Int J Clin Exp Pathol 2016;9(2):481-495
www.ijcep.com /ISSN:1936-2625/IJCEP0017471

Original Article

Effect of proliferation of neurons and astrocytes is regulated by glyoxylase 1

Mu He[1*], Jia Liu[2*], Zhao Qi[2], Zhao wei[2], Rang Liu[3], Wei Wang[3], Yang Xu[3], Chao You[1], Ting-Hua Wang[2,3]

[1]Department of Neurosurgery, Translational Neuroscience Center, State Key Lab of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China; [2]Institute of Neuroscience, Kunming Medical University, Kunming 650031, China; [3]Institute of Neurological Diseases, Translational Neuroscience Center, State Key Lab of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China. [*]Equal contributors.

Received October 8, 2015; Accepted November 20, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Spinal cord injury (SCI), one of the highest disability diseases, has well been documented in the world. However, the complicated mechanism underlying SCI is waiting to be elusive. Here, we established spinal cord transection (SCT, T10) model, and confirmed that glyoxylase 1 (Glo-1) was extensively downregulated in injured spinal cord by 2-DE, PDQuest analysis and western blotting. Moreover, to address the role of Glo-1 in injured spinal cord, we constructed HIV-Vector carrying shRNA to knockdown Glo-1 level. In C6 cell line, knockdown of Glo-1 could increase the cell viability, which indicated by MTT assay. Knockdown of Glo-1 not only decreased the cell size and neurite length of astrocytes, but also increased that of neurons. These findings therefore indicated that Glo-1, as a crucial neuroprotective factor, played a crucial role in improving proliferation in neurons and astrocytes. Our finding therefore paved a new strategy which suggested that Glo-1 might be a target naive protein for the treatment of SCI.

Keywords: Glyoxylase 1, spinal cord transection, knockdown, neurons and astrocytes

Introduction

Following the spinal cord injury, in the most of cases, the patients are faced to problematic neurological status, which mostly can cause the palsy of the limbs. And injured spinal cord shows little capacity for functional recovery involving regeneration of axonal protrusions. Results of some experiments which have been gained by recruit of stem cells, nerve and spinal cord segments, growth factors and scaffolds show that the regeneration of spinal cord fibers across the spinal cord lesion is probable. Meanwhile, it may provide new therapeutic measures [1-5].

But for the repair of spinal cord injury, currently the biggest problem is whether the regeneration of nerve fibers and recycled fibers could construct the new neural connections around the site of injury. The clinical use of neurotrophic factors is difficult due to side effects and elevated costs, but other molecules might be effective and more easily obtained [6]. So we revealed that the regeneration of injured spinal cord associated genes can be categorized into several groups, containing cytoskeletal proteins, neurotransmitter metabolizing enzymes, neuropeptides, growth factors, and signal transduction molecules in the spinal cord, as well as genes encoding proteins involved in inflammation, proliferation, and myelination in the spinal cord by proteomic analysis of the protein expression.

Another problem is that the recovery of spinal cord is interrelated to both neurons and astrocytes after injury. Previously studies indicated that astrocytes were considered as adverse effects on recovery of the injured spinal cord. But now some new results showed that reactive astrocytes provide essential activities that protect tissue and preserve function after mild or moderate SCI [7]. Astrocytes play a role in inflammation and one previous study revealed that tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death [8]. So we hypothesize that after injury, the recovery of spinal cord is relative to spinal cord neurons and

astrocytes by some molecules like GLO1, PKA and so on.

In the present study, using two-dimensional gel electrophoresis, we analyzed and identified the discrepancy protein expression profiles in spinal cord rostral to the injury. So we pay our attention to these proteins and the relationship between function and effect.

Method

Animals and animal feed

Twenty five adult female Sprague-Dawley (SD) rats, aging approximate 3 months and weighing 220-250 g, were provided by Animal Experimental Center of Si Chuan University. All animals were housed in clean cage and maintained on a 12 h on-12 h off light-dark cycle. Ambient temperature was kept at 25±2°C and humidity was kept at 55% to 70%. Food and water were given and ad libitum.

Surgery and spinal cord injury

All rats, randomly divided into two groups: Sham group and SCT group, were anaesthetized by intraperitoneal injection of 3.6% chloral-hydrate (36 mg/100 g). All rats were fixed on the operating table in the prone position, and the hair on the back area was shaved. A longitudinal incision was made through the back skin to expose the underlying musculature and the T9-T11 vertebrae identified. Rats in Sham group received only laminutesectomy; however, the cord was transected with iridectomy scissors at T10 in the other groups. The muscle and skin were sutured in layers with 3-0 silk. Then all animals were warmed, and when they became active, returned to the cages distinguished into two groups. All rats received an intraperitoneal injection of penicillin (12.5 mg/200 g) for two days after operation. Manual emptying of the bladders was performed third daily for a week, then two times per one day till sample harvesting in SCT group containing 3dpo, 14dpo, 28dpo, five rats in each group [9].

Two-dimensional (2D) gel electrophoresis

Proteins were precipitated by using the 2D Kit, and resuspended a mixture of 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 20 μg/ml DNase, 50 μg/ml RNase and $MnCl_2$ (isovolumetric with DNase). The computer analysis of the 2-DE image was carried out by using PDQUEST 7.40 (Bio-Rad) software. Samples of spinal cord weighing 200 mg were left on ice for 15 minutes followed by centrifugation at 4°C. The samples were centrifuged for 30 minutes at 12,000 r/min. The supernatant was discarded and protein content was measured by the Bradford Coomassie blue colorimetric assay. Samples were allowed to keep at room temperature for about 1 hour with DTT (0.02 g/ml) and Bio-lyte (0.0025 μl/ml), centrifuged for 10 minutes at 25,000 r/min. For overnight in-gel rehydration into IPG strips (18 cm, pH 3-10 NL) (BioRad, Hercules, USA), each lysate containing 150 μg protein was dissolved in 315 μl rehydration buffer (7 M urea, 2 M thiourea, 0.4% DTT, 4% CHAPS, 0.5% Bio-Lyte 3/10 ampholytes (BioRad, Hercules, USA), 0.001% bromphenol blue). Focusing was performed using the IEF-100 Isoelectric Focusing Unit (Hofer, Holliston, USA) to a total of 50000 Vh (50 V low 0.5 h, 250 V low 1 h, 500 V rapid 1.5 h, 1000 V rapid 1 h, 4000 V linear 3 h, 9000 V linear 3 h, 9000 V rapid 50000 Vh, 500 V rapid 30 minutes.). After isoelectric focusing, strips were equilibrated in 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl (pH 8.8) supplemented with 3% DTT for 15 minutes, and then with 2.5% iodoacetamide for additional 15 minutes. For second-dimension electrophoresis, IPG strips were subjected to SDS-PAGE using 12% polyacrylamide gels, until the dye front reached the end of the gel. The gels were stained by using CBB G-250. Only those spots that changed consistently and significantly (more than 1.5-fold) were selected by TOF/TOFTM analysis. Each of the paired samples was run three times to ensure the consistency of the data at 2-DE analysis [10].

In-gel tryptic digestion

Visual inspection of the gels was done to choose the best from the triplicates based on the highest image resolution and number of spots. The gel spots were destained twice with 0.1 ml of 50 mM NH_4HCO_3, and 50% acetonitrile in 20 minutes and dehydrated in 100% acetonitrile in 10 minutes. After complete destaining, gels were washed twice and shrunk by dehydration in ACN. Then the samples were puffed in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/ml trypsin at 48°C. After 30 minutes incubation, the gels were digested overnight at 37°C. Peptides were then extracted twice using 0.1% TFA in

Table 1. Proteomic identification information of rostral spinal cord at sham and 28dpo group

Name	ID	pI/MW (obversed)	pI/MW (calculated)	Score	Coverage
Glyoxylase 1	gi | 46485429	5.12/20806	5.15/24100	306	63%

Table 2. Information of antibodies

Antibody	Catalog	Supplier	Host	Application
NEUN	Ab104225	ABcom	Rabbit	1:50
GFAP	50	imunostar	Rabbit	1:100
GLO-1	6778-1	Epitmic	Rabbit	1:50
Cy3	17	Jackson	Goat anti-rabbit	1:200
Alexa 488	A11006	invitrogen	Goat anti-rat	1:100

50% ACN. At last mixtures of the peptide were redissolved in 0.8 μl of matrix solution which was a mixture of α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) in 0.1% TFA, and 50% CAN [11].

Mass spectrum and database search

MS analysis and protein identification were performed as reported previously [12, 13]. Spots with relatively higher intensities on 2-DE gels were excised, dried, analyzed on a 4700 proteomics analyzer (Applied Bio systems, Foster City, CA, USA). Data interpretation of acquired MS and MS/MS peptide spectra was carried out with Data Explorer™ software version 4.5 (Applied Bio systems) in default mode. The generated MS and MS/MS spectra were subsequently submitted to MASCOT (version 2.1, Matrix Science, London, UK) by GPS Explorer software (version 3.6, Applied Bio systems). On the basis of peptide-mass matching, peptide peaks obtained from mass mapping were identified using the NCBI database. All spots were examined by methionine oxidation, for iodoacetamide modification, and, with no-limitation, for pI. The confidence of identification was indicated by the number of matching peptides, and the percent of the acid-sequence coverage. Proteins were accepted on the basis of four or more peptides having matched.

PCR

The primer sequences and the expected sizes of PCR products were as follows: GLO1, sense 5'-CAGCGTCCAGTGGTCTT-3' and antisense 5'-CTGCGTCTCGTCATCTTC-3'; Total RNA was extracted using TRIzol reagent (Invitrogen), and RT-PCR was performed with conditions as follows: reverse transcription was done with the kit instruction (ferments). Denaturation at 95°C for 3 minutes; then amplification for 45 cycles at 95°C for 0.5 minutes, annealing at 52°C 0.5 minutes, and extension at 60°C for 0.5 minutes; and then a terminutesal elongation step at 60°C for 10 minutes and a final holding stage at 4°C. The PCR products were analyzed by electrophoresis through 1% agarose gel and visualized by GoldView with Alpha Innotech (Bio-Rad). For real-time PCR, the primer sequences described as follows: GLO1, sense 5'-TCAAGTTTGTGAAGAAGCCT-3'; antisense 5'-GTTGCCATTTTGTTAGGATT-3'; TM: 5'FAM-CTCCTCCTTCTTCTTGATGCC-TAMARA3'. PCR was performed in a DNA thermal cycler (ABI 7300) according to the standard protocol as the same as the RT-PCR.

Western blot analysis

Equal amounts of protein lysates (RIPA lysis buffer, Beyotime, Jiangsu, China) were separated by one dimensional SDS-PAGE and blotted onto PVDF membranes. Protein detection was performed by incubation with respective antibodies (**Table 2**), and proteins were visualized in Alpha Innotech (Bio-Rad) with ECL.

Immunofluorescence staining

The samples were processed for double immunofluorescence staining to find out whether GLO1 were co-localized with neurons or astrocytes. The free-floating sections of the medulla oblongata were simultaneously incubated with two primary antibodies, rat anti-Neun (ZSGB-BIO, 1:50)/rat anti-GFAP (ABcom, 1:100) or rabbit anti-GLO1 (EPITOMICS, 1:100). The same sections were concurrently incubated with two appropriate secondary antibodies, goat anti-rabbit IgG conjugated with Cy3 (1:200) for GLO1, and goat anti-rat IgG conjugated with Alexa 488 (1:100) for Neun or GFAP. Counterstaining was performed with DAPI (1:1,000) (Invitrogen) in PBS, and sections were coverslipped which were viewed under the laser scanning confocal microscope (Leica Microsystems, Germany) [14, 15].

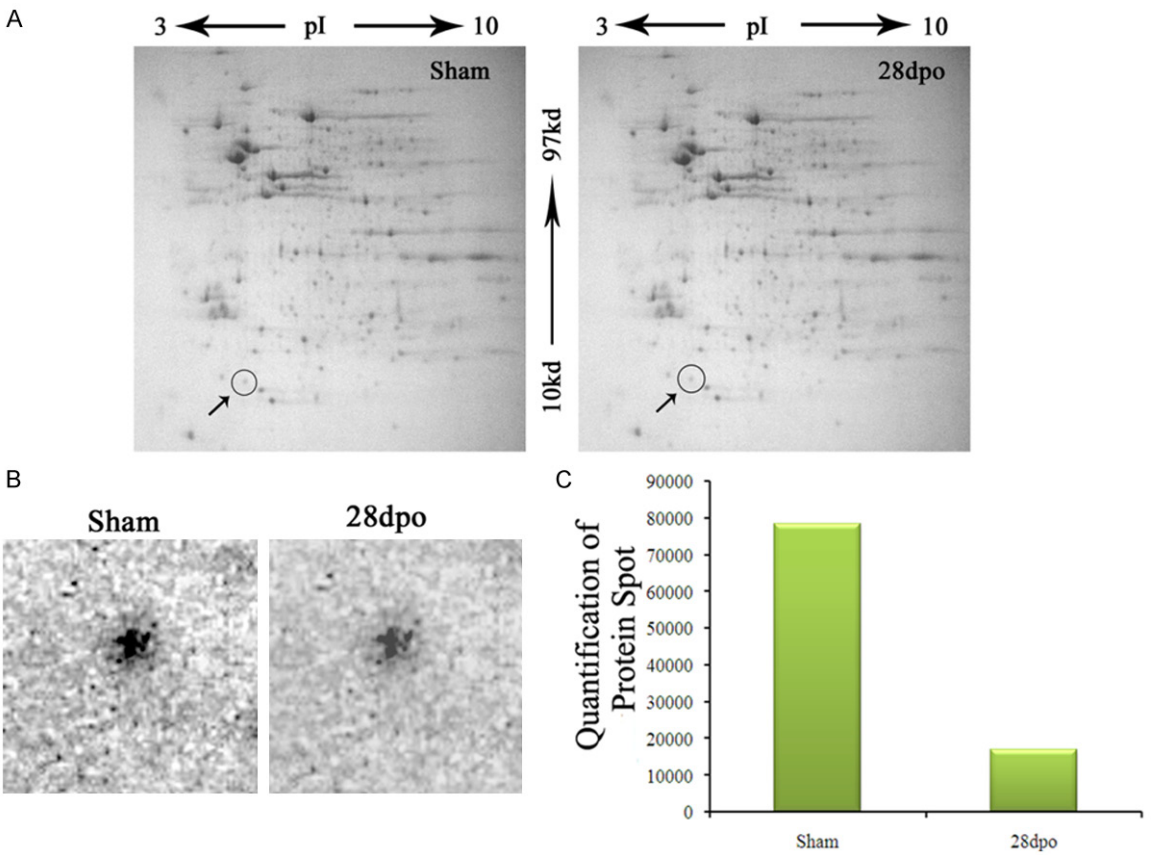


Figure 1. 2D protein profiles of sham group and 28dpo group of rostral spinal cord lesion from SCT rats. A. Localization of identified proteins on two-dimension electrophoresis (2-DE) gel. B. The up-related protein spot was amplified. C. Quantification of protein spot in sham group and 28dpo group SCT rats. Proteins were resolved by 2D-PAGE electrophoresis and stained with Coomassie Blue. Two parallel experiments were run. Horizontal axes, isoelectric points (pI); vertical axes, molecular masses (kDa).

Cell culture

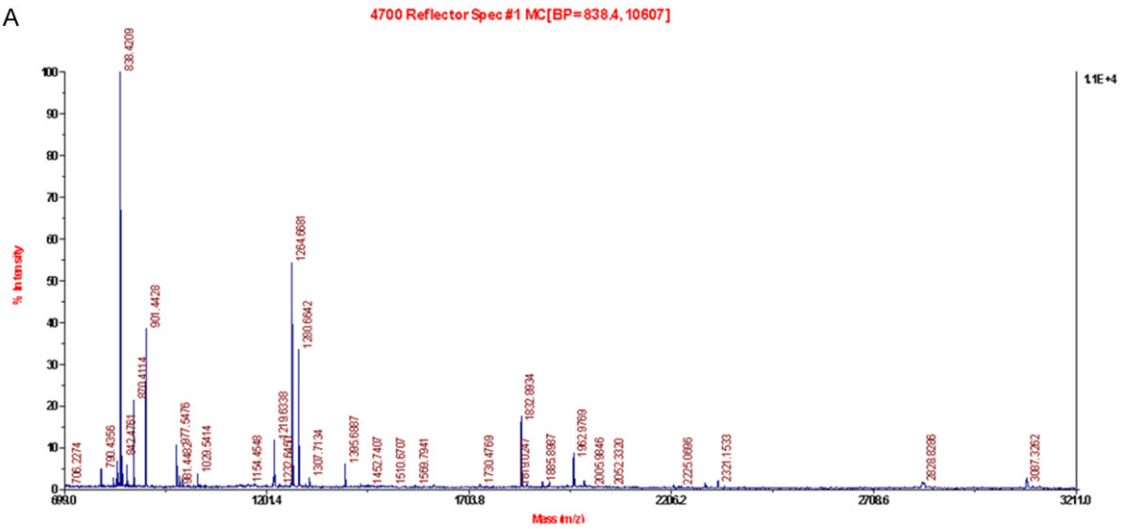
PC12 and 293 Tα cells were maintained in Dulbecco's modified Eagle medium (DMEM, Thermo Scientific, USA) containing 10% fetal bovine serum (FBS, Thermo Scientific, USA) and 1% antibiotic solution (mixture of 10 U/ml penicillin G and 10 mg/ml streptomycin). Cell cultures were maintained in 6-well plates at 37°C in a humidified atmosphere of 5% CO_2.

In brief, cells dissociated from the spinal cord of 2 day old postnatal rat Sprague-Dawley were seeded at a density of 2.4 × 10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific, USA) containing 10% FBS (Thermo Scientific, USA) and 1% antibiotic solution (mixture of 10 U/ml penicillin G and 10 mg/ml streptomycin) at 37°C, under a humidified 5% CO_2/95% air atmosphere. After 1 week, neurons and astrocytes were isolated and cultured and, plating at a final density of 2 × 10^5 cells/well on a 6 well culture plate. On the following day, cells were subjected to various treatments [16].

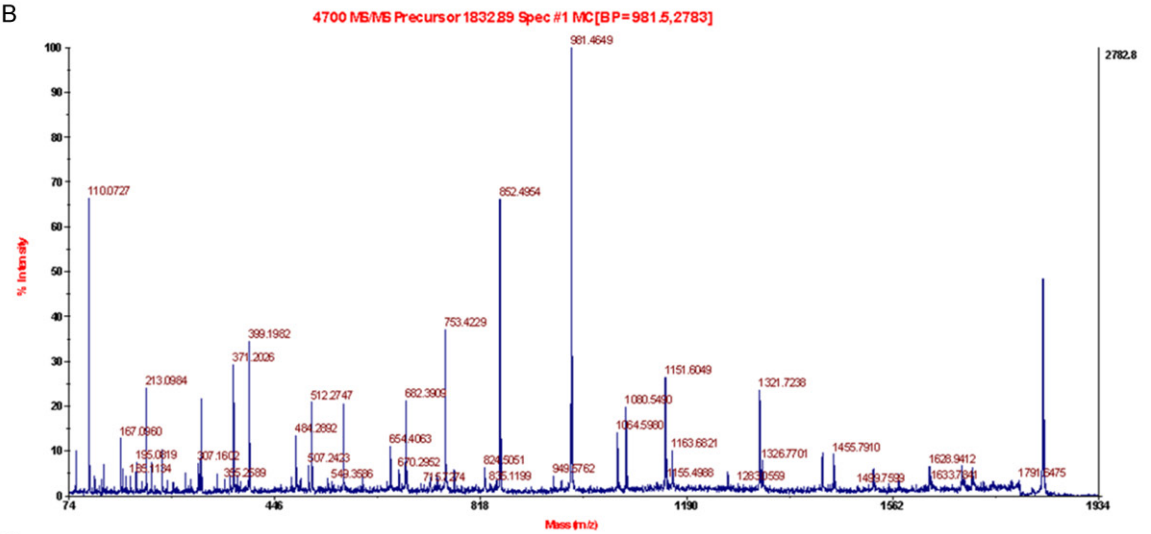
Lentivirus production and infection of cells

The cDNA sequence of GLO1 was obtained from GenBank (accession number: NM_207594). Four kinds of shRNA for GLO1 were inserted into the lentivirus expression plasmid psi-HIV-U6 (guangzhou genecopoeia, China). Non-silencing shRNA was used as a control. In order to generate GLO1-shRNA and control lentivirus, lentiviral shRNA plasmids targeting GLO1 or a control were transfected into 293Tα cells using EndoFectin Lenti reagent (guangzhou genecopoeia, China) together with the gag/pol packaging vector (guangzhou genecopoeia, China). For lentivirus infection, when the cells grew 60% (a density of 3 × 10^4 cells/well), the lentivirus of

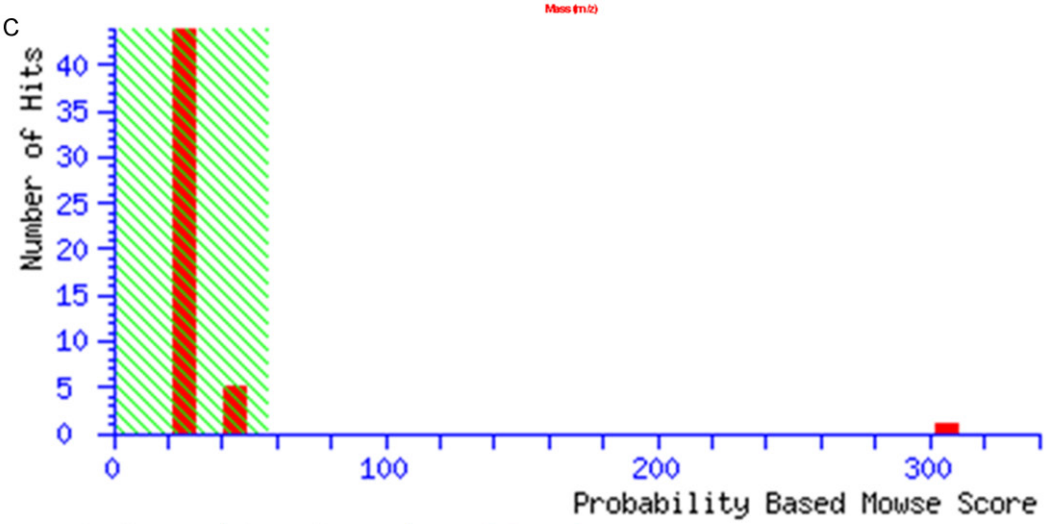
A



B



C



Matched peptides shown in **Bold Red**

```
  1 MAEPQPASSG LTDEAALSCC SDPDPSTKDF LLQQTMLRIK DPKKSLDFYT
 51 RVLGLTLLQK LDFPSMKFSL YFLAYEDKND IPKDKTERTA WAFSRKATLE
101 LTHNWGTEDD ETQSYHNGNS DPRGFGHIGI AVPDVYEACK RFEELGVKFV
151 KKPDDGKMKG LAFVQDPDGY WIEILNPNKM ATII
```

Figure 2. Mass spectra for protein identifications with searching score or single-peptide matching. A. PMF of the 2-DE spot glyoxylase 1. The arrow indicates the position of a precursor ion of *m/z* 838.4209. B. The MS/MS spectrum of precursor ion of *m/z* 838.4209. The database searching with this MS/MS spectrum results in the identification of peptide, which was also identified with a score of 306 based on PMF. C. The probability based on molecular weight search score. Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores being more than 56 are significant (P<0.05). Matched peptides are shown in Bold Red and the sequence coverage is 63%. So this protein is glyoxylase 1.

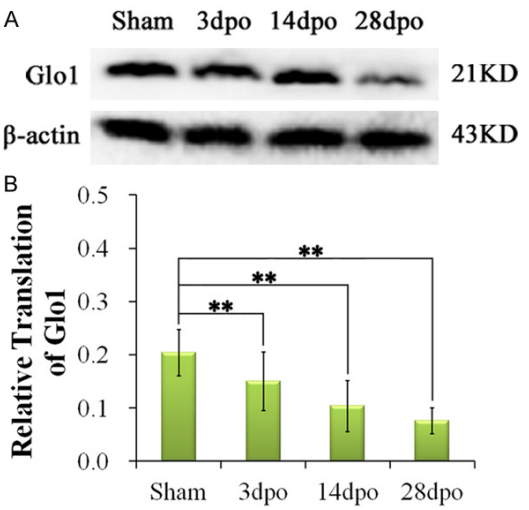


Figure 3. Validating the levels of glyoxylase 1 after SCT. A. Tissues from rostral to spinal cord lesion of sham, 3dpo, 14dpo and 28dpo group SCT rats were analyzed by Western blot. Each blotting strip was determined by Image J software. B. Analysis of verification of glyoxylase 1. The horizontal axes showed the average change fold of five parallel experimental runs. The results were quantified and normalized in relation to the β-actin content. Data are expressed as mean ± SD and were analyzed by One-Way ANOVA (*P<0.05, **P<0.01).

GLO1-shRNA or control lentivirus were transfected into these cells with 1% polybrene and 5% FBS in DMEN at a MOI of 2.3. 48 h after transfection, the medium was changed to the original medium. The pictures of growth of spinal cord neurons were photographed by microscope before transfection as 0 day, 2 day and 7 day after transfection [17].

Effective interference fragments screening

Transient transfection of PC12 cells for shRNA screening. PC12 cells were plated in 6-well plate and transfected with a total of 2 mg of GLO1-shRNA per well, using the SuperFectin™II DNA transfection reagent (Shanghai Pufei Biotech Co.). Culture medium was removed 12 hours after transfection and replaced with fresh medium. After 48 hours cell culture, TRIzol reagent was used to extract mRNA and continued to synthesis related cDNA for PCR. At last the production of PCR was run to Agarose gel electrophoresis and taken photos by Gel Imager. The software Image J was used to calculate the gray value. Statistical comparisons were done using an oneway ANOVA. Significance was accepted at P<0.05.

MTT cell viability assay

In brief, both uninfected and infected PC_{12} cells (4×10^4 cells/well) were seeded in 96-well plates. After 1, 2, 3, 4, or 5 days of infection, 20 μl MTT (5 mg/ml, Sigma, St. Louis, MO) was added, followed by incubation for another 4 h at 37°C. 150 μl of dimethylsulfoxide was added to the MTT-treated wells for 10-15 minutes and for colorimetric analysis the absorption was determined at 570 nm using an automated plate reader. The optical of sample bands were analyzed with Quantity One analysis software (Bio-Rad, USA). Each assay was repeated at least three times [18].

Results

Quantitative analysis of 2D protein maps and mass spectrum analysis of specific proteins

To study regeneration or apoptosis of spinal cord rostral to transection, we used proteome analysis to perform at sham and 28 day post-operation (dpo) after SCT to screen differential proteins expressed in the spinal cord. The proteome contained about 849 detectable protein spots staining in 2D gel by CBB R-250 [442 (sham group rats) and 407 (SCT rats)]. Comparing 28dpo to sham, we found a spot that was decreased in intensity (**Figure 1**). This spot was identified by mass spectrometry (**Figure 2**). Spectrum analysis showed that this differential protein spot was successfully identified and list in **Table 1**. And it was homologous between human and rat. This protein which may play a role in neuronal plasticity was abundantly ex-

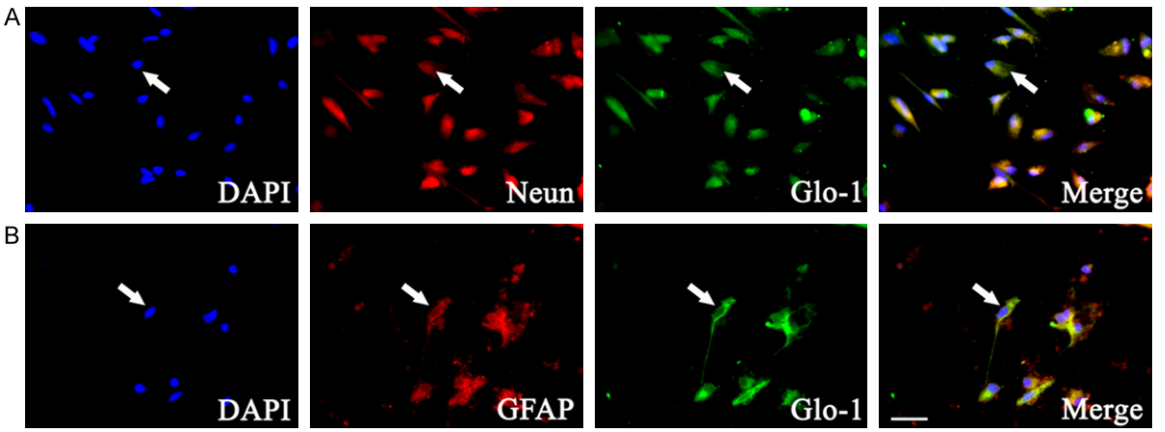


Figure 4. Various expressions of neuronal cells and astrocytes makers and glyoxylase 1 positive cells were mainly found at the neuronal cells and astrocytes. A. Glyoxylase 1 expressed in the neuronal cells. B. Glyoxylase 1 expressed in the astrocytes. Arrow indicates co-localization of glyoxylase 1 and Neun/GFAP expression. Scale bar 20 μm.

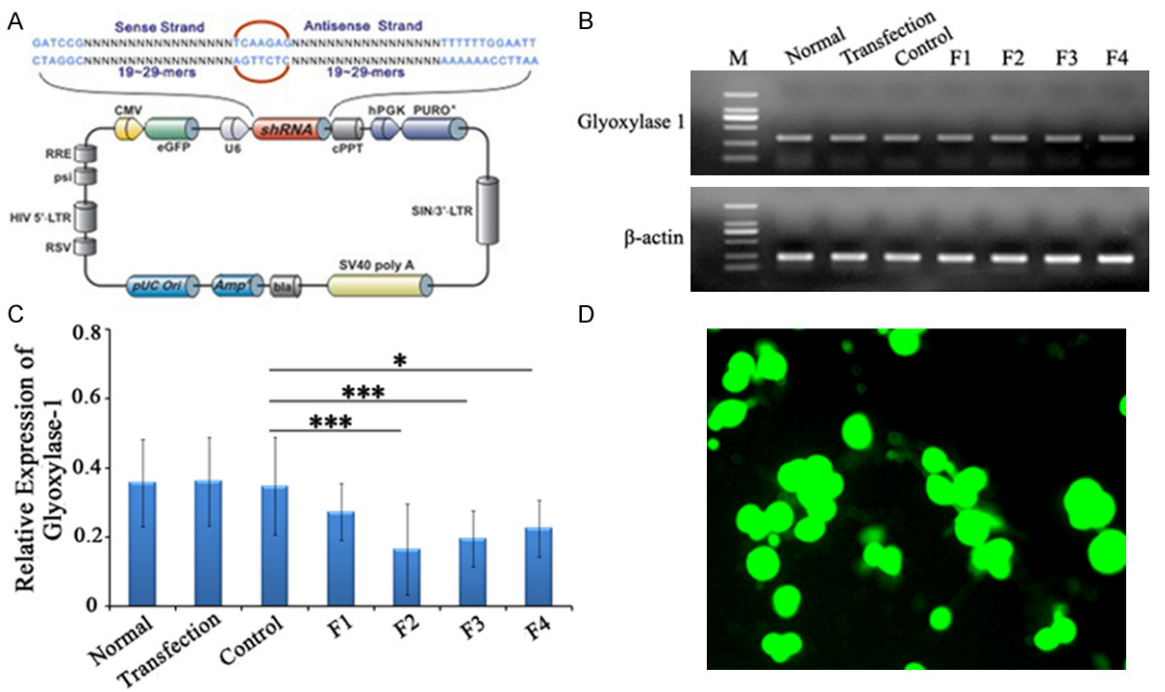


Figure 5. Screening of shRNAs for silencing expression of glyoxylase 1 and lentiviral production of glyoxylase 1. A. The skeleton information of SH glyoxylase 1. Enhance green fluorescent protein (eGFP) is reporter gene for identification. The framework also contains antibiotic: ampicillin and stable selection marker: puromycin. CMV and U6 are promoters for plasmid expression. B. The PCR product of the glyoxylase 1 (above) and β-actin (below) expression after transfected relative plasmids into PC12 cells. From left to right, each lane was DNA marker, normal, transfection, control, F1 (Interference Fragment 1), F2 (Interference Fragment 2), F3 (Interference Fragment 3), F4 (Interference Fragment 4). C. Results were expressed as the relative OD value of RT-PCR determined by Image J software after 48h transfection (Data were expressed as mean ± SD, $^{\#}P<0.001$, One-Way ANOVA). D. Lentiviral production of the shRNA expression plasmid of the glyoxylase 1 in 293Tα cells. The green fluorescent light showed the lentivirus of shRNA of the glyoxylase 1 was successfully produced.

pressed in the brain and spinal cord. This result led us to infer that this factor could have an important role in regeneration or apoptosis of spinal cord transaction.

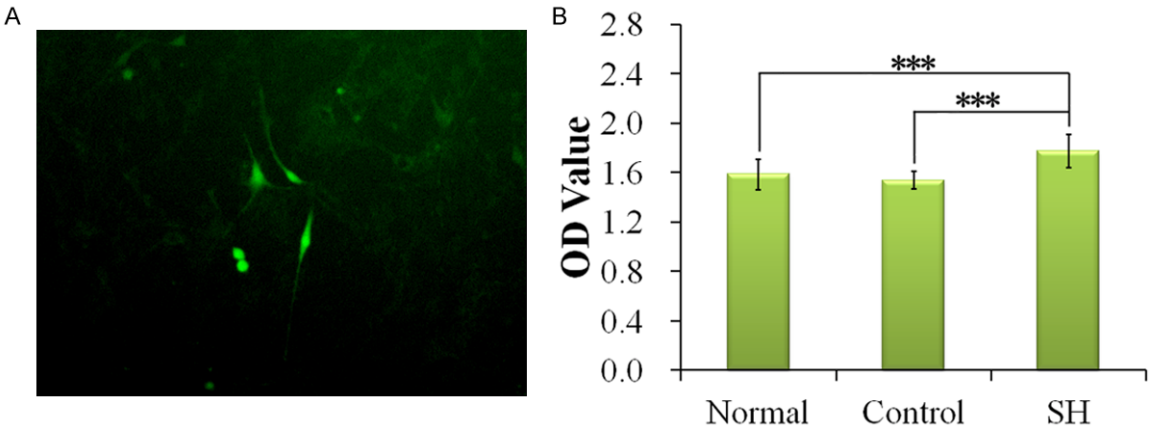


Figure 6. Results of function of glyoxylase 1 in vitro experiments. A. Fluorescence micrographs of formation of PC12 cell from MTT assay. B. The effect of lentiviral glyoxylase 1 SH on PC12 cell viability by MTT assay. The cell viability from three independent experiments is expressed as mean ± standard deviation.

Validating the levels of glyoxylase 1 by WB

To validate the changes of the protein level of glyoxylase 1, western blotting was used to determine the changes of glyoxylase 1 in translation levels. Western blot analysis showed that comparing to sham group, the protein level of glyoxylase 1 was significant lower in 3dpo, 14dpo and 28dpo ($P<0.01$). As time went on, the relative expression of protein level of glyoxylase 1 decreased (3dpo: 0.1499±0.0550, 14dpo: 0.1033±0.0478, 28dpo: 0.0757±0.0240) (**Figure 3**). And comparing 28dpo to sham, the expression of protein of glyoxylase 1 significantly decreased 2.7-fold ($P<0.01$).

Identification of the spinal cord neuron cells and spinal cord astrocytes expressing glyoxylase 1

The result of double immunofluorescence studies showed that performed with antibodies against glyoxylase 1 and the antibodies that recognize reactive neurons (NeuN) and astrocytes (GFAP) in spinal cord neuron cells (**Figure 4A**) and spinal cord astrocytes (**Figure 4B**). From the data we found that glyoxylase 1 was expressed in neurons and in astrocytes in the spinal cord.

Schematic of lentiviral plasmids used in this study

To construct a new transgene lentiviral vector that could interfere the target gene expression yet retaining gene transduction efficiency for following experiments, the coding sequence of rattus glyoxylase 1 was cloned into the optimized lentiviral backbone psiHIV-glyoxylase 1-eGFP-U6 (**Figure 5A**). The four lentiviral constructs harboring different shRNAs (F1, F2, F3 and F4) were used to transfect PC12 cells. In parallel, a negative control was run. After 48 h of transfection, the mRNA levels of glyoxylase 1 was measured by RT-PCR in the subgroups and the RT-PCR assay showed that all constructs, except F1, could significantly down-regulate glyoxylase 1 gene expression in PC12 cells (**Figure 5B**). The relative OD value was 0.3567±0.1253, 0.3612±0.1276, 0.3475±0.1409, 0.2731±0.0832, 0.1650±0.1312, 0.1953±0.0808, 0.2251±0.0832, 0.1650±0.1312, 0.1953±0.0808, 0.3612±0.1276, 0.3475±d by 52.52%, 43.80%, 35.22% excluding F1, $P<0.05$) (**Figure 5C**). The highest knockdown efficiency was achieved using F2 which was subsequently designated as psiHIV-glyoxylase 1-eGFP-U6-F3. The results suggest that the use of psiHIV-GLYOXYLASE 1-eGFP-U6-F3 is appropriate for the following experiments. The psiHIV-GLYOXYLASE 1-eGFP-U6 was used to transfect the 293T cells, at the same time transfecting packing mix plasmids containing gag, pol and env plasmids, and the viral particles were collected 48 hours later., the fluorescent proteins expressed by 293Tα cells were detected using the fluorescent microscopy. 293Tα cells transfected with SH plasmid demonstrate abundant cytoplasmic green fluorescence, consistent with expression of the green fluorescence fusion protein (**Figure 5D**).

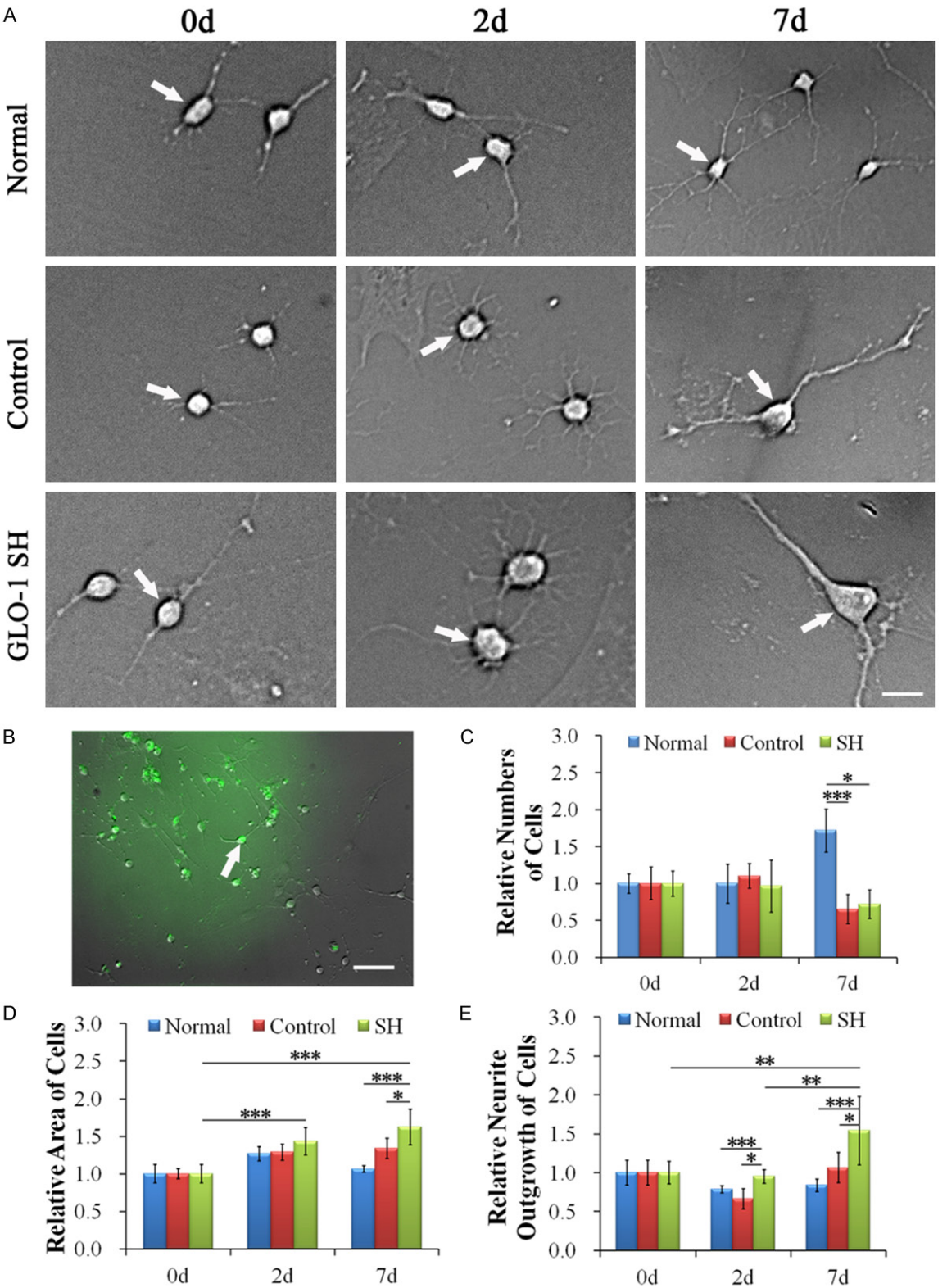


Figure 7. Glyoxylase 1 played an important role in growth of neuronal cells in vitro experiments. A. The picture showed the growth of the spinal cord neuron at 0 day, 2 day, 7 day, being transfected by glyoxylase 1 SH. B. After being transfected, green fluorescence was seen in the spinal cord neurons. That mean the lentivirus of glyoxylase 1 was successfully transfected into the neurons. C. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord neuron on the number of cells. The results of 0 day were expressed as standard and the rest results were

standardized with that of 0 day. The numbers from five independent experiments were expressed as mean ± standard deviation. D. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord neuron on the area of neuron. The results of 0 day were expressed as standard and the rest results were standardized with that of 0 day. The data from five independent experiments was expressed as mean ± standard deviation. E. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord neuron on the neurite outgrowth of cells. The results of 0 day were express as standard and the rest results were standardized with that of 0 day. The lengths from five independent experiments were expressed as mean ± standard deviation. All data were expressed as mean ± standard deviation and were analyzed by One-Way ANOVA ($^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.005$). Bar 15 μm.

Viability of PC12 cells after treatment with SH lentiviral production of glyoxylase 1

The cell viability was assessed by MTT assay. As shown in **Figure 6A**, green fluorescence was captured by the fluorescent microscopy that mean the lentivirus of glyoxylase 1 interference fragment was successfully transfected into PC12 cells. There was a dramatic increase in cell viability of glyoxylase 1 SH compared to that in controls and the OD (Optical density) value of cells increased from 1.5388±0.0687 to 1.7739±0.1330 ($P<0.001$) (**Figure 6**). It revealed that the SH could promote cell viability.

Interfering glyoxylase 1 increased neurite outgrowth and area of neuronal cellular body.

We wanted to know whether glyoxylase 1 promotes growth and area of cultured spinal cord neurons. Dissociated spinal cord neurons cultured from new born rats were treated with negative vectors lentivirus, sh vector of glyoxylase 1 lentivirus (**Figure 7A**, **7B**). As a result, comparing with control group, sh vector of glyoxylase 1 increased the neurite outgrowth and area of cellular body at 2 day and 7 day. At 2 day and 7 day, sh group significantly increased 43.7% and 44.6% of neurite outgrowth compared to control (**Figure 7E**, $P<0.05$). For the area of cellular body test, sh group significantly increased 21.5% of area of cellular body compared to control group at 7 day (**Figure 7D**, $P<0.05$). Thus, glyoxylase 1 inactivation significantly promotes neurite outgrowth and area of cellular body in spinal cord neurons under permissive conditions.

Interfering glyoxylase 1 decreased neurite outgrowth

On the other hand, whether glyoxylase 1 promotes growth and area of cultured spinal cord astrocytes is a key problem in vitro experiment. Dissociated spinal cord astrocytes cultured from new born rats were treated with negative vectors lentivirus, sh vector of glyoxylase 1 lentivirus (**Figure 8A**, **8B**). From the data of results, we got the information described as below: comparing with control group, sh vector of glyoxylase 1 decreased the number of cells, neurite outgrowth and area of cellular body at 2 day and 7 day. At 2 day and 7 day, sh group significantly decreased 14.8% and 22.3% of neurite outgrowth compared to control (**Figure 8E**, $P<0.05$). For the number of cells and area of cellular body test, sh group decreased compared to control group at 2 day and 7 day, but had no significances (**Figure 7C**, **7D**). Thus, glyoxylase 1 inactivation significantly inhibited neurite outgrowth in spinal cord astrocytes under permissive conditions.

Discussion

Right now in clinic, the patients who suffering from spinal cord injury have poor prognosis such as severance of axons, loss of neurons and glia, and demyelination. Many treatments of spinal cord injury have been established, but we don't find the best one. So the primary objective of this study is to apply proteomic analysis to identify changes in protein expression in response to spinal cord transection, one the most serious kind of SCI. And in these proteins we find the most important one and focus on it for our other aim to discover the relationship between the effect of this protein and the recovery of sensory and behavior function through regeneration or apoptosis.

The present study employed an electrophoretic technique, which allows rapid separation and visualization of many proteins within the proteome [19] including identification of possible post-translational modifications as suggested by changes in their pI/MW and/or molecular mass. This was important in the way of identification of several proteins, where the possibility of post-translational modifications was represented by identification of the same protein in different fractions [20]. From the proteomic analysis of the spinal cord segment rostral to

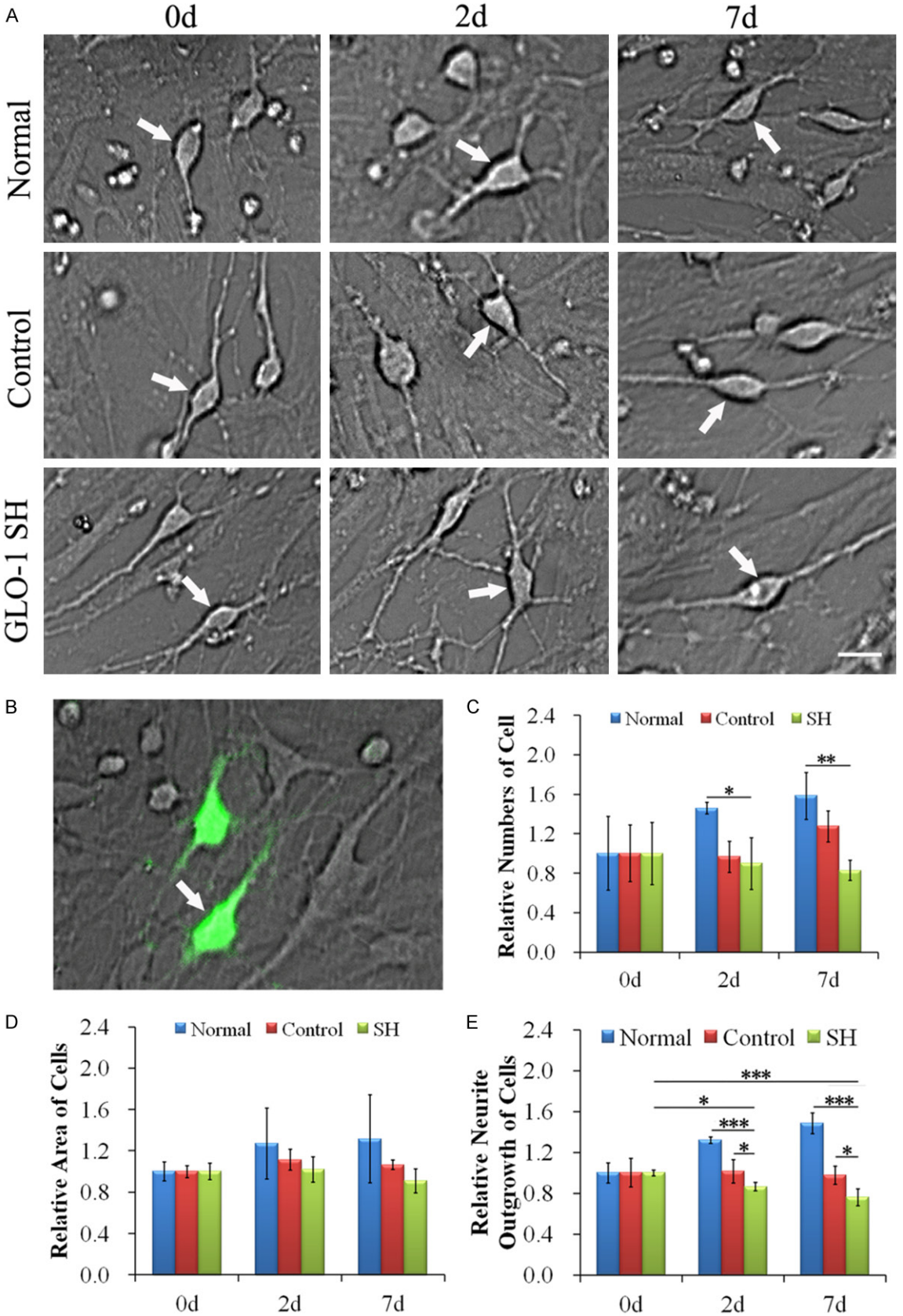

A
0d
2d
7d
Normal
Control
GLO-1 SH
B
C
Normal
Control
SH
Relative Numbers of Cell
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
**
0d
2d
7d
D
Normal
Control
SH
Relative Area of Cells
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0d
2d
7d
E
Normal
Control
SH
Relative Neurite Outgrowth of Cells
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
***
*
***
*
***
*
0d
2d
7d

Figure 8. Glyoxylase 1 played an important role in growth of astrocytes in vitro experiments. A. The picture showed the growth of the spinal cord astrocytes at 0 day, 2 day, 7 day, being transfected by glyoxylase 1 SH. B. After being transfected, green fluorescence was seen in the spinal cord astrocytes. That mean the lentivirus of glyoxylase 1 was successfully transfected into the astrocytes. C. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord astrocytes on the number of cells. The results of 0 day were expressed as standard and the rest results were standardized with that of 0 day. The data was from three independent experiments. D. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord astrocytes on the area of cells. The results of 0 day were expressed as standard and the rest results were standardized with that of 0 day. The data was from three independent experiments. E. The effect of lentiviral glyoxylase 1 SH transfected in primary spinal cord astrocytes on the neurite outgrowth of cells. The results of 0 day were express as standard and the rest results were standardized with that of 0 day. The data was from three independent experiments. All data were expressed as mean ± standard deviation and were analyzed by One-Way ANOVA ($^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.005$). Bar 15 μm.

the injury site, we found one unique proteins that responded to spinal injury and all results were validated by using western blotting.

From the result of proteomic analysis, we luckily focus on one of the most important proteins, glyoxalase I (Glo-1), which is an enzyme encoded by this gene being responsible for the catalysis and formation of S-lactoyl-glutathione from methylglyoxal condensation and reduced glutathione [21, 22]. In previous studies, more attention was put on the effect of glyoxalase I on metabolic diseases and cancer therapy such as that Glo-1 could be associated with the risk for breast cancer [23], Glo-I over-expression reduces hyperglycemia-induced levels of carbonyl stress, AGEs, and oxidative stress [24] and disturbed neuronal glyoxalase 1 activity under conditions of hyperglycaemia in the presence of impaired endothelium-dependent relaxation and cognitive function [25]. Another study showed that Glo-1 is a resistant factor to antitumor agent-induced apoptosis in human leukemia cells and that the Glo-1 inhibitor could be a drug resistance-reversing agent [26]. But in our previous researches, we found that Glo-1 not only expressed in spinal cord neurons but also expressed in spinal cord astrocytes. Interestingly, the changes of the expression of Glo-1 might have some relationship to the changes of recovery function after spinal cord injury.

So we assumed that Glo-1 played an important role on the function or mechanism of neurorecovery after spinal cord injury. This led us to the present study. Using proteome and shRNA interference, we found that after SCT Glo-1 in the rostral site to spinal cord lesion was down-regulated (**Figures 1** and **2**). When Glo-1 was down-regulated by shRNA treatment in vitro, we found that Glo-1 increase the neuronal neurite outgrowth and area of cellular body on neurons but have a decreasing trend of that on astrocytes (**Figures 7** and **8**). This finding should open a new arena for researches in the improvement of recovery function after SCT. More and more studies paid attention on the relationship between spinal cord injury and the formation of scar, because astrogliosis following spinal cord injury (SCI) involves an early hypertrophic response that serves to repair damaged blood-brain barrier and a subsequent hyperplastic response that results in a dense scar that impedes axon regeneration [27-29]. Luckily, in our study we also found that expression of Glo-1 was effected in astrogliosis which had an important role on the relationship among the scar, inflammation and the recovery of the injury of spinal cord. Glo-1 is a resistant factor to antitumor agent-induced apoptosis in human leukemia cells and that the Glo-1 inhibitor could be a drug resistance-reversing agent [26], so it's a chance to change this protein into a new drug in the future.

On the other hand, the other question is how the Glo-1 exerts its' function and which factors regulates the Glo-1. Though we didn't do this study, we could presume and make an inference from the previous studies. Glo-1 had its' peculiar signal transduction pathways which was relative to angiogenesis and antiapoptotic [30, 31]. One of evidences showed that the TNF-induced phosphorylation of GLO1 is the dominated factor for cell death [32, 33]. This phenomenon indicated that Glo-1 may have some effect in biological effects of astrocytes. But for spinal cord transection, there are no more evidences. Luckily in our study we discover that low expression of Glo-1 is beneficial for the proliferation of neurons after SCT and low expression of Glo-1 impedes that of the astrocytes. So it might be indicated that inhibiting Glo-1 could obstruct some kind of inflammation factors to reduce the formation of the scar in

injured lesion. Some studies revealed that Glo-1 had some neuroprotection through advanced glycation endproduct (AGE) pathway regulated by methylation [34-37]. So these data gave us a new direction to find out the mechanism of the Glo-1 in the recovery of spinal cord injury in our future researches.

Our finding reveals that Glo-1 is an important molecule in recovery of function on neurons and astrocytes. However, it is still unclear as to work on which particular signal pathways or neurotrophic factor in the rostral spinal cord lesion, which requires further investigation.

Conclusion

By using proteomics analysis, we revealed differentially expressed protein spots, in the rostra spinal cord lesion after spinal cord transaction (SCT) comparing with normal rats. Of these differential proteins, we focused on the effect of Glo-1 in recovery function following SCT. After SCT, Glo-1 was down-regulated and then the changes of expression of Glo-1 were relative to the effect on neurite outgrowth and area of neuronal cellular body in neurons and astrocytes. These finds may provide a novel strategy which is targeting Glo-1 for the treatment of SCI.

Acknowledgements

Animal Experimental Center of Si Chuan University approved this study. The protocol was approved by the Committee on the Ethics of Animal Experiments of Si Chuan University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Disclosure of conflict of interest

None.

Address correspondence to: Ting-Hua Wang, Institute of Neuroscience, Kunming Medical University, Kunming 650031, China; Institute of Neurological Diseases, Translational Neuroscience Center, State Key Lab of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China. E-mail: tinghua_neuron@263.net

References

[1] Iwashita Y, Kawaguchi S, Murata M. Restoration of function by replacement of spinal cord segments in the rat. Nature 1994; 367: 167-170.

[2] Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, Bregman BS. Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. J Neurosci 2001; 21: 9334-9344.

[3] Zaminutesy A, Shokrgozar MA, Sadeghi Y, Noroozian M, Heidari MH, Piryaei A. Transplantation of Schwann cells differentiated from adipose stem cells improves functional recovery in rat spinal cord injury. Arch Iran Med 2013; 16: 533-541.

[4] McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 1999; 5: 1410-1412.

[5] Wheaton BJ, Noor NM, Whish SC, Truettner JS, Dietrich WD, Zhang M, Crack PJ, Dziegielewska KM, Saunders NR. Weight-Bearing Locomotion in the Developing Opossum, Monodelphis domestica following Spinal Transection: Remodeling of Neuronal Circuits Caudal to Lesion. PLoS One 2013; 8: e71181.

[6] Perez M, Benitez SU, Cartarozzi LP, Del Bel E, Guimarães FS, Oliveira AL. Neuroprotection and reduction of glial reaction by cannabidiol treatment after sciatic nerve transection in neonatal rats. Eur J Neurosci 2013; 38: 3424-3434.

[7] Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive Astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 2004; 24: 2143-2155.

[8] Van Herreweghe F, Mao J, Chaplen FW, Grooten J, Gevaert K, Vandekerckhove J, Vancompernolle K. Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methylglyoxal-derived AGE. Proc Natl Acad Sci U S A 2002; 99: 949-54.

[9] Ek CJ, Habgood MD, Callaway JK, Dennis R, Dziegielewska KM, Johansson PA, Potter A, Wheaton B, Saunders NR. Spatio-Temporal Progression of Grey and White Matter Damage Following Contusion Injury in Rat Spinal Cord. PLoS One 2010; 5: e12021.

[10] Gündisch S, Schott C, Wolff C, Tran K, Beese C, Viertler C, Zatloukal K, Becker KF. The PAXgeneH Tissue System Preserves Phosphoproteins in Human Tissue Specimens and Enables Comprehensive Protein Biomarker Research. PLoS One 2013; 8: e60638.

[11] Clifton J, Huang F, Rucevic M, Cao L, Hixson D, Josic D. Protease inhibitors as possible pitfalls in proteomic analyses of complex biological samples. J Proteomics 2011; 74: 935-941.

[12] Yu M, Sun J, Peng W, Chen Z, Lin X, Liu X, Li M, Wu K. Protein expression in human trabecular meshwork: downregulation of RhoGDI by dexamethasone in vitro. Mol Vis 2010; 16: 213-223.
[13] Shang FF, Zhao W, Zhao Q, Liu J, Li DW, Zhang H, Zhou XF, Li CY, Wang TH. Upregulation of eIF-5A1 in the paralyzed muscle after spinal cord transection associates with spontaneous hindlimb locomotor recovery in rats by upregulation of the ErbB, MAPK and neurotrophin signal pathways. J Proteomics 2013; 91: 188-199.
[14] Chen J, Xia C, Wang J, Jiang M, Zhang H, Zhang C, Zhu M, Shen L, Zhu D. The Effect of Orexin-A on Cardiac Dysfunction Mediated by NADPH Oxidase-Derived Superoxide Anion in Ventrolateral Medulla. PLoS One 2013; 8: e69840.
[15] Shen Y, Sun A, Wang Y, Cha D, Wang H, Wang F, Feng L, Fang S, Shen Y. Upregulation of mesencephalic astrocyte-derived neurotrophic factor in glial cells is associated with ischemia-induced glial activation. J Neuroinflammation 2012; 9: 254.
[16] Kim JB, Choi JS, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Lee JK. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 2006; 26: 6413-6421.
[17] Zhang B, Li KY, Chen HY, Pan SD, Jiang LC, Wu YP, Liu SW. Spindle and kinetochore associated complex subunit 1 regulates the proliferation of oral adenosquamous carcinoma CAL-27 cells in vitro. Cancer Cell Int 2013; 13: 83.
[18] Peng W, Yu Y, Li T, Zhu Y, Chen H. The effects of small interfering RNA-targeting tissue factor on an in vitro model of neovascularization. Mol Vis 2013; 19: 1296-303.
[19] Rabilloud T. A comparison between low background silver diamminutese and silver nitrate protein stains. Electrophoresis 1992; 13: 429-439.
[20] Noor NM, Steer DL, Wheaton BJ, Ek CJ, Truettner JS, Dietrich WD, Dziegielewska KM, Richardson SJ, Smith AI, VandeBerg JL, Saunders NR. Age-Dependent Changes in the Proteome Following Complete Spinal Cord Transection in a Postnatal South American Opossum (Monodelphis domestica). PLoS One 2011; 6: e27465.
[21] Kim NS, Umezawa Y, Ohmura S, Kato S Human glyoxalase I. cDNA cloning, expression, and sequence similarity to glyoxalase I from Pseudomonas putida. J Biol Chem 1993; 268: 11217-21.
[22] Degaffe GH, Vander Jagt DL, Bobelu A, Bobelu J, Neha D, Waikaniwa M, Zager P, Shah VO. Distribution of glyoxalase I polymorphism among Zuni Indians: the Zuni Kidney Project. J Diabetes Complications 2008; 22: 267-72.
[23] Antognelli C, Del Buono C, Ludovini V, Gori S, Talesa VN, Crinò L, Barberini F, Rulli A. CYP17, GSTP1, PON1 and GLO-1 gene polymorphisms as risk factors for breast cancer: an Italian case-control study. BMC Cancer 2009; 9: 115.
[24] Brouwers O, Niessen PM, Ferreira I, Miyata T, Scheffer PG, Teerlink T, Schrauwen P, Brownlee M, Stehouwer CD, Schalkwijk CG. Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. J Biol Chem 2011; 286: 1374-80.
[25] Skapare E, Konrade I, Liepinsh E, Makrecka M, Zvejniece L, Svalbe B, Vilskersts R, Dambrova M. Glyoxalase 1 and glyoxalase 2 activities in blood and neuronal tissue samples from experimental animal models of obesity and type 2 diabetes mellitus. J Physiol Sci 2012; 62: 469-78.
[26] Sakamoto H, Mashima T, Kizaki A, Dan S, Hashimoto Y, Naito M, Tsuruo T. Glyoxalase I is involved in resistance of human leukemia cells to antitumor agent-induced apoptosis. Blood 2000; 95: 3214-8.
[27] Bhalala OG, Pan L, Sahni V, McGuire TL, Gruner K, Tourtellotte WG, Kessler JA. microRNA-21 regulates astrocytic response following spinal cord injury. J Neurosci 2012; 32: 17935-47.
[28] Lin B, Xu Y, Zhang B, He Y, Yan Y, He MC. MEK inhibition reduces glial scar formation and promotes the recovery of sensorimotor function in rats following spinal cord injury. Exp Ther Med 2014; 7: 66-72.
[29] Tao X, Minutesg-Kun Y, Wei-Bin S, Hai-Long G, Rui K, Lai-Yong T. Role of telomerase reverse transcriptase in glial scar formation after spinal cord injury in rats. Neurochem Res 2013; 38: 1914-20.
[30] Taniguchi H, Horinaka M, Yoshida T, Yano K, Goda AE, Yasuda S, Wakada M, Sakai T. Targeting the glyoxalase pathway enhances TRAIL efficacy in cancer cells by downregulating the expression of antiapoptotic molecules. Mol Cancer Ther 2012; 11: 2294-300.
[31] Ahmed U, Dobler D, Larkin SJ, Rabbani N, Thornalley PJ. Reversal of hyperglycemia-induced angiogenesis deficit of human endothelial cells by overexpression of glyoxalase 1 in vitro. Ann N Y Acad Sci 2008; 1126: 262-4.
[32] Birkenmeier G, Stegemann C, Hoffmann R, Günther R, Huse K, Birkemeyer C. Posttranslational modification of human glyoxalase 1 indicates redox-dependent regulation. PLoS One 2010; 5: e10399.
[33] de Hemptinne V, Rondas D, Vandekerckhove J, Vancompernolle K. Tumor necrosis factor induces phosphorylation primarily of the nitric-oxide-responsive form of glyoxalase I. Biochem J 2007; 407: 121-8.

[34] Bélanger M, Yang J, Petit JM, Laroche T, Magistretti PJ, Allaman I. Role of the glyoxalase system in astrocyte-mediated neuroprotection. J Neurosci 2011; 31: 18338-52.
[35] Jack M, Wright D. Role of advanced glycation endproducts and glyoxalase I in diabetic peripheral sensory neuropathy. Transl Res 2012; 159: 355-65.
[36] Distler MG, Plant LD, Sokoloff G, Hawk AJ, Aneas I, Wuenschell GE, Terminutesi J, Meredith SC, Nobrega MA, Palmer AA. Glyoxalase 1 increases anxiety by reducing GABAA receptor agonist methylglyoxal. J Clin Invest 2012; 122: 2306-15.
[37] Jia X, Chang T, Wilson TW, Wu L. Methylglyoxal mediates adipocyte proliferation by increasing phosphorylation of Akt1. PLoS One 2012; 7: e36610.