The TRIM protein Mitsugumin 53 enhances survival and therapeutic efficacy of stem cells in murine traumatic brain injury

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Abstract

Background: Traumatic brain injury (TBI) is a common neurotrauma leading to brain dysfunction and death. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) hold promise in the treatment of TBI. However, their efficacy is modest due to low survival and differentiation under the harsh microenvironment of the injured brain. MG53, a member of TRIM family protein, plays a vital role in cell and tissue damage repair. The present study aims to test whether MG53 preserves hUC-MSCs against oxidative stress and enhances stem cell survival and efficacy in TBI treatment.

Methods: In this study, we performed a series of in vitro and in vivo experiments in hUC-MSCs and mice to define the function of MG53 enhancing survival, neurogenesis, and therapeutic efficacy of stem cells in murine traumatic brain injury.

Results: We found that recombinant human MG53 (rhMG53) protein protected hUC-MSCs against H$_2$O$_2$-induced oxidative damage and stimulated hUC-MSC proliferation and migration. In a mouse model of contusion-induced TBI, intravenous administration of MG53 protein preserved the survival of transplanted hUC-MSCs, mitigated brain edema, reduced neurological deficits, and relieved anxiety and depressive-like behaviors. Co-treatment of MG53 and hUC-MSCs enhanced neurogenesis by reducing apoptosis and improving PI3K/Akt-GSK3β signaling.

Conclusion: MG53 enhances the efficacy of hUC-MSCs in the recovery of TBI, indicating that such adjunctive therapy may provide a novel strategy to lessen damage and optimize recovery for brain injury.

Keywords: Neuroprotection, Mitsugumin 53, Stem cells, Traumatic brain injury, PI3K-Akt-GSK3β

Introduction

Traumatic brain injury (TBI) is a common neural trauma that often initiates from an external force followed by a secondary neural injury, causing severe physical, psychological, and cognitive impairments [1, 2]. Several experimental and clinical studies have shown that stem cell transplantation can exert beneficial effects in TBI, acting via paracrine factors and providing cell replacement [3, 4]. Mesenchymal stem cells (MSCs) can be easily obtained from several tissues and characterized by self-renewal and differentiation potential. However, according to International Society for Cellular Therapy (ISCT) criteria, the isolated MSCs are heterogeneous, which contain stem cells, committed progenitors, and differentiated cells [5]. Though the nature of MSCs remains unclear, nonclonal stromal cultures currently serve as sources of putative MSCs, representing a promising therapy for neurodegenerative diseases. hUC-MSCs have advantages over embryonic stem cells, bone marrow, or adipose-derived mesenchymal stem cells in cell therapy for several reasons. hUC-MSCs are easily obtained and have fast proliferation rate and low

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immunogenicity, as well as low risk of teratoma forma-
tion [6, 7]. However, the migration and survival of trans-
planted stem cells are hindered by excessive reactive
oxygen species (ROS) generated under conditions of tis-
ue ischemia and inflammation [8, 9].

MG53, a member of the tripartite motif (TRIM) pro-
tein family, plays an essential role in cell membrane re-
pair and tissue damage recovery [10–13]. Genetic
ablation of MG53 results in defective membrane repair
and tissue regeneration capacity [10, 11, 14, 15]. Recom-
binit human MG53 (rhMG53) protein could protect
various cell types against membrane disruption and
ameliorate the pathologies associated with muscular dys-
 trophy [16], acute lung injury [17], myocardial infarction
[18], and acute kidney injury [19] in animal models.
Additionally, rhMG53 protects cultured neural cells
from injury in vitro, and intravenous delivery of rhMG53
penetrates the blood-brain barrier to protect against is-
chemic brain injury [20]. In muscle membrane repair,
MG53 binds to phosphatidylserine (PS) and interacts
with caveolin-3 (Cav-3) to mediate vesicle accumulation
at injury sites involved in patching the membrane [10,
11, 21]. In tissue repair, MG53 interacts with p85 as well
as CaV3 and activates the pro-survival RISK pathway
(including PI3K/Akt/GSK3β cascade and ERK1/2 path-
way) to protect ischemic brain injury and myocardial
damage [15, 20, 22]. However, it is unknown whether
rhMG53 can enhance the viability and neural repair of
hUC-MSCs in TBI and the mechanism remains unclear.

In this study, we tested whether rhMG53 can improve
the survival of hUC-MSCs for the treatment of TBI. Our
results showed that the combination of rhMG53 and
hUC-MSCs had synergistic effects in augmenting the
therapeutic benefits to mitigate brain edema and to im-
prove the cognitive function of mice subjected to TBI.
Moreover, the mechanism is through the activation of
PI3K/Akt-GSK3β signaling.

**Materials and methods**

**Isolation, cultivation, and identification of hUC-MSCs**

hUC-MSCs were isolated as previously described [23].
This study was approved by the Ethics Committees of
the Zhengzhou University. Briefly, human umbilical
cords were obtained postpartum from full-term healthy
infants delivered via normal vaginal delivery after in-
formed consent according to institutional guidelines
under an approved protocol. Cells were cultured in
DMEM/F12 containing 10% FBS, 1% penicillin-
streptomycin, and 10 ng/ml of basic fibroblast growth
factor in a 37 °C incubator with 5% CO2. Flow cyt-
ometer (Becton-Dickinson, USA) was used to identify
the immuno-phenotypic characterization of hUC-
MSCs. Passages 3–5 of the hUC-MSCs were used for
experiments.

**Preparation of recombinant human MG53 protein**

Purification of rhMG53 protein has been described pre-
viously [16]. rhMG53 was lyophilized and stored at 4 °C.
For intravenous injection of rhMG53, the protein was
diluted at 2 mg/ml in 0.9% sterile saline, filtered through
a 0.22-μm filter, and injected via the tail vein.

**Cell proliferation, apoptosis, and senescence-associated
β-galactosidase (SA-β-gal) detection**

hUC-MSCs were plated in 96-well plates at a density of
3 × 10^3 cells/well and incubated with various concentra-
tions of H2O2 within 60% confluence at 0, 8, 16, 24, 32,
and 40 h after H2O2 incubation; CCK-8 assay was per-
formed as previously described [24]. The effect of
rhMG53 at different concentrations (15, 30, 60, 120 μg/
ml) on hUC-MSC proliferation was also detected by
CCK-8 assay. The experiments were divided into 4
groups: CON group, MG53 group (30 μg/ml rhMG53),
H2O2 group (200 μM), and H2O2 + MG53 group (hUC-
MSCs were co-treated with 30 μg/ml rhMG53 and
200 μM H2O2 stimulation for 16 h). Annexin V/propio-
dium iodide (PI) staining was performed as previously
described [25]. The senescence of hUC-MSCs was evalu-
ated using a SA-β-gal kit (GenMed, USA) as described
previously [24].

**Transwell assay**

Cell migration was detected by a Transwell assay [25].
Briefly, hUC-MSCs treated with rhMG53 (30 μg/ml)
and/or H2O2 (200 μM, 16 h) were collected and resus-
pended in DMEM/F12 media without serum, and re-
plated in transwell with 8.0 μm holes at a density of 1 ×
10^5 cells/ml, 300 μl/well. Twenty-four hours later, the
cells were fixed with 4% paraformaldehyde for 10 min
and stained with crystal violet for 30 min. The cells in
the upper chamber were removed, and the migrated cells
were detected as purple in color and counted under a
microscope (Eclipse TE2000-E; Nikon, Japan).

**Detection of biomarkers of oxidative stress (GSH, SOD,
and MDA)**

In in vitro studies, hUC-MSCs were collected and super-
natants were harvested after cell lysed by ultrasonication.
In in vivo studies, peripheral blood of mice was har-
vested and the serum was collected and stored at –
80 °C. The SOD activity and concentration of GSH and
MDA in the cell supernatant or serum were measured
according to the kit manufacturer’s instruction (Jian
Cheng Biological Engineering Institute, China).

**Mouse model of TBI**

All procedures involving animals were conducted under
the National Guidelines for Care and Use of Laboratory
Animals and the Animal Care Guidelines issued by
Zhengzhou University. The C57BL/6 male mice (10–12 weeks, 23–25 g) were housed in cages and maintained at 24 °C with a normal 12 h/12 h light-dark schedule. The mice had free access to food and water. A weight-drop model of TBI was established as previously described [1]. A successful model was confirmed when the mice showed transient limb twitching and apnea after the injury and relieved themselves in a few seconds. The mice were kept on a heat mat to maintain a rectal temperature at 37 ± 0.5° during the operation and recovery.

**Experimental design**

A total of 210 mice were randomly divided into 5 groups: Sham, TBI, MG53, MSC, and MG53 + MSC (42 mice in each group). rhMG53 (3 mg/kg) and/or hUC-MSCs (1 × 10^6 cells) in 100 μl were injected intravenously via tail vein of TBI mice for 3 times at 6, 30, and 54 h after injury. The Sham and TBI group were treated with the same volume of 0.9% saline (100 μl). The timeline for animal experiments is showed in Fig. 3a.

**Western blotting**

Mice were scarified 3 days after TBI (n = 6/group). The brains were removed, and the lesion areas were isolated for protein preparation. Western blotting was carried out as previously described [22]. Equal amounts of protein (150 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad). The primary antibodies were MG53 (1:1000), Bcl-2 and Bax (Cell Signaling Technology, 1:200), Akt1, GSK-3β, ERK1/2, p-Akt1, p-GSK-3β, p-ERK1/2 (Cell Signaling Technology, 1:1000), and β-actin (Sango, 1:2000). The intensities of the resulting protein bands were quantified with Image J software (NIH, Bethesda, MD, USA).

**Modified neurological severity score (mNSS)**

mNSS was performed by observers blinded to the treatments at 0, 1, 3, 7, 14, 21, and 28 days after TBI as previously described [26]. The mNSS is a composite of motor, sensory, balance, and reflex tests which has been widely employed in TBI studies. This test is suitable to evaluate long-term neurological outcome after unilateral brain injury [3]. The test was performed double blindly by examiners.

**Brain water content (edema)**

The extent of brain edema after TBI was measured 3 days after TBI as described previously [27]. Briefly, whole brain specimens were harvested. Ipsilateral hemisphere and contralateral hemisphere were weighed immediately on an analytical balance to obtain the wet weight and dried for 24 h at 100 °C to obtain the dry weight. The formula for water content is as follows: percentage of water = (wet weight – dry weight)/wet weight.

**Morris water maze (MWM)**

MWM was used to evaluate spatial learning and memory at day 28 after TBI as previously described [28, 29]. Before experimentation, mice were trained four times in 20-min intervals with the start position randomly changed. The mice were gently placed in the apparatus and allowed for 60 s to find the platform and stay on it for 10 s. If the mice could not find the platform in 60 s, they were gently guided to the platform and stayed on the platform for 10 s, and the latency time was recorded as 60 s. On the 28th day, the platform was removed, and the mice were placed in the opposite quadrant to the one previously described. The mice were towel dried and placed into a heated cage after each trial. Results were expressed as swimming tracks, times to cross the platform, latencies to platform, and times spent in the goal quadrant.

**Novel object recognition (NOR) test**

Twenty-eight days post-TBI, we used an established protocol to assess each mouse with the NOR test [30–32]. The experiments were carried out in 27 days after TBI, and the mouse was placed in the box and habituated for 10 min. On the next day, two identical novel objects (black cubes, 4 × 4 × 4 cm^3) were placed in the box, and the mouse was allowed to explore the area for 10 min. After 1 h, one novel object (blue ball, 4 cm in diameter) and one old object (black cube) were placed in the box, and the mouse was allowed to explore for 5 min while being recorded by camera. We compared the total time spent exploring the old and new objects. The exploration time included time in direct contact with the objects and time within the object area; a discrimination index (total time spent with new object/total time devoted to exploration of objects) was also calculated for each mouse.

**Forced swim test (FST)**

FST was used to assess depression of the mice. On day 28 post-TBI, the mice underwent the FST as previously described [32]. Mice were placed individually in clear cylindrical tanks (height = 25 cm; diameter = 22 cm) with 15 cm of water at 23 ± 1 °C. The trial was conducted for 6 min, and the period of immobility during the last 4 min was measured. The immobility time was defined as the animal floating on the surface of the water, only making minimal movements necessary to remain afloat, typically slow paddling of one foot.
Tail suspension test (TST)
The TST was performed with each mouse 28 days post-TBI. Briefly, mice were suspended by the tail with a piece of adhesive tape (1 cm from the tip, 17 cm long) 55 cm above the desk. A camera corded the movement of the mouse for 3 min. Mice were considered immobile when they hung passively and motionless. The immobility time was calculated by subtracting the total amount of mobility time from the 180 s of test time. The method was according to a protocol described previously [33].

Sucrose preference test (SPT)
The sucrose preference test was carried out at 28 days after TBI. The test was performed as described previously with some slight modifications [32]. Before the test, mice were put to the cage having two bottles of 50 ml sucrose solution (1%, w/v) for 3 days; then, one bottle of sucrose was replaced with water. After 4 days, the respective weights of the sucrose solution and water consumed were recorded, and the percent sucrose preference was calculated by using the following formula: Sucrose preference = [sucrose consumption in g/(water + sucrose consumption in g)] × 100%. Diminished preference for the sweet drink indicates depression-like behavior.

Novelty suppressed feeding test (NSF)
At 28 days after TBI, NSF test was performed during a 5-min period [34]. The mice were weighed, and all food was removed from their cages. Water continued to be provided. Approximately 24 h after the removal of the food, the mice were transferred to the testing room, placed in a clean holding cage, and allowed to habituate for 30 min. The testing apparatus consisted of a Plexiglas box (45 × 45 × 20 cm) under an illuminated (approximately 1000 lx), soundproofed condition. The floor of the box was covered with 1 cm of wooden bedding. A small piece of mouse chow was placed in the center of the box on a white circular filter paper (11 cm in diameter). Each subject was placed in the corner of the testing arena, and the time until the first feeding episode was recorded. At the end of this period, the time to enter the center was counted.

Open-field test (OFT)
The open-field test was conducted at 28 days after TBI with apparatus that consisted in a 45 cm × 45 cm wooden square surrounded by a 20-cm high wall. The bottom of the black box was divided into 25 squares (7 cm × 7 cm) with 2 mm white lines. During the test, the mice were placed in one of the four corners of the box and facing the wall. The mice were recorded for the total distance traveled and number of rearings (vertical standing of mice on two hind legs) within 3 min [35].

In vivo PI (propidium iodide) staining
Three days after TBI, we diluted PI (10 mg/ml; Sigma-Aldrich, St Louis, MO, USA) in 0.9% NaCl and administered to mice by intraperitoneal injection at 0.4 mg/kg before sacrifice [29] to detect cell death in damaged areas of cerebral cortex of TBI mice. The sections were also stained with 4′, 6-diamidino-2-phenylindole (DAPI) to show total nuclei. Samples were observed and photographed under a fluorescence microscope (Eclipse TE2000-E; Nikon, Japan) at excitation and emission wavelengths of 535 nm and 617 nm, respectively.

Tissue processing and immunofluorescence staining
At 3, 7, 14, and 28 days after TBI, mice were deeply anesthetized with isoflurane and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde via cardiac puncture. All solutions were maintained at pH 7.4 and 4 °C. Brains were removed and stored in 4% paraformaldehyde overnight and then transferred to 30% sucrose in PBS for another 48 h. Frozen serial coronal brain sections were sliced at 10 μm on a cryostat (Leica, Germany).

Mouse anti-human nuclei monoclonal antibody (MAB1281) was applied for immunostaining to trace the migration of hUC-MSCs in the brain of TBI mice by 3 days post-TBI. Immunofluorescence staining for glial fibrillary acidic protein (GFAP), doublecortin (DCX), and neuronal nuclei (NeuN) was used to detect the degree of hippocampal neurogenesis in TBI mice as described previously [36]. After being blocked with 10% goat serum for 45 min, brain sections (n = 6 mice/group) were incubated with MAB1281 (1:50, Merck Millipore), GFAP (1:50, Cell Signaling Technology), DCX (1:50, Santa), and NeuN (1:50, Abcam) at 4 °C overnight and then with FITC/CY3-conjugated secondary antibody (1:1000, Sangon Biotech) for 1 h at room temperature. Stained sections were examined under a microscope (Eclipse TE2000-E; Nikon). The quantification of MAB1281, GFAP-, DCX-, or NeuN-positive cells was analyzed using Image J software (NIH, Bethesda, MD, USA) and divided by the total area in the image field (mm²).

Cresyl violet staining and lesion volume
On day 28 after TBI, coronal brain sections were stained with Cresyl violet to detect Nissl bodies as described previously [37]. Briefly, the brains were continuously coronally sectioned from the lesion with the edge of 2.0 mm. Each slice was 20 μm thick and randomly selected from 10 consecutive slices. Using the Image J software (NIH, Bethesda, MD, USA), the brain damage volume was calculated: brain damage volume (mm³) = average lesion area × number of brain slices (10) × 0.02 [37].
Polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR)
To confirm that hUC-MSCs could migrate to brain after intravenous injection, the presence of human-specific DNA (a 479-bp fragment of a highly repetitive α-satellite DNA sequence of the centromere region of human chromosome 17) in the hippocampus of TBI mice was detected 3 days after administration by using PCR technique. Genomic DNA was extracted from brain tissues using a DNA tissue Kit (Qiagen, USA) and quantified by a Nanodrop 2000 spectrophotometer (Thermo Fisher, USA). After PCR, the products were detected by 1% agarose gel electrophoresis, stained with ethidium bromide (Sigma, USA), and visualized using UV trans-illumination. Primers for human-specific PCR were as follows: forward-5′-GGGATAAATTTCACTGACTAAACAG-3′, reverse-5′-AAAACGTCCACACTGCTCTTAG-3′; GAPDH: for ward-5′-GGTTGAAAGTGTCGTTGAA-3′, reverse-5′-CTCTGACCTGTGCCGTTTGA-3′. The program consisted of 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C, and extension for 40 s at 72 °C [38].
For qRT-PCR, brains were collected at 28 days after TBI, and total RNA were isolated to check the transcription level of NGF and BDNF [39]. The primer sequences were as follows: NGF—forward-5′-TACAGGCAGAAC GGACAGA-3′, reverse-5′-CATAGACAAAA TGTCAGTTCTTAG-3′; GAPDH: forward-5′-GGTGAAGGTCGGTGTGAAC-3′, reverse-5′-CTCTGACCTGTGCCGTTTGA-3′. The program consisted of 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C, and extension for 40 s at 72 °C [38].

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Statistical analysis
Data are presented as mean ± SEM. Two-way ANOVA was used to analyze all behavioral tests between and among the treatment groups. In anatomical and biochemical studies, one-way or two-way ANOVA was used to compare multiple groups. A Bonferroni post hoc analysis was used to determine whether differences were significant. Differences between two groups were tested with the two-tailed Student’s t-test. The criteria for statistical significance were p < 0.05.

Results
rhMG53 protects cultured hUC-MSCs against oxidative damage and promotes cell migration
hUC-MSCs were derived from Wharton’s jelly and displayed fibroblast-like morphology approximately 2 weeks after primary culture (Fig. 1a–c). Flow cytometric analysis showed that the isolated hUC-MSCs were positive for CD73, CD90, and CD105 and very low or negative for hematopoietic lineage markers CD34, CD45, and HLA-DR (Fig. 1d).

In order to determine how sensitive hUC-MSCs were to oxidative stress, hUC-MSCs at third passage (P3) were treated with varying concentrations of H2O2. Results showed that H2O2 exhibited dose- and time-dependent inhibitory effects on the viability of hUC-MSCs at a concentration range of 0–400 μM (Fig. 2a, b). Based on the data, after treatment for 16 h, the half maximal inhibitory concentration of hUC-MSCs was 200 μM H2O2. So, 200 μM H2O2 was selected for the subsequent experiments to induce oxidative damage to hUC-MSCs.

In addition, the proliferation of hUC-MSCs was significantly improved as the concentration of rhMG53 increased (≥ 30 μg/ml) (p < 0.05, Fig. 2c). Considering the dose effect of rhMG53 and previous studies, 30 μg/ml rhMG53 was chosen for our further in vitro experiments [16]. The protective effects of rhMG53 on H2O2-induced oxidative damage to hUC-MSCs were then examined. CCK8 results showed that rhMG53 promoted proliferation of hUC-MSCs and also mitigated the inhibitory effects of H2O2 (p < 0.05, Fig. 2d). H2O2-induced cell apoptosis and senescence of hUC-MSCs were significantly recovered by rhMG53 (p < 0.05, Fig. 2e, f and Fig. 2g, i), while the reduction of cell migration triggered by H2O2 was partially improved by rhMG53 (p < 0.05, Fig. 2h, j).

Finally, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured to quantify the effects of H2O2 and rhMG53 treatments on oxidative damage to hUC-MSCs. Upon treatment with H2O2, SOD activity was significantly decreased (p < 0.05, Fig. 2k), and MDA content was significantly increased (p < 0.05, Fig. 2l), while rhMG53 could significantly reverse both effects (p < 0.05, Fig. 2k, l). These results showed that rhMG53 can protect cultured hUC-MSCs against oxidative damage and promote cell migration by decreasing oxidative stress induced by H2O2 in vitro.

rhMG53 enables hUC-MSC survival and entry into mouse brains
Before assessing the protective effects of rhMG53 and hUC-MSCs on TBI, whether rhMG53 could aid the homing of hUC-MSCs in the mouse brain was examined. The experimental design and timeline for animal experiments are shown in Fig. 3a. As shown in Fig. 3b and quantified in Fig. 3c, TBI induced the appearance of MG53 in the brain tissue, which was not detected in the brain under physiological conditions [20]. The expression of endogenous MG53 in the injured brain tissue likely reflected an increased permeability of blood-brain barrier (BBB). Further elevation of rhMG53 was observed after intravenous administration of the exogenous protein to mice subjected to TBI (Fig. 3b), indicating that rhMG53 can cross the BBB to target the injured
brain tissue, which is similar to our previous observation in a mouse model of ischemic brain injury [20].

The presence of human-specific DNA in the mouse brain tissues was used as a marker to determine the homing of hUC-MSCs by PCR analysis [4, 38]. As expected, no PCR product of human-specific DNA was detected in brain lesions derived from mice subjected to TBI, with or without rhMG53 treatment. In TBI mice receiving hUC-MSCs, abundant human-specific PCR product was detected (Fig. 3d). Moreover, TBI mice receiving co-treatment of rhMG53 + hUC-MSCs displayed significantly more human DNA in their brain lesions (p < 0.05, Fig. 3d, e).

Immunohistochemical staining was subsequently used to investigate the presence of hUC-MSCs in the mouse hippocampus. MAB1281 is an anti-human nuclear monoclonal antibody, which specifically reacts with the nucleus of all human cells [3]. MAB1281-positive cells were observed in the brain lesions derived from mice that received either hUC-MSCs or rhMG53 + hUC-MSCs within 3 days after hUC-MSC transplantation, but not in TBI mice receiving saline as control or rhMG53 alone (Fig. 3f). Quantitative analysis showed that the number of MAB1281-positive cells in rhMG53 + hUC-MSC group (63.5 ± 12.0 cells) was significantly higher than that in hUC-MSC group (31 ± 9.5 cells, p < 0.05, Fig. 3g). These results indicated that rhMG53 treatment either preserved the survival of hUC-MSCs in mouse brain following TBI or enhanced their ability to migrate into the brain by crossing the BBB.

rhMG53 and hUC-MSCs mitigate brain edema and improve survival signaling of brain tissues in mouse model of TBI

At 3 days post-TBI, brain edema of the ipsilateral hemisphere was significantly alleviated in the MG53, MSC, or
Fig. 2 (See legend on next page.)
MG53 + MSC group than that in TBI group (p < 0.05, Fig. 4a). However, none of the treatments had a significant impact on the degree of edema in the contralateral hemisphere when compared to TBI group (p > 0.05, Fig. 4a).

Fresh brain tissues derived from the injured lesions at 3 days after TBI were used for Western blotting analysis. As shown in Fig. 4b–d, improved cell survival signaling of p-Akt1 (Ser473), p-GSK3β (Ser9), and p-ERK1/2 (Thr202) was observed in MG53, MSC, or MG53 + MSC group (p < 0.05), whereas the total expressions of Akt1, GSK3β, and ERK1/2 were not affected (Fig. 4b–d, p > 0.05).

Then, the effects of the rhMG53 and hUC-MSC treatments on long-term brain structure were evaluated using Cresyl violet staining (Fig. 4e). At 28 days after TBI, results showed that the size of the lesion in the ipsilateral hemisphere was significantly reduced than those in the MG53 and MSC group. Moreover, co-treatment with rhMG53 and hUC-MSCs led to further reduction of the brain lesions (p < 0.05, Fig. 4f). Together, these results indicated that co-administration of rhMG53 and hUC-MSCs resulted in both short-term and long-term improvements of brain structure in the mouse model of TBI.

rhMG53 and hUC-MSCs alleviate oxidative stress, decrease neural apoptosis, and promote neurogenesis after TBI

Often, TBI and subsequent brain edema can lead to an increase in oxidative stress in the surrounding tissue, resulting in exacerbated brain damage [40]. To examine the potential anti-oxidative effects of rhMG53 and hUC-MSCs in the TBI mice brain, the concentrations and activities of oxidative markers (GSH, SOD, and MDA) in the hippocampus at 3 days after injury were quantified. rhMG53 or hUC-MSCs caused significant elevation of GSH and SOD (p < 0.05, Fig. 5a, b) and a decrease of MDA content (p < 0.05, Fig. 5c). But, the changes of GSH, SOD, and MDA in MG53 + MSC group were most significant (p < 0.05, Fig. 5a–c). These data suggest that co-treatment of rhMG53 and hUC-MSCs reduced the degree of oxidative stress in the hippocampus of mice with TBI.

PI staining was used to quantify the number of dying cells in the injured brain tissue at 3 days after TBI (Fig. 5d). The number of PI-positive cells in MG53 or MSC groups was significantly reduced than that of TBI mice. The most notable decrease of PI-positive cells occurred in MG53 + MSC group (p < 0.05, Fig. 5e), indicating that rhMG53 and hUC-MSCs ameliorated neuronal cell death. This result was further confirmed by Western blotting, which showed that the anti-apoptosis associated protein (Bcl-2) was increased while pro-apoptosis protein (Bax) was decreased in the MG53, MSC, and MG53 + MSC group (p < 0.05, Fig. 5f, g).

Furthermore, immunofluorescence staining was used to evaluate the potential benefits of rhMG53 and hUC-MSC treatment on neurogenesis following brain injuries. As shown in Fig. 5h–k, there were more GFAP+ cells at day 7, DCX+ neurons at day 14, and NeuN+ neurons at day 28 in MG53, MSC, and MG53 + MSC groups than those in TBI group (p < 0.05), but less than that in Sham group. qRT-PCR at 28 days post-TBI showed upregulated expressions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in all treated groups and most obviously in the MG53 + MSC group (p < 0.05, Fig. 5i). These data suggest that rhMG53 and hUC-MCSs not only reduced oxidative stress and subsequent cell death in injured brain tissue but also promoted neurogenesis.

rhMG53 and hUC-MSC treatment improve neurologic function of mice after TBI

Body weight was increased more in the MG53 + MSC group, but least in the TBI group compared with the other groups from day 14 to day 28 after TBI (p < 0.05, Fig. 6a). Compared with TBI group, mice in MSC, MG53, and MG53 + MSC group exhibited significantly lower mNSS scores from day 7 to day 28 after TBI, and lowest in MG53 + MSC group at day 21 to 28 (p < 0.05, Fig. 6b). These results indicated treatment with rhMG53 and hUC-MSCs improved resilience and recovery after TBI.
At day 28 post-TBI, the Morris water maze (MWM) was used to examine the spatial learning and cognitive function of the mice (Fig. 6c). Mice in MG53 and/or MSC groups performed significantly better than TBI group, as indicated by shorter latency to reach the platform (Fig. 6d), more crossing numbers (Fig. 6e), and more time spent in the target quadrant ($p<0.05$, Fig. 6f). In addition, compared with TBI mice, MG53 + MSC mice demonstrated a higher discrimination index in the NOR test, with
significantly improved cognition in MG53 + MSC group (p < 0.05, Fig. 6g).

**rhMG53 and hUC-MSCs relieve anxiety and depressive-like behavior in TBI mice**

Anxiety and depression were also assessed in the TBI mice at day 28 using forced swim test (FST), tail suspension test (TST), novelty suppressed feeding test (NSF), and open-field test (OFT). When subjected to rhMG53 and/or hUC-MSCs, TBI mice exhibited a significant decrease in immobility time in FST and the TST (p < 0.05, Fig. 6h, i), both of which are considered signs of depressive behavior [32]. SPT is another common method of assessing depression through anhedonia, the inability to feel pleasure [40]. Compared with TBI group, rhMG53 and/or hUC-MSC treatment significantly increased the sucrose preference index, indicating less anhedonia and vulnerability or resistance to stress (p < 0.05, Fig. 6j).

In addition, rhMG53- or hUC-MSC-treated mice exhibited decreased anxiety as demonstrated by a decrease in the time to enter the disk containing food, whereas that of co-treatment of rhMG53 and hUC-MSCs was least at 28 days after TBI (p < 0.05, Fig. 6k). Similarly, the total number of rearings and the total distance traveled in MG53 or MSC group were greatly increased in the OFT, and the most effective improvement was detected in MG53 + MSC group (p < 0.05, Fig. 6l, m). Taken together, our results indicated that rhMG53 and hUC-MSC treatment relieved anxiety and depressive-like behavior after TBI and the combination treatment resulted in the significant beneficial effects.

**Discussion**

According to the World Health Organization, TBI is a major health problem worldwide. Accumulating studies prove evidence that stem cell transplantation is a promising approach for TBI treatment [41]. It has been shown that intravenously administered MSCs can migrate to the brain lesion to improve neural function in TBI [42, 43]. However, the therapeutic efficacy of MSCs in TBI remains unsatisfactory due to limited migration and survival of transplanted stem cells in brain. rhMG53, a protein critical for membrane repair, was found to enhance the survival of hUC-MSCs after ox-LDL injury [44], as well as facilitate the repair of ischemia-reperfusion injury to the brain [20]. Our results presented evidence that rhMG53 could preserve survival of hUC-MSCs in vitro and in vivo and enhance therapeutic efficacy for TBI treatment.

Considering that rhMG53 has a short half-lifetime of ~1.5 h in the blood circulation after intravenous or
Fig. 5 (See legend on next page.)
subcutaneous administration [16, 19], repetitive administrations of rhMG53 are required. In our studies, rhMG53 and/or hUC-MSCs were respectively injected three times through intravenous administration after TBI.

TBI primarily leads to edema of the brain, which contributes to a range of complications and secondary injuries [1, 45, 46]. Our previous studies showed that mice treated by Wharton’s jelly tissue (which is able to produce hUC-MSCs) gained body weight than vehicle group during the 28 days after TBI [27]. We found that rhMG53 and hUC-MSCs could mitigate brain edema, and when applied together, edema was further alleviated in the TBI mice. Furthermore, rhMG53 and hUC-MSCs could reduce the lesion volume and severity of neurologic deficit with the mNSS assessment, as well as improving the whole body condition [47]. Other neurological behavior assessments, including the MWM, FST, TST, SPT, NSF, and OFT, indicated that TBI mice exhibited motor and cognitive dysfunction, and also present some psychiatric-like anxiety and depression; however, these impairments were significantly alleviated after intravenous administration of rhMG53 and hUC-MSCs. Given the relatively short half-life of MG53, the protective effect of rhMG53 in TBI might be limited to delay the progression of TBI. However, rhMG53 can increase the survival of hUC-MSCs at the injury site, thereby improving the long-term therapeutic effect of hUC-MSCs to TBI.

Common secondary injuries associated with TBI induce elevated oxidative stress and cell apoptosis [48], which lead to impairment of cognition, motor function, and neurological behavior [49]. Previous studies indicated that human MSCs reduced ROS production and enhanced wound closure in a TBI in vitro model [50] and elicited therapeutic effects on TBI through the secretion of neurotrophic factors and the inhibition of apoptosis [51]. In addition, rhMG53 protects cardiomyocytes from H2O2-induced oxidative stress [15] and protects the heart from ischemic injury [52]. Furthermore, rhMG53 reduces ischemia/reperfusion injury to hepatocytes in vivo and in vitro [53]. Here, we showed that rhMG53 suppressed oxidative stress in cultured hUCMSCs and TBI mice, accompanied by increased activity of SOD and decreased MDA content. We also found that rhMG53 and hUC-MSCs jointly rescued neural cells from apoptosis to alleviate brain injury.

GFAP is widely recognized as a specific biomarker for astrocytes which is now increasingly acknowledged as having fundamental and sophisticated roles in brain function and dysfunction by astrocyte-neuron interactions [54–57]. DCX is a microtubule-associated protein expressed in migrating and differentiating neurons, corresponding to the initial stage of neurogenesis [58]. NeuN is considered to be a reliable marker of mature neurons [59]. Our results showed that rhMG53 and hUC-MSCs boosted neurogenesis, with more GFAP+, DCX+, and NeuN+ cells presented in the hippocampus at different stages. Researchers have proposed that soluble factors secreted by MSCs facilitate the regenerative process of the injured neural tissue by enhancing proliferation, migration, and differentiation of native neural stem cells (NSCs) [60]. This was proved by the upregulated expression of neurotrophins (BDNF and NGF) in our study. However, the mechanism by which rhMG53 enhanced neurogenesis needs further exploration as there is limited knowledge about the way stem cells and rhMG53 target specific tissues.

PI3K/Akt-GSK3β signaling contributes to neuronal growth, proliferation, and differentiation of NSCs [61]; promotes hippocampal neurogenesis in a mouse model of Alzheimer’s disease [62]; and ameliorates the apoptosis of neuronal cells in a rat model of Parkinson’s disease [63]. ERK1/2 is a key regulator for neural differentiation [64], modulates neurogenesis [65] and neural apoptosis [66], and improves depression-like behaviors [67]. Activation of ERK and Akt pathways protected PC12 cells against oxidative stress [68], and MG53 activated PI3K/Akt-GSK3β and ERK1/2 signaling to elicit cardioprotective functions [15, 22]. In brain ischemia/reperfusion injury, rhMG53 increased the phosphorylation of Akt and GSK3β and exhibited neuroprotective function [20]. Exosomes secreted by MSCs could induce proliferation and migration of fibroblasts and H9C2 myocardial cells by activating Akt and ERK1/2 signaling cascades, which contributes to chronic wound healing and inflammation [69]. In the present study, we found that both rhMG53 and MSCs upregulated the phosphorylation of Akt, GSK3β, and ERK1/2 in the hippocampus of TBI mice, suggesting that activation of the pro-
Fig. 6 (See legend on next page.)
survival PI3K/Akt-GSK3β and ERK1/2 signaling might be one contributing factor for the beneficial effects of rhMG53 and hUC-MSCs for the TBI mice.

Although this study demonstrates that the co-treatment of rhMG53 and hUC-MCSs increases survival signaling in the TBI model, further studies are needed to assess the cellular mechanism that underlies reduced apoptosis, such as whether MG53 has an impact on the mitochondrial welfare of injured neurons under conditions of oxidative stress. Furthermore, hUC-MSCs secrete paracrine factors, which may be essential to understand how those paracrine factors may have a synergistic role with rhMG53 to mitigate TBI injury.

Conclusions

Our findings show that rhMG53 can protect hUC-MSCs against oxidative damage, accompanied by enhanced proliferation and migration, and reduced apoptosis and senescence in vitro. In vivo, hUC-MSCs combined with rhMG53 were more effective in reducing the neurological deficits by protecting against neural oxidative stress, improving neurogenesis, and brain function in TBI mice by activating PI3K/Akt-GSK3β signaling. These findings provide a novel strategy of cell-based therapy, combining rhMG53 with hUC-MSCs for TBI treatment.

Abbreviations

BBB: Blood-brain barrier; DCX: Doublecortin; FST: Forced swim test; GFAP: Glial fibrillary acidic protein; hUC-MSCs: Human umbilical cord-derived mesenchymal stem cells; MDA: Malondialdehyde; mNSS: Modified neurologic severity score; MWM: Morris water maze; NeuN: Neuronal nuclei; NOR: Novel object recognition; NSF: Novelty suppressed feeding test; OFT: Open-field test; PBS: Phosphate-buffered saline; PI: Propidium iodide; qRT-PCR: Quantitative real-time PCR; rhMG53: Recombinant human MG53; ROS: Reactive oxygen species; SOD: Superoxide dismutase; SPT: Sucrose preference test; TBI: Traumatic brain injury; TRIM: Tripartite motif; TST: Tail suspension test

Acknowledgements

We appreciate the assistance of Prof. David C. Spray and the late Dr. Herbert Tanowitz in Albert Einstein College of Medicine, USA, for their revisions of this manuscript.

Authors’ contributions

JM, FG, and SM conceived and designed the experiments. BY, XZ, and YY revised the final version of the paper. SM, TH, and KW performed the experiments. SM, TH, QX, and JS analyzed the data. SM, XW, TH, KG, TT, FG, and JM wrote the manuscript. PL, YZ, YL, and G-YY prepared the reagents and materials and performed part of the experiments. All authors reviewed the manuscript prior to submission. All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC 81601078), Science and Technology Research Project from Henan Province (15210231022).

Availability of data and materials

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

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Zhengzhou University. The animal procedures were conducted in strict accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable. All authors of this manuscript agreed to publication.

Competing interests

The authors declare that they have no competing interests.

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Received: 18 March 2019 Revised: 22 July 2019 Accepted: 30 September 2019 Published online: 28 November 2019

References

1. Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. Nat Rev Neurosci. 2013;14:128–42.
2. Chhor V, Moretti R, Le Charpentier T, Sigaut S, Lebon S, Schwendimann L, et al. Role of microglia in a mouse model of paediatric traumatic brain injury. Brain Behav Immun. 2017;68:197–209.
3. Guan J, Zhu Z, Zhao RC, Xiao Z, Wu C, Han Q, et al. Transplantation of human mesenchymal stem cells loaded on collagen scaffolds for the treatment of traumatic brain injury in rats. Biomaterials. 2013;34:5937–46.
4. Cui Y, Ma S, Zhang C, Cao W, Liu M, Li D, et al. Human umbilical cord mesenchymal stem cells transplantation improves cognitive function in Alzheimer’s disease mice by decreasing oxidative stress and promoting hippocampal neurogenesis. Behav Brain Res. 2017;320:291–301.
5. Squillaro T, Peluso G, Galdieri U. Clinical trials with mesenchymal stem cells: an update. Cell Transplant. 2016;25:829–48.
6. Wang S, Cheng H, Dai G, Wang X, Hua R, Liu X, et al. Umbilical cord mesenchymal stem cell transplantation significantly improves neurological function in patients with sequelae of traumatic brain injury. Brain Res. 2013;1532:76–84.
30. Bevins RA, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat Protoc. 2006;1:1306–11.

31. Zhu W, Gao Y, Chang CF, Wan JR, Zhu SS, Wang J. Mouse models of intracerebral hemorrhage in ventricle, cortex, and hippocampus by injections of autologous blood or collagenase. PLoS One. 2014;9:e97423.

32. Cheng T, Wang W, Li Q, Han X, Xing J, Qi C, et al. Cerebroprotection of fluvanof (–)-epicatechin after traumatic brain injury via Nr2f2-dependent and -independent pathways. Free Radiol Biol Med. 2016;9:215–28.

33. Zhu H-T, Bian C, Yuan J-C, Chu W-H, Xiang X, Chen F, et al. Curcumin attenuates acute inflammatory injury by inhibiting the TLR4/MyD88/NF-κB signaling pathway in experimental traumatic brain injury. J Neuroinflamm. 2014;11:59.

34. Fukushima K, Iijima M, Chaki S. Serotonin-1A receptor stimulation mediates effects of a metabolite glutamate 2/3 receptor antagonist, 2S-α-amino-2-(1S,5S)-2-carboxylicacidpropyl-1-y3-(xantho-9-y)-pyridine acid (LY341495), and an N-methyl-D-aspartate receptor antagonist, ketamine, in the novelty-suppressed feeding test. Psychopharmacology. 2014;231:1291–9.

35. Heredia L, Torrente M, Colomina MT, Domingo JL. Assessing anxiety in C57BL/6J mice: a pharmacological characterization of the open-field and light/dark tests. J Pharmacol Toxicol. 2014;69:108–14.

36. Tong J, Liu W, Han X, Han X, Hyrien O, Samadani U, et al. Inhibition of Nogo-66 receptor 1 enhances recovery of cognitive function after traumatic brain injury in mice. J Neurotrauma. 2013;30:247–58.

37. Tureykin K, Vemuganti R, Sailor KA, Dempsey RJ. Infant volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. J Neurosci Methods. 2004;139:203–7.

38. Liu L, Yu Y, Hou Y, Chai J, Duan H, Chu W, et al. Human umbilical cord mesenchymal stem cells transplantation promotes cutaneous wound healing of severe burned rats. PLoS One. 2014;9:e88498.

39. Yong KW, Pungguan-Murphy B, Xu F, Abas WA, Choi JR, Omar SZ, et al. Phosphorytic and functional characterization of long-term cryopreserved human adipose-derived stem cells. Sci Rep. 2015;5:5996.

40. Peng W, Sun J, Sheng C, et al. Systematic review and meta-analysis of efficacy of mesenchymal stem cells on locomotor recovery in animal models of traumatic brain injury (J). Stem Cell Res Ther. 2015;6:54.

41. Abdul-Muneeer PM, Chandra N, Haroah J. Interactions of oxidative stress and neurovascular inflammation in the pathogenesis of traumatic brain injury. Mol Neurobiol. 2015;51:1866–79.

42. Hasan A, Deeb G, Rahal R, Atwi K, Mondello S, Marei HE, et al. Mesenchymal stem cells in the treatment of traumatic brain injury. Front Neurol. 2017;8:28.

43. Xu C, Fu F, Li X, Zhang S. Mesenchymal stem cells maintain the microenviron of central nervous system by regulating the polarization of macrophages/microglia after traumatic brain injury. Int J Neuropsi. 2017;127:1124–35.

44. Li X, Xiao Y, Cai Y, Tan T, Narasimhalu CA, Hao H, et al. Cell membrane damage is involved in the impaired survival of bone marrow stem cells by oxidized low-density lipoprotein. J Cell Mol Med. 2014;18:2445–53.

45. Russo MV, McGavern DB. Inflammatory neuroprotection following traumatic brain injury. Science (New York, NY). 2016;353:783–5.

46. Skolnick BE, Maas AL, Narayan RK, van der Hoop RG, MacAllister T, Ward JD, et al. A clinical trial of progesterone for severe traumatic brain injury. N Engl J Med. 2014;371:2467–76.

47. Xing Z, Xia Z, Peng W, Li J, Zhang C, Fu C, et al. Xuefu Zhuyu decoction, a traditional Chinese medicine, provides neuroprotection in a rat model of traumatic brain injury via an anti-inflammatory pathway. Sci Rep. 2016;6:2040.

48. Dhowa KJ. Pathophysiology of traumatic brain injury. Phys Med Rehab Clin N Am. 2017;28:215–25.

49. Hall ED, Vaishnav RA, Mustafa AG. Antioxidant therapies for traumatic brain injury. Neurotherapeutics. 2010;7:51–61.

50. Torrente D, Avila MF, Cabezas R, Morales L, Gonzalez J, Samudio I, et al. Paracrine factors of human mesenchymal stem cells increase wound closure and reduce reactive oxygen species production in a traumatic brain injury in vitro model. Hum Exp Toxicol. 2014;33:673–84.

51. Kim HJ, Lee JS, Kim SH. Therapeutic effects of human mesenchymal stem cells on locomotor recovery in animal models of traumatic brain injury [J]. Stem Cell Res Ther. 2015;6:47.
55. Taupin P. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 2007;53:198–214.
56. Hill E, Nagel D, Parri R, Coleman M. Stem cell-derived astrocytes: are they physiologically credible? J Physiol. 2016;594:659–606.
57. Molofsky AV, Deneen B. Astrocyte development: a guide for the perplexed. Glia. 2015;63:1320–9.
58. Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, et al. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron. 1999;23:247–56.
59. Duan W, Zhang YP, Hou Z, Huang C, Zhu H, Zhang CQ, et al. Novel insights into NeuN: from neuronal marker to splicing regulator. Mol Neurobiol. 2016;53:1637–47.
60. Volkman R, Offen D. Concise review: mesenchymal stem cells in neurodegenerative diseases. Stem Cells (Dayton, Ohio). 2017;35:1867–80.
61. Li XT, Liang Z, Wang TT, Yang JW, Ma W, Deng SK, et al. Brain-derived neurotrophic factor promotes growth of neurons and neural stem cells possibly by triggering the phosphoinositide 3-kinase/AKT/glycogen synthase kinase-3beta/beta-catenin pathway. CNS Neurol Disord-Dr. 2017;16:828–36.
62. Zheng R, Zhang ZH, Chen C, Chen Y, Jia SZ, Liu Q, et al. Selenomethionine promoted hippocampal neurogenesis via the PI3K-Akt-GSKbeta-Wnt pathway in a mouse model of Alzheimer’s disease. Biochem Bioph Res Co. 2017;485:6–15.
63. Yue P, Gao L, Wang X, Ding X, Teng J. Intranasal administration of GDNF protects against neural apoptosis in a rat model of Parkinson’s disease through PI3K/Akt/GSKbeta pathway. Neurochem Res. 2017;42:1366–74.
64. Hosseini Farahabadi SS, Ghaedi K, Ghazvini Zadegan F, Karbalaie K, Rabie F, Nematiollahi M, et al. ERK1/2 is a key regulator of FndC5 and PGC1alpha expression during neural differentiation of mESCs. Neuroscience. 2015;297:252–61.
65. Lin YL, Persaud SD, Nheiu J, Wei LN. Cellular retinoic acid-binding protein 1 modulates stem cell proliferation to affect learning and memory in male mice. Endocrinology. 2017;158:3004–14.
66. Zhao B, Zheng Z. Insulin growth factor 1 protects neural stem cells against apoptosis induced by hypoxia through Akt/mitogen-activated protein kinase/extracellular signal-regulated kinase (Akt/MAPK/ERK) pathway in hypoxia-ischemic encephalopathy. Med Sci Monit. 2017;23:1872–9.
67. Li H, Linjuan L, Wang Y. G-CSF improves CUMS-induced depressive behaviors through downregulating Ras/ERK/MAPK signaling pathway. Biochem Bioph Res Co. 2016;479:827–32.
68. Ong Q, Guo S, Duan L, Zhang K, Collier EA, Cui B. The timing of Ras/ERK and Akt activation in protecting PC12 cells against oxidative stress. PLoS One. 2016;11:e0153487.
69. Shabbir A, Cox A, Rodriguez-Menocal L, Salgado M, Van Badavas E. Mesenchymal stem cell exosomes induce proliferation and migration of normal and chonic wound fibroblasts, and enhance angiogenesis in vitro. Stem Cells Dev. 2015;24:1635–47.

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