



MG53 protein rejuvenates hUC-MSCs and facilitates their therapeutic effects in AD mice by activating Nrf2 signaling pathway

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ABSTRACT

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) transplantation is a promising therapy for Alzheimer's disease (AD). However, hUC-MSCs cultured *in vitro* easily exhibit replicative senescence, which restricts their application. Although MG53 protein demonstrates multiple roles for a variety of cells and tissues repair, it remains unknown whether MG53 could rejuvenate senescent hUC-MSCs and enhance their efficacy in AD model. Here, we firstly presented that MG53 reinstated senescent hUC-MSCs via the activation of the Nrf2 signaling pathway by increasing cell proliferation and migration, ameliorating senescence and oxidative stress, and decreasing the release of senescence-associated secretory phenotype. *In vivo* studies showed that MG53 treatment improved the therapeutic effect of senescent hUC-MSCs in AD mice. Furthermore, MG53 combined with young hUC-MSCs transplantation alleviated cognitive deficit and depression-like behavior in AD mice, reduced A β deposition and Tau phosphorylation, promoted neurogenesis, and inhibited glia cells activation and oxidative stress by activating the Nrf2 signaling. Moreover, these neuroprotective effects mediated by MG53 and hUC-MSCs were partly reversed by Brusatol, a specific inhibitor of Nrf2 signaling. Taken together, our study revealed that MG53 could rejuvenate senescent hUC-MSCs and facilitate their efficacy in AD mice at least partly through activating Nrf2 signaling pathway, which suggest that the combined therapy of MG53 and hUC-MSCs may be a novel and effective strategy for AD.

1. Introduction

Senescence is the irreversible degradation of the structure and physiological functions in cells and tissues, which ultimately leads to the occurrence of aging-related diseases [1]. Alzheimer's disease (AD) is the most common age-related neurodegeneration that seriously affects human health and causes a heavy burden on society [2]. The predominant pathological manifestations of AD are characterized by amyloid- β (A β) plaques and hyperphosphorylated Tau-induced neurofibrillary tangles, which lead to progressive neuronal loss and memory decline [1]. Although modern medicine has made great progress in the past decades, the early diagnosis and treatment of AD are far from satisfactory.

Recently, mesenchymal stem cells (MSCs) based regenerative medicine has emerged as a novel alternative strategy for AD [3]. Accumulating preclinical studies have demonstrated that transplantation of human umbilical cord-derived MSCs (hUC-MSCs) improves the cognition in AD models by reducing A β deposition, protecting neuronal integrity and enhancing neurogenesis [4–6]. Besides, the safety of stem cell-based therapy has been evaluated in some clinical trials [7]. The enough cell amount and high quality of stem cells are the prerequisite for successful transplant [8]. However, stem cells also undergo senescent phenotypes like other cells, which will lead to the gradual decline of tissue function and then induce the occurrence of diseases [9,10]. For instance, with the increase of passages, hUC-MSCs cultured *in vitro* easily showed replicative senescence such as decreased proliferation and

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differentiation, and increased SA- β -gal-positive cells, which seriously affects their therapeutic effects [11]. In order to ensure cell vitality, most studies recommended using the young stem cells no more than five passages for transplantation. However, it is not always the case to reach the numbers as required, thus limiting the further efficient stem cell-based translational studies. Therefore, exploring novel strategy to rejuvenate senescent hUC-MSCs and increase their activity is urgently needed to ensure their efficacy after transplant.

Mitsugumin53 protein (MG53), known as TRIM72, has protective effects on muscle, lung, kidney, brain, and other cells or tissues [12–15]. It was reported that recombinant human MG53 protein (rhMG53) could protect bone marrow mesenchymal stem cells from low density lipoprotein-induced membrane damage [16]. Our previous work proved that rhMG53 could enhance the anti-oxidative and anti-inflammatory capacity of hUC-MSCs and promote the functional recovery of brain injury [17,18]. However, whether MG53 can rejuvenate senescent hUC-MSCs and improve their neuroprotective effects under chronic neuropathological condition of AD still needs to be clarified.

In the present study, replicative senescent hUC-MSCs were used as a model to investigate the role and mechanism of MG53 targeting cell senescence both *in vitro* and *in vivo*. Meanwhile, the synergistic therapeutic effects and underlying mechanism of MG53 protein combined with hUC-MSCs transplantation in AD mice were explored. We found that MG53 pretreatment rejuvenated senescent hUC-MSCs *in vitro* and enhanced their therapeutic effect in AD mice. Furthermore, MG53 facilitated the therapeutic efficiency of young hUC-MSCs in AD mice by partly activating the Nrf2 signaling pathway. Our results suggest that MG53 could rejuvenate senescent hUC-MSCs both *in vitro* and *in vivo*, and the combined MG53 and hUC-MSCs transplantation may be a novel and effective strategy for AD.

2. Materials and methods

2.1. Materials

rhMG53 protein was dissolved in cell culture medium and diluted to 30 μ g/mL for *in vitro* experiment. For intravenous injection, rhMG53 protein was diluted at 2 mg/mL in 0.9% sterile saline and filtered through a 0.22- μ m filter. Brusatol (Selleck, China) was dissolved in dimethylsulfoxide, and diluted to 0.5 μ mol/mL for intragastric administration.

2.2. Ethical approval

All the experimental procedures were approved by the Ethics Committees of Zhengzhou University. The animal use was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and by Animal Ethics Committee of Zhengzhou University.

2.3. Cell culture

hUC-MSCs were isolated from human umbilical cords obtained from healthy donors with prior informed consent. The isolation, identification, and culture of hUC-MSCs were performed as previously described [19]. hUC-MSCs were routinely cultured and collected at 5, 10 and 15 passages (P5, P10 and P15) for series of replicative cell senescence model.

2.4. Cell proliferation, migration, apoptosis, cell cycle and senescence detection

Then, P5, P10 and P15 hUC-MSCs were treated with 30 μ g/mL MG53 protein, and divided into 6 groups for the following experiments: P5 hUC-MSCs group (P5), P5 hUC-MSCs treated with MG53 group (P5+MG53), P10 group, P10+MG53 group, P15 group, and P15+MG53

group. CCK8 assay, transwell assay and flow cytometry analysis were respectively used to detect the cell viability, migration rate, and cell cycle changes as previously described [18]. Senescence-associated β -gal kit (GenMed, USA) was carried out to examine the senescence of hUC-MSCs as previously described [19].

2.5. Western blot

Total proteins in cells or tissues were extracted using the Cell Total Protein Extraction Kit (Sangon, China). After separated by SDS-PAGE and transferred to a poly(vinylidene fluoride) (PVDF) membrane, the proteins on membrane were blocked and then incubated with primary antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:5000, SA00001-2, Proteintech, China). The primary antibodies were MG53 (1:1000), P16 (1:1000, C610021, Sangon, China), P21 (1:1000, D220091, Sangon, China), P53(1:1000, 10442-1-AP, Proteintech, China), Tau (1:1000, D121297, Sangon, China), p-Tau (Ser396) (1:1000, D155045, Sangon, China), p-Tau (Ser231) (1:1000, D155301, Sangon, China) and p-Tau (Ser235) (1:1000, D155045, Sangon, China), Nrf2 (1:1000, WL02135, Wanlei, China), SOD1 (1:1000, WL01846, Wanlei, China), NQO1 (1:1000, WL04860, Wanlei, China) and Keap-1 (1:1000, WL03285, Wanlei, China), pCNA (1:1000, 10205-2-AP, Proteintech, China), Sirt1 (1:2000, 13161-1-AP, Proteintech, China), β -actin (1:2000, 20536-1-AP, Proteintech, China) and Histone-3 (1:2000, WL0984a, Wanlei, China). β -Actin and Histone-3 were used as internal reference of protein expression in the cytosol and nucleus, respectively.

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

The mRNA expressions of p16, p21, p53 and SIRT1 were detected by qRT-PCR according to previous studies [18,19].

2.7. Oxidative stress analysis

The activities of MDA and SOD were performed by using the commercial kits (A003-1 and A001-3, Jiancheng Bioengineering, China). Briefly, cells were homogenized by an ultrasonic equipment and the protein concentration was determined using a microspectrophotometer. Then, the detailed procedure for hUC-MSCs or serum samples were followed the manufacturer's protocol. Finally, the absorbance from different samples were measured by a full-wavelength microplate reader.

2.8. Reactive oxygen species (ROS) production

The production of intracellular ROS of hUC-MSCs was measured using DCFH-DA kit (Solarbio, China). Briefly, cells were stained with 10 μ M DCFH-DA and incubated for 20 min. After re-suspension, the average fluorescence of cells was detected by flow cytometry (Becton Dickinson, USA) as previously described [20]. For *in vivo* detection, the levels of ROS in the brain were measured by injecting dihydroethidium (DHE, a specific *in situ* marker for oxidative stress) 1 h before sacrificing the mice as previously reported [21].

2.9. Human inflammatory cytokine array

The release of senescence-associated secretory phenotype (SASP), such as IL-1 α , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, IFN γ and TNF- α in the supernatant of hUC-MSCs was measured by human inflammation array (RayBiotech, China) according to the manufacturer's protocol.

2.10. Animal experiments

A total of 76 AD mice (APP/PS1 transgenic mice, 6-month old) were divided into different groups and C57BL/6 mice were used as WT

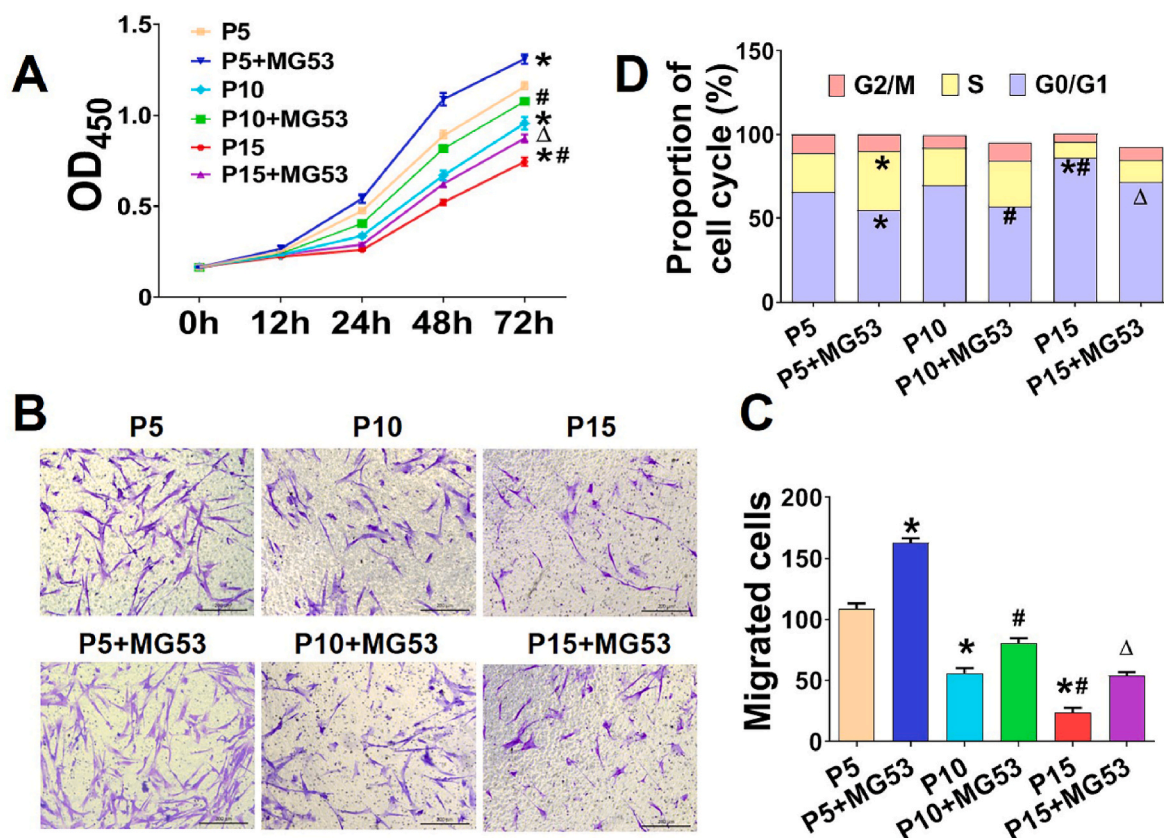


Fig. 1. Effects of MG53 on the proliferation and migration of hUC-MSCs in P5, P10 and P15. (A) Cell viability analyzed by CCK-8 assay. (B) Representative images of Transwell assay. (C) The migrated cell in each group. (D) Cell cycle proportion changes in different groups. Data were presented as mean \pm SEM. N = 3 per group. Scale bar = 200 μ m *: Compared with P5 group, $P < 0.05$; #: Compared with P10 group, $P < 0.05$; Δ : Compared with P15, $P < 0.05$.

control. For *in vivo* anti-aging study, 16 AD mice were respectively intravenously administered 0.9% saline, P5 hUC-MSCs (P5), P15 hUC-MSCs (P15) and MG53 pretreated P15 hUC-MSCs (MG53-P15) as previously reported [16]. In order to investigate the effects of combined therapy, 44 AD mice were divided into 4 groups: AD (AD mice injected with vehicle), MG53, MSCs, and MG53+MSCs. MG53 protein (3 mg/kg) and/or P5 hUC-MSCs (1×10^6 cells) were intravenously given to AD mice once a day for 3 consecutive days. For Nrf2 signaling-dependent rescue experiment, 16 AD mice were divided into 4 groups: AD (AD mice injected with vehicle), AD+BRU, MG53+MSCs, MG53+MSCs+BRU. MG53 and P5 hUC-MSCs were intravenously administered as mentioned above. 2 μ mol/kg BRU was gavaged once daily for 28 days.

2.11. Behavioral tests

Morris water maze test (MWM), novel object recognition (NOR), open field test (OFT), forced swim test (FST), tail suspension test (TST), and sucrose preference test (SPT) were performed to evaluate cognitive function, anxiety and depression behaviors as previously described [17, 22].

2.12. Tissue preparation

After behavioral tests were completed, the mice were anesthetized with 10% chloral hydrate and sacrificed. Then, the brains and blood were collected for the following experiments. After dehydration, the frozen brain tissues were embedded with OCT and serially cut to 10 μ m-thickness of coronal sections using a cryostat (Leica, Germany). The blood in each group was drawn and placed at room temperature for 24 h, then centrifuged at 2000 rpm for 15 min to obtain the serum.

2.13. Nissl staining

Coronal brain sections were stained with Cresyl violet to evaluate the neuronal loss and the integrity of Nissl bodies. Briefly, the frozen sections were gradually rewarmed and placed in Cresyl Violet stain dye (Solarbio, China) and incubated in 56 $^{\circ}$ C incubator for 1 h. After rinsed twice, the slices were added with Nissl differentiation solution for 1 min. Finally, the sections were dehydrated, transparentized and observed according to manufacturer's instructions.

2.14. TUNEL staining

TUNEL staining was carried out to detect the cell apoptosis in the brain sections by using In Situ Cell Death Detection Kit (KeyGEN, China) according to manufacturer's protocol as previously described [22].

2.15. Immunofluorescence staining

The cellular immunofluorescence staining for Lamin B1 (1:100, CL594-66095, Proteintech, China) was performed to detect cell senescence. For tissue immunofluorescence staining, after blocking, the frozen sections were incubated with primary antibodies against NeuN (1:1000, ab177487, Abcam, USA), A β (mouse anti-A β (1:200, SIG-39320, Covance), DCX (1:100, ab18723, Abcam, USA), MAB1281 (1:100, MAB1281, Merck, USA), GFAP (1:500, 16825-1-AP, Proteintech, China) and Iba1 (1:100, 10904-1-AP, Proteintech, China) overnight at 4 $^{\circ}$ C, and then incubated with Cy3 or FITC-conjugated anti-mouse or anti-rabbit IgG (1:200, AB0122, Abways, China) for 2 h. For EdU/DCX, EdU/NeuN, or EdU/Nestin double immunofluorescence staining, EdU solution (5 mg/kg) was intraperitoneally injected daily for three consecutive days before the mice were sacrificed, according to the

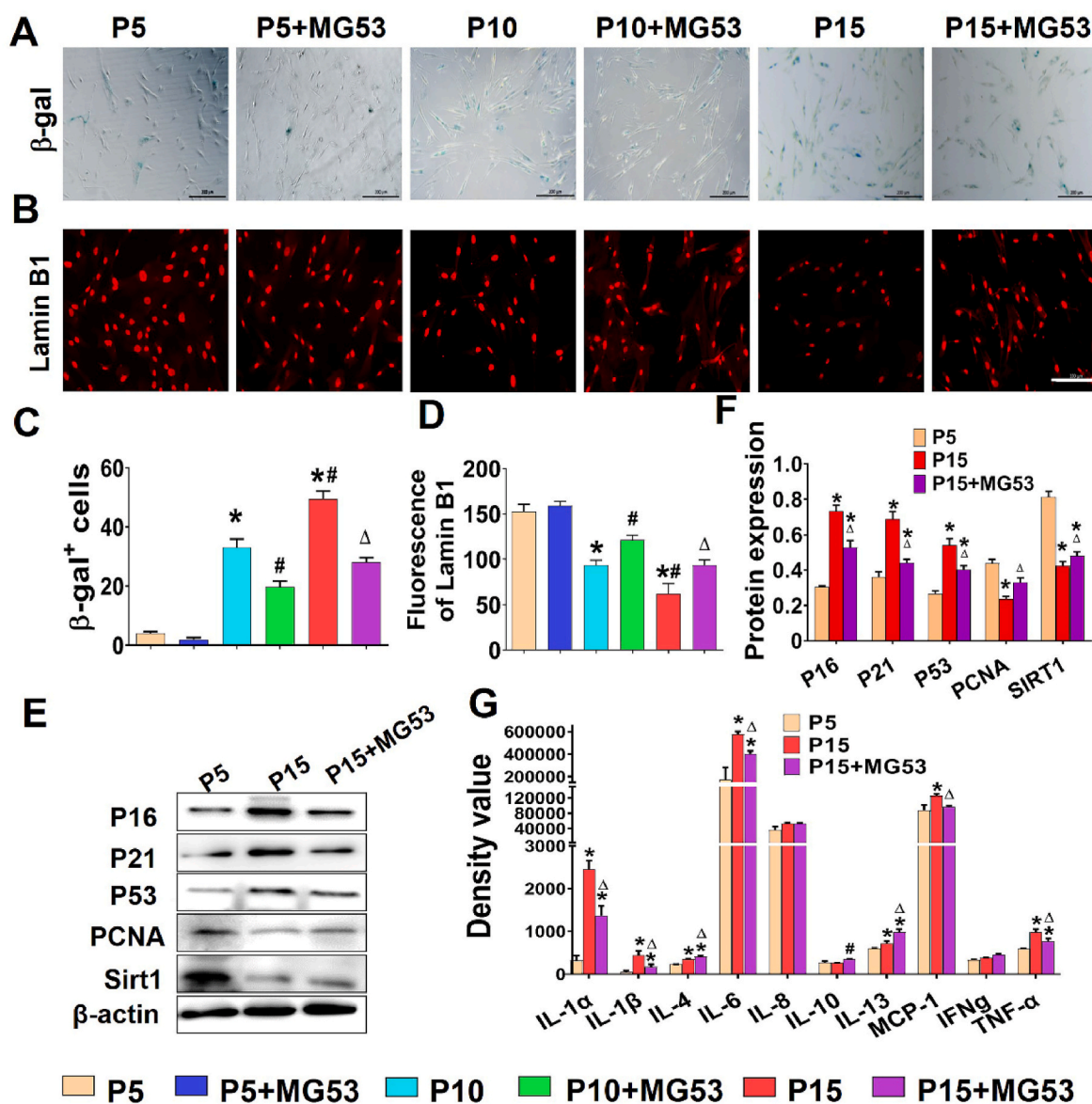


Fig. 2. MG53 rejuvenates senescent hUC-MSCs. (A) Representative images of β -gal staining. Scale bar = 200 μ m. (B) Representative images of Lamin B1 cellular immunofluorescence. Scale bar = 100 μ m. (C) Quantitative analysis of β -gal-positive cells. (D) Analysis of fluorescence of Lamin B1. (E) Western blots of P16, P21, P53, PCNA and Sirt1 protein. (F) Grey analysis of P16, P21, P53, PCNA and Sirt1 protein. (G) Human inflammation array was used to detect the release of SASP in different groups. Data were presented as mean \pm SEM. N = 3 per group. *: Compared with P5 group, $P < 0.05$; #: Compared with P10 group, $P < 0.05$; Δ : Compared with P15, $P < 0.05$.

instructions of the Cell-Light EdU Apollo567Kit (RiboBio, China). After Apollo staining, the slices were respectively incubated with DCX, NeuN or Nestin antibody (1:1000, 19483-1-AP, Proteintech, USA) overnight at 4 $^{\circ}$ C and incubated with FITC-conjugated IgG antibody (1:500, SA00003-2, Proteintech, USA) for 2 h. After DAPI (1:100) counterstaining, the sections were examined under a microscope (Leica, German), and the positive cells were analyzed using Image J software (Bethesda, USA).

2.16. Statistical analysis

All experiments were performed at least three times. The data were presented as mean \pm SEM, analyzed and plotted with GraphPad Prism 8 software. One-way analysis of variance (ANOVA) was used to analyze the difference between two groups. P value < 0.05 was considered statistically significant.

3. Results

3.1. MG53 protein promotes the proliferation and migration of hUC-MSCs

P5 and P15 hUC-MSCs were characterized by flow cytometry using their surface markers, and their karyotype, osteogenic or adipogenic differentiation capacity were confirmed as previously described [11]. We firstly examined the proliferation and migration of hUC-MSCs in P5, P10 and P15. As shown in Fig. 1A–C, compared with P5 MSCs, P10 and P15 MSCs had decreased cell viability and migration ability ($P < 0.05$). Besides, with the increase of passages, the proportion of G0/G1 phase gradually increased in P10 and P15 MSCs, while S phase decreased correspondingly ($P < 0.05$, Fig. 1D). MG53 was reported specifically located in skeletal and myocardial muscle [13]. In order to clarify whether hUC-MSC has endogenous MG53 expression or not, Western blot was carried out. As shown in Fig. S1, MG53 was not expressed in

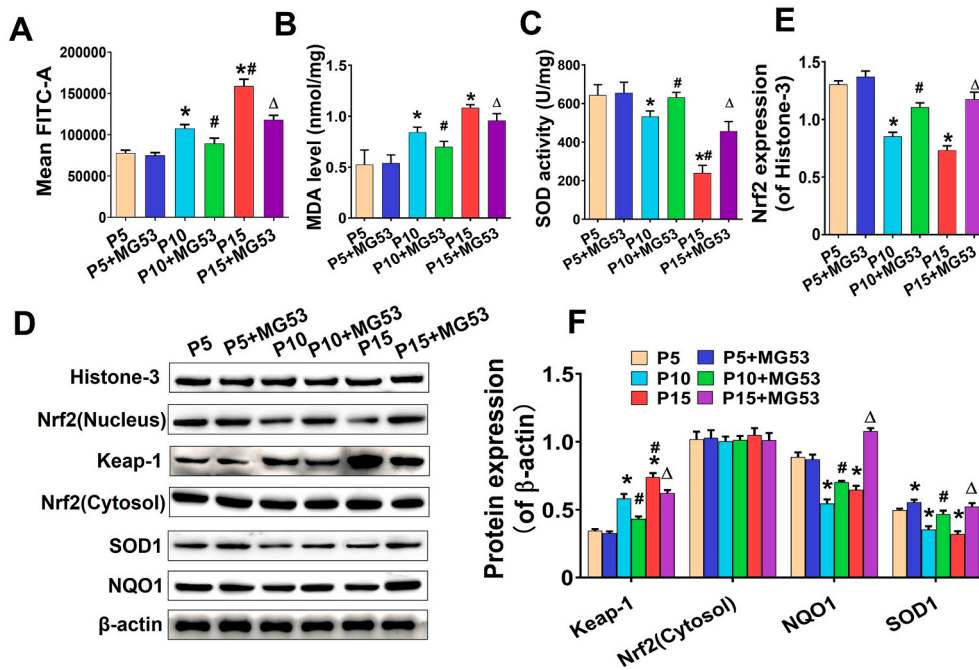


Fig. 3. MG53 reduces oxidative stress in senescent hUC-MSCs by activating the Nrf2 signaling pathway. (A) ROS production measured by DCFH-DA kit. (B) MDA level. (C) SOD viability. (D) Protein expression of Nrf2 signaling pathway in each group. (E) Densitometric analysis of Nrf2 expression in the nucleus. (F) Densitometric analysis of Keap1, NQO1, SOD1 and Nrf2 in the cytosol. Data were presented as mean \pm SEM. N = 3 per group. *: Compared with P5 group, $P < 0.05$; #: Compared with P10 group, $P < 0.05$; Δ: Compared with P15, $P < 0.05$.

hUC-MSCs, but was observed after rhMG53 pretreatment. Compared with the untreated group, pretreatment with MG53 protein significantly promoted the proliferation and migration of different passages of hUC-MSCs, and reduced the G0/G1 phase arrest ($P < 0.05$, Fig. 1).

3.2. MG53 protein rejuvenates senescent hUC-MSCs

Then, we examined the effects of MG53 on replicative senescence of hUC-MSCs in P5, P10 and P15. With the increase of passages, hUC-MSCs exhibited increased β -gal⁺ cells (Fig. 2A and C, $P < 0.05$), and elevated p16, p21 and p53 expression (Fig. S2 A, Fig. 2E and F, $P < 0.05$). Additionally, the expression of Lamin B1, another senescence indicator, was significantly inhibited in P15 hUC-MSCs compared with P5 hUC-MSCs (Fig. 2B and D, $P < 0.05$). Compared with the untreated group, MG53 treatment greatly reduced β -gal⁺ cells, P16, P21 and P53 expression, whereas it promoted Lamin B1, PCNA and Sirt1 expression (Fig. 2A–F, $P < 0.05$). Also, MG53 significantly suppressed the secretion of IL-1 α , IL-1 β , IL-6, TNF- α , MCP-1 and enhanced the release of the anti-inflammatory cytokines IL-4, IL-10 and IL-13, as evidenced by human inflammatory cytokine array (Fig. 2G, $P < 0.05$). Collectively, these data suggest that MG53 can inhibit the replicative senescence of hUC-MSCs and rejuvenate senescent hUC-MSCs.

3.3. MG53 reduces oxidative stress in senescent hUC-MSCs by activating the Nrf2 signaling pathway

Previous study showed that oxidative stress caused by excess ROS contributes to the senescence of MSCs [23]. And, Nrf2 signaling is one of the key molecular pathways that regulates aging, inflammation and oxidative stress [24]. Thus, we investigate whether MG53 could inhibit replicative senescence of hUC-MSCs by regulating oxidative stress via Nrf2 signaling pathway. As the passages increased, P10 and P15 hUC-MSCs showed higher ROS and MDA production, and lower SOD activity than those in P5 hUC-MSCs, which were obviously reversed by MG53 pretreatment (Fig. 3A–C, $P < 0.05$). In addition, MG53 significantly reduced the expression of Keap-1 and promoted expression of Nrf2 in nucleus, NQO1 and SOD1 in senescent hUC-MSCs detected by Western blot (Fig. 3D–F, $P < 0.05$). Taken together, our observations indicate that MG53 can reduce oxidative stress via the activation of Nrf2

signaling pathway.

3.4. MG53 pretreatment improves the therapeutic effect of senescent hUC-MSCs in AD mice

To define whether MG53 could improve the therapeutic effect of senescent hUC-MSCs *in vivo*, we intravenously injected MG53 pretreated P15 hUC-MSCs (MG53-P15 MSCs) into AD mice via tail vein. Firstly, MWM and NOR tests were used to evaluate the spatial learning and memory function of mice. As shown in Fig. 4A–D, mice in the P5 MSCs group exhibited shorter escape latency, more crossing times, more time spent in the target quadrant and higher discrimination index than those in AD group ($P < 0.05$). Compared with the P5 MSCs group, P15 MSCs transplantation had a very mild effects on AD mice, such as in MWM performance (Fig. 4A–C, $P > 0.05$). However, these behavioral indicators were partially restored in MG53-P15MSCs group (Fig. 4A–D, $P < 0.05$), suggesting that MG53-P15MSCs were superior to P15 MSCs in improving the memory of AD mice. Of note, the survival of neuronal cells and NeuN positive cells in the P15 group were significantly lower than those in the P5 group (Fig. 4E–H, $P < 0.05$). However, MG53-P15 MSCs group had increased surviving neural cells and NeuN positive cells than those in the P15 group (Fig. 4E–H, $P < 0.05$). And, the higher TUNEL positive cells in the P15 group was also attenuated in the MG53-P15 MSCs group (Fig. 4I and J, $P < 0.05$). Notably, compared with AD group, MG53-P15 MSCs could promote the expression of NeuN and reduce the apoptosis shown by less TUNEL-positive cells in hippocampus (Fig. 4G–J, $P < 0.05$). Overall, these results suggest that MG53 pretreatment can improve the therapeutic effect of senescent hUC-MSCs in AD mice.

3.5. Combined therapy of MG53 and hUC-MSCs transplantation synergistically alleviates memory impairment and depression-like behavior of AD mice

In order to further explore whether MG53 could improve the microenvironment and augment the efficacy of young hUC-MSCs (P5) in AD, we simultaneously applied MG53 injection and hUC-MSCs transplantation to treat AD mice. Compared with the AD group, mice in MG53, MSCs, and MG53+MSCs groups exhibited more crossings and

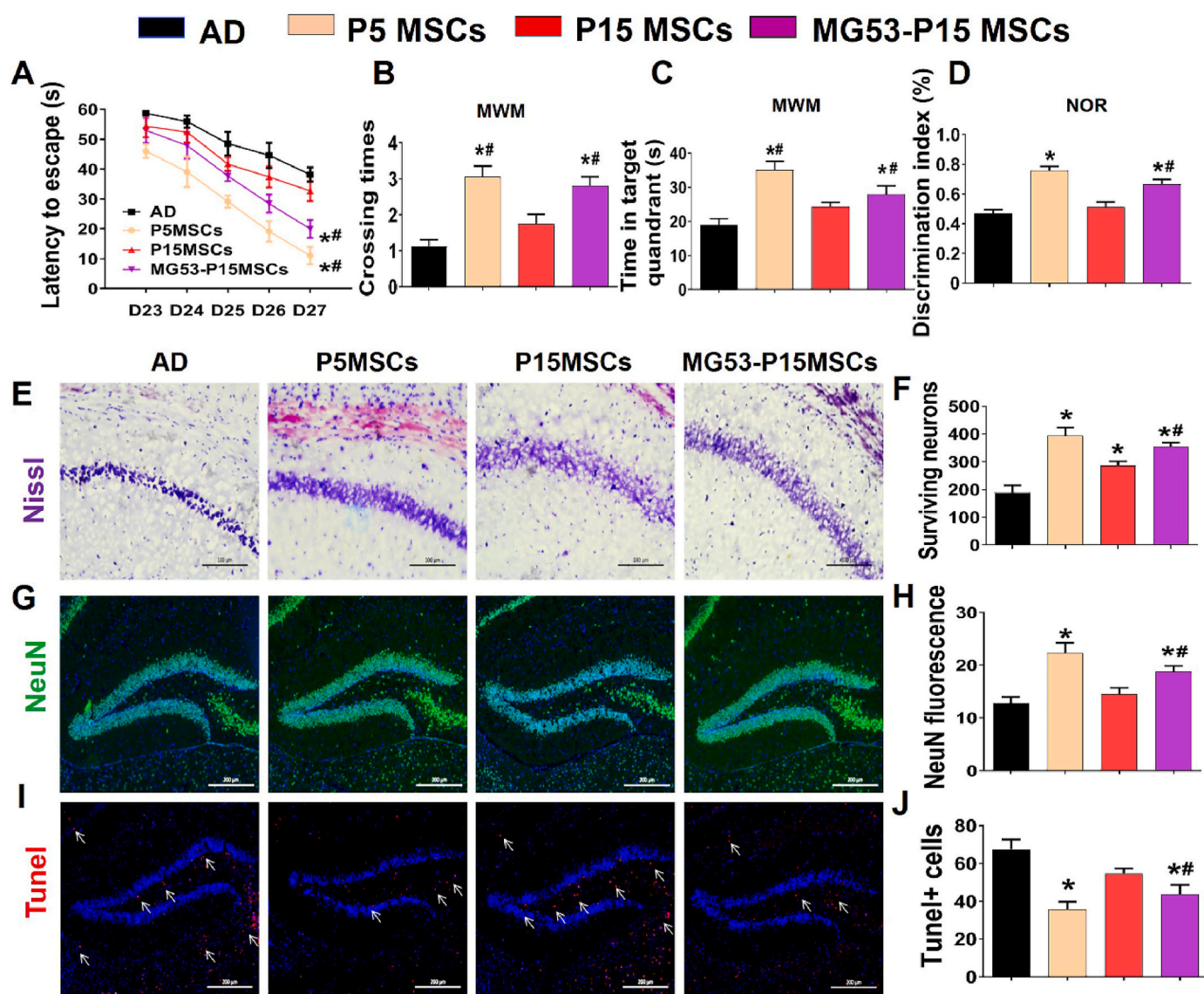


Fig. 4. MG53 pretreatment improves the therapeutic effect of senescent hUC-MSCs on AD mice. (A) Escape latency, (B) crossing numbers, (C) time spent in the target quadrant in MWM test, (D) Discrimination index in the NOR test. (E) Representative images of Nissl staining and (F) Quantitative analysis of surviving neurons. Scale bar = 100 μ m. (G) Representative immunofluorescence images of NeuN and (H) Quantitative analysis of NeuN+ cells. (I) Representative TUNEL staining images and (J) TUNEL + cells. Scale bar = 200 μ m. Cell nuclear (blue) was labeled by DAPI. Data were presented as mean \pm SEM. Data were presented as mean \pm SEM. N = 4 per group. *: Compared with AD group, $P < 0.05$; #: Compared with P15 MSCs group, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

time spent in the target quadrant in MWM test, and the performances in the MG53+MSCs group were most significant improved (Fig. 5A–C, $P < 0.05$). Given that most AD patients usually accompany some psychiatric symptoms such as anxiety and depression, mood behavior was also evaluated by FST, OFT, and NSF in our work. Compared with other three groups, the MG53+MSCs group showed the longest total distance, most total rearings in OFT, and the shortest immobility time measured by FST (Fig. 5D–F, $P < 0.05$). Besides, the feed latency was the lowest, while the total food consumption in NSF was the highest in the MG53+MSCs group (Fig. 5G and H, $P < 0.05$). Furthermore, the performance of the NOR, TST, and SPT in each treated group was better than that in the AD group (Fig. S3, $P < 0.05$). However, there was no significant difference between the MG53+MSCs group and MSCs treatment alone ($P > 0.05$). Collectively, these results indicate that MG53 and hUC-MSCs transplantation can synergically alleviate memory impairment and depression-like behavior in AD mice.

3.6. MG53 and hUC-MSCs transplantation reduces $A\beta$ deposition and Tau phosphorylation of AD mice

Amyloid- β ($A\beta$) deposition and neurofibrillary tangles caused by Tau hyperphosphorylation are the two main pathological features of AD [25]. So, we then explored the effects of MG53 and hUC-MSCs transplantation on $A\beta$ deposition and Tau phosphorylation examined by immunofluorescence and western blot, respectively. As shown in Fig. 6A and B, there was an obvious $A\beta$ plaque formation in the brain of AD mice, while MG53 and/or MSCs transplantation significantly reduced $A\beta$ deposition ($P < 0.05$). In addition, the expressions of p-Tau (Ser396), p-Tau (Ser231) and p-Tau (Ser235) were significantly inhibited by MG53 or MSCs alone, and MG53+MSCs combined group showed the least p-Tau (Ser231) expression than any single treatment of MG53 or MSCs groups (Fig. 6C and D, $P < 0.05$). However, there was no significant difference in total Tau levels among all groups ($P > 0.05$). Overall, our findings demonstrate that combined therapy of MG53 and

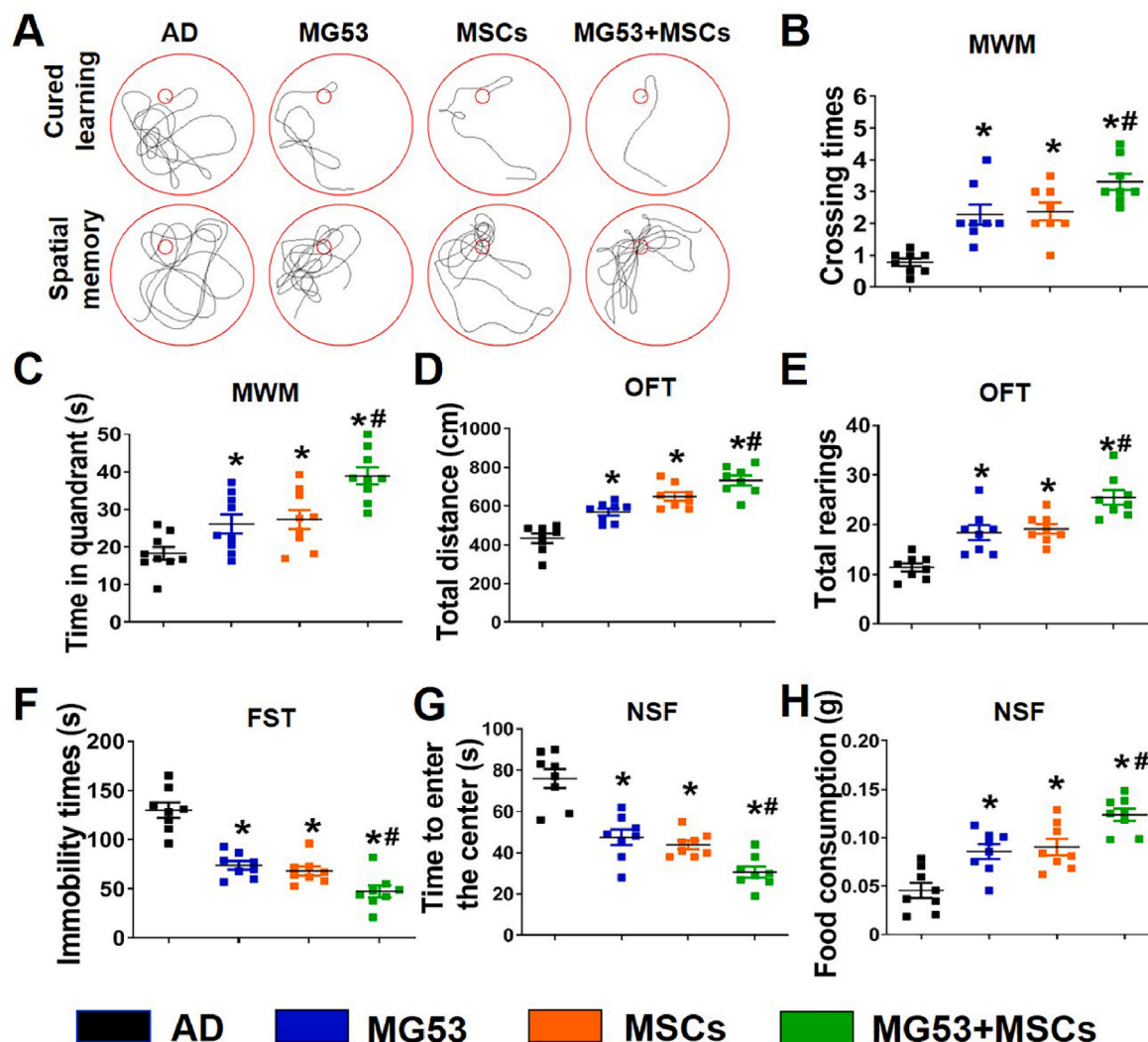


Fig. 5. MG53 and hUC-MSCs transplantation synergically alleviates memory impairment and depression-like behavior in AD mice. (A) The swimming trajectory path of MWM. (B) The crossing times of mice in each group. (C) Time spent in target quadrant. (D) Total distance and (E) total rearings of mice in OFT. (F) Immobility time in FST. (G) Time to enter the center and (H) the total food consumption in NSF. $N = 8$ per group. Data were presented as mean \pm SEM. *: Compared with the AD group, $P < 0.05$; #: Compared with the MSCs group, $P < 0.05$.

hUC-MSCs transplantation efficiently reduces A β burden and Tau hyperphosphorylation in AD mice.

3.7. MG53 combined with hUC-MSCs transplantation enhances neurogenesis in the brain of AD mice

Neuronal loss and neurogenesis in the hippocampus play a crucial role in the maintenance of homeostasis and cognitive function [26]. So, the neuronal apoptosis was detected by TUNEL staining. We found that MG53 and/or MSCs significantly inhibited neural apoptosis in the hippocampus (Fig. S4, $P < 0.05$). Then, we further detected the neurogenesis by double immunofluorescence staining of specific marker of neural cells. As illustrated in Fig. 7, the double positive staining of EdU⁺/DCX⁺ cells and EdU⁺/NeuN⁺ cells in the hippocampus of MG53+MSCs group were significantly increased compared with other three groups (Fig. 7A–C, $P < 0.05$). Besides, mice in the MG53+MSCs group exhibited the highest EdU⁺/Nestin⁺ cells (Fig. 7A and D, $P < 0.05$), a specific marker of neural stem cells (NSCs). In addition, MAB1281 was used to label the migrated and survived hUC-MSCs in the brain after transplantation. The MG53+MSCs group exhibited more MAB1281 positive cells than MSCs group (Fig. S5, $P < 0.05$). These results suggest that MG53 could promote the survival of hUC-MSCs *in*

in vivo and activate the endogenous NSCs. Thus, our results prove that MG53 combined with hUC-MSCs transplantation ameliorates neuronal loss and enhances neurogenesis in the brain of AD mice.

3.8. MG53 combined with hUC-MSCs transplantation reduces the activation of glia cells and oxidative stress in AD mice by activating the Nrf2 signaling pathway

Activation of glial cells and oxidative stress can initiate and accelerate AD pathology [27,28]. To determine whether MG53 can regulate the neuroinflammatory microenvironment in the brain of AD mice, immunofluorescence staining of GFAP and Iba-1 was used to detect the activation of astrocytes and microglia in hippocampus. Compared with the AD group, the number of GFAP⁺ or Iba-1⁺ cells in each treated group was significantly reduced, and the MG53+MSCs group had the lowest activation of astrocytes and microglia (Fig. 8A–C, $P < 0.05$). Meanwhile, the MG53+MSCs group had the least ROS production and MDA content, and the highest SOD activity compared with other three groups (Fig. 8A, D–F, $P < 0.05$). Our results indicated that MG53 combined with hUC-MSCs transplantation could attenuate the activation of glia cells and oxidative stress in AD mice. Furthermore, mechanistic studies demonstrated that the protein levels of Nrf2 in nucleus, NQO1

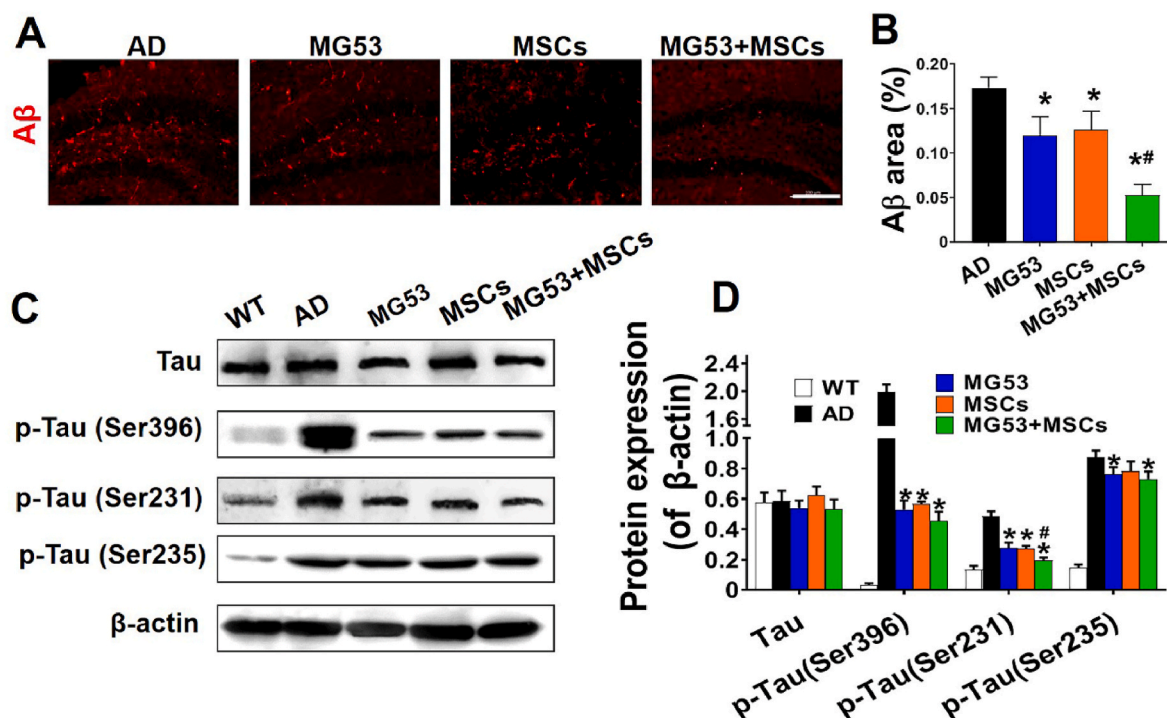


Fig. 6. MG53 combined with hUC-MSCs transplantation reduces A β burden and Tau phosphorylation in AD mice. (A) Representative immunofluorescence images of A β . Scale bar = 100 μ m. (B) Analysis of A β plaque area. (C) Expressions of Tau and p-Tau were detected by Western blot and (D) densitometry analysis. Data were presented as mean \pm SEM. N = 3 per group. *: Compared with the AD group, $P < 0.05$; #: Compared with the MSCs group, $P < 0.05$.

and SOD1 were significantly increased, while Keap-1 expression was downregulated in the MG53+MSCs group (Fig. 8G–I, $P < 0.05$). To conclude, our data manifest that MG53 combined with hUC-MSCs transplantation reduces glial cells activation and oxidative stress in AD mice by activating the Nrf2 signaling pathway.

3.9. BRU attenuates the therapeutic effects of MG53 and hUC-MSCs in AD mice

To further verify whether the neuroprotective effects of MG53 and hUC-MSCs is Nrf2 signaling-dependent, Brusatol (BRU), a specific inhibitor of the Nrf2 signaling [29], was simultaneously injected into AD mice. As expected, BRU markedly reversed the expression of Nrf2 in nucleus and Keap1 in cytosol mediated by MG53+MSCs transplantation (Fig. 9A and B, $P < 0.05$). Moreover, BRU significantly weakened the behavioral and mood improvement effects of MG53 combined with hUC-MSCs transplantation in AD mice (Fig. 9C–J, $P < 0.05$). Meanwhile, BRU also counteracted the increased Nissl bodies and DCX expression induced by MG53 and hUC-MSCs, and accelerated A β deposition (Fig. 9K–P, $P < 0.05$). Thus, MG53 and hUC-MSCs synergically played neuroprotective roles in AD mice mainly by activating the Nrf2 signaling pathway.

4. Discussion

Cell senescence is associated with the accumulation of SASP and oxidative stress, which in turn lead to irreversible replication inhibition, decreased differentiation potential, and impaired migratory ability [30, 31]. MSCs is the most widely used stem cells for clinic trial. However, it easily tends to become senescent after several passages of culture *in vitro*, which limits its quality and application. We and other researchers previously reported that with the increase of passages, hUC-MSCs cultured *in vitro* easily exhibit replicative senescence [11,32]. In our previous study, we found that P15 hUC-MSCs underwent morphological changes with swollen enlarged cell bodies [11]. Moreover, P15 hUC-MSCs

transplantation seriously affected their therapeutic effects in AD compared with the young MSCs [11]. Consistent with these observations, P10 and P15 hUC-MSCs displayed replicative senescence during *in vitro* passaging, which presented decreased proliferation and migration capacity, accelerated senescent cells, oxidative stress, and SASPs release. And, P15 MSCs performed more significant senescent characteristics than P10 MSCs. The rescued function of MG53 was proved in P10 and P15 MSCs in our experiments. In order to highlight the anti-aging effect of MG53, the more senescent P15 MSCs was chosen for further study.

Importantly, rejuvenating senescent MSCs to increase their therapeutic time window and efficacy in regenerative medicine is very pressing and challenging. Indeed, a series of novel strategies have been explored to restore senescent MSCs, such as targeting specific molecular pathways and mitochondrial metabolism [33]. Recently, rhMG53 protein was shown as a novel safe reagent to delay aging, which could improve heart function and the survival of the aging mice [34]. In the current study, we observed that pretreatment of MG53 protein promoted the proliferation and migration of hUC-MSCs, and rejuvenated senescent hUC-MSCs by reducing SA- β -gal positive cells, senescence associated protein expressions, and SASPs release. Besides, our data revealed that MG53 protein ameliorated replicative senescence of hUC-MSCs by decreasing oxidative stress.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates cellular oxidative stress and inflammatory response and plays a vital role in maintaining stem cell function and neural regeneration [24]. Under normal conditions, Nrf2 is continuously ubiquitinated by Keap-1, which leads to Nrf2 degradation through the ubiquitin-proteasome pathway. Upon oxidative stress, Nrf2 translocates to the nucleus and binds to antioxidant response element (ARE) sites, which activates the downstream genes expression, such as HO-1, SOD1, NQO1, etc [35]. In the present study, we found that MG53 significantly reduced oxidative stress in senescent hUC-MSCs by activating the Nrf2 signaling pathway. Moreover, MG53 pretreated-senescent hUC-MSCs demonstrated a greater capacity to improve the cognition, attenuate neuronal loss and promote neurogenesis in AD mice than the senescent

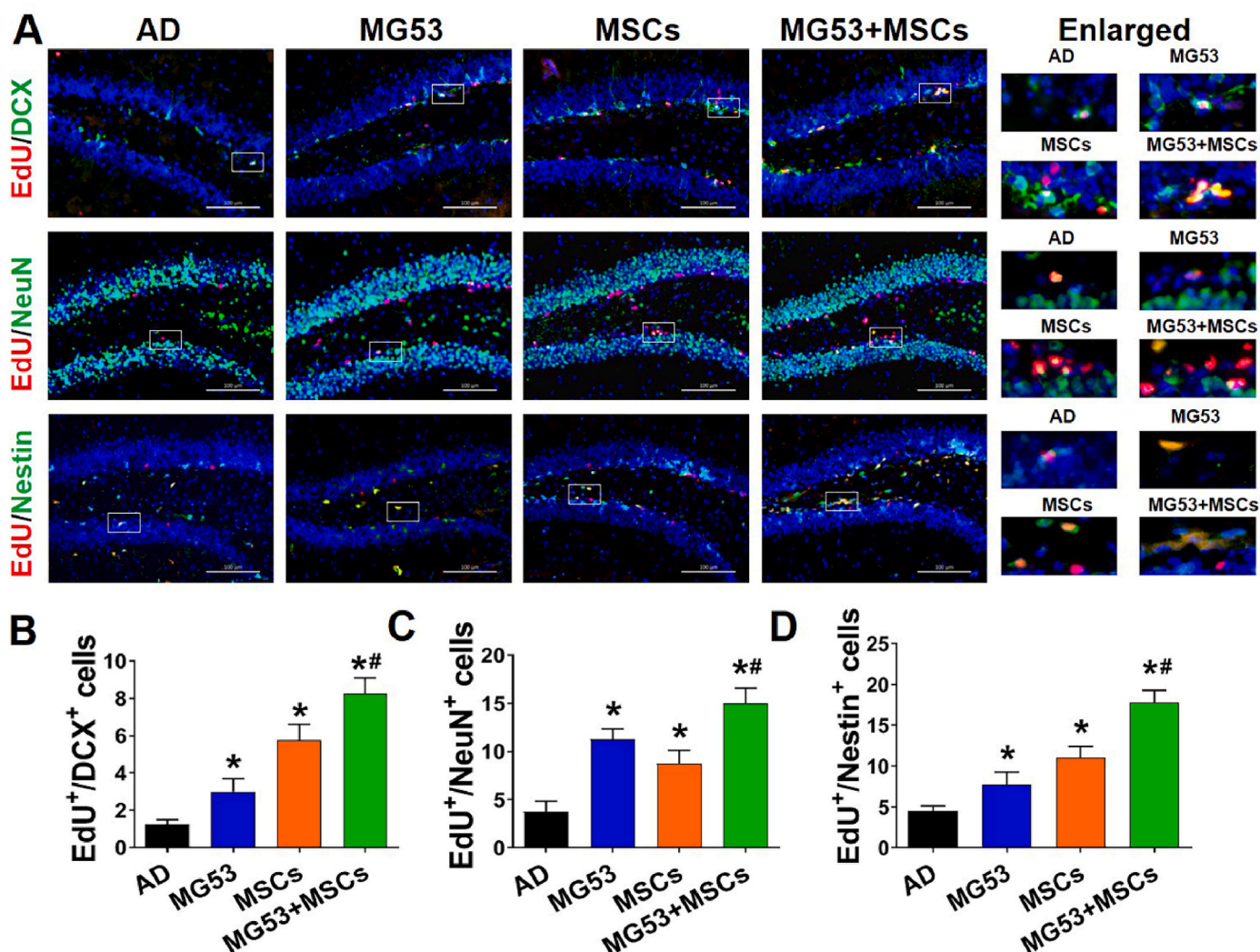


Fig. 7. MG53 combined with hUC-MSCs transplantation enhances neurogenesis in the brain of AD mice. (A) Representative immunofluorescence images of EdU/DCX, EdU/NeuN, and EdU/Nestin double staining. Scale bar = 100 μ m. (B-D) Quantification analysis of EdU⁺/DCX⁺ cells, EdU⁺/NeuN⁺ cells, and EdU⁺/Nestin⁺ cells. Cell nuclear (blue) was labeled by DAPI. Data were presented as mean \pm SEM. N = 3 per group. Compared with the AD group, $P < 0.05$; #: Compared with the MSCs group, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hUC-MSCs transplantation alone. These findings confirmed that MG53 could rejuvenate senescent hUC-MSCs both *in vitro* and *in vivo*. But, the exact underlying mechanism still needs further in-depth studies.

Although accumulating evidence suggested that stem cell therapy is a promising strategy for AD, the chronic pathologic microenvironment in the brain is not conducive to the survival of transplanted stem cells [36,37]. Therefore, it is urgent to explore and develop new approach to improve the efficacy of stem cell therapy. Increasing studies showed that pharmacological and non-pharmacological interventions improved the neuroprotective effects of transplanted MSCs in AD mice [38]. Wang et al. found that hUC-MSCs transplantation combined with resveratrol contributed to neuroprotection in AD mice than individual treatment [39]. Our previous work showed that MG53 could protect hUC-MSCs against LPS-induced inflammatory damage and facilitate their efficacy in LPS-treated C57/BL6 mice [18]. In this study, we found that combined therapy of MG53 and young hUC-MSCs produced better therapeutic effects than hUC-MSC or MG53 single treatment, which was evidenced by the attenuation of cognitive deficit and improvement of depression-like behavior in AD mice.

It is well-known that A β deposition and Tau phosphorylation are the two major pathological features of AD [25]. APP/PS1 mice displayed A β plaques in the brain at 3 months of age and developed rapidly afterwards

[40]. We found a considerable reduction in A β deposition by treating AD mice with MG53 and hUC-MSCs. In addition, compared with AD mice, MG53 and hUC-MSCs transplantation significantly reduced Tau hyperphosphorylation in AD mice. These findings convince that MG53 and hUC-MSCs synergistically alleviate the pathological features of AD.

It is worth mentioning that A β -induced neuronal loss is the main cause that leads to cognitive deficits in AD [25]. Our results showed that treatment with MG53 and hUC-MSCs ameliorated neuronal loss and preserved neuronal survival in AD mice. Notably, MG53 combined with hUC-MSCs transplantation also inhibit the senescence of neuronal cells, which was in line with the previous report [41]. Moreover, exogenous stem cell transplantation or endogenous NSCs activation can promote neurogenesis [42]. We found that MG53 could improve the survival of hUC-MSCs in the brain of AD mice. In addition, MG53 combined with hUC-MSCs transplantation increased EdU/DCX, EdU/NeuN, and EdU/Nestin positive cells in the hippocampus, which indicates that MG53 and hUC-MSCs combined therapy also promote neurogenesis via the activation of endogenous NSCs.

Mechanically, activated glial cells and oxidative stress are detrimental to the survival of neurons, which are associated with A β deposition. Excessive microglia and astrocytes activation induces persistent chronic neuroinflammation, leading to neurodegeneration [43]. We

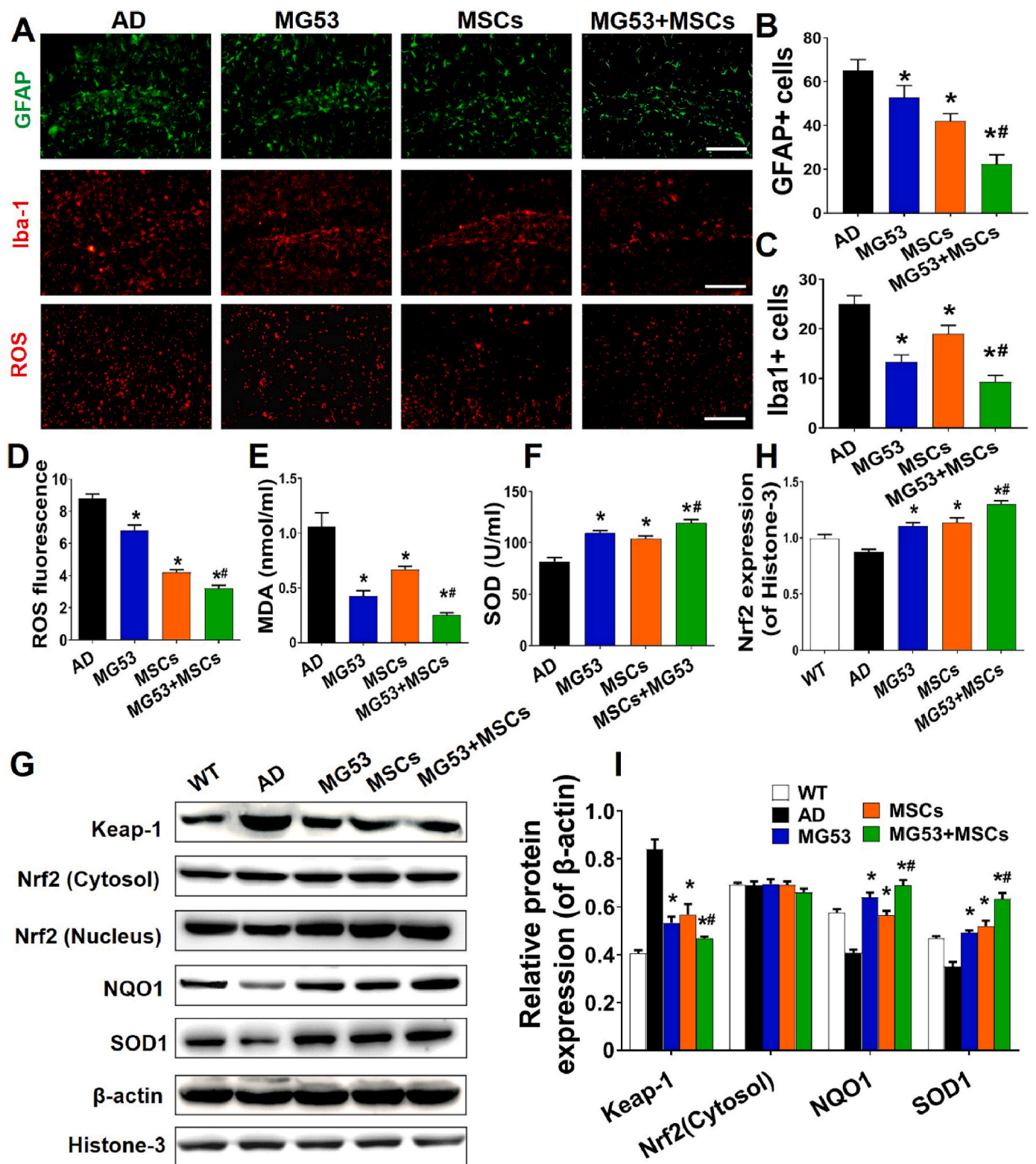
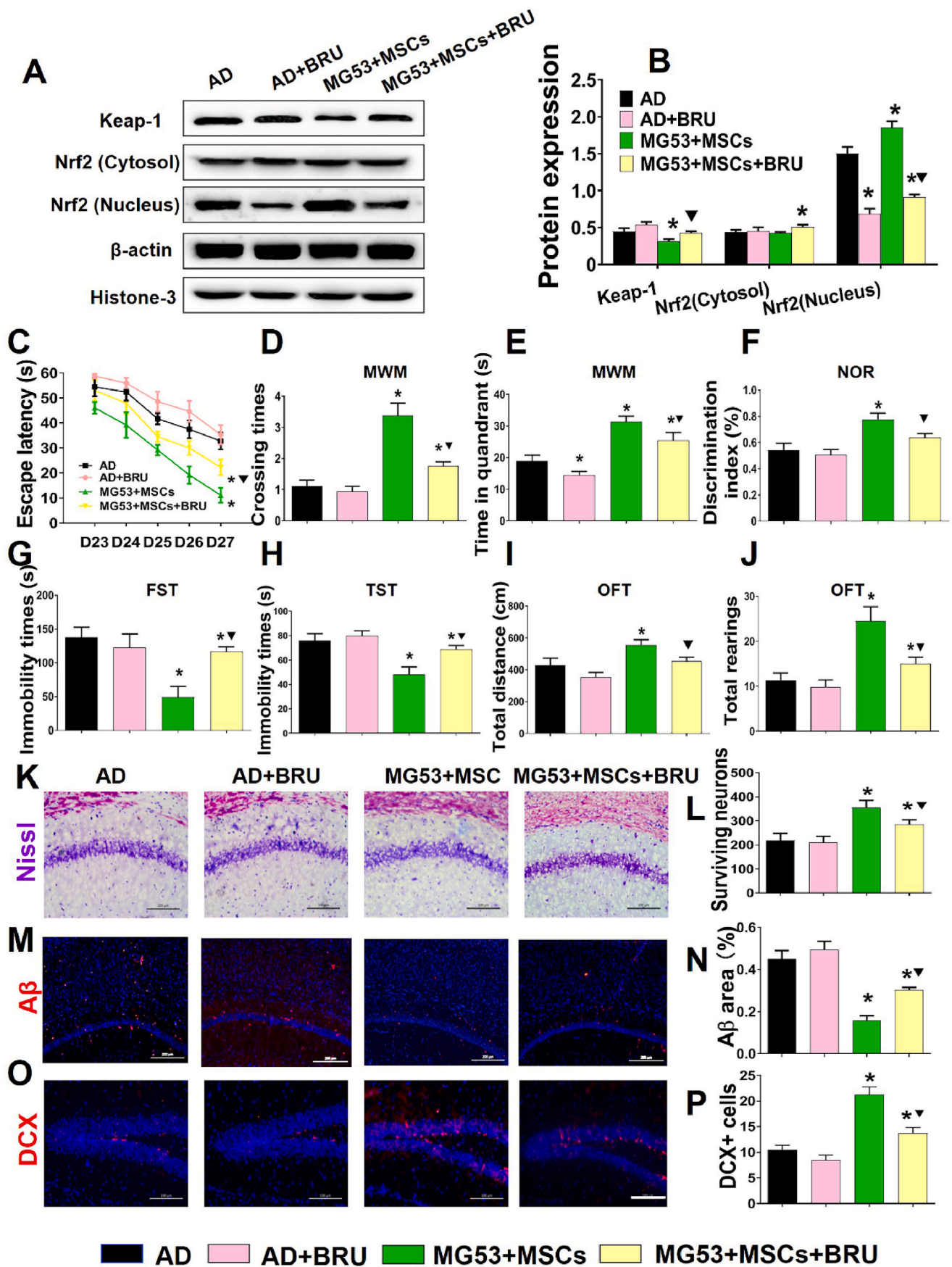


Fig. 8. MG53 and hUC-MSCs attenuate glia cells activation and oxidative stress in AD mice by targeting Nrf2 signaling pathway. (A) Representative immunofluorescence images of GFAP, Iba-1, and ROS staining. Scale bar = 100 μ m. (B) Quantification analysis of GFAP+ cells, (C) Iba1+ cells, and (D) ROS fluorescence. (E) MDA level. (F) SOD activity. (G) Immunoblots of key protein expressions of the Nrf2 signaling pathway and densitometric analysis (H–I). Data were presented as mean \pm SEM. N = 3 per group. *: Compared with the AD group, $P < 0.05$; #: Compared with the MSCs group, $P < 0.05$.



(caption on next page)

Fig. 9. BRU attenuates the therapeutic effects of MG53 combined with hUC-MSCs in AD mice. (A) Immunoblots of Keap-1 and Nrf2 protein and (B) densitometric analysis. (C) The swimming path of MWM. (D) The crossing times of mice in each group. (E) Time spent in target quadrant. (F) Discrimination index in NOR. (G) Immobility time in FST and TST (H). (I) Total distance and (J) total rearings of mice in OFT. N = 6 per group. (K) Representative images of Nissl staining and (L) quantitative analysis. Scale bar = 100 μ m. (M) Immunofluorescence images of A β and (N) analysis of A β plaque area. Scale bar=200 μ m. (O) Immunofluorescence images of DCX and (P) quantitative analysis of DCX⁺ cells. Cell nuclear (blue) was labeled by DAPI. Scale bar = 100 μ m. Data were presented as mean \pm SEM. N = 4 per group. *: Compared with the AD group, $P < 0.05$; \blacktriangleright : Compared with the MG53+ MSCs group, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

previously showed that MG53 protein mitigated LPS-induced neuroinflammation evidenced by lower IL-1 β and IL-6 production, and lessened microglia activation in the hippocampus of mice. In this study, our results indicated that MG53 combined with hUC-MSCs transplantation suppressed the activation of glial cells and oxidative stress in AD mice by promoting the expression of Nrf2, NQO1 and SOD1. Moreover, these therapeutic effects in AD mice were markedly attenuated by BRU, a specific Nrf2 signaling inhibitor. Collectively, our data suggest that MG53 could facilitate the neuroprotective efficacy of hUC-MSCs in AD mice at least partly by targeting the Nrf2 signaling pathway.

5. Conclusion

In this study, our results demonstrated that MG53 pretreatment rejuvenated senescent hUC-MSCs *in vitro* and enhanced the therapeutic effect of senescent hUC-MSCs in AD mice. Meanwhile, MG53 facilitated the therapeutic efficacy of young hUC-MSCs in AD mice manifested by improved cognition, decreased A β deposition and Tau hyperphosphorylation, and increased neurogenesis. Furthermore, the underlying mechanisms involved in the activation of Nrf2 signaling pathway. In conclusion, our results suggest that combined therapy of MG53 and hUC-MSCs may serve as an optimized approach for the treatment of AD.

Authors' contributions

Shanshan Ma: Conceptualization, Investigation, Methodology, Writing – original draft, Writing - review & editing. **Xinkui Zhou:** Methodology, Software, Investigation, Visualization, Validation. **Yaping Wang:** Investigation, Validation. **Zhe Li:** Software, Investigation, Validation. **Yingying Wang:** Formal analysis, Validation. **Jijing Shi:** Resources, Software. **Fangxia Guan:** Funding acquisition, Supervision, Project design and administration, Writing –review and editing.

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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

All the experimental procedures were approved by the Ethics Committees of Zhengzhou University. The animal use was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and by Animal Ethics Committee of Zhengzhou University. hUC-MSCs were isolated from human umbilical cords obtained from healthy donors with prior informed consent.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2022.102325>.

Abbreviations

AD	Alzheimer's disease
A β	amyloid- β
BRU	Brusatol
CCK8	cell counting kit-8
EdU	5-Bromo-2-deoxyUridine
GFAP	fibrillary acidic protein
hUC-MSCs	human umbilical cord derived mesenchymal stem cells
MG53	Mitsugumin53 protein
MDA	malondialdehyde
MWM	Morris water maze
Nrf2	Nuclear factor-erythroid 2-related factor 2
NOR	novel object recognition
NSF	novelty suppressed feeding test
OFT	open-field test
PFA	paraformaldehyde
ROS	reactive oxygen species
SAHF	senescence-associated heterochromatin foci
SOD	superoxide dismutase
TST	tail suspension test

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