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ORIGINAL PAPER

The Effect of SPTLC2 on Promoting Neuronal Apoptosis is Alleviated by MiR-124-3p Through TLR4 Signalling Pathway

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Abstract

To investigate the role and mechanism of microRNA-124-3p (miR-124-3p) and serine palmitoyltransferase long chain base subunit 2 (SPTLC2) in neuronal apoptosis induced by mechanical injury. Transient transfection was used to modify the expression of miR-124-3p and SPTLC2. After transfection, neuronal apoptosis was evaluated in an in vitro injury model of primary neurons using TUNEL staining and western blot. The correlation between miR-124-3p and SPTLC2 was identified through a dual luciferase reporter assay in HEK293 cells. A rescue experiment in primary neurons was performed to further confirm the result. To explore the downstream mechanisms, co-immunoprecipitation was performed to identify proteins that interact with SPTLC2 in toll-like receptor 4 (TLR4) signalling pathway. Subsequently, the relative expression levels of TLR4 pathway molecules were measured by western blot. Our results showed that increased miR-124-3p can inhibit neuronal apoptosis, which is opposite to the effect of SPTLC2. In addition, miR-124-3p was proved to negatively regulate SPTLC2 expression and suppress the apoptosis-promoting effect of SPTLC2 via the TLR4 signalling pathway.

Keywords miR-124-3p · SPTLC2 · Apoptosis · TLR4

Introduction

Traumatic brain injury (TBI) is a leading culprit of morbidity, disability, and mortality around the world. It has been established that neuron loss is the main cause of secondary brain injuries that result in disability after TBI [1]. Apoptosis occurs minutes to weeks following TBI, offering a potential window for therapeutic interventions [2]. Thus, finding effective ways to repress apoptosis during this period may improve the prognosis of TBI.

MicroRNA-124 (miR-124) is one of the most abundant brain-specific microRNAs (miRNAs) [3], and miR-124-3p is a broadly conserved subtype of the miR-124 family. MiR-124-3p has been reported to play a protective role in neurons after TBI [4] and to be involved in proliferation, differentiation, invasion, and apoptosis [5–7]. It has also been observed to target proviral integration site 1 to suppress astrocytoma [8], target signal transducerand activator of transcription 3 to attenuate neuronal injury [9], and target annexin a5 to inhibit the mitochondrial pathway of apoptosis [10]. Suppression of other target genes by miR-124-3p may have synergistic effects on neuronal apoptosis [11]. However, the mechanisms through which these effects would occur are still not clear and thus merit further investigation.

Serine palmitoyltransferases (SPTs) can initiate de novo biosynthesis of sphingolipids, which are important constituents of cell membranes. Sphingolipids are also involved in modulating apoptosis, cell proliferation and differentiation as second messengers [12]. Mammalian SPTs are composed of three different subunits, serine palmitoyltransferase long chain base subunit 1 (SPTLC1), SPTLC2 and SPTLC3 [13]. It has been found that the tissue distribution of SPTLC2 mRNA corresponds with the activity distribution of SPTs

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[14]. The amino acid sequence of SPTCL2 is similar to that of aminolevulinate synthase; both have a domain that forms a Schiff base with pyridoxal phosphate [15, 16]. Overexpression of SPTLC2 has been shown to decrease the viability of HEK293 and HepG2 cells and to induce apoptosis via elevation of cellular ceramide, sphingoid bases, and dihydroceramide [17]. However, the regulatory elements that control endogenous SPTLC2 gene transcription have not been identified.

Based on the biological prediction (TargetScan.org), the sequence "CACGGAA" of miR-124-3p could bind to the sequence "GUGCCUU" of SPTLC2. SPTLC2 is abundant in all cells [18] and miR-124-3p enriches in neurons [19], and both of them involve in cell apoptosis mentioned above. Meanwhile, miR-124-3p can regulate toll-like receptor 4 (TLR4) signalling pathway [20], which involves in process of apoptosis too [21]. Myeloid differentiation factor 88 (MYD88) and nuclear factor kappa B (NF-κB) are two key moleculars of TLR4 signalling pathway. According to GENEMANIA database, SPTLC2 may bond to MYD88 directly to regulate TLR4 pathway. A prior study showed that NF-κB binding sites were located in SPTLC2 promoter region [22]. So, the current study was aimed at examining their relationship in neuronal apoptosis.

In this study, we identify SPTLC2 as a direct target of miR-124-3p, finding that overexpression of miR-124-3p dramatically inhibits the expression of SPTLC2, attenuating neuronal apoptosis after injury. SPTLC2 is further observed to bind to MYD88. As the relative expression levels of miR-124-3p and SPTLC2 are changed, TLR4 signalling pathway related molecules expression levels also show corresponding changes. These results suggest that a miR-124-3p/SPTLC2/TLR4 pathway is involved in the regulation of neuronal apoptosis after injury.

Materials and Methods

Cell Culture

HEK293 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS, ScienCell, San Diego, CA, USA). The cells were maintained at 37 °C in an atmosphere of humidified air with 5% CO_2. The cells were randomly divided into six groups: miR-124-3p mimic negative control + pcDNA 3.1-SPTLC2 empty vector negative control (miR-NC + SPTLC2-NC), miR-124-3p mimic + SPTLC2-NC (miR-mimic + SPTLC2-NC), miR-NC + pcDNA 3.1-SPTLC2 wild type (miR-NC + SPTLC2-WT), miR-mimic + SPTLC2-WT, miR-NC + pcDNA 3.1-SPTLC2 mutant type (miR-NC + SPTLC2-MT) and miR-mimic + SPTLC2-MT (n = 3 in each group).

The primary cortical neurons were prepared from embryonic day 18–19 C57BL/6 mice (Laboratory Animal Center of the Air Force Military Medical University, Xi'an, China) as described [23, 24]. All relevant experiments on animals in this study were approved by the Ethics Committee for Animal Experimentation of the Air Force Military Medical University and were conducted under the guidelines for the care and use of laboratory animals. Neuronal cells were cultured in poly-L-lysine-coated 6-well plates at a density of 700,000/well in DMEM (Gibco) containing 10% FBS (ScienCell). After 6 h incubation, the medium was replaced with serum-free Neurobasal™ Medium (Gibco) supplemented with 2% B27 (Gibco), 0.5 mM glutamine (Gibco) and penicillin–streptomycin (Gibco). Half of the medium was changed every 3 days. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. The cells were randomly divided into six groups: Sham, Injury, Injury + SPTLC2-NC, Injury + pcDNA 3.1-SPTLC2 (Injury + SPTLC2), Injury + miR-NC and Injury + miR-mimic (n = 3 in each group).

Cell Transfection

After being cultured for 7 days, the primary neurons were transfected using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. MiR-124-3p mimics (100 nM) (Genomeditech, Shanghai, China) and Lipofectamine 2000 reagent (10 µl) (Invitrogen) were each diluted into 125 µl Neurobasal™ Medium (Gibco) for 5 min and then mixed for 20 min at room temperature. The neurons were treated with the transfection solutions following three washes with phosphate buffered saline (PBS). After 6 h of transfection, the transfection solutions were replaced by Neurobasal™ Medium (Gibco) supplemented with 2% B27 (Gibco) and 0.5 mM glutamine (Gibco). Analogously, a scrambled sequence control (100 nM), pcDNA3.1-SPTLC2 (2 µg) and pcDNA3.1 empty vector (2 µg) (Genomeditech) were individually transfected into neurons with Lipofectamine 2000 (Invitrogen) using the same method.

Mechanical Injury Model

After 24 h transfection, the primary neurons were subjected to mechanical injury. Traumatic axonal injury is a consistent component of TBI and has been recognized as a major pathology of TBI [25]. In the present study, we employed a plastic stylet to scrape the cells [26, 27]. Briefly, 35 mm Petri dishes were manually scratched with a 10 µl plastic stylet needle following a 9 × 9 square grid template (with 4 mm of space between each line). This scratch injury has

been used to model TBI in cortical neurons in vitro, as the scratch injury activates the damage of neurons at the primary site, which then extends to the entire population of neurons. The injured neurons were used for subsequent experiments.

Quantitative Real-Time PCR

At scheduled timepoints, total RNA was extracted using Trizol (Wanleibio) according to the manufacturer's instructions. The cDNA was synthesized using miRNA First Strand cDNA Synthesis Kit (Tailing Reaction) (Sangon Biotech). For RT-qPCR reaction, the MicroRNAs qPCR Kit (SYBR Green Method) (Sangon Biotech) was used along with a CFX96 real time-PCR detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The PCR conditions consisted of pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation 95 °C for 5 s and annealing at 60 °C for 30 s, with a final extension step at 95 °C for 5 min. U6 RNA included in the kit was used as an endogenous reference gene for normalizing the miR-124-3p gene expression. The results were calculated by the $2^{-\Delta\Delta Ct}$ method. The sequence of the primer used in this study is as follows: MiR-124-3p forward: 5′- TAAGGCACGCGGTGA ATGCC-3′. The sequences of the U6 RNA and the universal PCR reverse primer are proprietary information held by Sangon Biotech.

TUNEL Staining

Apoptotic cell percentage was showed by Tunel staining. The primary neurons in each group were washed with PBS, and then apoptosis was assessed using a TUNEL Cell Apoptosis Assay Kit (Wanleibio, Shenyang, China) according to the manufacturer's instructions. Fluorescent signals were observed with a fluorescence microscope (Olympus, Tokyo, Japan). Total cells were dyed by 4′,6-diamidino-2-phenylindole and showed bright blue cells on a black background. Apoptotic cells were detected as localized bright green cells. The total cells and apoptotic cells were counted by ImageJ software (v.1.45, National Institutes of Health, Bethesda, USA).

Western Blot Analysis

The samples were homogenized and digested in a homogenizer with a lysis buffer (Sangon Biotech, Shanghai, China). Bicinchoninic Acid Protein Assay kit (Beyotime, Shanghai, China) was used to analyse protein concentration. Sodium dodecyl sulfate (SDS) sample loading buffer was then added, and the samples were boiled at 100 °C for 5 min. Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Wanleibio) and then transferred onto nitrocellulose membranes (Sangon biotech). The membranes were blocked by 5% low-fat milk and then incubated with anti-SPTLC2 antibody (1:200, #sc-398704, Santa Cruz, Cambridge, USA), anti-MYD88 (1:100, #sc-74532, Santa Cruz), anti-interleukin 1 receptor associated kinase 1 (IRAK1) (1:200, #sc-5288, Santa Cruz), anti-tumor necrosis factor receptor associated factor 6 (TRAF6) (1:200, #sc-8409, Santa Cruz), anti-NF-κB (1:200, #sc-71675, Santa Cruz), anti-cleaved caspase 3 (1:500, #WL02117, Wanleibio), anti-B cell lymphoma 2 associated X protein (Bax) (1:2,000, #YM3619, ImmunoWay Biotechnology, Plano, TX, USA), anti-B cell lymphoma 2 (Bcl-2) (1:2,000, #YM3041, ImmunoWay Biotechnology) or anti-β-actin (1:8,000, #YM3028, ImmunoWay Biotechnology) overnight at 4 °C. After being washed 3 times, the membranes were incubated with mouse-IgGκ BP-horseradish peroxidase (HRP) (1:10,000, #sc-516102, Santa Cruz) or HRP-conjugated goat anti-rabbit IgG antibody (1:10,000, #WLA023a, Wanleibio) for 1 h at room temperature. The protein bands were visualized using Enhanced Chemiluminescence Solution (#WLA006a, Wanleibio) and analysed using ImageJ software (v.1.45, National Institutes of Health, Bethesda, USA).

Plasmid Construction and Luciferase Reporter Assay

The luciferase reporter assay was performed using HEK293 cells in 24-well plates. Different versions of the 3′-UTR region of SPTLC2, with either potential binding sites or mutant sites for miR-124-3p, were generated and then cloned into the luciferase reporter vector PGL3-CMV-LUC-MCS (Promega, Madison, WI, USA). We designated them as SPTLC2-WT and SPTLC2-MT, respectively. The inserts

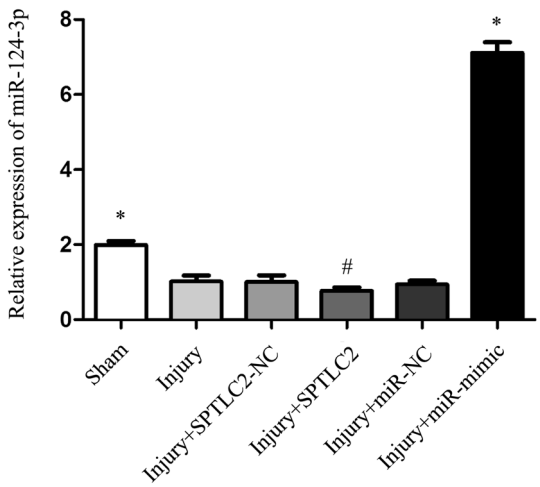
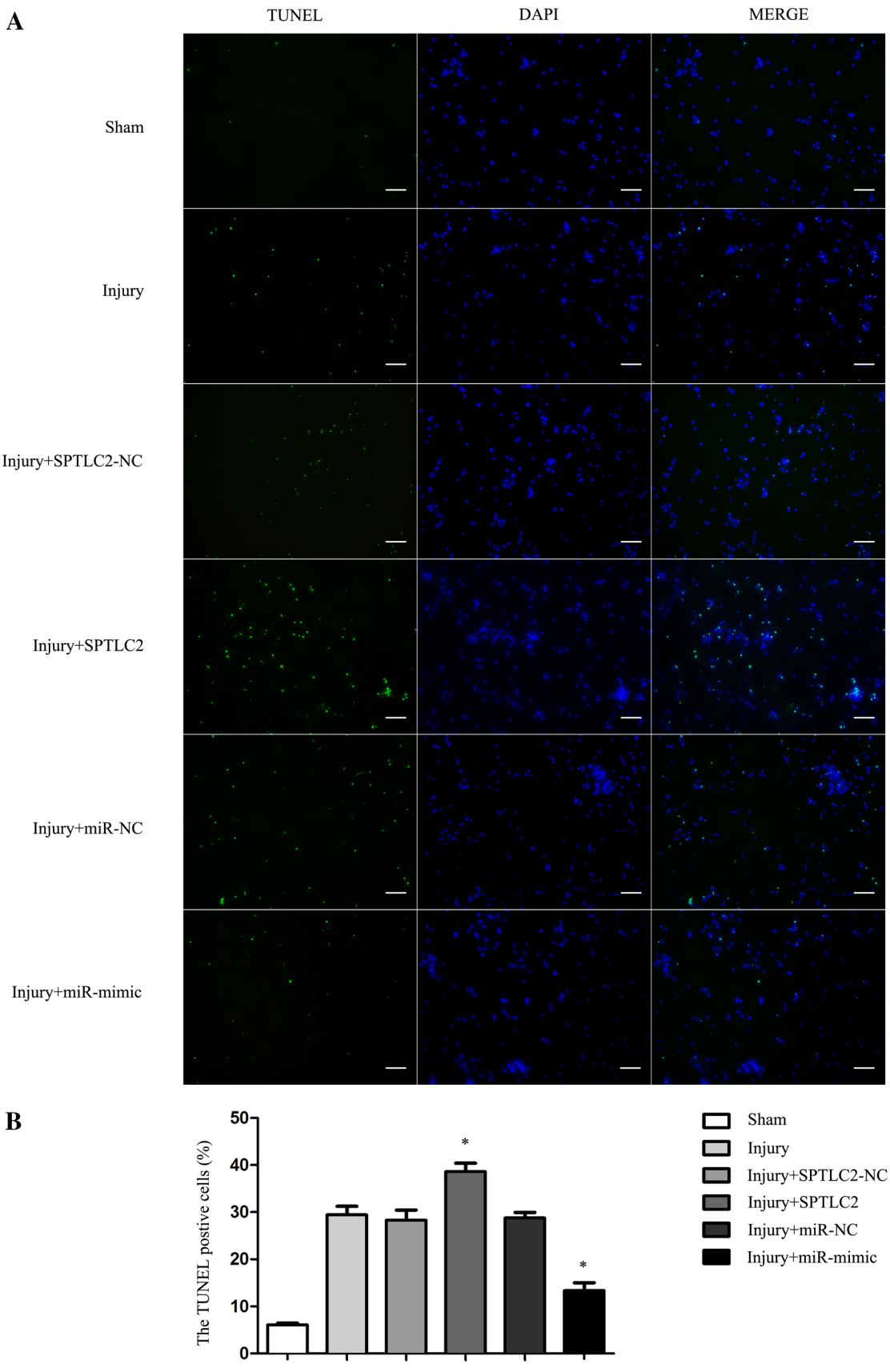


Fig. 1 The relative expression levels of miR-124-3p in primary cortical neurons 48 h after transfection and injury. MiR-124-3p expression levels were determined using qRT-PCR and normalised with U6 RNA. Values are presented as the means ± SD, n = 3 in each group, $^{*}P < 0.05$ versus Injury group. $^{\#}P > 0.05$ versus Injury group


A
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Injury+miR-mimic

◂**Fig. 2** TUNEL assay shows that miR-124-3p represses apoptosis of primary cortical neurons, while SPTLC2 promotes it. **a** Upregulation of miR-124-3p inhibited the apoptosis of primary neurons according to the TUNEL staining, whereas upregulation of SPTLC2 promoted the apoptosis. Scale bar = 50 μm. **b** Quantification of TUNEL staining. Values are presented as the means ± SD, n = 3 in each group, $^*P < 0.05$ versus Injury group

were amplified using the following primers: SPTLC2-WT forward: 5′- AGATCGCCGTGTGACCTGAAGATGGCC ACCTCCACTC-3′, and reverse: 5′-CTAGCACGCGTACAA GGACCCTGAATGAGCCAGAGG-3′, SPTLC2-MT forward: 5′-GCAATGTAAGGCTGCTGACTTCCCGCCCG-3′, and reverse: 5′-GCAGCCTTACATTGCTCCCTAGGG AGAGGGC-3′. 100 ng of SPTLC2-WT or SPTLC2-MT was co-transfected with 30 nM of either a miR-124-3p mimic or a scrambled sequence negative control (NC) along with 1.5 μl Lipofectamine 2000 (Invitrogen). After 48 h, cells were assayed by using a luciferase reporter assay kit (Genomeditech) in accordance with the manufacturer's guidelines. The relative luciferase activity was measured at 48 h after transfection and normalized to Renilla luciferase activity. The data are expressed relative to the fold change of the corresponding control groups.

Co-Immunoprecipitation (Co-IP)

The primary neurons were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 1 mM PMSF). Bicinchoninic acid was used to measure protein concentrations. Some protein from each group was used as the input control, and the remaining proteins were incubated with Protein A/G beads. The supernatants obtained following centrifugation were immunoprecipitated by incubation with anti-SPTCL2, anti-MYD88 antibodies (1–2 μg per 100–500 μg of total protein within 1 ml of cell lysate), followed by western blot with antibodies against SPTL2 and MYD88 respectively. Supernatant incubated with IgG was used as a control.

Statistical Analysis

All data were presented as the mean ± the standard deviation (SD) of at least three experiments with similar results. Western blot band intensities were determined using ImageJ software (v1.8.0, National Institutes of Health, Bethesda, MD, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA) and t-tests. The threshold of statistical significance was set at $P < 0.05$.

Results

Expression Levels of MiR-124-3p can be Readily Perturbed

In vitro experiments, transfection of primary neurons with miR-124-3p mimic was used to modify expression levels of the miRNA. Following transfection and injury, the miR-124-3p expression was examined using a miRNA qPCR array, and the expression profile of the miRNA was analysed. As shown in Fig. 1, the expression of miR-124-3p in Injury + miR-mimic gorup was 7.113 ± 0.165 times than that of the Injury group ($^*P < 0.05$).The expression of miR-124-3p in Sham gorup was 1.989 ± 0.064 times than that of the Injury group ($^*P < 0.05$). There were no statistically significant changes in miRNA expression in the Injury + SPTLC2 group ($\#P > 0.05$ vs. Injury group). Together, the results illustrate that miR-124-3p mimic can upregulate the expression of miR-124-3p in primary cortical neurons after scratch injury. The expression of miR-124-3p is reduced by injury. Transfection of pcDNA 3.1-SPTLC2 plasmid will not affect the expression of miR-124-3p.

MiR-124-3p Represses Apoptosis of Primary Neurons While SPTLC2 Promotes It

To evaluate the effects of miR-124-3p or SPTLC2 on neuronal apoptosis after injury, transient transfection and TUNEL staining was performed. As shown in Fig. 2a, b, the percentage of TUNEL-positive cells in the Injury + SPTLC2 group increased significantly ($^*P < 0.05$ vs. Injury group), while the miR-124-3p mimic notably decreased the percentage of TUNEL positive cells ($^*P < 0.05$ vs. Injury group).

It is well established that the expression levels of some specific proteins change significantly during neuronal apoptosis. Hence, an investigation of the degree of apoptosis was performed by assaying cleaved-caspase 3, Bax and Bcl-2 levels by western blot approximately 48 h after injury (Fig. 3a, b). The results indicated that SPTLC2 in Injury + miR-mimic group was inhibited and was increased observably in Injury + SPTLC2 group ($^*P < 0.05$ vs. Injury group). As the increased levels of SPTLC2, cleaved-caspase 3 levels were also significantly increased compared to the Injury group ($^*P < 0.05$). A rising trend in the BAX/BCL-2 ratio was also found ($^*P < 0.05$ vs. Injury group).

The sum of the evidence above indicates that overexpression of SPTLC2 does not affect the expression of miR-124-3p, but overexpression of miR-124-3p reduces neuronal apoptosis and that SPTLC2, in turn, can promote it.

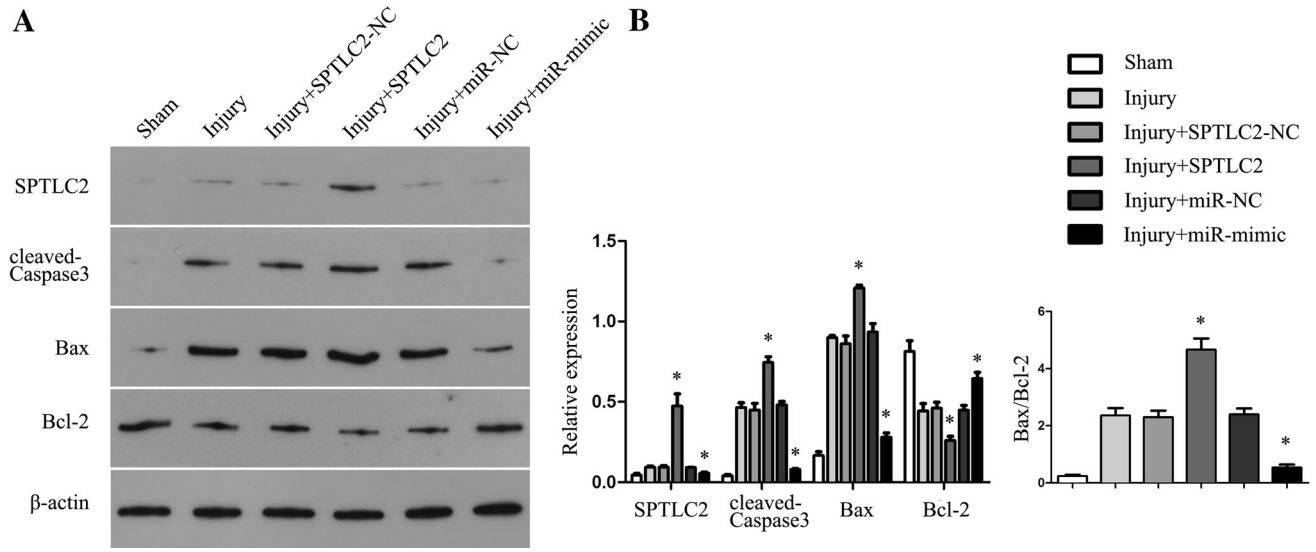

Fig. 3 Western blot shows the consistent results as TUNEL assay that miR-124-3p represses apoptosis of primary cortical neurons, while SPTLC2 promotes it. **a** Effects of upregulation of miR-124-3p or SPTLC2 on the expressions of apoptosis-related proteins were evaluated using western blot in primary cortical neurons 48 h after transfection and injury. **b** Quantifications of western blots. Values are presented as the means ± SD, n = 3 in each group, $^*P < 0.05$ versus Injury group

SPTLC2 is a Direct Target of MiR-124-3p

To further testify the relationship of miR-124-3p and SPTLC2, the online microRNA prediction program TargetScan (https://www.targetscan.org) was used to search for potential targets of miR-124-3p in mice and a conserved miR-124-3p binding site was found in the 3′UTR of SPTLC2 (Fig. 4a). To test whether SPTLC2 is a direct target of miR-124-3p, the 3′UTR of SPTLC2 was cloned into the XhoI site of a PGL3-CMV-LUC-MCS plasmid (SPTLC2 3′UTR WT). Co-transfection of SPTLC2 3′UTR WT with the miR-124-3p mimic significantly reduced luciferase activity compared to co-transfection with the miR-124-3p NC plasmid (Fig. 4b) ($^*P < 0.05$). When the putative miRNA-binding site of SPTLC2 was mutated ("GUGCCUU" to "UGUAAGG"), the inhibitory effect of miR-124-3p on luciferase activity disappeared. To further confirm SPTLC2 as a direct cellular target of miR-124-3p, we performed a rescue experiment in primary neurons. As shown in Fig. 4c, d, miR-124-3p mimic dramatically reduced the endogenous SPTLC2 protein levels in primary neurons compared with the SPTLC2 transfection group ($^*P < 0.05$).

These results suggest that miR-124-3p does target SPTLC2 mRNA directly at the predicted recognition sequence in the 3′UTR.

SPTLC2 can Interact with MYD88

Using a protein interaction database (https://genemania.org/), we searched for proteins in the TLR4 pathway that might interact with SPTLC2 and found MYD88 as promising candidate (Fig. 5a). To test this hypothesis, we used Co-IP. As shown in Fig. 5b, SPTLC2 was immunoprecipitated by anti-MYD88 antibody. Conversely, MYD88 was immunoprecipitated by the anti-SPTLC2 antibody. This result shows that SPTLC2 and MYD88 can form protein compound in natural state, but either this combination direct or indirect which needs further experiments to verify.

MiR-124-3p and SPTLC2 Regulate Neuronal Apoptosis via the TLR4 Pathway

The results above indicated that miR-124-3p inhibited neuronal apoptosis while STPLC2 promote it, SPTLC2 was a direct target of miR-124-3p and it could combine MYD88 in TLR4 signalling pathway. This part of experiment was performed to further investigate whether miR-124-3p and SPTLC2 regulated neuronal apoptosis by targeting TLR4 pathway. The expression levels of TLR4 signalling pathway molecules MYD88/IRAK1/TRAF6/NF-κB in primary neurons after transfection and injury were measured by western blot. Results showed that miR-124-3p mimic significantly decreased the expression levels of MYD88/IRAK1/TRAF6/NF-κB compared to Injury and Injury + SPTLC2groups (Fig. 6a, b) ($^*P < 0.05$). Meanwhile, when expression of SPTLC2 was promoted, expression levels of MYD88/IRAK1/TRAF6/NF-κB were significantly increased compared to Injury and Injury + miR-mimic groups ($^*P < 0.05$). Thus, miR-124-3p can target SPTLC2 to alleviate neuronal apoptosis through inhibiting the TLR4 signalling pathway after injury in vitro.

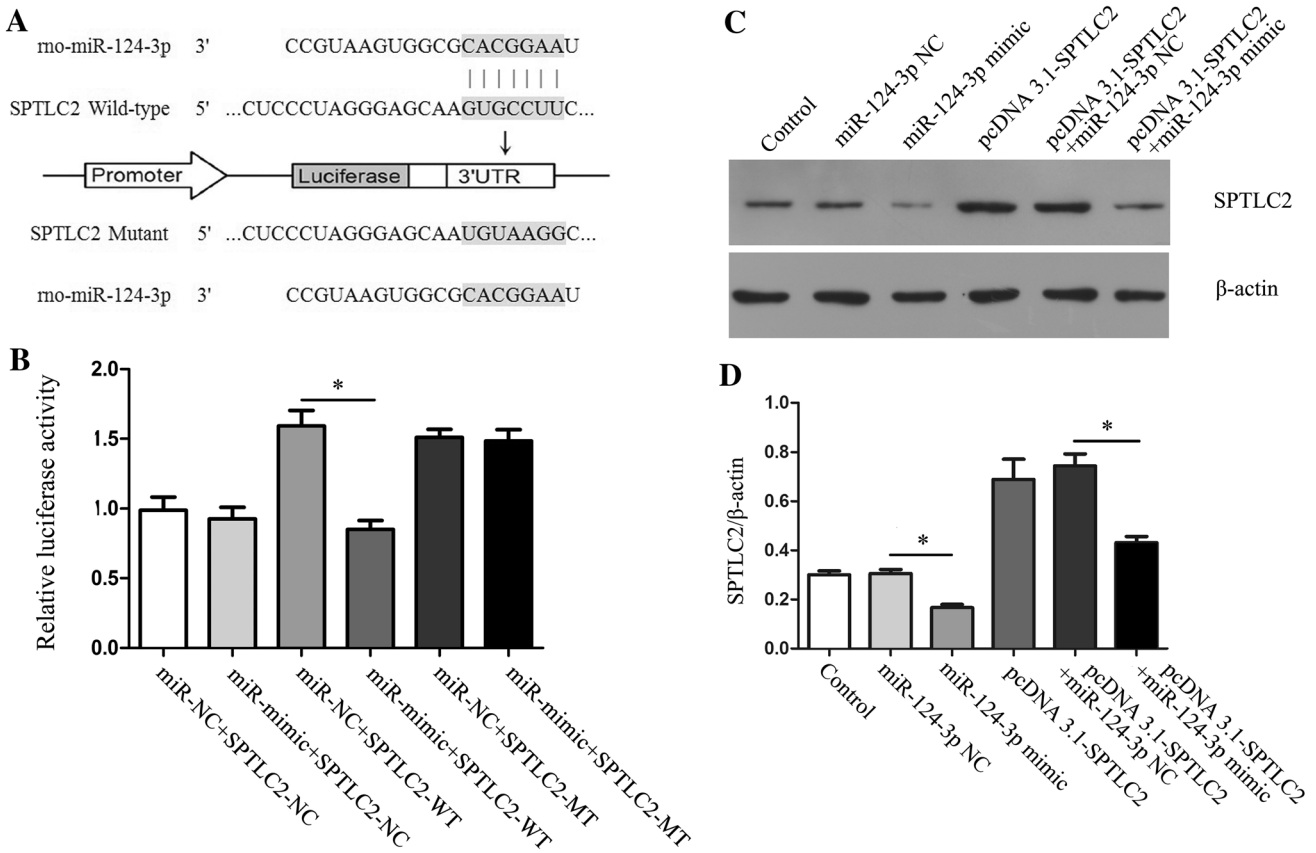

Fig. 4 SPTLC2 is a direct target of miR-124-3p. **a** The bind site between miR-124-3p and SPTLC2 was predicted using the TargetScan database. **b** Luciferase activity in HEK293 cells transfected with reporter constructs containing *NC* negative control, *WT* wild-type, *MT* mutated-type SPTLC2 3′ UTR. The cells were cotransfected with miR-124 mimics or NC, and normalised levels of luciferase activity are shown. **c** Western blot tested the SPTLC2 expression after primary cortical neurons transfected nothing, miR-124-3p mimic negative control, miR-124-3P mimic, SPTLC2 plasmid and SPTLC2 plasmid cotransfected with miR-124-3p mimic negative control or miR-124-3P mimic. **d** Quantification of western blot. Values are presented as the means ± SD, n = 3 in each group, $^*P < 0.05$

Discussion

The physiological and pathological processes involved in TBI include neuronal apoptosis, necrosis, autophagy, induction of an inflammatory response, and glial reaction, among others [28, 29]. The ischemic penumbra around the site of injury triggers mostly apoptotic responses, but the specific signalling pathways involved in this process are still unclear. Thus, reducing neuronal apoptosis and protecting neuronal function would constitute an effective intervention for the treatment of TBI.

A previous study found an antagonistic role of miR-124-3p in the inflammation of scratch-injured neurons [4]. Exosomal miR-124-3p from microglial cells promoted anti-inflammatory M2 polarization and suppressed the activity of mammalian target of rapamycin signalling to protect neurons from inflammation. Another study indicated that miR-124-3p could repress apoptosis by attenuating hyperphosphorylation of tau protein via a caveolin-1/phosphatidylinositol 3 hydroxy kinase/ protein kinase B/ glycogen synthase kinase 3β pathway [30]. In this study, we altered the expression of miR-124-3p and SPTLC2 by transfection in order to investigate their effects on neuronal apoptosis following injury. We found that neuronal apoptosis was increased after injury when SPTLC2 was overexpressed. This phenomenon was reversed when miR-124-3p was overexpressed. Using a luciferase reporter assay, western blots and a rescue experiment, we confirmed SPTLC2 as a direct target of miR-124-3p. Increasing the expression of miR-124-3p substantially reduced the expression of SPTLC2.

SPTLC2 is broadly expressed in most mammals and is a key enzyme in the synthesis of ceramide, an important participant in apoptosis. Ceramide can aggregate rapidly on the cell membrane, recruiting death receptors and ligands to promote apoptotic signal transduction and amplification. In the cytoplasm, ceramide is involved in many signalling pathways as a mediator that can regulate apoptosis [31, 32]. Within the cell nuclear membrane and the nucleus, it can

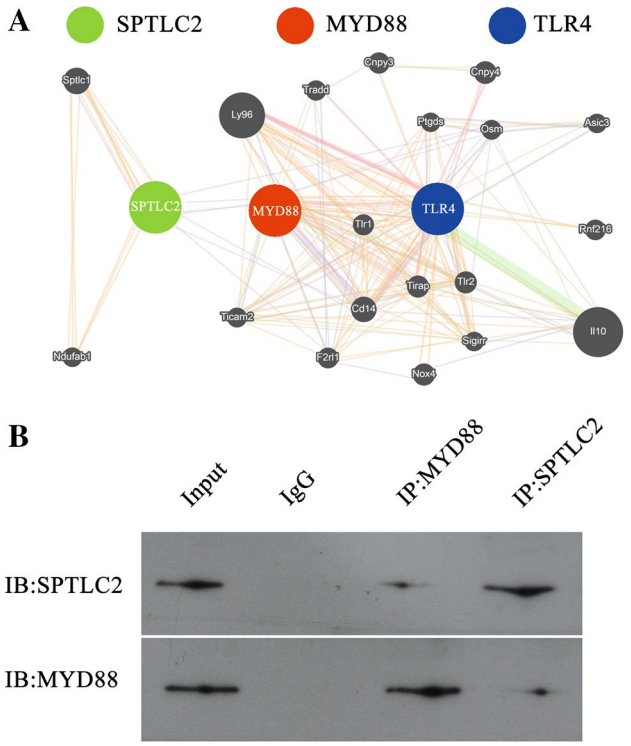

Fig. 5 SPTLC2 can interact with MYD88. **a** GeneMANIA database predicted SPTLC2 could bind to MYD88 in TLR4 pathway. **b** CO-IP experiment was carried out and western blot result showed that SPTLC2 interacted with MYD88

trigger cell proliferation or apoptosis via metabolic changes [33]. Another study shows that lipopolysaccharide activates inflammatory through SPTLC2 and that NF-κB binding sites are located in SPTLC2 promoter region [22]. In the present study, we observed that increasing SPTLC2 expression levels while holding miR-124-3p expression constant results in increased neuronal apoptosis following injury. Based on the predictions of databases such as GENEMANIA, we speculated that SPTLC2 might bind to MYD88 to affect neuronal apoptosis. MYD88 is considered as a key downstream protein for inflammation and immunity in TLR4 signalling pathway [34, 35]. Previous studies have also showed that TLR4 signalling pathway plays key roles in neurodegeneration, neural apoptosis and autophagy [36–38]. Moreover, researches have showed that miR-124 could negatively regulate the expression of TLR4 and its downstream molecules MYD88, TRAF6, IRAK1 and NF-κB [20, 39]. In the present study, Co-IP assay and western blots confirmed the prediction that SPTLC2 could bind to MYD88 to modulate neuronal apoptosis. Subsequently, we found TLR4 signalling pathway downstream molecules MYD88/IRAK1/TRAF6/NF-κB were changed as the changes of miR-124-3p and SPTLC2. These findings suggested to us that SPTLC2 could bind to MYD88 and induce conformational changes that initiate downstream apoptotic signalling. On account of these results, we proposed a reasonable assumption that SPTLC2 was a key enzyme but might also be a cytoplasmic messenger. This hypothesis expands our view of SPTLC2, however, the question remains unanswered and merits further study.

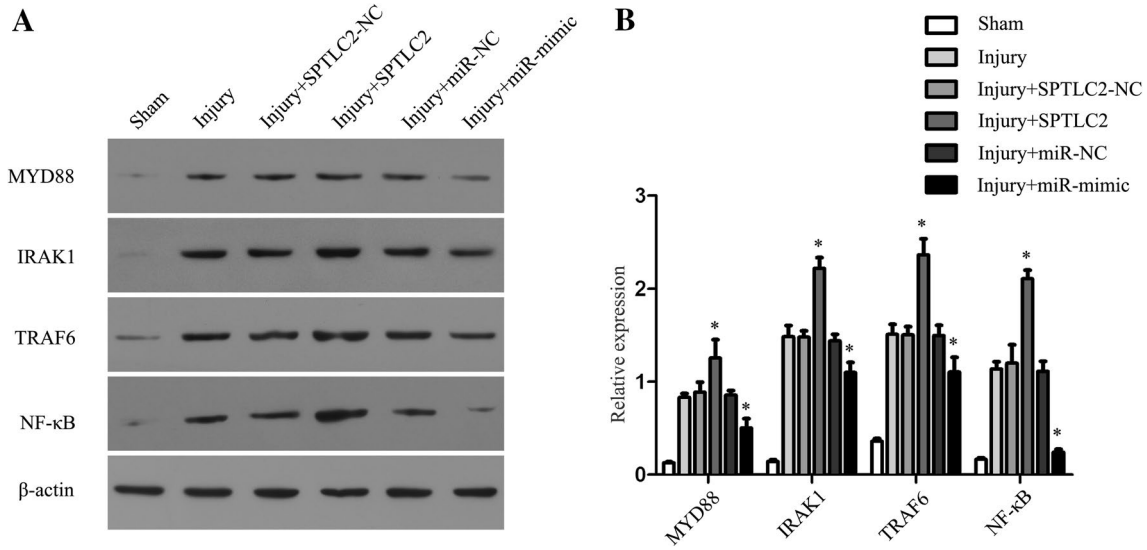

Fig. 6 MiR-124-3p and SPTLC2 regulate neuronal apoptosis via TLR4 pathway. **a** Western blot analysis showed different expression of MYD88/IRAK1/TRAF6/NF-κB. **b** Gray value analysis of each band was carried out and their ratios reported for quantification; the expression levels of MYD88/IRAK1/TRAF6/NF-κB decreased in miR-124-3p mimic group and increased in SPTLC2 plasmid transfection group. Values are presented as the means ± SD, n = 3 in each group, $^{*}P < 0.05$ versus Injury group

In summary, our findings suggest, for the first time, that SPTLC2 is a direct target of miR-124-3p in the regulation of neuronal apoptosis via TLR4 signalling pathway. This role of miR-124-3p is perhaps mediated through the binding of SPTLC2 to MYD88. We had also showed in another study of ours that injected exosomes with miR-124 through tail vein after TBI would significantly improve the prognosis of rats [40]. So, inhibiting neuronal apoptosis after injury is an effective approach to alleviate the symptoms of TBI, but the mechanisms underlying neuronal apoptosis are very complicated and thus need to be investigated further.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to disclose.

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