Epidermal growth factor receptor-extracellular-regulated kinase blockade upregulates TRIM32 signaling cascade and promotes neurogenesis after spinal cord injury

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Weimei Xue and Yannan Zhao contributed equally to this study.

Abstract
Nerve regeneration is blocked after spinal cord injury (SCI) by a complex myelin-associated inhibitory (MAI) microenvironment in the lesion site; however, the underlying mechanisms are not fully understood. During the process of neural stem cell (NSC) differentiation, pathway inhibitors were added to quantitatively assess the effects on neuronal differentiation. Immunoprecipitation and lentivirus-induced overexpression were used to examine effects in vitro. In vivo, animal experiments and lineage tracing methods were used to identify nascent neurogenesis after SCI. In vitro results indicated that myelin inhibited neuronal differentiation by activating the epidermal growth factor receptor (EGFR)-extracellular-regulated kinase (ERK) signaling cascade. Subsequently, we found that tripartite motif (TRIM) 32, a neuronal fate-determining factor, was inhibited. Moreover, inhibition of EGFR-ERK promoted TRIM32 expression and enhanced neuronal differentiation in the presence of myelin. We further demonstrated that ERK interacts with TRIM32 to regulate neuronal differentiation. In vivo results indicated that EGFR-ERK blockade increased TRIM32 expression and promoted neurogenesis in the injured area, thus enhancing functional recovery after SCI. Our results showed that EGFR-ERK blockade antagonized MAI of neuronal differentiation of NSCs through regulation of TRIM32 by ERK. Collectively, these findings may provide potential new targets for SCI repair.

KEYWORDS
EGFR, myelin, neural stem cell, neurogenesis, neuronal differentiation, spinal cord injury
1 | INTRODUCTION

Spinal cord injury (SCI), a common central nervous system (CNS) disorder resulting in the loss of sensation and voluntary movements below the level of the lesion, has become a serious problem for society. SCI can destroy spinal cord nerve fibers and lead to a series of cellular and molecular reactions at the injured site including axon breakage, immune reaction reactions, nutritional factor deficiency, and excitotoxicity. All these factors culminate in an inhibitory microenvironment that directly leads to difficulties in axon regeneration after SCI. As a result of such complicated pathophysiological processes, SCI therapy continues to be a considerable clinical challenge. Past strategies for treating SCI aimed to promote nerve fiber regeneration and provide a supportive substrate to guide neurogenesis, thus supporting the recovery of locomotor function. In the spinal cord, ependymal cells are a pluripotent cell type with the ability to differentiate. However, after SCI, ependymal neural stem cells (NSCs) rarely differentiate into neurons, which is an enormous challenge for exploiting their potential therapeutic efficacy.

Many studies have shown that molecules involved in myelin-associated inhibition such as Nogo, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein (OMgp) are major inhibitors of neurite regeneration after SCI. In addition, a previous study reported that myelin inhibited axonal regeneration through epidermal growth factor receptor (EGFR). Consistent with this result, EGFR activation was shown to inhibit neuronal differentiation and promote the occurrence of glia in NSCs. In addition, our previous study demonstrated that myelin can inhibit the neuronal differentiation of NSCs. Thus, EGFR appears to be involved in MAI of neuronal differentiation of NSCs. However, the underlying mechanism by which EGFR participates in fate determination of NSCs remains unknown.

Tripartite motif (TRIM)-NHL proteins are conserved stem cell regulators. Reportedly, TRIM32 can induce neuronal differentiation by mediating reductions in levels of c-Myc, a helix-loop-helix-leucine zipper oncoprotein that plays an important role in cell cycle regulation and proliferation. In addition, mitogen-activated protein kinase (MAPK) subtypes, extracellular-regulated kinase 1 and 2 (ERK1/2), have been shown to stabilize protein expression of c-Myc, which exerts its regulatory role in the cell cycle as a target gene of ERK. While TRIM32 can regulate c-Myc degradation and promote differentiation of NSCs into neurons, ERK can regulate c-Myc to promote cell proliferation. Therefore, we are particularly interested in understanding the relationship between ERK and TRIM32. To this end, we hypothesized that TRIM32 may be involved in regulating neuronal differentiation of NSCs in the MAI microenvironment.

In the present study, we first demonstrated that myelin inhibited neuronal differentiation through EGFR phosphorylation induced by protein kinase C (PKC) and Src-Pyk2 transactivation pathways. Next, we demonstrated the involvement of EGFR-ERK signaling pathway activation in myelin-mediated inhibition of neuronal differentiation. We also showed that TRIM32, a neuronal fate-determining factor, was inhibited during this process as a new downstream molecule of ERK. Additionally, injection of specific EGFR-ERK pathway inhibitors in vivo promoted TRIM32 expression, enhanced neurogenesis in the injured area, and improved the functional recovery after SCI. Subsequently, we found that EGFR-ERK inhibitors antagonized the myelin-mediated inhibition of neuronal differentiation of NSCs through regulation of TRIM32 by ERK. These findings potentially provide new targets for SCI repair.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (Bethesda, MD). All adult female Sprague-Dawley rats (180–200 g) and C57BL/6 mice (30 g) were housed in temperature- and humidity-controlled animal quarters under a 12-hours light/dark cycle.

2.2 | Preparation of myelin

Myelin was prepared from Sprague-Dawley rat brains according to a previously published protocol. Briefly, brain tissue was minced and suspended in 0.32 M sucrose and centrifuged at 27 000 rpm for 50 minutes. Samples from the interface were collected, placed in an ice bath for 30 minutes, and then centrifuged at 12 000g for 30 minutes. The resulting suspension was layered over 0.85 M sucrose and centrifuged again at 27 000 rpm. The interface extraction absorbed excess sugar in ice water. Crude myelin was obtained by centrifugation at 12 000g.

2.3 | NSC culture

The cells were obtained from postnatal (within 12 hours) rat telencephalons, which were digested in 0.25% trypsin containing 0.02% EDTA at 37°C for 25 minutes. The cell suspensions were plated and cultured in proliferation medium (Dulbecco's Modified Eagle's...
2.4 | Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 minutes and then treated with 0.1% Triton X-100 for 15 minutes at room temperature. Samples were washed three times with Dulbecco’s phosphate-buffered saline and then blocked with 5% bovine serum albumin (BSA) for 30 minutes. Primary antibodies were incubated overnight at 4°C (Supporting Information Table S1). Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen, Carlsbad, CA) were incubated at room temperature for 1 hour. Hoechst 33342 (1 mg/mL, DH164-1; Sigma–Aldrich, St. Louis, MO) was used for nuclei staining.

2.5 | Western blotting

Cells were collected and lysed in ice-cold RIPA buffer (WB-0071, China). Next, 40 μg protein was separated by SDS-PAGE and incubated with primary antibodies overnight at 4°C. All primary antibodies were diluted at 1:1000 (Supporting Information Table S2). Horseradish peroxidase-conjugated secondary antibodies were diluted at 1:3000 and incubated for 1 hour at room temperature. Protein bands were detected using an enhanced chemiluminescence Western blot detection reagent.

2.6 | Immunoprecipitation assay

NSC lysates were precleared by adding 1.0 μg of control IgG (normal mouse and normal rabbit) and 50 μL of protein A/G PLUS-Agarose (SC-2003, Santa Cruz Biotechnology, Dallas, TX). After incubation for 30 minutes at 4°C, samples were centrifuged at 1000g for 5 minutes at 4°C. Next, 50 μL of protein A/G PLUS-Agarose and 2 μg primary antibodies were added to lysates (Supporting Information Table S3); the last sample was reserved for input. Samples were incubated at 4°C on a rocker platform overnight, followed by centrifugation at 1000g for 5 minutes at 4°C. Supernatant of immunoprecipitations were washed four times with 0.5 mL of ice-cold RIPA buffer on a rocker platform at 4°C. Samples were boiled for Western blot assays.

2.7 | RNA-seq analyses

RNA extraction was performed with an Agilent 2100 RNA Nano 6000 Assay Kit (Santa Clara, CA), and the resulting mRNA was purified with Oligo(dT) magnetic beads. Purified double-stranded cDNA was then subjected to terminal repair with base A additions and joint sequence processing. Target fragments were then recovered by agarose gel electrophoresis and amplified by PCR to complete library preparation. Constructed libraries were sequenced using an Illumina HiSeq 2500 with the SE50 sequencing strategy. Reads reported in this article have been deposited in the National Center for Biotechnology Information BioProject database (accession no. PRJNA497959). Corrected P values <.05 and fold changes ≥2 for genes were used for the significant differential expression analysis.

2.8 | Quantitative real-time polymerase chain reaction

Total RNA was extracted from differentiated cells using Trizol and cDNA was reverse-transcribed using a kit (K1622; Thermo Fisher Scientific, Waltham, MA). mRNA abundance was detected with a kit (Bio-Rad, Hercules, CA) using 10 μL reaction volumes containing 2x SYBR Green Master Mix (A25741, Thermo Fisher Scientific). mRNAs were analyzed by the 2−ΔΔCt method. Primers are shown in Supporting Information Table S4.

2.9 | Lentivirus production and infection

The trim32 coding sequence was constructed using the CD511B vector and then transfected into 293T cells to produce lentivirus. The concentration (titer) of lentivirus used in our experiment was 10¹⁰ TU/mL. A green fluorescent protein (GFP) reporter gene is present in the construct. The lentivirus was first used to infect NSCs in vitro, and then for injection into normal spinal cords at both ends of the injury area of injured spinal cords to activate intrinsic NSC TRIM32 expression. The TRIM32 primer is shown in Supporting Information Table S5.

2.10 | Surgery and animal experiments

Surguries were performed following intraperitoneal anesthesia with sodium pentobarbital (50 mg/kg). A 2-mm section of spinal cord at the T8 site was removed by transection resection. After 10 minutes, gefitinib or PD98059 was injected into the injury site; for 3 consecutive days, intraperitoneal injections were injected for each group. For injury site injection, the concentration of gefitinib was 1 μM and 9–10 μL of gefitinib was injected into the injury site, thus producing an effective concentration of 50 μM/kg. The concentration of PD98059 is 1 μM and 3.6–4 μL of PD98059 was injected into the injury site, thus producing an effective concentration of 20 μM/kg. The concentration of cetuximab is 5 mg/mL and 4 μL cetuximab was injected into the injury site, which allowed the effective concentration of 100 μg/kg. For 3 consecutive days of intraperitoneal injections, the concentration of gefitinib was 30 mg/mL and 450–500 μL of gefitinib was injected, thus producing an effective concentration of 75 mg/kg. Similarly, the concentration of PD98059 was 20 mg/mL and 90–100 μL PD98059 was injected, thus producing an effective concentration of 10 mg/kg. Lentivirus solutions of 5 μL each were injected at two different locations (1.5-mm caudal and 1.5-mm rostral to the lesion epicenter) at a depth of 1.0 mm.

2.11 | Lineage tracing technology

Nestin-CreERT2 mice were purchased from Jackson Laboratory (Jax strain 016261; Bar Harbor, ME). Gt(ROSA)26Sortm2(CAG-LSL-DTR-EGFP) mice
were purchased from Beijing Biocytogen (Beijing, China). Nestin-Cre<sup>E<sub>RT2</sub>/Gt(ROS)<sub>A26S</sub>O262CAG-LSL-DTR, EGFR<sup>+</sup></sup> double-positive mice harbored Cre-mediated recombination ability under the nestin promoter following tamoxifen injection, which allows the daughter cells to retain their labels. Surgical and drug delivery processes were identical to those described for rats, but the injury distance was limited to 1 mm.

### 2.12 Functional analysis

Function assessments were evaluated weekly according to the Basso, Beattie, and Bresnahan (BBB) open field 21-point locomotion rating scale for rat<sup>25</sup> and Basso Mouse Scale (BMS) 9-point locomotion rating scale for mice.<sup>26</sup> Electrophysiological analyses were performed.
last. Two independent observers blinded to treatment conditions assessed the hindlimb movements of animals.

### 2.13 | Histological analysis

Embedded spinal cords were cut into 12-μm thick slices and mounted on slides, which were incubated in phosphate-buffered saline containing 5% BSA for 1 hour, followed by primary antibodies overnight at 4°C. The following day, samples were incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen) for 1 hour at room temperature. Hoechst 33342 (1 mg/mL) dye was used for nuclear staining. A set of images was collected under the same conditions using a Leica TCS confocal microscope. For the colocalization observation, the confocal z-axis layers scanning function was used to get the different layers of superposition of images, which allowed us to better observe the colocalization or marker genes expression from y- to z-axis and x- to z-axis similar to x- to y-axis.

### 2.14 | Rabies-virus-based retrograde tracing

Production of retrovirus was performed as described elsewhere. The glycoprotein-gene-deleted rabies virus stock (Dgp-mCh) was a generous gift from Dr. Weixiang Guo (Institute of Genetics and Developmental Biology). The production of pseudotyped rabies virus (Rabies-EnvA-mCh) was carried out as described previously. After SCI, mice were injected retrovirus (day 3) and Rabies-EnvA-mCh (week 7), respectively. Lastly, spinal cord sections (40 μm) were used to detect the connectivity of newborn neurons in the spinal cord (GFP/mCh double positive cells represent newborn neurons, mCh positive cells represent traced cells).

### 2.15 | Statistical analysis

Differences between the experimental groups were determined by two-tailed unpaired Student’s t test. Differences determined to be significant as follows: * represents a P value <.05, ** represents a P value <.01, and *** represents a P value <.001. All data are presented as mean ± SD and Prism 5.0 (GraphPad Software, San Diego, CA) was used to perform the statistical analyses.

## 3 | RESULTS

### 3.1 | EGFR pathway is involved in myelin-mediated inhibition of neuronal differentiation

To gain insight into the mechanism by which MAI of neuronal differentiation occurs and explore the role of EGFR in this process in vitro, the EGFR inhibitors cetuximab and gefitinib were added to differentiation medium under MAI conditions. The results showed that neuronal differentiation increased from 17.8% (±1.5%) in the myelin group to 31.1% (±3.8%) in the myelin + 15 μg/mL cetuximab group, and to 37.4% (±1.2%) in the myelin + 1 μM gefitinib group following 6 days of differentiation in the MAI microenvironment (Figure 1A,B). With the extension of the differentiation time (12 days), similar data showed that percentages of NeuN(+) cell were decreased from 9.6% (±1.0%) in control group to 1.9% (±0.3%) in myelin group, but were partially reversed to 6.8% (±0.8%) in the myelin + 15 μg/mL cetuximab group and to 7.3% (±2.6%) in the myelin + 1 μM gefitinib group (Supporting Information Figure S1A,B). During early differentiation (1 day), mRNA expression of the early neuron marker doublecortin (DCX) was lower in the myelin group compared with the control group. However, expression of DCX was higher in the myelin + cetuximab or gefitinib groups compared with the myelin group (Supporting Information Figure S1C). During later differentiation (6 and 12 days), Tuj-1 mRNA and NeuN mRNA were expressed as similar as DCX (Supporting Information Figure S1D,E). Next, we performed RNA-sequencing (RNA-Seq) of differentiated NSCs in the control and myelin inhibition groups. Differential expression genes are displayed in the form of a heat map (Supporting Information Figure S1F). Using GO pathway analysis of differentially expressed genes, we found that most genes were clustered into functional entries, such as neuronal differentiation and neurogenesis (Supporting Information Figure S1G). We found that mRNA expression of a comprehensive list of known neuron generation- and neuronal differentiation-related genes was inhibited during differentiation in the myelin-associated inhibitory (MAI) microenvironment; moreover, such expression was rescued upon adding cetuximab or gefitinib (Figure 1D,E). From these results, we inferred that EGFR inhibitors could antagonize MAI of neuronal differentiation of NSCs. To further investigate the signaling pathway involved in this process, we performed an in-depth analysis of the RNA-sequencing results, which indicated significant changes in the EGFR-MAPK signaling pathway (Figure 1C). Significantly, differentially expressed genes were identified and subsequently verified by

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**FIGURE 1** The epidermal growth factor receptor (EGFR) pathway involved in myelin-associated inhibition of neuronal differentiation. A. Effects of cetuximab and gefitinib on neuronal differentiation in a myelin-associated inhibitory microenvironment in vitro. B. Quantification of percentages of Tuj-1(+) cells among differentiated cells cultured with myelin, cetuximab, gefitinib, myelin + cetuximab, and myelin + gefitinib (n = 4). C. Hierarchical clustering analysis of differentially expressed genes involved in neuronal differentiation and neuron generation. In addition, differential expression of genes involved in the EGFR-mitogen-activated protein kinase (MAPK) pathway was analyzed (n = 3). D. Quantitative real-time polymerase chain (qRT-PCR) analysis of neuronal differentiation-related genes (n = 3). E. qRT-PCR analysis of neuron generation-related genes (n = 3). F. qRT-PCR analysis of EGFR-MAPK-related genes (n = 3). G. Detection of EGFR, Akt, and ERK phosphorylation by myelin stimulation preincubation with cetuximab or gefitinib for 1 hour. Tubulin was used as an internal control. H. Quantification of the EGFR, Akt, and ERK phosphorylation in (G; n = 3). Error bars represent mean ± SD; * represents a P value <.05, ** represents a P value <.01, and *** represents a P value <.001. All scale bars: 50 μm
quantitative real-time polymerase chain reaction (qRT-PCR) (Figure 1F). Based on previous studies, endogenous EGFR receptor phosphorylation was also assessed through Western blotting. The results showed that EGFR phosphorylation was increased by myelin, but decreased by cetuximab or gefitinib pretreatment (Figure 1G,H). Additional evidence was obtained by examining the phosphorylation of ERK and Akt proteins. Changes in ERK and Akt phosphorylation levels occurred in an EGFR-dependent manner (Figure 1G,H), thus supporting the notion that myelin can activate the EGFR signaling pathway. In summary, EGFR inhibitors promoted neuronal differentiation of NSCs in the presence of myelin, which mediates EGFR phosphorylation to play a significant role in this process.

3.2 Myelin inhibits neuronal differentiation through PKC-, Src-Pyk2-induced EGFR phosphorylation

To explore the mechanism of EGFR phosphorylation in MAI, four EGFR trans-activation signaling inhibitors were used. Go6976, PP2, GM6001, and EGTA, respectively, block PKC, Src and Pyk2 (non-receptor tyrosine kinases), metalloproteases, and calcium signaling molecules, all of which are reported EGFR transactivators.31-33 We examined whether the neuronal differentiation was improved after blocking EGFR trans-activation with these inhibitors in the context of a MAI microenvironment. NSCs cultured in differentiation medium for 6 days were subjected to immunofluorescence staining and qRT-PCR. Tuj-1 immunofluorescence results demonstrated that only the addition of Go6976 (PKC inhibitor, 100 nM) or PP2 (Src and Pyk2 inhibitor, 100 nM) promoted neuronal differentiation in the MAI microenvironment (from 13.1% ± 3.3% to 22.0% ± 6.6% in the Go6976 group and 24.5% ± 1.3% in the PP2 group; Figure 2A). Furthermore, qRT-PCR results indicated that blocking either PKC or Src-Pyk2 signaling pathway under MAI could promote Tuj-1 and NeuN mRNA expression to some extent compared with the myelin group (Figure 2B,C). In addition, inhibitors of PKC or Src-Pyk2 signaling were preincubated with starved cells for 1 hour in order to keep all cells in physiological state of the synchronization; subsequently, myelin was added to stimulate cells. Western blot results indicated reduced phosphorylation levels in preincubated cells treated with myelin compared with myelin-only treated cells (Figure 2D,E). Collectively, these data indicate that myelin inhibited neuronal differentiation through activation of EGFR phosphorylation by the PKC and Src-Pyk2 transactivation pathways, rather than metalloproteases or calcium signaling.
3.3 | Myelin inhibits neuronal differentiation by activating the EGFR-ERK signaling pathway

We aimed to explore the downstream signaling pathway involved in the regulation of neuronal differentiation by myelin. Previous reports suggest both ERK and Akt signaling pathways play vital roles downstream of EGFR during various biological processes. Therefore, we selected PD98059 and MK2206 2HCl as ERK and Akt signaling inhibitors, respectively. NSCs were treated with PD98059 (1 μM) and MK2206 2HCl...
represent mean ± SD; * represents a

myelin participates in cell proliferation. Thus, myelin may inhibit neuronal differentiation through EGFR-ERK signaling activation. Previous studies demonstrated that EGFR-ERK signaling participates in cell proliferation. Thus, myelin may inhibit neuronal differentiation by augmenting NSC proliferation through activation of EGFR-ERK signaling during the differentiation process. Ki67 and EdU staining data demonstrated that cell proliferation was promoted upon myelin addition, but inhibited when EGFR-ERK signaling was blocked by gefitinib (Figure 3D–F). Further examination showed that myelin promoted proliferation of nestin(+) cells, but not GFAP(+) cells (Figure 3G–I). These results suggested that, in the presence of myelin, EGFR-ERK signaling-mediated NSC proliferation may inhibit neuronal differentiation to some extent.

3.4 | EGFR-ERK-TRIM32 signaling cascade regulated myelin-inhibited neuronal differentiation of NSCs

The involvement of TRIM32 in inducing neuronal differentiation has been established. We speculated that TRIM32 may play a role in determining NSC fate as a downstream molecule of ERK. To this end, we examined the interaction between ERK and TRIM32. First, we determined whether TRIM32 expression was changed in the presence of myelin. The results indicated decreased TRIM32 protein levels with myelin stimulation, correlating with decreased Tuj-1 protein expression (Figure 4A,B). We also observed that with myelin and EGFR or ERK inhibitor treatment, TRIM32 protein levels were rescued, as were those of Tuj-1 (Figure 4A,B). Furthermore, our present study demonstrated that MAI of neuronal differentiation occurred through PKC-, Src-Pyk2-induced EGFR phosphorylation (Figure 2). Thus, EGFR trans-activation inhibitors were used to determine the effects on TRIM32 expression. Upon myelin treatment and inhibition of PKC- or Src-Pyk2-induced EGFR phosphorylation with Go or PP2, respectively, TRIM32 expression was improved despite the addition of myelin; other inhibitors examined did not elicit this effect (Figure 4C,D).

As these data indicated a positive correlation between neuronal differentiation and TRIM32 expression, the interaction between ERK and TRIM32 was explored further with immunoprecipitation. As shown in Figure 4E, ERK can interact with TRIM32 (Figure 4E). Furthermore, colocalized immunofluorescence results showed that ERK was expressed in the cytoplasm before nuclear entry and could colocalize with TRIM32, further indicating a direct interaction between ERK and TRIM32 (Figure 4F).

To explore the effect of myelin on differentiation of NSCs into neurons, we overexpressed TRIM32 in NSCs by lentivirus (Supporting Information Figure S2A–C). The results showed that in NSCs infected with empty vector (CD511B) lentivirus, the proportion of neuronal differentiation was 40.61% (±0.9%); however, this proportion was decreased to 16.56% (±3.0%) after myelin addition (Figure 4G). In contrast, in NSCs overexpressing TRIM32 (CD511B-TRIM32 lentivirus), the proportion of neuronal differentiation in the control group was 43.19% (±0.5%) and the proportion was 46.10% (±9.2%) after myelin addition, not decreased by myelin (Figure 4G,H). Notably, the percentage of NeuN(+) mature neurons was similar to Tuj-1(+) cells (Supporting Information Figure S2D). These results indicated the importance of TRIM32 during neuronal differentiation, and support the involvement of EGFR-ERK signaling in MAI of neuronal differentiation through inhibition of the ERK downstream molecule TRIM32.

3.5 | TRIM32 promotes neurogenesis in injury sites after complete transection SCI

After SCI, we observed decreased TRIM32 expression levels in the MAI microenvironment. However, TRIM32 expression was rescued by the addition of cetuximab or PD98059 after SCI (Figure 5A–D). To further examine whether TRIM32 plays a vital role in neurogenesis after SCI in vivo, we used a rat model of T8 complete transection SCI. A lentivirus carrying the trim32 coding sequence was injected into the normal spinal cords at both ends of the injury site. At 2 and 4 weeks, TRIM32 expression and new neuron occurrence at the injury site were observed. As predicted, TRIM32 colocalized with GFP in the vector, and TRIM32 expression was higher in the overexpression group than the control group at both 2 and 4 weeks (Figure 5E,F).
Additional results showed that the TRIM32 overexpression group displayed GFP/Tuj-1 double-positive cells that were not present in the empty vector (CD511B) group at 2 or 4 weeks; thus, GFP(+) TRIM32-overexpressing cells can differentiate into neurons in the injury site after complete transection SCI (Figure 5G,H). Moreover, we observed DCX (+) cells in the injury site in the TRIM32 overexpression group at 2 or 4 weeks, but none in the empty vector (CD511B) group (Figure 5I). Collectively, these results demonstrated that overexpression of TRIM32 can antagonize MAI to promote neurogenesis after complete transection SCI.

FIGURE 4  Legend on next page.
3.6 | EGFR-ERK inhibition promoted TRIM32 expression, increased neurogenesis, and enhanced functional recovery after complete transection SCI

To further analyze the effect of EGFR-ERK pathway inhibitors in treating SCI, we conducted animal experiments. After complete transection SCI, the motor function of rat hind limbs was completely lost, with a BBB score of 0 and undetectable electrophysiology signals were undetectable (Figure 6A,C). To test motor functions after treatment with EGFR or ERK signaling pathway inhibitors for 12 weeks, hindlimb locomotion was assessed using the BBB scale and inclined plane assays at 12 weeks. Rats in SCI + gefitinib (mean BBB score: 9.0 ± 1.2) and SCI + PD98059 (mean BBB score: 9.8 ± 0.9) groups exhibited significant improvements in BBB scale values compared with rats in the SCI group (mean BBB score: 6.6 ± 0.5) after 3 weeks postsurgery until sacrifice. Notably, the therapeutic effect of PD98059 was slightly better than that of gefitinib (Figure 6A). From weeks 3 to 9, the SCI group exhibited stable or limited spontaneous recovery. For rats that received EGFR-ERK inhibitors, initial spontaneous improvement was followed by sustained functional recovery until 11 weeks (Figure 6A; Supporting Information Movie S1) shows this in more detail. Inclined plane observations indicated statistically larger mean angles for rats in SCI + gefitinib (39.8° ± 2.7°) and SCI + PD98059 (39.2° ± 2.2°) groups compared with the SCI group (33.0° ± 3.5°). Normal rats maintained their body position on the inclined plane at 57.7° ± 2.5° (Figure 6B). These results demonstrated that motor function after severe SCI was improved by the injection of EGFR or ERK inhibitors. In the electrophysiology experiment, the amplitude of the spontaneous recovery of the electrophysiological wave was only 11.45 μV ± 10.7 μV and the latent period was 10.19 ms ± 4.0 ms in the SCI group, which is far from that of normal rats (amplitude: 153 μV; latent period: 1.47 ms; Figure 6C–E). In inhibitor-injection groups, amplitudes of SCI + gefitinib (24 μV ± 17 μV, two-tailed unpaired Student’s t test, P < .05) and SCI + PD98059 (20.8 μV ± 10.2 μV, two-tailed unpaired Student’s t test, P < .05) groups were larger and the latent periods of SCI + gefitinib (5.4 ms ± 1.2 ms, two-tailed unpaired Student’s t test, P < .01) and SCI + PD98059 (5.5 ms ± 1.0 ms, two-tailed unpaired Student’s t test, P < .001) groups were shorter compared with SCI spontaneous recovery group (Figure 6C–E). Thus, to a certain extent, both gefitinib and PD98059 can promote the improvement of the electrophysiology after SCI.

Twelve weeks later, we first assessed the TRIM32 expression level. In both gefitinib- and PD98059-injected groups, TRIM32 was more highly expressed in the injury site compared with the SCI group (Supporting Information Figure S3A,B). We further identified Tuj-1(+) neuronal populations and new neurons in both SCI + gefitinib and SCI + PD98059 groups, but not the SCI group (Figure 7A,C). In addition, NeuN(+) neuronal populations were occasionally observed in the injured site in treatment groups (Figure 7B,D), suggesting neurogenesis in these groups after complete transection SCI.

Previous studies reported that nestin(+) cells can be activated and migrate into the injury site after SCI to contribute to endogenous neurogenesis. To confirm that neurons detected within the treatment groups were newly born neurons from endogenous nestin(+) cells (rather than spared preexisting neurons) following complete transection SCI, we used a lineage-tracing method to trace the fate of endogenous nestin(+) cells. We performed tamoxifen-inducible genetic lineage tracing using Nestin-CreERT2 and Gt(ROSA)26Sortm2(CAG-LSL-DTR-EGFP) mice. After complete transection SCI (no neurons present in the injury site), tamoxifen was injected to label activated nestin(+) cells responding to the SCI stimulus (Figure 7E). Prior to experiments, normal mice were injected with tamoxifen to detect the specificity of the Nestin-Cre-loxP system. We only observed GFP(+) cells in the central canal, where nestin(+) cells existed in normal mice (Supporting Information Figure S4A). After SCI, some newly activated nestin(+) cells appeared to be labeled through tamoxifen injection after injury. Indeed, at 2 weeks postsurgery, GFP/DCX double-positive cells were observed in the injury sites of both SCI + gefitinib and SCI + PD98059 groups, but not in SCI group (Figure 7G). At 4 weeks postsurgery, GFP/Tuj-1 double-positive cells were also observed in the injury sites of both SCI + gefitinib and SCI + PD98059 groups, but not in SCI group (Figure 7F). However, no NeuN(+) mature neurons were detected in the injury site of any group at 4 weeks (Supporting Information Figure S4B). These results suggested differentiation of nestin(+) cells into new neurons in response to complete transection SCI promoted by EGFR-ERK inhibition in the injury site.

To detect whether functional recovery ability was improved on mice, we conducted functional recovery experiments in the mice model. As we can see, mice displayed better functional recovery in the SCI + gefitinib group (mean BMS score: 2.0) and SCI + PD98059 group (mean BMS score: 3.0) compared with that in the SCI group (mean BMS score: 1.0) after 5-week recovery (Supporting Information Figure S5A, Movie S2). Besides, the electrophysiology results showed higher amplitude and shorter latent period in the SCI + gefitinib group.
FIGURE 5  TRIM32 antagonized myelin-associated inhibition to promote neurogenesis after spinal cord injury (SCI). A–D, (A) and (C), In vivo, cetuximab and PD98059 were added to the injured site after SCI. Spinal cord samples harvested 1 mm from the damage site were lysed. Next, TRIM32 expression was detected by Western blotting. GAPDH and tubulin were used as internal controls. (B) and (D) show the quantification of TRIM32 proteins in (A) and (C; \( n = 3 \)). E, F, In vivo, lentiviruses carrying CD511B-TRIM32 or CD511B vectors were injected into the spinal cord after SCI. After 2 (E) or 4 weeks (F), the spinal cord was dissected and TRIM32 expression was determined by immunofluorescence staining. Scale bars: 50 μm. G, H, In vivo, lentiviruses carrying CD511B-TRIM32 or CD511B vectors were injected into the spinal cord after SCI. After 2 or 4 weeks, the spinal cord was dissected and the expressions of GFP and Tuj-1 were determined by immunofluorescence staining. Scale bars: 50 μm. I, After lentiviruses carrying CD511B-TRIM32 or CD511B vector injection, the DCX expression was detected by immunofluorescence staining. Scale bars: 25 μm. Scale bars in enlarged images: 7.5 μm. Error bars represent mean ± SD; ** represents a \( P \) value <.01, and *** represents a \( P \) value <.001.
Epidermal growth factor receptor (EGFR) and extracellular-regulated kinase (ERK) inhibitors promoted electrophysiological recovery and functional motor improvement after spinal cord injury (SCI). A, Basso, Beattie, and Bresnahan scores of rats in each group from surgery to 12 weeks postinjury. Animals were assessed weekly for their hindlimb movements by two independent observers blinded to treatment conditions. Rats in SCI + gefitinib and SCI + PD98059 groups showed better scores among all groups. B, Inclined plane assay of rats in each group. C, Motor evoked potential (MEP) results of normal and treatment group rats at 12 weeks post-treatment. D, Amplitude ratio of MEP in rats of each group. E, Latent periods of MEP in rats of each group (n = 10). Error bars represent mean ± SD; * represents a P value <.05, ** represents a P value <.01, and *** represents a P value <.001.
FIGURE 7   Epidermal growth factor receptor (EGFR) and extracellular-regulated kinase (ERK) inhibitors promoted neurogenesis in the lesion area after spinal cord injury (SCI). A, Immunostaining with Tuj-1 antibody to illustrate neurogenesis in the lesion area after 12-weeks SCI. Scale bars: 500 μm. B, Immunostaining with NeuN antibody to illustrate neurogenesis in the lesion area after 12-weeks SCI. Scale bars: 500 μm. C, Enlargement of (A). Left scale bars: 50 μm. Right enlargement scale bars: 10 μm. D, Enlargement of (B). Left scale bars: 50 μm. Right enlargement scale bars: 10 μm. E, Tamoxifen-inducible genetic lineage tracing mouse model used to trace the fate of neural stem cells. F, Immunostaining with Tuj-1 and GFP antibodies to illustrate neurogenesis in the lesion area after 4-weeks SCI in a nestin-CreERT2/Gt(ROSA)26Sortm2(CAG-LSL-DTR,-EGFP) double-positive mouse model. Scale bars in left images: 500 μm. Scale bars in middle enlarged images: 50 μm. Scale bars in right z-stack enlarged images: 25 μm. G, Immunostaining with DCX antibody and GFP antibody to illustrate neurogenesis in the lesion area after 2-weeks SCI. Scale bars in left images: 50 μm. Scale bars in right z-stack enlarged images: 10 μm
double-positive cells) were observed in the injured site and new connectivity of newborn neurons was also observed in the T6 and T7 normal spinal cord in both Gefitinib and PD98059 groups, but not in the SCI group (Supporting Information Figure S5G).

4 | DISCUSSION

The present study explored the mechanism by which myelin inhibits neuronal differentiation. We first performed in vitro studies focused on SCI repair through the promotion of neurogenesis by inhibition of EGFR-ERK signaling.

TRIM32 is a ubiquitously expressed E3 ubiquitin ligase and exerts its effects through its RING-domain at the N-terminus. This property of TRIM32 allows it to control cell fate not only in skeletal muscle progenitor cells, but also in the nervous system. Interestingly, TRIM32 has been demonstrated to play important roles in the neuronal differentiation of NSCs. Indeed, as a cell fate determinant, TRIM32 can regulate embryonic NSCs to differentiate into neurons as well as the occurrence of adult neurogenesis. TRIM32 ubiquitinates c-Myc, an essential factor for NSC proliferation. Here, we revealed a novel role for TRIM32 in regulating NSC differentiation as a new member of the EGFR-ERK downstream pathway. Indeed, TRIM32 may create a bridge between c-Myc and ERK by maintaining the balance between NSC proliferation and neuronal differentiation. However, these studies indicate that the functions of TRIM32 in regulation of cell fate are context-dependent. During mammalian CNS development, neurogenesis is completed through asymmetric divisions, whereas NSC self-renewal is accomplished by symmetric divisions, which are two opposing processes. Previous studies have clarified that symmetric division activities occur before asymmetrical division, which helps maintain an adequate number of NSCs to accomplish later fate determination. Importantly, cell cycle length is a major influencing factor during this process. As the ERK downstream target c-Myc plays a significant role in cell cycle regulation, we speculated that myelin might inhibit neuronal differentiation through effects on the cell cycle induced by TRIM32 inhibition, resulting from EGFR-ERK activation. Although our study sheds some light onto the complex regulatory mechanisms underlying MAI of neurogenesis in SCI, further studies are necessary to fully understand the entire process.

After severe SCI, the complex MAI microenvironment that arises at the injury site inhibits neurogenesis. In the spinal cord, ependymal cells are a pluripotent cell type with the potential to differentiate. However, nestin(+) cells that exist before SCI (mainly ependymal cells) are unable to migrate to the injury site after transection injury. Moreover, previous studies suggest that differentiation of ependymal cells into neurons is extremely difficult; indeed, this might be a major reason underlying the difficulty of SCI repair. However, previous studies have also reported that SCI can result in an increased number of NSCs, with numerous injury-induced nestin(+) cells appearing around and migrating to the site of injury. Furthermore, NT3-chitosan tubes contribute to endogenous neurogenesis by activated nestin(+) cells after SCI. In our in vivo study, we labeled injury-induced nestin(+) cells and induced these cells to differentiate into neurons by inhibiting EGFR-ERK signaling to antagonize myelin inhibition. These injury-induced nestin(+) cells were not only ependymal cells, but also nascently activated nestin(+) cells induced by SCI. However, as the source of these injury-induced nestin(+) cells with the ability to migrate to the injury site after complete transection SCI remains unknown, further study is necessary.

5 | CONCLUSION

Our data indicate that EGFR-ERK signaling activation by myelin plays an inhibitory role in neuronal differentiation. Moreover, we demonstrated that TRIM32 regulates neuronal differentiation through an ERK-TRIM32 interaction under MAI conditions. In vivo experiments confirmed that EGFR-ERK blockade promotes TRIM32 expression, enhances neurogenesis at the injury site, and facilitates functional recovery after SCI. Collectively, these findings would aid in the screening of specific small molecule drugs for SCI repair.

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CONFLICT OF INTEREST

The authors indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

modification; J.D.: conception and design, financial support, data analysis and interpretation, final approval of manuscript.

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

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REFERENCES

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.