

Research paper

Mitochondrial methionine sulfoxide reductase B2 links oxidative stress to Alzheimer's disease-like pathology

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ARTICLE INFO

Keywords:

Methionine sulfoxide reductase B2
Oxidative stress
APP/PS1 mice
APP
Transcription
Tau
Phosphorylation
JNK

ABSTRACT

Methionine sulfoxide reductase B2 (MSRB2) is a mitochondrial protein that protects cell from oxidative stress. The antioxidant activity suggests that MSRB2 may play a role in the pathophysiology of Alzheimer's disease (AD). Here, we report that in APP/PS1 mice, an animal model of AD, MSRB2 protein levels were decreased in the hippocampus at both young (6 mon) and old (18 mon) age, and in the cortex only at an old age, respectively. In HEK293 cells that stably express human full-length β -amyloid precursor protein (APP, HEK/APP), MSRB2 reduced the protein and mRNA levels of APP and β -amyloid converting enzyme 1 (BACE1), and the consequent amyloid beta peptide (A β) 1–40 and A β 1–42 levels. MSRB2 overexpression or knockdown also oppositely affected Tau phosphorylation at selective sites, with the concomitant alteration of the phosphorylated extracellular signal regulated kinase (p-ERK) and AMP-activated protein kinase (p-AMPK) levels. Moreover, in cells treated with long-term (24 h) hydrogen peroxide, the alterations of APP processing and Tau phosphorylation were reversed by MSRB2 overexpression. We further found that MSRB2-mediated regulation of APP transcription involved JNK and ERK signaling, as MSRB2 also reduced the levels of phosphorylated JNK (p-JNK), and JNK or ERK inhibitor attenuated the effect of MSRB2 on APP proteins and transcripts. Finally, MSRB2 reduced apoptosis-related proteins Bax and caspase3 and enhanced the anti-apoptotic protein Bcl2. These results indicated that the role for MSRB2 in AD-like pathology was closely associated with its antioxidant activity. By attenuating both amyloidogenesis and Tau phosphorylation, MSRB2 may serve as a potential therapeutic target for AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease. The pathological features include neuritic plaques which are mainly composed of amyloid β protein (A β), and neurofibrillary tangles due to abnormal phosphorylated Tau protein levels (Roland and Helmut, 2009). The etiology of AD is considered as multifactorial and includes oxidative stress, neuroinflammation and synaptic failure (Querfurth and LaFerla, 2010). Evidence has suggested that mitochondrial dysfunction and oxidative stress contribute to the accumulation of A β and the enhanced phosphorylation of Tau (Swerdlow, 2012). The elevated A β level, in turn, disrupts mitochondrial function (Picone et al., 2014; Wang et al., 2014). These events may occur early and chronically, leading to the hypothesis that mitochondrial dysfunction is a trigger of AD pathophysiology (Moreira et al., 2010; Schmitt et al., 2012).

Methionine sulfoxide reductases (MSR) include one MSRA and three

MSRBs (MSRB1, MSRB2, and MSRB3) proteins (Oien and Moskovitz, 2008; Zhang et al., 2011). They catalyze the reduction of free and protein-based methionine sulfoxides to methionine, and are involved in antioxidant mechanisms (Levine et al., 2000). The role for MSRA in defending oxidative stress has been well established in multiple cell lines (Kantorow et al., 2004; Moskovitz et al., 1998; Picot et al., 2005; Yermolaieva et al., 2004). MSRA knockout mice exhibit abnormal behavior and neurodegeneration (Moskovitz et al., 2001; Oien et al., 2008; Pal et al., 2007). In patients with AD, the decreased MSRA activity coincides with the oxidation of critical proteins (Gabbita et al., 1999). Different from MSRA, which is widely distributed in cellular compartments, MSRB2 is primarily located in mitochondrion (Kaya et al., 2010; Kim and Gladyshev, 2004). High levels of MSRB2 mRNA have been found in high energy-demand organs including the brain (Pascual et al., 2010). It is reported that MSRB2 protects cell damage from oxidative stress in cones, inner ear, lymphoblast and leukemia

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cells (Cabreiro et al., 2008; Cabreiro et al., 2009; Kwon et al., 2014; Pascual et al., 2010). Down regulation of MSRB2 increases oxidative stress-induced cell death in lens (Marchetti et al., 2005). Importantly, MSRB2 mutants also impair mitochondrial function in yeast, and conversely, MSRB2 overexpression preserves mitochondrial integrity in leukemia cells (Cabreiro et al., 2008; Kaya et al., 2010). Therefore, the cellular localization and the functional link to mitochondria suggest that MSRB2 may play a role in mitochondria-related oxidative stress, especially in the brain of AD (Chen and Zhong, 2014).

We hypothesized that the expression of MSRB2 in the brain of AD may be altered and MSRB2 is functionally associated with AD-like pathologies. In this study, we used APP^{sw}/PSEN1^{ΔE9} (APP/PS1) mice as an AD model. This model develop amyloidogenesis and Tau-positive neuritis in the brain, synaptic loss and mitochondrial dysfunction, resembling the pathological features of AD (Bilkei-Gorzo, 2014). In this model, we measured the expression pattern of MSRB2 in the cortex and hippocampus. In HEK293 cells stably expressing human full-length APP (HEK/APP), we investigated whether and how MSRB2 might be involved in amyloidogenesis and Tau phosphorylation in normal condition and oxidative stress.

2. Materials and methods

2.1. Animal models

APP/PS1 mice expressing Swedish APP and Presenilin1 delta exon 9 mutations (B6C3-Tg (APP^{sw}, PSEN1^{ΔE9}) 85Dbo/J, # 004462) were purchased from the Model Animal Research Centre of Nanjing University. All animals were housed in 12 h light/12 h dark cycles with free access to food and water in the Animal Center of Chongqing Medical University. All of the animal procedures conformed to the Ethics Committee of Chongqing Medical University.

2.2. Antibodies and reagents

Antibodies against MSRB2 (A8364; 1:1000 for Western blotting, 1:200 for immunostaining) was purchased from AbClonal (Boston, USA). Antibodies against p-AMPK (Thr172, 2535; 1:1000), AMPK (5831; 1:1000), γ -Secretase Antibody Sample Kit (5887; 1:1000), and DYKDDDDK Tag (14793S; 1:800 for immunofluorescence) were purchased from Cell Signaling Technology (Massachusetts State, USA). Those against ADAM10 (ab1997; 1:1000), BACE1 (ab2077; 1:1000), Non-phosphorylated Tau (ab64193 recognizes both non-phosphorylated and phosphorylated Ser262; 1:1000), p-TauT231 (ab151559; 1:1000), p-TauS262 (ab131354; 1:1000), p-TauS396 (ab109390; 1:1000), p-TauS404 (ab92676; 1:1000), GSK3 β (ab93926; 1:1000) and p-GSK3 β -Ser9 (ab75814; 1:1000), phospho-ERK1 (p-ERK1, T202/Y204)-phospho-ERK2 (p-ERK2, T185/Y187) (ab76299; 1:2000), and ERK1/ERK2 (ab184699; 1:2000) were purchased from Abcam (Cambridge, United Kingdom). The antibody against p-JNK (Thr183/Tyr185, SC-6254; 1:200), Tau-5 (sc-58,860; 1:200) and Tom20 (sc-17,764; 1:100 for immunofluorescence) were from Santa Cruz (California, USA); Anti-APP and CTFs (A8717; 1:1000) were from Sigma-Aldrich (St. Louis, MO). Anti-Cdk5 (10430-1-AP; 1:1000), Caspase3 (19677-1-AP; 1:1000), BAX (50599-2-Ig; 1:1000), Bcl2 (12789-1-AP; 1:1000) and primary antibody against GAPDH (60003-2-Ig; 1:4000) were from Proteintech (Wuhan, China). Those Western blotting horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies were purchased from Proteintech (Wuhan, China). Immunofluorescence secondary antibodies Alexa Fluor 488-labeled Goat Anti-Rabbit (A0423; 1:500) and Cy3-labeled Goat Anti-Mouse (A0521; 1:500) were purchased from Beyotime (Haimen, China). Immunohistochemistry experiment was performed with Rabbit hypersensitive two-step detection kit (PV-9001, ZSGB-BIO, Beijing, China).

2.3. Cell culture and pharmacologic treatments

Human neuroblastoma SH-SY5Y cells were cultured in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 mg/ml streptomycin and 100 U/ml penicillin G. Human embryonic kidney 293 cell line (HEK293, American Type Culture Collection, Rockville, MD) that stably expresses full-length human APP (HEK/APP), was generated as described previously (Hu et al., 2017). Cells were seeded on six-well plates for Western blotting and RT-PCR assays. Cell transfected with MSRB2 for 24 h and subsequently treated with 20 μ M SP600125, 20 μ M U0126 or 50 μ M hydrogen peroxide for 24 h, respectively.

2.4. Plasmid and short hairpin RNA (shRNA) transfection

The human MSRB2 plasmid (HG15713-CF) and control vector pCMV3 (CV013) were purchased from Sino Biological (Beijing, China). Cells were transfected with Lipofectamine™ 2000 Transfection Reagent (11668019, Thermo Fisher Scientific, Inc) and with Opti-MEM Reduced Serum Media (Thermo Fisher Scientific, Inc) according to manufacturer's protocol. The shRNA sequence for human MSRB2 (5'-GCGAGT CATTGCTTCTCTTAA -3') was selected from Invitrogen Block-iT RNAi Designer. This sequence was synthesized by Sangon Biotech (Shanghai, China) and designed as follows: 5'-GATCCCC GCGAGTCATTGCTTCTCTTAA TTCAAGAGA TTAAGAGAAGCAATGACTCGC TTTT-3' (sense); and 5'-AGTAAAAA GCGAGTCATTGCTTCTCTTAA TCTCTTGAA TTAAGAGAAGCAATGACTCGC GGG-3' (antisense). The protocol of shRNA was generated as described previously (Tang et al., 2018).

2.5. Western blotting

APP/PS1 mice and wild-type littermate at different age were anesthetized and decapitated. The brains were carefully removed, and the ipsilateral cortex and hippocampus regions were dissected and were frozen in liquid nitrogen. Animal tissues and culture cells were homogenized in ice-cold RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris) supplemented with protease inhibitors and phosphatase inhibitors (Beyotime, Haimen, China) mixture and centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentrations were measured using a BCA Protein Assay Kit (P0011, Beyotime, Haimen, China). Western blotting were performed as previously described (Hu et al., 2017; Liu et al., 2017; Tang et al., 2018). The samples were loaded onto SDS-PAGE (8–15% acrylamide) gels. The separated proteins were transferred to nitrocellulose membranes. The specific protein bands were visualized using ECL reagent (GE healthcare, UK) and the Fusion FX5 image analysis system (Vilber Lourmat, Marne-la-Vallee, France). Relative protein expression levels were calculated by Quantity One software (Bio-Rad, Hercules, CA) normalized to GAPDH.

2.6. Immunohistochemistry

Animals were humanely killed and systematically perfused with cold PBS to remove blood (Tang et al., 2018). Brain slices from mice (18-month-old) were paraffin-embed and were then deparaffinized in xylene for 30 min and rehydrated at 10 min intervals in a series concentration of ethanol. After washing with PBS (5 min \times 3), brain slices were permeabilized with 0.4% Triton-X 100 for 20 min before antigen retrieval with boiled sodium citrate buffer (10 mM, pH 6.0) for about 15 min. Slices were incubated with 3% H₂O₂ (PV-9001, ZSGB-BIO, Beijing, China) for 15 min at 37 °C, and were then incubated with the primary anti-MSRB2 antibody at 4 °C overnight. Sections were washed and incubated with reaction enhancement solution (PV-9001, ZSGB-BIO, Beijing, China) for 30 min at 37 °C and then with enhanced enzyme-labeled goat anti-rabbit IgG polymer (PV-9001, ZSGB-BIO, Beijing, China) for 30 min at 37 °C. Bound antibody was visualized

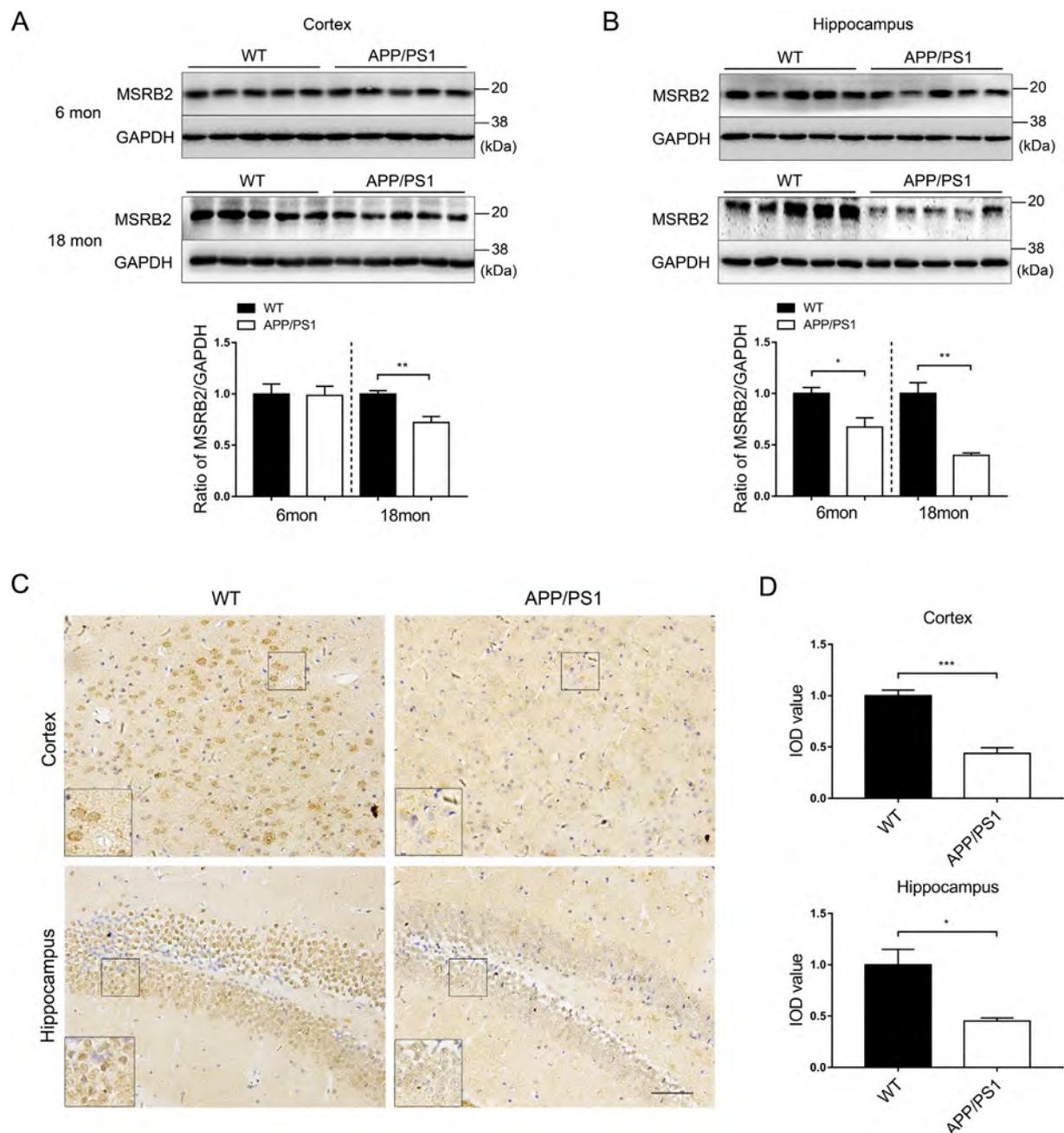


Fig. 1. Cerebral MSRB2 protein and immunoreactivity are decreased in APP/PS1 mice. (A&B) Representative Western blots (top) and quantification (bottom) of MSRB2 protein in the cortex (A) and hippocampus (B) of wild-type (WT) and APP/PS1 mice, at 6 mon ($n = 5$ in each group) and 18 mon ($n = 5$ in each group), respectively. (C) Representative immunohistochemical images probed by MSRB2 antibody in the cortex and the hippocampus at 18 mon mice. Compared with WT, APP/PS1 mice show the reduced MSRB2 immunoreactivity. Scale bar, 50 μm . (D) MSRB2 expression is quantified by Image-pro Plus 6.0 software. $n = 4$ in each group. * $P < .05$, ** $P < .01$, *** $P < .001$.

using 3,3'-diaminobenzidine (DAB, ZSGB-BIO, Beijing, China) for 3 min. Hematoxylin was used to counterstain nuclei for several minutes. Slices were dehydrated by lithium carbonate for 1 min and incubated with dimethylbenzene for 40 min, then covered and dried overnight. Quantification of immunohistochemistry was calculated by Image-pro Plus 6.0 software (Media Cybernetics, Bethesda, USA) as described previously (Liu et al., 2017).

2.7. Immunofluorescence

SH-SY5Y or HEK/APP cells incubated on coverslips were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at 37 °C. Cells were then permeabilized in 0.1% Triton X-100 for 20 min at

24 °C and incubated with the primary antibody against Tom20 and DYKDDDDK Tag in 5% BSA at 4 °C overnight. Coverslips were washed with PBS three times and incubated with Alexa Fluor 488-labeled Goat Anti-Rabbit and Cy3-labeled Goat Anti-Mouse secondary antibodies for 1 h at 24 °C. After washing with PBS for three times, the coverslips were mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, Alabama, USA). Images were acquired using a laser scanning confocal microscope (Leica TCS SP8 X, Germany).

2.8. Measurement of intracellular ROS production

The level of intracellular ROS was quantified using the Reactive Oxygen Species Assay Kit (S0033, Beyotime, Haimen, China). SH-SY5Y

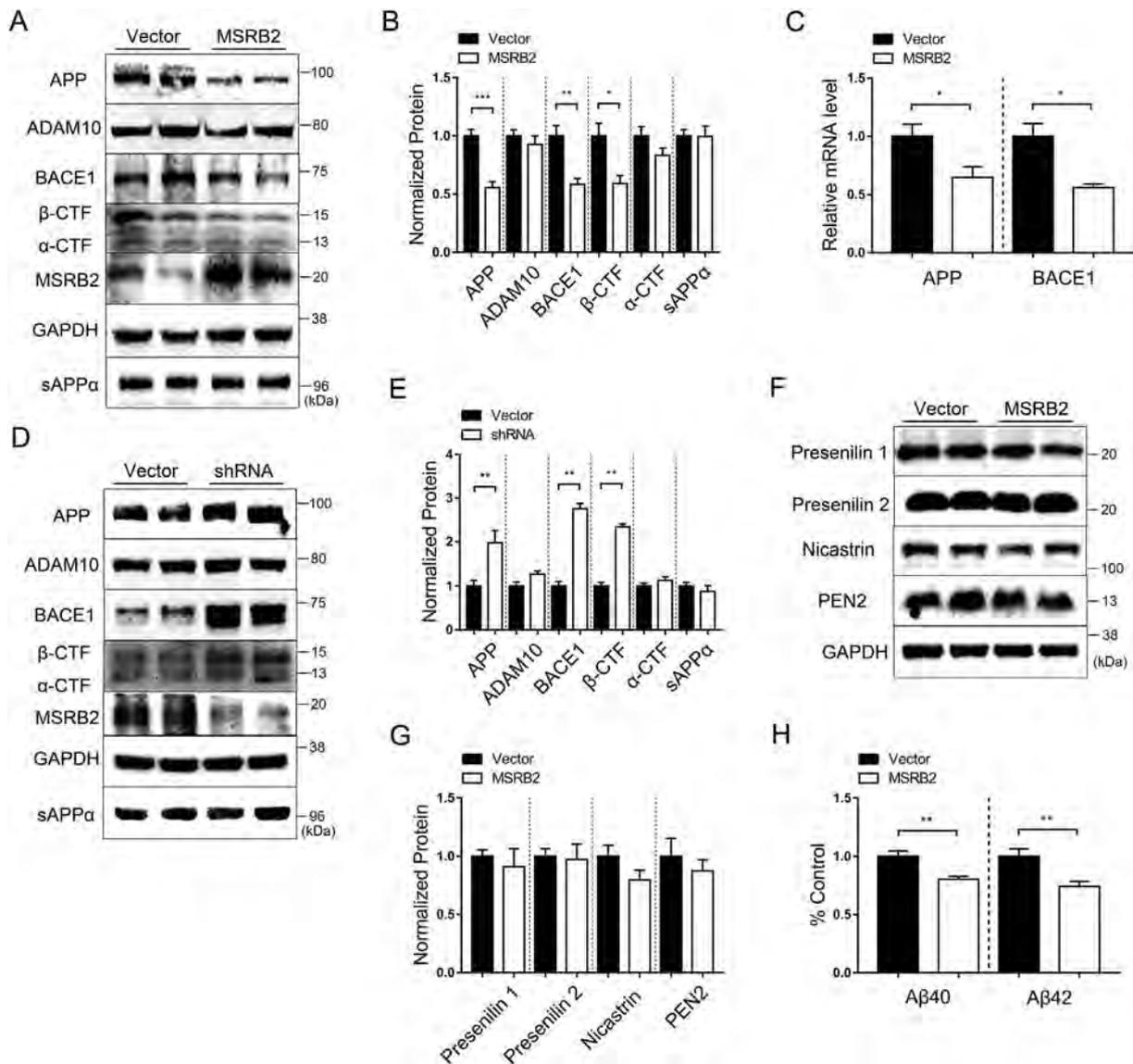


Fig. 2. MSRB2 affects APP processing in HEK/APP cells under basal condition. (A&B) Representative Western blots (A) and quantification (B) of APP, ADAM10, BACE1, α- and β-CTF, and sAPPα in HEK/APP cells transfected with Vector or MSRB2 for 48 h. All values were normalized to Vector control. (C) The mRNA levels of APP and BACE1 in HEK/APP cells transfected with Vector or MSRB2 for 48 h were measured by RT-qPCR. (D&E) Representative Western blots (D) and quantification (E) of APP, ADAM10, BACE1, α- and β-CTF, and sAPPα in HEK/APP cells transfected with Vector or MSRB2 shRNA for 48 h. (F&G) Representative Western blots (F) and quantification (G) of Presenilin 1, Presenilin 2, Nicastrin and PEN2 in HEK/APP cells transfected with Vector or MSRB2 for 48 h. (H) Protein levels of Aβ1–40 and Aβ1–42 in culture medium from HEK/APP cells transfected with Vector or MSRB2 for 48 h were measured by ELISA. *P < .05, **P < .01, ***P < .001.

cells were transfected with Vector or MSRB2 for 48 h before PBS washing for three times. Cell were then incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the dark. After PBS washing, DCF fluorescence images for Fig. 4B were acquired using a laser scanning confocal microscope (Leica TCS SP8 X, Germany). The fluorescent intensity for Fig. 4C was measured by EnSpire Multilabel Plate Reader (PerkinElmer, USA) with excitation wavelength at 488 nm and emission wavelength at 525 nm, respectively.

2.9. RNA extraction and quantitative RT-PCR

Total RNA was extracted using RNAiso plus (Takara, Dalian, Liaoning, China) according to manufacturer's instructions. The cDNA was synthesized by the 5 × HiScript II Select qRT Super Mix II (R233-01-AC, Vazyme, Nanjing, China) according to manufacturer's protocol. The mRNA expression levels of APP, BACE1 and GAPDH were detected by RT-qPCR. The primers for human APP, BACE1 and GAPDH were as

follows: APP, sense: 5'-TGGTGGGCGGTGTTGTCATA-3', antisense: 5'-TGGATTTTCGTAGCCGTTCTGC-3'; BACE1, sense: 5'-GCAAGGAGTACAACATGAC-3', antisense: 5'-AGCTTCAAACACTTCTTGG-3'; GAPDH, sense: 5'-AGAAGGCTGGGCTCATTG -3', antisense: 5'-TGC TGATGATCTTGAGGCTGTTG-3'. Reactions were performed with AceQ qPCR SYBR Green Master Mix (Q111-02, Vazyme, Nanjing, China). The reaction mixture (20 μl total) are consisted of 10 μl SYBR, 5.2 μl nuclease-free water, 0.4 μl each primer, and 4 μl diluted cDNA. Reactions were performed using the following steps: 1 cycles of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and the melting curve was run after RT-PCR. The Ct value of each sample was calculated, and the relative mRNA level was normalized to the GAPDH mRNA value as previously described (Zhu et al., 2016).

2.10. ELISA for Aβ1–40 and Aβ1–42

ELISA analysis of the human Aβ1–40 and Aβ1–42 levels in cultured

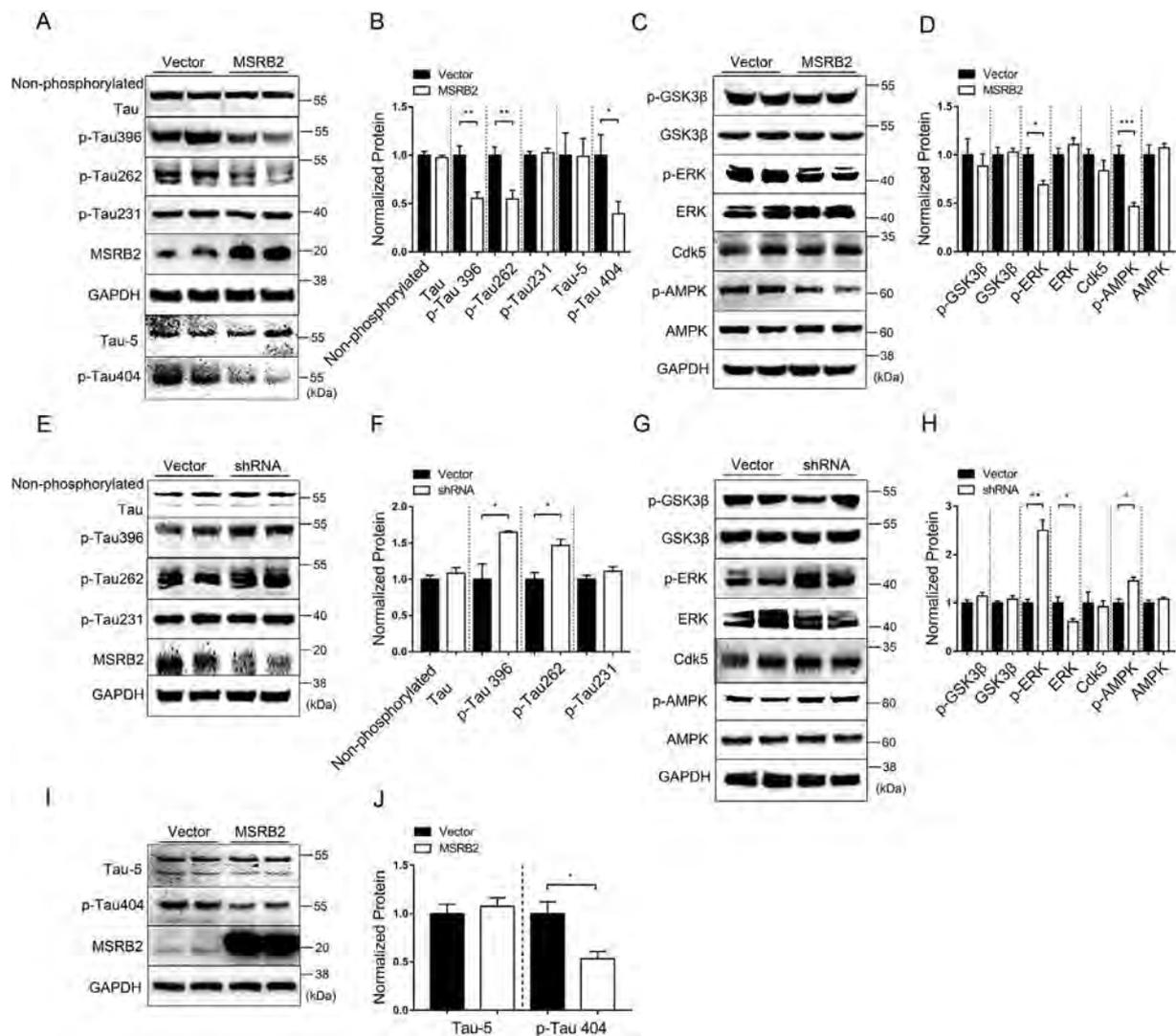


Fig. 3. MSRB2 affects Tau phosphorylation under basal condition. (A&B) Representative Western blots (A) and quantification (B) of non-phosphorylated Tau, p-Tau396, p-Tau262, p-Tau231, Tau-5 and p-Tau404 in HEK/APP cells transfected with Vector or MSRB2 for 48 h. (C&D) Representative Western blots and quantification of the p-GSK3β, GSK3β, p-ERK, ERK, Cdk5, p-AMPK and AMPK in HEK/APP cells transfected with Vector or MSRB2 for 48 h. (E&F) Representative Western blots and quantification of the non-phosphorylated Tau, p-Tau396, p-Tau262 and p-Tau231 in HEK/APP cells transfected with Vector or MSRB2 shRNA for 48 h. (G&H) Representative Western blots and quantification of the p-GSK3β, GSK3β, p-ERK, ERK, Cdk5, p-AMPK and AMPK in HEK/APP cells transfected with Vector or MSRB2 shRNA for 48 h. (I&J) Representative Western blots (I) and quantification (J) of the Tau-5 and p-Tau404 in SH-5Y5Y cells transfected with Vector or MSRB2 for 48 h. *P < .05, **P < .01, ***P < .001.

HEK/APP cells were processed as described previously (Tang et al., 2018). Briefly, concentrations of Aβ40 and Aβ42 in supernatant of cultured media was collected and centrifuged for 10 min at 4000 g at 4 °C was then quantitatively measured by ELISA according to the ELISA kits manufacturer's instructions (Cusabio, China). All samples were assayed in 3 duplicates, and each experiment was repeated 3 times.

2.11. Statistical analysis

Data were presented as mean ± standard error. Statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). Data were analyzed by unpaired two tailed Student's *t*-test, one-way and two-way ANOVA and Mann-Witney where it applied.

3. Results

3.1. Cerebral MSRB2 protein was decreased in APP/PS1 mice

To determine whether MSRB2 may be involved in the pathophysiology of AD, we first assessed MSRB2 protein levels in the cortex and hippocampus of 6 mon (young) and 18 mon (old) APP/PS1 mice (APP/PS1) relative to those in age-matched wild-type mice (WT). We found that in 6 mon APP/PS1 mice, MSRB2 protein levels were not altered in the cortex but were significantly decreased in the hippocampus (Fig.1A &B). However, in 18 mon APP/PS1 mice, MSRB2 protein was significantly decreased in both the cortex and hippocampus (Fig.1A&B). To further validate that MSRB2 protein was decreased in APP/PS1 mice by Western blotting, we assessed MSRB2 expression by immunohistochemical staining in 18 mon mice. As shown in Fig.1C&D, the density of MSRB2 was significantly decreased in the cortex and hippocampus of APP/PS1 compared with WT. These results indicated that in APP/PS1 mice MSRB2 proteins were decreased in the cortex in old age, and in the hippocampus regardless of age.

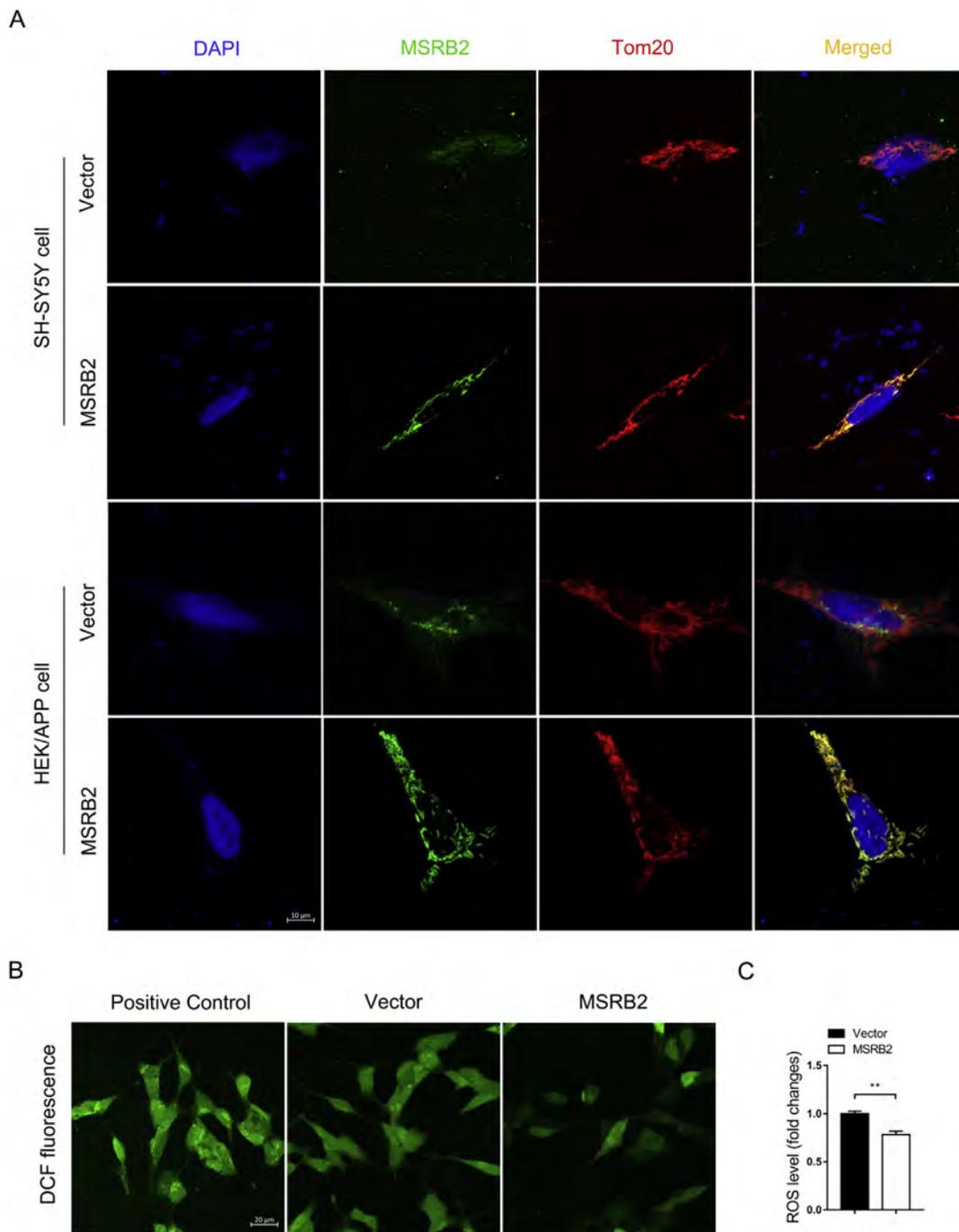


Fig. 4. Overexpression of mitochondrial MSRB2 reduces intracellular ROS level. (A) Immunofluorescent images show that MSRB2 colocalizes with mitochondrial marker Tom20, both in SH-SY5Y and HEK/APP cells transfected with Vector or MSRB2 for 48 h. Blue: DAPI (nucleus); Green: MSRB2; Red: Tom20 (mitochondrial membrane protein). (B) Cellular ROS level was detected by DCF fluorescence in SH-SY5Y cells transfected with Vector or MSRB2 for 48 h. Positive control: Rosup (included in the ROS kit). (C) Quantification of fluorescent intensity (ROS level) in SH-SY5Y cells transfected with Vector or MSRB2 for 48 h. $^{**}P < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. MSRB2 affected APP processing and Tau phosphorylation in HEK/APP cells under basal condition

The altered expression of MSRB2 prompted us to speculate that MSRB2 might be involved in AD-like pathology. The β -amyloid precursor protein (APP) can be sequentially cleaved by β -amyloid converting enzyme 1 (BACE1) and γ -secretase, resulting in increased β -COOH-terminal fragment (β -CTF) and $A\beta$ (De Strooper et al., 2010). Conversely, ADAM10 (a disintegrin and metalloproteinase 10) promotes the non-amyloidogenic cleavage of APP, resulting in the

increased sAPP α , α -CTF and the reduced $A\beta$ (Postina, 2012). Thus, we first assessed the effect of MSRB2 on APP processing in HEK293 cells that stably express human full-length APP (HEK/APP). As shown in Fig. 2A&B, MSRB2 overexpressing cells showed a significant reduction of APP, BACE1 and β -CTF protein levels, while the levels of ADAM10 and associated sAPP α and α -CTF were not significantly altered. Consistently, the mRNA levels of APP and BACE1 were also decreased in MSRB2 overexpressing cells (Fig. 2C). To further validate the effect of MSRB2 on APP processing, we performed MSRB2 knockdown experiments in HEK/APP transiently transfected with MSRB2 shRNA.

Compared with control, MSRB2 knockdown significantly increased protein levels of APP, BACE1 and β -CTF, whereas those of ADAM10, sAPP α and α -CTF were not significantly altered (Fig. 2D&E). The γ -secretase is a multiprotein complex comprised of four integral membrane proteins presenilin, nicastrin, Aph-1, and presenilin enhancer 2 (PEN2), which are essential for complete proteolytic activity (Yu et al., 2000). We found that these proteins were not significantly changed in MSRB2 overexpressing cells (Fig. 2F&G). Nonetheless, both A β 1–40 and A β 1–42 levels were significantly reduced in HEK/APP cells that overexpressed MSRB2 (Fig. 2H). These results indicated that MSRB2 reduced APP and BACE1 protein levels and A β generation, while without affecting ADAM10 and γ -secretase in HEK/APP cells.

Tau phosphorylation at Ser262 (p-Tau262), Ser396 (p-Tau396), Ser404 (p-Tau404) and Thr231 (p-Tau231) contributes to the pathogenesis of AD (Lee and Leurgers, 2012). The kinases responsible for these phosphorylation sites include glycogen synthase kinase-3 β (GSK3 β), cyclin-dependent kinase-5 (Cdk5), extracellular signal-regulated kinase (ERK), adenosine monophosphate-activated protein kinase (AMPK) (Lee and Leurgers, 2012; Wang et al., 2013). We next assessed the effect of MSRB2 on Tau phosphorylation in HEK/APP cells. As shown in Fig. 3A&B, overexpression of MSRB2 caused a decrease of protein levels of p-Tau396, p-Tau404 and p-Tau262, while those of non-phosphorylated Tau, total Tau (Tau-5) and p-Tau231 were not significantly altered. To further identify the potential kinases that may be involved in MSRB2-mediated regulation of Tau phosphorylation, we assessed the activities of GSK3 β (p-GSK3 β), ERK (p-ERK), Cdk5 and AMPK (p-AMPK) in MSRB2 overexpressing cells. As shown in Fig. 3C&D, overexpression of MSRB2 caused a significant reduction of p-ERK and p-AMPK protein levels, while having no significant effect on p-GSK3 β and p-Cdk5 protein levels. Conversely, knockdown of MSRB2 significantly increased protein levels of p-Tau396 and p-Tau262 and did not alter those of non-phosphorylated Tau and p-Tau231 (Fig. 3E&F). We also found a significant increase of p-ERK and p-AMPK protein levels in cells transiently transfected with MSRB2 shRNA, while p-GSK3 β and p-Cdk5 protein levels remained unchanged (Fig. 3G&H). Consistent with the findings in HEK/APP cells, overexpression of MSRB2 in SH-SY5Y cells caused a decrease of protein level of p-Tau404 while Tau-5 was not significantly altered (Fig. 3I&J). These results indicated that MSRB2 regulated Tau phosphorylation at selective sites and the kinase activity of ERK and AMPK, without affecting total Tau levels.

3.3. MSRB2 also attenuated AD-like pathology under oxidative stress

It is reported that MSRB2 protects cell from hydrogen peroxide-induced cell death and protein damage (Cabreiro et al., 2008). Indeed, MSRB2 co-localized with mitochondrial membrane protein Tom20 in both SH-SY5Y and HEK/APP cells (Fig. 4.A). And overexpression of MSRB2 significantly reduced cellular ROS (reactive oxygen species) level in SH-SY5Y cells (Fig. 4.B&C). To further determine whether the effect of MSRB2 on AD-like pathologies may be attributed to its antioxidant activity, we first assessed the effect of MSRB2 overexpression on APP processing in HEK/APP cells treated with 50 μ M H₂O₂ for 24 h. As shown in Fig. 5A-B, H₂O₂ alone caused a significant increase of APP and BACE1 proteins, with the concomitant increase of β -CTF. Although a significant decrease of ADAM10 protein was also found, the related α -CTF protein levels were not significantly changed, suggesting that oxidative stress might impair the catalytic activity of ADAM10 (Gasparini et al., 1997). In H₂O₂ treated cells, MSRB2 overexpression reduced the protein levels of APP, BACE1 and β -CTF (Fig. 5A&B), suggesting that MSRB2 scavenged the effect of ROS on the expression of the selected proteins. It is known that BACE1 transcription can be enhanced by oxidative stress (Mouton-Liger et al., 2012). Interestingly, the long-term H₂O₂ treatment (24 h) also increased APP mRNA levels (Fig. 5C), consistent with our previous finding (Tang et al., 2018). Consistently, MSRB2 overexpression led to the reduced levels of A β 1–40 and A β 1–42 (Fig. 5D).

Evidence has suggested that H₂O₂ alters Tau phosphorylation (Alavi Naini and Soussi-Yanicostas, 2015; Su et al., 2010; Yao et al., 2013). Consistently, we found that H₂O₂ treatment enhances Tau phosphorylation at all selected sites (p-Tau396 p-Tau262 and p-Tau231) in HEK/APP cells, whereas the non-phosphorylated Tau protein level were not changed (Fig. 5E&F). Importantly, overexpression of MSRB2 significantly reduced the protein levels of p-Tau396 and p-Tau262 in H₂O₂ treated cells, without altering those of non-phosphorylated Tau and p-Tau231 (Fig. 5E&F). Accordingly, H₂O₂ significantly increased the protein levels of p-GSK3 β , p-ERK and p-AMPK, but did not alter Cdk5 protein levels (Fig. 5G&H). In the presence of H₂O₂, MSRB2 overexpression led to a significant reduction of p-AMPK (Fig. 5G&H). Together, these results indicated that MSRB2 prevented ROS-induced alterations of APP processing and Tau phosphorylation.

3.4. MSRB2-mediated regulation of APP transcription involved JNK and ERK signaling

It is known that BACE1 transcription can be regulated by oxidative stress, which involves JNK signaling (Tamagno et al., 2009; Tong et al., 1996). Thus, we tested whether JNK might be involved in the regulation of APP transcription by MSRB2. As shown in Fig. 6A&B, MSRB2 overexpressing cells exhibited markedly decreased phosphorylated JNK (p-JNK) protein level, whereas knockdown of MSRB2 significantly increased p-JNK proteins (Fig. 6C&D). We further found that the basal protein and mRNA levels of APP was reduced in the presence of JNK inhibitor SP600125 (20 μ M), which further attenuated the effect of MSRB2 overexpression on APP protein and mRNA levels (Fig. 6E-G). Given that p-ERK was also affected by MSRB2 and oxidative stress (Figs. 3 & 5), we then tested whether ERK inhibitor U0126 (20 μ M) may affect MSRB2-mediated regulation of APP transcription. As shown in Fig. 6H-J, p-ERK levels were dramatically reduced in the presence of U0126, indicating that ERK activity was successfully inhibited by U0126 (Fig. 6H). U0126 alone did not alter the protein and mRNA levels of APP. However, in cells treated U0126, MSRB2 failed to significantly reduce APP protein and mRNA levels (Fig. 6H&J), suggesting that ERK was also involved in the regulation of APP transcription by MSRB2.

3.5. MSRB2 altered apoptosis-related proteins in HEK/APP cells

To determine whether MSRB2 might have impact on apoptosis, we assessed the apoptosis-related proteins Bax, Bcl2 and caspase-3 in HEK/APP cells (Jazvinscak Jembrek et al., 2015). As shown in Fig. 7A&B, overexpression of MSRB2 led to the decreased protein level of Bax and caspase-3 and the increased expression of Bcl2. MSRB2 knockdown resulted in quite opposite effects to MSRB2 overexpression: Bax and caspase-3 were increased and Bcl2, decreased (Fig. 7C&D). These results suggested that MSRB2 might have anti-apoptotic function in HEK/APP cells.

4. Discussion

Mitochondria harbor ATP generation where oxidative phosphorylation and the formation of ROS are inherently linked (Mailloux et al., 2013). The ROS is mostly decomposed by the mitochondria-embedded enzyme glutathione peroxidase (GPX), among others (Venditti et al., 2013). Impairment of the intrinsic defense system has been implicated in AD (Tonnie and Trushina, 2017; Wang et al., 2014). For instance, ablation of GPX4 in forebrain promotes cognitive impairment and hippocampal degeneration (Hambright et al., 2017). Overexpression of GPX facilitates cellular resistance to A β -induced neurotoxicity, whereas lack of GPX1 exacerbates A β -induced neuronal damage (Barkats et al., 2000; Crack et al., 1996). While most of these ROS-decomposing enzymes are associated with A β -induced toxicity or cognitive function, the mitochondria-targeted catalase is shown to reduce APP, BACE1 and

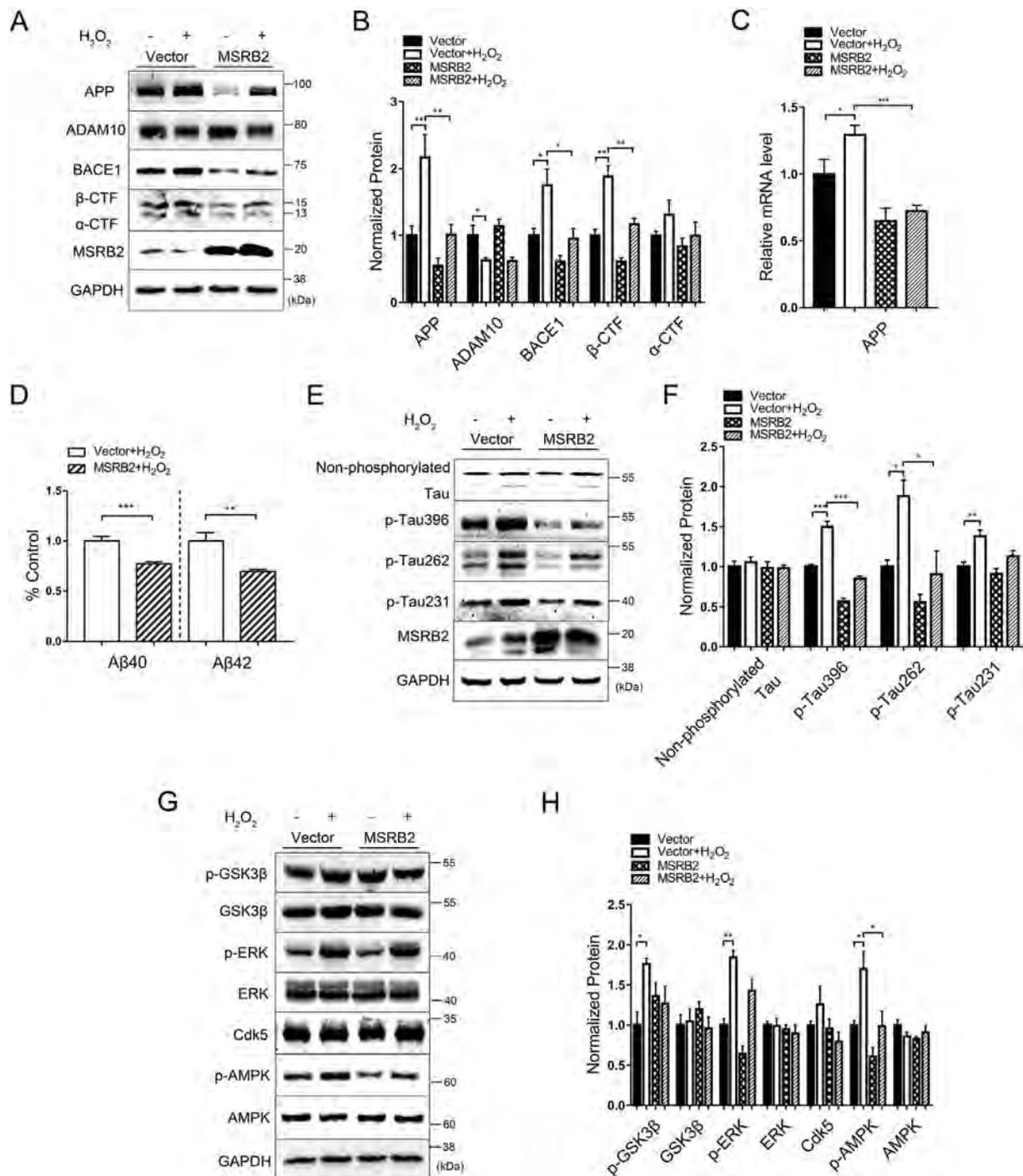


Fig. 5. Overexpression of MSRB2 attenuates AD-like pathology in oxidative stress. (A&B) Representative Western blots and quantification of APP, ADAM10, BACE1 and α- and β-CTF in HEK/APP cells transiently transfected with Vector or MSRB2 for 48 h, in the absence (Vector / MSRB2) or presence (Vector + H₂O₂ / MSRB2 + H₂O₂) of 50 μM H₂O₂ for 24 h. (C) The mRNA levels of APP in HEK/APP cells transfected with Vector or MSRB2 for 48 h, in the absence or presence of 50 μM H₂O₂ for 24 h. GAPDH was used as internal control. (D) Aβ_{1–40} and Aβ_{1–42} levels were measured by ELISA in the culture medium of HEK/APP cells transfected with Vector or MSRB2 for 48 h, presence (Vector + H₂O₂ / MSRB2 + H₂O₂) of 50 μM H₂O₂ for 24 h. All values are normalized to samples from Vector group. (E&F) Representative Western blots (E) and quantification (F) of the non-phosphorylated Tau, p-Tau396, p-Tau262 and p-Tau231 in HEK/APP cells transfected with Vector or MSRB2 for 48 h, in the absence or presence of 50 μM H₂O₂ for 24 h. (G&H) Representative Western blots and quantification of the p-GSK3β, GSK3β, p-ERK, ERK, Cdk5, p-AMPK and AMPK in HEK/APP cells transfected with Vector or MSRB2 for 48 h, in the absence or presence of 50 μM H₂O₂ for 24 h. *P < .05, **P < .01, ***P < .001.

Aβ levels in a mouse model of AD (Mao et al., 2012), suggesting a role of catalase in the pathogenesis of AD. The current study provides evidence that the mitochondrial protein MSRB2 also regulates AD-like pathology including APP processing, Aβ generation as well as Tau phosphorylation.

MSRA protein is reportedly decreased in the brain of AD patients (Gabbita et al., 1999). Interestingly, MSRB3 cellular distribution seems to be positively associated with the occurrence of pathological hallmarks in AD patients (Adams et al., 2017), suggesting that distinct MSR family proteins may be differentially involved in AD pathology. In our

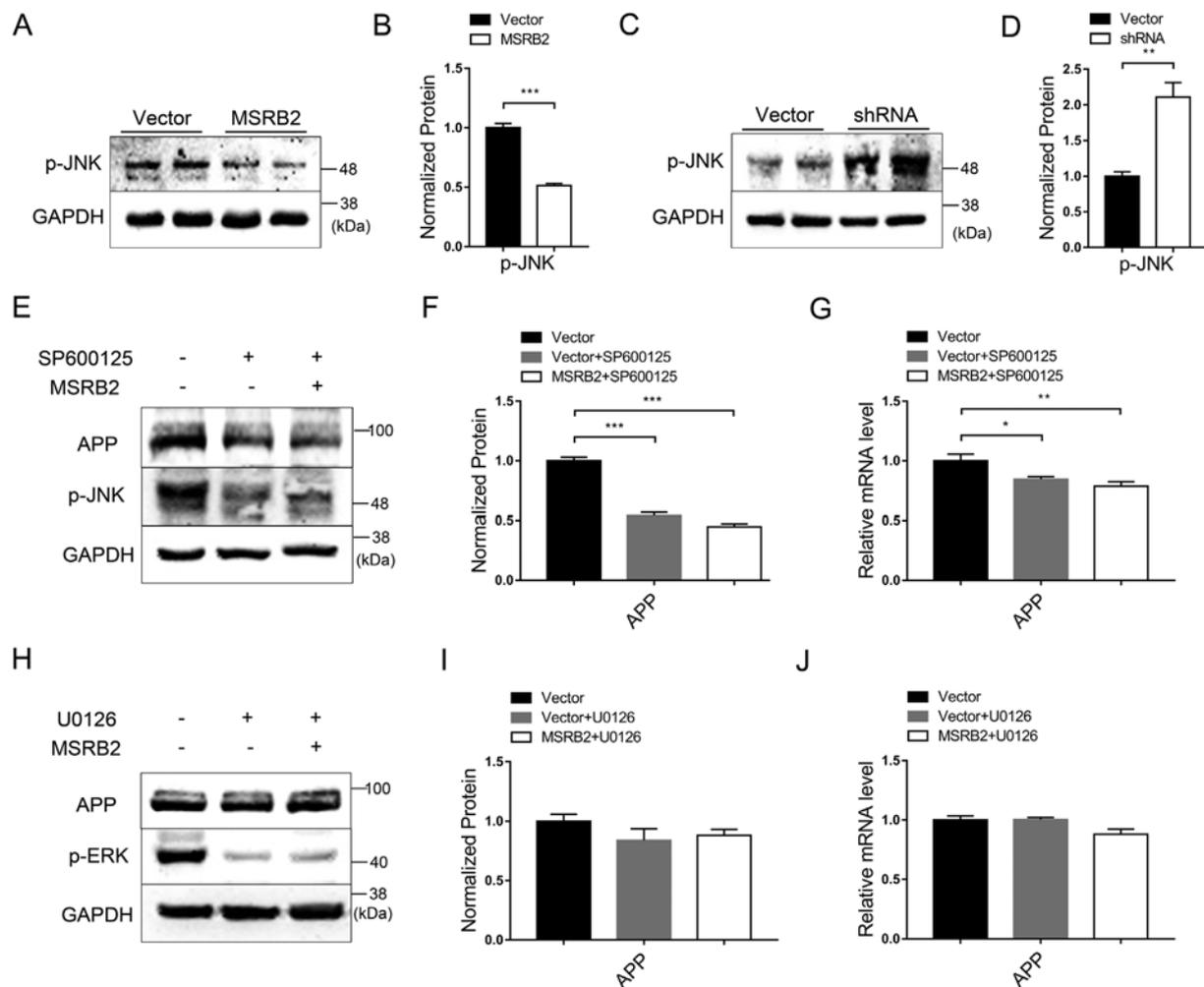


Fig. 6. MSRB2 affects APP transcription through JNK pathway. (A&B) Representative Western blots and quantification of p-JNK in HEK/APP cells transiently transfected with MSRB2 for 48 h. (C&D) Representative Western blots and quantification of p-JNK in HEK/APP cells transiently transfected with MSRB2 shRNA for 48 h. (E-G) Representative Western blots (E) and quantification of APP protein levels (F) and the corresponding mRNA levels (G) in HEK/APP cells transiently transfected with Vector or MSRB2 for 48 h, in the presence of JNK inhibitor SP600125 (20 μ M) for 24 h, respectively. (H-J) Representative Western blots (H) and quantification of APP protein levels (I) and the corresponding mRNA levels (J) in HEK/APP cells transiently transfected with Vector or MSRB2 for 48 h, in the presence of ERK inhibitor U0126 (20 μ M) for 24 h, respectively. * $P < .05$, ** $P < .01$, *** $P < .001$.

study, MSRB2 proteins are decreased earlier in the hippocampus than in the cortex. In line with this, the hippocampus has enhanced energy demand and mitochondrial stress relative to the cortex (Cabre et al., 2016). Evidence also suggests that oxidative stress is one of the prominent features in hippocampal aging (Blalock et al., 2003). In APP/PS1 mice, cerebral amyloidosis starts from \sim 2 mon (Bilkei-Gorzo, 2014), whereas impaired learning and memory occur in 6–8 mon (Webster et al., 2014). In our study, the reduced hippocampal MSRB2 protein levels at 6 mon might contribute to an enhanced ROS generation, which corresponds to cognitive impairment in APP/PS1 mice (Bilkei-Gorzo, 2014; Neves et al., 2008).

The current study provides evidence that MSRB2 reduces A β generation by reducing APP and BACE1 protein and mRNA levels, without affecting the protein levels of ADAM10 and γ -secretase. MSRB2 also fails to affect ADAM10 activity as measured by α -CTF and sAPP α (Fig. 2A&D). It is worth noting that ADAM10 protein expression does not always constitute a change in ADAM10 activity. Although H₂O₂ is shown to reduce ADAM10 protein in SH-SY5Y cells (Azmi et al., 2015), free radical generation leads to enhanced ADAM10 activity in human placenta cells (Yang et al., 2012) or increased sAPP α secretion in human neuroblastoma cells (Recuero et al., 2010). In our experimental condition, 50 μ M H₂O₂ for 24 h reduces ADAM10 protein but does not significantly alter α -CTF levels.

The effect of MSRB2 on APP and BACE1 preserves in cells treated with H₂O₂ (Fig. 5A-C), suggesting the antioxidant activity of MSRB2 (Cabreiro et al., 2006; Cabreiro et al., 2008). The role for oxidative stress in BACE1 transcription has been relatively well-documented (Chami and Checler, 2012). JNK pathways are closely associated with the pathogenesis of AD (Killick et al., 2014; Yarza et al., 2015; Zhu et al., 2001). Oxidative stress activates JNK and consequently enhances BACE1 expression through transcription factor activator protein-1 (AP-1) (Guglielmo et al., 2011; Tamagno et al., 2002; Tamagno et al., 2008; Tamagno et al., 2009). Interestingly, JNK also seems to regulate APP transcription in our study, as inhibition of JNK resulted in reduced APP mRNA levels (Fig. 6E-G). It is reported that APP transcription can also be stimulated by AP-1, which involves ApoE and ERK signaling (Huang et al., 2017). In addition, inhibition of JNK by SP600125 markedly reduces several pathological events including APP expression, A β production and Tau phosphorylation in traumatic brain and in APP/PS1 mice (Rehman et al., 2018; Zhou et al., 2015). In line with these observations, overexpression or knockdown of MSRB2 lead to the reduced or elevated p-JNK protein levels, respectively (Fig. 6A-D); and JNK inhibitor attenuated the effect of MSRB2 (Fig. 6E-G). Therefore, we speculate that JNK plays an important role in MSRB2-mediated regulation of APP and BACE1. On the other hand, ERK also seems to influence APP transcription to some extent. In the study by Huang et al.,

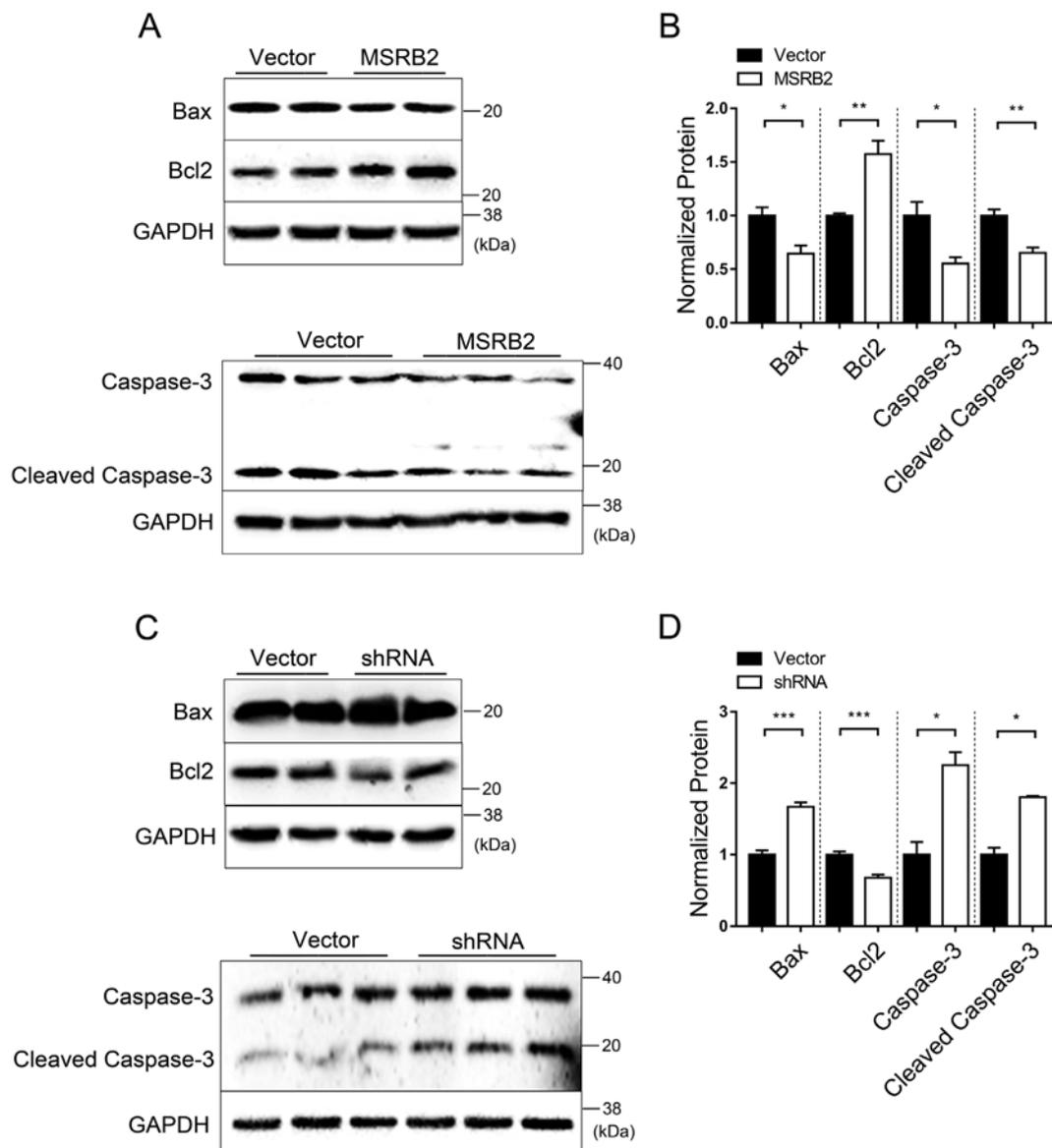


Fig. 7. MSRB2 affects the expression of apoptosis-related proteins. (A&B) Representative Western blots and quantification of Bax, Bcl2, Caspase-3 and Cleaved Caspase-3 in HEK/APP cells transiently transfected with MSRB2 for 48 h. All values were normalized to Vector control. (C&D) Representative Western blots and quantification of Bax, Bcl2, Caspase-3 and Cleaved Caspase-3 in HEK/APP cells transiently transfected with MSRB2 shRNA for 48 h. * $P < .05$, ** $P < .01$, *** $P < .001$.

ApoE enhanced APP expression and p-ERK protein levels (Huang et al., 2017). Unfortunately, direct evidence that ERK controls APP expression was not provided. In our study, ERK inhibitor U0126 alone does not affect basal APP expression, yet U0126 attenuates the effect of MSRB2 on APP transcription (Fig. 6H–J). One explanation may be that multiple co-factors control the promoter function of APP; these ERK-dependent co-factors are functional only when MSRB2 is overexpressed. Similar phenomenon has been demonstrated by other studies. CREB is a transcription factor. Knockdown of CREB does not affect basal LTP but blocks PKA-induced LTP (Pittenger et al., 2002); and knockdown of another transcription factor CEBP β has no effect on basal protein level of furin but prevents phorbol ester-induced enhancement of furin expression (Zha et al., 2017).

Deregulation of p-Tau262, p-Tau396 and p-Tau231 is closely associated with oxidative stress and AD pathology (Lee and Leugers, 2012; Mondragon-Rodriguez et al., 2013). Our study also suggests that MSRB2 links oxidative stress and downstream signaling to Tau phosphorylation. It is reported that AMPK promotes the phosphorylation of

Tau at Ser262 and Ser396 in cellular models under stress conditions (Domise et al., 2016; Thornton et al., 2011; Wang et al., 2013). Particularly, A β , as a trigger of oxidative stress, activates AMPK and promotes phosphorylation of Tau at Ser262 and Ser396 in primary neuronal cultures (Thornton et al., 2011). Evidence has suggested that p-Tau396 is also under the regulation by ERK and JNK (Wang et al., 2013). These kinases could be activated by oxidative stress and in the brain of AD patients (Chung, 2009; Sheng et al., 2009). In line with these observations, MSRB2 decreases p-Tau262 and p-Tau396 levels and related kinases under basal condition (Fig. 3); and in cells treated by H₂O₂, MSRB2 reversed the elevated levels of p-Tau262 and p-Tau396 and p-AMPK, and p-ERK to a lesser extent (Fig. 5). On the other hand, GSK3 β and Cdk5 are important kinases that phosphorylate Tau at large number of sites (Alavi Naini and Soussi-Yanicostas, 2015). While p-GSK3 β level is elevated in oxidative stress (Tang et al., 2018), Cdk5 protein levels do not alter (Kim et al., 2016). The failure of MSRB2 to influence p-GSK3 β and Cdk5 levels (Figs. 3 & 5), may partially explain the unchanged level of p-Tau231, which is under the regulation of

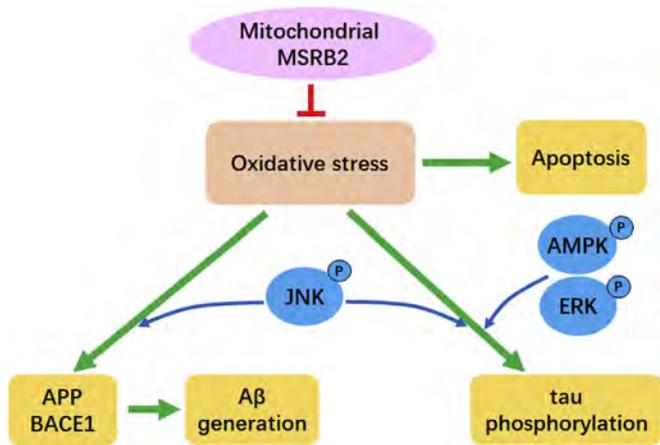


Fig. 8. Schematic diagram depicts the possible mechanisms through which MSRB2 regulates AD-like pathology. MSRB2 may attenuate AD pathology by antagonizing oxidative stress in following aspects: (Adams et al., 2017) reducing APP and BACE1 transcription through JNK signaling; (Alavi Naini and Soussi-Yanicostas, 2015) reducing Tau phosphorylation at selective sites (p-Tau262 and p-Tau 396) by inhibiting AMPK and ERK activities; and (Azmi et al., 2015) protecting cells from apoptosis.

GSK3 β and Cdk5 (Wang et al., 2013).

In summary, we describe the expression pattern of MSRB2 in APP/PS1 mice. We show that MSRB2 inhibits amyloidogenesis and Tau phosphorylation at selective sites in basal condition as well as in oxidative stress. We propose that MSRB2 may achieve these functions through its antioxidant activity (Fig. 8). On the one hand, MSRB2 inhibits the transcription of APP and BACE1 through JNK. On the other hand, MSRB2 reduces Tau phosphorylation by inhibiting AMPK and ERK. It is also not surprising that MSRB2 is anti-apoptotic. Therefore, the altered expression in APP/PS1 mice and the functional link to oxidative stress suggest that the role of mitochondrial MSRB2 in AD pathology is indispensable.

Acknowledgments

This work was supported by NSFC [grant numbers 81220108010]; Chongqing Science and Technology Commission [grant numbers cstc2018jcyjAX0067]; and Chongqing Yu-Zhong Science and Technology Project [numbers 001] to G-J Chen; and by National Key R & D Program of China [grant numbers 2018YFC1314700] to L-J Jin and G-J Chen.

Author contributions

G-J Chen designed the research; X-J Xiang performed the research and analyzed the data; L Song, X-J Deng, Y Tang, Z Min, B Luo, Q-X Wen, K-Y Li, J Chen, Y-L Ma and B-L Zhu provided assistance with the research; X-J Xiang and G-J Chen wrote the paper.

Conflict of interests

The authors have declared that no conflict of interest exists.

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