

## Original Article

# Therapeutic effects of Erbin inhibitor on spinal cord contusion in mice

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**Abstract:** Erbin has been shown to maintain the integrity of cell structure, regulate the proliferation and differentiation of cell and transconduct signals in the pathways. This study was conducted to assess the therapeutic effects of an Erbin inhibitor on spinal cord contusion in mice. Spinal contusion models of mouse were constructed and treated with an Erbin inhibitor. The experimental animals were divided into control (normal animal without any treatment), models with spinal cord injury (SCI), and models receiving Erbin inhibitor (Inhibitor). The contents of 5-hydroxytryptamine (5-HT) and reactive oxygen species (ROS) in the brain and spinal cord tissues were measured using ELISA. The expression of ERK1/2, MAPK, NF- $\kappa$ B and NRG1 was quantified using qRT-PCR, Western blot analysis and immunohistochemistry. Flow cytometry was used to determine the formation of macrophages. Erbin interference vector was constructed and its interference effect on the expression of these genes was characterized in cultured bone marrow cells. Spinal contusion models were successfully constructed. Administering Erbin inhibitor inhibited the expression of ERK1/2, MAPK and NF- $\kappa$ B and up-regulated the expression of NRG1. Flow cytometry showed that Erbin inhibitor induced the formation of a large number of macrophages, which are beneficial to the recovery of spinal cord injury. Experiments with Erbin interference vector showed similar impacts on the expression of genes at cellular level as the inhibitor did. Our work has demonstrated that the Erbin inhibitor is very effective to treat spinal cord contusion in mice. The possible mechanism of therapeutic effect is that the inhibitor suppresses the ERK1/2/MAPK and/or NF- $\kappa$ B/MAPK signal pathways and enhances the NRG1-ErbB signaling pathway by reducing the expression of Erbin, leading to the inhibition of apoptosis, promotion of proliferation and differentiation, and subsequent repair of the damaged spinal cord.

**Keywords:** Erbin, spinal cord injury, real-time fluorescence quantitative PCR, Erbin inhibitor, immunohistochemistry

## Introduction

Spinal cord injury has the characteristics of high incidence, high disability rate, high cost and low mortality. Commonly used treatment is spinal surgery that incises the spinal cord laterally to decompress the necrotic tissue [1]. Although this method has therapeutic effect, it is difficult to cure the disease completely.

Erbin is a newly discovered protein and plays a regulatory role in many signal transduction pathways. It recognizes the signaling downstream NLR2 and promotes the production of proinflammatory cytokines by blocking muramyl dipeptide (MDP) through the PDZ domain at the C terminal [2]. The biological functions of

Erbin have not been fully elucidated. Erbin is shown to interact with Sur8 to block the formation of the Sur8/Ras/Raf complex, leading to deactivation of the ERK signal pathway, which is one of the main survival signal pathways downstream Her2 [3]. Erbin also negatively regulates the activation of Nod2-mediated nuclear factor-kappa B (NF- $\kappa$ B) and TGF- $\beta$  signaling pathways [4]. However, as a multi-domain scaffold protein, its role in different signaling pathways has not been fully revealed [5].

Studies have shown that the expression of Erbin in the brain and spinal cord tissues of mice with spinal cord injury is higher than that in the normal tissue [6-8]. It is likely that the overexpressed Erbin may result in the apopto-

sis of the injured sites via some signal pathways [9]. Therefore, we treated mice with spinal cord injury with an Erbin inhibitor [10], and analyzed the changes after the treatment and analyzed the possible mechanism underling the therapeutic effect.

## Materials and methods

### *Experimental animals*

C57 mice were purchased from Cavens Experimental Animals, Changzhou, China (permit no. SCXK (Su) 2016-0010). All mice were housed under pathogen-free conditions and had access to standard rodent food and water ad libitum. Experiments were performed on mice between 7 and 10 weeks of age. This study protocols were approved by the Animal Research Ethics Board at Wuhan University.

### *Reagents and instruments*

DMEM/F-12 (cat. no. 1861453) was purchased from GIBICO, USA; FBS (cat. no. SKU 04-007-1A) was obtained from BI, USA; penicillin streptomycin mixture (cat. no. P1400), collagenase type II (cat. no. 1101D133) and trypsin digestion solution (0.25%, cat. no. T1300) were purchased from Solarbio, USA; rabbit polyclonal antibodies against mitogen activated protein kinase (MAPK8) (cat. no. A0288, 1:800) was from ABclonal, USA; against extracellular regulated protein kinase  $\frac{1}{2}$  (ERK1+ERK2) (cat. no. bs-0022R, 1:1100) was purchased from Bioss, USA; rabbit polyclonal antibody against NF- $\kappa$ B p65 (cat. no. ab3253, 1:900) and rabbit monoclonal antibody against NF- $\kappa$ B (cat. no. ab32536, 1:5000) were from Abcam, UK; rabbit polyclonal antibody against neuregulin1 (NRG1) (cat. no. 05217-1-AP, 1:1000) was from Proteintech, USA; rabbit polymerized HRP-labeled rabbit IgG (cat. no. SV0002, 1:1000) was from BOSTER Biologicals, USA; mouse monoclonal antibody against GAPDH (cat. no. TA-08, 1:2000), goat against mouse IgG (cat. no. ZB-2305, 1:2000) and goat against rabbit IgG (cat. no. ZB-23011:2000) were obtained from Zsbio, Beijing, China; mouse ROS (cat. no. ml037727) and 5-HT (cat. no. CK-E20010) ELISA detection kits were products of Mlbio, Shanghai, China. Trizon Reagent (cat. no. CW0580S), Ultrapure RNA Extraction kit (cat. no. CW0581M), HiFiScript first cDNA Stand Synthesis kit (cat. no. CW2569M), ultraSYBR

Mixture (cat. no. CW0957M) and BCA protein assay kit (cat. no. CW0014S) were purchased from CWBIO, Beijing, China. Cell lysis buffer (cat. not. C1053) was purchased from Applygen, Beijing, China. Ultrasensitive luminescence solution (cat. not. RJ239676) was from Thermo Fisher Scientific, USA. DAPI instant staining solution (cat. no. KGA215-50) was obtained from Keygen, Beijing, China. FITC-labeled antibody against CD45.1 (cat. no. 11-0453-81, 1:1000) and PE-labelled antibody against F4/80 (cat. no. 12-4801-80, 1:1000) were purchased from eBiosciences, USA. Erbin inhibitor, a peptide with 27 amino acids (YGRKKRRQ-RRRGPTAENPEYLGLDVPV) (cat. no. P14605) was obtained from SANGON Biotech, Beijing, China. PCR instrument (T100™), fluorescence quantitative PCR instrument (CFX Connect™) and ultrasensitive chemiluminescence imaging system (ChemiDocXRS+) were from Biorad, USA. Flow cytometry (NovoCyte 2060R) and plate reader (Rayto RT-6100) were products of BD, USA.

### *Spinal cord injury models and treatments*

Mice were divided into 3 groups, each consisting of 6 animals. Normal mice was used as control and models were treated with or without the Erbin inhibitor (designated as SIM and Inhibitor, respectively). Mice in SIM and Inhibitor groups were anesthetized by intraperitoneal injection of 0.6% pentobarbital sodium. After sterilized with iodophor on the back, the skin was gently cut open to expose the spinal cord. The cords were clipped for one min with a microhemostat clip to generate injury. The wound was cleaned and sutured. 10 h later, 25  $\mu$ L/mouse inhibitor (5 mM) was injected into the T2 spinal sheath. Two weeks later, the brain and spinal cord were isolated and fixed for subsequent analysis.

### *ELISA*

ELISA was performed using commercial kits according to manufacturer's instructions. After adding the chromogenic agents, the plates were read at 450 nm wavelength using a plate reader.

### *Immunohistochemistry*

The 2  $\mu$ m tissue sections were cut into coated slides, deparaffinised with xilol and rehydrated

**Table 1.** Primers for RT-PCR

Primer	Sequence
NRG1	F: TCTGCTTTTCCTCCCTTACA R: GATGCCGACCACCAACA
MAPK	F: CCCAAATGCTGACTCCAAA R: GAGCCTGTTCACCTCAATCC
NF-KB	F: CAAGACTACGACAGCCACATT R: GCCGCTTCCTCATATCCTC
ERK1/2	F: GGCTTTCTGACGGAGTATGTG R: GCTTGTTTGGGTGAAGGTAA
GAPDH	F: AAGAAGGTGGTGAAGCAGG R: GAAGGTGGAAGAGTGGGAGT

**Table 2.** siRNA sequences

siRNA	Sequence (5'-3')
Erbin-mus-1196	Forward: GCAGCUGCCGGAACUUAUUTT Reverse: AAUAGUUUCCGGCAGCUGCTT
Erbin-mus-762	Forward: GCUAGUGUGAACCCUUAUUUTT Reverse: AAUAGGGUUCACACUAGCTT
Erbin-mus-3461	Forward: GCAUUCUGCCAACAUGAAUUTT Reverse: AUUCAUGUUGGCAGAAUGCTT

through 90%, 80% and 70% ethanol. After washing in water, the slides were autoclaved for 3 min at 1.5 atmosphere in sodium citrate buffer (pH = 6 for ARID1A and PIK3CA and pH = 9 for cMET) for antigen retrieval. Endogenous peroxidase activity was blocked with hydrogen peroxidase for 5 min at room temperature. After rinsing with 1X tris buffered saline (TBS), the tissue sections were incubated with primary antibodies for 30 min. The sections were subsequently washed with 1× TBS and incubated with secondary antibodies at 37°C for 30 min. Diaminobenzidine (DAB) and haematoxylin chromogen (Dako, Glostrup, Denmark) method was used. The sections were subsequently examined by light microscopy.

#### Real-time quantitative PCR

Total RNA was isolated from the mouse tissues using the Trizol Reagent and Ultrapure RNA Extraction kit according to manufacturer's instructions and reversely transcribed into cDNA using HiFiScript first cDNA stand synthesis kit according to manufacturer's instructions. The PCR was carried out in a total volume of 10 µl containing 1.5 µl of cDNA, 10 µl of ultraSYBR Mixture and 1 µl of each fluorescence probe on fluorescence quantitative PCR instrument.

Mouse glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was used as internal control. The cycling conditions were 51°C for 2 min, 95°C for 10 min followed by 40 cycles, each one consisting of 15 s at 94°C and 1 min at 60°C. Primers used are listed in **Table 1**. Samples were run in triplicate and the mean value was calculated for each case.

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using comparative Ct method and obtaining the fold change value ( $2^{-\Delta\Delta Ct}$ ) according to previously described protocol [11].

#### Western blot analysis

After different treatments, the tissues were lysed with RIPA buffer that contains protease and phosphatase inhibitors cocktail and quantitated using BAC kit according to manufacturer's instructions. 50 µg protein was applied to polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and then detected by the proper primary and secondary antibodies before visualization with a chemiluminescence kit. The intensity of blot signals was quantitated using ultrasensitive chemiluminescence imaging system (Chemi-DocXRS+).

#### Flow cytometry

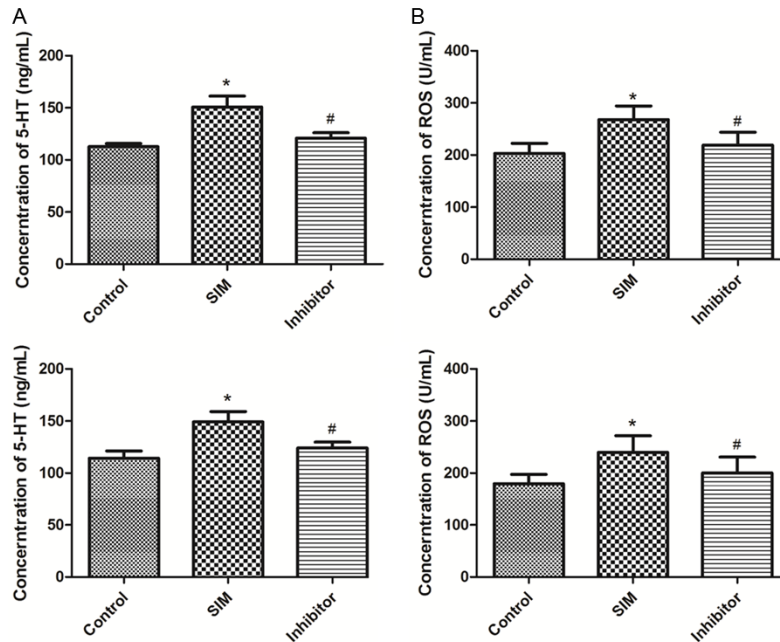
The spinal cord and brain tissue were grind into homogenate, collected and suspended in PBS, labeled with FITC-labeled antibody against CD45.1 and PE-labelled antibody against F4/80 following the manufacturer's instructions. The labelled cells were analyzed on flow cytometer.

#### Erbin interference RNA

The TRPM7 gene sequence was obtained from the NCBI database and used to design the siRNAs (**Table 2**), which were chemically synthesized at General Biologicals, Anhui, China.

#### Isolation and culture of bone marrow cells

Mice were sacrificed by cervical dislocation and the whole femurs were isolated. The femurs were punched at the end to wash out bone marrow cells using RPMI1640 medium. The single-cell suspension was adjusted to  $1 \times 10^5$  cells/



**Figure 1.** 5-HT (A) and ROS (B) contents of mice in spinal cord tissues (upper pane) and mouse brain tissue (lower pane) following spinal cord injury and Erbin inhibitor injection. \* and #denote  $P < 0.05$  vs control and SIM, respectively.

mL with RPMI1640 medium containing 20% horse serum, seeded in the wells of 6-well plate (4 ml/well) and inoculated at 37°C for 4 days in 5% CO<sub>2</sub> incubator. The medium were refreshed in three days and the cells were transfected with siRNA (Erbin-mus-3461) and empty vector at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, USA).

#### Immunofluorescence assay

Cells grown in culture plates were washed with PBS and fixed in 4% of polyformaldehyde for 15 min, cleared with 0.5% Triton X-100 (in PBS) at room temperature for 20 min and drop-added with 5% BSA at 37°C. The cells were incubated with antibodies against NRG1 and Erbin at 4°C overnight and then stained with fluorescence-labelled secondary antibody Cy3 (1:200) at 37°C for 30 min. After nuclear staining with DAPI in the dark at room temperature for 5 min, the slides were observed under fluorescence microscope.

#### Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., USA). All experiments were repeated at least three times and performed in tripli-

cate. Means were compared using the student's t-test or two-way ANOVA with the corresponding post-test. A  $p$ -value  $\leq 0.05$  was considered statistically significant.

## Results

### Spinal cord injury and gene expression

Two weeks after modelling, the spinal cord tissues were examined for 5-HT and ROS as indication of injury. Results showed that the contents of 5-HT and ROS in the spinal cord models were significantly increased (**Figure 1**), indicating that the animal were injured and the modelling was successful. After injection of the inhibitor, the contents of 5-HT

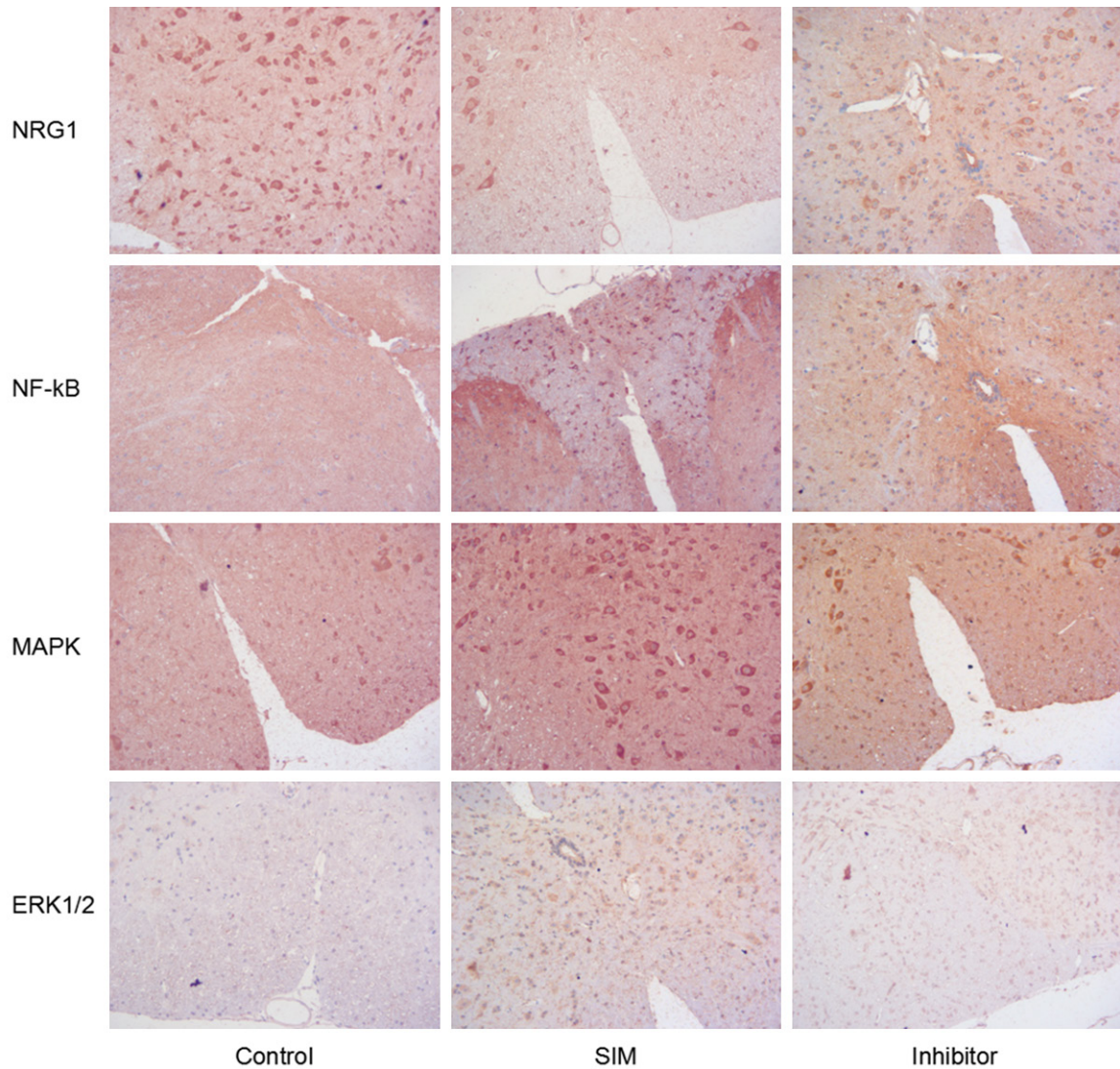
and ROS were significantly decreased compared with the contents in SIM control, indicating that Erbin inhibitor has therapeutic effect on the spinal cord injury.

We then examined the expression of several genes related to apoptosis. Immunohistochemistry analysis showed that the expression of ERK1/2, MAPK and NF- $\kappa$ B in SIM group was up-regulated as compared with control and down-regulated as compared with Inhibitor group (**Figure 2**). However, the NRG1 expression was upregulated (**Figure 2**). These results indicate that Erbin inhibitor can repair damaged spinal cord tissue. These results were further confirmed at mRNA and protein levels by RT-PCR and Western blot analyses (**Figures 3, S1**).

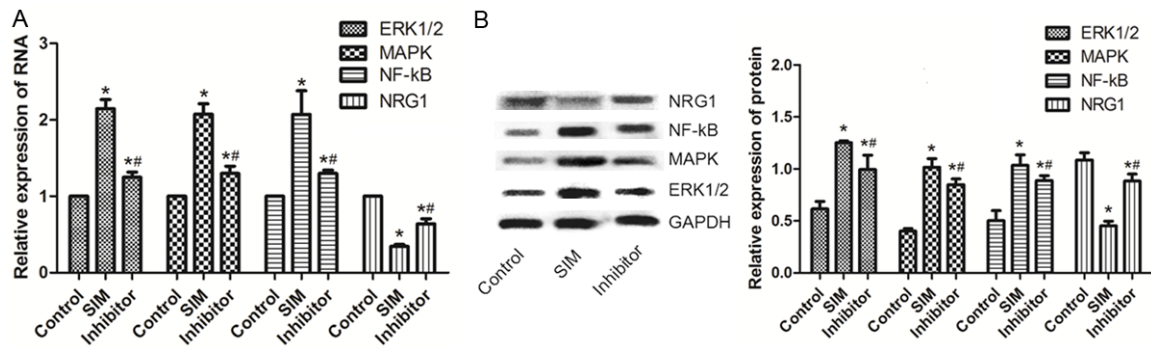
### Brain injury and gene expression

We then examined brain injury and the expression of these genes in the brain tissues. As shown in **Figures 4, 5** and **S2**, the brain injury and expression of these genes at mRNA level and protein level followed the same patterns as in the spinal cord tissue, suggesting that the Erbin inhibitor has the function of treating brain injury caused by spinal contusion, a further indication of its the therapeutic effect.

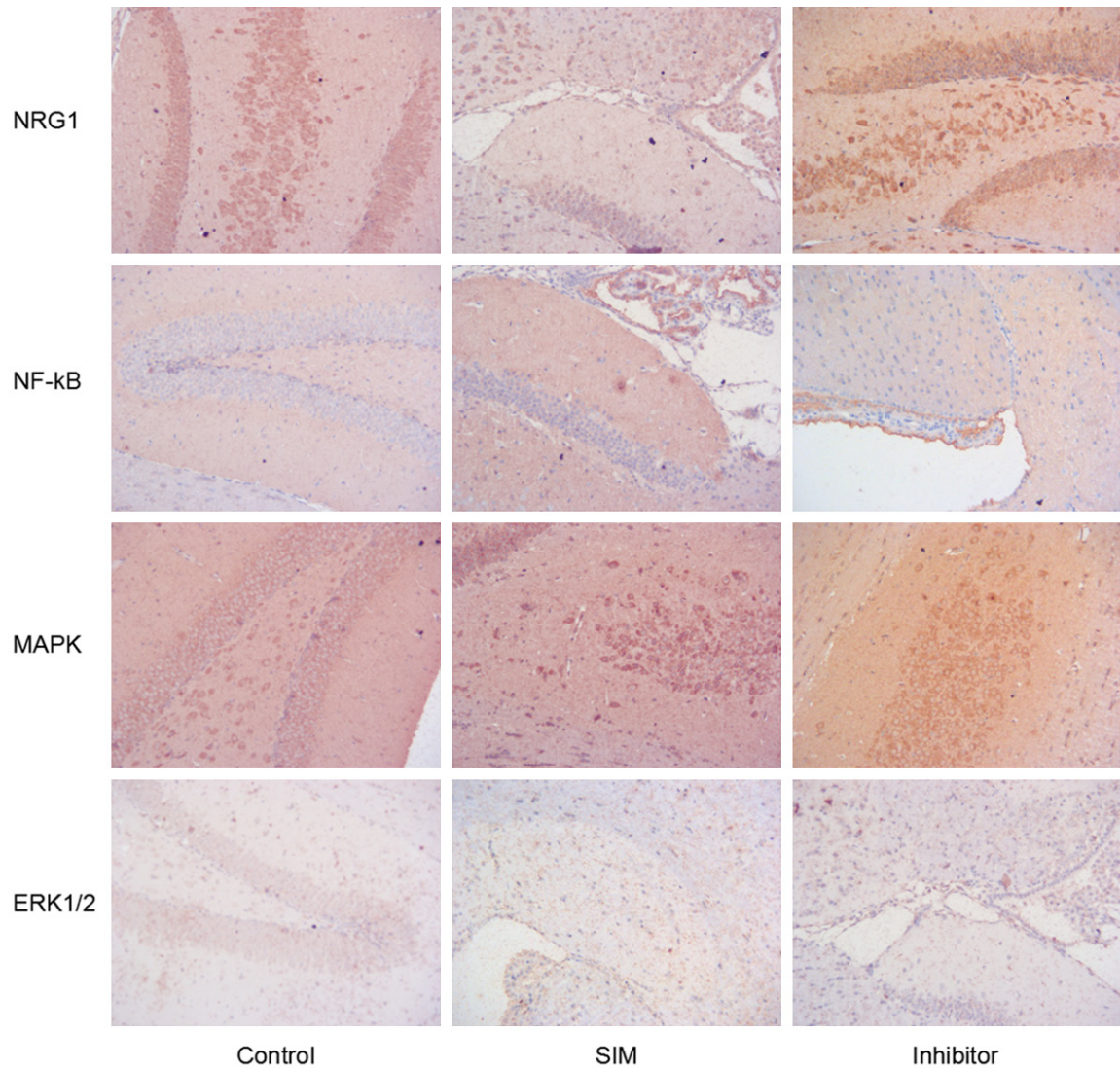




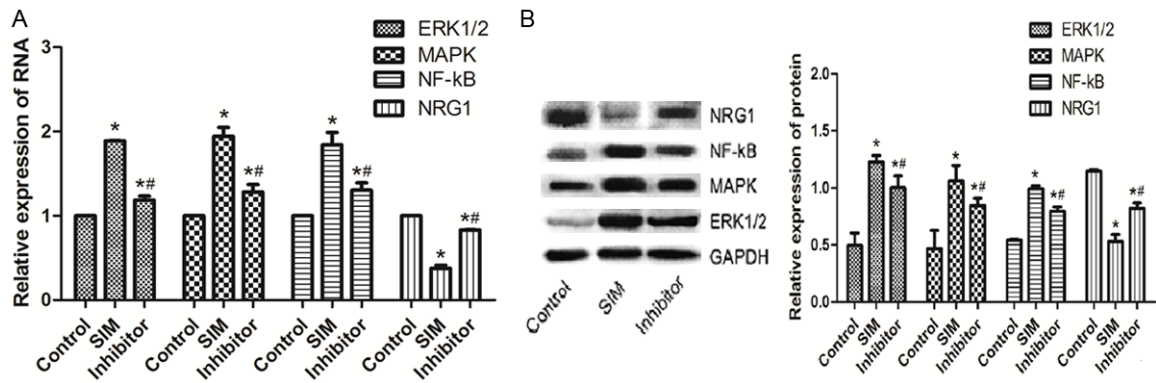
**Figure 2.** Immunohistochemistry analysis of ERK1/2, MAPK, NF-kB and NRG1 in spinal cord following spinal cord injury and Erbin inhibitor injection ( $\times 200$ ).



**Figure 3.** Relative mRNA (A) and protein (B) levels of ERK1/2, MAPK, NF-kB and NRG1 in the spinal cord following spinal cord injury and Erbin inhibitor injection. \* and # denote  $P < 0.05$  vs control and SIM, respectively.

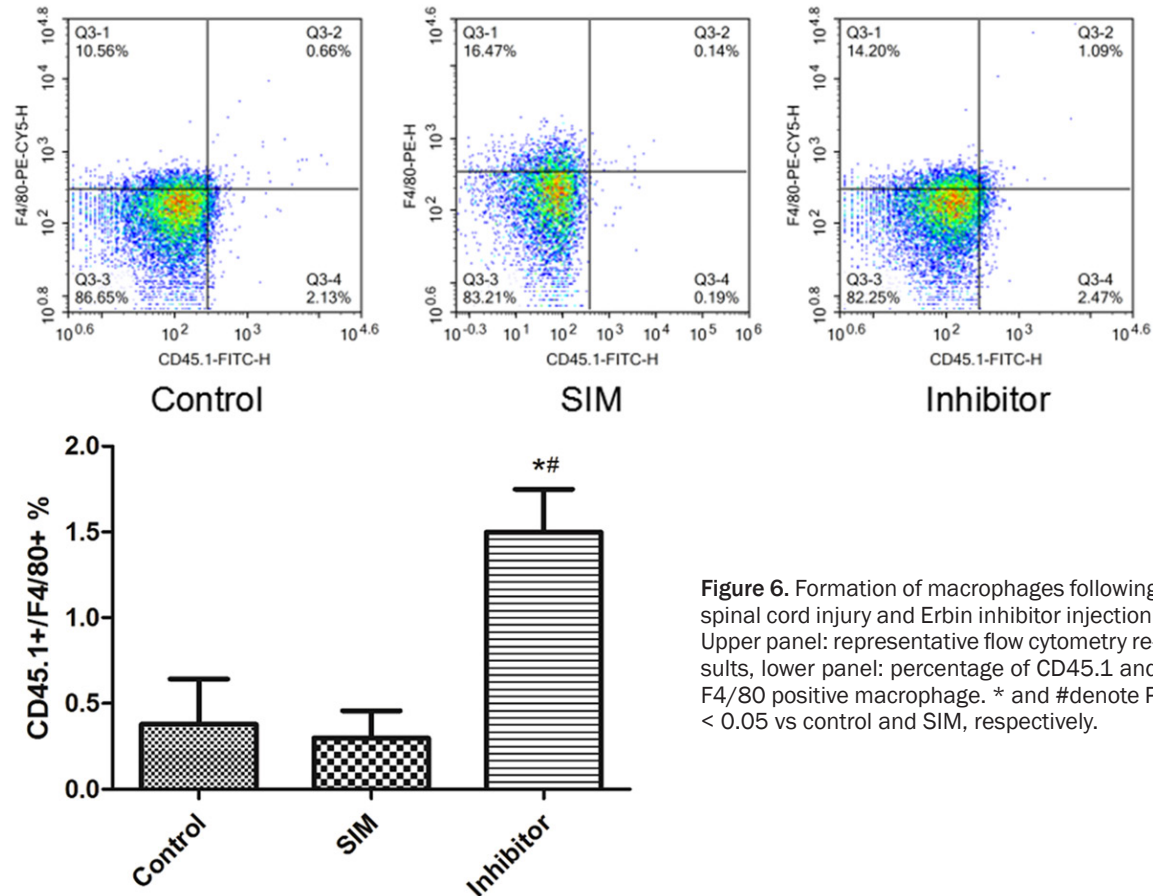


**Figure 4.** Immunohistochemistry analysis of ERK1/2, MAPK, NF-kB and NRG1 in brain tissue following spinal cord injury and Erbin inhibitor injection ( $\times 200$ ).



**Figure 5.** Relative mRNA (A) and protein (B) levels of ERK1/2, MAPK, NF-kB and NRG1 in the brain tissue following spinal cord injury and Erbin inhibitor injection. \* and # denote  $P < 0.05$  vs control and SIM, respectively.





**Figure 6.** Formation of macrophages following spinal cord injury and Erbin inhibitor injection. Upper panel: representative flow cytometry results, lower panel: percentage of CD45.1 and F4/80 positive macrophage. \* and #denote  $P < 0.05$  vs control and SIM, respectively.

#### Macrophage formation

The blood samples were assayed for macrophages. Flow cytometry studies showed that the blood from Inhibitor group had the highest counts of CD45.1- and F4/80-positive macrophage (**Figure 6**), suggesting that macrophages were stimulated to repair the injured tissue and this is an indirect indication that the Erbin inhibitor has a certain repair effect on the spinal cord injury.

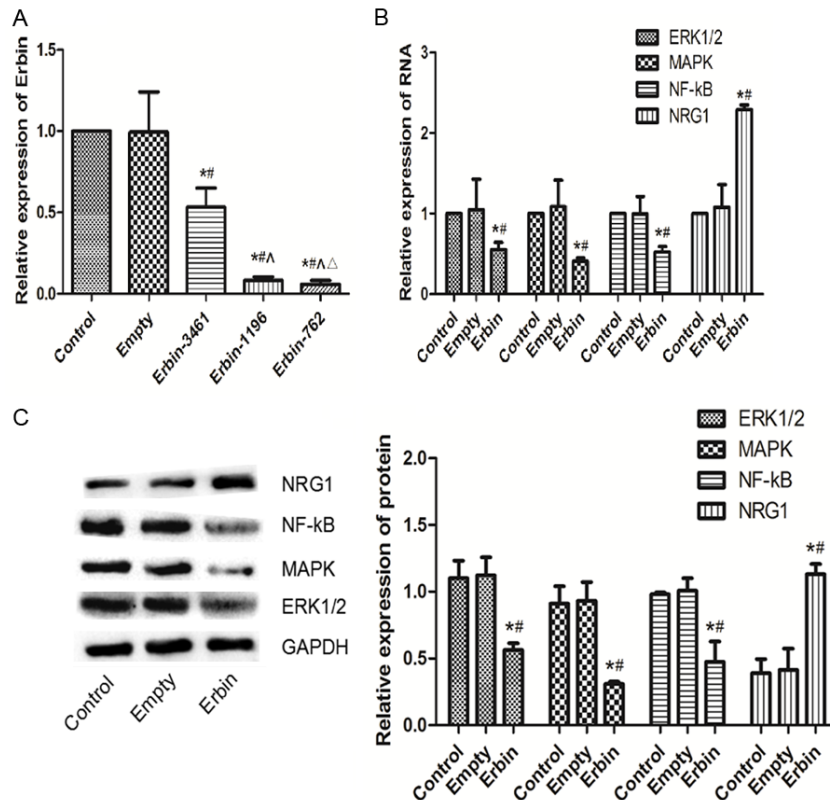
#### RNAi interference

The isolated bone marrow cells were used to investigate the effect of RNAi mediated-knock-down of Erbin. Based on Erbin expression, Erbin-3461-RNAi was most effective in down-regulating Erbin expression (**Figure 7A**) and it was used in subsequent experiments. qRT-PCR and Western blot analysis showed that after transfection with Erbin-3461-RNAi, the expression of Erbin, ERK1/2, MAPK and NF- $\kappa$ B was down-regulated and that of NRG1 was up-regu-

lated (**Figures 7B, 7C, S3**). These results were further confirmed by immunofluorescence assays showing that Erbin was up- and NRG was down-regulated following the transfection.

#### Discussion

With the development of economic level in the world, the incidence of spinal cord injury is increasing year by year [12]. Spinal cord injury is the most serious complication of spinal injury, which often leads to severe dysfunction of the lower limbs, bringing not only serious physical and psychological harm to the patient, but also a huge economic burden to the whole society [11]. Therefore, the prevention, treatment and rehabilitation of spinal cord injury have become a major topic in the medical field today. In this study, an Erbin inhibitor was attempted as a therapeutic drug in mouse model of spinal cord contusion for the first time. The first 12 amino acids of the peptide are trans-membrane sequence and the following 15 amino acids are the core domain of ErbB2 that interact with



**Figure 7.** Expression of Erbin (A), ERK1/2, MAPK, NF-kB and NRG1 mRNA (B) and protein (C) in cultured bone marrow cells following transfection with RNAi. \* and #denote  $P < 0.05$  vs control and empty vector, respectively.

Erbin [10]. Once bound with the peptide, Erbin would be inhibited to interact with other protein. The effects and possible models of action were studied at mRNA and protein expression levels in the spinal and brain tissues as well as isolated cells.

In the mouse model of spinal contusion, we found increased 5-HT and ROS in the brain and spinal cord tissues. 5-HT, also known as serotonin, is a biologically active molecule. Under the action of stimulating factors, 5-HT is released from the cells to the blood, and absorbed and stored in the platelets [13]. 5-HT is mainly distributed in the pineal gland and hypothalamus. It may participate in the regulation of several biological processes, such as pain, sleep and body temperature [9]. ROS is a group of reactive oxygen species, including  $O_2^-$  and  $H_2O_2$ , produced by cells during metabolism, which can induce apoptosis and tissue necrosis [7]. Therefore, after spinal cord injury, increased 5-HT and ROS contents indicate that the model has a certain degree of injury and

the modelling is successful. After injection of Erbin inhibitor, the contents of 5-HT and ROS was reduced, indicating that the Erbin inhibitor is effective in attenuating the spinal cord injury.

We also examined expression of several genes in the models. The results showed that Erbin inhibitor down-regulates the expression of ERK1/2, MAPK, NF-kB and up-regulates the expression of NRG1. Studies show that Erbin inhibitor excises action through MAPK signaling pathway [14, 15]. MAPK is an important transmitter that transmits signals from the cell surface to the nucleus, and participates in many important cell physiological and pathological processes

such as the regulation of cell growth, differentiation, stress adaptation to the environment, and inflammatory reactions [16]. ERK1/2 is a member of the MAPK family, involved in a number of biological processes such as cell proliferation and differentiation, maintenance of cell morphology, construction of cytoskeleton construction, cell apoptosis and malignant conversion of cell [15, 17]. Activated ERK1/2 would result in the activation of important effectors in other signaling pathway such as NF-kB and CREB, and important transcriptional factors such as bc1-2, bc1-XL and FasL, leading to uncontrolled cell proliferation [18-20].

Erbin is an ErbB2 binding partner, and NRG1-ErbB signaling pathway plays an important role in neural development, cell proliferation and differentiation and is closely related to the pathogenesis of neurodevelopmental abnormalities, as well as the development of diseases such as cancer, heart failure, and myocardial infarction [21, 22]. Study showed that overex-



pressed Erbin induces apoptosis and tissue necrosis via activating ERK1/2/MAPK or NF- $\kappa$ B/MAPK signaling pathways, while Erbin inhibitor downregulates the expression of Erbin to suppress MAPK-related signal transduction pathway to inhibit apoptosis [23].

Our study have showed that Erbin inhibitor upregulates the expression of NRG1, suggesting that it may also participate in NRG1-ErbB signaling pathway and is a negative regulator of this signal pathway. On other hand, Erbin inhibitor inhibits Erbin, resulting in enhanced NRG1-ErbB signaling pathway to repair cells in damaged spinal neurons.

We further have demonstrated that Erbin expression could be knockdown with siRNA at the cellular level, and the knockdown results in similar changes in the expression of these genes as the inhibitor did. This further reveals that model of action of Erbin inhibitor: it down-regulates the expression of Erbin to suppress the ERK1/2/MAPK and/or NF- $\kappa$ B/MAPK signaling pathways, and enhance the NRG1-ErbB signaling pathway, thus inhibiting cell apoptosis, promoting cell proliferation and differentiation, and repairing damaged spinal cord.

In conclusion, Erbin plays an important role in the pathogenesis and development of disease, but many studies have shown that Erbin are both positive and negative regulator in different tissues and cells [24, 25]. Our work shows that Erbin inhibitor has certain therapeutic effect on the spinal cord contusion in mice, but the specific mechanism of its action need further deliberation.

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## Disclosure of conflict of interest

None.

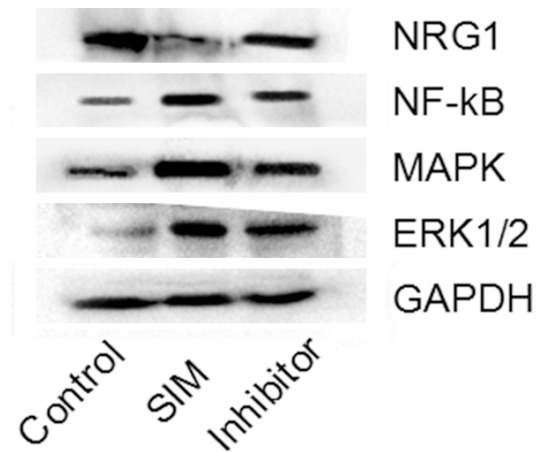
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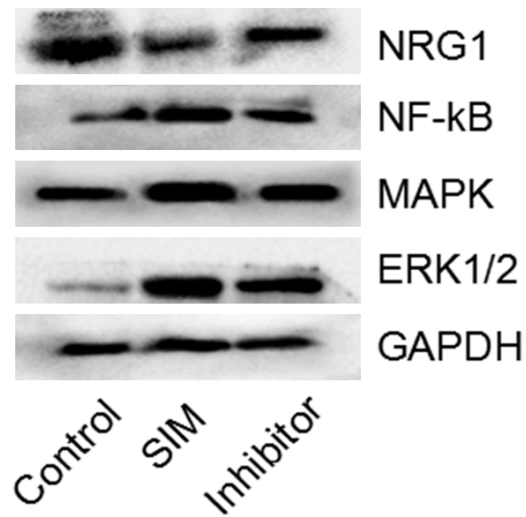
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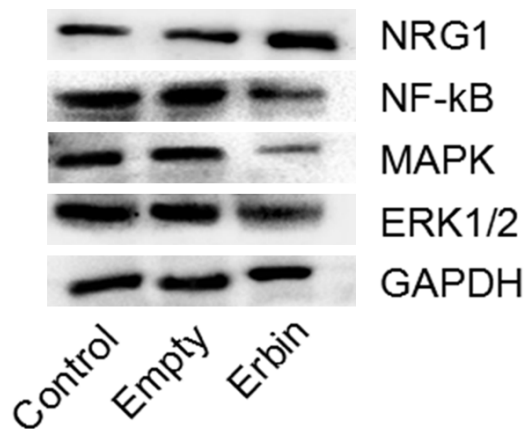
## Erbin inhibitor and spinal cord contusion



**Figure S1.** Representative Western blots showing relative protein levels of ERK1/2, MAPK, NF-kB and NRG1 in the spinal cord following spinal cord injury and Erbin inhibitor injection.



**Figure S2.** Representative Western blots showing relative protein levels of ERK1/2, MAPK, NF-kB and NRG1 in the brain tissue following spinal cord injury and Erbin inhibitor injection.



**Figure S3.** Representative Western blots showing relative protein expression of ERK1/2, MAPK, NF-kB and NRG1 in cultured bone marrow cells following transfection with RNAi vector.