lncRNA TNXA-PS1 Modulates Schwann Cells by Functioning As a Competing Endogenous RNA Following Nerve Injury

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As the major glia in PNS, Schwann cells play a critical role in peripheral nerve injury repair. Finding an efficient approach to promote Schwann cell activation might facilitate peripheral nerve repair. Long noncoding RNAs (lncRNAs) have been shown to regulate gene expression and take part in many biological processes. However, the role of lncRNAs in peripheral nerve regeneration is not fully understood. In this study, we obtained a global lncRNA portrayal following sciatic nerve injury in male rats using microarray and further investigated one of these dys-regulated lncRNAs, TNXA-PS1, confirming its vital role in regulating Schwann cells. Silencing TNXA-PS1 could promote Schwann cell migration and mechanism analyses showed that TNXA-PS1 might exert its regulatory role by sponging miR-24-3p/miR-152-3p and affecting dual specificity phosphatase 1 (Dusp1) expression. Systematic lncRNA expression profiling of sciatic nerve segments following nerve injury in rats suggested lncRNA TNXA-PS1 as a key regulator of Schwann cell migration, providing a potential therapeutic target for nerve injury repair.

Key words: competing endogenous RNA; long noncoding RNA; migration; peripheral nerve injury; Schwann cell

Significance Statement

The PNS has an intrinsic regeneration capacity after injury in which Schwann cells play a crucial role. Therefore, further exploration of functional molecules in the Schwann cell phenotype modulation is of great importance. We have identified a set of dys-regulated long noncoding RNAs (lncRNAs) in rats following sciatic nerve injury and found that the expression of TNXA-PS1 was significantly downregulated. Mechanically analyses showed that TNXA-PS1 might act as a competing endogenous RNA to affect dual specificity phosphatase 1 (Dusp1) expression, regulating migration of Schwann cells. This study provides for the first time a global landscape of lncRNAs following sciatic nerve injury in rats and broadens the known functions of lncRNA during nerve injury. The investigation of TNXA-PS1 might facilitate the development of novel targets for nerve injury therapy.

Introduction

Peripheral nerve injury could result in severe sensory and motor dysfunction by damaging the integrity of axon and Schwann cells,

affecting the quality of life (Wu and Murashov, 2013; Cheng et al., 2015). Although the traditional microsurgical approach may restore nerve continuity, limitations exist in nerve regeneration and functional recovery because of an insufficient understanding of neurobiology following peripheral nerve injury (Yu et al., 2015). Nerve regeneration is a complex biological process, orchestrated by multiple cells, growth factors, and extracellular matrix (Tang et al., 2013). Schwann cells, the major glia cells in PNS, provide a suitable microenvironment for nerve regeneration (da Silva et al., 2014). Following axonal degeneration by nerve injury, Schwann cells undergo a series biological processes, including dedifferentiation, proliferation, migration, removal of myelin debris, secretion of neurotrophic factors, and remyelination, facilitating nerve regeneration (Heinen et al., 2013). Schwann cell migration

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can precede and enhance axonal repair by guiding axon reinnervation and controlling synaptic formation (Son et al., 1996; Triguero et al., 1996; Forciniti et al., 2014).

With the development of high-throughput sequencing and array-based technologies, various kinds of noncoding RNAs (ncRNAs) have been identified. One major class of ncRNAs is long noncoding RNAs (lncRNAs), which are long transcripts (>200 nt) without obvious protein-coding potential (Geisler and Collier, 2013). Although many lncRNAs may be transcriptional noise, a growing number of lncRNAs have been reported to have biological functions. They could regulate various biological processes, including dosage compensation, genomic imprinting, cell cycle, differentiation, and development, at the epigenetic, transcriptional, and posttranscriptional levels (Wilusz et al., 2009; Chen and Carmichael, 2010; Yang et al., 2014). In addition, the dysregulation of lncRNAs appears to be involved in many diseases, especially in cancers (Xiang et al., 2014; Zhang et al., 2014). Emerging evidence highlights that lncRNAs are expressed in CNS and mediate neural cell activities, contributing to various CNS pathologies (Qureshi et al., 2010). However, the specific roles of lncRNAs in peripheral nerve injury, especially in Schwann cell phenotype modulation, are not well studied.

Our group has previously identified altered lncRNA expression in dorsal root ganglia (DRG) after sciatic nerve injury and found lncRNA uc.217 could affect neurite outgrowth in DRG neurons (Yu et al., 2013; Yao et al., 2015). Recently, Pan et al. (2017) reported time-dependent lncRNA alteration in mice by microarray. However, they did not fully explore the functions and mechanisms underlying certain lncRNAs. Here, we performed microarray aiming at detecting lncRNA expression alteration in sciatic nerve segment after sciatic nerve crush injury in rats and selected one specific lncRNA TNXA-PS1 for further investigation. We confirmed the downregulation of lncRNA TNXA-PS1 following peripheral nerve injury and found that silencing lncRNA TNXA-PS1 in Schwann cells could promote Schwann cell migration. Investigation of the mechanism responsible for this showed that lncRNA TNXA-PS1 might exert this role by competing with miRNAs to regulate the expression of dual specificity phosphatase 1 (Dusp1) in Schwann cells, providing a novel mechanism and a potential therapeutic target for peripheral nerve injury.

Materials and Methods

Animal surgery and tissue preparation. Twenty-four adult, male Sprague Dawley (SD) rats (180–220 g) were randomly divided into four groups of six rats each. All animals underwent surgery of sciatic nerve crush injury. Briefly, the sciatic nerve at 10 mm above the bifurcation into the tibial and common fibular nerves was crushed with a forceps at a force of 54 N for 3 times (a period of 10 s for each time). The proximal stumps of sciatic nerve (0.5 cm) were collected at 0, 1, 4, and 7 d after injury, respectively. The experiment was repeated three times. All the experimental procedures involving animals were conducted in accordance with institutional animal care guidelines and were approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

RNA extraction, microarray, and bioinformatic analyses. Total RNA was extracted from the sciatic nerve segment using TRIzol (Invitrogen) and RNA cleanup including a DNase I digestion step was performed using RNeasy spin columns (Qiagen). Total RNA from each sample was quantified using the Nano Drop 1000. We used the Array platform (Agilent Technologies) for microarray analyses. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols as described previously (Yu et al., 2013). lncRNA and mRNA expression profiles were compared and differentially expressed

genes were screened for different time points. The differentially expressed lncRNAs then underwent hierarchical clustering and expression pattern clustering analyses (Liao et al., 2011; Yu et al., 2013). In hierarchical clustering, the Z score was calculated from the expression of lncRNAs and the Euclidean distance measure was used to compute the distance (dissimilarity) in lncRNA and time. In expression pattern clustering, the significance analysis of expression tendency was screened for differentially expressed lncRNAs. Using a strategy for clustering short-time-series gene expression data, we defined some unique profiles. The expression model profiles are related to the actual or the expected number of genes assigned to each model profile. Significant profiles have higher probability than expected by Fisher's exact test and multiple-comparisons test.

A gene coexpression network was built according to the normalized signal intensity of specific expression lncRNAs and mRNAs (Liao et al., 2011). Gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses were performed to elucidate biological processes and signaling pathways associated with the correlated targets of specific lncRNAs.

qRT-PCR. RNA samples were reverse transcribed to cDNA with the Prime-Script RT reagent Kit (TaKaRa). qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa) on an ABI StepOne system (Applied Biosystems) according to standard protocols in triplicate for each sample. The relative expression level was calculated using the comparative $2^{-\Delta\Delta C_t}$ method.

In situ hybridization. Digoxigenin (DIG)-labeled rat lnc TNXA-PS1 probes were synthesized using the DIG RNA Labeling Kit (Roche). After treatment with proteinase K, the acetylated sciatic nerve segment sections were prehybridized for 2 h and then hybridized with DIG-labeled probes overnight at 37°C in a humid chamber. The sections were then blocked with AKP-conjugated Fab anti-DIG antibody (Roche) overnight at 4°C and stained by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Roche) for visualization and imaging.

5' and 3' rapid amplification of cDNA ends (RACE) analysis. RACE analyses were performed with total RNA extracted from sciatic nerve tissues using the SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. The RACE products were cloned into pGM-T vector (Tiangen Biotech) and sequenced.

Northern blotting. Northern blot was performed with the Dig Northern Starter Kit (Roche). Briefly, DIG-labeled rat lnc TNXA-PS1 probes were synthesized. Total RNA (10 μ g) of sciatic nerve segment tissues was subjected to formaldehyde gel electrophoresis and transferred onto a nylon membrane. After being prehybridized with northern blot hybridized buffer for 3 h, the membranes containing RNA were incubated with the labeled denatured probes at 60°C overnight. The following day, membranes were washed and detected.

Primary culture of Schwann cells and cell transfection. Schwann cells were isolated from sciatic nerves of 1-d-old SD rats and treated to remove the fibroblasts using anti-Thy1.1 antibody (Sigma-Aldrich) and rabbit complement (Invitrogen) as described previously (Mantuan et al., 2008). The final cell preparation consisted of 98% Schwann cells, as determined by immunostaining with the specific Schwann cell marker S100 β . Primary culture of Schwann cells was maintained in DMEM containing 10% fetal bovine serum (complete medium) at 37°C under humidified 5% CO₂. The cell culture was passaged no more than three times before the following tests.

Primary cultured Schwann cells were transfected with lnc TNXA-PS1 or Dusp1 siRNAs (Ribobio) with Lipofectamine RNAiMAX transfection reagent (Invitrogen). Schwann cell migration was examined using the Transwell migration assay as described previously (Mantuan et al., 2008). Transwell chambers (6.5 mm with 8 μ m pores; Costar) and the bottom surface of each membrane was precoated with 10 μ g/ml fibronectin. Briefly, Schwann cells in DMEM (100 μ l, 10⁶ cells/ml) were transferred to the top chamber of the Transwell and 600 μ l of complete medium was injected into the lower chamber. After incubating at 37°C in 5% CO₂ for 24 h, the upper surface of each membrane was cleaned with a cotton swab and cells adhering to the bottom surface were stained with 0.1% crystal violet. Each bottom surface was then imaged with >5 views

and counted with a DMR inverted microscope (Leica Microsystems). Assays were performed three times using triplicate wells.

Isolation of cytoplasmic and nuclear RNA. Cytoplasmic and nuclear RNA of Schwann cells were isolated and purified using the PARIS Kit (Life Technologies) according to the manufacturer's instructions. The relative expression of lnc TNXA-PS1 in each fraction was then detected by qRT-PCR.

FISH. FISH for lnc TNXA-PS1 was conducted on 4% PFA-fixed slides with monolayers of Schwann cells using a DIG-labeled probe at 1 μ g/ml concentration. Hybridization was performed at 42°C overnight, followed by incubating with anti-DIG antibody and secondary antibody. Slides were subsequently counterstained using Hoechst and observed under a microscope.

Competing endogenous RNA (ceRNA) target gene prediction. We used an approach termed mutually targeted miRNA response element (MRE) enrichment (MuTaME), as described previously (Tay et al., 2011), to search possible target genes of TNXA-PS1 as ceRNA. Briefly, MuTaME evaluates a candidate ceRNA target genes based on the number of miRNAs and the MREs shared by the target genes and TNXA-PS1. This bioinformatic analysis was conducted by rna22 microRNA target prediction algorithm (Miranda et al., 2006) to generate MuTaME scores (KangChen Bio-tech).

Luciferase reporter assay. The 3'-UTR sequence of Plxn3, Dusp1, Slit1, and the full length of TNXA-PS1 were constructed into pmirGLO vector and cotransfected with certain miRNAs into 293T cells. The relative luciferase activity was normalized to Renilla luciferase activity 48 h after transfection.

Western blot analysis. Protein extracts were prepared from primary cultured Schwann cells. Equal amounts of protein were electrophoresed on 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). Blots were probed with antibody against Dusp1 (Abcam). *In vivo tests for Schwann cell migration.* The *in vivo* Schwann cell migration was analyzed as described previously (Zhou et al., 2014). A silicone conduit (inner diameter 1.0 mm) was inserted into the 5 mm sciatic nerve gap between the proximal and distal stumps in a rat sciatic nerve transection model. Cy5-labeled lnc TNXA-PS1 siRNA or control (Ribobio) mixed with Matrigel (1:1 v/v) (BD Biosciences) was slowly injected into the conduit lumen using a precooled micropipette. The surgical incision was closed in a routine fashion and animals were housed in large cages with sawdust bedding to minimize the discomfort and possible painful mechanical stimulation.

At 10 d after surgery, rats were killed and the silicon conduits containing regenerated nerves were collected. The migration of Schwann cells and axon outgrowth were observed by immunostaining with anti-Sox10 (CST) and anti- β -tubulin III antibody (Sigma-Aldrich), respectively.

Statistical analysis. All data are presented as means $\pm$ SEM. Statistical comparison was performed with Student's *t* test using Prism 5 software (GraphPad). *p* < 0.05 was considered statistically significant.

Results

lncRNA expression profile in sciatic nerve after sciatic nerve injury

Following sciatic nerve crush injury, the sciatic nerve underwent degeneration with much axon and Schwann cell debris as shown by electron microscopic and immunohistochemistry analysis (Fig. 1*A,B*). After sciatic nerve injury, the layers of myelin sheath were condensed as normal in 1 d. However, at 4 d, sciatic nerve degenerated with the loosening of myelin sheath structure. Up to 7 d, the regenerated axons were remyelinated by Schwann cells. Rat sciatic nerve segments were then collected at 0, 1, 4, and 7 d. Using Arraystar Rat lncRNA microarray, we obtained a global landscape of lncRNAs and mRNAs following sciatic nerve injury, among which there was a total of 758 differentially expressed lncRNAs and 1387 mRNAs. Hierarchical clustering showed systematic variations in the expression of these lncRNAs at various time points and indicated that these lncRNA expression could be divided into two groups, Group 1 (0 and 1 d) and Group 2 (4 and

7 d) (Fig. 1*C*), which may represent the preregeneration phase and regeneration phase, respectively. We then summarized lncRNA expression with 26 model profiles. Among these, there were six profiles with significant *p*-values for expression tendency (Fig. 1*D*). Profile 1 contained 124 differentially expressed lncRNAs, which were downregulated following nerve injury (Fig. 1*E*). A gene coexpression network was constructed to cluster 124 lncRNAs of profile 1 and coding mRNA, suggesting that 268 coding mRNAs were coexpressed, positively or negatively, with these 124 lncRNAs during the time course (Fig. 1*F*). GO analysis for the coexpressed 268 coding genes showed that the biological processes of these genes were enriched in cell adhesion and regulation of cytoskeleton, suggesting that cell migration might change (Table 1).

lnc TNXA-PS1 expression and characteristics

We then chose one of the lncRNAs in profile 1, NR_024118 (lnc TNXA-PS1, tenascin XA, pseudogene 1), for further validation. Consistent with our microarray analysis, lnc TNXA-PS1 was downregulated following nerve injury as detected by qRT-PCR (Fig. 2*A*, 0 d: 0.216 ± 0.0106 ; 1 d: 0.131 ± 0.012 , *p* = 0.0057 vs 0 d; 4 d: 0.074 ± 0.001 , *p* = 0.0002 vs 0 d; 7 d: 0.107 ± 0.014 , *p* = 0.0033 vs 0 d; *n* = 3 for each group, Student's *t* test). *In situ* hybridization further indicated the downregulation of lnc TNXA-PS1 expression in Schwann cells at 4 d after sciatic nerve injury (Fig. 2*B*).

To investigate the functions and molecular mechanism of lncRNAs, a full length of the certain lncRNA is essential. Therefore, we tried to obtain the full sequence of TNXA-PS1 by RACE experiment and found that lnc TNXA-PS1 was 1548 nt in length (Fig. 2*C*). This transcript was located on chromosome 20p12 and comprised 11 exons, as shown in the USCS Genome Browser (Fig. 2*D*). Northern blots also revealed that the full-length of lnc TNXA-PS1 was ~1500 nt and was expressed stably in sciatic nerve segments (Fig. 2*E*).

We then isolated cytoplasmic and nuclear RNA from primary cultured Schwann cells and detected TNXA-PS1 expression in each fraction by qRT-PCR. The results shown that, compared with U6 (mostly expressed in nuclear), TNXA-PS1 is mainly expressed in cytoplasm (Fig. 2*F*, TNXA-PS1-nuclear: 22.5 ± 3.66 ; TNXA-PS1-cytoplasm: 77.5 ± 3.66 ; actin-nuclear: 20.6 ± 0.445 ; actin-cytoplasm: 79.4 ± 0.445 ; U6-nuclear: 50 ± 3.98 ; U6-cytoplasm: 50 ± 3.98 ; *n* = 3 for each group). Further FISH experiments on Schwann cells also confirmed the mainly cytoplasm localization of TNXA-PS1 (Fig. 2*G*).

Silencing of lnc TNXA-PS1 promotes Schwann cell migration both *in vitro* and *in vivo*

Specific TNXA-PS1 siRNAs were transfected into Schwann cells to explore the role of lnc TNXA-PS1 on Schwann cells. The mRNA expression of lnc TNXA-PS1 could be reduced significantly by both siRNA1 and siRNA2 to ~40% [Fig. 3*A*, negative control (NC): 1.027 ± 0.037 ; TNXA-PS1 siRNA1: 0.455 ± 0.153 , *p* = 0.0223 vs NC; TNXA-PS1 siRNA2: 0.393 ± 0.056 , *p* = 0.0007 vs NC; *n* = 3 for each group, Student's *t* test]. Transwell migration assays showed that silencing of lnc TNXA-PS1 expression in Schwann cells could promote their migration significantly compared with siRNA controls (Fig. 3*B*, NC: 1 ± 0.082 ; TNXA-PS1 siRNA1: 3.517 ± 0.167 , *p* < 0.0001 vs NC; TNXA-PS1 siRNA2: 2.628 ± 0.275 , *p* = 0.0013 vs NC; *n* = 4 for each group, Student's *t* test). In addition, TNXA-PS1 downregulation could also promote Schwann cell proliferation by approximately twofold.

Figure 1. Dys-regulated lncRNAs following peripheral nerve injury. **A**, Electron microscopic analysis of cross-sections of sciatic nerves at 0, 1, 4, and 7 d following sciatic nerve crush injury. Scale bar, 4 μ m. Magnification images are shown in the right corner. Scale bar, 0.2 μ m. **B**, Immunohistochemistry images of longitudinal sections of sciatic nerve at 0, 1, 4, and 7 d following sciatic nerve crush injury. The white dotted line indicates the injury site. Schwann cells were stained with anti-S100 β (green) and axons were stained with anti- β Tubulin III (red). Scale bar, 1000 μ m. Images on the right represent higher magnifications of the images on the left. Scale bar, 20 μ m. **C**, Heatmap of 758 significantly differentially expressed lncRNAs at four (Figure legend continues.)

Table 1. GO analysis

GO ID	GO terms	Count	p-value
GO:0007186	G-protein-coupled receptor protein signaling pathway	45	0.0001
GO:0032501	Multicellular organismal process	102	0.0002
GO:0051493	Regulation of cytoskeleton organization	10	0.0002
GO:0031109	Microtubule polymerization or depolymerization	5	0.0004
GO:0050877	Neurological system process	48	0.0008
GO:0007606	Sensory perception of chemical stimulus	33	0.002
GO:0046878	Positive regulation of saliva secretion	2	0.002
GO:0050774	Negative regulation of dendrite morphogenesis	2	0.002
GO:0044062	Regulation of excretion	3	0.002
GO:0046777	Protein autophosphorylation	7	0.002

We then wondered whether TNXA-PS1 could regulate Schwann cell migration *in vivo*. To investigate this, we injected Cy-5-labeled TNXA-PS1 siRNA and its negative control into the silicone-based nerve guidance conduit in a rat model of sciatic nerve injury. As detected by Sox10, a specific marker of Schwann cells, the distance of migrated Schwann cells was increased in the group of TNXA-PS1 siRNA injection (Fig. 3C,D, NC: 3212 ± 115.2 ; TNXA-PS1 siRNA1: 6543 ± 250.2 , $p = 0.0003$ vs NC; TNXA-PS1 siRNA2: 5573 ± 255.1 , $p = 0.0011$ vs NC; $n = 3$ for each group, Student's *t* test). Moreover, immunostaining with anti- β -tubulin III showed that downregulation of TNXA-PS1 expression could also enhance axon regeneration, as estimated by the distance of axon outgrowth (Fig. 3E,F, NC: 3156 ± 60.59 ; TNXA-PS1 siRNA1: 5156 ± 561.7 , $p = 0.024$ vs NC; TNXA-PS1 siRNA2: 4733 ± 468.1 , $p = 0.0288$ vs NC; $n = 3$ for each group, Student's *t* test). Therefore, these data suggested that TNXA-PS1 could also regulate Schwann cell migration *in vivo*, facilitating nerve regeneration.

lnc TNXA-PS1 functions as an endogenous miRNA sponge in Schwann cells

Next, we sought to explore the mechanisms behind the potent effect of lnc TNXA-PS1 on Schwann cell migration. Interactions with proteins have been recognized as a possible way that lncRNA to exerts its role (Moran et al., 2012). We then performed a RNA pull-down assay to identify proteins that are associated with lnc TNXA-PS1. However, we could not find any specific band between TNXA-PS1 and its antisense RNA (data not shown). Several recent reports also suggested that lncRNAs could function as ceRNA and affect the negative regulation of miRNAs on its target genes (Wang et al., 2013). Based on MRE enrichment (MuTaME) (Miranda et al., 2006; Tay et al., 2011), we identified 255 genes that could be the target gene regulated by TNXA-PS1 in the way of ceRNA. We then compared these genes with genes downregulated by TNXA-PS1 siRNAs as detected by microarray (1324 genes) and obtained 26 genes overlapped (Fig. 4A). Among them, nine genes (Ovol1, Ppargc1b, Plxna3, Bnpl, Aes, Dusp1, Sardh, Sfrp4 and Slit1) have been reported previously to be involved in cell migration and proliferation (Torigoe et al., 1996; Bellou et al., 2009; Fenster et al., 2010; Chen et al., 2011; Sun et al., 2014; Yuan

et al., 2014; Duque-Afonso et al., 2015; Quek et al., 2015; Furió-Tarí et al., 2016; Liu et al., 2016; Wang et al., 2016). After qRT-PCR validation, four genes (Plxna3, Slit1, Dusp1, and Ovol1) were downregulated by silencing TNXA-PS1 as the microarray analysis and were selected for further investigation (Fig. 4B, Plxna3: 0.317 ± 0.041 , $p = 0.0005$ vs NC; Slit1: 0.313 ± 0.032 , $p = 0.0002$ vs NC; Dusp1: 0.671 ± 0.087 , $p = 0.0321$ vs NC; Ovol1: 0.503 ± 0.045 , $p = 0.0016$ vs NC; $n = 4$ for each group, Student's *t* test). These genes were also downregulated following nerve injury, especially at day 1 (Fig. 4C, Plxna3: 1 d: 0.172 ± 0.003 , $p = 0.0002$ vs 0 d; 4 d: 0.318 ± 0.010 , $p = 0.0004$ vs 0 d; 7 d: 0.751 ± 0.064 , $p = 0.061$ vs 0 d; Slit1: 1 d: 0.273 ± 0.011 , $p = 0.0183$ vs 0 d; 4 d: 0.544 ± 0.011 , $p = 0.0447$ vs 0 d; 7 d: 0.544 ± 0.033 , $p = 0.0486$ vs 0 d; Dusp1: 1 d: 0.394 ± 0.008 , $p = 0.0045$ vs 0 d; 4 d: 0.415 ± 0.018 , $p = 0.0056$ vs 0 d; 7 d: 0.523 ± 0.008 , $p = 0.0073$ vs 0 d; Ovol1: 1 d: 0.306 ± 0.002 , $p = 0.0029$ vs 0 d; 4 d: 0.406 ± 0.015 , $p = 0.0046$ vs 0 d; 7 d: 1.046 ± 0.013 , $p = 0.3646$ vs 0 d).

We then sought to validate the relationship among TNXA-PS1, miRNAs, and target genes. As shown in Figure 4A, there were 11 miRNAs shared by TNXA-PS1 and the selected four genes. We constructed a luciferase plasmid of TNXA-PS1 (pmirGLO-TNXA-PS1). Luciferase assay showed that miR-24-3p, miR-320-3p, miR-485-5p, miR-152-3p, and miR-330-5p could reduce the luciferase activity of TNXA-PS1 significantly, suggesting that TNXA-PS1 may interact with these five miRNAs (Fig. 4D, miR-24-3p: 0.847 ± 0.014 , $p = 0.0084$ vs MC; miR-320-3p: 0.702 ± 0.047 , $p = 0.0245$ vs MC; miR-485-5p: 0.748 ± 0.011 , $p = 0.0019$ vs MC; miR-152-3p: 0.861 ± 0.017 , $p = 0.0148$ vs MC; miR-330-5p: 0.597 ± 0.034 , $p = 0.007$ vs MC; $n = 3$ for each group, Student's *t* test). The 3'-UTR of the predicted target gene of these miRNAs, Plxna3, Dusp1, and Slit1, were also constructed into luciferase vector and luciferase assay indicated that the 3'-UTR of Plxna3 and Dusp1 could bind with miR-24-3p/320-3p/485-5p and miR-24-3p/152-3p, respectively (Fig. 4E, Plxna3 group: miR-24-3p: 0.668 ± 0.018 , $p = 0.0029$ vs MC; miR-320-3p: 0.823 ± 0.029 , $p = 0.0251$ vs MC; miR-485-5p: 0.464 ± 0.031 , $p = 0.0034$ vs MC; Dusp1 group: miR-24-3p: 0.836 ± 0.026 , $p = 0.0246$ vs MC; miR-152-3p: 0.578 ± 0.026 , $p = 0.0039$ vs MC; miR-330-5p: 1.086 ± 0.067 , $p = 0.3247$ vs MC; $n = 3$ for each group, Student's *t* test). Dusp1 was selected for further validation. It was shown that miR-24-3p and miR-152-3p could decrease both mRNA and protein expression level of Dusp1 in Schwann cells (Fig. 4F,G, Dusp1 mRNA: miR-24-3p: 0.499 ± 0.037 , $p = 0.0054$ vs MC; miR-152-3p: 0.303 ± 0.039 , $p = 0.0031$ vs MC; Dusp1 protein: miR-24-3p: 0.346 ± 0.079 , $p = 0.0142$ vs MC; miR-152-3p: 0.273 ± 0.095 , $p = 0.0168$ vs MC; $n = 3$ for each group, Student's *t* test).

Dusp1 inhibited Schwann cell migration

We suspected that TNXA-PS1 might regulate Schwann cell migration through Dusp1 by sponging certain miRNAs, so we further detected the effect of Dusp1 on Schwann cell migration. The mRNA and protein expression of Dusp1 were both downregulated significantly by Dusp1 siRNAs (Fig. 5A, NC: 0.037 ± 0.003 ; Dusp1 siRNA1: 0.017 ± 0.004 , $p = 0.0105$ vs NC; Dusp1 siRNA2: 0.012 ± 0.001 , $p = 0.0004$ vs NC; $n = 4$ for each group, Student's *t* test). Transwell migration assay showed that interfering Dusp1 expression by siRNAs could promote Schwann cell migration (Fig. 5B; Dusp1 siRNA1: 1.634 ± 0.208 , $p = 0.056$ vs NC; Dusp1 siRNA2: 2.013 ± 0.169 , $p = 0.0078$ vs NC; $n = 3$ for each group, Student's *t* test). We also constructed plasmid of Dusp1 (pcDNA3.1-Dusp1) and transfected it into Schwann cells.

(Figure legend continued.) indicated time points after nerve injury. The color scale on the top illustrated the relative expression level of lncRNAs across all samples: red indicates expression >0 and green indicates expression <0 . D, Six profiles of significant *p*-values according to expression pattern clustering. E, Detailed expression changes of the 124 differentially expressed lncRNAs in sciatic nerve segment after nerve injury in Profile 1. F, Coexpression network of lncRNAs in Profile 1 with correlated target genes. lncRNAs and coding mRNAs are presented as red squares and blue circles, respectively. The solid line between lncRNAs and coding mRNAs indicates positive correlation in expression; the dotted line indicates negative correlation.

Figure 2. Expression and localization of Inc TNXA-PS1. **A**, Validation of TNXA-PS1 expression in sciatic nerve segments after nerve injury as shown by qRT-PCR. **B**, *In situ* hybridization of TNXA-PS1 on sciatic nerve tissue sections at 0 and 4 d after nerve injury. Scale bar, 10 μ m. **C**, Full sequence of TNXA-PS1 as detected by RACE. The nucleotide sequence of the full-length TNXA-PS1 identified by RACE is indicated in red. The reference sequence of TNXA-PS1 (NR_024118) is from the NCBI database. **D**, Scheme of TNXA-PS1 in rat genome searched by UCSC Genome Browser. The red line in the chromosome indicates the localization of IncRNA TNXA-PS1 in genome. Under the chromosome is the composition of TNXA-PS1. The black blocks indicate exons and the line between blocks represents introns. The arrows in line suggested the direction of transcription. RefSeq genes around IncRNAs TNXA-PS1 are also illustrated. **E**, Northern blot analysis of TNXA-PS1 in sciatic nerve tissue. Lines 1–3 represent sciatic nerve tissue RNA samples of three rats. **F**, Fractionation of Schwann cell lysates demonstrating cytoplasm expression of TNXA-PS1. β -Actin served as a positive control for cytoplasm gene expression; U6 served as a positive control for nuclear gene expression ($n = 4$ for each group). **G**, Subcellular localization analysis of Inc TNXA-PS1 by RNA FISH demonstrating localization of TNXA-PS1 in the cytoplasm. Scale bar, 10 μ m. Values are mean $\pm$ SEM. Statistical significance was assessed by two-tailed Student's t test, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. TNXA-PS1 affects Schwann cell migration. **A**, TNXA-PS1-specific siRNAs decreased TNXA-PS1 expression in Schwann cells as detected by qRT-PCR ($n = 3$ for each group). **B**, Both siRNA-1 and siRNA-2 of TNXA-PS1 promoted Schwann cell migration significantly compared with siRNA control. Representative images and quantification data of Transwell migration assay are shown. Scale bar, 50 μm ($n = 4$ for each group). **C**, Schwann cell migration in silicon conduits injected with TNXA-PS1 siRNA was faster compared with negative control (NC) as detected by immunostaining. Red: Sox10 positive Schwann cells; blue: cell nucleus. The white arrow indicates the edge of the proximal stump and the distance of Schwann cells migration from the edge of the proximal stump (white dotted line) were counted. Scale bar, 1000 μm . Images in the right represented the higher magnifications of the white square in the left images. Scale bar, 20 μm . **D**, Statistical comparison for the average distance of migrating Schwann cells in silicon conduits, $n = 3$ for each group. **E**, Axon outgrowth in the regenerative conduit injected with TNXA-PS1 siRNA or with NC. The proximal stump of the transection site is indicated by white arrows and the end of axon outgrowth is labeled with white dotted line. Axons were immunostaining by β -Tubulin III in green. Scale bar, 1000 μm . Images on the right represent higher magnifications of the white square in the left images. Scale bar, 20 μm . **F**, Average distance of axon outgrowth in silicon conduits ($n = 3$ for each group). Values are mean $\pm$ SEM. Statistical significance was assessed by two-tailed Student's t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. TNXA-PS1 might regulate Dusp1 expression by binding with miR-24-3p and miR-152-3p. **A**, Coverage of the downregulated genes by TNXA-PS1 siRNA and target genes of TNXA-PS1 as ceRNA. Four possible target genes were selected and the binding sites of miRNAs with TNXA-PS1 and target genes are illustrated on the right. **B**, Validation of the downregulation of target genes of TNXA-PS1 by TNXA-PS1 siRNA in Schwann cells ($n = 4$ for each group). **C**, Validation of target gene expression in sciatic nerve segment following nerve injury ($n = 4$ for each group). **D**, Luciferase analysis of the interaction between TNXA-PS1 and the related 11 miRNAs ($n = 3$ for each group). **E**, Luciferase analysis of the interaction between target genes and the related miRNAs ($n = 3$ for each group). **F**, mRNA expression of Dusp1 was downregulated by miR-24-3p and miR-152-3p, $n = 3$ for each group. **G**, Protein expression level of Dusp1 was downregulated by miR-24-3p and miR-152-3p. Top, Western blot of Dusp1. Bottom, Quantification of Western blot results ($n = 3$ for each group). Values are mean $\pm$ SEM. Statistical significance was assessed by two-tailed Student's t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. TNXA-PS1 regulate Schwann cell migration by Dusp1. **A**, Interfering efficiency of siRNAs on Dusp1 in Schwann cells as detected by qRT-PCR (left) and Western blot (right) ($n = 4$ for each group). **B**, Silencing Dusp1 promotes Schwann cell migration compared with siRNA control. Representative images and quantification data of transwell migration assay are shown ($n = 3$ for each group). Scale bar, 50 μm . **C**, mRNA (left) and protein (right) expressions of Dusp1 were elevated by overexpression plasmid pcDNA3.1-Dusp1 ($n = 5$ for each group). **D**, Overexpressing Dusp1 inhibited Schwann cell migration as detected by Transwell migration assay ($n = 3$ for each group). **E**, Overexpression of Dusp1 could antagonize the promotion effect of TNXA-PS1 siRNA on Schwann cell migration ($n = 3-8$). Scale bar, 50 μm . **F**, Schematic representation of the regulation of TNXA-PS1 suggesting that, after sciatic nerve injury, TNXA-PS1 was downregulated and there was less binding with miR-24-3p and miR-152-3p. These miRNAs then interacted with Dusp1 and reduced Dusp1 expression, encouraging Schwann cell migration. Values are shown as mean $\pm$ SEM. Statistical significance was assessed by two-tailed Student's t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

qRT-PCR and Western blot results showed that the mRNA and protein expression of Dusp1 were increased dramatically (Fig. 5C; pcDNA3.1: 0.157 ± 0.010 ; pcDNA3.1-Dusp1: 1.954 ± 0.139 , $p < 0.0001$ vs pcDNA3.1; $n = 5$ for each group, Student's t test).

Transwell migration assay indicated that Schwann cell migration was decreased by Dusp1 overexpression (Fig. 5D; pcDNA3.1-Dusp1: 0.5135 ± 0.060 , $p = 0.0089$ vs pcDNA3.1; $n = 3$ for each group, Student's t test). Additionally, overexpression of Dusp1

on Schwann cells, which silenced lnc TNXA-PS1, could antagonize the promotion effect of lnc TNXA-PS1 siRNAs on Schwann cell migration (Fig. 5E, siRNA NC+pcDNA3.1: 1 ± 0.1192 , siRNA NC+pcDNA3.1-Dusp1: 0.4065 ± 0.06548 , $p = 0.012$; TNXA-PS1 siRNA1+pcDNA3.1: 1.906 ± 0.1611 , TNXA-PS1 siRNA1+pcDNA3.1-Dusp1: 0.9944 ± 0.07413 , $p = 0.0001$; TNXA-PS1 siRNA2+pcDNA3.1: 1.974 ± 0.2974 , TNXA-PS1 siRNA2+pcDNA3.1-Dusp1: 1.029 ± 0.08391 , $p = 0.0018$; $n = 3-8$, Student's t test). These data suggested that lnc TNXA-PS1 might regulate Schwann cell migration by Dusp1.

Discussion

One major characteristic of the PNS is its ability to spontaneously regenerate following nerve injury. As we know, because of its growth-inhibitory environment, CNS lacks intrinsic growth capacity. However, there is a more permissive growth environment in the PNS due to an efficient and extensive removal of myelin debris and inhibitors by Schwann cells and macrophages (Christie and Zochodne, 2013). The dedifferentiation, proliferation, migration, and myelin clearance of Schwann cells, a vast majority of cells in the injured distal nerve stump, are evidently associated with successful nerve regeneration (Bosse, 2012). Research has shown that blocking Schwann cell proliferation and migration might attenuate axonal regeneration in transected nerves (Chen et al., 2005). Therefore, clarifying the mechanisms underlying the modulation of Schwann cell behavior and finding promising molecules for nerve injury therapy are of great importance. In addition to growth factors and extracellular matrix substrates (Akassoglou et al., 2003; Perlin et al., 2011; Wakatsuki et al., 2014), our group and others have shown that miRNAs are also involved in Schwann cell phenotype modulation (Verrier et al., 2010; Yu, Qian et al., 2012; Yu, Zhou et al., 2012; Zhou et al., 2014). Here, we have shown for the first time that lncRNA TNXA-PS1 can regulate Schwann cell migration as an miRNA sponge.

Because of recent technological advances, thousands of lncRNAs have been demonstrated to be transcribed pervasively in the genome (Moran et al., 2012). Although lncRNAs have no significant protein-coding capacity, they are involved in diversity cellular biological processes and diseases, highlighting a central role in cell homeostasis (Wu et al., 2013). In the CNS, lncRNAs have been shown to participate in brain development, neural differentiation and maintenance, synaptic plasticity, and so on (Wu et al., 2013). However, studies on the role of lncRNAs in the PNS are rare. lncRNA, a Kcna2 antisense RNA, could contribute to neuropathic pain by silencing Kcna2 in primary afferent neurons. In addition, we have previously detected altered lncRNA expression in DRG after rat sciatic nerve injury and confirmed that lncRNA uc.217 could affect neurite outgrowth (Yu et al., 2013; Yao et al., 2015). Recently, Xu et al. (2017) has identified differentially expressed lncRNAs between IL-22 and control groups on Schwann cells *in vitro*. Here, we investigated lncRNA alteration in sciatic nerve segment following peripheral nerve injury and identified lncRNA TNXA-PS1 as a negative regulator for Schwann cell migration.

lncRNAs could exert their function by interacting with DNA, RNA, and protein. According to recent reports, lncRNAs might function as ceRNAs, sponging and affecting the target genes of miRNAs (Cesana et al., 2011; Kallen et al., 2013). Considering the mainly cytoplasmic distribution of TNXA-PS1 in Schwann cells, as identified by qRT-PCR, we

speculated that TNXA-PS1 might also exert its regulatory role by sponging miRNAs. Therefore, in our study, we conducted bioinformatic prediction and found several possible miRNAs and target genes that could be regulated by TNXA-PS1 in the mechanism of ceRNA. Following luciferase assay validation, we confirmed that TNXA-PS1 could bind with miR-24-3p and miR-152-3p, both of which have the same target gene, Dusp1.

miR-24-3p and miR-152-3p are both involved in tumorigenesis and cell migration. miR-24-3p could regulate the proliferation and migration of human arterial smooth muscle cells by targeting platelet-derived growth factor receptor B and c-Myc, whereas forced miR-152 expression reduced the migratory and invasive capabilities of prostate cancer cells significantly *in vitro* (Zhu et al., 2013, 2015). Dusp1 is recognized as an inactivator of different MAPK isoforms and is involved in multiple cell biological behaviors (Abraham et al., 2006; Shen et al., 2016). Dusp1 suppression induced by IL-17 could activate p38 MAPK and ERK1/2, promoting mouse primary cardiac fibroblast proliferation and migration (Valente et al., 2012). In our study, overexpression or interference of Dusp1 affected Schwann cell migration significantly. We thus speculated that, TNXA-PS1 might weaken miR-24-3p/miR-125-3p-mediated Dusp1 suppression by sponging miR-24-3p/miR-125-3p. Following nerve injury, the expression of Dusp1 was decreased as a result of TNXA-PS1 downregulation, eventually promoting Schwann cell migration (Fig. 5F).

In conclusion, our study has shown for the first time global lncRNA in a sciatic nerve segment following peripheral nerve injury in rats. We further characterized and functionally evaluated one continuously downregulated lncRNA, TNXA-PS1. Our research showed that TNXA-PS1 might regulate Schwann cells migration by sponging miR-24-3p/miR-152-3p and affecting Dusp1 expression. Together, these findings suggested that lncRNAs play a regulatory role in Schwann cell behavior and might be a potential therapeutic target for peripheral nerve injury.

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