mfat-1 transgene protects cultured adult neural stem cells against cobalt chloride–mediated hypoxic injury by activating Nrf2/ARE pathways

Junfeng Yu† | Haiyuan Yang† | Bin Fang | Zhengwei Zhang | Ying Wang | Yifan Dai

1Jiangsu Key Laboratory of Xenotransplantation, Nanjing Medical University, Nanjing, People’s Republic of China
2Huaian First Hospital Affiliated to Nanjing Medical University, Hua’ian, People’s Republic of China

Correspondence
Dr. Yifan Dai and Dr. Ying Wang. Jiangsu Key Laboratory of Xenotransplantation, Nanjing Medical University, 101 Longmian Avenue, Nanjing, 211166, People’s Republic of China. Phone: 0086-25-86869477. Email: daiyifan@njmu.edu.cn and ywang@njmu.edu.cn.

Funding information
This work was supported by the National Natural Science Foundation of China (no. 81570402) and the Sanming Project of Medicine in Shenzhen, Fund for High Level Medical Discipline Construction of Shenzhen (no. 2016031638); Yifan Dai, Ying Wang, and Haiyuan Yang are Fellows at the Collaborative Innovation Center for Cardiovascular Disease Translational Medicine, Nanjing Medical University.

Abstract
Ischemic stroke is a devastating neurological disorder and one of the leading causes of death and serious disability in adults. Adult neural stem cell (NSC) replacement therapy is a promising treatment for both structural and functional neurological recovery. However, for the treatment to work, adult NSCs must be protected against hypoxic-ischemic damage in the ischemic penumbra. In the present study, we aimed to investigate the neuroprotective effects of the mfat-1 transgene on cobalt chloride (CoCl2)-induced hypoxic-ischemic injury in cultured adult NSCs as well as its underlying mechanisms. The results show that in the CoCl2-induced hypoxic-ischemic injury model, the mfat-1 transgene enhanced the viability of adult NSCs and suppressed CoCl2-mediated apoptosis of adult NSCs. Additionally, the mfat-1 transgene promoted the proliferation of NSCs as shown by increased bromodeoxyuridine labeling of adult NSCs. This process was related to the reduction of reactive oxygen species. Quantitative real-time polymerase chain reaction and Western blot analysis revealed a much higher expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream genes (HO-1, NQO-1, GCLC). Taken together, our findings show that the mfat-1 transgene restored the CoCl2-inhibited viability and proliferation of NSCs by activating nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response elements (ARE) signal pathway to inhibit oxidative stress injury. Further investigation of the function of the mfat-1 transgene in adult protective mechanisms may accelerate the development of adult NSC replacement therapy for ischemic stroke.

KEYWORDS

1 | INTRODUCTION

Ischemic stroke, also known as cerebrovascular accident, is one of the leading causes of death and serious long-term disability in adults across the world (Mozaffarian et al., 2016). There is currently no therapy that sufficiently improves clinical recovery after ischemic stroke (Sims & Muyderman, 2010; Stankowski & Gupta, 2011). Adult neural stem cell (NSC) replacement therapy is a promising treatment for neurological recovery both structurally and functionally: It includes endogenous NSC regeneration to replace damaged tissue or neural cells in adulthood and exogenous adult NSC transplant after injuries such as ischemic stroke (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002; Chung et al., 2015; Koch, Kokaia, Lindvall, & Brustle, 2009; Paradisi et al., 2014). Adult NSCs are clinically important not only because ischemic...
stroke occurs mainly in adults but also because they are easy to obtain, with no medical ethics problems and no tumorigenicity (Giusto, Donega, Cossetti, & Pluchino, 2014). In the ideal scenario, soon after ischemic stroke, endogenous or exogenous adult NSCs would exhibit proliferation, migration, and differentiation to repair neural function damage (Chung et al., 2015; Cramer, 2008). However, because of the loss of nutrients and oxygen in the ischemic penumbra, the majority of newly generated NSCs die soon after stroke, and their physiological function is lost (Azevedo-Pereira & Daadi, 2013; Bazan, Marcheselli, & Cole-Edwards, 2005; Rosenblum et al., 2015). Therefore, figuring out how to protect adult NSCs against hypoxic-ischemic injury after ischemic stroke is key to effective adult NSC replacement therapy.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have sparked clinical interest in their therapeutic application for promoting the survival, migration, proliferation, and differentiation of adult NSCs during replacement therapy in ischemic stroke. n-3 PUFAs are essential for human beings, helping to maintain cellular membrane structural and functional integrity (Antony, Vanni, Shindou, & Ferreira, 2015). Moreover, PUFAs are highly enriched in the brain and play a key role in brain development and repair under many conditions (Chang et al., 2012). Decades of research have provided insight into the elevation of the n-3/n-6 PUFA ratio, demonstrating that a higher ratio rather than n-3 PUFAs exerts beneficial effects in a variety of neurological disorders, including ischemic stroke (Belavev, Khoutorova, Atkins, & Bazan, 2009; Belavev et al., 2011; Hong, Belavev, Khoutorova, Obersaas, & Bazan, 2014; Hu et al., 2013; Liu et al., 2016; Zhang et al., 2010).

In this study, NSCs were cultured from an mfat-1 transgene mouse model that converts n-6 PUFAs to n-3 PUFAs in vivo, resulting in abundant endogenous n-3 PUFA, without changing total PUFA in their organs and tissue, through overexpressing the C. elegans n-3 fatty acid desaturase gene, mfat-1 (Wei et al., 2010). This mfat-1 transgene mouse model was firstly used to investigate whether overproduction of n-3 PUFAs could protect adult NSCs against hypoxic damage induced by cobalt chloride (CoCl2), which is a well-known hypoxia-mimetic agent. There are numerous reports that CoCl2 is widely used to mimic the hypoxic-ischemic microenvironment in various cultured cells (Chen, Zhao, & Huang, 2009; Lan et al., 2012; Sandner et al., 1997; Tan et al., 2009; Zou et al., 2001). Therefore, we used CoCl2-treated adult NSCs as an in vitro model to study the adult NSCs’ response to hypoxic-ischemic injury. And we demonstrated that elevation of the n-3/n-6 PUFAs ratio significantly enhanced the survival of adult NSCs in a CoCl2-mediated hypoxic injury model, but the underlying molecular protective mechanisms are still not fully understood. It has been reported that oxidative stress–induced neuronal apoptosis plays an important role in the pathogenesis of ischemic stroke (Chehalbi, Trabelsi, Mahdouani, & Slimane, 2016; Yamauchi et al., 2016). Brain tissues and cells are rich in PUFAs, which are more susceptible to oxidative stress injury (Guichardant et al., 2004). Cellular defense against oxidative stress injury is mainly mediated by antioxidative enzymes and detoxifying enzymes, including glutathione (GSH), heme-oxygenase-1 (HO-1), glutamate cysteine ligase catalytic subunit (GCLC), and NADPH quinone oxidoreductase 1 (NQO-1) (Dinkova-Kostova & Talalay, 2008; Zhang et al., 2013). The expression of antioxidative enzymes and detoxifying enzymes is regulated by nuclear factor erythroid 2-related factor 2/antioxidant response pathways (Nrf2/ARE). Then Nrf2-responsive genes are transcribed by Nrf2 through binding to ARE in the promoter region of the target gene, which leads to the expression of antioxidative enzymes and detoxifying enzymes. Our investigation showed that the Nrf2/ARE signal pathway is involved in this process. The results are of great importance not only for broadening the theory on adult NSCs’ protective mechanism after brain ischemic injury but also for innovating strategies for adult NSC clinical replacement therapy in ischemic stroke.

## 2 MATERIALS AND METHODS

### 2.1 Animals

In mfat-1 transgene mice, the coding region of C. elegans fat-1 cDNA was optimized to enhance the expression of fat-1 in mammalian cells. The fat-1 cDNA driven by a cytomegalovirus enhancer and chicken β-actin promoter linked with a muscle creatine kinase enhancer was introduced into C57BL/6 mice (RRID: IMSR_JAX:000664) by pronuclear microinjection (Wei et al., 2010). The fat-1 heterozygotes of C57/B6 and wild-type (WT) C57/B6 mice were crossed to produce fat-1 transgene mice and WT littermates. Both of them were bred in the Animal Core Facility of Nanjing Medical University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University, China. Every effort was made to minimize animal suffering. All experiments were performed in accordance with the approved guidelines for animal care and management of research projects.
2.2 Genomic DNA extraction and genotype identification

The adult (8-10 weeks) mfat-1 transgenic mice and WT littermates were identified by genotyping using polymerase chain reaction (PCR) amplification. Genomic DNA was extracted from mice tails using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The PCR primers used for the mfat-1 gene were forward 5’- GGAACCCTTGTTGAAAGAGCATCCG-3’ and reverse 5’-CGGTCCGAGAGCAGCAAC-3’. PCR product was 438 bp. The PCR conditions were as follows: 94°C for 5 min (1 cycle), 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s (35 cycles). The PCR products were separated by 1.5% agarose gel electrophoresis.

2.3 Primary adult mouse NSCs isolation and culture

The primary adult NSCs were isolated from the subventricular zone (SVZ) of adult mfat-1 transgenic mice and WT littermates at 8 to 10 weeks. Briefly, NSCs from 8- to 10-week-old adult mfat-1 transgenic mice and adult WT littermates were designated as NSCs\textsubscript{fat-1} and NSCs\textsubscript{WT}, respectively. First, under sterile conditions, the adult mouse brain was dissected out to a 60-mm tissue culture dish with cold phosphate buffer solution (PBS) (Gibco BRL, Grand Island, Ny, USA). Then, the coronal section of the brain was cut into 2- to 3-mm-thick slices with a new razor blade, and slices were placed in fresh PBS. The tissues were separated from SVZ with fine forceps under a dissecting microscope, then cut into small squares (approximately 1.0 mm\textsuperscript{3}) with forceps and put into 1.5-ml tubes containing SFM solution (described below). The tissues were then centrifuged for 5 min at 350g, followed by removal of the supernatant and lysing with 0.5 ml 1% trypsin (Gibco BRL, Grand Island, Ny, USA) at 37°C for 20 min. After centrifuging for 5 min at 350g, the supernatant was removed, 0.5 ml of trypsin inhibitor was added (Roche, Mannheim, Germany), and the sample was centrifuged for 5 min at 350g. The cells were suspended in 0.5 ml of SFM and triturated sufficiently to produce a single-cell suspension. After adding 1.5 ml of SFM containing the cells to each well of a 12-well plate (Corning, Ny, USA), all cultures were incubated at 37°C with 95% air and 5% CO\textsubscript{2}.

The adult stem cells were floating-cultured in serum-free medium and passaged every 4 to 6 days when the neurospheres grew to a diameter of approximately 150 \( \mu \)m. The SFM consisted of DMEM/F12 medium (1:1; Gibco BRL, Grand Island, Ny, USA) containing 28.5 \mu mol/ml NaHCO\textsubscript{3} (Sigma-Aldrich, St. Louis, MO, USA) 33.3 \mu mol/ml glucose (Sigma-Aldrich, St. Louis, MO, USA), 1 nmol/ml HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA), 0.2 nmol/ml progesterone (Sigma-Aldrich, St. Louis, MO, USA) 108.9 nmol/ml putrescine (Sigma-Aldrich, St. Louis, MO, USA), 2% B27 supplement (Molecular Probes; Invitrogen, Carlsbad, CA, USA), 1% insulin-transferrin-sodium selenite supplement (ITSS, Roche, Mannheim, Germany), 5 ng/ml basic fibroblast growth factor (FGF; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), and 1.83 \mu g/ml heparin (Sigma-Aldrich, St. Louis, MO, USA). SFM was filtered with a 0.22-\mu m-

2.4 Immunocytochemistry identification of NSCs\textsubscript{fat-1} and NSCs\textsubscript{WT}

The primary neurospheres were passaged with Accutase solution (Sigma-Aldrich, St. Louis, MO, USA), and the passaged cells were called first passage (P1). The third passage cells were used for all the subsequent experiments. For immunocytochemistry identification experiments, the third passage cells were triturated to single cells and planted at a density of \( 1 \times 10^{5} \) cells per milliliter on dishes that were coated with Poly-D-Lysine (Sigma-Aldrich, St. Louis, MO, USA) and sterile laminin (Roche, Mannheim, Germany). For differentiation studies, EGF was omitted and FGF was used at half the concentration of SFM, and then 1% FBS was added (Gibco BRL, Grand Island, Ny, USA). The cultures were fully differentiated for up to 7 days.

For immunofluorescent staining, cells were fixed with 4% paraformaldehyde for 30 min and washed in PBS (Gibco BRL, Grand Island, Ny, USA), pH 7.4, for 10 min, 3 times. After blocking in PBS containing 5% goat serum and 0.3% Triton X-100 at room temperature for 1 hr, the cells were incubated with primary antibodies at 4°C overnight. The primary antibodies used were as follows: mouse anti-nestin (BD Biosciences, 556309, RRID: AB_1645170), rabbit anti-NeuN (Abcam, ab177487, RRID: AB_2532109), chicken anti-II-III-tubulin (Millipore Bioscience Research Reagents, AB9354, RRID: AB_570918), mouse anti-glial fibrillary acidic protein (GFAP) (Millipore Bioscience Research Reagents, AB5804, RRID: AB_2109645), mouse anti-O4 (Millipore Bioscience Research Reagents, MAB345, RRID: AB_94872). After primary antibody incubation, the NSCs were rinsed 3 times with PBS and incubated in the corresponding fluorescent-conjugated secondary antibody (Table 2). Finally, cultures were counterstained with DAPI (Vector Laboratories Inc.) to label the nuclei.

2.5 Gas chromatography analysis of fatty acid profiles

Lipid extraction from integrated mouse brain tissues and appropriate cultured adult NSCs was performed according to the general technique reported previously (Lai et al., 2006). An Agilent 7890A (Agilent Technologies, Santa Clara, CA) did the gas chromatography. Fatty acid components were identified by comparison of retention times with those of authentic standards (Sigma-Aldrich, St. Louis, MO, USA). The ratio of n-3 to n-6 was calculated by the areas of peaks.

2.6 Adult NSCs hypoxic damage induced by CoCl\textsubscript{2}

CoCl\textsubscript{2} (Sigma-Aldrich, St. Louis, MO, USA), a chemical hypoxia-mimetic agent (Cheng et al., 2017; Naves, Jawhari, Jauberteau, Ratinaud, & Verdier, 2013), was dissolved in sterile deionized water to prepare 50 mM stock solution. Different terminal concentrations (0, 50, 100, 200, 300, 400 \mu M) were added to NSCs\textsubscript{WT} to develop the adult NSC hypoxia model in vitro (Tan et al., 2009). The NSCs\textsubscript{WT} were seeded
into 96-well plates at a density of $5 \times 10^4$ cells per well and were treated with CoCl$_2$ at different terminal concentrations for 24 hr (Cheng et al., 2017; Lan et al., 2012; Stenger, Naves, Verdier, & Rata", 2011). After the CoCl$_2$ treatment, cell viability was detected using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, Cell Titer-Glo regent equal to the volume of the cell culture medium was added to each well, and the culture was then incubated at room temperature for 10 min to stabilize the luminescence signal. The luminescence signal was recorded using a multimode microplate reader (BioTek, Synergy 2, CA). The outcomes were expressed as relative cell viability (%), and half the maximal inhibitory concentration of CoCl$_2$ was designated as the optimal condition to simulate adult NSC ischemia/hypoxia (Liu et al., 2014; Tan et al., 2009).

### 2.7 Adult NSCs viability assay
Dissociated NSCs$^{sr}$ and NSCs$^{WT}$ were precultured in opaque-walled 96-well plates at a density of $5 \times 10^4$ cells with 100 $\mu$l of SFM per well in quintuplicate, followed by hypoxic injury induced by CoCl$_2$ treatment. Then cell viability was investigated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The luminescent signal value was measured with a multimode microplate reader (BioTek, Synergy 2, CA, USA).

### 2.8 Adult NSCs caspase-3 activity assay
Caspase-3 activity was detected using a caspase-3 colorimetric assay kit (BIOBOX, China) according to the manufacturer’s instructions. Ac-DEVD-pNA was used as the substrate in this assay. Briefly, the Ac-DEVD-pNA complex was dissociated by active caspase-3, releasing fluorescent pNA; thus, the fluorescent pNA reflected the activity of caspase-3. After incubation with CoCl$_2$, cells were collected and lysed in lysis buffer on ice for 30 min. The protein concentrations of the supernatant fluids were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Intracellular GSH level assay was performed using a reduced GSH assay kit (Njjcbio, China). Briefly, third-passage adult NSCs were seeded into 6-well plates at a density of $1 \times 10^5$ cells per milliliter with five replicates in each group and subjected to the various treatments described previously. Subsequently, adult NSCs were collected and lysed on ice for 30 min. The protein concentrations of the supernatant fluids were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Intracellular GSH was then detected according to the protocol described by the manufacturer. Absorbance at 405 nm was measured with a microplate reader (BioTek, EON, CA, USA). The experiment was repeated independently 5 times.

### 2.9 Adult NSCs proliferation index by 5-bromodeoxyuridine labeling
Proliferation of cells was evaluated by 5-bromodeoxyuridine (BrdU) incorporation assay. Dissociated adult NSCs were planted at a density of $1 \times 10^5$ cells per milliliter into 24-well plates with coverslips, which were coated with Poly-D-Lysine (Sigma-Aldrich, St. Louis, MO, USA) and sterile laminin (Roche, Mannheim, Germany). After 4 hr, experimental groups were treated with CoCl$_2$ at 200 $\mu$M terminal concentrations for 22 hr and then incubated with 10 $\mu$M BrdU (Sigma-Aldrich, St. Louis, MO, USA) for 2 hr in each group. Immunocytochemical assay was used to determine the incorporation. The cultured cells were fixed in 4% paraformaldehyde for 15 min and treated with 2 M HCl for 20 min at room temperature; 0.1 M sodium borate (pH 8.5) was then added for 2 min. Blocked unspecific binding with 5% normal goat serum was performed for 30 min, followed by incubation with anti-BrdU monoclonal antibody (Abcam, ab2284, RRID: AB_302944) at room temperature for 2 hr; donkey anti-sheep IgG Alexa Fluor 594 (Invitrogen, A21099, RRID: AB_141474) was used as the secondary antibody. Nuclear DNA was labeled in blue with DAPI. The percentage of BrdU$^*$ cells was ascertained by randomly counting eight nonoverlapping microscopy fields of three coverslips for each condition; the average count was 120 cells per field. Independent experiments were done in triplicate.

### 2.10 Measurement of reactive oxygen species formation
Intracellular reactive oxygen species (ROS) levels were detected by ROS fluorescent probe–dihydroethidium (DHE) assay according to the manufacturer’s instructions. Briefly, the adult NSCs of each group were incubated with 1 $\mu$M DHE (Vigorous Biotechnology, Beijing, China) in the dark for 30 min. Fluorescence was measured with a Nikon ECLIPSE 80i fluorescence microscope and quantified using Image-Pro Plus analysis software (http://www.mediacy.com; RRID: SCR_007369).

### 2.11 Determination of reduced GSH generation
The intracellular GSH level assay was performed using a reduced GSH assay kit (Njjcbio, China). Briefly, third-passage adult NSCs were seeded into 6-well plates at a density of $1 \times 10^5$ cells per milliliter with five replicates in each group and subjected to the various treatments described previously. Subsequently, adult NSCs were collected and lysed on ice for 30 min. The protein concentrations of the supernatant fluids were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Intracellular GSH was then detected according to the protocol described by the manufacturer. Absorbance at 405 nm was measured with a microplate reader (BioTek, EON, CA, USA). The experiment was repeated independently 5 times.

### 2.12 Quantitative real-time PCR
Total RNA was extracted from cultured adult NSCs of each group using Qiagen RNaseasy Mini Kit (Qiagen, Germany). RNA samples were reverse transcribed to cDNA using Hiscript II Q RT SuperMix (Vazyme, Beijing, China). Nrf2, HO-1, NQO-1, and GCLC mRNA expression were quantified by real-time PCR (RT-PCR) with specific primers (Table 1). Samples consisted of 1 $\mu$l of cDNA per well of each reaction plate using an SYBR Green master mixture (Vazyme, Beijing, China) and normalized to $\beta$-actin. The LightCycler96 RT-PCR system (Roche, Mannheim, Germany) was used for quantitative RT-PCR analysis.

### 2.13 Western blot analysis
Adult NSCs were washed twice with precooled PBS and lysed using a Nuclear and Cytoplasmic Protein Extraction Kit (BIOBOX, Nanjing, China)
TABLE 1 | PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>TTTCTTCCACGACATCCACGACCCG</td>
<td>ACAGGCTTCAATACTGCCGTCCAG</td>
</tr>
<tr>
<td>HO-1</td>
<td>CAAGCCGGAATGCTGAGTTCA</td>
<td>GCAAGGGATGATTCTCTGCCCAG</td>
</tr>
<tr>
<td>NQO-1</td>
<td>GCGAGAAGAGCCCTGATTGATG</td>
<td>TCTCAACCCAGCTTTCCAGATGG</td>
</tr>
<tr>
<td>GCLC</td>
<td>ACATCTACACGCGAGTCAAGGACC</td>
<td>CTCAGAAGACCTGCTCCATTACAG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TCGTGGCTGACATTAAGAGAA</td>
<td>GTTGAGATGTTCTGCTGGATGC</td>
</tr>
</tbody>
</table>

Note: GCLC = glutamate cysteine ligase catalytic subunit; HO-1 = heme-oxygenase-1; NQO-1 = NADPH quinone dehydrogenase 1; Nrf2 = nuclear factor erythroid 2-related factor 2.

2.14 | Antibody characterization

All antibodies used in this study are listed in Table 2.

Mouse Anti-Rat Nestin, Clone 401 (BD, 556309, RRID: AB_1645170). Nestin is an intermediate filament protein that is abundantly expressed in neuroepithelial stem cells early in embryogenesis, but is absent from nearly all mature central nervous system cells. Staining pattern was identical to that reported in publications using the same antibodies (Frederiksen & Mckay, 1988).

Anti-O4, Clone 81 (Millipore, MAB345, RRID: AB_94872). O-antigens are sulfatides, which function as differentiation markers on the surface of oligodendrocytes of the central nervous system. O4 is formed postnatally and is a marker for cell bodies and processes of oligodendrocytes types I and II. The anti-O4 antibody has been routinely evaluated by immunocytochemistry, as demonstrated previously by others using the same antibody (Li et al., 2011; Schachner et al., 1981).

Anti-GFAP, Lot 2558352 (Millipore, AB804, RRID: AB_2109645). GFAP is the main constituent of intermediate filaments in astrocytes and serves as a cell-specific marker that distinguishes differentiated astrocytes from other glial cells during the development of the central nervous system. The staining pattern of cells and brain tissues was identical to those described in publications using the same antibodies (Benarroch et al., 2007).

Anti-Beta III Tubulin, Lot 2676286 (Millipore, AB9354, RRID: AB_570918). According to technical information from Millipore, class III beta-tubulin is a microtubule element expressed exclusively in neurons, and it is a popular marker specific for neurons in tissue. Specificity of the antibody was assessed by the manufacturer. The antibody against beta III tubulin has been used by others to visualize immature neurons of mice (Guo et al., 2011).

Anti-Nrf2 Clone EP1808Y (Abcam, ab62352, RRID: AB_944418). Transcription activator that binds to ARE elements in the promoter regions of target genes. Important for the coordinated upregulation of genes in response to oxidative stress. The antibody was evaluated by Western blotting, and results were as expected from previous descriptions (Turpaev & Drapier, 2009).

Anti-NQO1 Clone A180 (Abcam, ab28947, RRID: AB_881738). NQO1 belongs to the NAD(P)H dehydrogenase (quinone) family and is located in the cytoplasm. The Western blot of NQO1 in neural cells was consistent with that reported by others (Siegel et al., 1998).

Anti-HO-1 Clone EPR1390Y (Abcam, ab68477, RRID: AB_11156457). According to technical information from Abcam, this antibody recognizes mouse, human, and rat HO-1, which is suitable for Western blot and immunohistochemistry. HO-1 exhibits cytoprotective effects since an excess of free heme sensitizes cells to undergo apoptosis. Our Western blot results were consistent with those from other published articles using the same antibody (He et al., 2014; Maruyama et al., 2014).

Anti-GCLC (Abcam, ab80841, RRID: AB_2107804). This antibody recognizes the heavy subunit of GCLC. The GCLC antibody detected only the expected protein (73kD) on Western blot of mouse brain. The results of Western blot with GCLC were as expected from foregoing descriptions.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Description of immunogen</th>
<th>Source, host species, catalog no., clone or lot no., RRID</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Rat(E15) spinal cord extracts</td>
<td>BD mouse IgG1, Cat.# 556309, Clone Rat 401, RRID: AB_1645170</td>
<td>1:500</td>
</tr>
<tr>
<td>O4</td>
<td>Homogenate of white matter of corpus callosum from bovine brain</td>
<td>Millipore mouse monoclonal, Cat.# MAB345, RRID: AB_94872</td>
<td>1:25</td>
</tr>
<tr>
<td>GFAP</td>
<td>Purified bovine GFAP</td>
<td>Millipore rabbit polyclonal, Cat.# AB9504, RRID: AB_2109645</td>
<td>1:500</td>
</tr>
<tr>
<td>Beta III tubulin</td>
<td>Synthetic peptide from human/rat beta III tubulin</td>
<td>Millipore chicken polyclonal, Cat.# AB9354, RRID: AB_570918</td>
<td>1:500</td>
</tr>
<tr>
<td>NeuN</td>
<td>Synthetic peptide within Human NeuN aa 1-100 (Cysteine residue).</td>
<td>Abcam rabbit monoclonal, Cat.# ab177487, RRID: AB_2532109</td>
<td>1:500</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine coupled to keyhole limpet hemocyanin (KLH)</td>
<td>Abcam sheep polyclonal, Cat.# ab2284, RRID: AB_302944</td>
<td>1:500</td>
</tr>
<tr>
<td>Nurf2</td>
<td>Synthetic peptide within human Nrf2 aa 550 to the C-terminus</td>
<td>Abcam rabbit monoclonal, Cat.# ab62352, RRID: AB_944418</td>
<td>1:1,000</td>
</tr>
<tr>
<td>HO-1</td>
<td>Synthetic peptide within Human Heme Oxygenase 1aa 1-100 (N terminal)</td>
<td>Abcam rabbit monoclonal, Cat.# ab68477, RRID: AB_11156457</td>
<td>1:1,000</td>
</tr>
<tr>
<td>NQO1</td>
<td>Recombinant full-length proteinof human NQO1</td>
<td>Abcam mouse polyclonal, Cat.# ab28947, RRID: AB_881738</td>
<td>1:1,000</td>
</tr>
<tr>
<td>GCLC</td>
<td>Synthetic peptide corresponding to Rat GCLC aa 295-313</td>
<td>Abcam rabbit polyclonal, Cat.# ab80841, RRID: AB_2107804</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Histone H3</td>
<td>Synthetic peptide within human histone H3 aa 1-100 conjugated to keyhole limpet hemocyanin</td>
<td>Abcam rabbit polyclonal, Cat.# ab9050, RRID: AB_306966</td>
<td>1:1,000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Recombinant protein</td>
<td>Proteintech mouse IgG2b, Cat.# HRP-60004, RRID: AB_2107436</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>SANTA CRUZ goat anti-rabbit IgG secondary antibody, HRP conjugate Cat.# sc-2004, RRID: AB_631746</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>SANTA CRUZ goat anti-mouse IgG secondary antibody, HRP conjugate, Cat.# sc-2005, RRID: AB_631736</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Mouse IgG(H+L)</td>
<td>Abcam goat anti-mouse IgG(H+L) secondary antibody, Alexia Fluor 594 conjugate, Cat.# ab307-21, RRID: AB_10897916</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Sheep IgG</td>
<td>Invitrogen donkey anti-sheep IgG secondary antibody, Alexia Fluor 594 conjugate, Cat.# A21099, RRID: AB_141474</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Rabbit IgG(H+L)</td>
<td>Abcam goat anti-rabbit IgG(H+L) secondary antibody, FITC conjugate, Cat.# ab6718, RRID: AB_955551</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Rabbit IgG(H+L)</td>
<td>Abcam goat anti-rabbit IgG(H+L) secondary antibody, Alexia Fluor 594 conjugate, Cat.# ab96901, RRID: AB_10679699</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Chicken IgY(H+L)</td>
<td>Thermo Fisher scientific goat anti-chicken IgY(H+L) secondary antibody, Alexia Fluor 594 conjugate, Cat.# SAS-10072, RRID: AB_2556652</td>
<td>Invitrogen goat anti-mouse IgG secondary antibody, Alexia Fluor 488 conjugate, Cat.# A21121, RRID: AB_141514</td>
<td>1:5,000</td>
</tr>
</tbody>
</table>
2.15 Statistical analysis

Comparisons between two groups were subject to a two-tailed Student test. The differences between means of multiple groups were assessed by ANOVA followed by the Bonferroni/Dunn post hoc test. Statistical analysis was performed in SPSS 23 statistical software (RRID: SCR_002865; IBM, Armonk, NY). All data were presented as the mean ± SD of at least three independent experiments, and statistical significance was defined as \( p < .05 \).

3 RESULTS

3.1 Identification of NSCs\textsuperscript{fat-1} and NSCs\textsuperscript{WT} in vitro

Tail samples were taken from adult mfat-1 transgenic mice and WT littermates. The genotypes of fat-1 transgenic mice and WT littermates were identified by PCR amplification. The PCR analysis showed high expression of the fat-1 gene in mfat-1 transgenic mice (lanes 1, 2, and 3) and negative expression in WT mice (lanes 4, 5, and 6) (Figure 1A). NSCs are capable of self-renewal and have a proliferative and multipotent capacity through...
TABLE 3  Analysis of n-6 and n-3 PUFA composition

<table>
<thead>
<tr>
<th>Sample</th>
<th>LA</th>
<th>AA</th>
<th>Total n-6</th>
<th>EPA</th>
<th>DPA</th>
<th>Total n-3</th>
<th>n-3/n-6 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCsWT</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.73</td>
<td>0.04</td>
<td>0.72</td>
<td>0.07</td>
</tr>
<tr>
<td>Fat-1 brain</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
<td>0.85</td>
<td>0.07</td>
<td>0.92</td>
<td>0.08</td>
</tr>
<tr>
<td>WT brain</td>
<td>0.04</td>
<td>0.02</td>
<td>0.06</td>
<td>0.85</td>
<td>0.07</td>
<td>0.92</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Note: The cultured third-passage NSCs and whole mouse brains were collected from fat-1 transgenic mice and WT control littermates. Compositions of n-3 or n-6 PUFAs were expressed using relative percentages—that is, the distribution areas of n-3 or n-6 PUFA peaks divided by the total peak areas of all detectable saturated and unsaturated free fatty acids (from the same sample) resolved from the gas chromatography column. Data are mean ± SD; n = 3. *Student t test, p < .001 compared with NSCsWT. #acid; DHA = docosahexaenoic acid; ALA = α-lipoic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; AA = arachidonic acid. Data are mean ± SD; n = 3.

3.2 | Expression of the mfat-1 transgene elevated the n-3/n-6 PUFA ratio

Fatty acid analyses of mfat-1 transgenic mice and WT littermates were performed using GC-MS. The whole mouse brains and cultured third-passage NSCs were detected. As shown in Table 3, NSCs<sup>fat-1</sup> exhibited a decreased proportion of n-6 PUFAs, including AA, and increased expression of n-3 PUFAs, including EPA, DPA, and DHA, which resulted in sharp elevation of n-3/n-6 ratio relative to those in the NSCs<sup>WT</sup> (1.28 ± 0.06 in NSCs<sup>fat-1</sup> vs. 0.36 ± 0.02 in NSCs<sup>WT</sup>; Student t test, n = 3, *p < .001). Compared with NSCs, the ratio of n-3/n-6 in the mouse brain was modestly increased (0.74 ± 0.02 in fat-1 brain vs. 0.67 ± 0.01 in WT brain; Student t test, n = 3, *p < .001).

3.3 | NSCs<sup>fat-1</sup> activity was enhanced against CoCl<sub>2</sub>-induced hypoxic injury

Cultured cells exposed to CoCl<sub>2</sub> for 24 hr are widely used to establish a hypoxic injury model (Li et al., 2013; Tan et al., 2009; Zhang, Qian, Pan, Li, & Zhu, 2012). To identify the optimal CoCl<sub>2</sub> concentration, NSCs<sup>WT</sup> were treated with different CoCl<sub>2</sub> concentrations (0, 50, 100, 200, 300, and 400 μM) for 24 hr, and cell viability was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega). As shown in Figure 2A, NSC viability was robustly reduced in a dose-dependent manner. Exposure to 200 μM CoCl<sub>2</sub> for 24 hr resulted in a cell viability loss of about 50%, which was identified as the optimal experimental condition for an adult NSC hypoxic injury model in vitro (Liu et al., 2014).

We then investigated the protective effects of the high ratio of n-3/n-6 PUFA on NSCs<sup>fat-1</sup> that were treated with the adult NSC hypoxic injury model. After 200 μM CoCl<sub>2</sub> exposure for 24 hr, as shown in Figure 2B, the cell viability rate of NSCs<sup>fat-1</sup> + CoCl<sub>2</sub> was significantly increased compared with NSCs<sup>WT</sup> + CoCl<sub>2</sub> (68.2% ± 5.9% in NSCs<sup>fat-1</sup> + CoCl<sub>2</sub> vs. 49.3% ± 3.1% in NSCs<sup>WT</sup> + CoCl<sub>2</sub>; Student t test, n = 3, *p < .001). The results indicate that NSCs<sup>fat-1</sup> were protected against CoCl<sub>2</sub>-induced hypoxic injury.

3.4 | mfat-1 transgene inhibited CoCl<sub>2</sub>-mediated NSCs<sup>fat-1</sup> apoptosis

After 200 μmol/l CoCl<sub>2</sub> treatment for 24 hr, we detected the caspase-3 activity of each group. Only a small amount of intrinsic apoptosis was detected in cultured NSCs<sup>fat-1</sup> and NSCs<sup>WT</sup> without CoCl<sub>2</sub> treatment, and caspase-3 activity was almost the same between the two groups.
NSCsfat-1 showed a significant decrease in caspase-3 activity (32.7% ± 2.9%) compared with NSCsWT+CoCl2(24h) (51.4% ± 3.1%) (**p < .01; Figure 2C). NSCsfat-1 exhibited anti-hypoxic injury properties. These findings indicate that a high n-3/n-6 PUFA ratio could protect NSCs against CoCl2-mediated hypoxic injury.

3.5 | *mfat-1* transgene promoted proliferation of adult NSCs against CoCl2-mediated injury

To further investigate the anti-hypoxic damage effects of a high n-3/n-6 PUFA ratio in adult NSCs, we used the BrdU labeling assay to quantify the adult NSC proliferation rate. BrdU staining showed no
significant difference between the NSCfat-1 and NSCWT groups before CoCl2-mediated hypoxic injury (37.6% ± 1.8% in NSCfat-1 vs. 38.2% ± 3.1% in NSCWT; Student t test, n = 5, p > .05; Figure 3A,B). Around 24.58% ± 2.61% of cells were found to be BrdU+ in the NSCWT + CoCl2(24h) group, while the rate of BrdU positivity was significantly decreased in the NSCfat-1 + CoCl2(24h) group, in which only 17.13% ± 2.44% of cells were found to be BrdU+ (**p < .01; Figure 3A,B). The results suggest that a high n-3/n-6 PUFA ratio protected cultured adult NSCs from CoCl2-mediated hypoxic injury and promoted the proliferation of adult NSCs against CoCl2-induced hypoxic injury.

3.6 | mfat-1 transgene improved CoCl2-mediated adult NSC injury through antioxidative damage

It has been reported that oxidative stress–induced adult NSC apoptosis plays a critical role in the pathogenesis of ischemic stroke and its complications (Chehaibi et al., 2016; Yamauchi et al., 2016). Oxidative stress is a primary factor that has been shown to affect adult NSC function, differentiation, and survival (Wang, Liu, et al., 2014). To determine whether the protective role of the mfat-1 transgene on adult NSCs in hypoxic-ischemic damage might relate to oxidative stress, we...
measured intracellular superoxide formation by DHE assay and then examined intracellular GSH levels with a reduced GSH assay kit. The DHE assay consists of fluorogenic probes designed to reliably measure ROS in live cells, whose signal is localized primarily in the nucleus. As shown in Figure 4B, when NSCs^{fat-1} and NSCs^{WT} were exposed to 200 μmol/l CoCl_{2} for 3 hr, a significant difference existed between NSCs^{fat-1} + CoCl_{2}(3h) and NSCs^{WT} + CoCl_{2}(3h) (124% ± 7.9% vs. 148% ± 9.8%, *p < .05); when NSCs^{fat-1} and NSCs^{WT} were exposed
FIGURE 5. NSCsfat-1 upregulated the Nrf2-ARE signal pathway and increased the expression of downstream genes and phase II detoxification genes (HO-1, NQO-1, GCLC). (A) In CoCl2-treated groups, quantitative RT-PCR indicated that NSCsfat-1 induced significant increases in the mRNA expression level of Nrf2 and its downstream genes and phase II detoxification gene transcripts HO-1, NQO-1, and GCLC compared with NSCsWT + CoCl2. β-actin was used as an internal control. Values represent the mean ± SD of three independent experiments. *p < .05, **p < .01 vs. the same-time CoCl2-treated group. NSCsfat-1 were treated with 200 μM CoCl2 or vehicle for 24 hr, and were lysed and fractionated to isolate nuclear and cytosolic fractions as indicated. (B, C) Western blotting analysis and quantification of cytoplasmic and nuclear Nrf2 protein. Cytoplasmic and nuclear proteins were normalized by GAPDH and histone H3, respectively (n = 3). (D, E, F) Western blotting analysis and quantification of HO-1, NQO-1, and GCLC protein expression separately (n = 3). Data are presented as mean ± SD; *p < .05, **p < .01, and ***p < .001 vs. control. ##p < .01 and ###p < .001 vs. NSCsWT + CoCl2(24h) group. [Color figure can be viewed at wileyonlinelibrary.com]
to 200 μmol/l CoCl₂ for 24 hr, the intracellular ROS level (mean fluorescence intensity) significantly increased (248% ± 4.54% of control, ***p < .001), and there was a significant difference between the two groups (173% ± 11.72% in NSCsfat-1 + CoCl₂(24h) vs. 248% ± 9.54% in NSCsWT + CoCl₂(24h), **p < .01), revealing that NSCsWT exposed to CoCl₂ displayed intense fluorescence after being stained with DHE probe reagent, while intracellular ROS accumulation resulting from CoCl₂ exposure was remarkably reduced in NSCsfat-1 (Student t test, n = 3, *p < .05, **p < .01; Figure 4A,B).

To further explore possible mechanisms underlying the beneficial effects of a high n-3/n-6 PUFA ratio, a reduced GSH assay kit was used to evaluate the expression of GSH, a potent ROS scavenger, in cultured NSCsWT and NSCsfat-1 after CoCl₂ insult. Production of GSH was detected at different time points in NSCsfat-1 and NSCsWT after CoCl₂ insult. As shown in Figure 4C, a statistical difference existed between NSCsWT and NSCsfat-1 after CoCl₂ insult for 3 hr. We also investigated the 24-hr time point. The results show that the production of GSH increased notably in NSCsfat-1 compared with NSCsWT after CoCl₂ insult for 24 hr (10.63 ± 0.71 in NSCsfat-1 + CoCl₂(24h) vs. 5.24 ± 1.18 in NSCsWT + CoCl₂(24h)); Student t test, n = 5, ***p < .001; Figure 4C). Collectively, oxidative stress is implicated in the pathogenesis of CoCl₂-mediated hypoxic injury in vitro, and a high n-3/n-6 PUFA ratio exhibits a protective effect on adult NSCs by increasing GSH levels and enhancing the capacity of scavenging ROS.

3.7 | Nrf2-ARE signal pathway is involved in protective effects of NSCsfat-1

Several recent studies have shown Nrf2 to be a critical transcription factor that regulates a battery of antioxidant enzymes in the response to oxidative stress (Ma, 2013). Therefore, to study the antioxidative mechanisms, we first investigated whether Nrf2, the principal transcription factor that regulates the basal and inducible expression of a battery of antioxidant enzymes, was upregulated. Quantitative RT-PCR analysis showed that in the 24-hr CoCl₂-treated groups, NSCsfat-1 robustly increased the protein expression of nuclear Nrf2 compared with NSCsWT (Figure 5B), while the expression level of cytosolic Nrf2 in NSCsfat-1 was significantly decreased compared with NSCsWT (Figure 5C). In addition, the expression of Nrf2-related antioxidant defense enzymes such as HO-1, NQO-1, and GCLC was substantially increased (Figure 5D–F). These results indicate that the mfat-1 transgene promoted the nuclear translocation of Nrf2 and upregulated endogenous antioxidants through an Nrf2-dependent signaling pathway in CoCl₂-mediated adult NSC hypoxic injury.

4 | DISCUSSION

The cell injury induced by hypoxia is a primary concern in various clinical fields, such as ischemic stroke and NSC transplantation (Chen, Zhang, Gu, & Guo, 2016). CoCl₂-induced cell hypoxic damage in NSCs can serve as a simple and convenient in vitro model (Chen et al., 2009; Sandner et al., 1997; Tan et al., 2009; Zou et al., 2001), which provides a chance to investigate the effect of a high n-3/n-6 PUFA ratio on adult NSCs against hypoxic-ischemic damage in vitro and to elucidate the underlying molecular mechanisms.

Decades of research have shown that a high n-3/n-6 PUFA ratio could protect the neural system against hypoxic-ischemic damage. Hu et al. (2013) reported that n-3 PUFA supplementation is a potential neurogenic and oligodendroglione treatment to naturally improve post-stroke brain repair and long-term functional recovery. Using a mouse model of transient focal cerebral ischemia, researchers concluded that n-3 fatty acids could potentially play a role in poststroke cerebrovascular remodeling (Wang, Shi, et al., 2014). The n-3 PUFA exerted protective effects on neurons against ischemic injury both in vitro and in vivo, partly through inhibiting ROS activation (Shi et al., 2016). However, little information is available on the role of n-3 PUFA in protecting adult NSCs from hypoxic injury after CoCl₂-induced hypoxic damage at a cellular level.

In the present study, we first investigated the effects of CoCl₂ on adult NSCs and successfully established an adult NSC hypoxic injury model in vitro. To explore the protective effects of the high ratio of n-3/n-6 PUFAs on adult NSCsfat-1 subjected to the adult NSC hypoxic injury model, the cell viability and cell apoptosis were investigated by CellTiter-Glo Luminescence Cell Viability Assay (Promega) and caspase-3 colorimetric assay (BIOBOX, China), respectively. The results show that a high n-3/n-6 PUFA ratio could exert protective effects against CoCl₂-induced cell viability loss and also decreased the apoptotic rate throughout the experiment. Next, an experiment was performed to detect the proliferation index of adult NSCs against CoCl₂-mediated injury, which showed that the rate of BrdU positivity was significantly increased in the NSCsfat-1 + CoCl₂(24h) group compared with the NSCsWT + CoCl₂(24h) group. Collectively, the results indicate that mfat-1 transgenic adult NSCs with a high n-3/n-6 PUFA ratio were protected from hypoxic damage induced by CoCl₂.

The mechanisms underlying the protective effect of the mfat-1 transgene against hypoxic damage to adult NSCs from CoCl₂ are still
not fully understood. Previous studies have reported that oxidative stress--induced neuronal apoptosis plays an important role in the pathogenesis of ischemic stroke (Chehabil et al., 2016; Yamashima et al., 2016). Recent investigations have indicated that overproduction of n-3 PUFAs is highly effective in protecting the brain, and that there are protective mechanisms involved in Nrf2 activation and HO-1 upregulation (Zhang et al., 2014). One study also found that n-3 PUFAs enhanced the phosphorylation of Akt after ischemic stroke by regulating phosphatidyserine (Akbar, Calderon, Wen, & Kim, 2005). However, these factors may not clarify the adult NSCs' protective mechanism. In the present study, we demonstrated that oxidative stress is implicated in the pathogenesis of CoCl2-mediated hypoxic injury in vitro, and that a high n-3/n-6 PUFA ratio exerts a protective effect on NSCs by increasing GSH levels and enhancing the capacity of scavenging ROS. Subsequently, we investigated the Nrf2/ARE signal pathway, a key pathway of antioxidative stress. Nrf2 is a transcription factor that plays a key role in cytoprotection against oxidative stress (Dou et al., 2016; Ma, 2013). Under basal conditions Nrf2 is mostly retained in the cellular cytosol by binding of its Neh2 domain to Keap1, which in turn is anchored to actin cytoskeleton (Kobayashi et al., 2004). Keap1 is a cysteine-rich cytosolic protein that functions as an adaptor, leading to the proteasomal degeneration of Nrf2 via ubiquitination. Under stressful conditions, the Nrf2–Keap1 complex is dissociated and Nrf2 rapidly undergoes nuclear translocation, initiating the expression of antioxidative and detoxifying enzymes (Dou et al., 2016). The activated Nrf2 upregulates the transcription of numerous antioxidant and phase II detoxification genes to provide protective effects in many neurological diseases (Nanou et al., 2013; Zhang et al., 2014). Our quantitative RT-PCR analysis showed that a much higher expression of Nrf2 and its downstream genes (HO-1, NQO-1, GCLC) was found in the NSCsfat-1 + CoCl2(24h) group than in the NSCsWT + CoCl2(24h) group. Subsequently, Western blot results showed that a high n-3/n-6 PUFA ratio enhanced Nrf2 translocation from the cytoplasm to the nucleus of cultured adult NSCs after CoCl2 treatment for 24 hr and substantially increased the expression of Nrf2-related antioxidant defense enzymes such as HO-1, NQO-1, and GCLC. In summary, our results reveal that a high n-3/n-6 PUFA ratio has a neuroprotective effect on adult NSCs against CoCl2-mediated hypoxic injury, and the underlying molecular mechanism may involve the activation of the Nrf2/ARE pathway. Although our study has revealed the neuroprotective mechanism of mtfat-1, the potential antioxidant effects of mtfat-1 have yet to be established clearly, but they may be important. The fat-1 gene can convert n-6 to n-3 PUFAs, leading to a higher n-3/n-6 PUFA ratio. Fluidity of plasma membrane plays important roles for cellular functions such as signal transduction, cell recognition, membrane order and the function of membrane receptors; flexibility of plasma membrane could increase membrane stability, which can adapt to challenge of different conditions (Lafourcade et al., 2011; Yamashima, 2008). The ratio of n-3/n-6 has a positive correlation with membrane fluidity and flexibility, which can be involved in antioxidative effects and upregulate the expression of antioxidant enzyme genes in NSCsfat-1 to enhance the capacity of scavenging ROS (Hasadsri et al., 2013; Lee et al., 2014; Ying, Feng, Agrawal, Zhuang, & Gomez-Pinilla, 2012).

In recent years, NSC replacement therapy has emerged as a promising potential treatment for ischemic stroke (Kokaia & Darsalia, 2011). Because of their many advantages (Giusto et al., 2014), adult NSCs have been regarded as an excellent source of replacement therapy for human ischemic stroke. However, the primary obstacle to clinical application is the loss of physiological function of endogenous NSCs and exogenous adult NSCs resulting from hypoxic-ischemic injury in the ischemic penumbra (Azevedo-Pereira & Daadi, 2013; Bazan et al., 2005; Rosenblum et al., 2015). Our results provide valuable information for the development of effective adult NSC replacement therapy for ischemic stroke. With regard to endogenous adult NSC replacement therapy, the n-3/n-6 PUFA ratio can be increased by dietary or other means, resulting in increased protection of adult NSCs from hypoxic-ischemic damage to get better support for self-repair of neural function damage. For exogenous adult NSC replacement therapy, the n-3/n-6 PUFA ratio of adult NSCs could be elevated with an engineered gene for mtfat-1. A combination of the above two therapies could achieve optimal restoration of neurological function in ischemic stroke.

ACKNOWLEDGMENTS

The authors thank their colleagues in the Jiangsu Key Laboratory of Xenotransplantation, Nanjing.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Y.W., J.F.Y. Acquisition of data: J.F.Y., Y.W., B.F. Analysis and interpretation of data: J.F.Y., Y.W., H.Y.Y. Drafting of the manuscript: J.F.Y., Y.W. Critical revision of the article for important intellectual content: Y.W., H.Y.Y. Statistical analysis: J.F.Y., H.Y.Y. Obtained funding: Y.F.D. Technical and material support: J.F.Y., Y.W. Study supervision: Y.W.

REFERENCES


