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MicroRNA-130b inhibits cerebral ischemia/reperfusion induced cell apoptosis *via* regulation of IRF1

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Abstract. – OBJECTIVE: Cerebral ischemia/reperfusion (CIR) frequently causes serious disabilities and correlates with certain neurological processes. Some studies have shown that microRNAs (miRNAs) exert a neuroprotective effect by modulating the inflammatory process in CIR. However, the biofunction and the mechanism of miR-130b in CIR need to be fully elucidated.

MATERIALS AND METHODS: An oxygen-glucose deprivation/reperfusion (OGD/R) model was constructed using SH-SY5Y cell line to analyze the function of miR-130b in CIR. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to examine the expression levels of miR-130b and IRF1. Western blot was performed to detect the protein levels of IRF1, Bax, and Bcl-2. Cell viability was determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays. Dual-Luciferase reporter assay was conducted to confirm the target gene of miR-130b.

RESULTS: In this study, we found that miR-130b level was prominently decreased after treatment with OGD/R. Through gain and loss assays, we concluded that miR-130b restoration promoted cell proliferation and inhibited cell apoptosis in OGD/R-treated cells. Moreover, we also identified IRF1 as an important target of miR-130b. Additionally, IRF1 knockdown remarkably abrogated the protection mediated by miR-130b against the injuries in OGD/R-treated cells.

CONCLUSIONS: Taken together, our results suggested that miR-130b facilitated cell viability and suppressed cell apoptosis of CIR via negatively regulating of IRF1.

Key Words:
Cerebral ischemia/reperfusion, MiR-130b, Apoptosis, IRF1.

Introduction

Cerebral ischemia injury, which arises from the insufficient blood supply to the brain tissues, is one of the death-associated brain diseases with high incidence and mortalities in adults[1]. For cerebral ischemia patients, rapid reperfusion is the standard therapy[2]. However, cerebral ischemia reperfusion (CIR) can lead to brain grave damages, resulting in glutamate excitotoxicity in neurons, apoptosis and oxidative stress, which may seriously impair the neurological functions[3,4]. Currently, clinical treatments for CIR remain limited as its development is dominated by environmental factors and genes jointly[5]. The underlying mechanism involved in the pathogenesis of CIR remains elusive. Therefore, further investigation of the precise mechanisms may be useful for providing novel therapeutic strategies for the prevention and treatment of CIR injury.

Ruike et al[6] have demonstrated that microRNAs (miRNAs) regulated a variety of pathological and physiological processes *via* negative regulation of gene expressions through base paring with the 3′-UTR of target mRNA. MiRNA plays an important role in almost all biological processes, including cell growth, metastases, differentia-

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tion, and apoptosis[7,8]. Especially, in recent years, growing investigations have demonstrated the important functions of miRNAs in developments of central nerve systems[9] and cerebral diseases, such as stroke[10]. The inhibition of miR-200a exerted protective functions in neural stem cell against CIR injury[11]. Moreover, miR-93 inhibited cell apoptosis and inflammatory response of CIR by regulating IRAK4[12]. Additionally, Tang et al[13] indicated that miR-138 could inhibite the CIR induced injuries in rats. Therefore, miRNA has been emerging as a novel therapeutic tool for CIR injury. However, a fully understanding of the functional effects and mechanisms of miR-130b in CIR injury remains obscure, and the target genes of miR-130b deserve further identification.

The interferon regulatory factor-1 (IRF1), a transcription factor, was initially considered to be an important regulator of IFNs and IFN-inducible genes[14]. More recently, it has shown that IRF-1 participates in various physiological processes, such as cellular response to programmed cell death and inflammation[15]. Furthermore, IRF1 has been demonstrated[16,17] to be implicated in the regulation of tumor, immune, and other related diseases. Alexander et al[18] revealed that the knockdown of IRF1 was beneficial to the post-ischemic brain, implying that expressions of IRF1 in neuron took important parts in ischemic neuronal deaths. Iadecola et al[19] found that IRF1 was upregulated after CIR and made great contribution to ischemic brain injury. Therefore, it is vital to identify the underlying regulatory mechanisms of IRF1 in CIR.

Materials and Methods

Cell Culture

Human SH-SY5Y cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) which contained 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a humidified incubator at 37°C containing 5% CO_2.

Oxygen-Glucose Deprivation/ Reperfusion (OGD/R) Model

For OGD/R model, SH-SY5Y cells were incubated in glucose-free DMEM at 37°C for 4 h in hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2). Then, the medium was replaced with glucose containing medium supplemented with 10% FBS again and the cells were cultured under normal conditions (95% air and 5% CO_2) for reperfusion. OGD/R-free cells which were cultured in regular medium under normoxic conditions were used as control.

Cell Transfection

MiR-130b mimics or inhibitor, IRF1 siRNA and the corresponding negative control (NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). Then, Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to transfect them into SH-SY5Y cells in strict line with the manufacturers' instructions. Subsequently, the transfected cells were subjected to OGD/R treatment, and then, be harvested for further analysis.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Then, a Prime Script RT reagent kit (TaKaRa, Otsu, Shiga, Japan) was utilized to reversely transcribe RNA into complementary deoxyribose nucleic acid (cDNA). qRT-PCR reactions were carried out with ABI 7900 Real Time-PCR Detection System (Applied Biosystems; Carlsbad, CA, USA) by SYBR® Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an endogenous control for genes while U6 for miRNAs. The relative expressions were detected by $2^{-\Delta\Delta Ct}$ method. The sequences of qRT-PCR primers were as follows: miR-130b: 5'-CTG GTAGGGTACAGTACTGTGATA-3', miR-130b-R: 5'-CTGGTGTCGTGGAGTCGGC-3'; IRF1-F: 5'-ACCCTGGCTAGAGATGCAGA-3', IRF1-R: 5'- GCTTTGTATCGGCCTGTGTG-3'; U6-F: 5′-CTCGCTTCGGCAG CACA-3′, U6-R: 5′-AACGCTTCACGAATTTGCGT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-F: 5'-GGGAGCC AAAAGGGTCAT-3', GAPDH-R: 5'-GAGTCCTTCCACGATACCAA-3'.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

MTT assays were performed to determine cell proliferation ability. The treated SH-SY5Y cells (treatment with transfection and OGD/R) were plated into 96-well plate and incubated for 0 h,

24 h, 48 h, or 72 h at 37°C. After that, 20 µl MTT solution was added into each well and incubated at 37°C for 4 h. Subsequently, dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Then, a microplate reader (BioTek, Winooski, VT, USA) was used to measure at the absorbance of 490 nm.

Dual-Luciferase Reporter Assay

The wildtype (WT) or mutant (MUT) IRF13'UTR (3′-untranslated region) containing the putative binding sequences of miR-130b were purchased from GenePharma, Co., Ltd. (Shanghai, China) and inserted into pGL3 plasmids (Promega, Madison, WI, USA). SH-SY5Y cells were co-transfected with miR-130b mimics and IRF13'UTR-WT or IRF13'UTR-MUT by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' proposals. The Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was utilized to detect the Luciferase activities at 48 h after transfections. Each experiment was independently repeated at least three times.

Western Blot

To analyze the protein expression levels of specific genes, the treated cells were lyzed in the ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc, Waltham, MA, USA.) was utilized to quantify the protein concentration. Subsequently, the proteins were separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the proteins were subjected to being transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was blocked in Tris-Buffered Saline-Tween (TBST) with 5% skim milk for 2 h at room temperature. After that, the membrane was then incubated at 4°C overnight with specific primary antibodies against IRF1 (1:1000, Abcam, Cambridge, MA, USA), Bax (1:2000, Abcam, Cambridge, MA, USA), Bcl-2 (1:1000, Abcam, Cambridge, MA, USA), GADPH (1:1000, Abcam, Cambridge, MA, USA). Subsequently, the membrane was incubated with an HRP-conjugated secondary antibody (1:2000, Abcam, Cambridge, MA, USA) at room temperature for 2 h. The proteins were visualized using enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAPDH was used as an internal control.

Statistical Analysis

All experiments were repeated at least 3 times. Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA). The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was indicated to be statistically significant.

Results

MiR-130b Expressions were Significantly Decreased in the OGDR Model

We first measured the expression of miR-130b in OGD/R model using qRT-PCR. The results revealed that miR-130b was prominently downregulated in OGD/R model in comparison to the non-OGDR model (Figure 1).

MiR-130b Overexpression Promoted Cell Proliferation

As we confirmed that miR-130b was dramatically downregulated in OGD/R model, we further investigated the effects of miR-130b on SH-SY5Y cell viability by performing MTT assays. Firstly, we overexpressed or inhibited

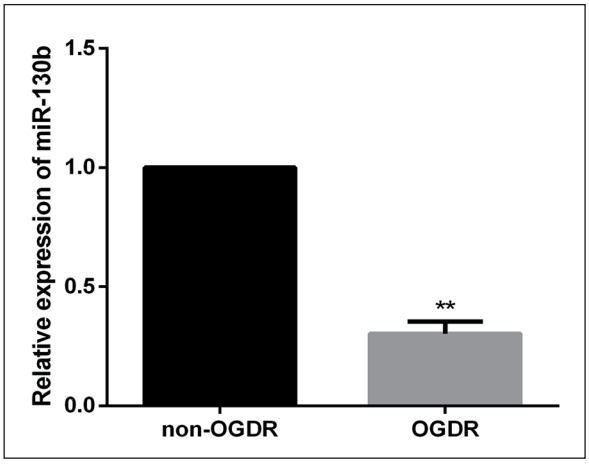

Figure 1. MiR-130b was significantly downregulated in OGD/R-treated SH-SY5Y cells. $^{**}p<0.01$.

the miR-130b expressions in treated (OGD/R) SH-SY5Y cells by transfecting with miR-130b mimics or inhibitor into SH-SY5Y cells, and the transfection efficiencies were estimated by qRT-PCR. As shown in Figure 2A and 2B, miR-130b was successfully overexpressed or suppressed in SH-SY5Y cells. Then, MTT was carried out to evaluate the functions of miR-130b overexpression or inhibition in SH-SY5Y cell viability. It was found that SH-SY5Y cells subjected to OGD/R presented significantly declined proliferation abilities when compared with the non-OGD/R group. In the meantime, the decreased viability of OGD/R SH-SY5Y cells was markedly reversed by miR-130b overexpression (Figure 2C). On the contrary, miR-130b inhibition significantly inhibited the decreased cell viability caused by OGD/R (Figure 2D). These above results indicated that overexpression of miR-130b could reverse effects of OGD/R on SH-SY5Y cell viability.

MiR-130b Upregulation Inhibited Cell Apoptosis

Subsequently, we further investigated the roles of miR-130b in cell apoptosis. In brief, the expressions of apoptosis-associated genes (Bax and Bcl-2) were examined by qRT-PCR and Western blot. The results demonstrated that OGD/R model could significantly enhance the expression of Bax while inhibited Bcl-2 expressions (Figure 3A and 3B). Moreover, we also found that miR-130b restoration remarkably impaired the alteration in Bax and Bcl-2 expressions caused by OGD/R model (Figure 3A and 3B). Furthermore, it was also detected that miR-130b inhibition could facilitate the apoptosis induced by OGD/R model *via* significantly upregulating Bax expressions and downregulating Bcl-2 expression (Figure 3C and 3D). Taken together, we drew the conclusion that miR-130b overexpression could repress cell apoptosis induced by OGD/R model.

IRF1 was a Direct Target Gene of MiR-130b

We, then, explored the potential mechanisms underlying the suppressive functions of miR-130b in OGD/R-induced apoptosis. Firstly, bioinformatics analysis was conducted to explore the potential targets of miR-130b. As shown in Figure 4A, highly conserved miR-130b targeting sites were predicted in IRF1 3′-UTR. Then, Dual-Luciferase reporter assays were performed to confirm the interaction between miR-130b and IRF1. Data revealed that the Luciferase ac-

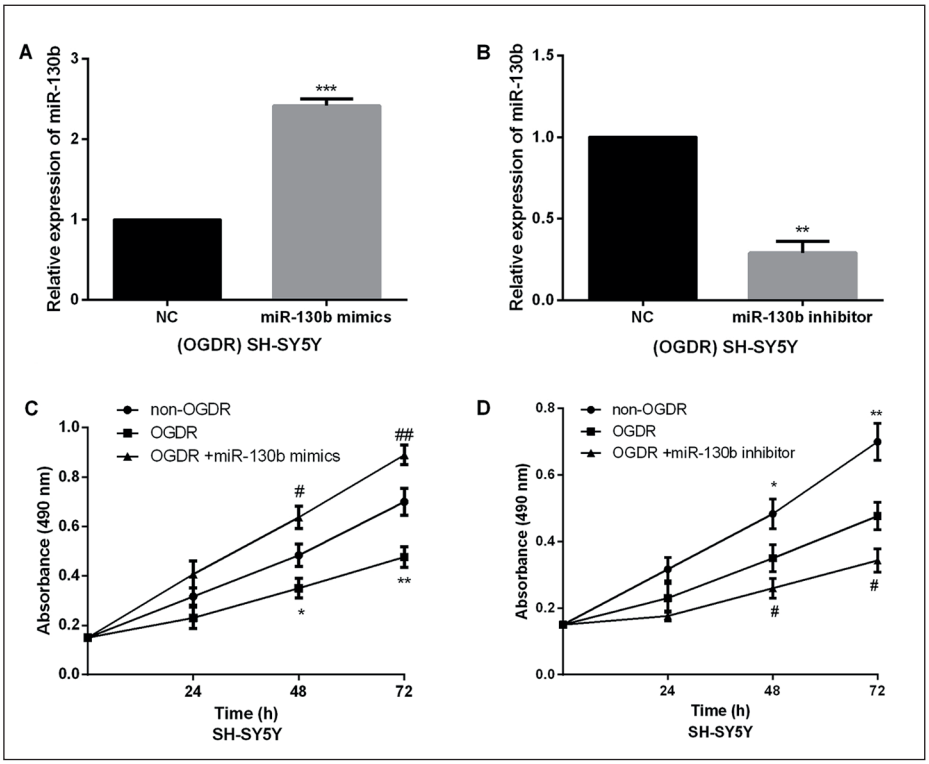

Figure 2. MiR-130b upregulation promoted cell viability. **A, B,** qRT-PCR was performed to observe the overexpression and inhibition in OGD/R-treated SH-SY5Y cells (***p<0.001, **p<0.01). **C, D,** MTT assays were carried out to determine the effects of miR-130b on the viability of OGD/R-treated SH-SY5Y cells. *(p<0.05) and **(p<0.01) represent OGDR *vs.* non-OGDR; #(p<0.05) and ##(p<0.01) represent OGD/R *vs.* OGD/R + miR-130b mimics/inhibitor.

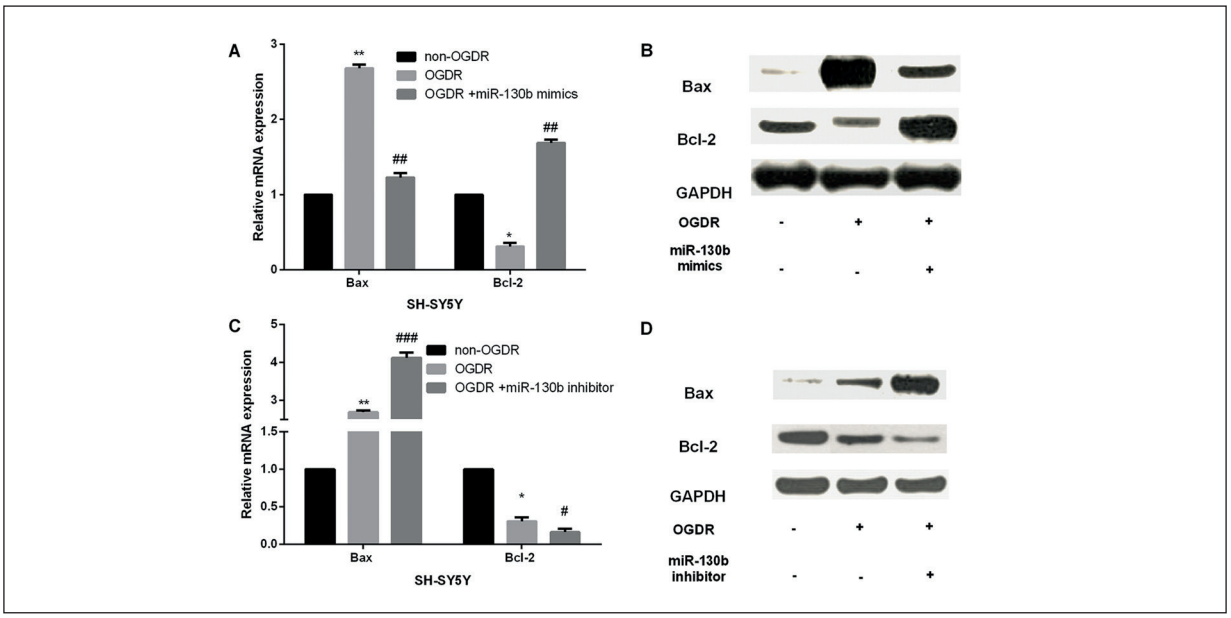

Figure 3. MiR-130b overexpression repressed cell apoptosis. **A, B,** qRT-PCR and Western blot were used to examine the influence of miR-130 mimics on the expressions of apoptosis- associated genes. *(p<0.05) and **(p<0.01) represent OGD/R *vs.* non-OGD/R; ##(p<0.01) represents OGD/R *vs.* OGD/R + miR-130b mimics. **C, D,** The functions of miR-130 inhibition in cell apoptosis were analyzed using qRT-PCR and Western blot. *(p<0.05) and **(p<0.01) represent OGD/R *vs.* non-OGD/R; #(p<0.05) and ###(p<0.001) represent OGD/R *vs.* OGD/R + miR-130b inhibitor.

tivity of the IRF1-3'UTR-WT was significantly suppressed by miR-130b overexpression whereas the IRF1-3'UTR-MUT had no evident effect on the Luciferase activity (Figure 4B). Moreover, we also investigated the regulatory functions of miR-130b on the expressions of IRF1. The data of qRT-PCR indicated that the overexpression of miR-130b evidently suppressed the IRF1 expression while miR-130b inhibition significantly enhanced the IRF1 expression (Figure 4C and 4D). All these findings suggested that IRF1 was a target gene of miR-130b.

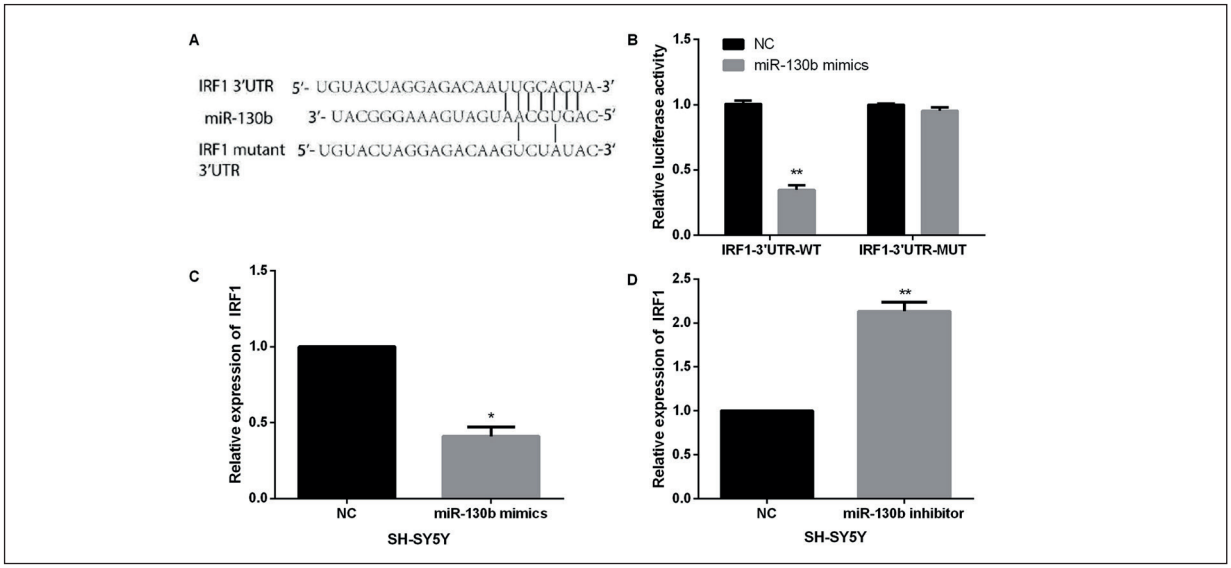

Figure 4. IRF1 was a target of miR-130b. **A,** The predicted binding sites of miR-130b on IRF1 3'UTRs. **B,** The Luciferase activities were detected. **C, D,** The regulatory functions of miR-130b in IRF1 expressions were determined. **p<0.01, *p<0.05.

IRF1 Downregulation Reversed the Protection Effects on OGD/R Model Mediated by MiR-130b

We further investigated whether the functions of miR-130b in regulating OGD/R was modulated by IRF1. In brief, IRF1 siRNAs and miR-130b inhibitor were co-transfected into SH-SY5Y cells with OGD/R-treated. The interfering efficiencies of IRF1 siRNA were assessed by qRT-PCR and western blot analysis. The results showed that IRF1 siRNAs could prominently suppressed the expression of IRF1 both on mRNA and protein levels as shown in Figure 5A and 5B. Then, MTT assays were carried out to determine the impact of IRF1 on cell viability. As shown in Figure 5C, the knockdown of IRF1 markedly reversed the suppressive function in OGD/R-treated SH-SY5Y cell viability mediated by miR-130b inhibitor. Moreover, we also estimated the influence of IRF1 on cell apoptosis by performing Western blot. The results demonstrated that the promoting effects of miR-130b inhibitor on cell apoptosis of OGD/R-treated SH-SY5Y cells were markedly attenuated by IRF1 knockdown (Figure 5D). These findings verified that the OGD/R protective roles of miR-130b were mediated by inhibition of IRF1 expressions.

Discussion

CIR-induced cerebral injury is significantly more severe than ischemic-induced injury[20]. The deficits of neurological functions resulted from CIR have been observed in experimental animals and clinical[21]. Neuronal cell death represents an important aspect of stroke pathophysiologies, and there is evidence[22] suggesting that miRNAs regulate this process, which includes apoptosis and autophagy. For decades, despite being one of the most common injury-induced neurological diseases and exhibiting high mortality and disability rates, ischemic stroke is not effectively treated. Hence, it will be crucial to explore promising therapeutic targets for CIR injury research. Apoptosis is an important form of cell death and plays key

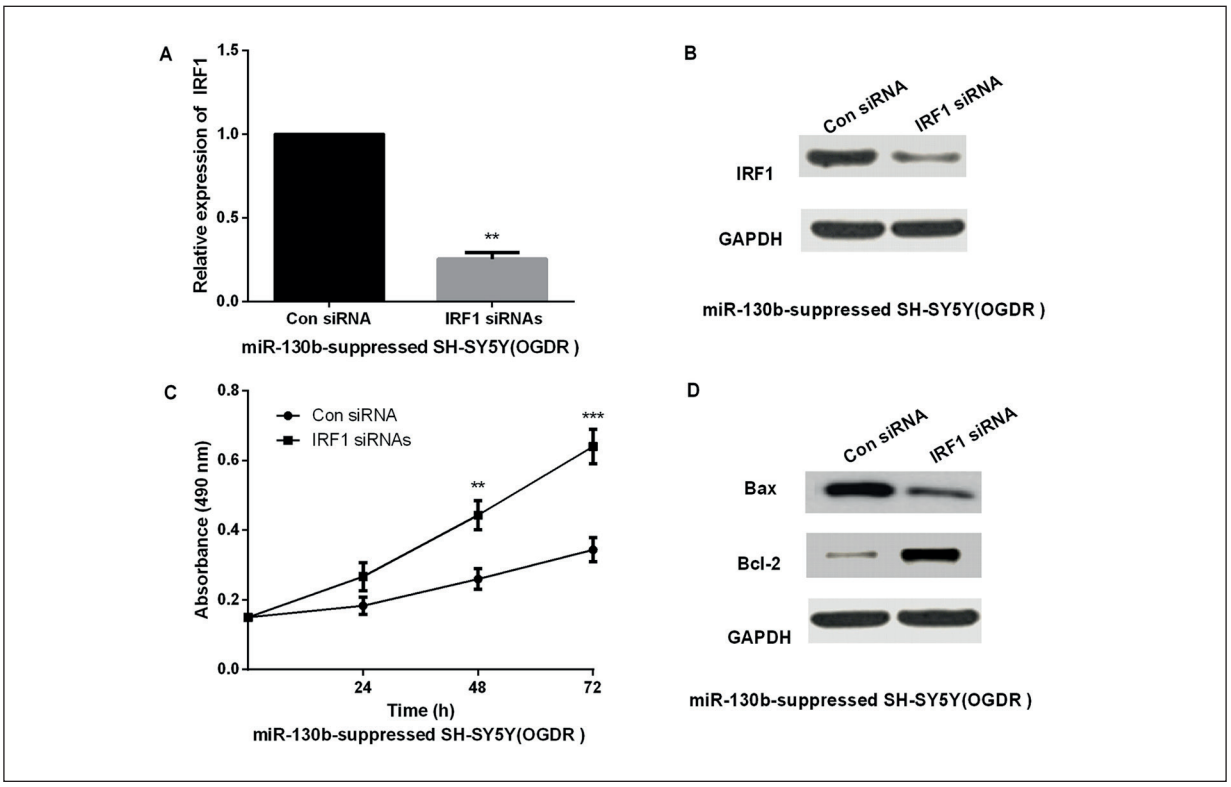

Figure 5. IRF1 knockdown impaired the protective functions of miR-130b. **A, B,** qRT-PCR and Western blot were used to detect the IRF1 expressions. **C,** The impact of IRF1 siRNA on cell viability was assessed using MTT assays. **D,** Western blot was conducted to determine the effects of IRF1 siRNA on cell apoptosis. ***p<0.001, **p<0.01.

roles in CIR injury *via* the activations of the protein cascade associated with apoptosis[23]. There is increasing evidence suggesting that miRNA regulates neuronal death during CIR, which includes autophagy and apoptosis[22]. Jiang et al[24] showed that miR-210 can regulate vagus nerve stimulation-induced anti-apoptosis reaction and antioxidant stress following CIR injury in rats. Zhang et al[25] revealed that miR-25 negatively mediated CIR injury-induced cell apoptosis by Fas/FasL pathway. Fang et al[26] found that miR-544 suppressed cell apoptosis and inflammatory response after CIR by regulating IRAK4. Therefore, elucidating biological functions of miR-130b in CIR may be critical in the identification of diagnostic tools and clinical therapies.

The present study has explored how miR-130b influenced CIR injury, which is an attempt to find a novel therapeutic target for CIR. Here, we have constructed an OGD/R model and found that the level of miR-130b was decreased in OGD/R-treated SH-SY5Y cells. Moreover, miR-130b upregulation prominently promoted cell proliferation of OGD/R-treated SH-SY5Y cells. We also found that miR-130b overexpression exerted inhibitory functions in OGD/R induced cell apoptosis. All these results suggested that miR-130b provided protective roles against CIR.

The effect of miR-130b on cell growth that we observed in SH-SY5Y cells treated with OGD/R was consistent with its role in gastric cancer[27]. To further investigate the role of miR-130b in OGD/R-induced cell apoptosis, we speculated that miR-130b might target a downstream gene to regulate this process. Recently, a growing body of target gene of miR-130b has been identified, including CYLD[28], NKD2[29] and VGLL4[30]. Herein, a new target gene IRF1 was identified as a direct target of miR-130b. Researchers have demonstrated that IRF1 was the target of miR-383[31] and miR-345[32]. However, the relationship between miR-130b and IRF1 in CIR was still controversial. Massa et al[33] indicated that IRF1 played important roles in neural cells. In the present study, we demonstrated that the expression of IRF1 was negatively associated with the miR-130b levels. In addition, IRF1 knockdown could reverse the protective functions mediated by miR-130b in OGD/R-treated SH-SY5Y cells. All these data provided strong basis to affirm that miR-130b played a protective role in CIR by negatively regulating IRF1 expression.

Conclusions

In summary, miR130b was significantly downregulated in SH-SY5Y cells treated with OGD/R. Moreover, miR-130b overexpression could dramatically alleviate OGD/R-induced cell injuries *via* the negative regulation of IRF1 expression. Our results demonstrated that miR-130b may be a potential therapeutic target for CIR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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