Irisin regulates the expression of BDNF and glycometabolism in diabetic rats

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Abstract. Irisin is a proteolytic product of the fibronectin type III domain-containing protein 5. The aim of the present study was to verify whether irisin is involved in the pathogenesis of diabetic mild cognitive impairment and elucidate the associated mechanisms. Diabetic rats were divided into four groups: Control, Model, Irisin (overexpression of irisin) and Irisin-short hairpin (sh)-RNA (irisin interference). The levels of irisin, brain-derived neurotrophic factor (BDNF), glycosylated hemoglobin A1c (GHbA1c) and advanced glycated end products (AGEs) in the serum were determined using ELISA. The expression of BDNF in the hippocampal tissue was evaluated by immunohistochemical analysis. Compared with the Control group, the levels of irisin and BDNF were markedly decreased in the Model and Irisin-shRNA groups, whereas those of GHbA1c and AGEs were markedly increased. However, the levels of irisin and BDNF in the Irisin group were significantly higher than those in the Model group, whereas the levels of GHbA1c and AGEs in the Irisin group were significantly lower. Irisin-shRNA significantly downregulated the expression of irisin and BDNF, and upregulated the levels of GHbA1c and AGEs, compared with those in the Model group. Rat primary hippocampal nerve cells were isolated and identified by microtubule-associated protein-2 labeling. The vitality of primary cells from diabetic rats, evaluated using a methyl thiazolyl tetrazolium assay, was markedly decreased and further reduced following the injection of irisin-shRNA, however, it was markedly improved following irisin treatment. The mRNA and protein levels of BDNF in the primary cells were evaluated by fluorogenic reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively, following the exposure of cells to different concentrations of glucose: 0 (control), 5.5, 15 and 25 mmol/l for 12, 24 and 48 h. The mRNA and protein expression levels of BDNF in the primary cells following exposure to glucose were significantly lower than those observed in the control. Further exposure to glucose led to a significant decrease in the expression of BDNF. In conclusion, irisin may regulate the expression of BDNF and glycometabolism in diabetic rats.

Introduction

Mild cognitive impairment (MCI) is characterized by mild impairment in cognitive function or memory, however, its clinical features are not entirely consistent with the diagnostic criteria for dementia. Patients with MCI retain the ability to manage the majority of aspects of daily life. MCI is a transitional state between normal cognitive function and dementia. Its etiology and pathogenesis remain to be fully elucidated.

Previous studies have shown that there is a close association between MCI and type 2 diabetes (1-3). Glucolipotoxicity, insulin resistance, hypoglycemia and Ca^{2+} homeostasis failure may markedly contribute to the complex pathogenesis of diabetic MCI (4-6). Investigating the pathogenesis and identifying novel therapeutic targets are key to understanding cognitive function in diabetes.

Irisin is a type of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)-dependent muscle factor. PGC-1α mediates energy metabolism, and regulates the expression of uncoupling protein (UCP) 1 and heat production in brown adipose tissue. In addition, it controls mitochondrial biosynthesis and oxidative metabolism in multiple cells (7). PGC-1α stimulates the expression of fibronectin type III domain-containing protein 5 in muscle tissue (8). Following proteolysis, this protein becomes irisin, which is released into the blood (8). Irisin enables the development of brown adipose tissue from white adipose tissue via the activation of UCP1 (9). UCP is a mitochondrial inner membrane protein, which can eliminate the transmembrane proton concentration difference on both sides of the mitochondrial inner membrane, slowing down oxidative phosphorylation driven by proton concentration difference, and hindering the normal production of adenosine triphosphate (9). A previous report revealed that irisin influenced the expression of UCP2-5 in different regions of the brain, including the hypothalamus, pituitary gland,

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hippocampus, cerebellum, striatum and cortex, which may have an effect on certain brain functions (9). At present, irisin is reported to have the following characteristics: i) Exercise can increase the expression level of irisin (8,10,11); ii) irisin can promote energy release *in vitro* and *in vivo* (12); iii) irisin can effectively reduce obesity and insulin resistance (13); and iv) irisin is involved in glycolipid metabolism and is closely associated with brain tissue (14,15).

Therefore, irisin may be involved in the pathogenesis of diabetic MCI. In the present study, in order to confirm this hypothesis and elucidate the associated mechanisms, a diabetic animal model was established. The effects of irisin on the levels of glycosylated hemoglobin A1c (GHbA1c), brain-derived neurotrophic factor (BDNF) and advanced glycated end products (AGEs) in serum, and on the level of BDNF in hippocampal tissues, were investigated using gene silencing and via overexpressing irisin. In addition, primary hippocampal nerve cells were isolated from rats, in order to investigate the effects of irisin on cell viability and the association between glucose concentration and expression levels of BDNF in these cells.

Materials and methods

Materials and animals. Cy3-conjugated goat anti-rabbit IgG (cat. no. CW0159), TRIzol reagent (cat. no. CW0580), the HiFiScript cDNA synthesis kit (cat. no. CW2569) and UltraSYBR mixture (cat. no. CW0957) were purchased from CWBio (Beijing, China). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel; cat. no. 04-007-1A). High-carbohydrate and high-fat diets were purchased from Hunan SLAC Jingda Laboratory Animal Co., Ltd. (Hunan, China; cat. no. M01-20170128). Horseradish peroxidase-conjugated anti-rabbit immuno-globulin G (IgG) was purchased from Boster Biological Technology (Pleasanton, CA, USA; cat. no. SV0002). Neurobasal™ medium (cat. no. 21103049), serum-free B-27™ supplement (50X, cat. no. 17504044) and SuperSignal® chemiluminescent substrate (cat. no. 34077) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Rabbit anti-BDNF monoclonal antibody (cat. no. ab108319) and rabbit anti-microtubule-associated protein-2 (MAP-2) polyclonal antibody (cat. no. ab32454) were purchased from Abcam (Cambridge, MA, USA). Rat BDNF ELISA kit (cat. no. ml302829), rat irisin ELISA kit (cat. no. ml0373721), rat GHbA1c ELISA kit (cat. no. ml024079) and rat AGEs ELISA kit (cat. no. ml003305) were obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd., (Shanghai, China). Recombinant mouse β-nerve growth factor was obtained from Sino Biological, Inc., (Beijing, China; cat. no. 50385-MNAC). Streptozotocin (STZ; cat. no. 415G0316) and L-glutamine (cat. no. G0200) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

A total of 24 male Sprague-Dawley (SD) rats (4 weeks old) were obtained from Shanghai Super-B&K Laboratory Animal Co., Ltd. [License no. SCXK(HU)2013-0016; Shanghai, China]. The animals were fed in a room at 18-26°C, with 40-70% relative humidity, and a natural light/dark cycle. The animals had free access to food and water. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Fujian Medical University (Fuzhou, China).

Establishment of the type 2 diabetes animal model. The 24 SD rats were provided with a normal diet for 1 week. Following acclimatization, the rats were randomly divided into two groups (normal diet group, and high-carbohydrate and high-fat diet group) according to the proportion of 1:3. The rats in the latter group (n=18) were administered with the high-carbohydrate and high-fat diet for 3 months. Subsequently, 1% STZ was intraperitoneally injected into the rats at a dosage of 35 mg/kg. The rats in the former group (n=6) were administered with a normal diet for 3 months, following which normal saline was intraperitoneally injected into the rats. The blood glucose levels were measured and rats were considered to be diabetic when levels were >16.7 mmol/l.

Construction of overexpression and interference vectors. The gene sequences of irisin were searched on NCBI (https://www.ncbi.nlm.nih.gov/nuccore/?term=irisin) and restriction enzyme sites (*Cla*I/*Cla*I) were introduced into the sequences. The irisin genes were ligated to a PDS166_pAd-CMV-GFPa1-IRES vector (Huayueyang Bio Technology Co., Ltd., Beijing, China) to construct an irisin overexpression vector. In addition, a pair of short hairpin (sh)-RNA primers were designed as follows, according to the gene sequences of irisin: Forward 5'-GATCCCCCTCTGTGAACATCATCAAACTCGAGTTT GATGATGTTCACAGAGGGTTTTTG-3' and reverse 5'-AAT TCAAAAACCCTCTGTGAACATCATCAAACTCGAGTTT GATGATGTTCACAGAGGGG-3'. The interference segment was ligated to a pGenesil-1.2-irisin-shRNA plasmid (Huayueyang Bio Technology Co., Ltd.), following which the pShuttle-Basic-E GFP-irisin-shRNA recombinant shuttle and pAdxsi-GFP-irisin-shRNA adenovirus vector (Huayueyang Bio Technology Co., Ltd.) were constructed successively.

Animal grouping. The 24 rats were randomly divided into four groups (n=6): Control, Model, Irisin and Irisin-shRNA. Normal diet-fed rats served as the Control group. The Irisin and Irisin-shRNA groups contained diabetic rats injected with the irisin overexpression and interference vectors, respec-tively. The Model group contained diabetic rats that had not undergone vector injection. The rats in the Control and Model groups were injected with normal saline. Following anesthesia via an intraperitoneal injection of 1% pentobarbital sodium, the skin on the head of the rats was shaved and smeared with iodophor. The skin was then cut open. Subsequently, the hippocampal region was identified according to the coordi-nates AP=4.8, 5 and 4.9, marked and then drilled. The irisin overexpression and interference vectors were injected using a microinjector at a dose of 5 μl per rat. The skin was then sutured and the rats were raised for a further 3 weeks prior to sample collection. Blood from the heart was collected and centrifuged at 2,000 x g and room temperature for 10 min to prepare the serum. The weights of the rats at the onset and end of the experiments are presented in Table I.

ELISA assay. The reagents in the ELISA kits were incubated at room temperature for 30 min prior to use. The ELISA assay was

Table I. Weights of rats at the onset and end of experiments.

Group	Onset (g)						End (g)					
	1	2	3	4	5	6	1	2	3	4	5	6
Control	230	240	230	240	240	230	410	460	378	460	425	492
Model	230	220	230	230	230	240	258	264	311	294	328	301
Irisin	220	220	220	220	230	220	323	302	289	295	309	311
Irisin-shRNA	210	210	220	230	220	220	348	321	329	301	353	301

shRNA, short hairpin RNA.

performed according to the manufacturer's protocol. Briefly, 50 μl of standard and diluted serum were added into the respective wells of plates. Subsequently, 100 μl of enzyme-linked reagents were added into each well. The plates were incubated at 37˚C for 60 min. The mixture in each well was discarded and the developing agents were added. The plates were placed in the dark for 20 min and the absorbance of each well was determined at 450 nm using a microplate reader (RT-6100; Rayto Life And Analytical Sciences Co., Ltd., Shenzhen, China). Zero was set based on blank control wells, which were not exposed to standards, samples or enzyme-linked reagents.

Immunohistochemical analysis. The hippocampal tissues were collected, fixed in paraformaldehyde at 4˚C overnight, embedded with paraffin and cut into slices (4 μm) on a microtome (BQ-318D; Bona Medical Technology, Hubei, China). Following incubation at 75˚C for 1.5 h, the slices were immersed in xylene for 10 min and then in fresh xylene for a further 10 min. The slices were then successively incubated in 100, 95 or 80% ethanol and purified water for 3 min each. Following this, the slices were incubated in citrate buffer solution (antigen retrieval buffer) in a box. Once the antigen retrieval buffer was discarded, the slices were eluted with phosphate buffer solution (PBS) and incubated in fresh 3% hydrogen peroxide in a wet box at room temperature for 10 min to remove endogenous peroxidase blocking buffer. The slices were sufficiently eluted with PBS and normal goat serum (Thermo Fisher Scientific, Inc.) was added dropwise onto the slices at room temperature for 30 min. Excess solution was discarded. Diluted BDNF antibody (1:1,000) was added dropwise onto each slice. Following incubation at 4˚C overnight, the slices were sufficiently rinsed with PBS. Secondary antibody buffer (1:2,000) was added dropwise onto each slice. Following incubation at room temperature for 1 h, the slices were rinsed, stained, dehydrated, mounted and examined under a microscope (CX41; Olympus Corporation, Tokyo, Japan). Quantitative analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Isolation and culture of primary hippocampal nerve cells. The diabetic rats were disinfected with 75% ethanol. The brain was collected following decapitation and immersed in precooled Hank's balanced salt solution (HBSS). The bilateral hippocampus was isolated and the pia mater and blood vessels were removed microscopically. The isolated hippocampus was immersed in 0.125% trypsin and agitated in a water bath at 37˚C for 15 min. Once trypsin was removed, the hippocampus was washed twice with HBSS and suspended in DMEM (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) containing 10% FBS (Biological Industries). The cell suspension was sieved using a 70-μm sieve and then seeded into high-carbohydrate DMEM containing 10% FBS, 1% sodium pyruvate and 1% glutamine. Following culture at 37˚C for 4 h, the medium was replaced by Neurobasal™ medium containing 2% B-27™ and 1% glutamine for further culture. Half of the medium was replaced every 3 days.

Immunofluorescent analysis. Coverslips with growing cells in plates were washed three times in PBS for 3 min each time. The cells growing on the coverslips were fixed in 4% paraformaldehyde for 15 min and washed in PBS. They were then incubated in 0.5% Triton X-100 at room temperature for 20 min. Following washing with PBS, residual PBS was blotted using absorbent paper and normal goat serum was added dropwise onto the coverslips at room temperature for 30 min. The blocking buffer was also blotted using absorbent papers. Diluted primary antibodies (MAP-2 antibody, 1:200) were added dropwise. Following incubation at 4˚C overnight, the coverslips were washed in PBS- 0.05% Tween-20 (PBST) and the residual solution was blotted with absorbent papers. Diluted secondary antibody buffer (1:200) was added dropwise. Following incubation at 37˚C for 1 h, the coverslips were washed in PBST and incubated with 4',6-diamidino-2-phenylindole (DAPI) in the dark for 5 min. The coverslips were subsequently washed in PBST and the residual solution was blotted with absorbent papers. The coverslips were mounted with fluorescent mounting medium and evaluated using a fluorescence microscope (742BR1154; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell viability. The cells were seeded in 96-well plates. When cell confluence reached 80%, 50 μl of methyl thiazolyl tetrazolium (MTT, 0.5 mg/ml) was added into each well. Following culture for a further 4 h, the culture media was discarded and 200 μl DMSO was added. Once the crystal was fully dissolved by vibration for 10 min, the absorbance was determined at 490 nm using a microplate reader (Infinite F200/M200; Tecan Group, Ltd., Männedorf, Switzerland). The relative cell viability was then calculated.

Fluorogenic reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. When the cells grew to 80% confluence, they were cultured for 48 h in media containing different concentrations of glucose: 0 (Control), 5.5, 15 and 25 mmol/l. Total RNA from multiple cells was extracted using TRIzol reagent and reverse transcribed into cDNA using the HiFiScript cDNA synthesis kit, according to the manufacturer's protocol. The primer sequences of the BDNF and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers are listed in Table II. PCR system (25 μl) included RNase free dH2O 9.5 μl, cDNA 1 μl, forward primer 1 μl, reverse primer 1 μl, and 2XULtraSYBR Mixture 12.5 μl (cat. no. CW0957; CWBIO, Beijing, China). The reaction parameters were set as follows: Pre-denaturation for 10 min at 95°C, denaturation for 10 sec at 95°C, annealing for 30 sec at 51.5°C, elongation for 30 sec at 72°C, for 40 cycles. The dissociation curve was analyzed as follows: 15 sec at 95°C, 1 min at 51.5°C, 15 sec at 95°C, 15 sec at 51.5°C, 15 sec at 51.5°C, and measured stepwise from 95°C every 0.5°C. The analysis was eventually performed on a fluorogenic qPCR detection system (CFX Connect™; Bio-Rad Laboratories, Inc.). GAPDH served as the internal control.

The level of irisin in primary hippocampal nerve cells was also determined. Total RNA from the primary hippocampal nerve cells was extracted using TRIzol reagent and reverse transcribed into cDNA using the HiFiScript cDNA synthesis kit, according to the manufacturer's protocol. The primer sequences of irisin and GAPDH are listed in Table II. The reaction parameters were set as follows: Pre-denaturation for 10 min at 95°C, denaturation for 10 sec at 95°C, annealing for 30 sec at 57°C, elongation for 30 sec at 72°C, for 40 cycles. The dissociation curve was analyzed as follows: 15 sec at 95°C, 1 min at 57°C, 15 sec at 95°C, 15 sec at 57°C, 15 sec at 57°C, and measured stepwise from 95°C every 0.5°C. The analysis was performed on a fluorogenic qPCR detection system (CFX Connect™; Bio-Rad Laboratories, Inc.). GAPDH served as the internal control. Relative expression levels of genes were calculated by using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. The cells were 37°C cultured for 48 h in media containing different concentrations of glucose: 0 (Control), 5.5, 15 and 25 mmol/l. The cells were incubated in a lysis buffer in an ice-bath for 30 min, and the resulting lysate was centrifuged at 9,000 x g and 4°C for 10 min. The supernatant was carefully collected to acquire total protein. A BCA protein assay kit was used to measure protein concentration. The protein was denatured and 10 μl protein was loaded into gel lanes to perform SDS-PAGE (12%) for 1-2 h. Polyvinylidene fluoride membrane transfer was performed using the wet transfer method for 30-50 min, and the membrane was subsequently incubated in anti-BDNF antibody buffer (1:2,000) at 4°C overnight. It was then rinsed and incubated in secondary antibody buffer (dilution 1:2,000) at room temperature for 1-2 h. Following the addition of chemiluminescent substrate, the membrane was exposed on a gel imaging system (ChemiDoc™ XRS+; Bio-Rad Laboratories, Inc.). Quantitative analysis was performed using Quantity One software (v4.62, Bio-Rad Laboratories, Inc.). GAPDH served as the internal control.

Table II. Primers for polymerase chain reaction analysis.

Gene	Primer (5'-3')
BDNF	Forward: GCTTATCCTGGTCTTCGG
	Reverse: CTGGGTTGAATGACCTGTT
Irisin	Forward: AAGTGGTCATTGGCTTTGC
	Reverse: GTTGTTATTGGGGCTCGTTGT
GAPDH	Forward: GCAAGTTCAACGGCACAG
	Reverse: CGCCAGTAGACTCCACGAC

BDNF, brain-derived neurotrophic factor.

Table III. Blood glucose levels in the rats of the control, Model, Irisin, and Irisin-shRNA groups.

Group	Blood glucose level (mmol/l)					
	1	2	3	4	5	6
Control	4.6	4.5	3.4	3.1	3.4	3.7
Model	23.9	21.6	31.0	25.9	28.6	27.4
Irisin	25.3	26.0	25.1	26.4	28.6	29.1
Irisin-shRNA	32.9	30.2	21.6	25.6	29.2	24.3

shRNA, short hairpin RNA.

Statistical analysis. The experiments were repeated in triplicate. Data are presented as the mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance followed by a Tukey's post hoc test, using SPSS software (version 19.0, IBM Corps., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Blood glucose levels. The blood glucose levels of rats in the Control, Model, Irisin and Irisin-shRNA groups are presented in Table III. The glycemic concentration in all rats in the Model, Irisin and Irisin-shRNA groups was >16.7 mmol/l, suggesting the successful establishment of the diabetic model.

Serum levels of irisin, BDNF, GHbA1c and AGEs. The levels of irisin, BDNF, GHbA1c and AGEs in the serum of the rats in the Control, Model, Irisin and Irisin-shRNA groups, as determined by ELISA assays, are shown in Fig. 1. It was revealed that, compared with the Control group, the levels of irisin and BDNF in the Model and Irisin-shRNA groups were significantly decreased, and the levels of GHbA1c and AGEs were significantly increased (all P<0.05). However, the levels of irisin and BDNF in the Irisin group were significantly higher than those in the Model group, and the levels of GHbA1c and AGEs in the Irisin group were significantly lower (all P<0.05). Irisin-shRNA significantly downregulated the expression levels of irisin and BDNF and upregulated those of
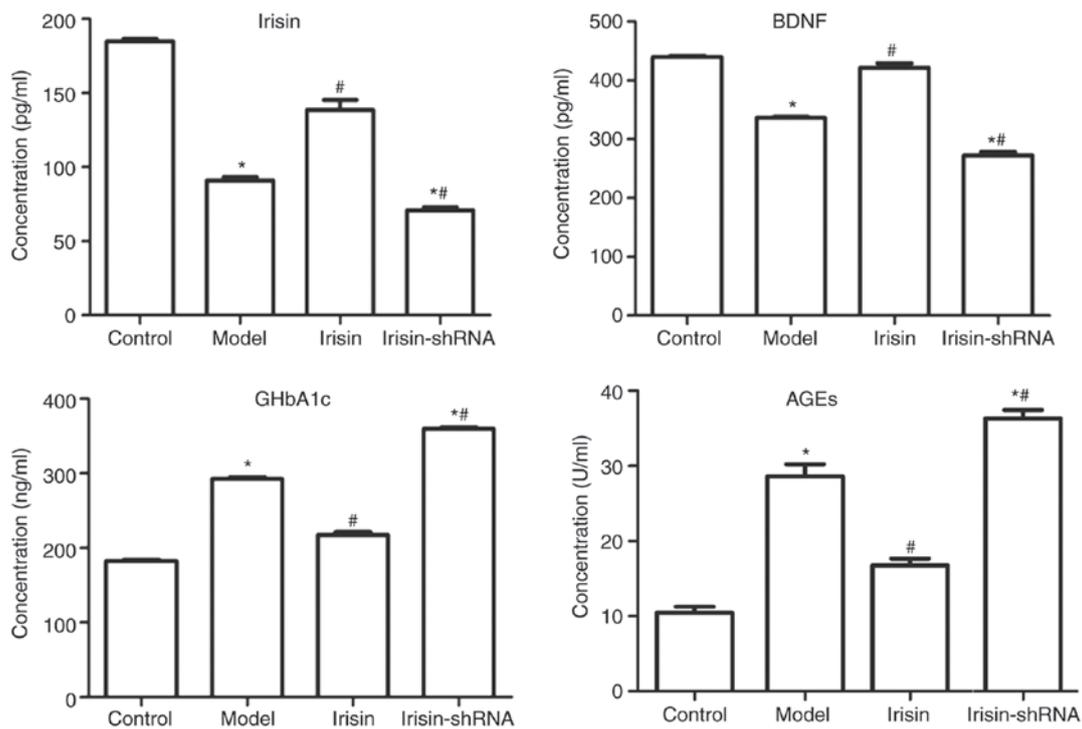
Figure 1. Levels of irisin, BDNF, GHbA1c and AGE in the serum of rats in the Control, Model, Irisin and Irisin-shRNA groups, as determined by ELISA. *P<0.05, vs. the Control group; #P<0.05, vs. the Model group. BDNF, brain-derived neurotrophic factor; GHbA1c, glycosylated hemoglobin A1c; AGE, advanced glycated end product; shRNA, short hairpin RNA.

GHbA1c and AGEs, compared with those in the Model group (all P<0.05).

Expression of BDNF in the hippocampal tissue. The expression of BDNF in the hippocampal tissues of rats in the Control, Model, Irisin and Irisin-shRNA groups, as evaluated by immunohistochemical analysis, is shown in Fig. 2. Brown staining represents the expression of BDNF. High expression of BDNF was observed in the Control and Irisin groups, whereas expression was low in the Model and Irisin-shRNA groups. The quantitative result in the hippocampal tissue was consistent with that observed in the serum. Compared with the Control group, the expression levels of BDNF in the Model and Irisin-shRNA groups were markedly downregulated (P<0.05). However, compared with that in the Model group, the expression of BDNF in the Irisin group was markedly upregulated (P<0.05). Following irinsin-shRNA treatment, the expression of BDNF was significantly downregulated, compared with that in the Model group (P<0.05).

Isolation and identification of primary hippocampal nerve cells. Images of the isolated primary hippocampal nerve cells from diabetic rats, as identified by immunofluorescent analysis, are shown in Fig. 3. The isolated primary cells grew well. MAP-2 protein is a specific protein of the hippocampal neuron. Red fluorescence indicates positive protein expression of MAP-2, and DAPI-emitted blue fluorescence indicates the cellular nuclei. It was demonstrated that there was >98% overlap (pink) of red and blue fluorescence following merging, indicating that the purity of the primary hippocampal nerve cells was >98%. Therefore, primary hippocampal nerve cells were successfully isolated and cultured.
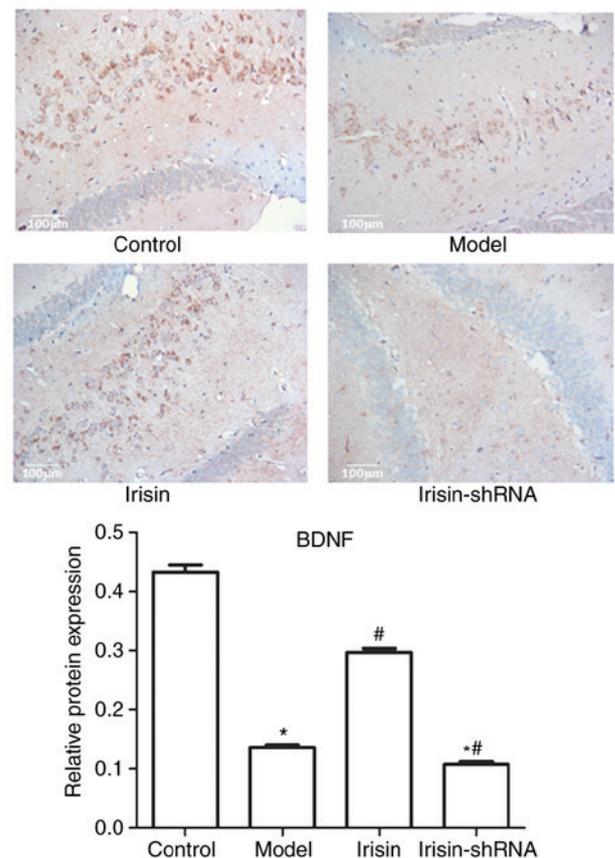
Figure 2. Expression of BDNF in the hippocampal tissue of rats in the Control, Model, Irisin and Irisin-shRNA groups, as evaluated by immunohistochemical analysis. Brown staining indicates BDNF expression. *P<0.05, vs. the Control group; #P<0.05, vs. the Model group. BDNF, brain-derived neurotrophic factor; shRNA, short hairpin RNA.
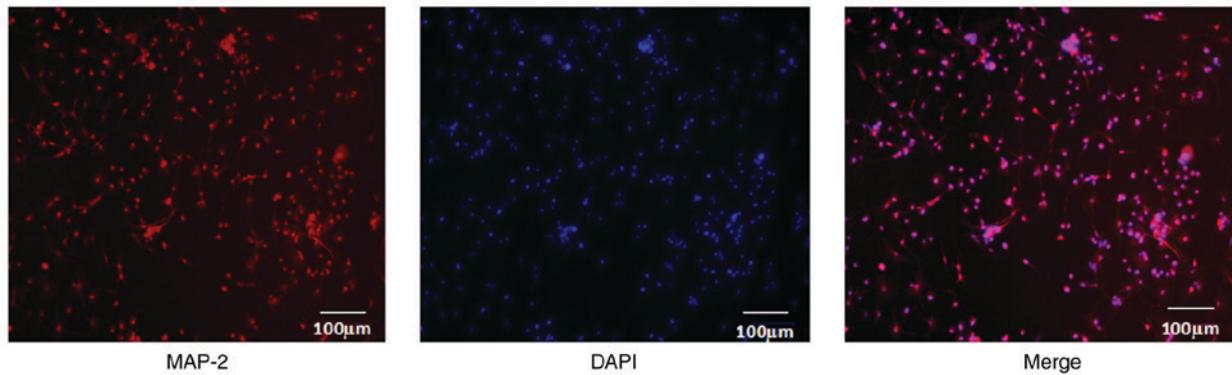
Figure 3. Images of the isolated primary hippocampal nerve cells from diabetic rats, as identified by immunofluorescent analysis. Red fluorescence indicates protein expression of MAP-2, and DAPI-emitted blue fluorescence represents cellular nuclei; >98% overlap (pink) of red and blue fluorescence was observed following merging. MAP-2, microtubule-associated protein-2; DAPI, 4',6-diamidino-2-phenylindole.
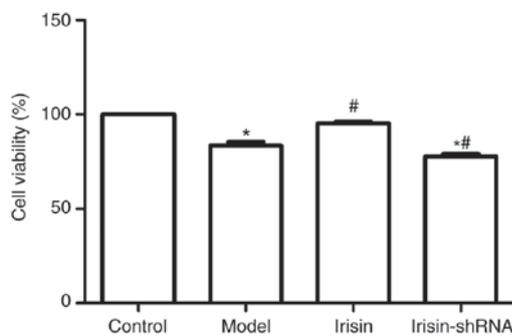
Figure 4. Cell viability in the Control, Model, Irisin and Irisin-shRNA groups, as evaluated via methyl thiazolyl tetrazolium assay. *P<0.05, vs. the Control group; $^\#$P<0.05, vs. the Model group. shRNA, short hairpin RNA.

Figure 6. mRNA levels of BDNF in primary hippocampal nerve cells of diabetic rats, as evaluated by fluorogenic reverse transcription-quantitative polymerase chain reaction analysis. The cells were exposed to different concentrations of glucose: 0 (Control), 5.5, 15 and 25 mmol/l for 12, 24 and 48 h. *P<0.05, vs. the control; $^\#$P<0.05, vs. 5.5 mmol/l; $^+$P<0.05, vs. 15 mmol/l. BDNF, brain-derived neurotrophic factor.

Figure 5. mRNA levels of irisin in the primary hippocampal nerve cells, as evaluated by fluorogenic reverse transcription-quantitative polymerase chain reaction analysis. *P<0.05, vs. the control. shRNA, short hairpin RNA.

Cell viability. Cell viability in the various groups, as determined by the MTT assay, is shown in Fig. 4. The viability of primary hippocampal nerve cells from the diabetic rats was markedly decreased (P<0.05), and was further reduced following the irisin-shRNA injection (P<0.05). However, a marked improvement in cell viability was observed following irisin treatment (P<0.05).

Irisin levels in primary hippocampal nerve cells. Irisin levels in the primary hippocampal nerve cells, as determined by fluorogenic RT-qPCR analysis, is shown in Fig. 5. Following

the administration of irisin and irisin-shRNA, the levels of irisin in the primary hippocampal nerve cells were markedly increased and decreased, respectively (P<0.05). This indicated the successful transfection of irisin and irisin-shRNA genes.

Expression of BDNF in primary hippocampal nerve cells. The expression of BDNF in the primary hippocampal nerve cells of diabetic rats, as determined by fluorogenic RT-qPCR and western blot analyses, is presented in Figs. 6 and 7, respectively. The primary hippocampal nerve cells were exposed to different concentrations of glucose: 0 (Control), 5.5, 15 and 25 mmol/l for 12, 24 and 48 h. Data from fluorogenic RT-qPCR analysis exhibited that, compared with the control, glucose exposure (5.5, 15 and 25 mmol/l) for 12, 24 and 48 h markedly decreased the mRNA production of BDNF in the primary hippocampal nerve cells of rats (all P<0.05). In addition, the mRNA level of BDNF was significantly reduced with the increase of glucose concentration, particularly at the 24- and 48-h time-points (P<0.05). Similar results were observed by the
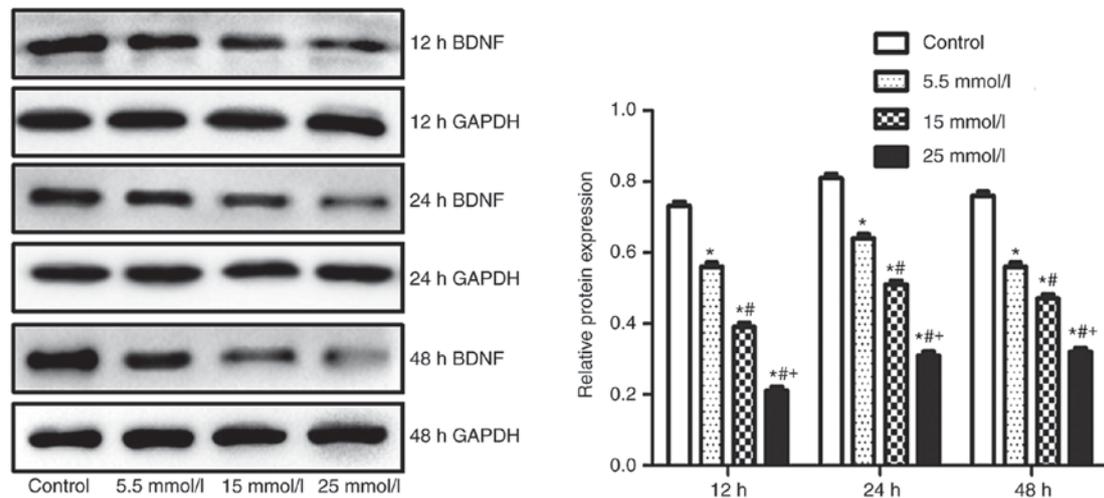
Figure 7. Protein levels of BDNF in primary hippocampal nerve cells of diabetic rats, as evaluated by qualitative and quantitative western blot analyses. The cells were exposed to different concentrations of glucose: 0 (Control), 5.5, 15 and 25 mmol/l for 12, 24 and 48 h. *P<0.05, vs. the control; $^#$P<0.05, vs. 5.5 mmol/l; $^+$P<0.05, vs. 15 mmol/l. BDNF, brain-derived neurotrophic factor.

qualitative and quantitative western blot analyses. The protein expression of BDNF in the primary hippocampal nerve cells of rats following glucose exposure (5.5, 15 and 25 mmol/l) for 12, 24 and 48 h was observed to be significantly lower than that of the control (all P<0.05). Further glucose exposure for 12, 24 and 48 h was shown to significantly decrease the protein expression of BDNF (all P<0.05).

Discussion

MCI is a cognitive disorder syndrome. Compared with normal individuals of a similar age and educational background, patients with MCI exhibit mild cognitive decline, although this does not have an apparent effect on their daily lives (17). The main symptom of MCI is the impairment of cognitive function. According to etiology or location, brain damage can affect one or more cognitive functions, including memory, executive function, language, application and visuospatial structure. As a member of the neurotrophin family, BDNF maintains the integrity of adult neurons and provides important neurotrophic support. It is important in the regulation of synaptic structure, neurotransmission, and the maintenance and consolidation of long-term potentiation, thereby affecting the processes of learning and memory (18). Under normal circumstances, it is abundant in the hippocampus and cerebral cortex (18). An autopsy study indicated that the protein level of BDNF was clearly decreased in the hippocampus and cortex of patients with MCI (19), and an animal study confirmed that the level of BDNF in the brain tissue of humans was positively correlated with that in the serum (20). The expression level of BDNF in the serum of patients with Alzheimer's disease was observed to be decreased, suggesting that a change in BDNF levels in the serum can, to a certain extent, reflect its change in the brain tissue (21). Exercise can not only increase the expression of irisin, but also effectively improve cognitive dysfunction, which has been revealed to be associated with the upregulation of BDNF (22).

GHbA1c is a binding product of hemoglobin in red blood cells and blood glucose. The level of GHbA1c can usually reflect the blood glucose control of patients from the past 8-12 weeks. The generation of GHbA1c from the binding of blood glucose and hemoglobin is irreversible and directly proportional to blood glucose concentration (23). In elderly patients with type 2 diabetic MCI, the levels of irisin and BDNF in the serum were decreased and, with the exception of hypertension and age, GHbA1c was identified as an independent risk factor for cognitive impairment (24,25). GHbA1c usually serves as a biomarker for cognitive impairment (24,25). Therefore, in the present study, the level of GHbA1c was investigated in order to determine cognitive impairment.

AGEs are known to be associated with cognitive function in patients with diabetes mellitus. The non-enzymatic glycosylation of various proteins in the body, induced by continuous hyperglycemia and the resulting AGEs, are important in the pathogenesis of chronic diabetes complications (26). The level of AGEs in diabetic rats is increased (26). AGEs cause changes in the molecular structure and composition of extracellular matrix, resulting in alterations in matrix function (27). In a previous study, immunohistochemical analysis indicated the appearance of AGEs in thickened mesangium and glomerular basement membrane (27).

In the present study, the diabetic model in SD rats was established by an intraperitoneal injection of STZ combined with a high-carbohydrate and high-fat diet; a glycemic concentration >16.7 mmol/l confirmed successful establishment of the diabetic model. The levels of BDNF and irisin in the serum of diabetic rats were decreased, whereas the levels of GHbA1c and AGEs were increased. In addition, it was found that, when the irisin level was enhanced, the above results were reversed. The results from gene silencing and overexpression technologies indicated that irisin was able to positively regulate the expression of BDNF and negatively influence the levels of GHbA1c and AGEs, suggesting that irisin influences cognitive dysfunction in rats with type 2 diabetes by regulating the expression of BDNF and glycometabolism. Of note, not injecting control (scrambled) RNA into the rats of the Model group was a limitation of the present study.

In order to further elucidate the mechanism underlying the effect of irisin on cognitive dysfunction in rats with type 2 diabetes, the effects of irisin on the viability of the primary hippocampal nerve cells was investigated, and the association between glucose concentration and the expression of BDNF in primary hippocampal nerve cells of diabetic rats was examined. The primary hippocampal nerve cells were successfully isolated and identified through the double-labeling of specific protein MAP-2 and cellular nuclei. The vitality of the primary hippocampal nerve cells from diabetic rats was markedly decreased; however, marked improvement was observed following irisin treatment. Furthermore, the expression of BDNF in the primary hippocampal nerve cells was observed to decrease as glucose concentration and glucose exposure time increased. The results of the present study were consistent with those of a previous report which revealed that, when glucose concentration increased, the ratio of paraventricular nucleus (PVN) neurons decreased, resulting in a decrease in the expression of BDNF (28). BDNF had direct independent effects on PVN neurons, which were regulated by local glucose concentration (28). These results demonstrated that irisin in the serum enhance the vitality of primary hippocampal nerve cells and affect glycometabolism in rats with type 2 diabetes.

However, certain signaling pathways may differ between rats and humans, therefore, investigations on human serum are required. The repetition and validation of the findings of the present study are also required, due to possible rat reactivity to specific factors. In addition, the western blot analysis results were semiquantitative and, as ELISA is an example of a quantitative technique, future investigations on human serum require quantification using ELISA rather than western blot analysis.

In conclusion, irisin was shown to regulate the expression of BDNF and glycometabolism in rats with type 2 diabetes. Irisin may serve as a promising novel target for the treatment of diabetic MCI. The development of irisin-targeting drugs and therapies may assist in preventing the occurrence of MCI in type 2 diabetes. The results of the present study are of positive significance for the prevention of diabetic dementia.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LH and LY designed study and wrote paper. SY and LL collected and analyzed data. All authors performed study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Fujian Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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