT and AST levels in the LIRI group were significantly higher than those in the sham operation group. However, this effect was reversed in the LIRI+PMSC group and even more significantly so in the LIRI+PMSC-Lin28 group (Figure 5C). Similarly, mice in the LIRI group developed severe liver injury, with a significant increase in the necrotic area of liver tissue and Suzuki score. In contrast, the degree of liver damage was significantly lower in the LIRI+PMSC and LIRI+PMSC-Lin28 groups, and especially in the LIRI+PMSC-Lin28 group (Figure 5D,E). These observations suggested that the overexpression of Lin28 can increase the protective effects of PMSCs against LIRI. Given that the inflammatory response is also an important factor in LIRI, we examined inflammation-related parameters such as the neutrophil infiltration ratio using MPO staining. In the LIRI+PMSC-LIN28 and LIRI+PMSC groups, the neutrophilic infiltration was reduced, with the effect being more obvious in the LIRI+PMSC-LIN28 group (Figure 5F,G), suggesting that the overexpression of Lin28 could increase the effectiveness of PMSC in reducing the inflammatory response during LIRI. In addition, the protective effect of PMSCs was also examined using TUNEL staining. As shown in the figure, the LIRI group had the most TUNEL -positive cells. After PMSC and PMSC-LIN28 treatment, TUNEL -positive cells decreased in number, especially after PMSC-LIN28 treatment (Figure 5H,I). Hence, we concluded that Lin28 overexpression could increase the protective effect of PMSCs against the apoptosis of liver cells.

First, Lin28 binds to microRNA Let-7 family precursors, so microRNA Let-7 family are down-regulated. Second, microRNA Let-7 binds to the PIK3CA promoter region to influence its transcription, PI3K phosphorylates AKT and activates AKT, which promotes the expression of LDHA. LDHA increases the glucose metabolism of PMSC and makes PMSC produce more energy in the absence of oxygen, increasing the protection offered by PMSC against LIRI.

This diagram were created with BioRender.com

3. Discussion

Orthotopic liver transplantation is now emerging as a frontrunner for treating end-stage liver disease. However, LIRI remains one of the most important and common causes of postoperative declines in hepatic function, inflammatory infiltration, and apoptosis, and its severity is tightly linked to the postoperative recovery of patients [29,30].Many studies have focused on the treatment of LIRI [31,32]. MSCs are considered to be effective in reducing LIRI and consequently protecting liver function [33-35]The findings of the present study also prove their effectiveness. However, the mechanism by which MSCs protect the body against LIRI is unclear. Currently, it is hypothesized that these effects are related to the inhibition of reactive oxygen species overproduction by mitochondria, improvement of mitochondrial function [36], regulation of miRNA [37], and regulation of immune cells [38] by extracellular vesicles. Changes in macrophage activation can prevent inflammation [39]. However, during liver ischemia, MSCs are also in a state of ischemia and hypoxia. It is unclear if these conditions change their cellular functions and what mechanisms could underlie these changes.

in the present study, we first constructed a hypoxia model in PMSCs and observed an increase in Lin28 protein levels. Subsequently, we transfected Lin28 into PMSCs using a lentivirus. We showed that Lin28 can increase anaerobic fermentation in PMSCs in a hypoxic environment and increase energy production in an anaerobic state, enabling their survival. Next, we sought to identify the pathway via which Lin28 exerted this effect. To this end, we examined a large number of proteins related to glycolysis, including LDHA, PDK1, PKM2, HEX I, HEX II, and PFKFB2, all of which are key regulators of glycolysis [40,41]. Our results confirmed that these enzymes were significantly upregulated under hypoxic conditions, but only LDHA showed a significant increase in PMSCs overexpressing Lin28. Meanwhile, we also found that the overexpression of Lin28 had little effect on the expression of mitochondrial complex proteins. Together, these results indicated that LDHA may be a key enzyme mediating the effects of Lin28 on anaerobic fermentation in PMSCs.

Next, we studied the effects of Lin28 on LDHA. Lin28 can downregulate members of the microRNA Let-7 family by binding to the microRNA Let-7 precursor [42,43]. Our experiment also verified these reports. Further, the increased expression of LDHA under hypoxia helps cells switch their glucose metabolism from oxidative phosphorylation to anaerobic glycolysis mainly through the activation of the PI3K-Akt pathway [44,45]. Our experiments, as expected, showed that the PI3K-LIN28 pathway was activated in PMSCs after transfection with Lin28. To verify that the effects of Lin28 on LDHA expression are mediated by this pathway, we used the AKT inhibitor MK2206 2Hcl, which can effectively inhibit the phosphorylation of Ser473 in AKT [46]. After treatment with this inhibitor, we found that the promoting effects of Lin28 overexpression on aerobic glycolysis, energy generation in an anoxic environment, and LDHA levels were significantly inhibited, despite no changes in Lin28 expression. This demonstrated that AKT inhibitors can attenuate the Lin28-mediated regulation of LDHA without affecting Lin28 expression, implying that Lin28 regulates LDHA via the PI3K-Akt pathway, thereby increasing the ability of PMSCs to perform anaerobic glycolysis. These results suggest that Lin28 overexpression can increase energy production in PMSCs even in an anoxic environment. Hence, we speculated that the overexpression of Lin28 could enable PMSCs to play greater protective roles against LIRI.

We designed an in vitro cellular model of hypoxia using AML12 cells co-cultured with PMSCs and examined whether Lin28-overexpressing PMSCs were more effective than normal PMSCs in protecting hepatocytes from hypoxia. We found that Lin28-overexpressing PMSCs significantly reduced the apoptosis rate in AML12 cells after anoxia, conferring a better protective effect than normal PMSCs. To confirm this effect in vivo, we constructed a mouse LIRI model. Studies have shown that most MSCs disappear around 2 hours after their caudal intravenous infusion, and only a few surviving MSCs reach the liver, resulting in failed protection against LIRI [47]. This also explains why several studies have focused on extracellular vesicles from MSCs in preventing LIRI instead of the MSCs themselves. If MSCs are infused after reperfusion, they may be unable to prevent the damage caused by ischemia to liver cells. Hence, we chose the method involving the 1-h portal vein infusion of PMSCs before ischemia, which allows MSCs to accumulate in the liver and protects stem cells from ischemia-reperfusion injury [48]. We noted that after LIRI, there was an increase in serum ALT and AST levels and liver damage in mice, but these changes were attenuated by PMSC treatment and even more effectively by PMSC-Lin28 treatment. The neutrophil infiltration (MPO staining) was lowest in the LIRI+PMSC-LIN28 group, followed by the PMSC group and the LIRI group, suggesting that Lin28 can increase the relieving effect of PMSCs on the LIRI -induced inflammatory response. In our study,TUNEL staining showed that Lin28 transfection increases PMSC and decreases liver cell apoptosis after LIRI.

Although Lin28 has been shown to activate the PI3K-Akt pathway [49], there are few reports on how Lin28 regulates the PI3K-Akt signaling pathway (Figure 6). We analyzed the gene promoter region of PI3K, and found that the promoter region of PIK3CA gene (whose expression product is PI3K α subunit) has a region that can bind to microRNA Let7 family, and we speculated that it might be microRNA The Let7 family binds to the promoter region of PIK3CA and inhibits the transcription of PIK3CA leading to the decreased expression of PI3K, which is consistent with the previous results. We will investigate these mechanisms in subsequent experiments.

In conclusion, our study shows that Lin28 can improve the glycolytic ability of PMSCs under hypoxia through the PI3K-Akt pathway, enabling better energy production even under hypoxia, thus increasing the protection offered by PMSC against LIRI. To our knowledge, our study is the first to show that Lin28 alters the glucose metabolism of PMSCs via the PI3K-Akt pathway, thereby regulating the protection offered by these cells against LIRI. Despite having some limitations, our study provides new insights into the molecular regulation of glycolysis in PMSCs, which pave the way for the development of more effective MSC therapies and prevention of LIRI.

4. Materials And Methods

2.1. Animals

We purchased male C57BL/6J mice (6–8 weeks old) from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed in the Animal Facility of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All animal experiments were

conducted after approval from the Institutional Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology, and all procedures were in compliance with the relevant guidelines and regulations of the Chinese Council on Animal Care.

2.2. Partial LIRI Model

Male C57BL/6J mice (20–22 g) were randomly assigned to one of four groups: Sham; LIRI; LIRI+PMSC; and LIRI+PMSC-Lin28. The partial LIRI model was developed using methods described in an earlier study [27]. The mice in sham group underwent the same operative procedure, without any blood vessel blocking. The LIRI+PMSC and LIRI+PMSC-Lin28 groups received injections with PMSCs and PMSCs-Lin28 into the portal vein (100 μL, 107 cells/mL) 1 hour prior to hepatic ischemia. After 1 hour of ischemia and 6 hours of reperfusion, mice were sacrificed and serum and liver tissue samples were collected.

2.3. Serum Biochemistry Assay

Blood collected from mice was first chilled on ice (30 min) and then centrifuged at 8000 RPM (15 min). We diluted the supernatant as appropriate and used a standard automatic analyzer (Mindray BS-200) to detect serum ALT and AST levels.

2.4. Cell Culture and Hepatocyte Hypoxia Model

Human PMSCs (Shanghai Tongji University) and murine hepatocyte cells (AML12 cell line; ATCC®CRL-2254TM) were cultured in Dulbecco's Modified Eagle Medium/F-12 (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 40 ng/mL dexamethasone (Sigma-Aldrich, D4902), and ITS Liquid Media Supplement (Sigma-Aldrich, I3146). The AML12 cells and PMSCs were placed in normoxic and anoxic environments, respectively, at 37°C. A humidified incubator containing 5% CO2 was used to maintain a normoxic environment, whereas a hypoxia incubator (Whitley H35 Hypoxystation) containing 5% CO2, 1% O2, and 94% N2 was used for maintaining an anoxic environment. To induce the ischemic and hypoxic cellular model, cells were cultured to a density of approximately 70%, Subsequently, old medium was removed and serum-free medium was added; cells were incubated in anoxic conditions for 24 h. Additionally, in the inhibitortreatment group, MK2206 (Sellect, S1078) was added to cells (final concentration, 3 μM). Based on MK2206 treatment, four treatment groups were established: PMSC, PMSC-Lin28, PMSC+MK2206 2Hcl, and PMSC-Lin28+MK2206. We maintained AML12 cells under hypoxic conditions for 24 h to construct a cellular model of ischemia and hypoxia. The cells were divided into three groups: AML12 group (AML12), AML12 co-cultured with PMSCs (AML12+ PMSC group), and AML12 co-cultured with PMSCs-Lin28 (AML12+ PMSC-Lin28 group).

2.5. Overexpression of Lin28 in Cells

When cells reached a density of approximately 70%, they were cultured in OPTI-MEM Reduced Serum Medium (Gibco) with a Lin28 overexpression lentivirus (10μL/mL; with green fluorescence; obtained from

Hanbio Biotechnology Co., Ltd.) for 72 hours. Subsequently, fluorescence microscopy was used to detect transfection efficiency.

2.6. Cellular Glucose Metabolism Assay

Glucose (Solarbio,BC2500), lactic acid(LA) (Solarbio,BC2230), ATP (Solarbio,BC0300), and NADPH (Solarbio,BC1100) detection kits were used to detect the intracellular glucose levels, LA levels, ATP levels, and NADPH/NADP+ ratio, respectively. ECAR was detected using the Seahorse XF Glycolysis Stress Test Kit (103020-100).

2.7. Pathology

2.7.1. Hematoxylin and Eosin (H&E) Staining

After 6 hours of ischemia, liver tissue was fixed in 4% paraformaldehyde, dehydrated using an increasing ethanol concentration gradient, and embedded in paraffin. Subsequently, 4 -μm -thick sections were obtained and subjected to H&E staining. The stained samples were examined by two independent pathologists, who assessed hepatic necrosis based on Suzuki's scores [28].

2.7.2. TUNEL assay

A TUNEL Kit (Servicebio, G1501) was used based on manufacturer's instructions. First, tissue sections were deparaffinized using protease K. Subsequently, membranes were disrupted broken, the reaction solution was added, and nuclei were stained with DAPI. Finally, the sections were imaged using an upright fluorescence microscope (NIKON ECLIPSE C1, Japan). The proportion of TUNEL-positive cells was calculated using five randomly selected fields.

2.7.3. Myeloperoxidase (MPO) staining

MPO immunohistochemistry was performed using mouse anti-MPO (Servicebio, GB12224) and HRP -labeled goat anti-mpO (Servicebio, G23301) antibodies, as well as the histochemical DAB chromogenic agent kit (Servicebio, GB12224) based on manufacturer's instructions. The fixed tissue was first sealed with serum, followed by the addition of primary antibody, secondary antibody and developer, and then the nucleus was stained with DAP. Finally, the tissue was examined under a microscope; images were then captured and analyzed. The hematoxylin-stained nuclei appeared blue, whereas positive mPO expression was indicated by a brownish yellow color.

2.8. Cell Viability Assay

The CCK-8 kit (Dojindo) was used to detect cell viability. Before hypoxia treatment, 10,000 cells were planted in each well of a 96-well plate, and three holes were made in each well. After 24 hours of culture, the medium was replaced and 10μL of the CCK-8 reagent was added to each well (incubation, 37°C, 2 h). Finally, a microplate reader was used to measure the absorbance at 450 nm.

2.9.Flow Cytometry Analysis of Apoptosis

An Annexin V-FITC and propidium iodide staining kit (MULTISCIENCES, Hangzhou, China) was used to examine cell apoptosis using manufacturer's instructions. Using a Flow Cytometer (BD FACSCelesta) and FlowJo software for data analysis, we measured the percentages of apoptotic cells.

2.10.qRT-PCR

The Total RNA Rapid Extraction Kit (Fastagen, Shanghai, China) was used to isolate the total mRNA from PMSCs, which was then reverse transcribed using a high-capacity cDNA reverse transcription kit (TAKARA, Shiga, Japan) based on manufacturer's instructions. Target gene expression was evaluated with qRT-PCR, performed using a SYBR Green kit (Takara). StepOne software (Thermo Fisher Scientific) was used to analyze the data. For examining microRNA levels, the high-capacity microRNA reverse transcription kit (TAKARA, Shiga, Japan) was used; the other steps remained the same as those used for mRNA analysis. GADPH and U6 were chosen as internal controls for mRNA and microRNA, respectively. Each experiment was performed at least in triplicate. Primer sequences are shown in the table below.

2.11. Western Blot

IP lysis buffer (Beyotime, Shanghai, China) with protease inhibitors was used to extract the total protein from cell lysates. A Microplate Reader (BioTek) and BCA Protein Assay Kit (Beyotime, Shanghai, China) were used to measure the protein concentration. We performed 10% SDS-PAGE to separate protein samples, which were then transferred onto polyvinylidene fluoride membranes (Millipore, 0.22μm). The membranes were sealed with 5% skim milk in TBST and then incubated with primary antibodies for 16 h. Antibodies against β-catenin (ab32572), LDHA(ab52488), AKT(ab179463), pAKT(ser473), Lin28(ab191881), Total OXPHOS (ab110431), HEX I (ab150423), HEX II (ab209847), PKM2(ab85555), PDK1(ab202468), PFKFB2(ab234865) purchased from Abcam were used. Subsequently, the membranes were blocked with either the goat anti-rabbit IgG H&L or goat anti-mouse IgG H&L antibody labeled with horseradish peroxidase (Abcam, ab6721 and ab6789) at room temperature (20-25℃) for 1 hour. The ECL reagent (Beyotime, Shanghai, China) was used to quantify the expression of these proteins based on chemiluminescent detection, and the Chemiluminescence Imaging System (GeneGnome, Shanghai, China) was used to detect protein bands. Image-Pro Plus software (Media Cybernetics) was used for analysis. Proteins levels were normalized based on β-catenin concentration, and all experiments were performed at least thrice.

2.12. Statistical Analysis

All statistical analyses were performed using Prism software (GraphPad). All data were expressed as means±standard error of the mean. One-way ANOVA was used when multiple groups were being compared, whereas unpaired t-tests were used when two groups were being compared. P-values <0.05 were statistically significant.

5. Conclusions

This section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

Declarations

Ethics approval and consent to participate: All animal experiments were conducted after approval from the Institutional Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology, and all procedures were in compliance with the relevant guidelines and regulations of the Chinese Council on Animal Care.

Consent for publication: Not applicable

Availability of data and material: All data included in this study are available upon request by contact with the corresponding author.

Competing Interests: The authors declare that they have no competing interests.

Funding: This work was funded by the National Natural Science Foundation of China (81800580 and 81770652).

Author Contributions: All authors had full access to all the data in the study. and take responsibility for the integrity of the data and the accuracy of the data analysis. XZ participated in experimental design, animal model, cell experiment, molecular experiment, draft writing, data analysis and collation. JL, JW, HY, JW and XX assisted in cell culture, animal experiments and revised the manuscript critically for important intellectual content. MY, YZ took part in study design and performed the statistical analysis. ZC and BZ were the study supervisor, designed the study and acted as the corresponding author. All authors approved the final version.

Acknowledgments: Thanks to Professor LAN Peixiang for his constructive suggestions on experimental design, and thanks to MJEditor(www.mjeditor.com) for its linguistic assis- tance during the preparation of this manuscript.Conflict of interest: The authors have declared that no conflict of interest exists.

References

1. Busuttil R. Liver ischaemia and reperfusion injury. Br J Surg. 2007;94(7):787-788. doi:10.1002/bjs.5921
2. Adelmann D, Roll GR, Kothari R, et al. The Impact of Deceased Donor Liver Extraction Time on Early Allograft Function in Adult Liver Transplant Recipients. Transplantation. 2018;102(11):e466-e471. doi:10.1097/TP.0000000000002380

3. Maheshwari A, Maley W, Li Z, Thuluvath PJ. Biliary complications and outcomes of liver transplantation from donors after cardiac death. Liver Transpl. 2007;13(12):1645-1653. doi:10.1002/lt.21212
4. Quesnelle KM, Bystrom PV, Toledo-Pereyra LH. Molecular responses to ischemia and reperfusion in the liver. Arch Toxicol. 2015;89(5):651-657. doi:10.1007/s00204-014-1437-x
5. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol. 2011;26 Suppl 1:173-179. doi:10.1111/j.1440-1746.2010.06592.x
6. Hoek JB, Pastorino JG. Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol. 2002;27(1):63-68. doi:10.1016/s0741-8329(02)00215-x
7. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Piña E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res. 2008;147(1):153-159. doi:10.1016/j.jss.2007.06.015
8. Han Z, Li Y, Yang B, et al. Agmatine Attenuates Liver Ischemia Reperfusion Injury by Activating Wnt/β-catenin Signaling in Mice. Transplantation. 2020;104(9):1906-1916. doi:10.1097/TP.0000000000003161
9. Tan R, Tian H, Yang B, et al. Autophagy and Akt in the protective effect of erythropoietin helix B surface peptide against hepatic ischaemia/reperfusion injury in mice. Sci Rep. 2018;8(1):14703. Published 2018 Oct 2. doi:10.1038/s41598-018-33028-3
10. Rao J, Cheng F, Zhou H, et al. Nogo-B is a key mediator of hepatic ischemia and reperfusion injury. Redox Biol. 2020;37:101745. doi:10.1016/j.redox.2020.101745
11. Maqsood M, Kang M, Wu X, Chen J, Teng L, Qiu L. Adult mesenchymal stem cells and their exosomes: Sources, characteristics, and application in regenerative medicine. Life Sci. 2020;256:118002. doi:10.1016/j.lfs.2020.118002
12. Xiao K, He W, Guan W, et al. Mesenchymal stem cells reverse EMT process through blocking the activation of NF-κB and Hedgehog pathways in LPS-induced acute lung injury. Cell Death Dis. 2020;11(10):863. Published 2020 Oct 15. doi:10.1038/s41419-020-03034-3
13. Wang Y. Mesenchymal stem cells (MSC) delays the occurrence of graft-versus-host disease(GVHD) in the inhibition of hematopoietic stem cells in major histocompatibility complex semi-consistent mice by regulating the expression of IFN-γ/IL-6. Bioengineered. 2021;12(1):4500-4507. doi:10.1080/21655979.2021.1955549
14. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008;8(9):726-736. doi:10.1038/nri2395
15. Kim JY, Park S, Lee HJ, Lew H, Kim GJ. Functionally enhanced placenta-derived mesenchymal stem cells inhibit adipogenesis in orbital fibroblasts with Graves' ophthalmopathy. Stem Cell Res Ther. 2020;11(1):469. Published 2020 Nov 5. doi:10.1186/s13287-020-01982-3
16. Na J, Song J, Kim HH, et al. Human placenta-derived mesenchymal stem cells trigger repair system in TAA-injured rat model via antioxidant effect. Aging (Albany NY). 2020;13(1):61-76. doi:10.18632/aging.202348

17. Aghayan HR, Payab M, Mohamadi-Jahani F, Aghayan SS, Larijani B, Arjmand B. GMP-Compliant Production of Human Placenta-Derived Mesenchymal Stem Cells. Methods Mol Biol. 2021;2286:213-225. doi:10.1007/7651_2020_282
18. Wang J, Sun M, Liu W, Li Y, Li M. Stem Cell-Based Therapies for Liver Diseases: An Overview and Update. Tissue Eng Regen Med. 2019;16(2):107-118. Published 2019 Feb 21. doi:10.1007/s13770-019-00178-y
19. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? [published correction appears in Trends Biochem Sci. 2016 Mar;41(3):287] [published correction appears in Trends Biochem Sci. 2016 Mar;41(3):287]. Trends Biochem Sci. 2016;41(3):211-218. doi:10.1016/j.tibs.2015.12.001
20. Balzeau J, Menezes MR, Cao S, Hagan JP. The LIN28/let-7 Pathway in Cancer. Front Genet. 2017;8:31. Published 2017 Mar 28. doi:10.3389/fgene.2017.00031
21. Ali A, Bouma GJ, Anthony RV, Winger QA. The Role of LIN28-let-7-ARID3B Pathway in Placental Development. Int J Mol Sci. 2020;21(10):3637. Published 2020 May 21. doi:10.3390/ijms21103637
22. Thornton JE, Gregory RI. How does Lin28 let-7 control development and disease?. Trends Cell Biol. 2012;22(9):474-482. doi:10.1016/j.tcb.2012.06.001
23. Mayr F, Heinemann U. Mechanisms of Lin28-mediated miRNA and mRNA regulation–a structural and functional perspective. Int J Mol Sci. 2013;14(8):16532-16553. Published 2013 Aug 9. doi:10.3390/ijms140816532
24. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004;30(2):193-204. doi:10.1016/j.ctrv.2003.07.007
25. Heras-Sandoval D, Pérez-Rojas JM, Hernández-Damián J, Pedraza-Chaverri J. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell Signal. 2014;26(12):2694-2701. doi:10.1016/j.cellsig.2014.08.019
26. Xie Y, Shi X, Sheng K, et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). Mol Med Rep. 2019;19(2):783-791. doi:10.3892/mmr.2018.9713
27. Li Y, Ma D, Wang Z, Yang J. MicroRNA-155 Deficiency in Kupffer Cells Ameliorates Liver Ischemia-Reperfusion Injury in Mice. Transplantation. 2017;101(7):1600-1608. doi:10.1097/TP.0000000000001765
28. Li Y, Ma D, Wang Z, Yang J. MicroRNA-155 Deficiency in Kupffer Cells Ameliorates Liver Ischemia-Reperfusion Injury in Mice. Transplantation. 2017;101(7):1600-1608. doi:10.1097/TP.0000000000001765
29. Zhai Y, Petrowsky H, Hong JC, Busuttil RW, Kupiec-Weglinski JW. Ischaemia-reperfusion injury in liver transplantation–from bench to bedside. Nat Rev Gastroenterol Hepatol. 2013;10(2):79-89. doi:10.1038/nrgastro.2012.225
30. Casillas-Ramírez A, Mosbah IB, Ramalho F, Roselló-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. Life Sci. 2006;79(20):1881-

1894. doi:10.1016/j.lfs.2006.06.024

31. Huang J, Xie P, Dong Y, An W. Inhibition of Drp1 SUMOylation by ALR protects the liver from ischemia-reperfusion injury. Cell Death Differ. 2021;28(4):1174-1192. doi:10.1038/s41418-020-00641-7
32. Yi Z, Deng M, Scott MJ, et al. Immune-Responsive Gene 1/Itaconate Activates Nuclear Factor Erythroid 2-Related Factor 2 in Hepatocytes to Protect Against Liver Ischemia-Reperfusion Injury. Hepatology. 2020;72(4):1394-1411. doi:10.1002/hep.31147
33. Saidi RF, Rajeshkumar B, Shariftabrizi A, et al. Human adipose-derived mesenchymal stem cells attenuate liver ischemia-reperfusion injury and promote liver regeneration. Surgery. 2014;156(5):1225-1231. doi:10.1016/j.surg.2014.05.008
34. Pan GZ, Yang Y, Zhang J, et al. Bone marrow mesenchymal stem cells ameliorate hepatic ischemia/reperfusion injuries via inactivation of the MEK/ERK signaling pathway in rats. J Surg Res. 2012;178(2):935-948. doi:10.1016/j.jss.2012.04.070
35. Sun CK, Chang CL, Lin YC, et al. Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats. Crit Care Med. 2012;40(4):1279-1290. doi:10.1097/CCM.0b013e31823dae23
36. Zheng J, Chen L, Lu T, et al. MSCs ameliorate hepatocellular apoptosis mediated by PINK1-dependent mitophagy in liver ischemia/reperfusion injury through AMPKα activation. Cell Death Dis. 2020;11(4):256. Published 2020 Apr 20. doi:10.1038/s41419-020-2424-1
37. Zare MA, Zare A, Azarpira N, Pakbaz S. The protective effect of bone marrow-derived mesenchymal stem cells in liver ischemia/reperfusion injury via down-regulation of miR-370. Iran J Basic Med Sci. 2019;22(6):683-689. doi:10.22038/ijbms.2019.32670.7812
38. Zheng J, Lu T, Zhou C, et al. Extracellular Vesicles Derived from Human Umbilical Cord Mesenchymal Stem Cells Protect Liver Ischemia/Reperfusion Injury by Reducing CD154 Expression on CD4+ T Cells via CCT2. Adv Sci (Weinh). 2020;7(18):1903746. Published 2020 Aug 20. doi:10.1002/advs.201903746
39. Li C, Jin Y, Wei S, et al. Hippo Signaling Controls NLR Family Pyrin Domain Containing 3 Activation and Governs Immunoregulation of Mesenchymal Stem Cells in Mouse Liver Injury. Hepatology. 2019;70(5):1714-1731. doi:10.1002/hep.30700
40. Vaupel P, Multhoff G. Revisiting the Warburg effect: historical dogma versus current understanding. J Physiol. 2021;599(6):1745-1757. doi:10.1113/JP278810
41. Kierans SJ, Taylor CT. Regulation of glycolysis by the hypoxia-inducible factor (HIF): implications for cellular physiology. J Physiol. 2021;599(1):23-37. doi:10.1113/JP280572
42. Varela-Rodríguez H, Abella-Quintana DG, Espinal-Centeno A, et al. Functional Characterization of the Lin28/let-7 Circuit During Forelimb Regeneration in Ambystoma mexicanum and Its Influence on Metabolic Reprogramming. Front Cell Dev Biol. 2020;8:562940. Published 2020 Nov 19. doi:10.3389/fcell.2020.562940

43. Tan FE, Sathe S, Wheeler EC, Yeo GW. Non-microRNA binding competitively inhibits LIN28 regulation. Cell Rep. 2021;36(6):109517. doi:10.1016/j.celrep.2021.109517
44. Xu K, Yin N, Peng M, et al. Glycolysis fuels phosphoinositide 3-kinase signaling to bolster T cell immunity. Science. 2021;371(6527):405-410. doi:10.1126/science.abb2683
45. Xu K, Yin N, Peng M, et al. Glycolytic ATP fuels phosphoinositide 3-kinase signaling to support effector T helper 17 cell responses. Immunity. 2021;54(5):976-987.e7. doi:10.1016/j.immuni.2021.04.008
46. Magbanua MJM, Swigart LB, Wu HT, et al. Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. Ann Oncol. 2021;32(2):229-239. doi:10.1016/j.annonc.2020.11.007
47. Saat TC, van den Engel S, Bijman-Lachger W, et al. Fate and Effect of Intravenously Infused Mesenchymal Stem Cells in a Mouse Model of Hepatic Ischemia Reperfusion Injury and Resection. Stem Cells Int. 2016;2016:5761487. doi:10.1155/2016/5761487
48. Kanazawa H, Fujimoto Y, Teratani T, et al. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. PLoS One. 2011;6(4):e19195. Published 2011 Apr 29. doi:10.1371/journal.pone.0019195
49. Zhu H, Shyh-Chang N, Segrè AV, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell. 2011;147(1):81-94. doi:10.1016/j.cell.2011.08.033Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name***Year**, *Volume*, page range.

Figures

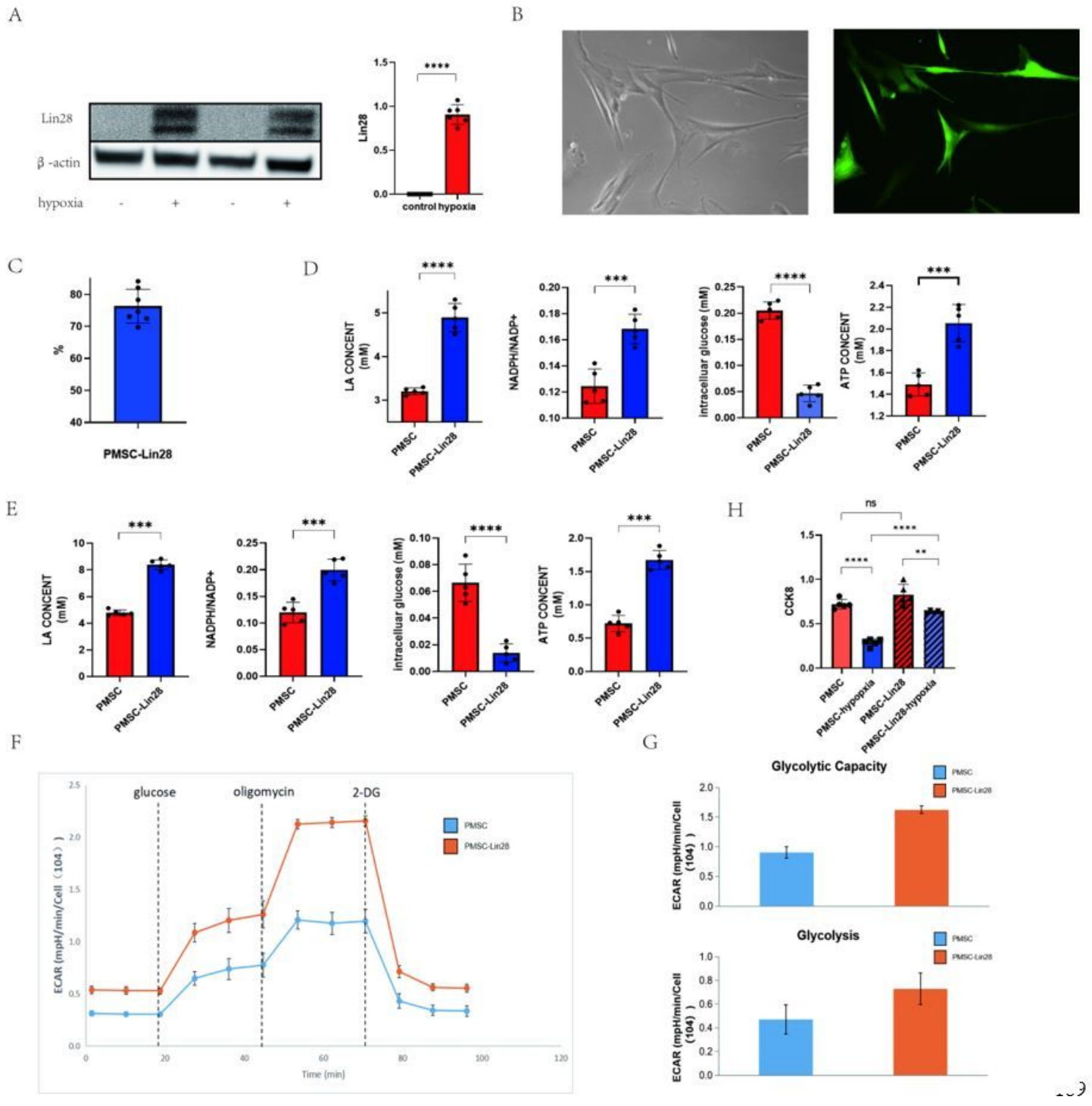

Figure 1

Lin28 enhance the glycolytic ability of PMSCs A.The protein levels of Lin28 in PMSCs after hypoxia were analyzed using western blots. B.PMSCs were transfected with lentiviral constructs for green fluorescence-tagged Lin28 and observed using fluorescence microscopy. C.The proportion of green fluorescent cells (transfection ratio) was calculated by randomly selecting different fields. D.Lactic acid (LA), intracellular glucose,NADP+/NADPH, and ATP levels were detected under normoxic conditions in PMSCs and PMSCs overexpressing LIN28. E.Lactic acid (LA), intracellular glucose,NADP+/NADPH, and ATP levels were detected under hypoxic conditions in PMSCs and PMSCs overexpressing LIN28. F.Glucose metabolism in

PMSC and PMSCs-Lin28 was measured using CAR, and glucose, oligomycin (ATP synthase inhibitor), and 2-Deoxy-d-glucose (2-dg) were added sequentially. G.Basal glucose metabolism levels and the glycolytic ability of cells were calculated using ECAR, and significant differences were observed. ***p<0.001; ****p<0.0001

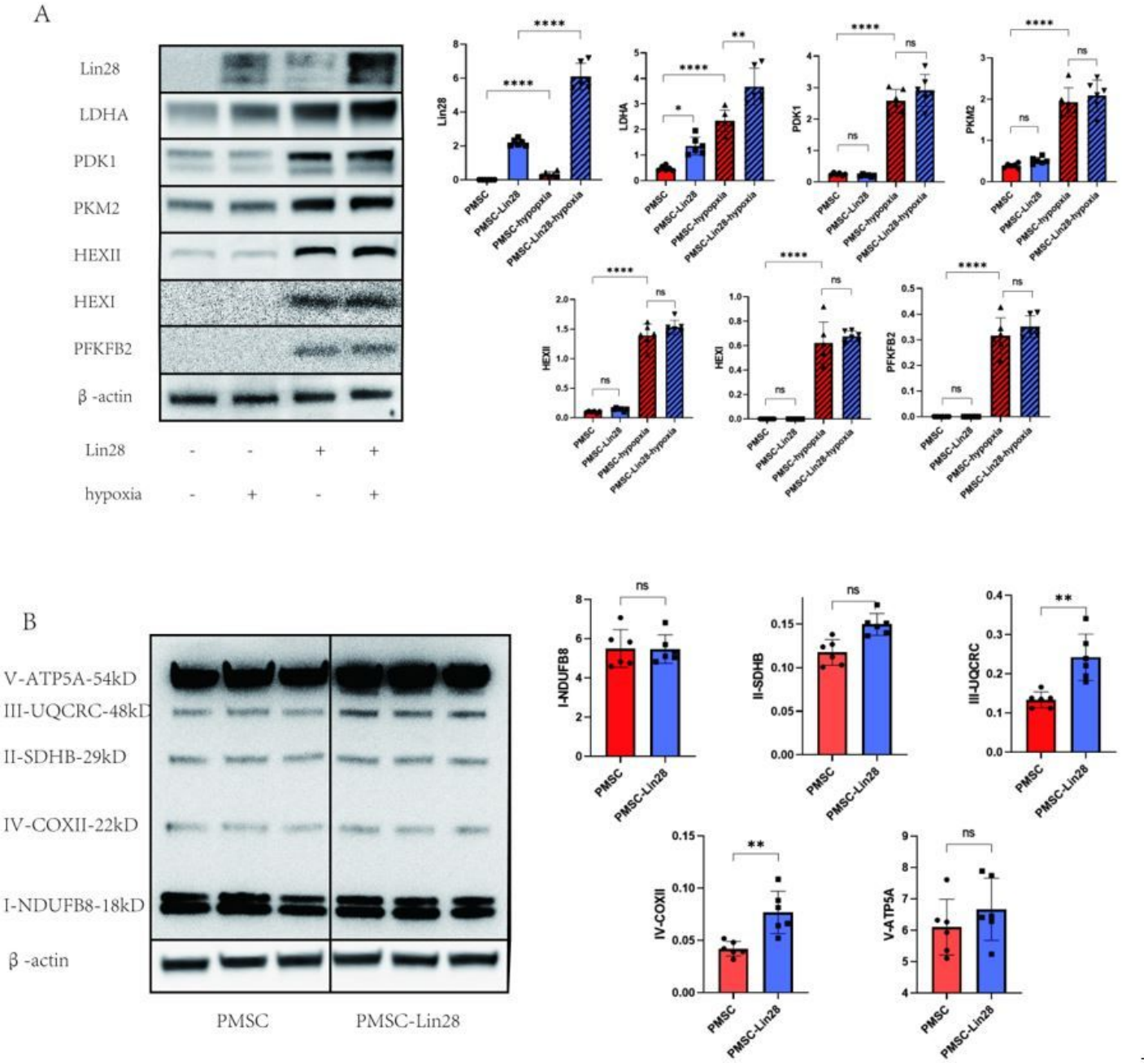

Figure 2

Expression of glucose metabolism-related proteins in PMSCs and PMSCs-Lin28 under normoxic and hypoxic conditions A. The protein expression of Lin28, lactate dehydrogenase A(LDHA), 3-phosphoinositide dependent kinase-1(PDK1), M2-pyruvate kinase (PKM2), Hexokinase I and II (HEX I and HEX 2), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) analyzed using western blots. B. The expression of proteins involved in oxidative phosphorylation, including NDUFB8 (18 kD),

SDHB (29 kD), UQCRC (48 kD), COXII (22 kD), and ATP5A (54 kD) analyzed using western blots. ns, p>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

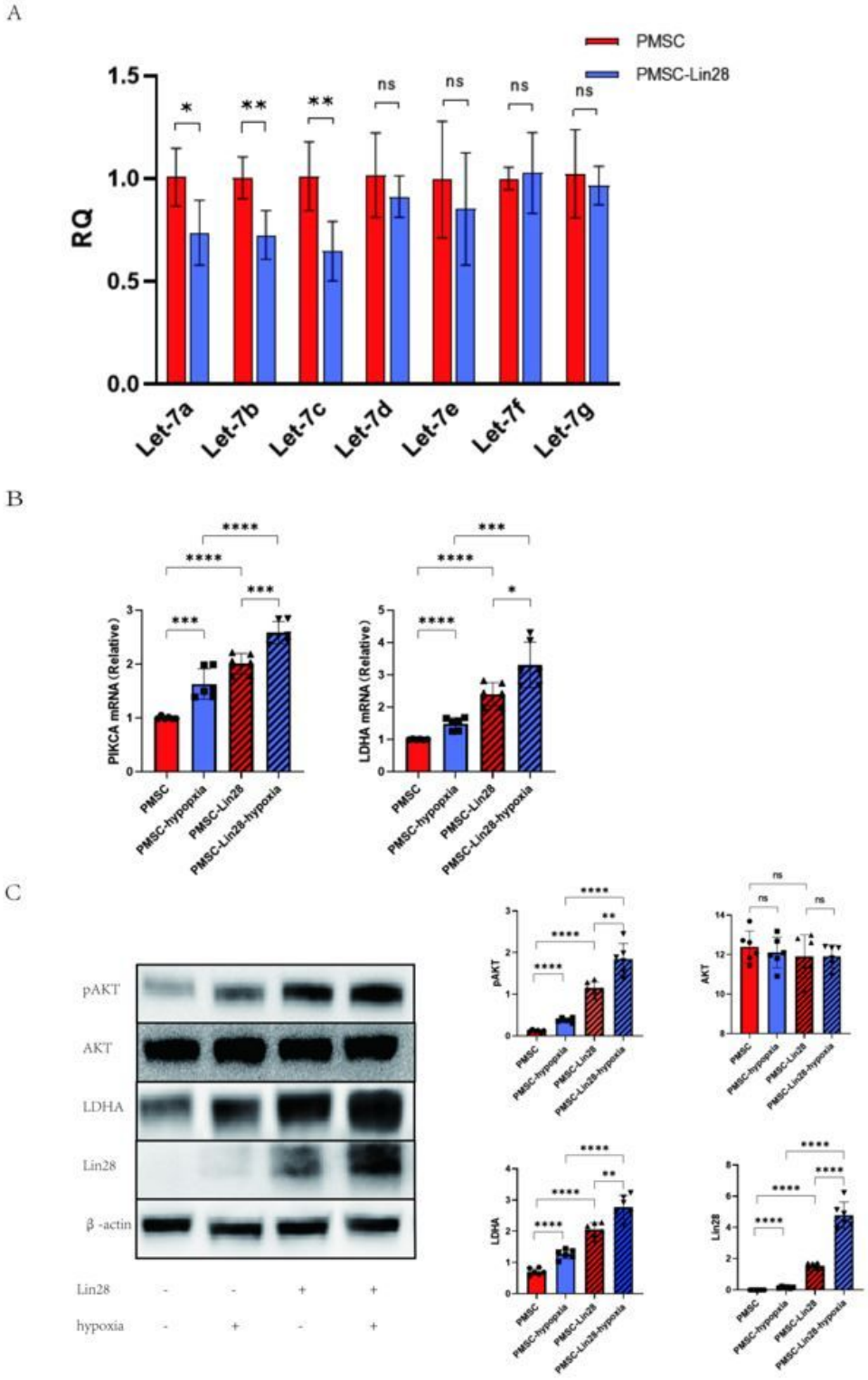

Figure 3

Lin28 regulates LDHA through the Let7-PI3K-Akt pathway A.The microRNA levels of Let-7 family members were measured using real-time polymerase chain reaction . B.The mRNA levels of PI3K and LDHA were measured using RT-PCR. C.The protein expression of pAKT(ser 473), AKT, LDHA, and Lin28 in

PMSCs and PMSCs-Lin28 under normoxic and hypoxic conditions was analyzed using western blotting. ns, P>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

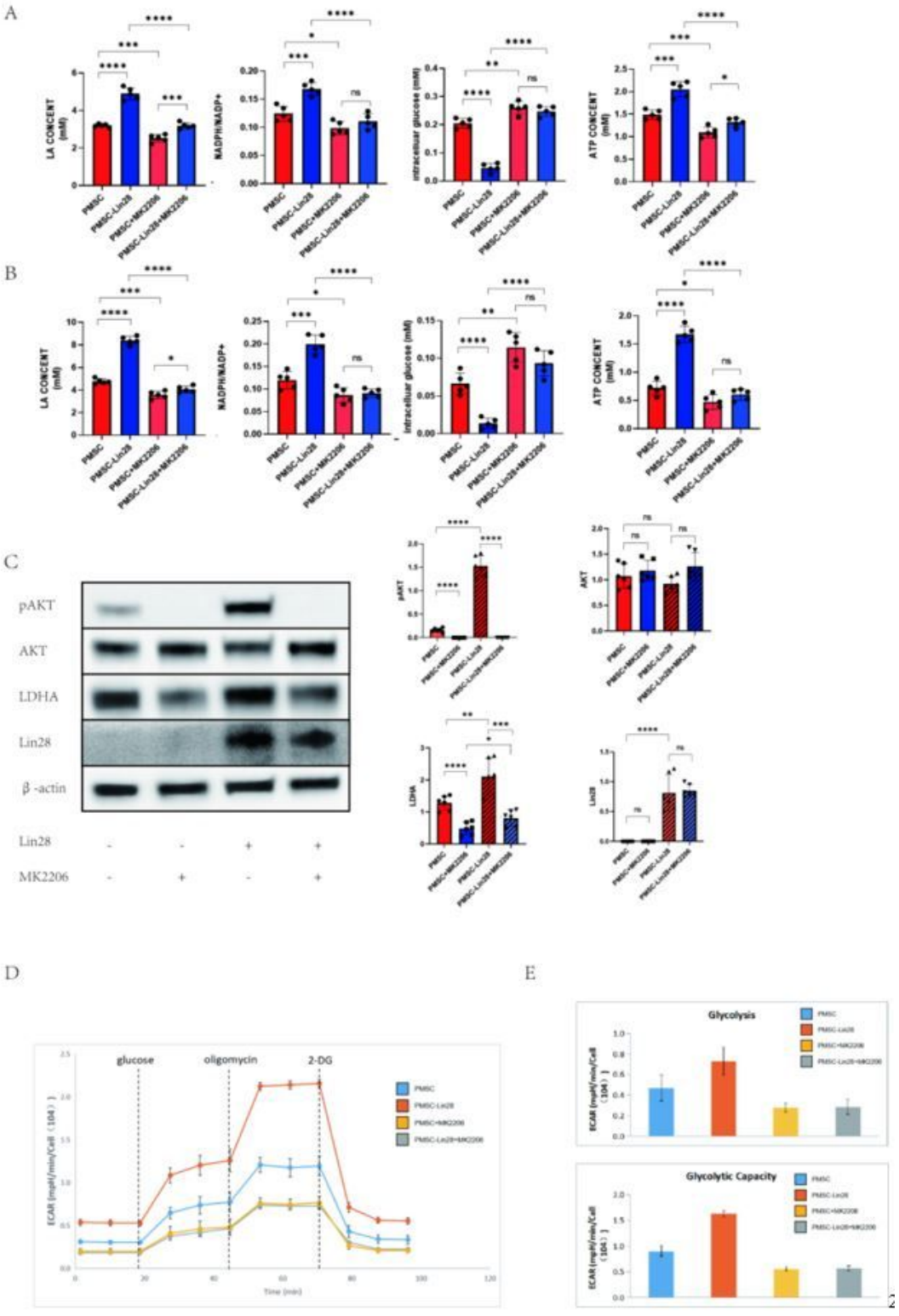

Figure 4

Effect of Lin28 on glucose metabolism in PMSCs after treatment with MK2206, an inhibitor of the PI3K-Akt pathway A.Lactic acid (LA), intracellular glucose,NADP+/NADPH, and ATP levels detected in PMSCs and PMSCs-LIN28 treated with MK2206 under normoxic conditions B.Lactic acid (LA), intracellular

glucose,NADP+/NADPH, and ATP levels detected in PMSCs and PMSCs-LIN28 treated with MK2206 under hypoxic conditions C.The protein expression of pAKT(ser 473), AKT, LDHA, and Lin28 was examined in PMSCs and PMSCs-Lin28 treated with MK2206 under normoxic and hypoxic conditions using western blot. D.Glucose metabolism was examined in PMSCs and PMSCs-Lin28 treated with MK2206 using CAR, and glucose, oligomycin (ATP synthase inhibitor), and 2-Deoxy-d-glucose (2-dg) were added sequentially. E.Basal glucose metabolism levels and the glycolysis ability of PMSCs and PMSCs-Lin28 were calculated using ECAR, and significant differences were observed. ns, $p>0.05$; *$p<0.01$; **$p<0.01$;***$p<0.001$; ****$p<0.0001$

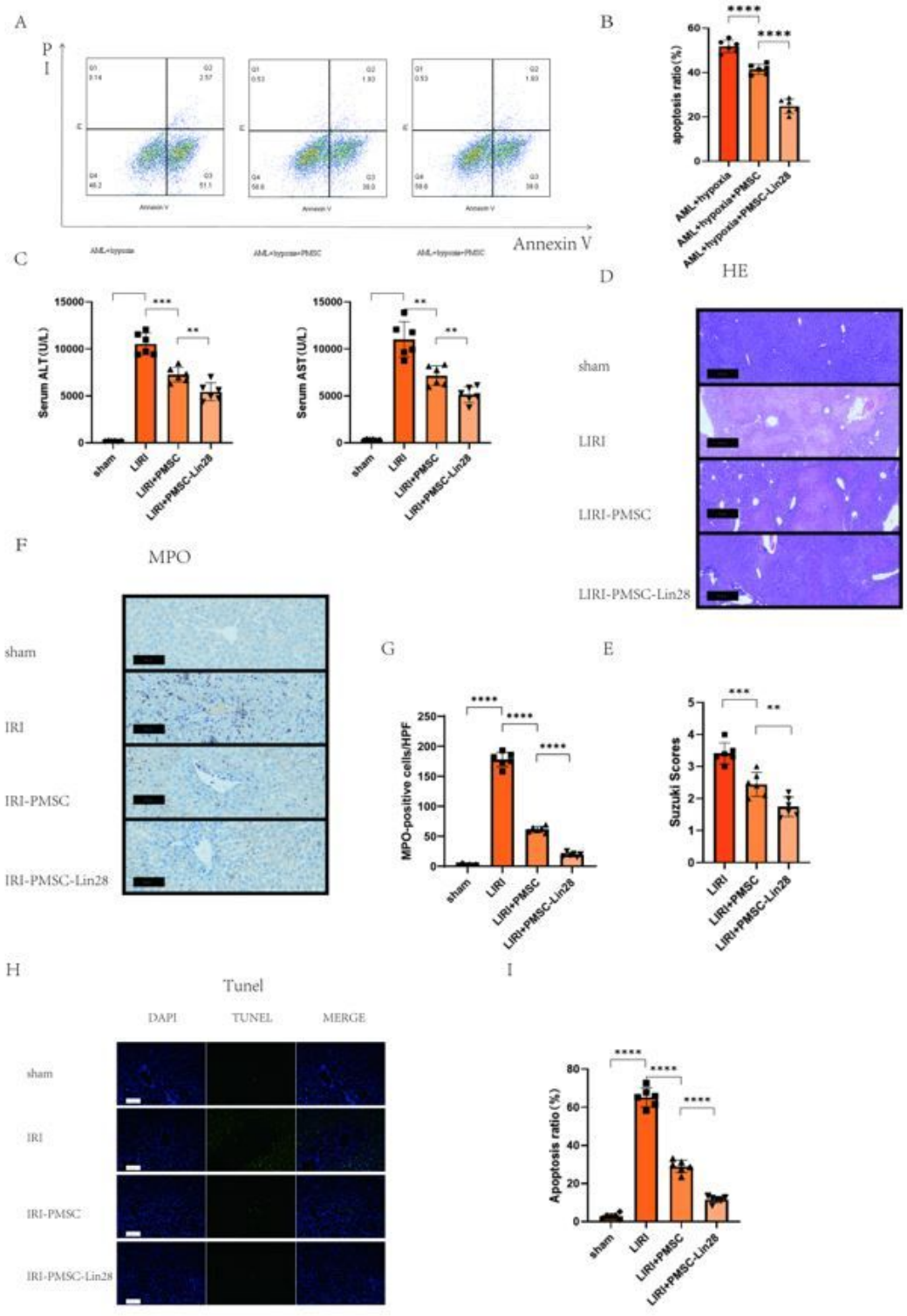

Figure 5

Lin28 enhanced the protective effect of PMSC against LIRI in vitro and in vivo. A,B. Apoptosis rates examined with Annexin V/PI staining. C. Serum ALT and AST levels were examined in mice after 6 h of reperfusion. D. Hematoxylin and eosin-stained liver tissues (×100). Scale bar = 100 μm. E. Extent of liver injury graded based on Suzuki's scores. F. MPO staining of liver sections from mice (×200). Scale bar = 50 μm. G. Quantification of MPO-positive cells. H. TUNEL assay of the liver sections from mice(×200).

Scale bar = 100 μm. I. Quantification of TUNEL-positive cells (×200). LIRI, liver ischemia –reperfusion injury;PI, propidium iodide; MPO, myeloperoxidase All numerical data were obtained from n = 6 mice per group. **p<0.01; ***p<0.001; ****p<0.0001

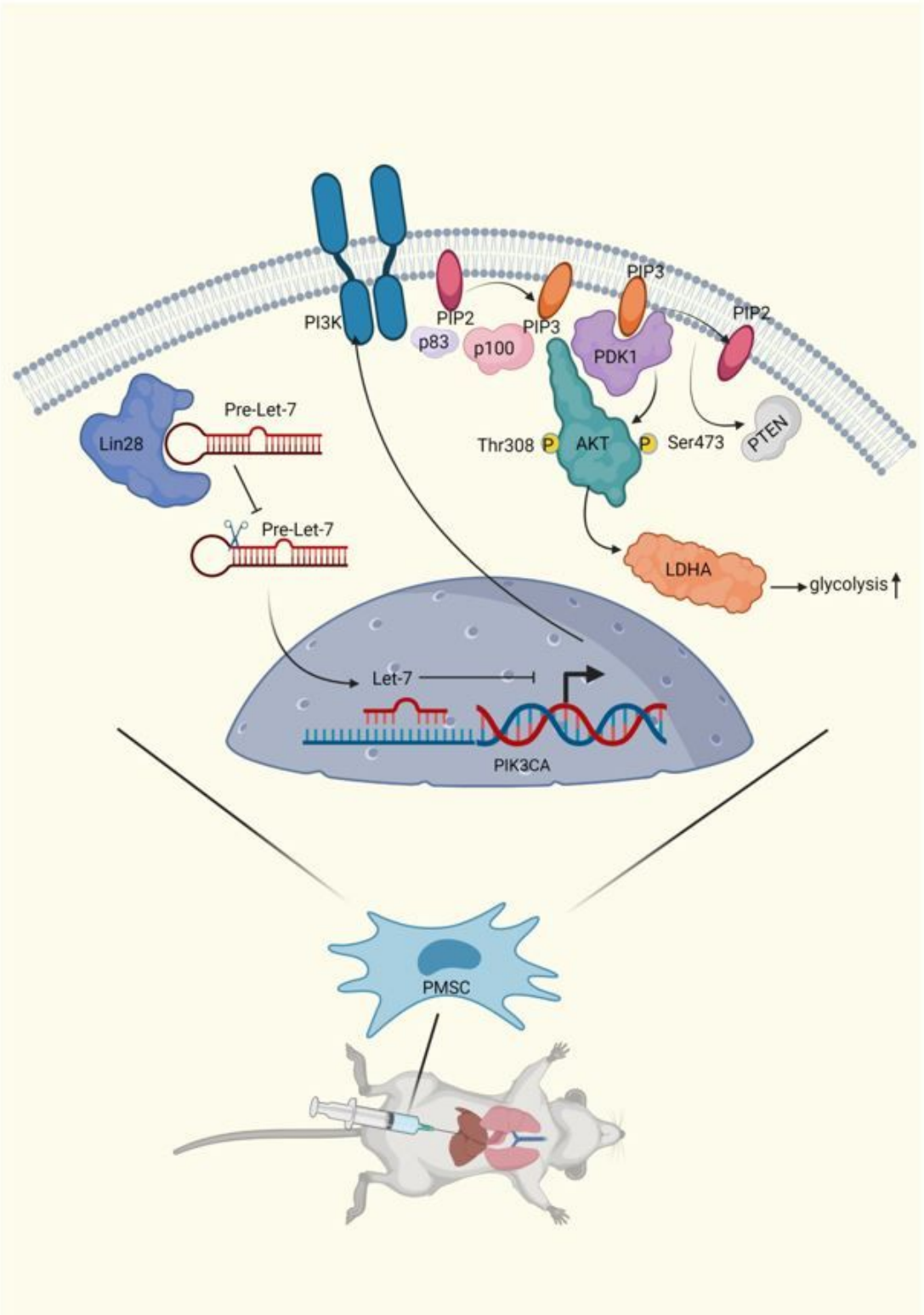

Figure 6

Lin28 increase the protection offered by PMSC against LIRI.