Overexpression of Rictor in the injured spinal cord promotes functional recovery in a rat model of spinal cord injury

Ningning Chen¹ | Pengxiang Zhou² | Xizhe Liu³ | Jiachun Li¹ | Yong Wan³ | Shaoyu Liu¹ | Fuxin Wei¹

¹Department of Orthopedics, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China
²Department of Physical Diagnostic, Daqing Longnan Hospital, Daqing, China
³Department of Spine Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Correspondence
Yong Wan, Department of Spine Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. Email: yongwanphd@163.com
Shaoyu Liu and Fuxin Wei, Department of Orthopedics, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, 628 Zhenyu Road, Shenzhen 518107, China. Email: gzshaoyuliu@163.com (S. L.) and fuxinweiphd@163.com (F. W.)

Funding information
This work was supported by grant funds from the National Natural Science Foundation of China Youth Found (No. 81801907), Shenzhen Committee of Science and Technology, China (No. JCYJ2018307 145215811), Sun Yat-sen University Youth Teacher Training Project

Abstract
Rictor is an essential component that directly activates the mammalian target of rapamycin (mTOR) activity, which contributes to the intrinsic axon growth capacity of adult sensory neurons after injury. However, whether its action also applies to regeneration after spinal cord injury (SCI) remains unknown. In this study, rats were given spinal cord contusion at the T9-10 level to establish the SCI model and were subsequently treated with intraspinal cord injection of a Rictor overexpression lentiviral vector to locally upregulate the Rictor expression in the injured spinal cord. Thereafter, we investigated the therapeutic effects of Rictor overexpression in the injured spinal cords of SCI rats. Rictor overexpression not only significantly attenuated the acute inflammatory response and cell death after SCI but also markedly increased the shift in macrophages around the lesion from the M1 to M2 phenotype compared to those of the control lentiviral vector injection-treated group. Furthermore, Rictor overexpression dramatically increased neurogenesis in the lesion epicenter, subsequently promoting the tissue repair and functional recovery in SCI rats. Interestingly, the mechanism underlying the beneficial effects of Rictor overexpression on SCI may be associated with the Rictor overexpression playing a role in the anti-inflammatory response and driving macrophage polarization toward the M2 phenotype, which benefits resident neuronal and oligodendrocyte survival. Our findings demonstrate that Rictor is an effective target that affects the generation of molecules that inhibit spinal cord regeneration. In conclusion, localized Rictor overexpression represents a promising potential strategy for the repair of SCI.

Abbreviations: BBB, Basso, Beattie and Bresnahan; BSA, bovine serum albumin; CD, cluster of differentiation; DAPI, 4′-6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; FG, fluorogold; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GLT-1, glial glutamate transporter 1; HE, hematoxylin-eosin; IFN-γ, interferon-gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; mTOR, mammalian target of rapamycin; NF200, neurofilament 200; NO, nitric oxide; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PVDF, polyvinylidene difluoride; Rictor, rapamycin-insensitive companion of mammalian target of rapamycin; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; SCI, spinal cord injury; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, Tris-HCl-based buffer with Tween 20; TNFR1, tumor necrosis factor receptor 1; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Ningning Chen, Pengxiang Zhou, and Xizhe Liu are contributed equally to this work.
1 | INTRODUCTION

Spinal cord injury (SCI) is a devastating traumatic injury that immediately induces the catastrophic disability and disrupts the quality of family and social life.\(^1\) SCI is characterized by an initial injury followed by a secondary pathological cascade, such as inflammation, ischemia, apoptosis, and cell death that further leads to expansion of damage to the spinal cord.\(^2\) Current clinical treatments have poor therapeutic efficacy in SCI patients.\(^3\) Many translational medicine studies are focused on exploring the potential targets for improving the functional recovery after SCI.\(^4\)

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that controls the cell and organismal growth in response to various kinds of stimulation. mTOR assembles into two distinct, multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).\(^5\) Several lines of evidence indicate that targeted inhibition of mTORC1 activity via rapamycin promotes the functional recovery after spinal cord injury in rats through autophagy promotion, anti-inflammation, and neuroprotection.\(^6\) In contrast, the function of mTORC2 is much less well defined; to date, recent studies have indicated a role of mTORC2 in the regulation of actin cytoskeletal dynamics.\(^7\) One recent work suggested that activation of mTORC2 contributes to the intrinsic axon growth capacity in adult sensory neurons after injury.\(^8\) Rictor is an essential and unique component of mTORC2 that directly activates the mTORC2 function.\(^9\) However, whether mTORC2 plays a role in the process of SCI and whether activation of mTORC2 promotes the axonal regeneration in the lesion after SCI remain unclear.

Autophagy is a lysosome-dependent catabolic pathway that functions to degrade the cytoplasmic proteins and organelles and is essential in maintaining the cellular homeostasis and protecting against SCI.\(^10\)\(^,\)\(^11\) However, autophagy dysfunction also contributes to neuronal cell death after SCI, which is due to the dysregulation of macrophage polarization.\(^12\) Accumulating evidence suggests that macrophages are one of the bridges connecting autophagy and immunity.\(^13\)\(^,\)\(^14\) The polarization of macrophages results in the acquisition of distinct functional phenotypes in response to different environmental stimuli.\(^15\) M1 macrophages represent a pro-inflammatory phenotype after SCI. These macrophages exhibit increased phagocytic and antigen-processing activity, as well as increased production of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), nitric oxide (NO), and reactive oxygen species (ROS), which cause dramatic damage to the injured spinal cord. Conversely, M2 macrophages are referred to as anti-inflammatory macrophages and exhibit decreased production of a number of pro-inflammatory molecules, thus promoting neurite outgrowth after SCI. Macrophages can convert between the M1/M2 phenotypes in response to varied microenvironments.\(^16\)\(^,\)\(^17\)

Evidence indicates that mTORC2 and its related pathways regulate the M2 macrophage differentiation and function in various pathophysiological responses\(^18\)\(^,\)\(^19\); however, so far, the precise role of the mTORC2 adaptor protein Rictor in macrophage differentiation and function after SCI has yet to be defined.

In this study, lentivirus encoding Rictor cDNA to induce Rictor overexpression was injected into the injured spinal cord of SCI rats to investigate the effects of this treatment on tissue repair in vivo. We then explored the anti-inflammatory effects of Rictor overexpression and the potential mechanisms underlying these effects. Our findings not only define a role for Rictor in the anti-inflammatory effect but also substantiate the critical role of Rictor in promoting neurogenesis and functional recovery in SCI. To this end, the present study suggests that Rictor promisingly represents a gene target to enhance the therapeutic process by facilitating the plasticity of neural circuits after SCI.

2 | MATERIALS AND METHODS

2.1 | Lentiviral vector construction

Based on our previous study,\(^22\) to overexpress Rictor, human Rictor cDNA (NM_001285439) was cloned into the lentiviral vector (lenti-Rictor) and constructed by GeneChem, Inc (Shanghai, China), a negative control gene lentiviral vector (lenti-control) was generated using a nontargeted cDNA sequence. The final lentiviral vector concentration was \(1 \times 10^9\) TU/mL.

2.2 | The rat model of SCI

All procedures performed on experimental animals were approved by the Animal Care and Use Committee of Sun Yat-sen University and were conducted in accordance with the Guide to the Care and Use of Experimental Animals by the National Research Council (1996, USA).

The number of animals in all studies was determined by power analysis (power of 0.9 with an alpha value of 0.05). A total of 120 adult female Sprague Dawley rats (weighing
200-220 g, supplied by the Experimental Animal Center of Sun Yat-sen University, China) were used in a randomized completely block design and were randomly assigned to three groups for this study: the sham group (n = 40), the Rictor overexpression group (lenti-Rictor, n = 40), and the control vector-treated group (lenti-control, n = 40). Briefly, the animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and kept on a heating pad to prevent hypothermia. Afterward, the spinal cord was exposed at the 10th thoracic vertebral level (T10) via laminectomy. The exposed cord was contused with an NYU weight-drop device by dropping a 10-g rod with a flat circular impact surface from a precalibrated height of 12.5 mm. After hemostasis during the operation, animals in the Rictor overexpression group received 2 µL of Rictor lentivirus (final titer of 1 × 10^9 IU/mL), which was slowly injected into the ipsilateral spinal cord at five locations 1 mm rostral to the lesion epicenter using a Hamilton syringe with a pulled glass micropipette, and animals in the control group received vehicle lentivirus at the same final titer.

The animals in the sham group underwent spinal cord exposure but no injury. The muscle and skin were sutured using 5-0 sutures, meloxicam (1 mg/kg) was given as an analgesia after the operation, and 1 mL of saline was intraperitoneally injected daily for 1 week to protect against dehydration. The bladders of the rats were manually expressed twice daily until urinary function was restored. The number of rats in each experiment is indicated in the figure legends.

2.3 | Functional assessment

The 22-point (0-21) Basso, Beattie, and Bresnahan (BBB) open-field locomotor test was used to assess the hindlimb locomotor function, including joint movement, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion, as observed in the sham rats. All animals underwent behavioral testing, and the duration of each session was 5 minutes per rat. Finally, an overall score was calculated.

2.4 | Spinal cord-evoked potential recording

Spinal cord-evoked potential (SCEP) recordings were used to evaluate the electrophysiological restoration of the injured spinal cord as described in our previous report (N = 10 per group). To obtain high-quality waveforms for the SCEP signals, one hundred SCEP responses were averaged for each rat. The evaluation was performed by observers who were blinded to the treatment group of the tested animals.

2.5 | Axonal tract tracing

After behavioral testing, the animals (N = 10 per group) were used for retrograde labeling of host axonal tracts as previously described. After anesthesia as described above, a dorsal laminectomy was performed at T12, and 0.5 µL of fluorogold (FG, Biotium, USA) was injected into the spinal cord using a Hamilton syringe. The animals were euthanized 1 week later by an overdose of xylazine and then, transcardially perfused with ice-cold 0.9% saline followed by ice-cold 4% PFA. The T8 segment of the spinal cord was removed, cryopreserved in graded sucrose solutions, and sliced into 20-µm frozen sections. The labeled cell bodies of neurons rostral to the lesion epicenter were observed via fluorescence microscopy (Olympus, Tokyo, Japan) to detect and count FG-labeled neurons in the ventricornu. The number of FG-labeled axons was checked by two independent examiners who were blinded to the treatment status of the rats.

2.6 | Histopathological analysis

At 8 weeks after SCI, all rats were deeply anesthetized with an overdose of xylazine and were transcardially perfused with 250 mL of 0.9% normal saline. Then, 1-cm samples of spinal cord tissue containing the lesion were promptly extracted from the animals and used for RT-PCR and Western blot analysis. Other animals were further perfused with 300 mL of 4% PFA (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The T8-T11 cord segments were dissected based on the dorsal spinal root count, postfixed overnight in 4% PFA, and soaked at 4°C overnight in 10% sucrose followed by 30% sucrose. The specimens were embedded in optimal cutting temperature (OCT) compound, frozen at −20°C, and sliced at a thickness of 20 µm in the longitudinal or transverse plane.

To visualize the cavity area, animals (N = 10 per group) were sacrificed for hematoxylin-eosin (HE) staining. The T8-T11 longitudinal and transverse spinal cord sections from each group were stained with HE according to standard protocols and observed under a brightfield microscope. For motor neuron counting, the anterior horn of the injured spinal cord in transverse sections (N = 10 per group) was used to stain neurons for NeuN. Ten sections at 1, 3, and 5 mm rostral and caudal to the lesion epicenter were counted for each rat. The numbers of positively stained cells were counted and averaged per section in a blinded manner.

2.7 | Evaluation of remyelination

Animals (N = 10 per group) were sacrificed for electron microscopy analysis as described previously. The spinal
cord tissues that contained the injury epicenter were fixed with glutaraldehyde and osmium tetroxide and then, embedded in Epon. Transverse semi-thin (1 mm) sections were generated and stained with toluidine blue (Sigma Aldrich, St. Louis, MO, USA). Ultrathin sections were sliced at 50 nm and double-stained with uranyl acetate and lead citrate. Myelinated axons were counted in five different fields from the dorsal column in the lesion epicenter for each sample. A minimum of five sections per rat was counted, and all analyses were performed under blinded conditions.

2.8 | Immunofluorescence

Tissue sections from rats (N = 10 per group) were fixed in 4% PFA for 30 minutes and permeabilized with 0.3% Triton X-100 for 30 minutes. Blocking was performed with 5% normal goat serum for 1 hour. The tissue sections and the cells were incubated overnight at 4°C in primary antibodies against the following antigens: active caspase-3 (1:100, Abcam, Cambridge, UK), iNOS (1:100, Abcam, Cambridge, UK), GFAP (1:100, CST, Danvers, MA, USA), and neurofilament 200 (NF200) (1:200, Sigma Aldrich, St. Louis, MO, USA). After washing three times with PBS, the primary antibodies were probed with Alexa Fluor 488 goat anti-rabbit (1:500, Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 594 goat anti-mouse (1:500, Invitrogen, Carlsbad, CA, USA) secondary antibodies for 1 hour at room temperature. Finally, the coverslips were washed with PBS three times and mounted using Prolong Gold Antifade Reagent containing 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). The targeted marker-positive cells in each visual field were counted under a fluorescence microscope (Carl Zeiss Axio Observer Z1, Oberkochen, Germany). Five randomly chosen fields from the lesion epicenter in each section and 10 sections from each group were examined.

2.9 | Western blot assay

Protein lysates were extracted from the animals at various times after SCI (N = 10 per group), and 1-cm samples of spinal cord tissue encompassing the lesion were perfused with normal saline and ground with a mortar and pestle. Afterward, the protein concentration was determined using a bicinechonic acid assay (Beyotime Biotechnology, Shanghai, China) and equalized before loading. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% Bis-Tris gel), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA), and blocked with 5% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA) for 1 hour followed by incubation with primary antibodies at 4°C overnight. The primary antibodies used at a 1:1000 dilution were Rictor (Abcam, Cambridge, UK), iNOS (Abcam, Cambridge, UK), Arginase-1 (Abcam, Cambridge, UK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (CST, Danvers, MA, USA). After washing in TBST (Tris-HCl-based buffer with 0.2% Tween 20, pH 7.5), the membrane was incubated with peroxidase-conjugated secondary antibody for 1 hour at room temperature, washed in TBST, and developed using an ECL system (Millipore, Bedford, USA).

2.10 | RNA extraction and real-time polymerase chain reaction

The mRNA expression was measured by real-time polymerase chain reaction (RT-PCR) as previously described. Total RNA from animals (N = 10 per group, tissues were separated from the homogenate prior to Western blot analysis) and cells (N = 10 per group, harvested at 3 days after transfection) was extracted using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using a reverse transcription system (Promega, Madison, WI, USA). RT-PCR was performed on an ABI 7900 PCR detection system using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Parallel amplification of the GAPDH gene was used to normalize the gene expression. The relative expression level of target mRNA was calculated using the ΔΔCt method. PCR primer sequences used in the study are listed in Table 1.

2.11 | TUNEL assay

Apoptotic cells in the lesion site were identified and quantified using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer’s instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche, Switzerland) as previously described (N = 10 per group). Five randomly chosen fields from the lesion epicenter in each section and 10 sections from each group were examined.

2.12 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc, USA). All data in the study were first tested for normality using the Shapiro-Wilk test, which revealed a normal distribution. All data are expressed as the means ± SD and were analyzed via one-way
ANOVA followed by Bonferroni post hoc tests for multiple comparisons or Student’s *t* test for pairwise comparisons. A value of *P* < .05 was considered to be statistically significant.

### 3 | RESULTS

#### 3.1 Rictor overexpression altered the inflammatory response and attenuated SCI-induced spinal cord apoptosis at the lesion site in the acute phase after SCI

We evaluated the efficacy of injection of the lentiviral vector encoding Rictor cDNA in the injured spinal cord by Western blotting. As shown in Figure 1A,B, the protein expression of Rictor increased after injection, peaked at 3 days after SCI, and then, steadily sustained the Rictor overexpression over time. Then, we determined the effect of Rictor overexpression on inflammatory activity after SCI. We used qRT-PCR to assess the expression of multiple inflammatory cytokines/chemokines (TNF-α, IL-1β, IL-6, and IFN-γ) in the injured spinal cord tissue at different time points after SCI (1, 3, 5, and 7 days). The expression of these cytokines increased immediately after SCI, and three of them (TNF-α, IL-1β, and IL-6) peaked at 1 day after SCI, while IFN-γ peaked at 3 days after SCI and then, reduced slowly (Figure 1C). Notably, in the Rictor-overexpression group, the expression of the abovementioned cytokines at any time point was significantly suppressed (Figure 1C, *P* < .05), indicating that Rictor overexpression attenuated the acute inflammatory response by immediately reducing the inflammatory cytokine infiltration after SCI and may have eventually contributed to the favorable microenvironment in the lesion. Furthermore, we evaluated apoptotic cells via activated caspase-3-positive cell staining and TUNEL staining in the lesion epicenter at Day 7 after SCI. As shown in Figure 2A, extensive activated caspase-3-positive cell staining at the injury site was observed. The number of activated caspase-3-positive cells in the lesion was significantly less in the Rictor-overexpression group (32.5 ± 3.4 cells/mm²) than in the control vector-treated group (61 ± 5.5 cells/mm²) (Figure 2B). Moreover, pronounced apoptotic cell death, as determined by TUNEL staining, was observed in the lesion of the injured spinal cord (Figure 2A), but interestingly, the percentage of TUNEL-positive cells in the Rictor overexpression group (27.4 ± 3.2% of control, *P* < .05) was significantly less than that in the control vector-treated group (Figure 2C). Taken together, these results clearly demonstrate that Rictor overexpression attenuated SCI-induced spinal cord apoptosis at the lesion site.

#### 3.2 Rictor overexpression reduced M1 macrophages and increased M2 macrophages at the lesion site in the acute phase after SCI and contributed to the persistence of M2 polarization in the injured spinal cord

To determine the effect of Rictor overexpression on the polarization of M1 and M2 macrophages in the injured spinal cord, macrophage phenotype was assessed at Day 7 after SCI. CD11b positivity is a typical macrophage marker, and iNOS and Arginase-1 are used to distinguish between M1 and M2 subpopulations, respectively, when the phagocytic response of macrophages reaches its maximum.21 Double immunofluorescence labeling of tissue sections in the Rictor overexpression and control groups showed that compared to the control group, the Rictor overexpression group had a significantly decreased the percentage of iNOS-expressing M1 macrophages (70.6% ± 6.8% vs 36.3% ± 5.6%; *P* < .05) and an increased proportion of Arginase-1-expressing M2 macrophages (29.4 ± 6.7% vs 63.7 ± 5.9%, *P* < .05) among the CD11b-positive cells (Figure 3A,B). Furthermore, the expression of iNOS and Arginase-1 at the lesion site was directly measured via Western blot analysis. As shown in Figure 3C, in the Rictor overexpression group, the levels of iNOS expression were significantly decreased, but the levels of Arginase-1 expression were significantly increased.

### Table 1 Real-time PCR primers used in the study

| Gene      | GenBank accession number | Forward primer 5′–3′                      | Reverse primer 5′–3′                      |
|-----------|--------------------------|------------------------------------------|------------------------------------------|
| TNF-α     | NM_012675.3              | CTCAAGCCCTGTTATGAGCC                     | GGCTGGTGTAAGAAACGGATG                    |
| IL-1β     | NM_031512.2              | GGCAACTGTCCTCCTGAAC                     | TCCACAGCCACAATGAG                       |
| IL-6      | NM_012589.2              | TCCTACCCCAACTTCCAATGC                   | TAGCACACTAGGTGTTTGCCAG                  |
| IFN-γ     | NM_138880.2              | CTGGCAAAGGAGCCTGAAC                     | TGGCTGGAATCGTGAGAAGG                    |
| iNOS      | S71597.1                 | TTGCTCTGTCCTAATGCGG                     | AAGGCGATGCTGAAACAGA                     |
| CD116     | NM_000569                | CCTCCTGTCATTGTCCGG                      | TCGAGCAACCTGTACCATTG                    |
| CD206     | NM_002438                | GGCTGCTATACCTCCTATGCC                   | TTTTCTGTCTGTGGCGCAGT                    |
| Arginase1 | NM_000045                | GGGAAACCTTGCGAGAACAC                    | AATCTGCCATCGGAGATC                      |
| GAPDH     | NM_014364                | CTACCCCGATGCACACATGT                   | CGCGTTCCTCACAAATGTC                    |
compared with those in the control vector-treated group. Moreover, we also investigated the mRNA expression of iNOS, CD16/32, arginase 1, and CD206 by real-time RT-PCR analysis. The results (Figure 3D) showed that Rictor overexpression induced a marked shift in expression of M1 macrophage marker mRNA (iNOS and CD16/32) (2.3-fold decrease vs control vector treatment, \( P < .05 \)) to M2 macrophage marker mRNA (arginase-1 and CD206) (2.4-fold increase vs control vector treatment, \( P < .05 \)). These findings suggest that Rictor overexpression contributes to promoting M2 macrophage polarization in the injured spinal cord.

### 3.3 Rictor overexpression enhanced residual motor neuron and oligodendrocyte survival surrounding the lesion, suppressed astrocytic scar formation, and promoted neurite outgrowth in the lesion

The death of neurons and oligodendrocytes occurs after SCI.\(^2\) We examined whether Rictor overexpression improves neuronal survival in the ventral horn in the injury epicenter (Figure 4A). As shown in Figure 4A,B, the number of NeuN-positive cells in the Rictor overexpression group was approximately three times higher than that in the control vector-treated group (25.1 ± 3.8 vs 9.3 ± 2.7; \( P < .01 \)). Subsequently, the marked improvement in neuronal survival prompted us to evaluate the survival of residual oligodendrocytes at the injured site. As expected, in the Rictor overexpression group, significantly more CNPase-positive oligodendrocytes were found than in the control vector-treated group (86.2 ± 4.7 vs 44.3 ± 5.1; \( P < .01 \)) (Figure 4A,C). These results suggest that Rictor overexpression enhanced residual motor neuron and oligodendrocyte survival around the lesion.

Moreover, the peripheral rim of the injured site is largely associated with hypertrophic glial cells and glial processes and the formation of a glial barrier to axonal regeneration.\(^2\) To assess the response of activated astrocytes after SCI, we examined the distribution of the glial filament marker GFAP at 7 days after SCI, which is associated with the characteristics of the astrocytic response. A strongly reactive region of GFAP staining was observed surrounding the lesion epicenter after SCI (Figure 4D). Fortunately, GFAP staining intensity in the Rictor overexpression group was significantly lower than that in the control group (Figure 4E, \( P < .01 \)), which suggests that Rictor overexpression suppressed astrocytic scar formation around the lesion center.

Furthermore, staining for NF200, an axonal marker, showed neurofilament outgrowth of the host tissue across the injury site at 8 weeks after SCI. NF200 density (nerve fiber...
growth) in the injured spinal cord was markedly weaker than that in the sham group (Figure 4D). However, as shown in Figure 4F, NF200 density in the Rictor overexpression group significantly increased compared to that in the control group (P < .01). These results indicate that Rictor overexpression was conducive to neurogenesis after SCI.

### 3.4 Rictor overexpression enhanced remyelination and neural circuit plasticity across the lesion center

To explore whether Rictor overexpression promotes spinal cord remyelination, we detected the myelination level using toluidine blue staining. Loss of myelin in the injured axon was observed at the lesion epicenter, whereas more myelinated and/or remyelinated axons were visualized in the Rictor overexpression group (Figure 5A). As shown in Figure 5D, the percentage of myelinated axons was markedly increased in the Rictor overexpression group compared to that in the control vector-treated group (P < .01). Furthermore, through electron microscopy analysis (Figure 5B), we detected that the lamellae of the myelin sheath were obviously separated from each other in the SCI groups. However, both the number and the percentage of demyelinated sheaths in the Rictor overexpression group were markedly decreased compared to those in the control group (Figure 5E, P < .01).
In rat SCI models, the extent of locomotor recovery correlates closely with the cross-sectional area of residual myelinated fiber tracts present in the injured site through the lesion epicenter. Therefore, we examined whether the remyelinated axons contributed to neural circuit plasticity via retrograde tracing using FG. In the sham animals, nearly all neurons in the spinal cord at the T8 level were labeled with FG at 8 weeks after SCI. In contrast, only a small percentage of neurons in the spinal cord at the T8 level were FG-positive in the SCI groups (Figure 5C). Notably, as shown in Figure 5F, the number of FG-labeled neurons in the Rictor overexpression group was approximately fourfold higher than that in the control vector-treated group (P < .01). Together, these findings suggest that Rictor overexpression promoted remyelination and neural circuit plasticity during the repair process.

3.5 | Rictor overexpression reduced the lesion volume in the injured spinal cord and enhanced functional recovery after SCI

The size of the lesion cavity was calculated in HE-stained sections at 8 weeks postinjury to detect tissue repair. In rats that were treated with lentivirus encoding Rictor overexpression, the lesion volume in either the sagittal or transverse plane was significantly decreased compared to that in the control vector-treated group (Figure 6A). The total...
cavity area in the Rictor overexpression group was approximately 45% smaller in the sagittal plane or 50% smaller in the transverse plane than in the control group (Figure 6B, \( P < .01 \)).

To determine whether Rictor overexpression in the injured spinal cord after SCI produces beneficial effects, behavioral tests of locomotor function (BBB scores) were conducted to assess the therapeutic effect. As shown in Figure 6C, the day after SCI, all but the sham rats received a score of 0, confirming the association of hindlimb paralysis with acute trauma. BBB scores in the SCI groups were significantly below those of the sham group, with no significant difference within the
first week after surgery. However, the Rictor overexpression group had a significant increase in BBB scores compared with those of the control vector-treated group after one week, and the difference between the two groups became increasingly outstanding from the second to the eighth week ($P < .05$). Furthermore, we verified whether the improvement in locomotor function was associated with the electrophysiological restoration of SCEP responses. At the end of the eighth week, the SCEPs in the control vector-treated and Rictor overexpression groups were both weak, the SCEP latencies were prolonged, and the SCEP amplitudes were significantly decreased compared to those in the sham group (Figure 6D,E). However, in the Rictor overexpression group, the SCEP latencies were significantly shorter, and the SCEP amplitudes were notably higher than those in the control vector-treated group (Figure 6D,E, $P < .05$). These results demonstrate that Rictor overexpression promoted locomotor function and neurological recovery after SCI.

### DISCUSSION

Our current work was the first to identify Rictor as a novel molecular target that is responsible for improving the functional recovery after SCI. Overexpression of Rictor in the injured spinal cord alleviated inflammation-induced cell death and promoted macrophage polarization to the M2 phenotype and the SCI neural repair process. Therefore, Rictor overexpression promisingly represents an effective strategy to enhance the therapeutic process for SCI in the future.

Pro-inflammatory cytokines (TNF-α and IL-1β) are immediately released during the earliest stage of SCI, subsequently increasing the expression of inflammatory factors such as IL-6 and IFNγ and inducing the recruitment of neutrophils and monocytes/macrophages into the lesion, causing secondary damage, which induces apoptosis of neuronal cells in the injured spinal cord. The use of either an anti-TNF-α antibody or IL-1 receptor antagonist significantly alleviates the inflammatory response and cell death after SCI. Rictor suppresses the response by inhibiting the inflammatory factor (TNF-α, IL-6, and MIP1) expression. Consistently, our study showed that Rictor overexpression downregulated the expression of other pro-inflammatory cytokines (IL-1β and IL-6) and contributed to cell survival in the injured spinal cord.

Accumulated evidence has demonstrated that macrophage activation in response to inflammatory stimulation participates in the process of SCI. Activated macrophages have either detrimental or beneficial effects on neural regeneration after SCI based on their M1/M2 polarization. M1 macrophages within the epicenter of the injured site are induced by pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, exacerbate inflammatory responses and bring about a hostile environment, which leads to further devastating neuronal cell death. In contrast, M2 macrophages suppress the excessive inflammatory response by producing anti-inflammatory cytokines, including IL-14, IL-10, and IL-13, during acute inflammation. Previous studies have reported that infiltration of macrophages and microglial cells peaks at approximately 7 days postinjury. In agreement with previous findings, the decreased expression of pro-inflammatory cytokines caused by Rictor overexpression in this study contributed to a notable decrease in the number of M1 (iNOS/CD11b) macrophages and a marked increase in the number of M2 (Arginase-1/CD11b) macrophages at 7 days postinjury. Therefore, regulation of macrophage differentiation into an M1 or M2 phenotype in the microenvironment determines whether infiltrating macrophages exacerbate secondary injury or promote tissue repair.

However, previous studies have demonstrated that Rictor/mTORC2 promotes M2 macrophage activation in other diseases, such as infection, kidney fibrosis, and tumor growth. Fortunately, our study for the first time shows that Rictor overexpression promotes M2 macrophage polarization around the lesion after SCI. Moreover, further investigations are required to explore the underlying molecular mechanism by which Rictor regulates macrophage polarization to strengthen our primary findings.

M2 macrophage activation facilitates spinal cord repair in SCI, which is associated with the promotion of neuronal survival. Integrating the previous findings, the current observations that Rictor overexpression reduced apoptosis in
FIGURE 5  Rictor overexpression promoted the plasticity of neural circuits in the lesion site
8 wk after SCI. A, Sections of toluidine blue staining showed myelinated and demyelinated axons in the sham, lenti-control-treated and lenti-Rictor-treated groups, respectively (scale bar = 20 um). B, Electron microscopic visualization of viable demyelinated axons in the sham, lenti-control- and lenti-Rictor-treated groups (red arrows point loose and separated layers of damaged myelin sheaths, scale bar = 1um). C, Immunohistochemistry showing Fluorogold-labeled neurons in the T8 segment spinal cord, which indicates that neurogenesis is capable of bridging the lesion epicenter (Scale bar = 50 mm). D, Quantification of toluidine blue-stained sections, **P < .01 vs the lenti-control-treated group. E, Quantification of the relative density of viable myelinated sheaths in the lenti-Rictor-treated group vs the lenti-control-treated group. ***P < .01 vs the lenti-control-treated group. F, Quantification of the relative rate of FG labeled neurons in different groups. Lenti-control: the control vector treated, lenti-Rictor: Rictor overexpression treated. n = 10 rats/group, *P < .05 vs the sham group, comparison of the data between lenti-control-treated and lenti-Rictor-treated groups. **P < .01
host neurons and oligodendrocytes in the injured lesion may benefit the neurite outgrowth and synaptic plasticity after traumatic SCI.\textsuperscript{38}

Glial scars are formed by reactive astrocytes and are widely regarded to inhibit neurite outgrowth and hinder outcomes after SCI. The microenvironment around the injured site induces glial proliferation, which further drives reactive gliosis.\textsuperscript{39} It is important to note that Rictor inactivation transforms quiescent astrocytes into reactive astrocytes by upregulating glial glutamate transporter 1 (GLT-1).
expression. In contrast, our current study showed markedly increased the neurofilament outgrowth (NF200 density) and decreased the glial scar formation (GFAP density) due to Rictor overexpression. This suggests that Rictor overexpression inhibits astrocyte reactivity and accounts for these intriguing contributions to neurite outgrowth after SCI.

The observation of neurite outgrowth motivated us to explore axonal regeneration after SCI. Axonal dieback emerges at early stages; therefore, axonal regeneration is severely inhibited after SCI. A previous study on the therapeutic potency of Rictor in rodent models of peripheral nervous system (PNS) injury associated axonal regeneration with dorsal root ganglion (DRG) neuron preservation, which encouraged us to explore Rictor-mediated promotion of axonal regeneration in CNS injury. Although axonal regrowth and re-establishment of connections are thus hindered, in our work, we found that the Rictor overexpression group had significantly enhanced axonal regeneration via toluidine blue staining, electron microscopy analysis, and FG retrograde tracing, despite the complexity of the environment in vivo. Moreover, our results first underscore the importance of Rictor overexpression in axonal regeneration post-SCI, presumably by contributing to the restoration of conductivity in spared and/or regrown axons, restructuring the neural circuits.

Effective tissue repair of the injured spinal cord is closely related to functional recovery after SCI. The motor recovery analysis using BBB scores in SCI model rats clearly show that an increased score reflects a biologically significant change. In the current study, we observed a more significant increase in BBB scores in Rictor-overexpressing rats, supporting the importance of Rictor overexpression in the recovery of locomotion after traumatic SCI. We hypothesize that the difference between these two points in the context of a human patient suffering from SCI might translate into notable differences in quality of life and would greatly reduce the possibility of comorbidities associated with injury severity. Second, SCEP is a sensitive indicator of spinal cord biophysical activity and highly correlates with the restoration of neuronal pathways in rats with transected spinal cords. The increased amplitude of SCEP in Rictor-overexpressing rats in the spinal cord suggests that Rictor overexpression significantly improved the functional status of motor and sensory axonal conduction. As confirmed by HE staining, Rictor overexpression significantly reduced the lesion volume, which directly reflected tissue repair and contributed to functional recovery after SCI.

Based on our current findings, we demonstrated for the first time that Rictor overexpression is an effective strategy for SCI repair. More importantly, the underlying mechanism might be associated with Rictor overexpression alleviating the inflammatory response, driving macrophages toward M2 polarization and enhancing neurogenesis after SCI. However, it is well known that endogenous neural stem cells play an indispensable role in the repair process following SCI, such as migration to the lesion site and multilineage differentiation into neurons, oligodendrocytes and astrocytes for SCI repair. Interestingly, our parallel research has found that Rictor overexpression activates ENSC migration and neuronal differentiation in vitro (data not shown). Thus, the exact molecular mechanism underlying the effect of Rictor overexpression on functional recovery after SCI does not remain entirely understood. Further research focused on the molecular mechanism by which Rictor overexpression promotes the functional recovery in SCI is required.

In summary, our work provides strong evidence that Rictor overexpression in the injured spinal cord facilitates neurogenesis and functional recovery after SCI and suggests an attractive strategy for the repair of SCI.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Ningning Chen designed and performed experiments, analyzed data, generated figures, and wrote the manuscript; Pengxiang Zhou and Jiachun Li performed experiments, analyzed data, helped generate figures, and commented on the manuscript; Xizhe Liu performed the additional experiments, analyzed data, helped generate figures; Yong Wan revised the manuscript; Shaoyu Liu contributed key experimental reagents and commented on the manuscript; and Fuxin Wei designed experiments, contributed to writing the manuscript, and had final approval of the manuscript.

REFERENCE

1. New PW, Lee BB, Cripps R, Vogel LC, Scheinberg A, Waugh MC. Global mapping for the epidemiology of paediatric spinal cord damage: towards a living data repository. Spinal Cord. 2019;57:183-197.
2. Alkabie S, Boileau AJ. The role of therapeutic hypothermia after traumatic spinal cord injury—a systematic review. World Neurosurg. 2016;86:432-449.
3. Tran AP, Warren PM, Silver J. The biology of regeneration failure and success after spinal cord injury. Physiol Rev. 2018;98:881-917.
4. Silva NA, Sousa N, Reis RL, Salgado AJ. From basics to clinical: a comprehensive review on spinal cord injury. Prog Neurobiol. 2014;114:25-57.
5. Liu GY, Sabatini DM. mTOR at the nexus of nutrition, growth, ageing and disease. Nat Rev Mol Cell Biol. 2020. https://doi.org/10.1038/s41580-019-0199-y.
6. Chen HC, Fong TH, Hsu PW, Chiu WT. Multifaceted effects of rapamycin on functional recovery after spinal cord injury in rats through autophagy promotion, anti-inflammation, and neuroprotection. J Surg Res. 2013;179:e203-e210.
7. Goldshmit Y, Kanner S, Zacs M, et al. Rapamycin increases neuronal survival, reduces inflammation and astrocyte proliferation after spinal cord injury. Mol Cell Neurosci. 2015;68:82-91.
8. Sekiguchi A, Kanno H, Ozawa H, Yamaya S, Itoi E. Rapamycin promotes autophagy and reduces neural tissue damage and locomotor impairment after spinal cord injury in mice. *J Neurotrauma*. 2012;29:946-956.

9. Thomanetz V, Angliker N, Cłoetta D, et al. Ablation of the mTORC2 component rictor in brain or Purkinje cells affects size and neuron morphology. *J Cell Biol*. 2013;201:293-308.

10. Chen W, Lu N, Ding Y, et al. Rapamycin-resistant mTOR activity is required for sensory axon regeneration induced by a conditioning lesion. *eNeuro* 2016;3:0358-16.

11. Benavides-Serrato A, Lee J, Holmes E, et al. Specific blockade of Rictor-mTOR association inhibits mTORC2 activity and is cytotoxic in glioblastoma. *PLoS ONE*. 2017;12:e0176599.

12. Liu S, Sarkar C, Dinizo M, et al. Disrupted autophagy after spinal cord injury is associated with ER stress and neuronal cell death. *Cell Death Dis*. 2015;6:e1582.

13. Saraswat Ohri S, Bankston AN, Mullins SA, et al. Blocking autophagy in oligodendrocytes limits functional recovery after spinal cord injury. *J Neurosci*. 2018;38:5900-5912.

14. Zhou T, Zheng Y, Sun L, et al. Microvascular endothelial cells engulf myelin debris and promote macrophage recruitment and fibrosis after neural injury. *Nat Neurosci*. 2019;22:421-435.

15. Clarke AJ, Simon AK. Autophagy in the renewal, differentiation and homeostasis of immune cells. *Nat Rev Immunol*. 2019;19:170-183.

16. Shibutani ST, Saitoh T, Nowag H, Munz C, Yoshimori T. Autophagy and autophagy-related proteins in the immune system. *Nat Immunol*. 2015;16:1014-1024.

17. Gensel JC, Kopper TJ, Zhang B, Orr MB, Bailey WM. Predictive screening of M1 and M2 macrophages reveals the immunomodulatory effectiveness of post spinal cord injury azithromycin treatment. *Sci Rep*. 2017;7:40144.

18. Ma SF, Chen YJ, Zhang JX, et al. Adoptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury. *Brain Behav Immun*. 2015;45:157-170.

19. Kobashi S, Terashima T, Katagi M, et al. Transplantation of M2-deviated microglia promotes recovery of motor function after spinal cord injury in mice. *Mol Ther*. 2019.

20. Byles V, Covarrubias AJ, Ben-Sahra I, et al. The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun*. 2013;4:2834.

21. Hallowell RW, Collins SL, Craig JM, et al. mTORC2 signaling regulates M2 macrophage differentiation in response to helminth infection and adaptive thermogenesis. *Nat Commun*. 2017;8:14208.

22. Chen N, Cen JS, Wang J, et al. Targeted inhibition of leucine-rich repeat and immunoglobulin domain-containing protein 1 in transplanted neural stem cells promotes neuronal differentiation and functional recovery in rats subjected to spinal cord injury. *Crit Care Med*. 2016;44:e146-157.

23. Maybhate A, Hu C, Bazley FA, et al. Potential long-term benefits of acute hypothermia after spinal cord injury: assessments with somatosensory-evoked potentials. *Crit Care Med*. 2012;40:573-579.

24. Menezes K, de Menezes JR, Nascimento MA, Santos Rde S, Coelho-Sampaio T. Polyaminin, a polymeric form of laminin, promotes regeneration after spinal cord injury. *FASEB J*. 2010;24:4513-4522.

25. Wu HF, Cen JS, Zhong Q, et al. The promotion of functional recovery and nerve regeneration after spinal cord injury by lentiviral vectors encoding Lingo-1 shRNA delivered by Pluronic F-127. *Biomaterials*. 2013;34:1686-1700.

26. Pineau I, Lacroix S. Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J Comp Neurol*. 2007;500:267-285.

27. Esposito E, Cuzzocrea S. Anti-TNF therapy in the injured spinal cord. *Trends Pharmacol Sci*. 2011;32:107-115.

28. Nesci O, Xu GY, McAdoo D, High KW, Hulsebosch C, Perez-Pol R. IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. *J Neurotrauma*. 2001;18:947-956.

29. Bhattacharya I, Dragert K, Albert V, et al. Rictor in perivascular adipose tissue controls vascular function by regulating inflammatory molecule expression. *Arterioscler Thromb Vasc Biol*. 2013;33:2105-2111.

30. Kim JH, Kim JY, Mun CH, Suh M, Lee JE. Agmatine modulates the phenotype of macrophage acute phase after spinal cord injury in rats. *Exp Neurobiol*. 2017;26:278-286.

31. Yao A, Liu F, Chen K, et al. Programmed death 1 deficiency induces the polarization of macrophages/microglia to the M1 phenotype after spinal cord injury in mice. *Neurotherapeutics*. 2014;11:636-650.

32. Ji XC, Dang YY, Gao HY, et al. Local injection of Lenti-BDNF at the lesion site promotes M2 macrophage polarization and inhibits inflammatory response after spinal cord injury in mice. *Cell Mol Neurobiol*. 2015;35:881-890.

33. Wang C, Zhang L, Ndung JC, et al. Progranulin deficiency exacerbates spinal cord injury by promoting neuroinflammation and cell apoptosis in mice. *J Neuroinflammation*. 2019;16:238.

34. Oh MH, Collins SL, Sun IH, et al. mTORC2 signaling selectively regulates the generation and function of tissue-resident peritoneal macrophages. *Cell Rep*. 2017;20:2439-2454.

35. Ren J, Li J, Feng Y, et al. Rictor/mammalian target of rapamycin complex 2 promotes macrophage activation and kidney fibrosis. *J Pathol*. 2017;242:488-499.

36. Shrivastava R, Asif M, Singh V, et al. M2 polarization of macrophages by Oncostatin M in hypoxic tumor microenvironment is mediated by mTORC2 and promotes tumor growth and metastasis. *Cytokine*. 2019;118:130-143.

37. Gaudet AD, Mandrekar-Colucci S, Hall JC, et al. miR-155 deletion in an injury model of subarachnoid hemorrhage affects tissue-resident peritoneal macrophages. *Arterioscler Thromb Vasc Biol*. 2019;40:6997-7008.

38. Hallowell RW, Collins SL, Craig JM, et al. mTORC2 signaling regulates the generation and function of tissue-resident peritoneal macrophages. *Cell Rep*. 2017;20:2439-2454.

39. Ji XC, Dang YY, Gao HY, et al. Local injection of Lenti-BDNF at the lesion site promotes M2 macrophage polarization and inhibits inflammatory response after spinal cord injury in mice. *Cell Mol Neurobiol*. 2015;35:881-890.

40. Jing YF, Zhou L, Xie YJ, et al. Upregulation of glutamate transporter GLT-1 by mTOR-Akt-NF-κB cascade in astrocytic oxygen-glucose deprivation. *Glia*. 2013;61:1959-1975.
41. Hesp ZC, Goldstein EZ, Miranda CJ, Kaspar BK, McTigue DM. Chronic oligodendrogenesis and remyelination after spinal cord injury in mice and rats. *J Neurosci*. 2015;35:1274-1290.

42. Ni S, Luo Z, Jiang L, et al. UTX/KDM6A deletion promotes recovery of spinal cord injury by epigenetically regulating vascular regeneration. *Mol Ther*. 2019;27:2134-2146.

43. Stenudd M, Sabelstrom H, Frisen J. Role of endogenous neural stem cells in spinal cord injury and repair. *JAMA Neurol*. 2015;72:235-237.

**How to cite this article:** Chen N, Zhou P, Liu X, et al. Overexpression of Rictor in the injured spinal cord promotes functional recovery in a rat model of spinal cord injury. *The FASEB Journal*. 2020;34:6984–6998. [https://doi.org/10.1096/fj.201903171R](https://doi.org/10.1096/fj.201903171R)