Electroacupuncture-Regulated miR-34a-3p/PDCD6 Axis Promotes Post-Spinal Cord Injury Recovery in Both In Vitro and In Vivo Settings

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Electroacupuncture (EA) could enhance neuroregeneration and posttraumatic conditions; however, the underlying regulatory mechanisms remain ambiguous. PDCD6 (programmed cell death 6) is an established proapoptotic regulator which is responsible for motoneuronal death. However, its potential regulatory role in post-spinal cord injury (SCI) regeneration has remained largely unknown. Further investigations are warranted to clarify the involvement of PDCD6 post-SCI recovery and the underlying mechanisms. In our study, based on bioinformatics prediction, we found that miR-34a-3p might be an upstream regulator miRNA for PDCD6, which was subsequently validated through combined utilization of the qRT-PCR, western blot, and dual-luciferase reporter system. Our in vitro results showed that miR-34a-3p might promote the in vitro differentiation of neural stem cell (NSC) through suppressing PDCD6 and regulating other important neural markers such as fibroblast growth factor receptor 1 (FGFR1), MAP1/2 (MAP kinase kinases 1/2), myelin basic protein (MBP), βIII-tubulin Class III β-tubulin (βIII tubulin), and glial fibrillary acidic protein (GFAP). Notably, in the post-SCI rat model, exogenous miR-34a-3p agomir obviously inhibited the expression of PDCD6 at the protein level and promoted neuronal proliferation, motoneurons regeneration, and axonal myelination. The restorations at cellular level might contribute to the improved hindlimbs functions of post-SCI rats, which was manifested by the Basso-Beattie-Bresnahan (BBB) locomotor test. The impact of miR-34a-3p was further promoted by EA treatment in vivo. Conclusively, this paper argues that a miR-34a-3p/PDCD6 axis might be a candidate therapeutic target for treating SCI and that the therapeutic effect of EA is driven through this pathway.
1. Introduction

Spinal cord injury (SCI) is caused by traumatic physical damage or chronic degenerative diseases depending on the etiology [1]. SCI is pathophysiologically manifested by excessive inflammation [2] and neuronal apoptosis [3]. Clinical consequences of SCI include either temporary or permanent loss of normal spinal functions [1] and, in severe cases, complete dependency and shortened life span [4], which severely affect the wellbeing of the sufferers in physical and emotional aspects and bring about heavy social and economic burden [4].

Over years, endeavors have been made to develop novel strategies for promoting functional rehabilitation following SCI, among which transplantation neural stem cells (NSCs) have emerged as a promising approach in the field of regenerative therapy [5]. NSCs are pluripotent cells with the potential to differentiate into various mature cells constituting the mammalian central nervous system (such as neurons, astrocytes, and oligodendrocytes) [6]. Due to their important neurological roles, NSCs are widely distributed throughout the spinal cord and respond differently under homeostatic condition or external stimulations [7]. An animal study showed that transplantation of NSCs stemmed from adult spinal cord facilitated the post-SCI recovery in rodents [7]. Acupuncture, a traditional Chinese medicine, has found a broad application in the treatment of various neurological diseases. As an improved variant of acupuncture approach, electroacupuncture (EA) had been reported to be efficacious in treating various SCI-related symptoms [8]–[13]. Combined treatment of EA and curcumin improves the recovery of motor function and regulates oxidative stress in rats after traumatic SCI [14]. EA regulation of ApoE and Nrf2 to inhibit inflammation and oxidative stress in SCI has been also reported [15]. EA stimulation was reported to regulate the function of SCs, which favors axonal recovery post-SCI [16]. Through regulating Notch signaling pathway, EA was found to promote the recovery following SCI in rats [9]. The effectiveness of EA in treating trauma has also been demonstrated [17], but its possible mechanism in SCI still needs to be revealed. Moreover, whether EA would potentiate NSCs-induced post-SCI regeneration and the underlying mechanism needs to be explored.

PDCD6 (programmed cell death 6), or apoptosis-linked gene-2 (ALG-2), is an established regulator of apoptosis [18]. Mechanistically, PDCD6 works in concert with human death-associated protein kinase 1 (DAPK1) to trigger caspase-3 dependent signaling [19]. However, in the context of neuronal death, another interactor of PDCD6, namely ALG-2 interacting protein 1 (AIP1; also designated programmed cell death 6-interacting protein, Alix), has drawn more attention. First, AIP1 (ALG-2-interacting protein 1) itself was recognized as an important regulator of neuronal cell death [20]. Second, previous reports showed that PDCD6 could mediate motoneuronal death by forming a complex with AIP1 [21]. Furthermore, the binding between PDCD6 and AIP1 was crucial in the activation of both caspase-dependent and caspase-independent pathways that determined the survival of cerebellar granule neurons [22]. Given the foregoing, although the proapoptotic property of PDCD6 has been extensively studied in various contexts, its potential role in SCI has remained elusive. We speculated that PDCD6 might be responsible for SCI-associated neuronal death and aimed at clarifying this speculation.

miRNAs, endogenous noncoding RNAs composed of approximately 20 nucleotides [23], exert their multifunctional regulatory roles mainly through acting as epigenetic modulators [24]. By binding to their targeted mRNAs’ 3’ UTR (3’ untranslated region), miRNAs profoundly affect the posttranscriptional gene degradation or silencing [25]. In such a manner, the expression of the majority of protein-coding genes and the corresponding biological processes are under the control of miRNAs [26]. In previous reports, miRNAs were proposed to be essential for repairing SCI [26]; for example, bone marrow mesenchymal stem cells (BMSCs) displayed potential for repairing damaged spinal cord tissue in an in vivo rat model whereas reduced miR-127-5p expression was found to exacerbate SCI via activation of MAPK1 (mitogen-activated protein kinase 1) [27]. Aside from these regulatory roles in SCI, the importance of miRNA in SCI-induced apoptosis is frequently reported; miR-411 could alleviate post-SCI apoptosis and damage induced by inflammation [28]. Similarly, overexpression of miR-129-5p was found to alleviate SCI-related inflammation [29]. Specifically, in SCI rat model, miR-34a-3p was proposed to suppress the expression of CD47, thereby triggering PI3K/AKT signaling and ameliorate SCI-associated apoptosis [30]. In a more recent in vivo study, a reduced miR-34a-3p level was found following induction of SCI. By contrast, upregulating miR-34a-3p validly reduced inflammatory microglia count, accompanied by improved neural structures [31].

Collectively, both miR-34a-3p and PDCD6 were reported to be implicated in the pathophysiology of SCI through modulating apoptosis, and as far as we are aware, there is currently no evidence regarding the joint effect of miR-34a-3p and PDCD6 on post-SCI regeneration. Moreover, EA was found to enhance posts ischemic stroke neurobehavioral function via targeting of SOX2-mediated axonal regeneration by microRNA-132 [32]. Also, EA pretreatment is protective against ischemia/reperfusion injury via miR-214 [33] and miR-124 [34]. However, the underlying mechanism of EA and miR-34a-3p/PDCD6 axis in post-spinal cord injury recovery has never been studied. Hence, the motivation and novelty of the study is to focus on elucidating the potential miR-34a-3p/PDCD6 regulatory axis in NSCs, in order to provide novel perspective for SCI treatment and research. We also aimed to explore the regulatory effect of EA on this pathway in vivo.

2. Materials and Methods

2.1. Target Prediction Using Bioinformatics Tools. To provide a basis for subsequent investigations, we retrieved the 3’ UTR sequence of PDCD6 from the NCBI Gene database. Moreover, the sequence of miR-34a-3p was retrieved from Targetscan (URL: http://www.targetscan.org/). The prediction of binding sites between miR-34a-3p and PDCD6 was carried out by online LncTar noncoding RNA target site
2.2. Separation and Cultivation of NSCs as Neurospheres (NSs). NSCs were extracted from diencephalon and telencephalon from rat at embryonic day 14.5 obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) by dissecting microscope, mechanical pulverisation, and filtering with a 70 mm mesh filter. The extracted NSCs were seeded in a serum-free medium comprising DMEM/F12 (Invitrogen, Grand Island, NY), 100 IU/mL penicillin-streptomycin (Invitrogen, Grand Island, NY), 1% N2 supplement (Invitrogen), 20 ng/mL bFGF (Sigma-Aldrich), and 20 ng/mL EGF (PeproTech, Rocky Hill, NJ). The cells were seeded as detached NSs in low attachment six-well plates, with 4 mL medium/well; 2-3 mL of the medium was substituted each day. NSCs were subcultured at a frequency of 3-5 days via Accutase (Millipore, Bedford, MA) digestion in the medium. The cultures were performed in a 5% CO2 incubator at 37°C.

2.3. Immunocytochemistry Analysis to Characterize NSs. Immunocytochemistry was performed to characterize NSs in vitro with anti-Nestin antibodies (Abs) (a marker for NSCs, 19483-1-AP, ProteinTech), glial fibrillary acidic protein (GFAP) (a marker for astrocytes, 60190-1-lg, ProteinTech), βIII-tubulin (a marker for immature neurons, 11224-1-AP, ProteinTech), and Olig2 (a marker for immature oligodendrocytes, MABN50, MERCK). The nuclei in the NSs were treated with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA) counterstaining. PE- and FITC-conjugated secondary Abs (Jackson Laboratories, West Grove, PA, USA) were added, followed by imaging of NSs with confocal microscopy (Leica sp8, Germany).

2.4. Dual-Luciferase Reporter Assay. The in silico prediction of the binding site between miR-34a-3p and the 3'UTR of PDCD6 was retrieved from bioinformatics website TargetScan (URL: http://www.targetscan.org). Wild-type (WT) PDCD6 3'UTR fragment containing the putative miR-34a-3p binding sequence, along with the mutated version (MUT), was ordered from Sangon Biotech (Shanghai, China). The above sequences were cloned into the downstream of pLenti-UTR-Dual-Luciferase Cloning Vector (Applied Biological Materials, BC, Canada) to generate PDCD6-WT and PDCD6-MUT recombinant vectors. NSCs were plated in 6-well plates for cotransfection with PDCD6-WT/PDCD6-MUT plasmids and miR-34a-3p mimic. After 48 h of transfection, luciferase activity was quantified using a Spectramax® L Microplate Luminometer (Molecular Devices, CA, USA). Relative luciferase activity was calculated by normalizing the intensity of Renilla luciferase against the internal control firefly luciferase.

2.5. In Vitro miRNA Mimic or Inhibitor Transfection. miR-34a-3p mimics or inhibitor, synthesized by Sangon Biotech (Shanghai, China), was used to induce transient in vitro or in vivo miR-34a-3p overexpression or knockdown. For in vitro miRNA transfection, the suspended NSCs (passage < 3) were seeded onto the Costar® 24-well plate (Corning, NY, USA) at a density of 10,000 cells per well and maintained in complete culture medium for 2 d until the NSCs monolayer reached a confluency of 50%. Then, Lipofectamine 2000™ reagent (Thermo Fisher Scientific, USA) was used as a vector for miRNA transfection. Next, NSCs in different wells underwent transfection with miR-34a-3p mimics or inhibitor or the corresponding negative control (NC) (sequence-scrambled oligonucleotides). The cell specimens were harvested at 48 h posttransfection for various downstream analyses unless specified otherwise.

2.6. Western Blotting (WB) Analysis. WB analysis was employed to quantify the protein expression of various neural markers in NSCs and spinal tissues from SCI model. Briefly, each sample was first lysed by RIPA buffer (Beyotime, Shanghai, China) and the resulting lysate was harvested through centrifugation at 4°C, 12000g for 5 min. After boiled water bath with sample loading buffer, the denatured total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) isolation according to their differences in molecular weight. Next, the proteins were electrically transferred onto polyvinylidene difluoride (PVDF, Millipore, CA, USA) membranes. After blocking with skimmed milk for 2 h in room temperature, the PVDF membranes underwent incubation with primary Abs at 4°C overnight, which was followed by incubation with secondary Abs (1:1000) under the same condition. Protein bands were visualized via SuperSignal™ WB Enhancer (Thermo Fisher Scientific, USA). The intensity of each protein band was quantified using the ImageJ software (NIH Image); NIH, Bethesda, MD). The expression of proteins of interest was normalized with β-actin. The level of protein phosphorylation was calculated by normalizing against the corresponding total protein. Aside from anti-MEK1/2 (9122S) and anti-phospho-MEK1/2 (9121S) that were supplied by cell signaling technology (Danvers, MA, USA) and NF-200 (N1412) purchased from Millipore (Temecula, CA, USA), the other Abs used in this study were obtained from Abcam (Cambridge, UK), including anti-PDCD6 (ab109181), anti-βIII-tubulin (ab18207), anti-GFAP (ab7260), anti-MBP (ab209328), and anti-FGFR1 (ab76464).

2.7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). qRT-PCR was employed to evaluate miR-34a-3p expression in differentiating NSCs and in the specimens isolated from the spinal cord of post-SCI model. Total RNA extraction was carried out with TRIzol reagent (Invitrogen, CA, USA) in compliance with the supplier’s instruction. Next, the purified RNA was treated with reverse transcription using the PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). The generated cDNA was employed in qRT-PCR which was executed using the SYBR Green qPCR Master Mix (QIAGEN, Hilden, Germany). The primer pairs specific for miR-34a-3p/U6 (internal control for miRNA) were ordered from Sangon Biotech (Shanghai, China). 2−ΔΔCt [35] calculated the miR-34a-3p expression (normalized against U6). The primer pairs used in qRT-PCR were as follows:
miR-34a-3p: F: 5′-GGGATCCCGTCATATGAAC-3′; R: 5′-GGGATCCCGTCATATGAAC-3′; U6: F: 5′-TTGTGAGTCGGTCTTCGGCA-3′; R: 5′-TTGTGAGTCGGTCTTCGGCA-3′.

2.8. Immunofluorescence Assay. Immunocytochemistry was used to visualize the intracellular localization of the proteins of interest. NSCs were subjected to fixation with 4% paraformaldehyde (PFA, TCI technology, Shanghai, China) and subsequent permeabilization in 0.3% Triton X-100 (Thermo Scientific, CA, USA). For frozen spinal cord tissues pretreatment, samples were first homogenized in room temperature for approximately 15 min and were subsequently soaked in precooled PBS for about 10 min; the antigenicity was then restored by incubation with proteinase K (Thermo Fisher, USA). After pretreatment, 5% Gibco™ bovine serum albumin (Gibco, USA) was used to block to nonspecific binding sites in both cell and tissue samples. The incubation of primary Abs was carried out overnight at 4°C. After PBS rinsing, the signal of the primary Abs was amplified by Alexa Fluor 488 (for GFAP)/568 (for NF200)-conjugated secondary Abs (1:500; Life Technologies, USA), with the simultaneous counterstaining of nucleus using DAPI. An Olympus BX63 fluorescence microscope (Olympus, Tokyo, Japan) was utilized to observe the localization of proteins of interest.

2.9. EdU Proliferation Measurement. NSCs were divided into 1 d and 3 d groups according to the time from miRNA mimic or inhibitor transfection. After reaching confluency, the monolayer NSCs were detached, resuspended, and seeded onto a 96-well plate (Sigma-Aldrich, MO, USA) at a density of 10,000 per well. 20 μM 5-ethynyl-2′-deoxyuridine (EdU, Thermo Fisher Scientific) was placed into the plate. Following a 1 d incubation period, NSCs were fixed by precooled phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA, HEAD biotechnology, Beijing, China). Subsequently, the fixed NSCs underwent incubation with Apollo® reaction cocktails for 30 min under room temperature (22 ± 2°C), followed by DAPI counterstaining. NSCs were then captured in randomly selected cages under a specific field of interest. NSCs were subjected to immunofluorescence Assay which was performed on GV4 (Mingmen), posterior midline underneath the spinous process of the 2nd lumbar vertebra) and GV14 ("Dazhui," posterior midline underneath the spinous process of the 7th cervical vertebra). Briefly, the rats were firmly fixed on wooden boards. Two acupuncture needles (HANS-200E, Jisheng Medical Instruments, China) (0.3 × 25 mm) were inserted to a depth of 5~7 mm at each acupoint, whereby in a continuous-wave of 2 Hz frequency, 0.4 mA intensity was produced. The EA were applied on a daily basis, 5 days per week for a total of 4 consecutive weeks, each time lasting for 20 min. All the above procedures were conducted after isoflurane (1%oxygen + 5%isoflurane).

2.11. Acupuncture Treatment. Electroacupuncture (EA) is a modern version of a traditional Chinese medical therapy, which has been widely utilized for various research purposes [39] [40, 41]. The animals achieved physical stabilization 3 d after the SCI operation and were subjected to an EA stimulation which was performed on GV4 ("Mingmen," posterior midline underneath the spinous process of the 2nd lumbar vertebra) and GV14 ("Dazhui," posterior midline underneath the spinous process of the 7th cervical vertebra). Briefly, the rats were firmly fixed on wooden boards. Two acupuncture needles (HANS-200E, Jisheng Medical Instruments, China) (0.3 × 25 mm) were inserted to a depth of 5~7 mm at each acupoint, whereby in a continuous-wave of 2 Hz frequency, 0.4 mA intensity was produced. The EA were applied on a daily basis, 5 days per week for a total of 4 consecutive weeks, each time lasting for 20 min. All the above procedures were conducted after isoflurane (1%oxygen + 5%isoflurane).

2.12. In Vivo miRNA Transplantation. miR-34a-3p-agomir (and the corresponding NC-agomir), which was specifically designed as an enhanced version of miR-34a-3p agomir to ensure the biostability and miRNA-inhibitory efficacy in an in vivo setting, was used to investigate the in vivo biological impact of miR-34a-3p on post-SCI recovery. Briefly, miR-34a-3p-agomir and the corresponding NC were treated with saline solution dilution to reach a final concentration of 15 nmol/mL. The miR-34a-3p or NC-agomir solutions were filled into the abovementioned minipumps, allowing for the continuous delivery of the solution (at a rate of 1 μL per
hour) into the spinal cord through the mini-pump
cconnected catheter [42]. The treatment was performed on
a daily basis for three consecutive days. After the implant-
tation, the incisions were sutured immediately, and rats
were allowed to recover from the operation under an
appropriate environment.

2.13. Basso-Beattie-Bresnahan (BBB) Score Assessment. The
BBB locomotor test is an evaluation standard designed spe-
cifically for lumbar injury animal models, which assigns a
score (proportional to the degree of recovery of hindlimb
function) to each subject. The BBB test was carried out to
estimate the post-SCI recovery of hindlimb functions at 1,
3, 7, 14, and 28 days post SCI. SD rats from each group were
observed individually in a quiet, open-field setting that per-
mitted free movement. The locomotor functions at various
sites including the hip joints, ankle, and knee were examined
by blinded trained observers according to the open-field
BBB locomotion test [43] for 5 min to evaluate each rat’s
BBB score. The records for each group of animals were
subjected to further statistical analyses. The control group
was the sham-operated animals without functional impair-
ment (BBB score: 21).

2.14. Fluoro-Gold Retrograde Tracing. For Fluoro-Gold (FG)
retrograde tracing, the SD rats first underwent anesthesia,
before a dorsal laminectomy was carried out between the
T11 and 12 vertebral level. Approximately 3
fluoro-gold™ (FG; Biotium, CA, USA) was slowly injected into the
cord caudal to the initial SCI lesion). On the second day post
operation, the FG-labeled SD rats were perfused, allowing
for separation of a spinal segment (10 mm in length) contain-
ing the transection site at T10 vertebral level. The separated
specimens were longitudinally sectioned into slices via a freez-
ing microtome (Thomas Scientific, NJ, USA). Then, the FG-
labeled cells were inspected and counted at five different sites.

2.15. Toluidine Blue Staining Analysis. The spinal cord tis-
iues embedded in paraffin were sectioned into 4 μm slices
and then stained with toluidine blue staining. The tissue sec-
dions were dewaxed into distilled water. Toluidine blue
working solution (5 mL 1%toluidine blue stock solution + 45
mL 1% sodium chloride solution) was used for dying for
2 min, followed by washing with distilled water for three times.
Next, treatment with 0.5% acetic acid for 1 min was performed,
followed by rinsing, dehydration, clearing, and sealing.

2.16. Transmission Electron Microscopy (TEM). To evaluate
myelination, rats (n = 6 per group) were anesthetized for
2.5% glutaraldehyde (Kemiou, Tianjing, China; 25% glutaraldehyde: 4%PFA = 1 : 9) perfusion eight weeks post-operatively. The collected tissue samples were then sequential-
ly subjected to 2.5% glutaraldehyde fixing, 1% osmium
tetroxide treatment, and cleaning in a series of propylene
oxide post dehydration. Subsequently, 50 nm ultrathin
sections were double-stained with 2% lead citrate and uranyl-
lacetate for TEM (HITACHI, HT7800) observation. The
morphology of myelin was observed under TEM in a
blinded manner.

2.17. Statistics and Analysis. All statistical analyses were per-
formed by GraphPad Prism8 (CA, USA), and the data were
presented as mean ± standard deviation (SD). The one-way
ANOVA approach was used to measure the statistical signifi-
cance of one variable among three or more groups, followed
by Tukey’s post hoc multiple comparison test, while the two-
way ANOVA was applied for two or multiple variables in
multiple-groups comparisons, followed by Šidák’s multiple
comparisons test. As to the BBB score, two-way ANOVA
(repeated ANOVA) followed by the Tukey’s multiple com-
parison test was carried out. Analysis results with p < 0.05
were assumed to be statistically significant.

3. Results
The results of the statistical tests performed in this study are
reported in Additional File 1. The results of the experiments
are presented below.

3.1. Characterization of NSCs. NSC characterization was
performed using immunocytochemical staining for Nestin,
GFAP, βIII tubulin, and Olig2 (Figure 1(a)). The cells were
grown in NSs, and the immunohistochemical staining indi-
cated that cells were strongly Nestin- and GFAP-positive,
but low expression levels of βIIItubulin and Olig2 were
recorded (Figure 1(a)), confirming that the isolated cells
were NSCs.

3.2. miR-34a-3p Might Regulate In Vitro Differentiation of
NSC via Suppressing PDCD6. The majority of miRNAs exert
regulatory roles via binding to their target sites in 3’UTR of
miRNAs [44]. We first predicted the direct interaction
between miR-34a-3p and PDCD6 and found a consensus
sequence as a binding site of miR-34a-3p in the 3’UTR of
PDCD6 (Figure 1(b)). To further verify whether miR-34a-
3p regulates PDCD6, we cloned the wild-type (WT) or
mutant (MUT) PDCD6 3’UTR segments containing the
predicted miR-34a-3p binding site (Figure 1(b)) into the
dual-luciferase reporter vector downstream region. Lucifer-
ase activity assay indicated, as shown in Figure 1(c), that
cotransfection of miR-34a-3p mimic and WT PDCD6 3’
UTR significantly decreased the luciferase activity by com-
parison with the control and PDCD6-WT groups, and no
difference was found between the MUT PDCD6 3’UTR+
miR-34a-3p mimic cotransfection and the control and
PDCD6-WT groups, which confirmed a direct interaction
between miR-34a-3p and PDCD6. This result was further
corroborated by WB analysis at the protein level; and as
shown in Figures 1(d) and 1(e), miR-34a-3p mimic obvi-
ously suppressed the expression of PDCD6.

We next examined whether miR-34a-3p could induce
time-course dependent effects on NSC differentiation. To
this end, proliferating NSCs (day 0) and differentiating NSCs
(day 1, 3, 5, or 7) were collected from the culture flask for
subsequent qRT-PCR and WB analyses. miR-34a-3p expres-
sion was increased in differentiating NSCs compared to the
proliferating cells, with high expression levels in the early
stages of differentiation (1 d–5 d) (Figure 1(f)). Moreover,
the WB analysis revealed the gradual increase of PDCD6
Figure 1: Continued.
protein expression during NSC differentiation, reaching a plateau at approximately 5 d (Figures 1(g)–1(h)). The opposite trend between PDCD6 and miR-34a-3p expression sign-posted that miR-34a-3p might regulate NSC differentiation via targeting PDCD6.

3.3. miR-34a-3p Promotes NSC Proliferation and Neuronal Differentiation In Vitro. To investigate the proliferative impact of miR-34a-3p, we used the EdU assay to evaluate the proliferation of miR-34a-3p mimics or inhibitor transfected NSCs at 1 d and 3 d of differentiation (Figure 2(a)). At both time points, the ratio of EdU (red)-positive cells was calculated (Figure 2(b)). Compared to the NCs, the percentage of EdU-positive cells was increased by miR-34a-3p mimic while the opposite was observed with the miR-34a-3p inhibitor at both time points (Figures 2(a) and 2(b)). These results indicated the proproliferative effect of miR-34a-3p on NSCs. Subsequently, we examined the potential role played by miR-34a-3p in regulating the self-renewal and differentiation ability of NSCs. Three important markers of the differentiation NSCs, including neuron-specific marker Class III β-tubulin (βIII tubulin), astrocyte-specific marker GFAP, and oligodendrocyte specific marker myelin basic protein
MBP, were evaluated in miR-34a-3p mimic or inhibitor transfected NSCs at 5 d. The results of immunostaining against βIII tubulin or GFAP are shown in Figure 2(c), along with the quantification results (Figure 2(d)); we found that in the miR-34a-3p mimic group, oligodendrocyte differentiation (manifested by the expression of βIII tubulin) was significantly increased while astrocyte differentiation (manifested by the expression of GFAP) was significantly inhibited; the opposite results were found in the miR-34a-3p inhibitor group compared to the NC groups. Moreover, WB results (Figures 2(e) and 2(f)) indicated that βIII tubulin and MBP were increased by miR-34a-3p overexpression.
but decreased by miR-34a-3p inhibitor; the contrary trends were observed for GFAP. These evidences jointly indicated that miR-34a-3p was involved in the differentiation of NSCs toward neural cells or oligodendrocytes but not toward astrocytes.

3.4. SCI-Associated Signaling Pathways Were Regulated by the miR-34a-3p/PDCD6 Axis. Since we have confirmed the inverse regulation of miR-34a-3p on PDCD6 expression, we further investigated the contribution of miR-34a-3p on PDCD6 expression, and at the same time, inhibited the phosphorylation of MEK1/2.

The results are shown in Figures 3(a) and 3(b), miR-34a-3p mimics induced a significant upregulation of FGFR1, and at the same time, inhibited the phosphorylation of MEK1/2 and downregulated PDCD6 expression; the miR-34a-3p inhibitor induced opposite effects. These evidences suggested that miR-34a-3p may promote the post-SCI recovery mainly through suppressing PDCD6 and promoting FGFR expression while inhibiting MEK1/2 phosphorylation. The subsequent treatment of miR-34a-3p mimic-transfected NSCs with PD089828 (a competitive inhibitor of FGFR1)
demonstrated that PD089828 significantly reversed miR-34a-3p-induced increase of FGFR1 (Figures 3(c) and 3(d)).

3.5. EA Promotes miR-34a-3p/PDCD6 Axis-Facilitated Post-SCI Neural Regeneration in Rats. Our in vitro study suggested that PDCD6 is a pivotal target for miR-34a-3p; hence, we further investigated these observations in vivo. As shown in Figure 4(a), we first found a time-course- (3 d and 7 d after SCI induction) dependent decrease of miR-34a-3p expression in post-SCI spinal cord tissue, which coincided with a gradually increased PDCD6 expression at protein level (Figures 4(b) and 4(c)). Given the notably lower miR-

![Figure 4](image_url)

**Figure 4:** miR-34a-3p effectively facilitated post-SCI axonal regeneration and functional recovery via targeting PDCD6. (a) qRT-PCR quantification of miR-34a-3p expression at different post-SCI time points (3 d and 7 d); the comparison was also made against forced miR-34a-3p overexpression (as miR-34a-3p dropped markedly at 7 d post-SCI). The experiment was performed in triplicate from each of the six animals in each group. The average values obtained from each animal were used in statistical analysis and plotting. (b, c) Western blotting analysis and PDCD6 quantification in SCI rats. The western blotting analysis and quantification revealed that PDCD6 expression was elevated in a time-dependent fashion after the induction of SCI; the obvious miR-34a-3p overexpression at 7 d obviously reversed the SCI-induced PDCD6 elevation. Actin was used as an internal control. The experiments were performed in triplicate from 6 different animals (n = 6). One-way ANOVA followed by Tukey’s multiple comparisons test was utilized to identify differences. **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns: nonsignificant among the compared groups.
34a-3p expression in SCI-7D group compared with SCI-3D group, we selected this time point for miR-34a-3p agomir treatment. As expected, at the 7th day after SCI induction, we found that SCI upregulation of PDCD6 was abolished by the treatment with miR-34a-3p agomir (Figures 4(b) and 4(c)), further confirming the *in vitro* results of the
**Figure 6: Continued.**
interaction between miR-34a-3p and PDCD6. In the subsequent combined treatment of SCI injury with EA and miR-34a-3p-agomir, we found that both EA and miR-34a-3p-agomir stimulated miR-34a-3p expression compared to the SCI group, and this effect was further promoted by the combined treatment of EA and miR-34a-3p-agomir (Figure 5(a)). Moreover, both EA and miR-34a-3p-agomir displayed inhibitory effects on PDCD6 expression at protein level (Figures 5(b) and 5(c)), and the effect of their combination treatment was greater than individual treatments. As reflected by BBB score (Figure 5(d)), the post-SCI hindlimb function was greatly restored after miR-34a-3p-agomir treatment. At the end of the 4-week-long observational period, NF-200 immunostaining showed the continuity of neurofilament in the SCI lesion center. Regenerating neuron fibers were rarely observed in SCI and SCI+NC-agomir groups (Figures 5(e) and 5(f)). In contrast, treatment with EA or miR-34a-3p agomir, either separately or in combination, increased the number of neuron fibers compared to the SCI and SCI+NC-agomir groups, and the combined effect of EA and miR-34a-3p agomir outperformed the treatment with EA or miR-34a-3p agomir single separated treatments (Figure 5(f)). By using FG retrograde tracing, we also examined the plasticity of axons extending from regenerated neurons. As shown in Figures 5(g) and 5(h), a relatively limited number of FG-labeled neurons were observed in both SCI and SCI+NC-agomir groups compared to that in EA- and miR-34a-3p agomir-treated groups. EA and miR-34a-3p exhibited a synergistic effect in promoting motor neuron regeneration (Figure 5(h)).

3.6. EA Promotes miR-34a-3p-Agomir-Facilitated Axonal Myelination. Axonal myelination is pivotal for the normal functioning of the central nerve system [47]. To
comprehensively evaluate the potential of miR-34a-3p as a remyelinating agent, we measured the post-SCI expression of MBP, an established marker for myelination [48], as well as other two important markers NF-200 and GFAP, at protein level. As shown in Figures 6(a)–6(c), the post-SCI expression of MBP and NF-200 was increased in response to EA or miR-34a-3p treatments while the reduced GFAP expression was observed after EA or miR-34a-3p treatments (Figures 6(a) and 6(d)). Immunofluorescence assay results were almost consistent with the WB analyses and indicated that both EA and miR-34a-3p treatments significantly increased the number of axons with positive NF-200 staining and reduced that with positive GFAP staining when compared with the SCI group. These observations (Figures 6(a)–6(g)) suggested that miR-34a-3p may facilitate post-SCI axonal myelination through promoting the expression of NF-200 and MBP and suppressing GFAP. Finally, we visualized post-SCI remyelination in response to miR-34a-3p through TBS. As shown in Figure 6(h), an obvious loss of myelination was observed in both SCI and SCI+NC-ago- mir groups when comparing the uninjured controls. However, the myelinated cells were regained after miR-34a-3p or EA treatments, either individually or in combination (Figure 6(h)). Consistent with the above data, miR-34a-3p exhibited a promyelinating role which was similar to the effect of EA stimulation (Figure 6(i)). The effect of miR-34a-3p and EA combination treatments were stronger than individual treatments (Figure 6(i)). As shown in the electron microscope images (Figure 6(j)), myelin compaction was observed in post-SCI rats that received either EA or miR-34a-3p treatments; in contrast, fragmented myelin was found in post-SCI rats without the preceding treatments (Figure 6(j)). Our current study described a potential miR-34a-3p/PDCD6 axis that promotes the post-SCI recovery by reducing apoptosis (through MEK1/2) and promoting various neuron functions such as neuronal proliferation, motoneurons regeneration, and axonal myelination (through FGFR1) (Figure 7).

4. Discussion

The homeostasis of numerous miRNAs is perturbed after the induction of SCI. Depending on their downstream targets, dysregulation of these epigenetic regulators might result in either alleviated or aggravated post-SCI condition [49]. In the current research, we first found that miR-34a-3p might target PDCD6 and miR-34a-3p-regulated NSCs differentiation via suppressing PDCD6. Considering the proapoptotic nature of PDCD6 [18], we hypothesized that miR-34a-3p might exert an opposite effect by negatively regulating PDCD6, in consistency with previous reports [30, 31]. Our subsequent dual-luciferase reporter assay and WB analysis confirmed our conjecture. Based on these evidences, we further proposed that miR-34a-3p could be beneficial to post-SCI regeneration in the SD rat model.

EA has been proven to facilitate post-SCI neurologic and functional recoveries, but the mechanism remains to be clarified. In this study, we evaluated the involvement of the miR-34a-3p/PDCD6 regulatory axis in the therapeutic effect of EA treatment in an animal SCI model. The results revealed that miR-34a-3p promoted NSC proliferation and neuronal differentiation and regulated SCI-associated signaling pathways by targeting the PDCD6 axis. Interestingly, EA regulation of the miR-34a-3p/PDCD6 axis facilitated post-SCI neural regeneration and axonal myelination in rats. All these suggest that miR-34a-3p plays a critical part in EA therapy for SCI and that miR-34a-3p may be an attractive therapeutic target for SCI treatment.

To further clarify the involvement of neuron-specific markers in the miR-34a-3p/PDCD6 regulating network, we evaluated the expression of FGFR1, MEK1/2, GFAP, MBP, and βIII-tubulin in response to forced (transient) miR-34a-3p overexpression and found that aside from suppressing PDCD6 expression, miR-34a-3p promoted FGFR1, βIII tubulin, and MBP protein levels, while suppressing the protein expression of GFAP and activation of MEK1/2. Aside from its important role in SCI [46], activation of MEK1/2 has long been considered accountable for apoptosis in SCI, because ERK1/2, an important upstream regulator of various apoptotic factors (including caspase 9 and BCL2-family protein Bim), was directly activated by MEK1/2 [50]. The abovementioned evidences suggested that in our current study, the promoted post-SCI regeneration and neural proliferation might be attributed to the miR-34a-3p-induced inhibition of the MEK1/2 signaling. In contrast, delivery of FGFR1 ligand to

![Figure 7: Mechanism of the effect of EA on the miR-34a-3p/PDCD6 axis in SCI.](Image 315x432 to 546x726)
the lesion site of the SCI was shown to be beneficial to post-SCI recovery and neural proliferation [45]. Importantly, FGFR1 is essential for neural proliferation and was upregulated in differentiating neural cells [51]. These observations agreed with our current study showing that miR-34a-3p-induced FGFR1 elevation was accompanied by improved neuronal proliferation, motoneurons regeneration, and axonal myelination.

Currently, there are no solid evidence for the interaction between PDCD6/miR-34a-3p and the preceding neuron markers. However, in cancer cell lines, PDCD6 could mediate the inhibition of ASK1/JNK pathway through Raf-1 (Raf protooncogene, serine/threonine kinase), thereby increasing tumor sensitivity to chemotherapy, and Raf-1 was required for MEK2 activation under proapoptotic conditions [52]. Therefore, Raf-1 might serve as a bridge that connect PDCD6 and the activation of MEK1/2, whereby they work in concert to regulate apoptosis.

The therapeutic efficacy of EA has been validated in various SCI rat model, including promoting motor function [53], improving neuronal function [54], and facilitating the proliferation and differentiation of oligodendrocyte precursor cells [55]. However, the mechanism underlying the therapeutic effect EA is not well elucidated. A previous study investigated the expression profiles of miRNAs involved in EA-treated SCI rats and demonstrated the potential mechanism and functional role as well as the potential as therapeutic targets of miRNAs in SCI rats [56]. Studies have also indicated that EA downregulates the expression of miR-449a to increase NSCs proliferation and promote neuron survival [17]. The miR-214 was also found to be a target of EA in SCI [33]. Herein, we evaluated whether the therapeutic potential of EA could be driven through the miR-34a-3p/PDCD6 axis in post-SCI recovery. We demonstrated that EA as well as the miR-34a-3p/PDCD6 axis facilitates post-SCI neural regeneration and axonal myelination in rats and these effects were further promoted by the combined treatment with EA and miR-34a-3p-agomir, indicating that the miR-34a-3p/PDCD6 axis mediates the therapeutic effect of EA. This study is the first of its kind to establish the therapeutic regulatory role of EA on miR-34a-3p in post-SCI recovery.

In summary, we demonstrated that this pathway is a target for EA. These results might provide a novel perspective for developing therapeutic strategies against SCI and shed light on the pathogenesis and contribute in the in-depth researches on post-SCI recovery. However, this study still has room for improvement. miRNAs may present differential expression in different cell types, which can directly or indirectly regulate target mRNAs. In addition, a miRNA may have multiple target mRNAs, resulting in the difficulty in evaluating the interaction between its target mRNAs. In addition, accurate acupoint positioning is also crucial for EA. Therefore, further studies with a larger sample size are needed to clarify the relationship between EA, miRNA changes, protein expression, and spinal cord injury recovery.

**Data Availability**

All relevant data are within the paper.

**Ethical Approval**

All experimental procedures involving experimental animals were carried out in compliance with the local animal welfare laws/policies, and the approval from the laboratory animal ethics committee of Taizhou Enze Medical Center (Approval No. tzy-20180078) was obtained.

**Conflicts of Interest**

The authors declare no competing interests.

**Authors’ Contributions**

QD, LLM, and LZM wrote the manuscript and interpreted the data. YY and TC contributed to the drafting of the manuscript and data analysis. QD designed the study. All the authors have read and agreed to submit the final version of the manuscript. Lili Ma and Lizhong Ma contributed equally to this work and are co-first authors.

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