Knockdown of polypyrimidine tract binding protein facilitates motor function recovery after spinal cord injury

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Abstract
After spinal cord injury (SCI), a fibroblast- and microglia-mediated fibrotic scar is formed in the lesion core, and a glial scar is formed around the fibrotic scar as a result of the activation and proliferation of astrocytes. Simultaneously, a large number of neurons are lost in the injured area. Regulating the dense glial scar and replenishing neurons in the injured area are essential for SCI repair. Polypyrimidine tract binding protein (PTB), known as an RNA-binding protein, plays a key role in neurogenesis. Here, we utilized short hairpin RNAs (shRNAs) and antisense oligonucleotides (ASOs) to knock down PTB expression. We found that reactive spinal astrocytes from mice were directly reprogrammed into motoneuron-like cells by PTB downregulation in vitro. In a mouse model of compression-induced SCI, adeno-associated viral shRNA-mediated PTB knockdown replenished motoneuron-like cells around the injured area. Basso Mouse Scale scores around the injured area but also by modestly reducing the density of the glial scar without disrupting its overall structure. Together, these findings suggest that reactive spinal astrocytes from mice were directly reprogrammed into motoneuron-like cells by PTB downregulation in vitro and improves motor function recovery after SCI.

Key Words: antisense oligonucleotides; astrocytes; glial scar; motoneuron-like cells; motor function; neurogenesis; neuron-like cells; polypyrimidine tract binding protein; short hairpin RNAs; spinal cord repair

Introduction
Spinal cord injury (SCI) is a devastating central nervous system injury that often results in paralysis below the injury site (Sofroniew, 2018; Fouad et al., 2021). After SCI, fibroblasts and microglia form fibrotic scars that fill the lesion core. Simultaneously, astrocytes rapidly activate and proliferate, which results in the formation of a dense glial scar around the lesion core. An increasing number of studies have confirmed that the glial scar has dual effects on SCI repair (Yang et al., 2020a). Favorable effects include alleviating further development of inflammatory responses, limiting the spread of fibroblastic scars, and forming astrocytic bridges that contribute to axonal regeneration (Anderson et al., 2016; Sofroniew, 2018). However, the glial scar is also regarded as a physical barrier that hinders axons from regenerating through the lesion site and serves as a chemical barrier because of the secretion of molecules that suppress neural regeneration (Li et al., 2020; Escartin et al., 2021). Therefore, the dual role of glial scars makes SCI repair difficult. Our previous study confirmed that 2 weeks was a suitable time point for manipulating the glial scar after SCI (Yang et al., 2020b). In SCI repair, this time point may alleviate the detrimental effects of the glial scar while supporting its favorable effects. During and after SCI, impaired neurons are lost because of necrosis or apoptosis, and the remaining damaged neurons have little regenerative capacity (O’Shea et al., 2017). These adverse factors coordinate with each other and, ultimately, complicate the SCI repair process. To date, there are no effective approaches for facilitating nerve regeneration and motor function restoration after SCI.

Direct conversion of somatic cells is a promising strategy for clinical use in regenerative medicine. Astrocytes are widely distributed throughout the spinal cord and have high plasticity (Yu et al., 2020). Reactive astrogliosis occurs after SCI, and reactive astrocytes express high levels of the neural stem cell marker nestin and display some characteristics of neural progenitor cells (Liddelow and Barres, 2017). Some studies (Su et al., 2014; Puls et al., 2020; Yang et al., 2020b; Liu et al., 2021) have shown that overexpression of neural transcription factors (TFs) can directly reprogram spinal astrocytes into diverse neuronal phenotypes both in vitro and in vivo. However, these studies mainly used glutamatergic, GABAergic, or mature neurons (Su et al., 2014; Puls et al., 2020; Yang et al., 2020b; Liu et al., 2021). Moreover, these approaches showed limited roles in motor function restoration. Therefore, the strategy for gaining more motor neurons and allowing effective restoration of motor function after SCI requires further investigation.

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Poly pyrimidine tract binding protein (PTB, also known as PTBP1), encoded by the PTBP1 gene in mice, is an important RNA-binding protein that widely participates in RNA metabolism (Xue et al., 2009). PTB can regulate translation initiation or suppression that leads to neuronal vulnerability (Rousarrie et al., 2020). Moreover, PTB plays a key role in neuronal induction. During neurogenesis, the expression level of PTB is naturally decreased (Hu et al., 2018). Studies have demonstrated that knockdown of PTB was able to directly reprogram reactive spinal astrocytes into neural-like cells in vivo, which were replenished by down-regulation of PTB, promote the recovery of visual responses in mice with retinal injury (Zhou et al., 2020). Maimon et al. (Maimon et al., 2021) confirmed that downregulation of PTB by an antisense oligonucleotide (ASO) significantly reprogrammed reactive astrocytes into neurons in vitro. Treatment with a PTB-ASO generated neurons that integrated into the hippocampal circuit and ameliorated the behavioral deficits in aging mice (Maimon et al., 2021).

The above studies show that suppression of PTB expression is a powerful strategy for treating various disorders caused by neuronal loss. However, the function of PTB knockdown in SCI repair has not yet been explored. In this study, short hairpin RNAs (shRNAs) and ASOs were used to knock down the expression of PTB to determine its role both in vitro in primary murine reactive spinal astrocytes and in vivo using a mouse model of compression-induced SCI.

Methods

ASOs and viral vectors

ASOs were produced by RIBOBIO Biotechnologies (Guangzhou, China). The sequence of the mouse Ptbp1 ASO (PTB-ASO) was 5ʹ-GGG TGA AGA TCC TGT TCA G 3ʹ. The Cy3 fluorochrome was linked to the 5ʹ end of the ASO.

To construct adeno-associated virus (AAV) vectors, the same sequence of shPTB was inserted into the pAAV-GFAP-EGFP-MIR155 vector to construct pAAV-GFAP-EGFP-shPTB-MIR155 (Genechem Co., Ltd.; Shanghai, China). The shRNA sequence targeting mouse Ptbp1 (shPTB) was 5ʹ-GGG TGA AGA TCC TGT TCA GC 3ʹ. The CMV promoter of the pAAV vector was replaced by the human cytomegalovirus (CMV) promoter. The CMV-EF1α-EGFP vector (OBiO Biotechnologies, Shanghai, China) was subcloned into the pCLenti-GFAP-shRNA vector (NC)-CMV-EGFP-WPRE vector (OBIO Biotechnologies, Shanghai, China).

Primary murine spinal astrocyte culture and activation

All animal experiments were approved by the Institutional Animal Ethics Committee of Nantong University (approval No. S20201015-402) on October 20, 2020. All experiments were designed and reported according to the ARRIVE guidelines: Reporting of In Vivo Experiments (ARRIVE) guidelines (Perice du Sert et al., 2020). Primary murine astrocytes were prepared from the spinal cords of 1–2-day-old Institute of Cancer Research (ICR) mice (Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020)). Primary murine spinal astrocyte culture and activation were performed as previously described above (20,000 cells/well). The conditioned culture medium was replaced every 3 days. After 7 days, primary microglial cells were removed by shaking. The next day, the medium was changed to induction medium, which consisted of equal volumes of DMEM/F12 and neurobasal medium (Gibco) supplemented with sodium selenite (30 nM; Sigma–Aldrich), insulin (25 μg/mL; AbMole, USA), putrescine (100 nM; APEXBio; Houston, TX, USA), progesterone (20 nM; Sigma–Aldrich), 2% fetal bovine serum, 0.4% B-27 supplemented with L-glutamine, ascorbic acid, thiamine, pyridoxine, biotin, vitamin C, phenol red, sodium selenite, insulin, and transferrin (ScienCell, San Diego, CA, USA). The medium was cultured for 20 days after induction medium for 48 hours for further transdifferentiation.

SCI mouse model

Eight-week-old female ICR mice (specific pathogen-free grade, 23–28 g, Animal Center of Nantong University; license No. SYXK (Su) 2017-0046) were anesthetized through the surgery with isoflurane (1–1.5%, flow rate: 200 μL/min) in oxygenized air supplied by a standard small animal anesthesia device (RWD; Shenzhen, China). SCI leads to urine retention in mice, and male mice are more likely to suffer from urethritis and die than female mice. In addition, the experimental period was up to 12 weeks in this study. Considering the survival rate of mice after SCI, female mice were selected (Wu et al., 2021).

The spinal cord was exposed by a T9–T10 laminectomy. A complete compression injury was induced for 10 seconds by bilateral compression with No. 5 Dumont forceps (Fine Science Tools, Foster City, CA, USA) (Anderson et al., 2018). The following day, dexamethasone was closed with 0.9% normal saline and used to close the skin incision. The SCI mice were randomly divided into four groups: AAV-shCtrl, AAV-shPTB, Control AOS, and PTB-AOS groups, of which included 25 mice.

AAV and ASO injection

AAVs or ASOs may require several days to take effect in vivo, and 2 weeks after SCI was considered an appropriate time point to manipulate the glial scar (Shomer et al., 2020). Therefore, we performed injections intrathecally immediately after SCI. AAV-shPTB was diluted to the same titer as AAV-shCtrl (2.71 × 1011 VG/mL) with phosphate-buffered saline (PBS). AAs were injected at two oblique points on opposite sides of the lesion site (1.0 mm caudal and rostral to the lesion site), with 15 μL per injection, using a 32G needle with a 33G needle (Hamilton, Basel, Switzerland). The method for injecting ASOs (1 μg/mL, 1.0 μL per injection) was the same as that for injecting AAVs. At 12 weeks after SCI, mice were deeply anesthetized with intraperitoneally administered ethyl carbamate (Tongpharm Chemical Reagent Co. Ltd., Shanghai, China) and then sacrificed. The spinal cords of the mice were collected.

Western blot analysis and quantitative reverse transcription-polymerase chain reaction

For measuring protein expression levels of PTB following PTB silencing in reactive spinal astrocytes, the reprogrammed cells were lysed in cell lysis buffer containing cOmplete™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany). After quantification, equivalent amounts of reprogrammed cell protein from each treatment group were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA). One-half volume of the medium was replaced every 3 days.

For western blot analysis, the membrane was incubated with the primary antibody (1:1000 dilution) for 1 hour at room temperature. Membranes were incubated with the secondary antibody (1:5000 dilution) for 1 hour at room temperature. Immunoreactions were visualized with a chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) and a fully automated ChemiDoc system (RWD; Shenzhen, China). The primary antibodies and dilutions were as follows: rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10,000 dilution), rabbit anti-PTB (1:1000), and rabbit anti-β-actin (1:1000; AbCional, Wuhan, China, Cat. A3487, RRID: AB_2863069). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; Thermo Fisher Scientific, Cat# 31466, RRID: AB_10960844). ImageJ 1.47v software (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012) was used to quantify the protein band signals.

Total RNA from reprogrammed cells or spinal cord was extracted using TRI reagent (Sigma–Aldrich) and reverse transcription was performed using a complementary DNA (cDNA) synthesis kit (Thermo Fisher Scientific). Polymerase chain reaction (PCR) was performed using a QuantStudio SYBR Green PCR System. Amplification was performed on a CFX96 Real-Time PCR instrument (Hercules, CA, USA). The quantitative reverse transcription PCR mixture (20 μL) contained 6 μL cDNA, 9 μL Taqman Universal PCR Master Mix (Applied Biosystems), and 2 μL SYBR Green. Reaction conditions were: pre-denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 30 seconds. The relative expression of target genes was normalized to G6PD. The primer sequences are listed in Table 1.
**Table 1**

| Gene   | Sequence                        | Product length (bp) |
|--------|---------------------------------|---------------------|
| Ptbp1  | Forward: 5'-AGA GGA GGC TCG CAA CAC TA-3' | 286                 |
|        | Reverse: 5'-GGT GAA GGT CGG TGT GAA CGG-3' |                     |
| Caspase-3 | Forward: 5'-GAC GTC AGC GTA CTA-3' | 118                 |
|        | Reverse: 5'-GGT GTA CAT TCT TAC TCT-3' |                     |
| Caspase-9 | Forward: 5'-GAT GTC GCA GAA CCA GGT-3' | 84                  |
|        | Reverse: 5'-GTA TTC CCG GGA TCC TTC TC-3' |                     |
| Gfap   | Forward: 5'-CGG AGA GGC ATC ACC TCT G-3' | 126                 |
|        | Reverse: 5'-AGG GAG TGG AGG ACT TCG-3' |                     |
| Gpdh   | Forward: 5'-GGT GAA GGT CGG TGT GAA CGG-3' | 143                 |
|        | Reverse: 5'-TCA TAC TGG AAC ATG TAG ACC-3' |                     |

**Immunocytochemistry**

To measure astrocyte differentiation in vitro, reprogrammed cells were collected after 3 and 4 weeks of lentivirus-shPTB-mediated PTB knockdown or 5 weeks of PBS ASO-mediated PTB knockdown and fixed with 4% paraformaldehyde for 10 minutes. Cells were permeabilized in PBS containing 1% Triton X-100 for 15 minutes and then washed three times with PBS. Cells were blocked with 3% bovine serum albumin in PBS for 30 minutes and incubated with specific primary antibodies diluted in PBS containing 3% bovine serum albumin for 16 hours at 4°C. The primary antibodies included mouse anti-microtubule-associated protein 2 (MAP2; 1:200; Abcam), Cambridge, MA, USA, Cat# ab11266, RRID: AB_297886), goat anti-green fluorescent protein (GFP; 1:1000; Abcam, Cat# ab5450, RRID: AB_304987), rabbit anti-choline acetyltransferase (ChAT; 1:400; Abcam, Cat# ab181023, RRID: AB_267893), and goat anti-giall fibrillary acidic protein (GFAP; 1:1000; Abcam, Cat# ab185544, RRID: AB_880202). After washing three times with PBS, reprogrammed cells were incubated for 2 hours at room temperature with the following secondary antibodies: Alexa Fluor 488–labeled donkey anti–goat IgG (1:1000; Thermo Fisher Scientific, Cat# A11055, RRID: AB_2534102), Alexa Fluor 488–labeled donkey anti–rabbit IgG (1:1000; Thermo Fisher Scientific, Cat# A12106, RRID: AB_2535792), Alexa Fluor 568–labeled donkey anti–mouse IgG (1:1000; Thermo Fisher Scientific, Cat# A10337, RRID: AB_2534013), or Alexa Fluor 647–labeled donkey anti–rabbit IgG (1:1000; Thermo Fisher Scientific, Cat# A31573, RRID: AB_2536183). Nuclei were labeled with Hoechst 33342 (Beyotime) by incubation for 15 minutes at 37°C. Images were acquired using a confocal microscope (Leica, Wetzlar, Germany). MAP2, GFP, ChAT, GFAP, and Hoechst’s cells were counted.

**Immunohistochemistry**

To measure the localization of shPTB delivered by AAV in the spinal cord of mice, spinal cord staining was conducted 1 week after injection. To explore the role of PTB knockdown in vivo, spinal cord staining was conducted 12 weeks after SCI. Mice were anesthetized with isoflurane and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde. The T9–T11 vertebrae of mice were excised and postfixed with 4% paraformaldehyde for 60 minutes and washed two times with PBS. Sections were permeabilized in PBS containing 0.5% Triton X-100 for 5 minutes at room temperature. After washing two times with PBS, the sections were incubated with the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) detection liquid (Beyotime) for 1 hour at 37°C. Nuclei were labeled with a confocal microscope, and the numbers of TUNEL+ cells were counted. The percentages were calculated as the number of TUNEL+ cells divided by the total number of Hoechst’s cells.

**Behavioral experiments**

Motor recovery was measured at 1 week post-SCI and then weekly for 12 weeks post-SCI.

**Basso Mouse Scale score**

The motor function of SCI mice was evaluated using the Basso Mouse Scale (BMS) scoring system (Huang et al., 2020). Two trained evaluators scored the mice in a double-blinded manner. Mice were evaluated for at least 5 minutes after scoting the open field environment for 20 minutes. The score was from 0 to 9. A higher score represented improved function of both hindlimbs.

**Forced swim test**

The swimming ability of each SCI mouse was evaluated using the forced swim test. A rectangular plexiglass container (42 cm × 30 cm × 35 cm) filled with water was used for the test. The water depth was adjusted to the mouse temperature at approximately 23°C (Yang et al., 2020b). The swim test was performed for 1 minute each time and repeated three times at 15 minutes intervals. An HD camera was utilized to photograph the swimming process. The swimming frequency of the hindlimb was determined every 10 seconds. The maximum swim frequency during each measurement was used, and the mean value of three tests was calculated.

**Inclined plate test**

The muscle strength of both hindlimbs of SCI mice was evaluated using the inclined plate test (Yang et al., 2020b), which consisted of a rectangular plexiglass box (42 cm × 7 cm × 8 cm) with a rough wooden surface at the bottom. When the box was inclined, the mice would slide down from the rough surface because of gravity. We gradually raised the tilt of the box until the mice could no longer maintain position for 5 seconds without sliding down. The recorded result was generated from the maximum height of the top of the box perpendicular to the horizontal plane. The test was repeated three times, and the maximum height was used as the result.

**Cold allodynia**

The sensory recovery of SCI mice was assessed by the cold allodynia test. Cold allodynia was scored by the aceton solution method (Burton et al., 1999). 50 μL of aceton solution was placed onto the plantar skin surface of the paw through a blunt needle attached to a syringe. The reaction of the mouse was observed within 20 seconds: adaphoria, scored 0; minor response, scored 1; moderate reaction—the hindpaw was lifted but did not touch any surface when sprayed with aceton, scored 2; strong response—the paw was licked, bit, and shaken, scored 3. The test was repeated three times at 15 minutes intervals, and the mean value was recorded.

**Hot plate test**

The hot plate test was used to assess the sensory function of SCI mice (Luszczki and Czuczwara, 2008). Mice were placed in a clear 25 cm × 25 cm chamber with a height of 40 cm. The chamber contained a metal plate, and the surface temperature of the plate was set to 50.5°C. The cumulative time that the mouse lifted its hindpaw from the hot plate and licked the paw within a 30 second interval was calculated. The experiment was repeated three times at 15 minutes intervals, and the mean value of the three experiments was calculated.

**Statistical analysis**

The numbers of repeated samples or mice are listed in the figure legends. Degrees of freedom were used to predetermine sample sizes for the in vivo experiments (Charan and Kantharia, 2013), and sample sizes were estimated according to our previous studies for similar behavioral, immunofluorescence, and TUNEL staining analyses (Yang et al., 2020b). Outcome assessors were blinded to the assignment. All data are presented as the means ± standard error of mean (SEM). All statistical analyses were performed with GraphPad Prism 6.01 software (GraphPad Software, San Diego, CA, USA; www.graphpad.com). The Shapiro-Wilk test was utilized to measure data normality. Western blot, RT-PCR, immunocytochemistry, and immunohistochemistry reactivity were analyzed using two-way analysis of variance. Student’s *t*-test and *F*-test were used for statistical analysis. All statistical analyses were performed using repeated measures two-way analysis of variance with Tukey’s post hoc tests. A value of *P* < 0.05 was considered statistically significant.

**Results**

ShPtB mediates direct conversion of reactive spinal astrocytes from mice into motoneuron-like cells in vitro.

Given the demonstrated direct neuronal conversion of cultured mouse cortical astrocytes after shPTB treatment (Qian et al., 2020), we explored whether PTB silencing led to the conversion of reactive mouse spinal astrocytes into motor neurons in vitro. In a reactive astrocyte model, we treated primary murine spinal astrocytes with lipopolysaccharide (Additional Figure 2A & B). Next, we transduced reactive spinal astrocytes from mice with a lentiviral vector containing a Gfp promoter driving the expression of an shPTB against mouse Ptbp1. When astrocytes were transduced with the Ptbp1 significantly downregulated both the protein expression of PtB and mRNA expression of Ptbp1 (Figure 1A–C). At 2 weeks post-reprogramming, shPTB-infected astrocytes showed complete axon outgrowth, and, by 4 weeks, these cells displayed obvious neuronal morphology, whereas cells transduced with the...
To explore the potential beneficial function of the PTB-ASO in SCI mice, we determined whether injection of the PTB-ASO into the injured spinal cord replenished neurons, including motor neurons. This strategy may be helpful for sensitizing reprogramming function of ASOs after massive loss of mature neurons in the lesion area. We found that control ASO-injected mice exhibited an obvious decrease in NeuN+ and ChAT+ cells in the region proximal to the lesion area compared with the distal region at 12 weeks after SCI (Figure 7A). In PTB-ASO-injected mice, the number of NeuN+ and ChAT+ cells in the region proximal to the lesion at a level similar to that observed in the distal site (Figure 7A). Quantitatively, the number of NeuN+ and ChAT+ cells in the region proximal to the lesion area was greater in PTB-ASO-injected mice compared with control ASO-injected mice (Figure 8A). Furthermore, the stained spinal and distal spinal tissue exhibited lower expression levels of the apoptotic markers caspase-3 and caspase-9 in PTB-ASO-injected mice compared with those in control ASO-injected mice (Figure 8C). These findings revealed that the PTB-ASO reduced glial scar density without disrupting its overall structure, replenished motoneuron-like cells around the SCI lesion area, and decreased apoptotic cell death in the injured spinal cord.

PTB-ASOs promote motor function recovery in SCI mice

The function of the PTB-ASO was further evaluated by behavioral tests in SCI mice. Strikingly, compared with the control ASO, the PTB-ASO enhanced motor function recovery from 6–12 weeks post-injury (Figure 9A and B). The PTB-ASO-treated mice reached a higher height than control ASO-treated mice in the inclined plate test at 12 weeks post-SCI (Figure 9C). Compared with control ASO-treated mice, PTB-ASO-treated mice showed a tendency to use their forelimbs more than control mice after SCI. In the sensory system, cold- and heat-induced sensory responses were not different between the PTB-ASO-treated and control ASO-treated mice from 6–12 weeks post-injury (Figure 9E). These findings demonstrated that PTB-ASOs promoted motor function recovery in SCI model mice.

Discussion

SCI is a serious neurological disorder with the pathological characteristics of fibrinoid fibroblast-mediated cavitation, astroglial-mediated glial scar formation around the lesion area, and loss of motor neurons in the injured area. SCI ultimately causes failure of axonal regeneration in the injured area. At present, research on SCI repair mainly focuses on inhibiting astroglial-fibrous scar formation, supplementing neurons, and improving nerve regeneration. However, glial cell transplantation therapies, such as the possibility of the activator of transcription 3 knockout, leads to the exhaustive ablation of the glial scar, which results in poor functional recovery after SCI (Anderson et al., 2016; Sofroniew, 2018; Gu et al., 2019). Moreover, many approaches that replenish neurons survive in the injured area may not achieve satisfactory functional recovery after SCI (Huang et al., 2020; Yang et al., 2020b). Thus, the identification of efficacious therapeutic approaches for SCI is an extremely urgent need. As mentioned earlier, PTB can regulate the inclusion or skipping of exon 10, which leads to the beneficial regulation of neuronal survival (Ming et al., 2020). In addition, PTB has an important effect on neuronal induction, and its expression level is naturally reduced during neurogenesis (Hu et al., 2018). Our current study is the first to explore the function of silencing PTB in SCI.

As previously described, the glial scar has dual effects on SCI repair. An appropriate method to treat the glial scar needs to be discovered to alleviate the adverse effects of the scar. In this study, we report that PTB mediated by ASOs may be a scavenger of the scar. Our previous studies confirmed that 2 weeks post-SCI is a suitable time point for manipulating the glial scar because the formation of the scar is almost complete by this time after injury (Yang et al., 2020b). Because gene therapies may take additional days to stably alter gene expression, we postponed the in situ injection for 7 days after SCI. Thus, the effects of gene therapy began around 2 weeks after SCI, which coincided precisely with the appropriate time point to manipulate the glial scar. In our study, we found that ASOs, which modestly reduced the density of the glial scar and maintained the structure of the scar after SCI in contrast to exhaustive scar ablation.

Somatic cell reprogramming is an alternative to cell replacement therapy and shows great potential in SCI repair. As some previous studies have shown, spinal astrocytes can be reprogrammed into neurons. Our team previously reported that the SOX2 and Isl1 gene programs glial scar-forming astrocytes into neurons in a mouse model of SCI (Yang et al., 2020b). More recently, activation of the endogenous neuron-related genes Ngn2 and Is1l was shown to reprogram astrocytes into motor neurons in the spinal cord of adult mice (Zhao et al., 2021). These programs can efficiently reprogram gliotic astrocytes into neurons in vitro (Kempf et al., 2021). The Ngn2 gene programs spinal astrocytes into neurons both in vitro and in vivo (Kempf et al., 2021; Liu et al., 2021). The pronuclear OCT4 and KLF4 TFs can facilitate motor recovery in a combination program of SCI (Kempf et al., 2021). The OCT4 and KLF4 TFs have been found to reprogram spinal astrocytes into motor neurons, while most other strategies result in astrocyte-derived glutamatergic, GABAergic, or mature neurons. Thus, the identification of new key TFs or genes involved in motor neuron replenishment is expected to be a new breakthrough.
Figure 1 | Direct reprogramming of primary murine reactive spinal astrocytes into motoneuron-like cells by PTB knockdown in vitro.

(A) Western blot bands and (B) quantification of the protein expression levels of PTB following PTB silencing with short hairpin (sh)PTB RNA in reactive mouse spinal astrocytes for 2 days. The results were normalized to the sh control (shCtrl) group. (C) Relative RNA levels after PTB knockdown for 2 days. The results were normalized to the shCtrl group. (D) Representative photographs of the morphological changes following PTB silencing for 2 and 4 weeks. After PTB silencing for 2 weeks, shPTB-infected cells showed complex neurite outgrowth, and these cells displayed obvious neuronal morphology by 4 weeks, whereas cells transduced with shCtrl displayed an astrocyte-like flattened and polygonal morphology. Scale bar: 40 μm. (E) Reactive mouse spinal astrocytes were immunostained for the neuronal marker MAP2 (red, Alexa Fluor 488) and ChAT (green, Alexa Fluor 488) cells that stained positive for MAP2, whereas no shCtrl-transduced GFP cells were positive for MAP2. Scale bar: 40 μm. (F) Quantification of the number of MAP2+ cells among transduced GFP cells at 3 weeks. (G) Reprogrammed motoneuron-like cell morphology and expression of ChAT (cyan, Alexa Fluor 647) after PTB silencing for 4 weeks. There were some shPTB-transduced GFP cells that stained positive for ChAT, whereas no shCtrl-transduced GFP cells stained positive for ChAT. Scale bar: 40 μm. (H) Quantification of the number of MAP2+ cells and ChAT+ cells among transduced GFP cells at 4 weeks. Data are shown as the means ± SEM. The experiments were repeated three times. *P < 0.05, vs. shCtrl (two-tailed Student’s t-test). ChAT: Choline acetyltransferase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP: green fluorescent protein; MAP2: microtubule-associated protein 2; n.d.: not detected; PTB: poly-pyrimidine tract-binding protein; Ptbp1: poly-pyrimidine tract-binding protein.

Figure 2 | Localization of shPTB delivered by AAV in the spinal cord of mice.

At 1 week post-injection, immunohistochemical staining showed that GFP (green, Alexa Fluor 488) cells colocalized with GFAP+ (red, Alexa Fluor 568) astrocytes (A) but not with ChAT+ (cyan, Alexa Fluor 647) motor neurons (B). The lower panels are magnifications of the white dashed box areas in the upper panels to improve clarity. The arrowheads in (A) indicate representative co-labeled spinal astrocytes, and the swallowtail arrowheads in (B) denote endogenous motor neurons (n = 3 mice, four sections per mouse). Scale bars: 50 μm. (C) Quantitative analysis of the percentage of GFAP+ and ChAT+ cells colocalizing with GFP+ cells. The data are presented as the means ± SEM (n = 3 mice/group) and were analyzed by two-tailed Student’s t-test. AAV: Adeno-associated virus; GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; MAP2: microtubule-associated protein 2; n.d.: not detected; PTB: poly-pyrimidine tract-binding protein.

Figure 3 | PTB knockdown replenished motoneuron-like cells around the injured area after SCI in mice.

(A) Longitudinal sections at 12 weeks post-SCI. In the AAV-shPTB group, some of the GFP+ (green, Alexa Fluor 488) cells were colocalized with NeuN+ (red, Alexa Fluor 568) and ChAT+ (cyan, Alexa Fluor 647) cells. The arrows show representative supplementary motoneuron-like cells, and the arrowheads indicate representative endogenous motor neurons. In the AAV-shCtrl group, there were no co-labeled GFP+/NeuN+ cells or GFP+/ChAT+ cells (n = 3 mice, four sections per mouse). Scale bars: 200 μm. In each group, the lower panels are magnifications of the white dashed box areas in the upper panels to improve clarity. (B) Replenished neuron-like cells (GFP+ [green, Alexa Fluor 488] and MAP2+ [red, Alexa Fluor 568]) and motoneuron-like cells (GFAP+/ChAT+) (cyan, Alexa Fluor 647) were detected in the AAV-shPTB group 12 weeks after SCI. The arrows denote representative replenished motoneuron-like cells, and the arrowheads indicate representative endogenous motor neurons. There were no GFP+/MAP2+ or GFP+/ChAT+ cells in the AAV-shCtrl group (n = 3 mice, four sections per mouse). Scale bars: 200 μm. (C) Quantitative analysis of the percentage of NeuN+, MAP2+, and ChAT+ cells among the GFP+ cells (n = 3 mice). (D) Quantitative analysis of total NeuN+, MAP2+, and ChAT+ cells around the injured spinal cord areas in the AAV-shCtrl and AAV-shPTB mouse groups. The data are shown as the means ± SEM (n = 3 mice/group). #P < 0.05, vs. AAV-shCtrl (two-tailed Student’s t-test). AAV: Adeno-associated virus; ChAT: choline acetyltransferase; GFP: green fluorescent protein; MAP2: microtubule-associated protein 2; PTB: poly-pyrimidine tract-binding protein; SCI: spinal cord injury.
The experimental timelines.

(A) At 12 weeks post-SCI, the GFAP (green, Alexa Fluor 488) fluorescence intensity was lower and the number of NeuN (cyan, Alexa Fluor 647) cells in the Control ASO group was much greater around the lesion area than in mice that received the AAV-shCtrl. (B) AAV-shPTB-injected mice maintained their position at an inclined plate test showed that AAV-shPTB-injected mice had a greater height than AAV-shCtrl mice at 12 weeks post-SCI. The data are shown as the means ± SEM (n = 6 mice/group). *P < 0.05, vs. AAV-shCtrl (two-tailed Student’s t-test). (C) Quantitatively, the efficiencies of neuron-like and motoneuron-like cell reprogramming were calculated based on the percentages of MAP2+ and ChAT+ cells among total Hoechst+ cells. The experiments were repeated three times. The data are shown as the means ± SEM. **P < 0.01, vs. control ASO (two-tailed Student’s t-test). ASO: Antisense oligonucleotide; ChAT: choline acetyltransferase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MAP2: microtubule-associated protein 2; n.d.: not detected; PTB: polypyrimidine tract-binding protein; Ptbp1: polypyrimidine tract-binding protein; SCI: spinal cord injury; TUNEL: TdT-mediated dUTP Nick-End Labeling.

Figure 5 | PTB knockdown facilitated motor function recovery after SCI.
(A) BMS scores. Motor function recovery was better in mice with AAV-shPTB-mediated PTB knockdown from 6 to 12 weeks after SCI than in mice that received the AAV-shCtrl. (B) The swim, (C) inclined plate, (D) cold allodynia, and (E) hot plate tests. No obvious differences were observed in the swim, cold allodynia, and hot plate tests between the AAV-shCtrl and AAV-shPTB groups. The inclined plate test showed that AAV-shPTB-injected mice maintained their position at a greater height than AAV-shCtrl mice at 12 weeks post-SCI. The data are shown as the means ± SEM (n = 6 mice/group). *P < 0.05, vs. AAV-shCtrl (repeated measures two-way analysis of variance followed by Tukey’s post hoc test). AAV: Adeno-associated virus; BMS: Basso Mouse Scale; PTB: polypyrimidine tract-binding protein; SCI: spinal cord injury; w: week(s).

Figure 6 | The PTB-ASO mediated the reprogramming of murine reactive spinal astrocytes to motoneuron-like neurons in vitro.
The protein expression levels of PTB were measured by (A) western blot analysis after PTB-ASOs were transfected into murine reactive spinal astrocytes for 2 days. (B) Quantification of PTB protein. The results were normalized to the Control ASO group. (C) The mRNA expression levels of Ptbp1 following PTB silencing for 2 days. The results were normalized to the Control ASO group. (D) Representative images of the expression of ChAT (green, Alexa Fluor 488) by immunocytochemical staining after PTB-ASO-mediated PTB knockdown for 5 weeks. In the PTB-ASO group, MAP2+ (red, Alexa Fluor 568) cells stained positive for ChAT (green, Alexa Fluor 488), but there were no MAP2+ and ChAT+ cells in the Control ASO group. Scale bar: 40 μm. (E) Quantitatively, the efficiencies of neuron-like and motoneuron-like cell reprogramming were calculated based on the percentages of MAP2+ and ChAT+ cells among total Hoechst+ cells. The experiments were repeated three times. The data are shown as the means ± SEM. **P < 0.01, vs. control ASO (two-tailed Student’s t-test). ASO: Antisense oligonucleotide; ChAT: choline acetyltransferase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MAP2: microtubule-associated protein 2; n.d.: not detected; PTB: polypyrimidine tract-binding protein; Ptbp1: polypyrimidine tract-binding protein.

Figure 7 | The PTB-ASO reduces the density of the glial scar and replenishes motoneuron-like cells around the spinal cord lesion.
(A) At 12 weeks post-SCI, the GFAP (green, Alexa Fluor 488) fluorescence intensity was lower and the number of NeuN (red, Alexa Fluor 568) and ChAT (cyan, Alexa Fluor 647) cells was much greater around the lesion area in PTB-ASO-injected mice than in the Control ASO group. The arrows indicate the glial scar area. The yellow swallowtail arrowheads and lines surrounding the area denote the NeuN+ cells in the region proximal to the lesion area. The white swallowtail arrowheads and lines surrounding the area denote the ChAT+ cells around the damaged area. The yellow arrowheads and lines surrounding the area indicate NeuN+ cells in the region distal to the damaged area. The white arrowheads and lines surrounding the area denote ChAT+ cells in the region distal to the damaged area (n = 3 mice; four sections per mouse). Scale bars: 200 μm. In each group, the lower panels are magnifications of the white dashed box areas in the upper panels to improve clarity. (B) Quantitative analysis of the fluorescence intensity of GFAP in the glial scar area. (C) Quantitative analysis of the numbers of NeuN+ and ChAT+ cells per 0.1 mm² in the region proximal to the lesion area. The data are shown as the means ± SEM (n = 3 mice/group). **P < 0.01, vs. control ASO (two-tailed Student’s t-test). ASO: Antisense oligonucleotide; ChAT: choline acetyltransferase; GFAP: glial fibrillary acidic protein; PTB: polypyrimidine tract-binding protein; SCI: spinal cord injury.
PTB participates in the regulation of RNAs involved in neural differentiation. Neurons have a positive RE1-silencing transcription factor (REST)/miR-124 loop in which REST downregulates the expression of many neural-specific TFs and miR-124 (Xue et al., 2013; Hu et al., 2018). Thus, the regulation of PTB plays a key role in the cell fate decision of the neuronal lineage. Various cells, including MEFS, N2A, NT2, and SH-SY5Y cells can be gradually reprogrammed into functional neurons via activation of the REST/miR-124 feedback loop induced by PTB knockdown (Xue et al., 2013). Notably, astrocytes have the same regulated signaling loop for neuronal maturation as neurons; thus, PTB knockdown alone leads to the activation of a positive REST/miR-124 feedback loop (Qian et al., 2020). This characteristic increases the feasibility of one-step reprogramming of astrocytes into neurons by reducing PTB expression. Qian et al. (2020) confirmed that a single one-step strategy that suppressed PTB significantly induced reprogramming of miobrain astrocytes into functional DA neurons in vitro. In a mouse model of Parkinson’s disease, after PTB knockdown for 3 months, this strategy improved motor function by not only replenishing DA neurons but also by rebuilding the striatal dopamine circuit (Qian et al., 2020). Maimon et al. (2021) demonstrated that ASO-mediated PTB knockdown reprogrammed astrocytes into neurons in vivo. In aging mice, this PTB-ASO replenished neurons in the brain and modified behavioral dysfunction (Maimon et al., 2021). Moreover, many studies have reported that the region-specific status of astrocytes strongly affects the cell conversion process. Astrocytes from different regions have different transcriptional and proteomic environments and, potentially, region-specific neural TFs that lead to conversion into different subtype-specific neurons (Mattugini et al., 2019; Herrero-Navarro et al., 2021; Kempf et al., 2021). The property of regionalization is maintained during astrocyte-to-neuron conversion both in vitro and in vivo. Because the spinal cord contains an abundance of motor neurons, we successfully reprogrammed reactive spinal astrocytes into motoneuron-like cells through PTB knockdown in vivo. Interestingly, a challenge has recently been raised in the field (Wang et al., 2021), where PTB knockdown failed to reprogram brain astrocytes into neurons in vivo over a relatively short period of time, and endogenous neurons were the source of the presumed astrocyte-derived neurons. Some scholars believe that astrocytic reprogramming is a long and gradual developmental process that requires a relatively long time to be observed in vivo, and the conversion rate may be related to the appropriate virus concentration and type. In our mouse model of compression-induced SCI, we found that PTB silencing for approximately 3 months not only reduced the density of the glial scar without disrupting its overall structure but also replenished motoneuron-like cells around the injured area and decreased apoptotic cell death in the injured spinal cord, which ultimately led to the promotion of motor function recovery. Based on the present studies (Qian et al., 2020; Wang et al., 2021), we speculate that the supplemented motoneuron-like cells may be induced by astrocytic reprogramming, originate from endogenous neurons because of the neuronal protective effect of PTB knockdown, or are derived from Ptbp1 knockdown-mediated residual neural crest cell differentiation. Thus, the origin of the supplemented motoneuron-like cells in SCI mice after PTB silencing requires confirmation by further studies.

In this study, we used AAVs and ASOs as gene therapeutics to target Pb1. We are the first to use an shPTB and a PTB-ASO to reprogram spinal astrocytes into motoneuron-like cells in vitro. In our study, AAV-mediated delivery of shPTB and ASO-mediated knockdown of PTB were shown for the first time to replenish motoneuron-like cells around the injured area after SCI. Moreover, after SCI, the PTB-ASO replenished the motoneuron-like cells in the lesion area, decreased apoptotic cell death in the injured spinal cord, and modestly reduced the density of glial scar without destroying its overall structure. The use of AAVs as viral vectors for gene delivery is clinically feasible because of their minimal immunogenicity, wealth of engineered tissue-specific serotypes, and minimal insertion mutagenesis rate (Wu et al., 2006). Recently, some AAV-mediated clinical gene therapeutic approaches have been verified, such as GlaxoSmithKline’s severe combined immunodeficiency therapy (Naldini, 2015). AAVs are a better choice than other viral vectors for shRNA delivery in vivo. Reconstructed shRNA expressed steadily and steady expression within 1 week (Mason et al., 2010). The formation of glial scars almost completely ceased 2 weeks post-SCI, and thus, 1 week post-SCI is a better time point for injection of AAVs into the injured area. AAV-mediated shRNA expression can be sustained for months even years in vivo with low toxicity (Aguiar et al., 2017). Moreover, in 2016, an ASO gene technology for spinal muscular atrophy therapy was approved by the U.S. Food and Drug Administration (Tosolini and Sleigh, 2017), which contributes to gene therapy of motor neurons. ASOs are synthetic chemically modified single strands of 15–25 nucleotides that efficiently reduce the expression...
level of target genes by binding to specific sequences of pre-mRNA or mRNA (Amado and Davidson, 2021). When injected into the central nervous system, ASOs can be distributed widely (Rigo et al., 2014; Tosolini and Sleigh, 2017). Moreover, ASOs have drug-like features and are easy to regulate (Kordasiewicz et al., 2012), and the delivery method of these properties make ASOs suitable for therapy of some medical diseases. However, ASOs have more off-target effects and cause more inflammatory reactions and toxicities than AAVs (Rao et al., 2009). Although both AAV-mediated shRNA and ASOs are reported to knock down specific genes efficiently, the type of strategy must be carefully selected to improve the therapeutic effect. The determination of efficacy differences between these two strategies for SCI repair will require further investigations.

In summary, we found that PTB knockdown replenished motoneuron cells around the injured area, decreased apoptotic cell death in the injured spinal cord, and moderately reduced the density of the glial scar without disrupting its overall structure, thereby facilitating motor function recovery after SCI. In addition, a limitation of this approach was that the functional recovery extended only to improved motor ability but did not include improvement in sensory perception. As a result, further improvements of this therapeutic approach will be required. The serious consequences of SCI are a result of many factors. Future studies should consider strategies for SCI repair from multiple aspects, such as combining PTB knockdown strategies with other engineering techniques, with the aim of further enhancing functional recovery after SCI.

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Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials: All data generated or analyzed during this study is included in this published article and its supplementary information file.

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Additional files:
Additional Figure 1: Schematic diagram of pAAV-GEF-EGFP-MIR155(Bptbp1) (left) and pC lent-GFP-shRNA(Pbp1)-CMV-EGFP-WPRE.
Additional Figure 2: Establishment of a cell model of mouse reactive spinal astrocytes.

Additional Figure 3: Localization of neural stem cells in the injured spinal cord of mice.

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Additional Figure 1 Schematic diagram of pAAV-GFAP-EGFP-MIR155(Ptbp1) (left) and pClenti-GFAP-shRNA(Ptbp1)-CMV-EGFP-WPRE.
Created with SnapGene® software (from Insightful Science; available at snapgene.com).
Additional Figure 2 Establishment of a cell model of mouse reactive spinal astrocytes.

(A) Primary murine spinal astrocytes were identified via immunocytochemical staining for the astrocyte-specific marker GFAP (green, Alexa Fluor 488). Scale bar: 40 μm. (B) Relative GFAP mRNA level in primary murine spinal astrocytes after lipopolysaccharide treatment for 24 hours. The results were normalized by Control group. The data are presented as the mean ± SEM. The experiments were repeated three times. *P < 0.05 (two-tailed Student’s t-test). GFAP: Glial fibrillary acidic protein; LPS: lipopolysaccharide.
Additional Figure 3 Localization of neural stem cells in the injured spinal cord of mice.

(A) At 1 week post-injection, immunohistochemical staining showed that FoxJ1⁺ (red, Alexa Fluor 568) cells were localized near the lesion core, with the arrowheads indicating some representative residual neural stem cells and dotted line indicating the lesion core, but there was a distance between FoxJ1⁺ cells and AAVs infected GFP⁺ (green, Alexa Fluor 488) cells (n = 3 mice, four sections per mouse) Scale bar: 50 μm. (B) Schematics of the location of residual neural stem cells and the injection sites of AAVs after SCI. AAV: Adeno-associated virus; GFP: green fluorescent protein.