Neurourology and Urodynamics

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BASIC SCIENCE ARTICLE OPEN ACCESS

Mitochondrial Fusion Protein 2-Modified Bone Marrow Mesenchymal Stem Cells Improved Hyperglycemia-Induced Schwann Cell Injury via Regulating Mitochondria-Associated Endoplasmic Reticulum Membranes

Housheng Fu[1,2] | Zhewen Ou[1,2] | Fei Wang[1,2] | Weifu Wang[1,2] | Zhongyao Wang[1,2]

[1]Department of Urology, Hainan Hospital Affiliated to Hainan Medical University, Haikou City, Hainan Province, China | [2]Kidney Disease Center, Hainan General Hospital, Haikou City, Hainan Province, China

Correspondence: Zhongyao Wang (wangzhongyao2023@163.com)

Received: 17 June 2024 | **Revised:** 1 March 2025 | **Accepted:** 15 April 2025

Funding: The present study was supported by the Hainan Province health industry research project (No. 22A200188).

Keywords: diabetic bladder dysfunction | mitochondria-associated endoplasmic reticulum membranes | mitochondrial fusion protein 2

ABSTRACT

Objective: High glucose damages rat Schwann cells (SCs), which is closely related to the dysfunction of mitochondria-associated endoplasmic reticulum membranes (MAMs). Therefore, the present study aimed to investigate the protective effects and mechanisms of modified bone marrow mesenchymal stem cells (BMSCs) and mitochondrial fusion protein 2 (Mfn2) modified BMSCs against SCs injury.

Methods: The Mfn2-modified BMSCs were constructed after culturing with neural-induced differentiation solution. MAP-2 (microtubule-associated protein-2, neuron marker) and GFAP (glial fibrillary acidic protein, astrocytes marker) immunofluorescence staining was used to observe changes in the differentiation potential of neural-like BMSCs. SCs (RSC96) cells cultured under high glucose conditions were cocultured with Mfn2-modified BMSCs. Changes in functional protein expression of MAMs were detected by Western Blot. Transmission electron microscopy (TEM) was used to observe the microscopic morphology of MAMs, mitochondria and endoplasmic reticulum.

Results: The expression level of Mfn2 was significantly increased in BMSCs transfected with Mfn2. The fluorescence densities of MAP-2 and GFAP were significantly upregulated in Mfn2-BMSCs after induction by neural inducible differentiation solution. When RSC96 was incubated with high glucose and Mfn2-modified/non-modified BMSCs, the expression level of Mfn2 in RSC96 was significantly increased, while PERK, IP3R and Drp1 expressions were significantly reduced. And the Mfn2-modified BMSCs showed more significant effects comparing to Mfn2-non-modified BMSCs. The TEM showed the structural integrity of MAMs, clear structure of mitochondrial cristae and obvious and structurally intact extension of endoplasmic reticulum in Mfn2-BMSC group.

Conclusions: Mfn2 transfection promoted neural-like cell differentiation in BMSCs. Mfn2-modified BMSCs modulated the structural and functional homeostasis of MAMs by regulating the expression levels of MAMs functional proteins.

Housheng Fu and Zhewen Ou contributed equally to this study.

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1 | Introduction

Diabetes mellitus and its complications represent an extremely high economic and social burden on human health and social development. According to the Diabetes Atlas released by the International Diabetes Federation (IDF) in 2021, about 5.37 billion people aged 20–79 years worldwide have diabetes [1]. Diabetic bladder dysfunction (DBD) is a common complication of the lower urinary tract in patients with diabetes mellitus, with a prevalence of more than 80% in diabetic patients [2, 3]. It manifests as a dual impairment of bladder storage and voiding function, with clinical manifestations including urinary frequency, dysuria, bladder sensory disturbance and urinary retention [4]. The early symptoms of DBD are not obvious and are easily overlooked, and patients often have bladder dysfunction during diagnosis [3]. Studies have shown that the impact of DBD on patients' quality of life is no less than that of chronic diseases such as AIDS [3, 5–7]. However, due to the lack of unified diagnostic criteria, complex and diverse pathological mechanisms, and the absence of large-scale, prospective clinical trials to explore its therapeutic effects, research on DBD have been relatively slow.

Current studies on the pathogenesis of DBD mainly consider different aspects such as neuropathy, forced urethral muscle injury and abnormal urethral function [6]. There is an association between peripheral neuropathy and bladder dysfunction due to diabetes mellitus [8]. Bladder sensory dysfunction induced by type 2 diabetes mellitus is characterized by slowed bladder afferent nerve conduction and increased bladder capacity [9]. Schwann cells (SCs) are a type of myelinating cell found in the peripheral nervous system, and controlling SCs damage in peripheral nerves is one of the effective ways to alleviate DBD [10]. Mitochondria-associated endoplasmic reticulum membranes (MAMs) are the physical and biochemical connections that exist between mitochondria and the endoplasmic reticulum. MAMs are involved in and regulate a wide range of cellular life processes such as phospholipid metabolism, calcium homeostasis, mitochondrial function, endoplasmic reticulum stress, and many other processes [11, 12]. Previous studies by our group have confirmed that the structure and function of MAMs are disrupted in high glucose-induced nevus cell injury [13]. It is suggested that Mfn2 is involved in regulating the distance between the endoplasmic reticulum and mitochondria and in regulating the structure and function of MAMs [14].

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stromal cells with immunomodulatory properties and differentiation potential, playing a pivotal role in cellular repair and novel disease treatments [15]. BMSCs have been proven effective in ameliorating bladder dysfunctions caused by diabetes [16] and various factors [17–19]. Genetically modified or engineered BMSCs exhibit enhanced therapeutic potential for bladder repair [20, 21]. Transplantation of integrin-linked kinase-modified BMSCs significantly enhances bladder functional recovery and repair in diabetic cystopathy rodent model through activating AKT/GSK-3β pathway, promoting the process of angiogenesis and protecting cells from high glucose-associated apoptosis [21, 22]. A recent study found that when co-culturing BMSCs with neurons, it transfers functional mitochondria to injured neurons via gap junctions to improve bioenergetics, reduce apoptosis, and promote cell survival [23]. Co-culturing BMSCs with other cells may mitigate cellular damage and enhance recovery through multiple mechanisms such as intercellular communication, paracrine signaling, mitochondrial transfer, epigenetic regulation, and immunoregulation [23–25]. Therefore, we intended to investigate the effect of Mfn2-modified BMSCs on MAMs in high glucose-induced SCs injury in this study. The overexpression of Mfn2 in BMSCs might increase the expression of Mfn2 in co-cultured SCs, working together to exert protective effects. We also aimed to investigate the mechanism of Mfn2-modified BMSCs in mitigating injured SCs to further indicate potential strategy for DBD treatment.

2 | Materials and Methods

2.1 | Reagents

Rat BMSCs, RSC96 (Wuhan Pricella Biotechnology Co. Ltd., China). PBS buffer, APS (Beyotime, China). APS、RIPA、PMSF、TEMED (Beyotime, China). 30% Acr/Bic、Tris-Base、TBS buffer (Biosharp, China). SDS, Glycine, Tween 20, skimmed milk (BioFroxx, China). BCA protein assay kit, BSA protein standard, 5xSDS-PAGE buffer, ECL luminescent solution AB (NCM Biotech, China). Prestained Protein Marker II (G2058-250UL) (Servicebio, China). Western Antibody GAPDH-Loading Control (bsm-33033M), Goat Anti-Rabbit IgG H&L/HRP (bs-0295G-HRP), Goat Anti-Mouse IgG H&L/HRP (bs-0296G-HRP) (Bioss, China). Anti-DRP1 antibody [EPR19274] (ab184247), Anti-IP3 receptor antibody [EPR4537] (ab108517), Anti-PERK antibody [EPR19876-294] (ab229912), Anti-Mitofusin 2 antibody [NIAR164] (ab124773) (Bioss, China).

2.2 | Experimental Apparatus

The experimental apparatuses are shown in Table 1.

2.3 | Cell Culture and Grouping

BMSCs and RSC96 cells were cultured under standard conditions using DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 50 units/mL streptomycin. The cultures were maintained at 37°C in a humidified incubator with 95% humidity and 5% CO_2.

Mfn2-modified BMSCs were constructed by plasmid transfection. The pLV-CMV-eGFP-Neo-Ad-Mfn2 was constructed by inserting the target gene sequence of Ad-Mfn2 into the transfer plasmid pLV-CMV-eGFP-Neo. In the design and production process of lentiviral vectors, the following operations were adopted: co-transfection of 293 T cells with packaging plasmid psPAX2, envelope plasmid pMD2.G, and constructed transfer plasmid pLV-CMV-eGFP-Neo-Ad-Mfn2. The specific steps are as follows: Mix the three plasmids in a 4:3:1 ratio (psPAX2: pMD2.G: Plv-CMV-eGFP-Neo-Ad-Mfn2), use Lipofectamine 3000 transfection reagent, and transfect the plasmid mixture

TABLE 1 | The experimental apparatus used in the experiment.

Experimental apparatus	Equipment model and manufacturer
Optical microscope	Shanghai Optical Instrument No.1 Factory, China
Ultra-low temperature refrigerator	Haier, China
Real-time fluorescence quantitative PCR instrument	CFX96 Touch, BIO-RAD, 1855195
Western blotting system	Model: Criterion electrophoresis tank, Trans-blot transfer tank (Bio-Rad, USA).
EVOS M5000 fluorescence microscope	Themo Fisher, USA
Orbital shaker TS-100	Kylin-Bell, China
JP-K6000 chemiluminescence analyzer	Shanghai Jiapeng, China
10T Transfection kit	Nanjing Jiangyuan Biotechnology Co. Ltd., China

into 293T cells according to the manufacturer's instructions. After 48 h of transfection, collect the cell culture supernatant containing lentivirus particles, filter the supernatant through a 0.22 μm filter membrane to obtain a pure lentivirus solution, and then use a PEG-it virus concentration kit to concentrate the virus solution and measure the virus titer. After obtaining the lentivirus, it was divided into negative control group (BMSCs group) and Ad-Mfn2 overexpression vector transfection group (Mfn2-BMSCs group). The viral stock solution was diluted and added to the well plates, which were incubated at 37°C with 5% CO_2. After 12 h, the medium was discarded and added to the culture medium for 3 days. The transfection efficiency was measured. The prepared BMSCs and Mfn2-BMSCs were cultured with neurogenic induction differentiation fluid for 7 days, and the induction fluid was replaced every 2 days. Neurogenic induction differentiation medium contains 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 2% DMSO, 200 μM buhydroxyanisole, 25 mM KCl, 2 mM sodium valproate, 10 mM trichothroxine, 1 mM hydrocortisone.

For the high glucose model, RSC96 cells were cultured in DMEM containing 150 mmol/L glucose under the same conditions of temperature, humidity, and CO_2 concentration. Then, Mfn2-modified BMSCs and RSC96 co-culture system was established using a 6-well Transwell insert system. RSC96 cells were seeded in the lower chambers of the Transwell plates, and BMSCs were seeded in the upper chambers of the Transwell plates. In the BMSCs + HG-SC group, BMSCs were co-cultured with RSC96 cells in DMEM containing 150 mmol/L glucose in a 1:1 ratio for 24 h, allowing for intercellular communication and interaction. In this group, the BMSCs were not genetically modified and served as the control. For the Mfn2-BMSCs + HG-SC group, Mfn2-modified BMSCs were co-cultured with RSC96 cells cultured in 150 mmol/L glucose. These Mfn2-modified BMSCs were genetically engineered to overexpress Mfn2, and the co-culture was maintained in the same 1:1 ratio. Both cultures were maintained at 37°C in a humidified incubator with 95% humidity and 5% CO_2.

2.4 | Western blot Detection of Protein Expression

The total protein was extracted with RIPA lysate and the protein quantification was carried out by the BCA method. Proteins were separated by SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The membrane was then immersed in 5% skim milk for 2 h at room temperature. Then incubated with the following primary antibody overnight at 4°C: Anti-Mfn2; Anti-PERK; Anti-IP3R; Anti-Drp1; GAPDH. After three-time-wash with TBST, incubation was performed by adding secondary antibodies Goat Anti-Rabbit IgG H&L/HRP or Goat Anti-Mouse IgG H&L/HRP. The membranes were developed using a chemiluminescence imaging system and analyzed in grey scale using ImageJ software (Table 2).

TABLE 2 | Antibody were diluted as follows.

Antibodies	Dilution (application)
Anti-Mfn2	1:500 (WB)
Anti-PERK	1:500 (WB)
Anti-IP3R	1:4000 (WB)
Anti-Drp1	1:1000 (WB)
GAPDH	1:10000 (WB)
Goat Anti-Rabbit IgG H&L(HRP)	1:20000 (WB)
Goat Anti-Mouse IgG H&L(HRP)	1:20000 (WB)

2.5 | Immunofluorescence Single Staining to Observe the Differentiation Ability of BMSCs

BMSCs or Mfn2-BMSCs were inoculated at 2×10^5 cells per well and cultured at 37°C, 5% CO_2 for 24 h. Supernatants were discarded and washed with PBS, fixed with 1 mL of 4% paraformaldehyde, permeabilized with 1 mL of 0.25% Triton-X100 and blocked with 1 mL of goat serum. The cells were incubated with the following primary antibody overnight at 4°C: MAP2 Antibody [EPR19691] (Abcam, ab183830, 1:500); GFAP Antibody (Abcam, ab7260, 1:5000). After three-time-wash with TBST, incubation was performed by adding Goat anti-Rabbit IgG(H + L) Cross-Adsorbed Secondary Antibody (Alexa Fluor 568, Invitrogen, A-11011, 1:1000) or Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody (Alexa Fluor 488, Invitrogen, A-11008, 1:1000), followed by DAPI staining, and the cells were photographed under a fluorescence microscope to observe the fluorescent expression of MAP-2 and GFAP.

2.6 | Transmission Electron Microscopy Observation of Cell Ultra Morphology

Firstly, cells were fixed in 3% glutaraldehyde and then re-fixed in 1% osmium tetroxide. Next, dehydrate in acetone and embed in Epon812. After positioning, the semi thin slices were prepared into ultra-thin slices of 70 nm. double staining was performed using uranyl acetate and lead citrate. Randomly read 3 fields of view from each group and observe the ultrastructure of RSC96 cells.

2.7 | Data Analysis

Data were analyzed and plotted using Graphpad Prism 9 (version 9.5.0). All graphs are expressed as mean ± SD, and statistical differences between two groups were analyzed using T-test, and statistical differences between more than two groups were analyzed using one-way test, with $p < 0.05$ considered significantly different ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3 | Results

3.1 | Mfn2 Transfection of BMSCs Promotes Their Neural-Like Cell Differentiation

To verify the efficiency of Mfn2 transfection, we detected the expression level of Mfn2 in BMSCs after transfection by Western-Blot. As shown in Figure 1A,B, the expression of Mfn2 protein was significantly increased after transfection with OE-Mfn2 plasmid ($p < 0.001$). We then induced Mfn2-BMSCs by neural-induced differentiation solution for 7 days and observed the expression of MAP-2 and GFAP by immunofluorescence staining. As can be seen in Figure 1C–F, the expression of MAP-2 ($p < 0.01$) and GFAP ($p < 0.001$) was significantly increased after transfected Mfn2 BMSCs were induced to differentiate into neurons compared to the control group. The above results demonstrated the potential of Mfn2 transfection in enhancing the capability of BMSCs to differentiate into neurons and glial cells.

3.2 | Mfn2 Modification of BMSCs Improves Mams Function in RSC96 Cells

To investigate the effect of Mfn2-BMSCs on the function of MAMs in high glucose-induced RSC96 cells, we examined the proteins expression levels of mitochondrial dynamics, endoplasmic reticulum stress by Western Blot. As shown in Figure 2, Mfn2 protein expression was significantly increased ($p < 0.001$) and PERK, IP3R, and Drp1 protein expression was significantly decreased ($p < 0.01$) in the HG-SC (BMSCs-HG-SC) group after co-culture of BMSCs compared with that in the high glucose-induced group (HG-SC). Mfn2-modified BMSCs co-cultured with HG-SCs (Mfn2-BMSCs + HG-SC) group showed a significant increase in the expression of Mfn2 protein ($p < 0.001$) and a significant decrease in the expression of PERK ($p < 0.01$), IP3R ($p < 0.05$), and Drp1 ($p < 0.01$) proteins compared with the BMSCs + HG-SC group.

3.3 | Mfn2 Modification of BMSCs Improves Mam Structure in RSC96 Cells

To investigate the effect of Mfn2-BMSCs on high glucose-induced RSC96 cells, we observe the microstructure of MAMs by TEM (Figure 3). Compared with HG-SC and BMSCs + HG-SC, the mitochondrial crest structure of Mfn2-BMSCs + HG-SC was clearer, the endoplasmic reticulum expansion was more obvious, and the structure was more complete. The experimental results showed that Mfn2 modification of BMSCs improved the structure of MAMs in RSC96 cells, affecting the morphology and distance of mitochondria and endoplasmic reticulum.

4 | Discussion

In this study, we found that MAP-2 and GFAP fluorescence levels increased significantly after Mfn2-transfected BMSCs were cultured in neural-induced differentiation solution, suggesting that Mfn2 promotes the differentiation of BMSCs into neural-like cells. Furthermore, we found that Mfn2-modified BMSCs improved the protein expression levels of Mfn2, PERK, IP3R, and Drp1 in high glucose-induced RSC96. This suggests that our Mfn2 BMSCs may be involved in the regulation of ER stress, cytosolic calcium conductance and mitochondrial dynamics, which are closely related to the structure and function of MAMs. We also observed the microstructure of RSC96 cells and found that Mfn2 BMSCs improved the number of MAMs, mitochondrial and endoplasmic reticulum morphology under high glucose-induced RSC96 injury.

The therapeutic mechanism of BMSCs in neurogenic bladder can be summarized as migration, homing, differentiation and paracrine effects, which can promote tissue repair and regeneration, and has become a research hotspot for DBD treatment in recent years [26–29]. BMSCs have a continuous self-renewal capacity and the potential to differentiate into neuron-like cells. Meanwhile, BMSCs have the ability to chemotaxis to the site of injury and can migrate to the damaged tissue of the bladder to participate in repair. BMSCs can differentiate into urinary tract epithelium, nerve fibers and bladder smooth muscle cells. They have been shown to have the ability to differentiate directionally into urothelium of endodermal origin and to develop into mature bladder tissue in the appropriate environment [30, 31]. BMSCs can also effectively promote angiogenesis in the transplanted area through paracrine effects, improve bladder tissue ischemia and promote bladder smooth muscle cell regeneration [22]. In addition, BMSCs can restore the immune environment of the bladder and inhibit hypoxia-induced inflammatory and fibrotic pathways in bladder smooth muscle cells [32].

Clinical treatment of diabetic neurogenic bladder is still based on symptomatic management, and the combination of transgenic and cellular engineering technologies has important potential in the treatment of DBD. Zhu et al. [33] revealed that nerve growth factor (NGF) gene transfected BMSCs survived for a long period of time in vivo in a rat model of DBD and promoted stable expression of NGF and improved bladder function. Huang et al. [22] showed that integrin-linked kinase (ILK) gene-modified BMSCs promoted the activation of the AKT/

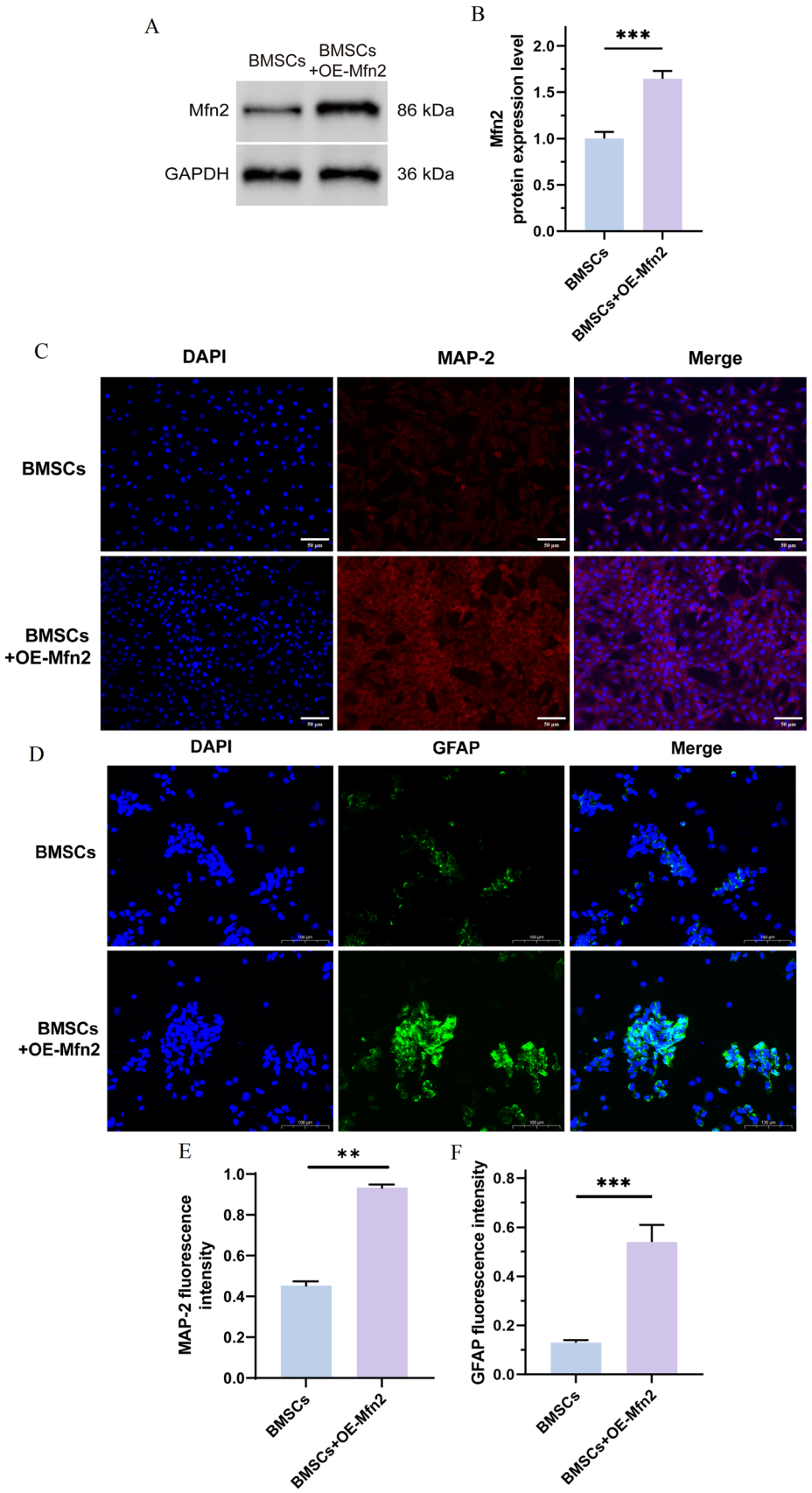


FIGURE 1 | Validation of the transfection efficiency of Mfn2-modified BMSCs and exploration of the pro-neural-like cell differentiation potential of BMSCs. Western blot detection of Mfn2 transfection efficiency (A and B); immunofluorescence staining for MAP-2, GFAP and quantification (C–F), $^{**}p < 0.01$, $^{***}p < 0.001$. $N = 3$.

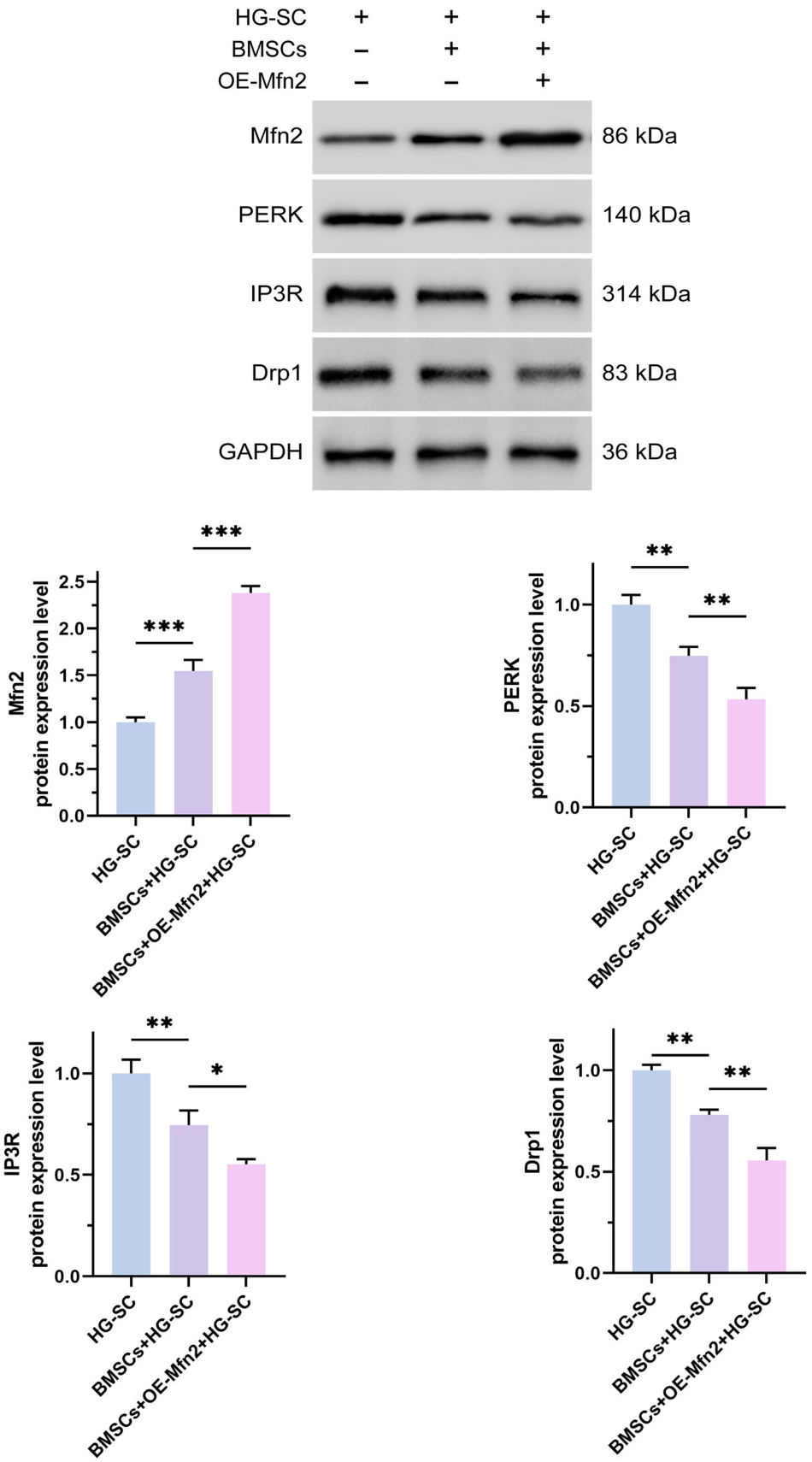
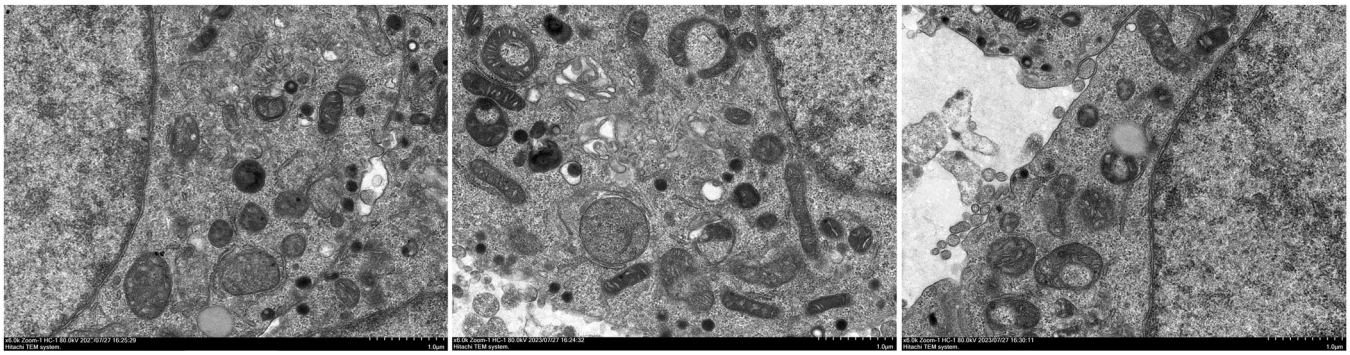


FIGURE 2 | Protein expression levels of Mfn2, PERK, IP3R, and Drp1. Western blot detection of Mfn2, PERK, IP3R, and Drp1. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. $N = 3$.



A HG-SC B BMSC+HG-SC C Mfn2-BMSCs+HG-SC

FIGURE 3 | The submicroscopic structures of MAMs, mitochondria and endoplasm were analyzed by TEM. The microstructure of MAMs was observed by TEM (A–C). $N = 3$.

GSK-3β pathway, which in turn improved the survival of BMSCs. In the STZ-induced DBD rat model, ILK BMSCs were shown to be effective in promoting the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and stromal cell-derived factor-1 (SDF-1). This in turn promotes the angiogenic process and protects the cells from high glucose-associated apoptosis to restore bladder function and histological structure.

Our study provides preliminary evidence that regulating the function of MAMs in peripheral nerve cells may be an effective method for ameliorating high glucose-induced damage. Although our research lacks direct connection with in vivo or in vitro urinary system models related to diabetes bladder dysfunction and peripheral nerve injury, our results provide a research basis for BMSC based gene modification strategies in the treatment of DBD. Future studies focusing on the in vivo models to further validate the therapeutic potential of Mfn2-modified BMSCs in DBD are warranted. Gene modification strategies based on BMSCs might have significant potential and broad application prospects in the treatment of DBD, therefore, exploring the long-term effects of such treatments and their mechanisms of action in more detail are also needed. We expect to invest more research resources into this interesting field in the future.

4.1 | Conclusion

In conclusion, we explored the ameliorative effects of Mfn2 gene modification of BMSCs on high glucose-injured glial cells, which may exert biological effects by modulating the structure and function of MAMs. This study provides preliminary evidence that modulating the function of MAMs in peripheral nerve cells may be an effective way to ameliorate high glucose-induced injury. And it suggests that our BMSC-based gene modification strategy has important potential for the treatment of DBD.

Acknowledgments

The authors are grateful to all participants in the present study. The present study was supported by the Hainan Province health industry research project (No. 22A200188).

Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data involved in the present study can be provided under reasonable request.

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