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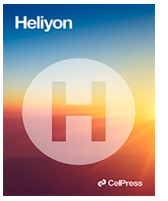

Research article

LncRNA OIP5-AS1 regulates ferroptosis and mitochondrial dysfunction-mediated apoptosis in spinal cord injury by targeting the miR-128-3p/Nrf2 axis

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ABSTRACT

Background: Ferroptosis is an important way of neuronal cell death in acute phase and participates in the inflammatory cascade after spinal cord injury (SCI). It is reported that microRNA (miRNA) and long non-coding RNA (lncRNA) are key mediators in the regulation of ferroptosis. This study will explore the inhibitory effect of LncRNA OIP5-AS1 on ferroptosis and mitochondrial dysfunction-mediated apoptosis in SCI.

Methods: The ferric ammonium citrate (FAC)-induced cell model and the SCI rat model were established. The expression of LncRNA OIP5-AS1, miR-128-3p and Nrf2 were transfected to evaluated the effect on the viability and apoptosis of FAC-induced cell. The interaction between LncRNA OIP5-AS1 and miR-128-3p or miR-128-3p and Nrf2 were analyzed. In addition, expressions of markers related to ferroptosis and mitochondrial dysfunction were analyzed in vitro and in vivo. Histopathologic slide staining was used to analyze spinal cord injury in vivo.

Results: LncRNA OIP5-AS1 expression was abnormally down-regulated in FAC-induced SCI cell model and SCI rats. The LncRNA OIP5-AS1 deficiency induced decreased Nrf2 level by less sponging miR-128-3p, thus, aggravating spinal cord injury and inducing more apoptosis, ferroptosis and mitochondrial dysfunction in neural stem cells with SCI. However, overexpression of LncRNA OIP5-AS1 inhibited apoptosis, ferroptosis and mitochondrial dysfunction, thus effectively ameliorating spinal cord injury.

Conclusion: This finding demonstrates that LncRNA OIP5-AS1 overexpression could enhance the recovery of spinal cord injury by regulating the miR-128-3p/Nrf2 axis.

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1. Introduction

Spinal cord injury (SCI) is a kind of central nervous system (CNS) disease which induces high mortality, high rate of disability rate

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and high medical cost [1]. With the development of modern social economy and transportation construction, the incidence of SCI is increasing annually [2]. Thus, SCI has attracted a large number of attentions of doctors and scholars. SCI can be divided into primary SCI and secondary SCI, in which primary SCI is mostly spinal cord in the traumatic violence against the results are irreversible and secondary SCI is a secondary injury that causes axonal, vascular, and neurological dysfunction that lasts for weeks or even months after primary SCI [3]. The pathological development of SCI includes many factors, including excessive oxidative stress injury, reactive oxygen free radical injury, inflammatory cascade injury and nerve cell death, which make the complete tissue around the initial lesion undergo self-destructive lesions to further deepen the degree of injury and expand the scope of injury [4,5]. The key to spinal cord injury repair is how to effectively inhibit a series of damage caused by secondary injury [6].

After SCI, the imbalance of microenvironment induced by excessive oxidative stress injury, inflammatory cascade injury and a large number of deaths of nerve cells in the acute phase is the most important reason why SCI is difficult to be repaired [7]. The cell apoptosis is mainly induced by the loss of mitochondrial membrane potential ($\triangle\psi m$) which makes cytochrome C (Cyt C) released from the mitochondrial membrane space into the cytoplasm and thus, triggers cell apoptosis [8]. In addition, ferroptosis is a recently discovered form of programmed cell death which is iron-dependent [9] and plays critical roles in central nervous system degeneration and traumatic injury [10,11]. It has been reported that ferroptosis is one of the important ways of neuronal cell death in the acute phase and participates in the inflammatory cascade after spinal cord injury [12]. The long non-coding RNA (LncRNA) possesses capacities in regulating gene expression and has been proved to play roles in various human diseases [13]. It should be noted that the LncRNA has been found essential in the regulation of ferroptosis and abnormally expressed in SCI [14]. While whether and how LncRNA would regulate ferroptosis after SCI remains unclear. Since Riley RS et al. have proved that LncRNA OIP5-AS1 inhibited ferroptosis and promoted the progression of prostate cancer [15], we hypothesized the regulation of LncRNA OIP5-AS1 on ferroptosis in SCI.

In the present study, based on available online database and related experimental assays, it was predicted and verified that the nuclear factor erythroid 2-related factor 2 (Nrf2) was one of targets of miR-128-3p that was meanwhile targeted by lncRNA-OIP5-AS1. A large number of studies have shown that the suppressed Nrf2 expression or activation was associated with the increased level of ferroptosis in nerve cells and various tumor cells [16,17]. Accordingly, we further proposed whether and how LncRNA OIP5-AS1/miR-128-3p/Nrf2 mediated ferroptosis and mitochondrial dysfunction and thus, regulated the death of nerve cells after SCI.

In the present study, we systematically studied the effect of LncRNA OIP5-AS1 on ferroptosis and apoptosis mediated by mitochondrial dysfunction after SCI and how the underlying mechanisms was related to miR-128-3p/Nrf2 pathway, providing a theoretical basis for further understanding of new therapeutic researches in the process of SCI.

2. Materials and methods

2.1. Murine model of spinal cord injury (SCI)

SPF level healthy adult male SD rats (220 ± 20g, 8 weeks) obtained from Shandong First Medical University Affiliated Provincial Hospital were selected to make moderate SCI model with the modified Allen percussion device [18]. Rats in the sham operation group received the surgery of only removing vertebral lamina with the spinal cord not being hit. Then, LncRNA OIP5-AS1 virus overexpression vector and LncRNA OIP5-AS1 antisense nucleic acid group (ASO) were constructed and injected into the tail vein twice a week. Rats were injected with an overdose of sodium pentobarbital on the 21st day Then, the tissues obtained from rats were made into paraffin sections for histopathological analysis. This study was approved by Ethics Committee of Shandong First Medical University Affiliated Provincial Hospital (approval no. 2021-243).

2.2. Histopathological analysis

Paraffin-embedded sections were treated with hematoxylin and eosin (H&E) staining or Nissl staining, and were examined under a light microscope.

Cyt C polyclonal antibody was used to examine the mitochondrial in spinal cord tissue. The positive reaction of Cyt C was mitochondrial pattern, that is, discrete spots were distributed in the cytoplasm, and the negative reaction was diffuse cellular immunostaining without obvious spots [19].

2.3. Isolation and culture of rat neural stem cells

Under sterile conditions, the 15-day-old fetal rats in the abdomen of SD pregnant rats were taken out. The subventricular zone, ventricular campground membrane and hippocampus of the fetal rats were isolated under a stereomicroscope, cut into pieces on ice, digested with 0.25 % membrane protease for 30 min, passed through 40 μm cell sieve, and terminated with DMEM/F12 complete medium containing fetal bovine serum (FBS). The cells were inoculated in a Petri dish pre-coated with gelatin at a cell concentration of 1×10^8/L. The cell morphology was observed by inverted microscope. The cells were cultured in DMEM/F12 medium containing 20 μg/L epidermal growth factor, 10 μg/L basic fibroblast growth factor, 1 % B27 and 100 U/mL penicillin-streptomycin at 37 °C in a 5 % CO_2 incubator. The medium was changed every 3 days and passaged every 7 days. Rat neural stem cells were isolated and cultured in serum-free medium under hypoxic conditions for 6 h, and then cultured in complete medium containing 100 μM ferric ammonium citrate (FAC) for 24 h to mimic spinal cord injury hemorrhage and ferroptosis. LncRNA OIP5-AS1 overexpression plasmid and shRNA or miR-128-3p mimic and miR-128-3p inhibitor were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). The Nrf2 inhibitor ML385 (1 μM; HY-100523, MedChemExpress, Monmouth Junction, NJ, USA) was added in cell culture medium for

inhibiting Nrf2 [20].

2.4. Cell viability

Cells were seeded in 96-well plates (07–6096, Biolglx) at a density of 1×10^4 cells/well. 5 mg/ml MTT solution (MA0218-5, meilunbio) was added and the cells were cultured for 4 h using incubator BPN-80CH. Then, 150 μL DMSO was replaced added to dissolve formazan crystals, and the absorbance was measured at 490 nm on a microplate reader (MOLECULAR DEVICES, CMax Plus).

2.5. Flow cytometry

The flow cytometry analysis was performed according to protocols [21]. Apoptotic cells were double stained with FITC Annexin V and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit (AP101, Hangzhou Lianke Biotechnology Co., Ltd.) and the apoptosis rates were analyzed using Flow Cytometry CytoFLEX (BECKMAN).

2.6. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay

Trizol kit (DP424, Tiangen Biochemical Technology (Beijing) Co., Ltd.) was employed to extract total RNA from tissues and cells. Then, the RNA was reversely transcribed into cDNA using a reverse transcription kit (KR116, Tiangen Biochemical Technology (Beijing) Co., Ltd.). Fluorescence quantitative analysis was assayed employing the SuperReal Quantitative fluorescence prex reagent (FP205, Tiangen Biochemical Technology (Beijing) Co., Ltd.). qRT-PCR was conducted on the real-time fluorescent quantitative PCR (LightCycler96, Roche). U6 and Actin were applied as normalizer. The $2^{-\Delta\Delta CT}$ was employed for quantifying transcriptional level of genes. The primer sequence is shown in Table 1.

2.7. Western blot assay

Proteins were extracted using lysate (P0013J, Beyotime Biotechnology) [22] and quantified using BCA protein concentration assay kit (P0010S, Beyotime Biotechnology). Then, the proteins were separated and transferred to membranes. The membranes were incubated overnight at 4 °C with primary antibody against GAPDH (1:1000, TA-08, Zhongshan Golden Bridge), Caspase 3 (1:1000, 19677-I-AP, Proteintech), Bax (1:1000, 50599-2-Ig, Proteintech), Bcl-2 (1:1000, 12789-I-AP, Proteintech), Nrf2 (1:1000, ab137550, Abcam), GPX4 (1:1000, ER1803-15, HUABIO) and FIH1 (1:1000, 10646-1-AP, Proteintech). Subsequently, samples were incubated with secondary antibody (1:2000, ZB-2305, ZB-2301, Zhongshan Golden Bridge) at room temperature and then observed via Tanon-4600 (Beijing Yuan Pinghao Biotechnology Co., LTD), using alphaEaseFC (Alpha Innotech) to determined gray value of strip.

2.8. Rationale prediction and verification

The potential targeted miRNA of LncRNA OIP5-AS1 and the predicted binding sites were analyzed by the online ENCORI platform (https://rnasysu.com/encori/). The regulatory effect of the predicted LncRNA OIP5-AS1 target miR-128-3p on Nrf2 and the binding sites were analyzed by the online database TargetScan (https://www.targetscan.org/vert_70/). LncRNA OIP5-AS1-WT and LncRNA OIP5-AS1-MUT pmirGLO reporter vectors or Nrf2-WT and Nrf2-MUT consisting of miR-NC and miR-128-3p mimic were constructed and transfected into neural stem cells for 48 h to verify the predicted relations by determining luciferase activity. Moreover, how LncRNA OIP5-AS1 regulated miR-128-3p expression level and how miR-128-3p regulated Nrf2 expression level were also determined for verifying their underlying relations.

2.9. Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) in neural stem cells cells was determined using the fluorescence probe rhodamine-123.

Table 1
Sequence of the primers for fluorescence quantification.

Name	Primer sequences (5′-3′)
LncRNA OIP5-AS1-F	AACAGGTGCTTAGGTGGTGG
LncRNA OIP5-AS1-R	TGGCACTGCATGAGGGATTT
Nrf2-F	GCAGCCTTGAGCTCTCTTGA
Nrf2-R	AGTGACTGAAACGTAGCCGA
Actin-F	GGCTGTATTCCCCTCCATCG
Actin-R	CCAGTTGGTAACAATGCCATGT
MiR-128-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAGAG
MiR-128-3p-F	CGCGTCACAGTGAACCGGT
MiR-128-3p-R	AGTGCAGGGTCCGAGGTATT
U6-F	GCTTCGGCAGCACATATACT
U6-R	GTGCAGGGTCCGAGGTATTC

Cells were stained with 2 μM rhodamine-123 at 37 °C for 15 min. The fluorescence intensity was detected using fluorescence spectrophotometer (excitation wavelength: 488 nm, emission wavelength: 525 nm). The MMP was expressed as the relative percentage of fluorescence intensity versus 0 μM group.

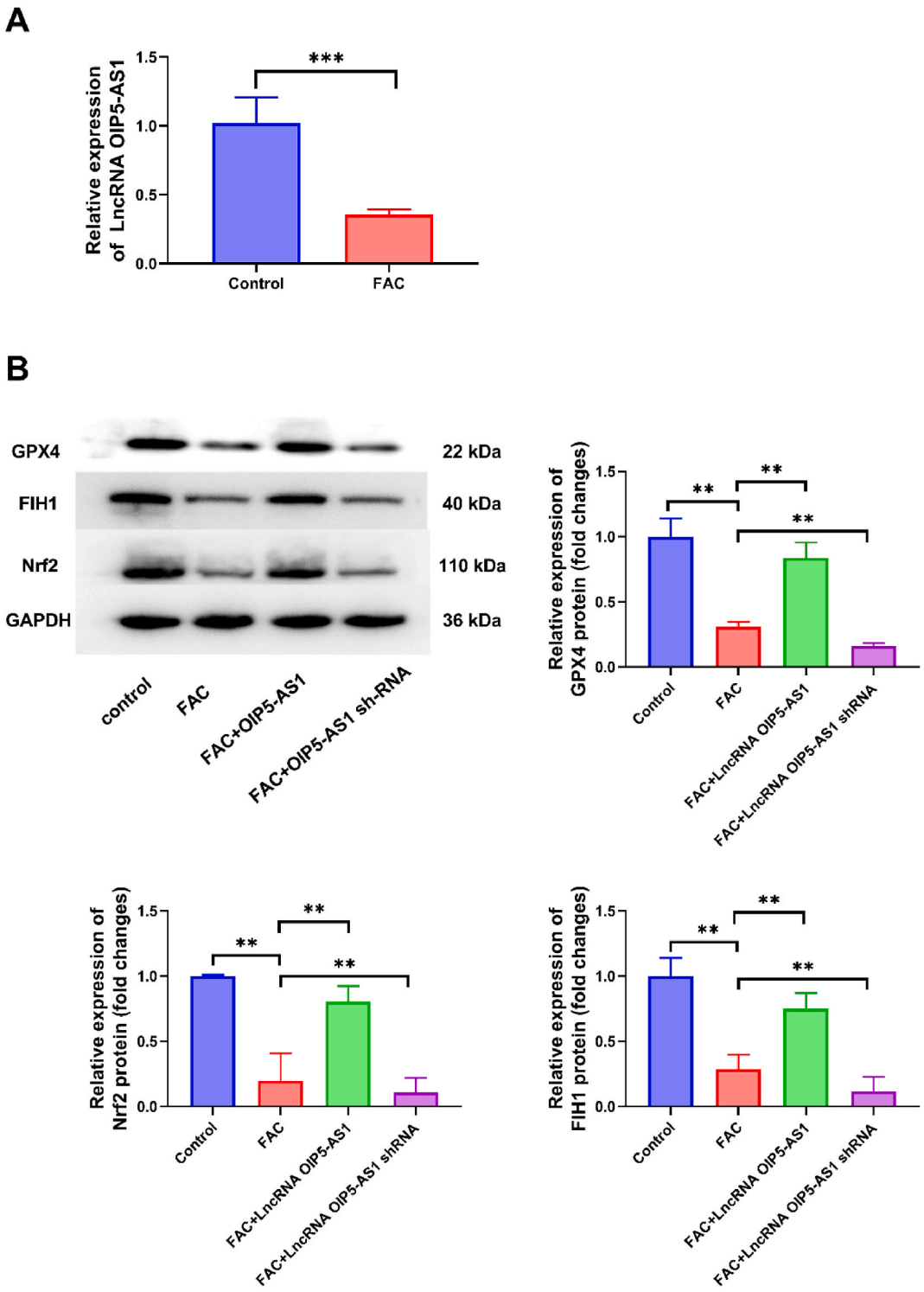

Fig. 1. (A) The expression of LncRNA OIP5-AS1 in neural stem cells after SCI. (B) Effect of LncRNA OIP5-AS1 on Nrf2, GPX4 and FIH1 protein expression. n = 3, **P < 0.01, ***P < 0.001.

2.10. Reactive oxygen species (ROS) level

Cellular ROS level was measured by incubating cells with 10 μM dichlorofluorescein-diacetate (DCFH-DA) at 37 °C for 30 min in the dark. Then, the cells were analyzed using fluorescence microplate reader (excitation wavelength: 485 nm, emission wavelength: 520 nm). The ROS level was expressed as the fluorescence intensity of dichlorofluorescein (DCF), and observed by fluorescence microscope.

2.11. Basso-Beattie-Bresnahan (BBB) scoring

Before the formal testing, ensure that the mice have a period of acclimation in the testing environment. Typically, mice need to be allowed to acclimate to the laboratory environment before testing to minimize interference caused by changes in their environment. Place the mice on a standardized locomotion test surface, usually a smooth, horizontal plane. Observe and record the mice's locomotor behaviors under different conditions, including walking, standing, gait, and motor coordination.

The BBB scoring system rates the mice's locomotor ability on a scale from 0 to 21, where 0 indicates complete paralysis and 21 represents normal gait.

0 points: Complete paralysis.
1-2 points: Very limited movement capability, able to make very limited movements.
3-7 points: Some limited movement, such as occasional gait or brief standing, but unable to walk normally.
8-13 points: Limited gait, may require support or show some instability.
14-19 points: Gait is gradually approaching normal but still shows slight motor impairment.
20-21 points: Near-normal or normal gait and locomotor ability.

Score each mouse based on the observed locomotor behavior. Summarize and analyze the data to assess the extent of spinal cord injury and its impact on the mice's motor function.

2.12. Basso mouse scale (BMS) scoring

Place the mouse on the testing surface and allow it to move freely. Observe its hind limb movements and overall locomotor behavior. Evaluate the mouse's locomotor performance according to the following BMS scoring criteria, with scores ranging from 0 to 9:

0: No movement or spontaneous activity.
1: No coordinated movement, but some spontaneous activity.
2: Some movement attempts, but no coordinated movement.
3: Limited stepping movement with severe impairment; occasional uncoordinated stepping.
4: Improved stepping, but still severe impairment; frequent dragging of hind limbs.
5: Better stepping with less hind limb dragging; some weight-bearing but still impaired.
6: Fair locomotion with good weight-bearing and coordination; occasional uncoordinated movements.
7: Good locomotion, close to normal; occasional uncoordinated movements.
8: Near-normal gait with smooth and coordinated movement.
9: Normal gait with no observable impairment.

Score each mouse based on the observed performance. Scoring should be performed by two observers.

2.13. Statistical analyses

The data of all experiments were analyzed through GraphPad 8.0.2 (San Diego, California, USA https://www.graphpad-prism.cn/) and multi-group comparisons were performed using the analysis of variance (ANOVA) with Tukey's post hoc test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. LncRNA OIP5-AS1 inhibits ferroptosis of neural stem cells after SCI

Fig. 1A showed that LncRNA OIP5-AS1 was abnormally decreased in the simulated SCI cell model in vitro ($P < 0.001$) and the FAC-induced SCI caused ferroptosis presented by the decreased levels of Nrf2, GPX4 and FIH1 protein (Fig. 1B, $P < 0.01$), while these decreased protein levels were reversed by increased LncRNA OIP5-AS1 expression ($P < 0.01$) and further decreased by the inhibition of LncRNA OIP5-AS1 expression ($P < 0.01$).All these results demonstrated that LncRNA OIP5-AS1 inhibited the ferroptosis in SCI cell model.

3.2. LncRNA OIP5-AS1 ameliorates the mitochondrial dysfunction

Fig. 2A suggested that the neural stem cells after SCI showed the loss of mitochondrial membrane potential ($P < 0.01$), which indicated that SCI could cause mitochondrial dysfunction, leading to an abnormal increase in ROS level (Fig. 2B, $P < 0.01$). However,

LncRNA OIP5-AS1 overexpression recovered the mitochondrial membrane potential (Fig. 2A, $P < 0.01$) and reduced ROS overproduction (Fig. 2B, $P < 0.01$) while LncRNA OIP5-AS1 further reduced mitochondrial membrane potential (Fig. 2A, $P < 0.01$) and increased ROS level (Fig. 2B, $P < 0.01$). These findings proved the effect of LncRNA OIP5-AS1 on recovering mitochondrial function and consequently limiting ROS production in SCI cell model.

3.3. LncRNA OIP5-AS1 inhibits apoptosis of neural stem cells after SCI

As shown in Fig. 3A, the cell viability of neural stem cells after SCI is significantly reduced ($P < 0.01$), which was reversed by LncRNA OIP5-AS1 overexpression ($P < 0.01$) while further exacerbated by LncRNA OIP5-AS1 downregulation ($P < 0.01$). Moreover, cell apoptosis was increased in FAC-induced SCI cell models (Fig. 3B–C, $P < 0.01$), which was reversed by LncRNA OIP5-AS1 overexpression ($P < 0.01$) while further increased by LncRNA OIP5-AS1 downregulation ($P < 0.01$). The cell apoptosis-related BCL-2 was increased while BAX and Caspase-3 were decreased in FAC-induced SCI cell models (Fig. 3D, $P < 0.01$), which was further changed flowing the existing trends y LncRNA OIP5-AS1 downregulation ($P < 0.05$) while was reversed by LncRNA OIP5-AS1 overexpression ($P < 0.05$). Accordingly, LncRNA OIP5-AS1 was proved to inhibit the apoptosis of SCI neural stem cells.

3.4. LncRNA OIP5-AS1 regulates miR-128-3p and targets Nrf2

As shown in Fig. 4A, there are several binding sites that could interact between miR-128-3p and Nrf2, suggesting the capacity and potential of Nrf2 binding with miR-128-3p. Subsequent results of luciferase reporter exhibited that ectopic expression of miR-128-3p significantly reduced luciferase activity in the Nrf2-WT reporter ($P < 0.05$), but had no significant difference in Nrf2-MUT reporter compared with miR-NC transfection (Fig. 4B), suggesting that miR-128-3p and Nrf2 could interact with each other through putative binding sites and miR-128-3p overexpression decreased the level of Nrf2 (Fig. 4C, $P < 0.01$) while miR-128-3p downregulation increased the level of Nrf2 ($P < 0.01$).

As shown in Fig. 4D, there were several binding sites that could interact between LncRNA OIP5-AS1 and miR-128-3p, suggesting LncRNA OIP5-AS1 potentially bind with miR-128-3p. Furthermore, luciferase reporter exhibited that ectopic expression of miR-128-3p significantly reduced luciferase activity in the OIP5-AS1-WT reporter ($P < 0.05$), but had no significant difference in OIP5-AS1-MUT reporter compared with miR-NC transfection (Fig. 4E), proving that miR-128-3p and LncRNA OIP5-AS1 could interact with each other through putative binding sites. Thus, we took miR-128-3p as the target mRNA for subsequent experiments. As shown in Fig. 4F, the level of miR-128-3p in neural stem cells after SCI with OIP5-AS1 transfection was lower than that of FCA group, but silencing of OIP5-AS1 enhanced the level of miR-128-3p ($P < 0.05$), indicating that LncRNA OIP5-AS1 could regulated miR-128-3p.

We further verified whether LncRNA OIP5-AS1 regulates Nrf2 expression by sponging miR-128-3p. qRT-PCR showed that LncRNA OIP5-AS1 silencing reduced the expression of Nrf2, while further transfection with miR-128-3p detected a lower expression of Nrf2 (Fig. 4G). In conclusion, OIP5-AS1 promotes Nrf2 expression through sponge miR-128-3p.

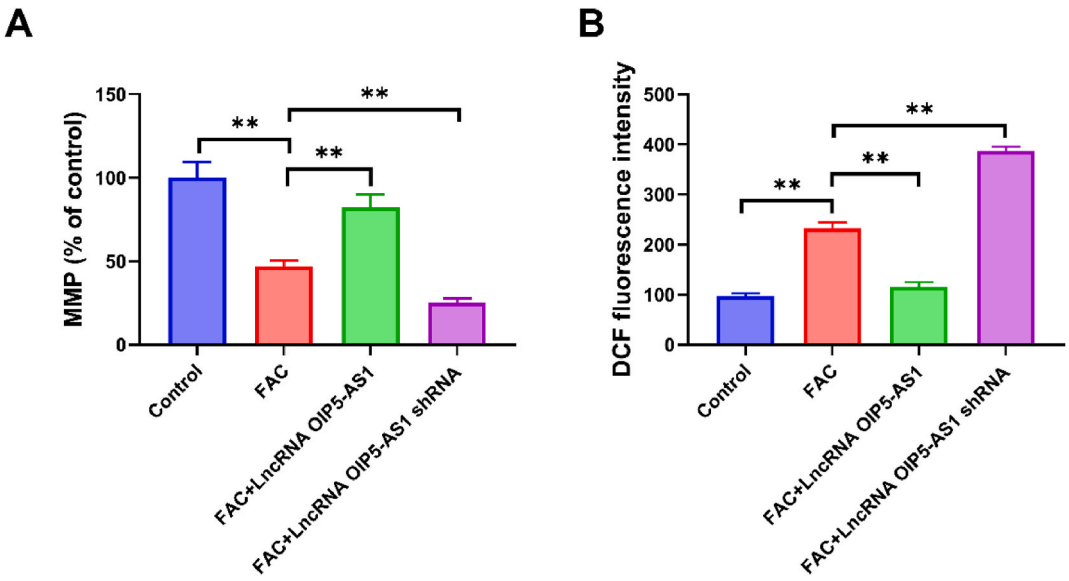

Fig. 2. (A) Mitochondrial membrane potential in neural stem cells after SCI transfected with LncRNA OIP5-AS1. (B) DCF fluorescence intensity of intracellular ROS levels in neural stem cells after SCI transfected with LncRNA OIP5-AS1 detected by flow cytometry. $n = 3$, $^{**}P < 0.01$.

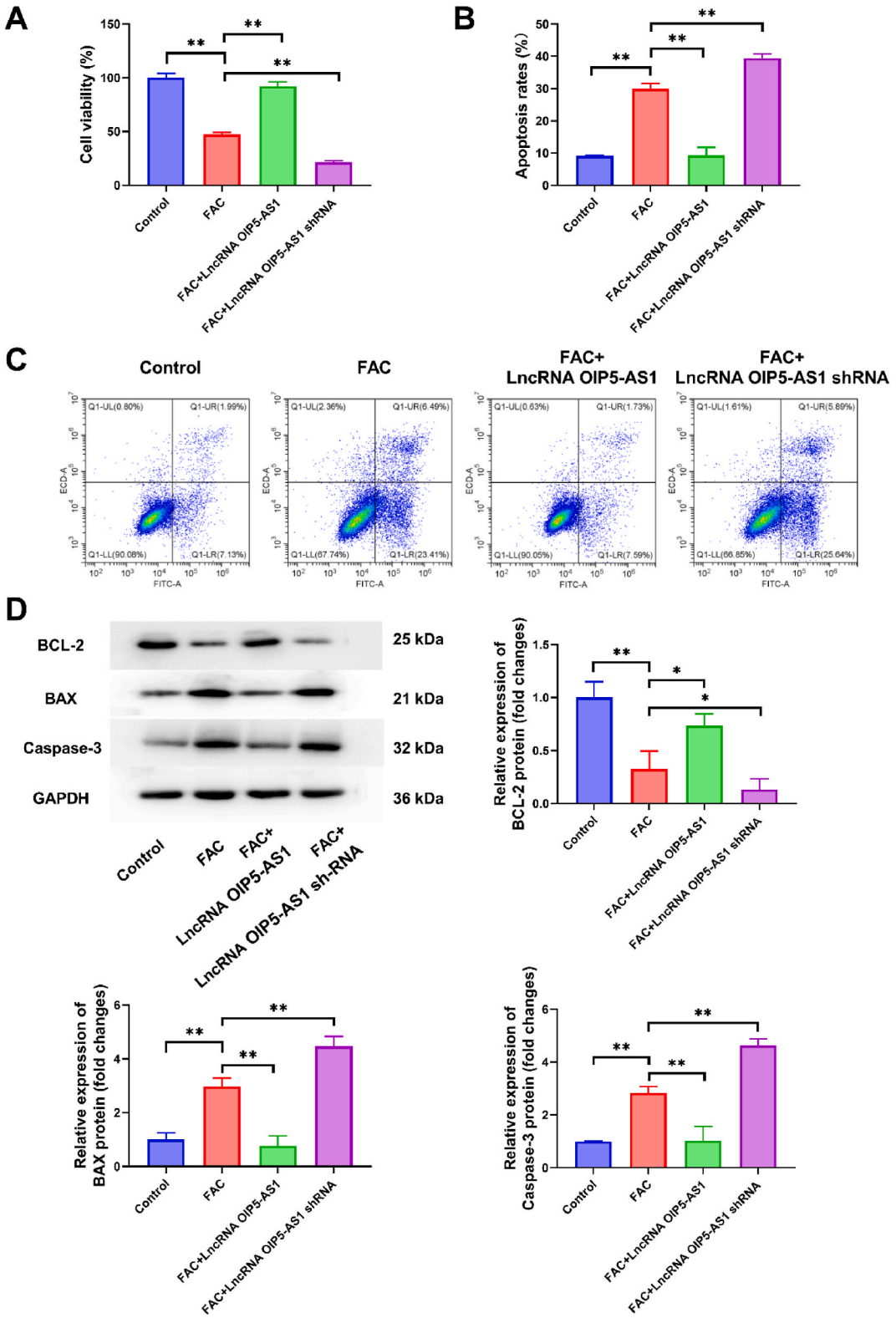

Fig. 3. (A) Cell viability in neural stem cells after SCI transfected with LncRNA OIP5-AS1. (B and C) Apoptosis rate in neural stem cells after SCI transfected with LncRNA OIP5-AS1. (D) The levels of BCL-2, caspase-3 and BAX in neural stem cells after SCI transfected with LncRNA OIP5-AS1. n = 3, *P < 0.05, **P < 0.01.

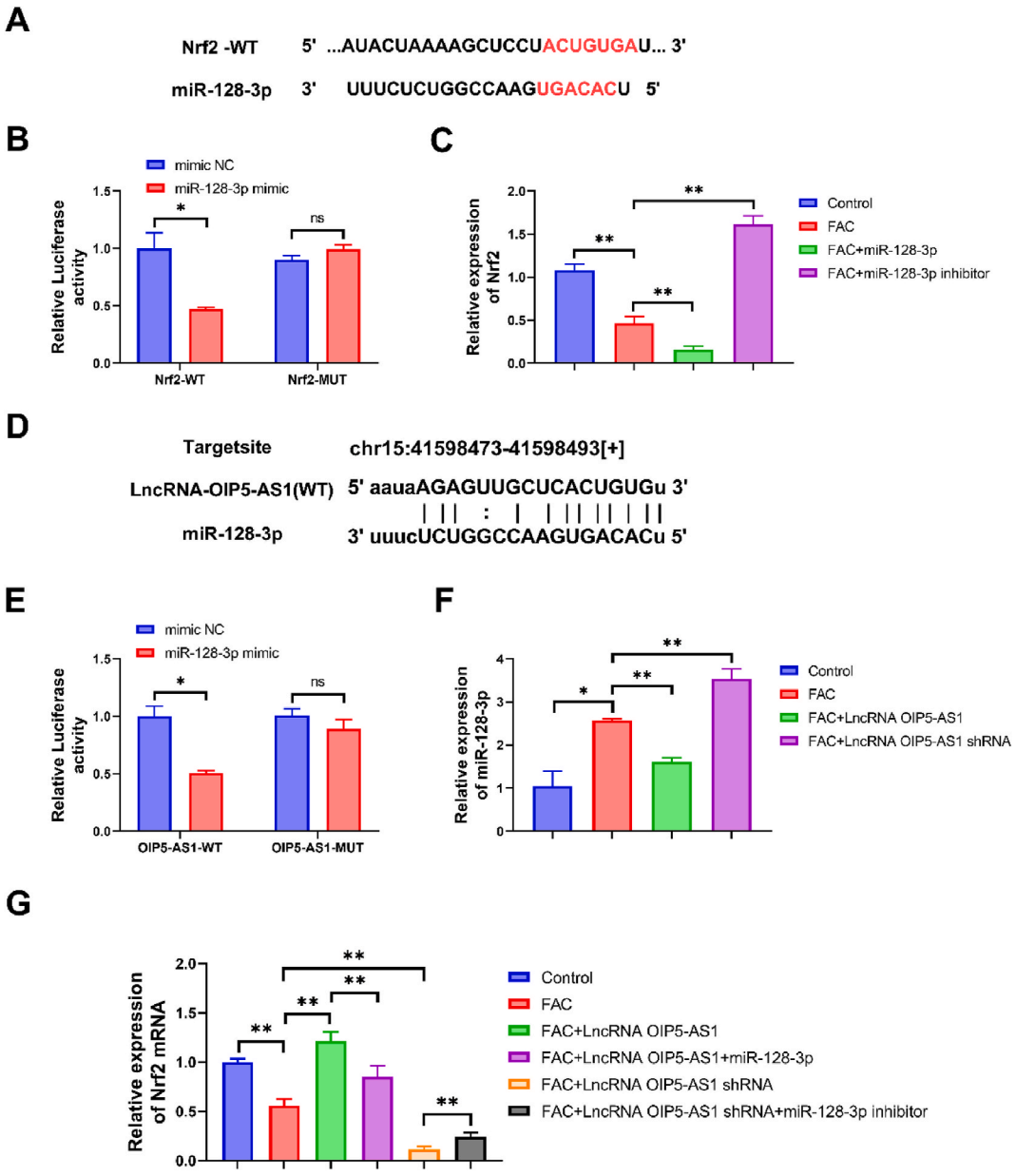

Fig. 4. (A) Predicted binding sites between miR-128-3p and Nrf2, and mutant sites of Nrf2 in reporter. (B) Luciferase activities in neural stem cells after SCI after transfecting with genes. (C) The level of Nrf2 in neural stem cells after SCI. (D) Schematic showed the predicted binding site between LncRNA OIP5-AS1 and miR-128-3p. (E) Luciferase activities in neural stem cells after SCI after transfecting with genes. (F) The level of miR-128-3p in neural stem cells after SCI. (G) The expression of Nrf2 in in neural stem cells transfected with genes. n = 3, *P < 0.05, **P < 0.01.

3.5. LncRNA OIP5-AS1 regulates spinal cord injury by targeting miR-128-3p in vitro

We found that LncRNA OIP5-AS1 overexpression could inhibit the occurrence of ferroptosis presented by the increased levels of Nrf2, GPX4 and HIF1 proteins (Fig. 5A–D, P<0.01) and ameliorate mitochondrial dysfunction and oxidative stress presented by increased MMP and decreased DCF intensity (Fig. 5E–F, P < 0.01). The addition of miR-128-3p reversed the promoting effect of LncRNA OIP5-AS1 overexpression on Nrf2, GPX4 and HIF1 proteins (Fig. 6A–D, P<0.01). Moreover, the transfection of miR-128-3p reduced MMP and promoted ROS production (Fig. 6E–F, P < 0.01). On the other hand, the inhibitive effect of LncRNA OIP5-AS1 downregulation on Nrf2, GPX4 and HIF1 expression was reversed by the miR-128-3p inhibition (Fig. 6A–D, P < 0.05). The miR-128-3p inhibition also reversed the promotion of mitochondrial dysfunction and ROS production induced by LncRNA OIP5-AS1 downregulation (Fig. 6E–F, P < 0.01). These results demonstrated that LncRNA OIP5-AS1 inhibited ferroptosis of SCI cells by downregulating miR-128-3p. Moreover, the cell viability (Fig. 6A) and apoptosis-related BCL-2 (Fig. 6D) were decreased in SCI cell

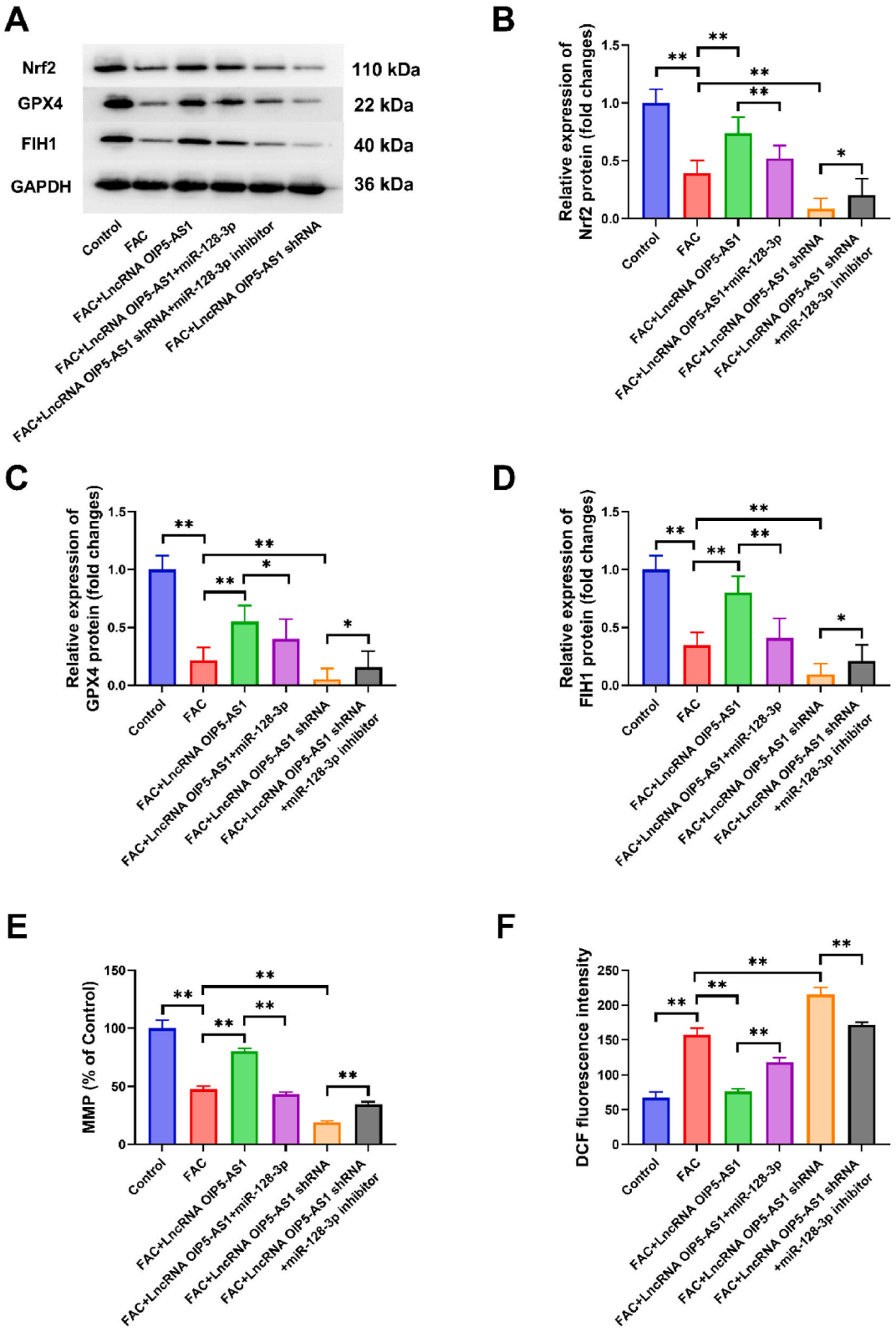

Fig. 5. (A–D) Effect of LncRNA OIP5-AS1 on Nrf2, GPX4 and FIH1 protein expression measured by Western blot. (E) Mitochondrial membrane potential in neural stem cells after SCI transfected with LncRNA OIP5-AS1. (F) DCF fluorescence intensity of intracellular ROS levels in neural stem cells after SCI transfected with LncRNA OIP5-AS1. n = 3, *P < 0.05, **P < 0.01.

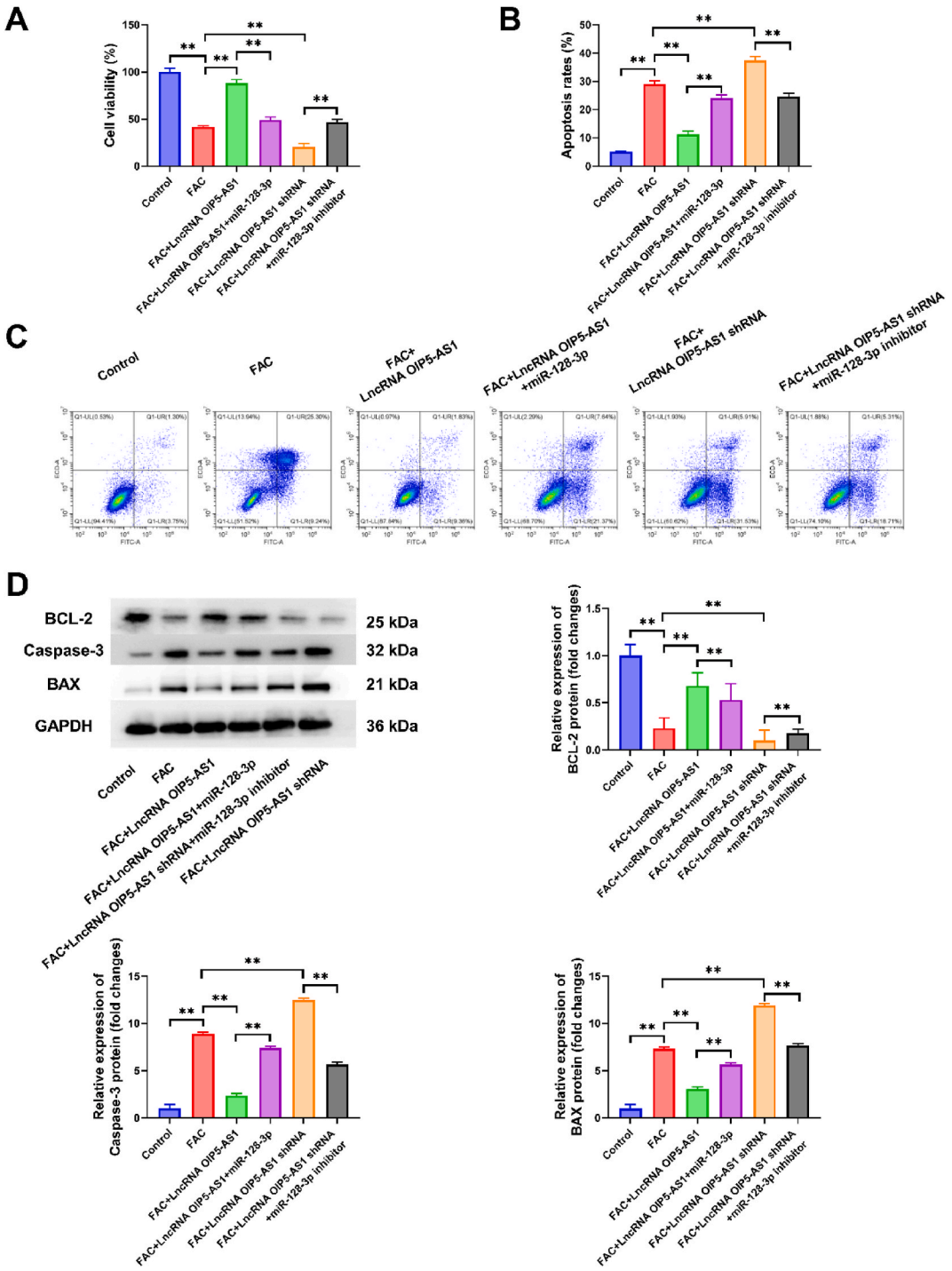

Fig. 6. Cell viability (A) and apoptosis rate (B and C) in neural stem cells after SCI transfected with genes. (D) The expression of BCL-2, caspase-3 and BAX in neural stem cells after SCI transfected with genes. n = 3, *P < 0.05, **P < 0.01.

model (P < 0.01). The LncRNA OIP5-AS1 overexpression reversed these decreases (P < 0.01), which was reversed again by the addition of miR-128-3p (P < 0.01). And LncRNA OIP5-AS1 downregulation further decreased levels of cell viability and BCL-2 (P < 0.01), which was reversed by the miR-128-3p inhibition (P < 0.01). The cell apoptosis (Fig. 6B and C) and apoptosis-related BAX and Caspase-3 (Fig. 6D) were increased in SCI cell model (P < 0.01). The LncRNA OIP5-AS1 overexpression reversed these increases (P < 0.01), which was reversed by the addition of miR-128-3p (P < 0.01). And LncRNA OIP5-AS1 downregulation further increased levels of cell apoptosis, BAX and Caspase-3 (P < 0.01), which was reversed by the miR-128-3p inhibition (P < 0.01). These results indicated

that the LncRNA OIP5-AS1 suppressed cell apoptosis by downregulating miR-128-3p. To sum up, LncRNA OIP5-AS1 inhibited ferroptosis and apoptosis related to mitochondrial dysfunction and oxidative stress, and enhanced cell viability after cells were received SCI by downregulating miR-128-3p, protecting neural cells.

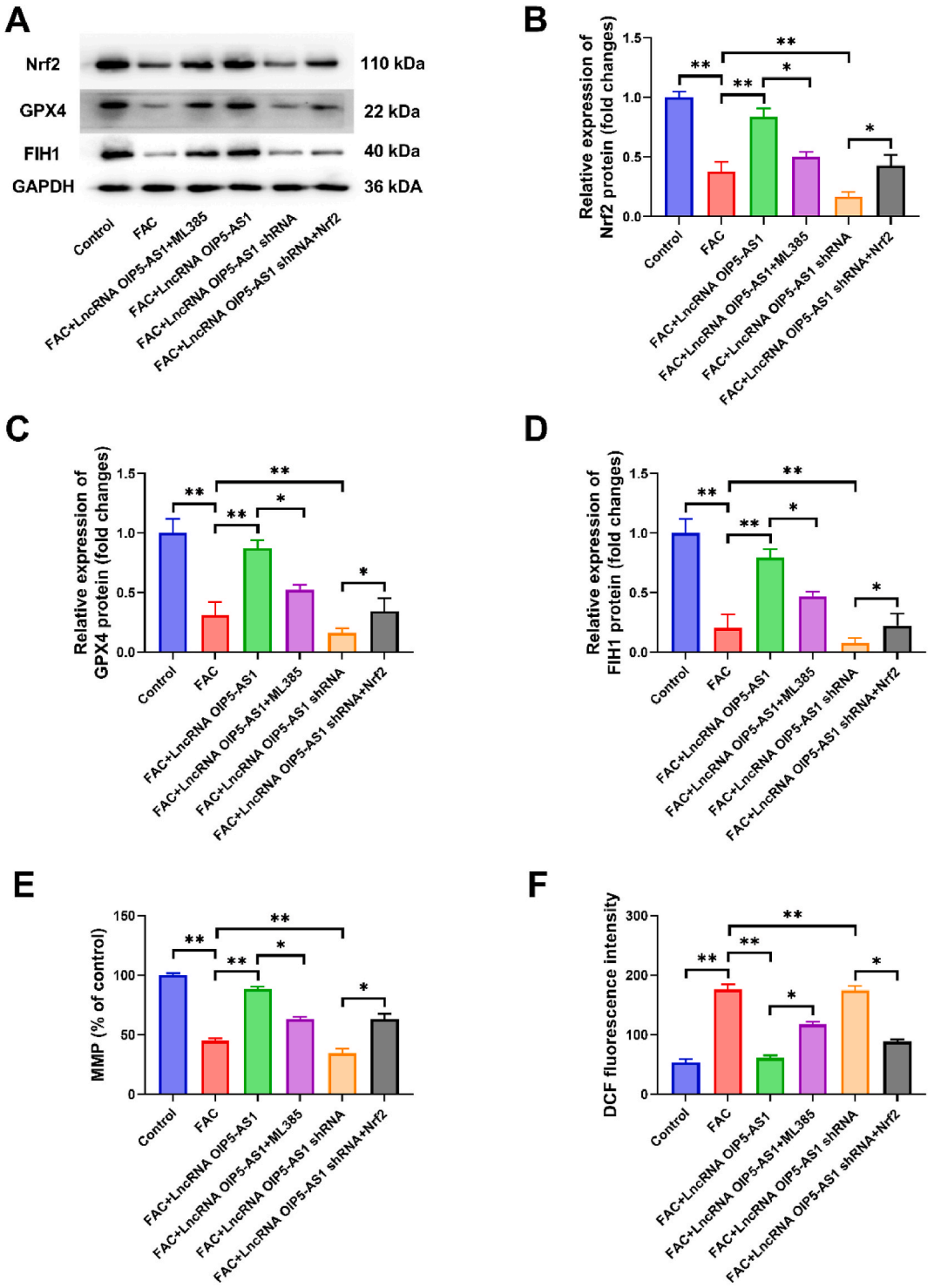

Fig. 7. (A–D) Effect of LncRNA OIP5-AS1 on Nrf2, GPX4 and FIH1 protein expression measured by Western blot. (E) Mitochondrial membrane potential in neural stem cells after SCI transfected with genes. (F) DCF fluorescence intensity of intracellular ROS levels in neural stem cells after SCI transfected with LncRNA OIP5-AS1. n = 3, *P < 0.05, **P < 0.01.

3.6. LncRNA OIP5-AS1 regulates spinal cord injury by targeting Nrf2 in vitro

The Nrf2 (Fig. 7A and B) was decreased in SCI cell model ($P < 0.01$). The LncRNA OIP5-AS1 overexpression increased Nrf2 level in SCI cells ($P < 0.01$), which was decreased by Nrf2 inhibitor ML385 ($P < 0.05$). And LncRNA OIP5-AS1 downregulation decreased Nrf2

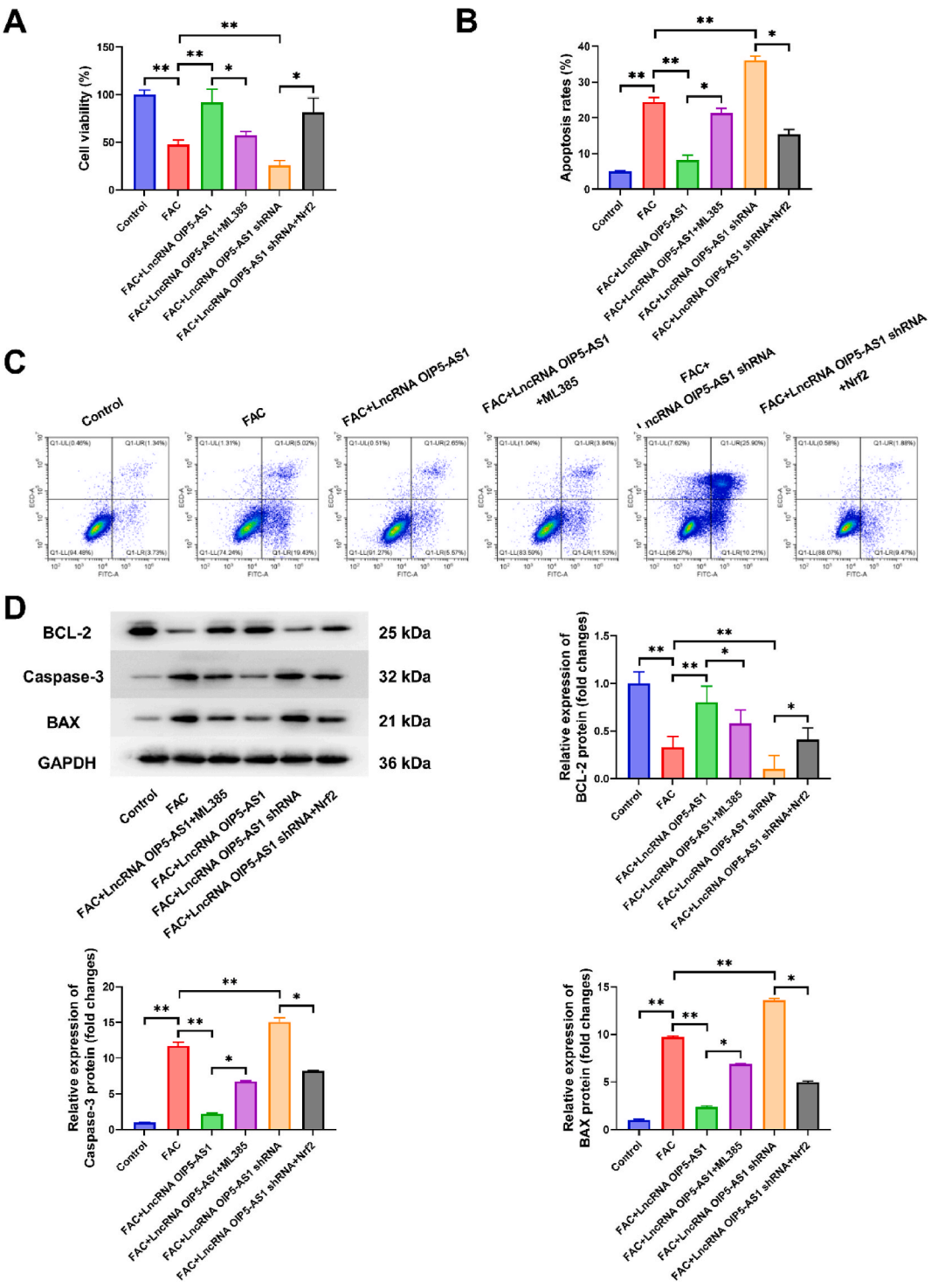

Fig. 8. Cell viability (A) and apoptosis rate (B and C) in neural stem cells after SCI transfected with genes. (D) The levels of BCL-2, caspase-3 and BAX in neural stem cells after SCI transfected with genes. n = 3, *P < 0.05, **P < 0.01.

level in SCI cells ($P < 0.01$), which was increased by addition of Nrf2 protein ($P < 0.05$). The Nrf2 inhibitor ML385 reversed the LncRNA OIP5-AS1 overexpression induced increase of GPX4 (Fig. 7A and C, $P < 0.05$) and FIH1 (Fig. 7A and D, $P < 0.05$). On the other hand, the LncRNA OIP5-AS1 downregulation induced decrease of GPX4 (Fig. 7A and C, $P < 0.05$) and FIH1 (Fig. 7A and D, $P < 0.05$) expression was reversed by the Nrf2 ($P < 0.05$). The Nrf2 inhibitor disrupted the elevation of MMP and reduction of ROS level (Fig. 7E–F, $P < 0.05$) while Nrf2 reversed the promotion of mitochondrial dysfunction and ROS production induced by LncRNA OIP5-AS1 downregulation (Fig. 7E–F, $P < 0.05$). These results demonstrated that LncRNA OIP5-AS1 inhibited ferroptosis of SCI cells by activating Nrf2. Moreover, the Nrf2 inhibitor reversed the increased cell viability (Fig. 8A, $P < 0.05$) and BCL-2 level (Fig. 8D, $P < 0.05$) induced by LncRNA OIP5-AS1 overexpression while the Nrf2 reversed the decreased cell viability (Fig. 8A, $P < 0.05$) and BCL-2 level (Fig. 8D, $P < 0.05$) induced by LncRNA OIP5-AS1 downregulation. Additionally, the cell apoptosis (Fig. 8B and C) and apoptosis-related BAX and Caspase-3 (Fig. 8D) were increased in SCI cell model ($P < 0.01$). The LncRNA OIP5-AS1 overexpression reversed these increases ($P < 0.01$), which was reversed by the Nrf2 inhibitor ($P < 0.05$). And LncRNA OIP5-AS1 downregulation further increased levels of cell apoptosis, BAX and Caspase-3 ($P < 0.01$), which was reversed by the Nrf2 ($P < 0.05$). These results indicated that the LncRNA OIP5-AS1 suppressed cell apoptosis by upregulating Nrf2. To sum up, LncRNA OIP5-AS1 inhibited ferroptosis and mitochondrial dysfunction mediated apoptosis, and enhanced cell viability after cells were received SCI by upregulating Nrf2.

3.7. *LncRNA OIP5-AS1 regulates spinal cord injury by targeting miR-128-3p/Nrf2 axis in SCI rats*

Basso-Beattie-Bresnahan (BBB) and Basso mouse score (BMS) scales were used to evaluate the therapeutic effect of LncRNA OIP5-AS1 on SCI. Fig. 9A and B showed that ats motor function decreased significantly in SCI rats compared with rats received sham treatments ($P < 0.01$), demonstrating the successful establishment of SCI rat models. While compared with the SCI group, motor

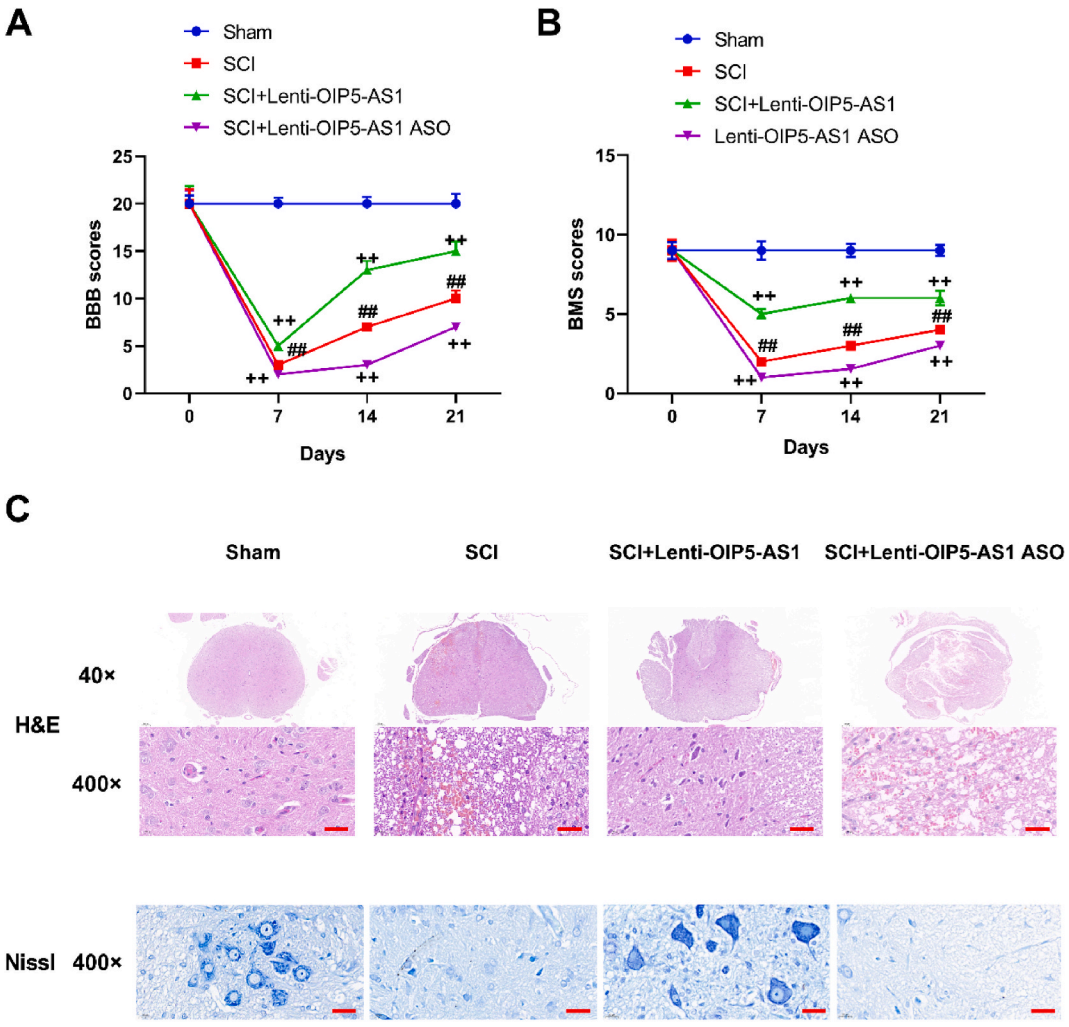

Fig. 9. The BBB (A) and BMS (B) scores of the different groups. (C) Representative images from H&E and Nissl staining. Scale bar: 200 μm (40 ×). Scale bar: 20 μm (400 ×). n = 6, $^{\#\#}P < 0.01$ vs. Sham. $^{++}P<0.01$ vs. SCI.

function of rats in Lenti-OIP5-AS1 therapy group improved significantly ($P < 0.01$) and motor function of rats in Lenti-OIP5-AS1 ASO therapy group reduced significantly ($P < 0.01$). In addition, we further assessed the damage of peripheral white matter and central gray matter after SCI using H&E staining (Fig. 9C). Consistent with the motor assessment, more damage in the lesion area was observed in SCI group, further demonstrating that the SCI rat models were established. The tissues from Lenti-OIP5-AS1 therapy group exhibited less damage in the lesion area and retained the motor neurons in the anterior horn while Lenti-OIP5-AS1 ASO therapy group exhibited more damage, indicating that LncRNA OIP5-AS1 overexpression protected the spinal cord from severe injury during SCI.

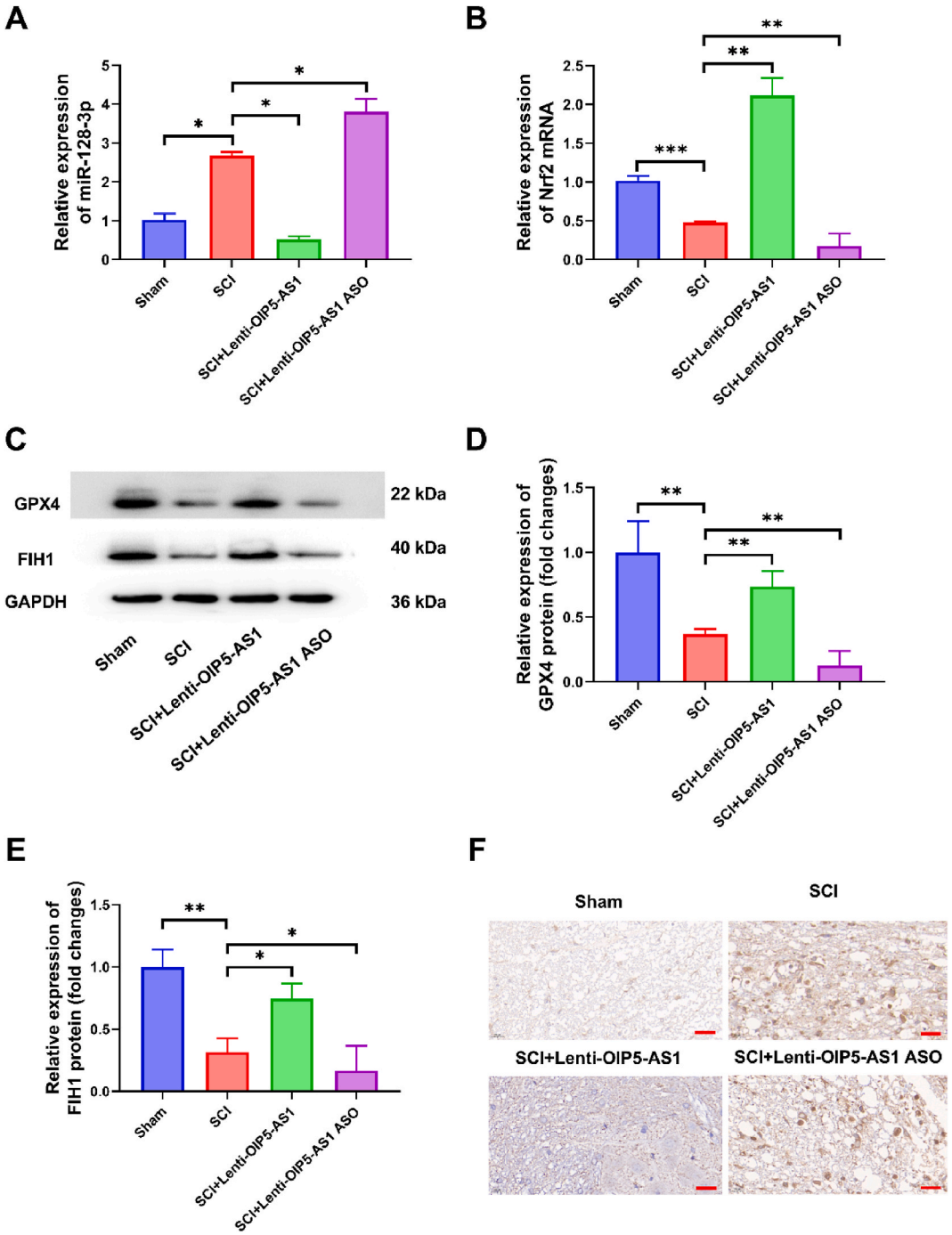

Fig. 10. Relative expression of miR-128-3p (A) and Nrf2 (B) of the different groups. (C–E) The GPX4 and FIH1 protein expression of the different groups measured by Western blot. (F) Representative illustrations of immunohistochemical staining of cytochrome C (Cyt C) in SCI rats. Scale bar: 20 μm (400 ×). n = 6, *P < 0.05, **P < 0.01, ***P < 0.001.

Lenti-OIP5-AS1 therapy reduced miR-128-3p level (Fig. 10A, $P < 0.05$) and enhanced Nrf2 protein expression (Fig. 10B, $P < 0.01$), effectively inhibited the occurrence of ferroptosis presented by the increased levels of GPX4 and FIH1 (Fig. 10C–E, $P < 0.05$) and ameliorated mitochondrial dysfunction presented by decreased cytochrome C release (Fig. 10F). On the other hand, the Lenti-OIP5-AS1 ASO therapy increased miR-128-3p level (Fig. 10A, $P < 0.05$) and decreased Nrf2 protein expression (Fig. 10B, $P < 0.01$), promoted the ferroptosis presented by the decreased levels of GPX4 and FIH1 (Fig. 10C–E, $P < 0.05$) and exacerbated mitochondrial dysfunction presented by increased cytochrome C release (Fig. 10F). Therefore, LncRNA OIP5-AS1 facilitated the survival of motor neurons and protect brains from SCI, through downregulating miR-128-3p and subsequently upregulating Nrf2.

4. Discussion

In the past, studies on neuronal cell death in SCI mainly focused on apoptosis, necrosis and autophagy [23,24]. Ferroptosis is a recently discovered iron-dependent, non-apoptotic cell necrosis characterized by the accumulation of ROS and lipid peroxides (lipid ROS) [25]. The occurrence of ferroptosis ultimately reduces the antioxidant capacity of cells by directly or indirectly inhibiting the activity of glutathione peroxidase 4 (GPX4) [26], resulting in the oxidation of membrane polyunsaturated fatty acids (PUFAs) out of control, lipid ROS accumulation, irreparable damage to membrane structure and permeability changes, mitochondrial dysfunction, and ultimately leading to ferroptosis [25]. Qi W et al. found that ferroptosis inducer could promote the expression of lncRNA GABPB1-AS1, leading to oxidative stress damage and ferroptosis by inhibiting GABPB1 protein [27]. In the study of patients with cerebral infarction, it was found that the expression of lncRNA PVT1 in plasma increased and the expression of miR-214 decreased [28]. LncRNA PVT1 regulated the occurrence and development of neuronal ferroptosis through miR-214-mediated TFR1 and TP53 expression [28]. Hou J et al. confirmed that LncRNA OIP5-AS1/GPX4 promoted esophageal cancer (EC) development and relieved ferroptosis [29]. All these previous studies presented the effect of LncRNA on regulating ferroptosis in many diseases and neuronal health through mediating miRNA. Our study suggested that in SCI cell models, LncRNA OIP5-AS1 were abnormally reduced while LncRNA OIP5-AS1 overexpression in SCI cells was accompanied by a decrease in GPX4 and FIH1 expression, indicating that LncRNA OIP5-AS1 were involved in suppressing ferroptosis. In addition, the lost mitochondrial membrane potential and the enhanced release of ROS were all reversed in the LncRNA OIP5-AS1-treated neural stem cells after SCI, thereby suppressing apoptosis, accompanied by a decrease in BAX and Caspase-3 expression. Accordingly, LncRNA OIP5-AS1 could protect cells from SCI-induced ferroptosis and apoptosis.

However, the mechanism underlying the effect of LncRNA OIP5-AS1 on SCI has not been reported. The analysis of this study showed that LncRNA OIP5-AS1 could bind to miR-128-3p and regulate the expression of its target Nrf2 protein to affect the development of SCI. The addition of miR-128-3p disrupted the effect of LncRNA OIP5-AS1 on inhibiting ferroptosis and apoptosis. In addition, miR-128-3p overexpression could decrease the level of Nrf2, while enhancing the expression of Nrf2 offset the role of miR-128-3p, thus inhibited the occurrence of ferroptosis and cell apoptosis. Zhang Y et al. previously proved that LncRNA OIP5-AS1 promoted prostate cancer progression and suppressed ferroptosis through miR-128-3p/SLC7A11 signaling [30], which was partly consistent with and support what we have found in this study that LncRNA OIP5-AS1 suppressed ferroptosis through regulating miR-128-3p. In order to verify the therapeutic effect of LncRNA OIP5-AS1 on SCI, we then built a SCI rat model and injected Lenti-OIP5-AS1 for treatment, which confirmed the experimental results in vitro. Lenti-OIP5-AS1 therapy increased the survival rate of neurons, thus effectively relieving spinal cord injury and motor function. qRT-PCR and Western blot results showed that Lenti-OIP5-AS1 therapy significantly reduced the expression of miR-128-3p and increased the level of Nrf2. In addition, Lenti-OIP5-AS1 therapy effectively inhibited iron death and cytochrome C release from mitochondria, indicating that Lenti-OIP5-AS1 could regulate iron death and restore mitochondrial function, which were helpful to alleviate SCI.

There were also limitations in this study. Firstly, more neural cells could be exploited for more primary verification of the effect of LncRNA OIP5-AS1 on SCI. In addition, more in vivo experiments, including more indicators for ferroptosis and mitochondrial dysfunction, should be performed as supplementary for further verifying the existing findings. In the future more in-depth studies, these questions would be delt with at the first time in order to build the firm foundation for further potential clinical usage.

5. Conclusion

These findings provide a baseline for LncRNA-based approaches to regulate SCI, offering new clues of LncRNA OIP5-AS1 in suppressing ferroptosis and protecting neural cells from SCI through miR-128-3p/Nrf2 axis, which may be valuable in future clinical applications.

Ethics approval and consent to participate

This study was approved by Ethics Committee of Shandong First Medical University Affiliated Provincial Hospital (approval no. 2021-243).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Zhensong Jiang: Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Data curation, Conceptualization. **Weimin Zhang:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jianru Zhang:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37704.

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