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Loc680254 regulates Schwann cell proliferation through Psrc1 and Ska1 as a microRNA sponge following sciatic nerve injury

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Abstract

Peripheral nerve injury triggers sequential phenotype alterations in Schwann cells, which are critical for axonal regeneration. Long noncoding RNAs (lncRNAs) are long transcripts without obvious coding potential. It has been reported that lncRNAs participate in diverse biological processes and diseases. However, the role of lncRNA in Schwann cells and peripheral nerve regeneration is unclear. Here, we identified an lncRNA, loc680254, which is upregulated in rat sciatic nerve after peripheral nerve injury. The loc680254 knockdown inhibits Schwann cell proliferation, enhances apoptosis, and hinders cell cycle, while loc680254 overexpression has the opposite effect. Mechanically, we found that loc680254 might act as a microRNA sponge to regulate the expression of mitosis-related gene, spindle and kinetochore associated complex subunit 1 (Ska1) and proline/serine-rich coiled-coil 1 (Psrc1). Silencing of Psrc1 or Ska1 attenuates the effect of loc680254 overexpression on Schwann cell proliferation. Finally, we repaired the rat sciatic nerve gap with chitosan scaffolds loaded with loc680254-overexpressing Schwann cells and evaluated axon regeneration and functional recovery. Our results indicated that loc680254 is a new potential modulator for Schwann cell proliferation, which could be targeted to develop novel therapeutic strategies for peripheral nerve repair.

KEYWORDS
cell proliferation, competing endogenous RNA, lncRNA, peripheral nerve injury, Schwann cells

1 | INTRODUCTION

Different from the central nervous system (CNS), the peripheral nervous system (PNS) has a regenerative ability following injury attributed to a growth-permissive environment, which was partially provided by Schwann cells (SCs; Cattin & Lloyd, 2016; Jessen

et al., 2015). As the pivotal glial cells in the PNS, highly specialized myelinating and non-myelinating Schwann cells have supportive function for axonal physiology. Upon injury, both myelinating and non-myelinating Schwann cells in the distal stump are triggered to de-differentiate and proliferate, and then are re-programed to become specialized repair Schwann cells (Jessen & Arthur-Farraj, 2019; Jessen & Mirsky, 2016). They upregulate transcription factors such as Jun and Sox2 to re-program gene expression (Arthur-Farraj

et al., 2012; Patodia & Raivich, 2012; Roberts et al., 2017). They express neurotrophic factors to support neuronal survival and promote axonal regeneration (Gordon, 2009). Schwann cells in the distal nerve stump also activate myelinophagy to actively digest myelin (Gomez-Sanchez et al., 2015). They express cytokines and activate local innate immune response for myelin and axonal debris clearance (Jessen et al., 2015; Jessen & Arthur-Farraj, 2019; Martini et al., 2008; Zigmond & Echevarria, 2019). Moreover, the repair Schwann cells reorganize the structure and elongate their cell processes to form regeneration tracks (Gomez-Sanchez et al., 2017). Upon the contact with regenerated axons, Schwann cells re-differentiate and re-myelinate regenerated axon (Nocera & Jacob, 2020).

Long noncoding RNAs (lncRNAs) are long RNAs (>200 nt in length) without protein-coding potential. Emerging evidence indicates that lncRNAs carry out critical roles in diverse biological processes through multiple mechanisms in epigenetic, transcriptional, and post-transcriptional levels (Repana et al., 2006; Wu et al., 2013). Working as a competing endogenous RNA (ceRNA) to sponge microRNAs (miRNAs) and affect gene expression is one of the lncRNA regulatory mechanisms (Hirata et al., 2015; Thomson & Dinger, 2016). In the nervous system, lncRNAs participate in brain development, functional diversification, and neurodegenerative diseases (Johnson, 2012; Wu et al., 2013). However, the role and the molecular mechanism of lncRNAs in peripheral nerve regeneration are unclear (Yao & Yu, 2019). A conserved lncRNA, named Kcna2 antisense RNA, was up-regulated in the injured rat dorsal root ganglion (DRG) following peripheral nerve injury, contributing to neuropathic pain by decreasing Kcna2 expression (Zhao et al., 2013). Our laboratory has previously performed microarray analysis to identify differentially expressed lncRNAs in rat DRGs following sciatic nerve injury, and we found that lncRNA uc.217 was able to regulate neurite outgrowth (Yao et al., 2015; Yu et al., 2013). As for Schwann cells, it was reported that IL-22 might affect Schwann cell proliferation and apoptosis by regulating lncRNA-TF-gene pathways, suggesting that lncRNAs could modulate Schwann cell phenotype (Xu et al., 2017). Our group has identified altered lncRNAs in sciatic nerves after peripheral nerve injury by lncRNA microarray and found that lncRNA TNXA-PS1 and BC088259 regulate Schwann cell migration (Yao et al., 2018; Yao et al., 2020).

Compared with microarray analysis, RNA-sequencing (RNA-seq) provides a more precise measurement of transcript expression (Gong et al., 2016). We performed RNA-seq analysis and obtained a landscape of differentially expressed mRNAs in the distal nerve stump following sciatic nerve injury (Yi et al., 2015). In this study, we further identified differentially expressed lncRNAs in the distal nerve stump after sciatic nerve injury by RNA-seq analysis. We show that loc680254 was upregulated after peripheral nerve injury. It could modulate Schwann cell proliferation and apoptosis. Mechanism analysis uncovered that loc680254 might exert its regulatory role as a ceRNA to affect gene expression involved in mitosis. We also repaired the rat sciatic nerve gap with nerve conduit loaded with loc680254-overexpressing Schwann cells and evaluated axon regenerative outcomes by histological and functional assessments.

2 | METHODS

2.1 | Animal treatment and tissue collection

Adult male Sprague–Dawley (SD) rats (180–220 g) were randomly divided into five groups according to different time points and subjected to surgery for sciatic nerve crush injury as previously described (Yi et al., 2015). Briefly, the sciatic nerve was crushed with forceps at a force of 54 N for three times at 10 mm above the bifurcation site of the tibial and common fibular nerves (a period of 10 s for each time). After surgery, the animals were housed and fed routinely. At 0, 1, 4, 7, and 14 days after surgery, the 0.5-cm-long stumps in the distal site of the sciatic nerve were collected, respectively. All experimental procedures involving animals were conducted in accordance with the institutional animal care guidelines of Nantong University and approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

2.2 | RNA extraction, RNA-sequencing, and bioinformatic analyses

Total RNA was extracted from the sciatic nerve segments using Trizol reagent (Invitrogen, Carlsbad, California) and purified with RNeasy spin columns (Qiagen, Valencia, California). After RNA quality and quantity evaluation, transcriptome sequencing was performed using Illumina HiSeq 2000 (Beijing Genomics Institute, BGI-Shenzhen, China). The expression levels of mapped lncRNAs and genes were calculated using the Reads per kilobase transcriptome per million mapped reads (RPKM) method to normalize gene expression levels (Yi et al., 2015). Differentially expressed lncRNAs were screened at different time points using SAM (Significance Analysis of Microarrays) software (Tusher et al., 2001). Hierarchical clustering and expression pattern clustering analyses were then performed (Liao et al., 2011). Gene-coexpression networks based on the normalized signal intensity of specific expression lncRNAs and co-expressed mRNAs were constructed according to the previous method (Liao et al., 2011). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted to illustrate the enriched biological processes and signaling pathways associated with co-expressed genes of specific lncRNAs.

2.3 | Quantitative real-time polymerase chain reaction

Prime-Script RT Reagent Kit (TaKaRa, Dalian, China) was used to synthesize cDNA from RNA samples. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Premix Ex Taq (TaKaRa) on an ABI Stepone system (Applied Biosystems, Foster City, California) in triplicate for each sample. The specific primers were listed in Table S10. The relative expression level was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and normalized against GAPDH level.

2.4 | In situ hybridization

Digoxigenin (DIG)-labeled rat loc680254 probes were synthesized using DIG RNA Labeling Kit (Roche, Mannheim, Germany). After acetylation and prehybridization, the sciatic nerve segment sections were hybridized with the specific 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.

2.5 | 5′ rapid amplification of cDNA ends analysis

The 5′ end of cDNA was acquired using the SMAR RACE cDNA amplification kit (Clontech, Palo Alto, California) from the total RNA of sciatic nerve segments. The specific primers used were presented in Table S10. The 5′ rapid amplification of cDNA ends (RACE) products were then cloned into a pGEM-T-easy vector (Tiangen Biotech CO., LTD, Beijing, China) for further sequencing. The obtained sequences were blasted in UCSC (http://genome.ucsc.edu/).

2.6 | Primary Schwann cell culture and transfection

Schwann cells were isolated from sciatic nerves of 1-day-old SD rats and treated with anti-Thy1.1 antibody (Sigma, St Louis, Missouri) and rabbit complement (Invitrogen) to remove the fibroblasts as previously described (Mantuano et al., 2008). The final cell preparation consisted of 98% Schwann cells as determined by Schwann cell marker S100β (Sigma). Purified Schwann cells were maintained in DMEM containing 10% FBS, 2 μM forskolin and 10 ng/mL HRG at 37°C in a humidified 5% CO_2 incubator. The cell culture was passaged no more than three times before the following treatment.

Primary cultured Schwann cells were seeded and transfected with specific siRNAs (Ribobio, Guangzhou, China) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). To overexpress loc680254, primary Schwann Cells were infected with lentivirus encoding the full length of loc680254 (Lv-254) constructed by Shanghai GeneChem. After 48 h transfection, the cells were underwent following experiments.

2.7 | Cell proliferation assay

Schwann cell proliferation was tested by 5-ethynyl-2′-deoxyuridine (EdU) assay using a Cell-Light EdU Apollo567 in Vitro Kit (Ribobio). Briefly, 100 μL complete medium containing 50 μM EdU solution was added to transfected Schwann cells in each well of a 96-well plate. After 24 h incubation, cells were washed twice with PBS and then fixed with 4% paraformaldehyde (PFA). After that, cells were stained with Apollo dye solution (Ribobio) for proliferating cells and

Hoechst 33342 for nucleus. Images were taken using a fluorescence microscope.

2.8 | TUNEL analysis

Apoptotic DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated biotinylated UT nick end labeling (TUNEL) with an In Situ Cell Death Detection Kit (Roche). In brief, cells were fixed with 4% PFA and incubated in permeabilization solution. After washing twice with PBS, cells were incubated with 50 μL TUNEL reaction solution for 1 h in a humidified chamber at 37°C in the dark. Then, cells were rinsed with PBS and stained the nucleus with Hoechst 33342. Images were taken using a fluorescence microscope.

2.9 | Flow cytometry

To evaluate cell cycle, flow cytometry was performed. Transfected Schwann cells were harvested, fixed in 70% ethanol, washed, and then stained with PI solution following the manufacture's protocol of the Cell cycle kit (Beyotime). All labeled cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, California).

2.10 | RNA distribution analyses

The PARIS Kit (Life Technologies, Carlsbad, California) was used to isolate and purify cytoplasmic and nuclear RNA of Schwann cells. The relative expression of loc680254 in each fraction was then detected by qRT-PCR. Fluorescent in situ Hybridization (FISH) for loc680254 was performed on 4% PFA fixed slides with monolayers of Schwann Cells. The slides were hybridized with DIG-labeled loc680254 probes at 42°C overnight. After incubating with anti-DIG antibody and secondary antibody, the slides were subsequently counterstained with Hoechst and observed under microscopes.

2.11 | Microarray analysis and competing endogenous RNA (ceRNA) target gene prediction

Primary Schwann cells transfected with 254-si-3 or si-NC were collected for RNA extraction and underwent microarray analysis using Agilent Whole Rat Genome Microarray (Shanghai Biotechnology Corporation). Raw data were normalized by Quantile algorithm, limma packages in R. Genes with the absolute ratio of normalized intensities (FC (abs)) >2, p value <0.05 were considered as differentially expressed genes (DEGs). The potential miRNAs that interacted with loc680254 were predicted as described previously (Tay et al., 2011). The target genes of these miRNAs with FC (abs) >2 down-expressed after loc680254 interfering were considered as potential ceRNA

target genes of loc680254. We then further selected candidate genes with the criterion that FC (abs) >3 and miRNA align site >1. GO and KEGG analyses were conducted using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatic resources (https://david.ncifcrf.gov/) to illustrate the enriched biological processes and signaling pathways associated with DEGs (FC (abs) >2, p-value <0.05) after loc680254 silencing.

2.12 | Luciferase reporter assay

The 3′-UTR sequence of Psrc1, Ska1, and the full length of loc680254 were constructed into the pmirGLO vector, respectively. The 293T cells were co-transfected with the constructed pmirGLO plasmid and certain miRNAs. The relative luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 48 h after transfection.

2.13 | Expression of target genes after miRNA overexpression

Primary Schwann cells were transfected with certain miRNA mimics and miRNA mimic negative control (MC; Ribobio) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacture's protocol. Forty-eight hours later, the cells were harvested and underwent qRT-PCR analysis to detect the mRNA expression of Psrc1 and Ska1, respectively.

2.14 | RNA immunoprecipitation

For argonaute 2 (AGO2) immunoprecipitation, certain miRNA mimics and miRNA mimic negative control were transfected into primary Schwann cells. Forty-eight hours after transfection, AGO2 immunoprecipitation was performed using a Magna RIP Kit (Millipore) according to the manufacturer's instructions. Antibodies for RNA immunoprecipitation (RIP) assays were AGO2-specific antibody (Abcam). An IgG antibody was used as a negative control. Beads were subsequently washed six times in lysis buffer and the RNA was extracted using Trizol reagent. The coprecipitated RNA was detected by qRT-PCR.

2.15 | In vivo tests for loc680254 overexpression

Adult male SD rats (180–220 g) underwent sciatic nerve transection injury as previously described (Gu et al., 2017). A segment of the sciatic nerve was transected and removed, leaving a 10 mm-long gap after retraction of the nerve ends. The animals were randomly divided into three groups (each group has nine rats). Chitosan scaffolds injected with PBS, Lv-control Schwann cells (Lv-con SCs), and Lv-loc680254 Schwann cells (Lv-254 SCs) were used to bridge the

10 mm sciatic nerve gap in rats, respectively. After surgery, the animals were housed and fed routinely. At 10 days post-surgery, three rats in each group were killed and the scaffolds containing regenerated nerves were collected. The axon outgrowth was observed by immunostaining with NF200 antibody (Sigma). To evaluate the recovery of locomotive function, the gait parameters of rats at 12 weeks post-surgery were recorded with the CatWalk system (Noldus Information Technology; Bozkurt et al., 2008). The movement of the toes was evaluated as previously described (He et al., 2018), which scored 0, 1, and 2 for no spreading, intermediate spreading, and full spreading, respectively. For electrophysiological assessment, at 12 weeks after surgery, the sciatic nerve on the injured side of the remaining rats in each group was re-exposed under anesthesia. The electrical stimuli (10 mA in strength, 0.01 ms in pulse width) were sequentially applied to the proximal and distal nerve stumps. The compound muscle action potentials (CMAPs) and the conduction velocity of motor nerves were recorded and calculated using an MYTO electromyographic machine and Galileo NT System software (Esaote, Genua, Italy; Gu et al., 2017).

2.16 | Statistical analysis

All data are presented as means ± SEM. Statistical comparison was performed with Student's t-test or Two-way ANOVA for multiple comparisons with the aid of Prism5 software (GraphPad, San Diego, California). The $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Differentially expressed lncRNAs following sciatic nerve injury

Thirty differentially expressed lncRNAs were identified by RNA-seq and subsequent analysis in rat sciatic nerve following crush injury (Figure 1(a) and Table S1). We then obtained 10 lncRNA profiles according to their expression trend. The expression trend Profile 8 includes eight lncRNAs, all of them were upregulated following injury with high expression level at 4 and 7 days post-injury (Figure 1 (b)). Two hundred and seventy-nine differentially expressed mRNAs were identified which correlate with the expression of eight lncRNAs in profile 8, as shown in the gene co-expression network (Figure 1(c) and Table S2). GO and KEGG functional enrichment analysis indicated that these genes are involved in the signaling pathways of cell apoptosis and proliferation (Figure 1(d), Tables S3 and S4).

3.2 | loc680254 is upregulated following sciatic nerve injury

Following above analysis, we chose lncRNA loc680254 in profile 8 for further investigation. qRT-PCR of sciatic nerve segments showed that
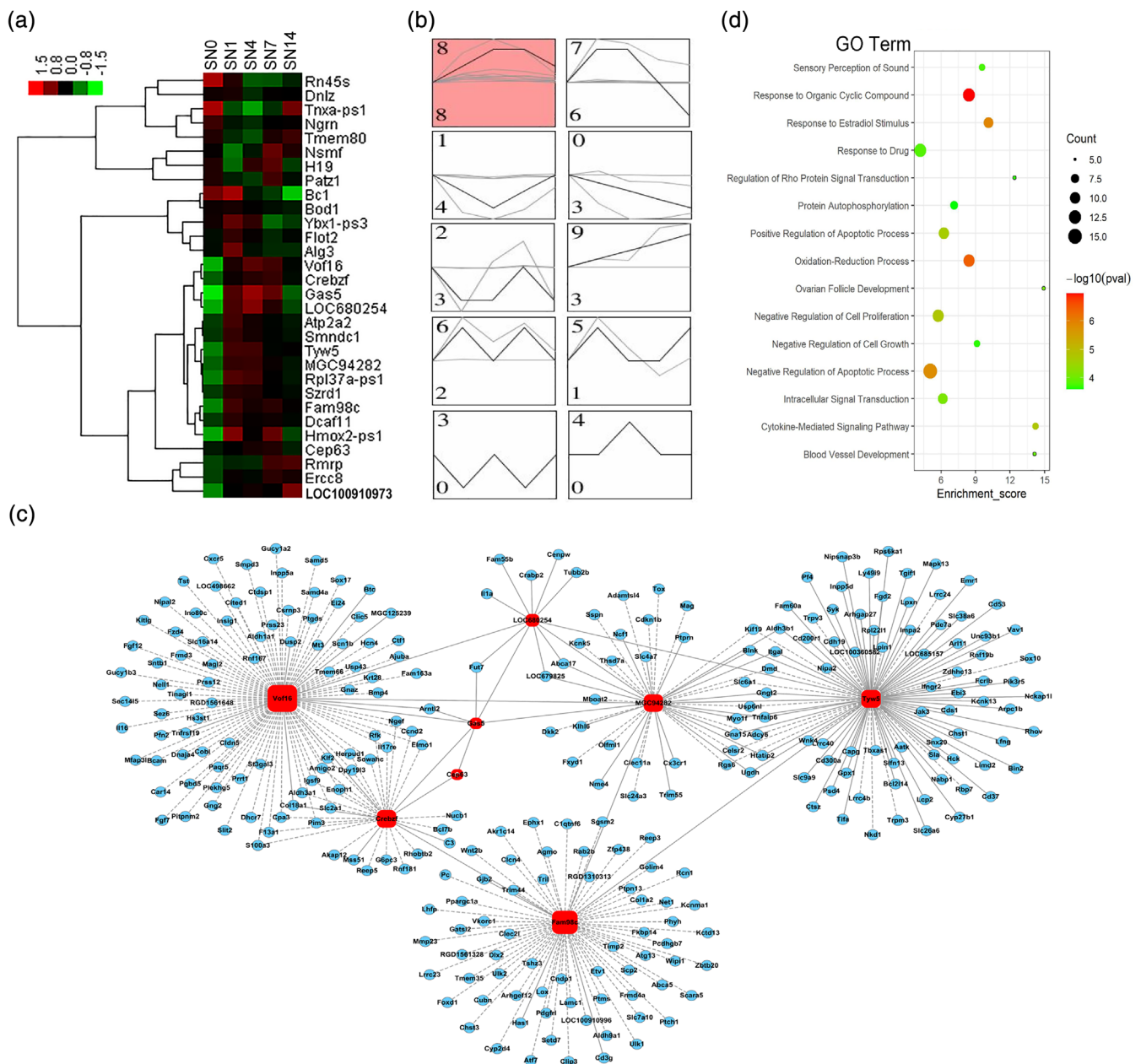
FIGURE 1 Differentially expressed lncRNAs after sciatic nerve crush injury by RNA-seq analysis. (a) Hierarchical clustering heatmap of differentially expressed lncRNAs at five time points after sciatic nerve crush injury. The color scale on the top illustrates the relative expression level of lncRNAs across all samples: Red denotes expression >0 and green denotes expression <0. (b) Expression trend profiles of differentially expressed lncRNAs. The number on the top left is the profile name and the number on the bottom left represents the number of lncRNAs in each profile. (c) Coexpression network of lncRNAs in profile 8 with correlated target genes. Red squares represent lncRNAs, and blue circles represent coding mRNAs. Solid lines between lncRNAs and coding mRNAs indicate positive correlation in expression; dotted lines indicate negative correlation. (d) Top 15 GO biological process terms enriched by genes coexpressed with lncRNAs in profile 8. Green to red color represents −log10 (p-value) of each GO term. The circle size reflects gene numbers involved in each GO term [Color figure can be viewed at wileyonlinelibrary.com]

loc680254 was continuously upregulated following nerve injury and reached the peak expression at 7 days, which is consistent with the expression trend of profile 8 (Figure 2(a)). In situ hybridization of loc680254 in the sciatic nerve section also confirmed the increased expression of loc680254 in sciatic nerve tissue at 4 days after nerve injury (Figure 2(b)). Since the full sequence of lncRNA is critical for its functional exploration, we performed RACE to obtain the full length of loc680254. After sequencing and blasting with the rat genome in UCSC, we identified that loc680254 was about 790 nt in length and localized in rat chromosome 13q22, consisting of 3 exons (Figure 2 (c)). Cellular distribution analysis showed that loc680254 mainly expressed in Schwann cell cytoplasm, compared with U6 and β-actin (Figure 2(d)). FISH experiments in Schwann cells further confirmed this result (Figure 2(e)).
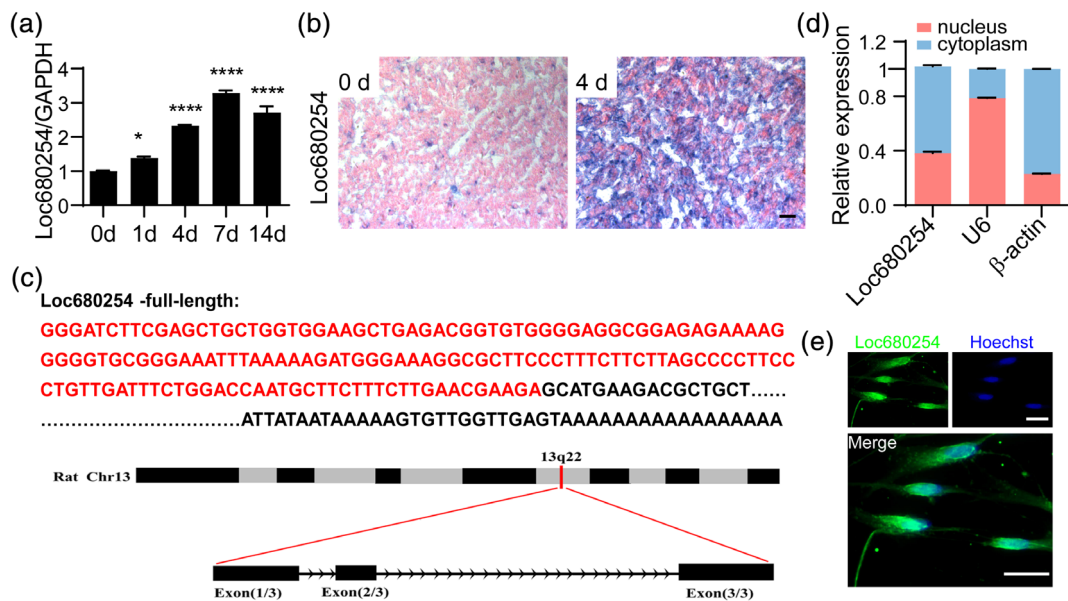
FIGURE 2 The expression and characteristics of loc680254. (a) Relative loc680254 expression in sciatic nerve tissues at the indicated time points after sciatic nerve crush injury as detected by qRT-PCR. (b) In situ hybridization of loc680254 on sciatic nerve tissue sections at 0 and 4 days after sciatic nerve crush injury. Scale bar: 50 μm. (c) Full length of loc680254 and the scheme of loc680254 in rat genome searched by UCSC Genome Browser. (d) Distribution of loc680254 in Schwann cell nucleus and cytoplasm as detected by qRT-PCR. U6 and β-actin were positive controls of the nucleus and cytoplasm, respectively. (e) Loc680254 expressed in the nucleus and cytoplasm of Schwann cells as detected by FISH experiments. Scale bar: 20 μm. $N = 3$ for each group, $*p < 0.05$, $****p < 0.0001$ [Color figure can be viewed at wileyonlinelibrary.com]
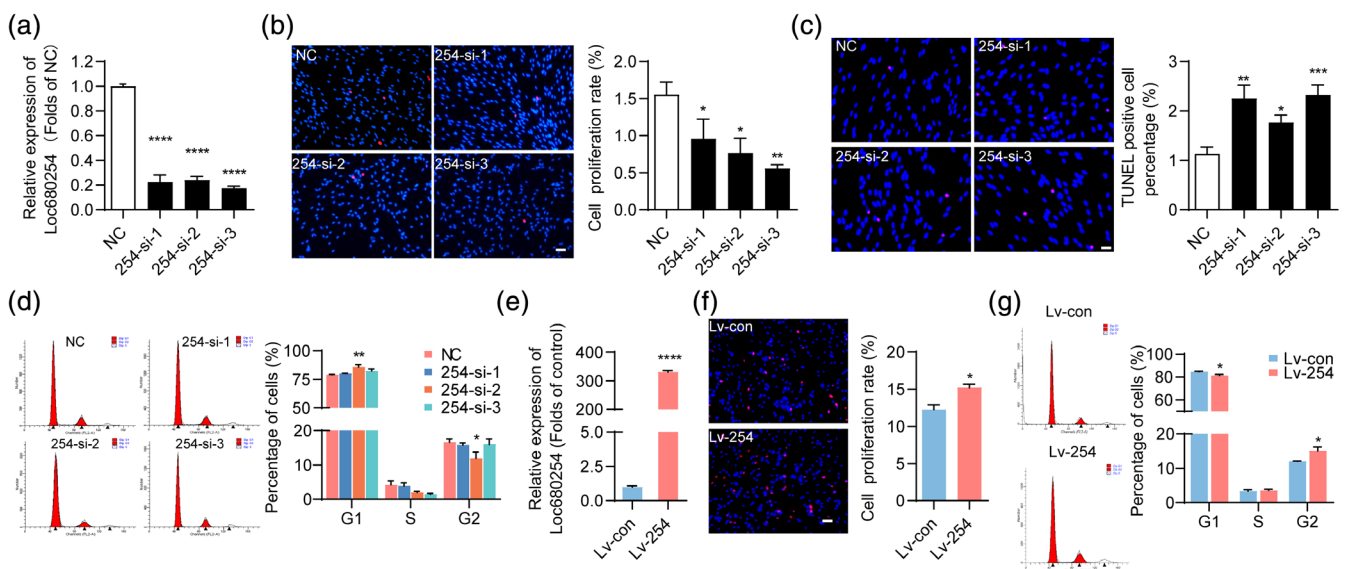

FIGURE 3 Loc680254 modulates Schwann cell proliferation, apoptosis, and cell cycle. (a) Expression of loc680254 after loc680254 siRNAs (254-si-1, 254-si-2, 254-si-3) and random siRNA negative control (NC) treatment. $N = 4$ for each group, $****p < 0.0001$. (b) Interfering loc680254 reduced Schwann cell proliferation as detected by EdU assay. Representative images (left) and quantification data of EdU positive cells (right) were shown. NC: siRNA negative control. Scale bar: 50 μm. $N = 4$ for each group, $*p < 0.05$, $**p < 0.01$. (c) Detection of Schwann cell apoptosis after loc680254 siRNA treatments by TUNEL assay. Representative images (left) and quantification data of TUNEL positive cells (right) were shown. NC: siRNA negative control. Scale bar: 25 μm. $N = 5$ for each group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (d) Cell cycle analyses by flow cytometry. Representative images (left) and the percentage of cells in G1, S, and G2 phases (right) were shown. NC: siRNA negative control. $N = 3$ for each group, $*p < 0.05$, $**p < 0.01$. (e) Expression of loc680254 in Schwann cells infected by loc680254 lentivirus (Lv-254) and control (Lv-con). $N = 3$ for each group, $****p < 0.0001$. (f) Edu proliferation assay results of Schwann cells infected by Lv-254 and Lv-con. Representative images (left) and quantification data of EdU positive cells (right) were shown. Scale bar: 100 μm, $n = 3$ for each group, $*p < 0.05$. (g) Cell cycle analyses of Schwann cells overexpressed loc680254 by flow cytometry. Representative images (left) and the percentage of cells in G1, S, and G2 phases (right) were shown. $N = 3$ for each group, $*p < 0.05$ [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | loc680254 affects Schwann cell proliferation in vitro

To investigate the role of loc680254 in Schwann cells, we designed three specific siRNAs for loc680254. All of them could effectively reduce loc680254 expression to almost 20% in Schwann cells (Figure 3 (a)). EdU proliferation assay showed that interfering loc680254 in Schwann cells significantly reduced the percentage of EdU positive cells

(Figure 3(b)). TUNEL assays indicated that downregulation of loc680254 significantly induced cell apoptosis (Figure 3(c)). In addition to above observations, interfering loc680254 also resulted in retardation of cell cycle progression, with a decreased percentage of cells in S and G2 phases by 254-si-2 (Figure 3(d)). It was interesting to reveal that, although 254-si-1 and 254-si-3 had a more effective impact in Loc680254 suppression, only 254-si-2 affects the cell cycle. We also overexpressed loc680254 in Schwann cells by lentivirus infection.
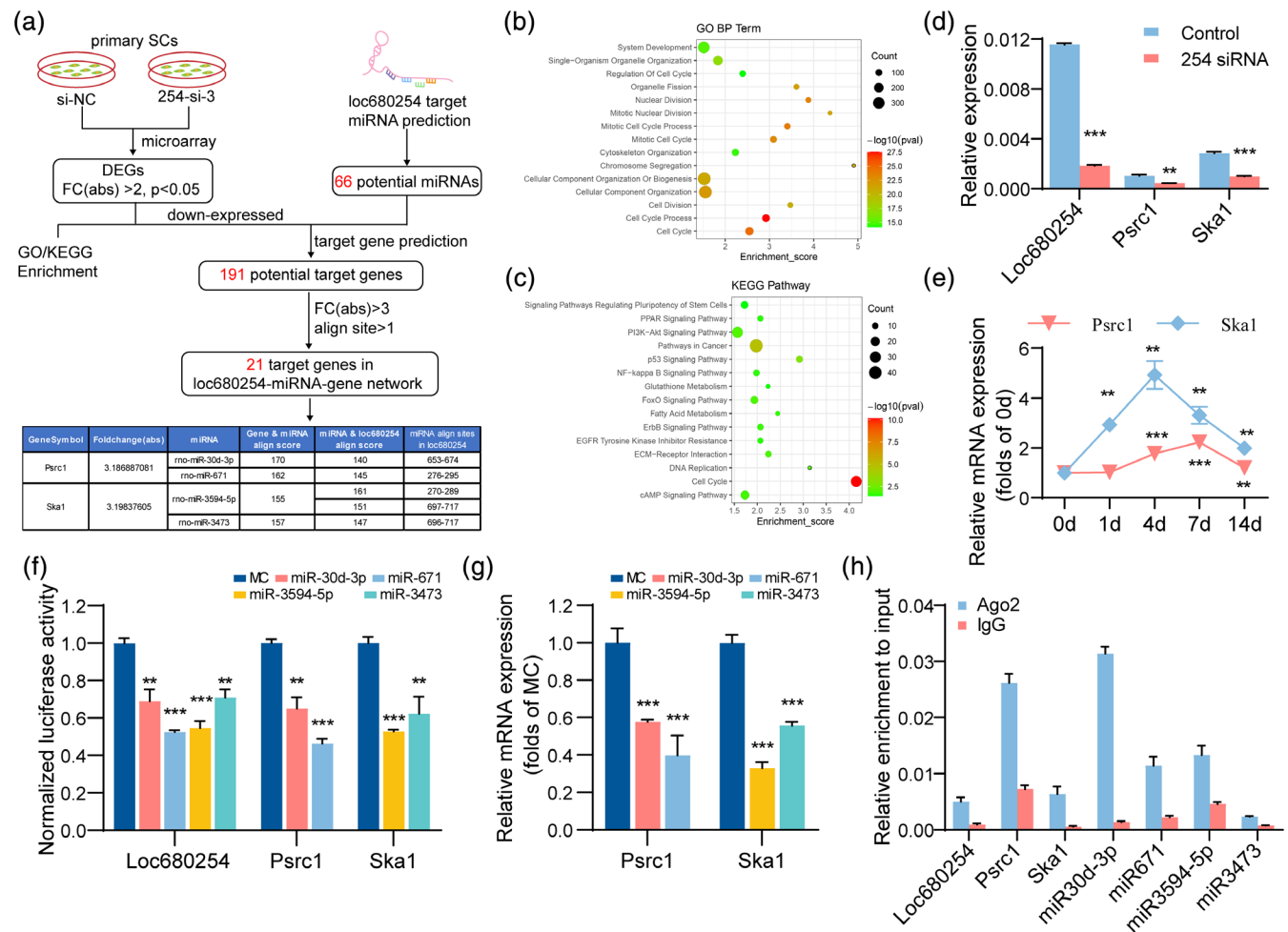

FIGURE 4 Loc680254 acted as a ceRNA to interact with Prsc1 and Ska1. (a) A schematic diagram for loc680254 target gene selection and aligned information of loc680254, miRNA, and target genes. Briefly, primary Schwann cells were transfected with loc680254 siRNA3 (254-si-3) or negative siRNA control (si-NC). Microarray analysis was performed to obtain differentially expressed genes (DEGs) after loc680254 silencing. DEGs with absolute fold change (FC (abs)) > 2 and $p < 0.05$ were selected for GO/KEGG functional enrichment analysis and the results were shown in b and c. meanwhile, potential miRNA targets of loc680254 were predicted. The coverage genes between down-regulated DEGs after loc680254 silencing and miRNA target genes were considered as potential target genes in the loc680254-miRNA-gene ceRNA regulation network. We then reduced target gene numbers with the criterion that FC (abs) >3 and align site >1. Target genes with high align scores were shown in the table below. (b) GO biological process enrichment of differentially expressed genes after loc680254 siRNA3 (245-si-3) treatment in Schwann cells. Top 15 GO biological process terms enriched were illustrated. The circle color represents −log10 (p-value) of each GO term. The circle size represents the gene numbers involved in each GO term. (c) KEGG pathway enrichment of differentially expressed genes after loc680254 siRNA3 (245-si-3) treatment in Schwann cells. Top 15 KEGG pathway terms enriched were illustrated. The circle color represents −log10 (p-value) of each KEGG pathway term. The circle size represents the gene numbers involved in each KEGG pathway term. (d) The mRNA expression of Psrc1 and Ska1 in loc680254 siRNA or siRNA negative control (NC) treated Schwann cells. (e) Relative mRNA expression of Psrc1 and Ska1 in sciatic nerve segments following sciatic nerve crush injury. (f) Luciferase results between loc680254, Psrc1 3′-UTR, Ska1 3′-UTR, and certain miRNAs, respectively. MC: miRNA mimic negative control. (g) qRT-PCR results showed that overexpressing corresponding miRNAs decreased the mRNA expression of Psrc1 and Ska1, respectively. MC: miRNA mimic negative control. (h) Ago2 immunoprecipitation of loc680254, miRNAs, and target genes. $N = 3$ for each group, ***$p < 0.001$; **$p < 0.01$ [Color figure can be viewed at wileyonlinelibrary.com]
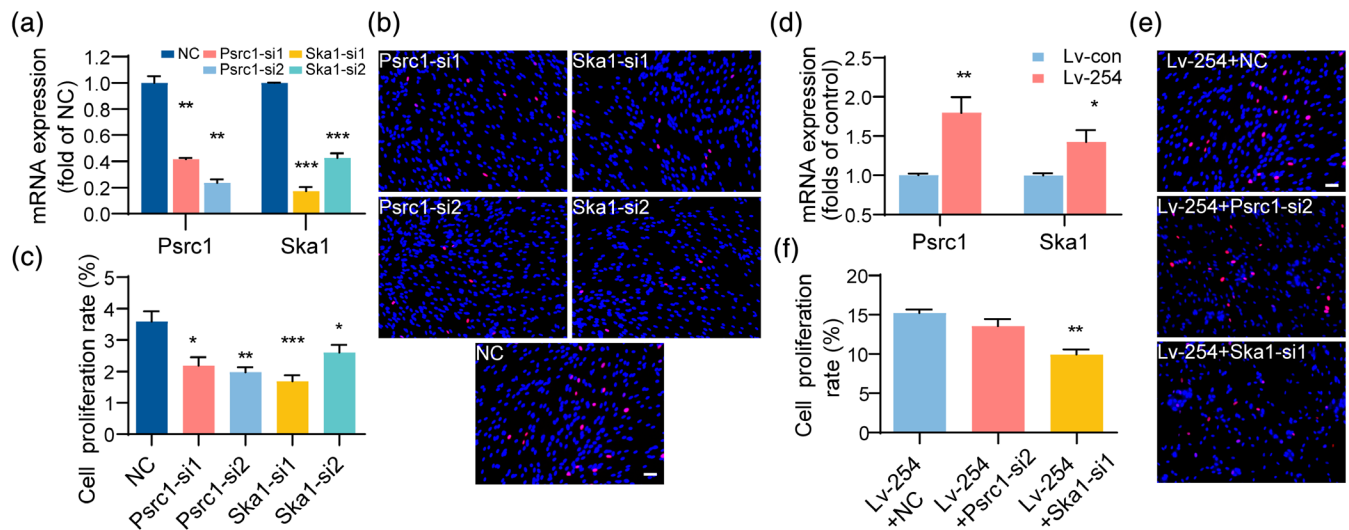
FIGURE 5 Loc680254 modulated Schwann cell proliferation through Prsc1 and Ska1. (a) The interfering effects of Psrc1 siRNAs (Psrc1-si1, Psrc1-si2) and Ska1 siRNAs (Ska1-si1, Ska1-si2) in Schwann cells as detected by qRT-PCR. NC: siRNA negative control. (b) Representative images of EdU proliferation assay after Psrc1 or Ska1 silencing in Schwann cells. Scale bar: 50 μm. (c) Quantification of cell proliferation rate as EdU positive cell percentage in Schwann cells after Psrc1 or Ska1 silencing. (d) mRNA expression of Psrc1 and Ska1 after loc680254 overexpression (Lv-254) and lentivirus negative control (Lv-con). (e) Representative images of EdU proliferation assay after Psrc1 or Ska1 silencing in loc680254-overexpressed Schwann cells. Scale bar: 50 μm. (f) Quantification of cell proliferation rate in loc680254-overexpressed Schwann cells after Psrc1 or Ska1 silencing. NC: siRNA negative control. $N = 3$ for each group. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ [Color figure can be viewed at wileyonlinelibrary.com]

qRT-PCR results showed that Lv-254 treatment dramatically enhanced loc680254 expression in Schwann cells compared with Lv-con control (Figure 3(e)). Cell proliferation rate was elevated following loc680254 overexpression (Figure 3(f)). In addition, more cells were accumulated in G2 phase after loc680254 overexpression (Figure 3(g)).

3.4 | Loc680254 serves as a ceRNA to regulate Psrc1 and Ska1 expression

Since loc680254 mainly distributed in the cytoplasm, we speculated that it might work as a miRNA decoy, which is a principal regulatory mechanism for cytoplasmic lncRNAs according to previous reports (Noh et al., 2018). We then performed microarray analysis to identify genes affected by loc680254 silencing in Schwann cells. Briefly, Schwann cells transfected with loc680254 siRNA3 (254-si-3) or random siRNA negative control (si-NC) were collected for microarray (Figure 4(a)). There were 1106 differentially expressed genes (DEGs; FC (abs) >2 and $p < 0.05$) after loc680254 silencing in Schwann cells (Table S5). GO and KEGG functional enrichment analysis showed that these genes were involved in cell cycle (Figure 4(b,c), Tables S6 and S7), which is consistent with our cell proliferation assay results. We then screened the potential mRNA targets of loc680254 as a ceRNA by bioinformatic prediction as illustrated in Figure 4(a) (Tables S8 and S9) and found 21 potential candidate genes. Among them, spindle and kinetochore associated complex subunit 1 (Ska1) and proline/serine-rich coiled-coil 1 (Psrc1) could cooperate each other to regulate spindle dynamics, and affect cell cycle and proliferation (Park et al., 2016). Thus, in the following studies, we focused on the investigation of Ska1 and Psrc1 expression and function in Schwann cells.

The loc680254 interfering reduced mRNA expression of Psrc1 and Ska1 in Schwann cells (Figure 4(d)). Following peripheral nerve injury, Psrc1 and Ska1 expression increased in the sciatic nerve tissues, with a similar trend as loc680254 (Figure 4(e)). These results confirmed that Psrc1 and Ska1 expression were associated with loc680254 up-regulation. To validate the interaction between loc680254, miRNAs and target genes, we performed the luciferase assay. As shown in Figure 4(f), all predicted potential miRNAs (miR-30d-3p, miR-671, miR-3594-5p, and miR-3473) could bind with loc680254. In addition, miR-30d-3p/miR-671 and miR-3594-5p/miR-3473 could interact with the 3′-UTR of Psrc1 and Ska1 (Figure 4(f)), and reduce the mRNA expression of these genes, respectively (Figure 4(g)). To further validate the association of loc680254, miRNAs, and target genes, we also conducted RNA binding protein immunoprecipitation experiments with the antibody against Ago2, a component of RNA-induced silencing complex (RISC). As expected, loc680254, Psrc1, Ska1, and miRNAs were enriched in Ago2 immuno-precipitate compared with control IgG experiment (Figure 4(h)).

3.5 | loc680254 modulates Schwann cell phenotype through regulating Psrc1 and Ska1 expression

To explore whether loc680254 regulates Schwann cell proliferation through Psrc1 and Ska1, we transfected Psrc1 and Ska1 siRNAs into Schwann cells. As shown in Figure 5(a), these siRNAs efficiently decreased Psrc1 (Psrc1-si1, Psrc1-si2) and Ska1 (Ska1-si1, Ska1-si2) mRNA expression in Schwann cells, respectively. As expected, Psrc1
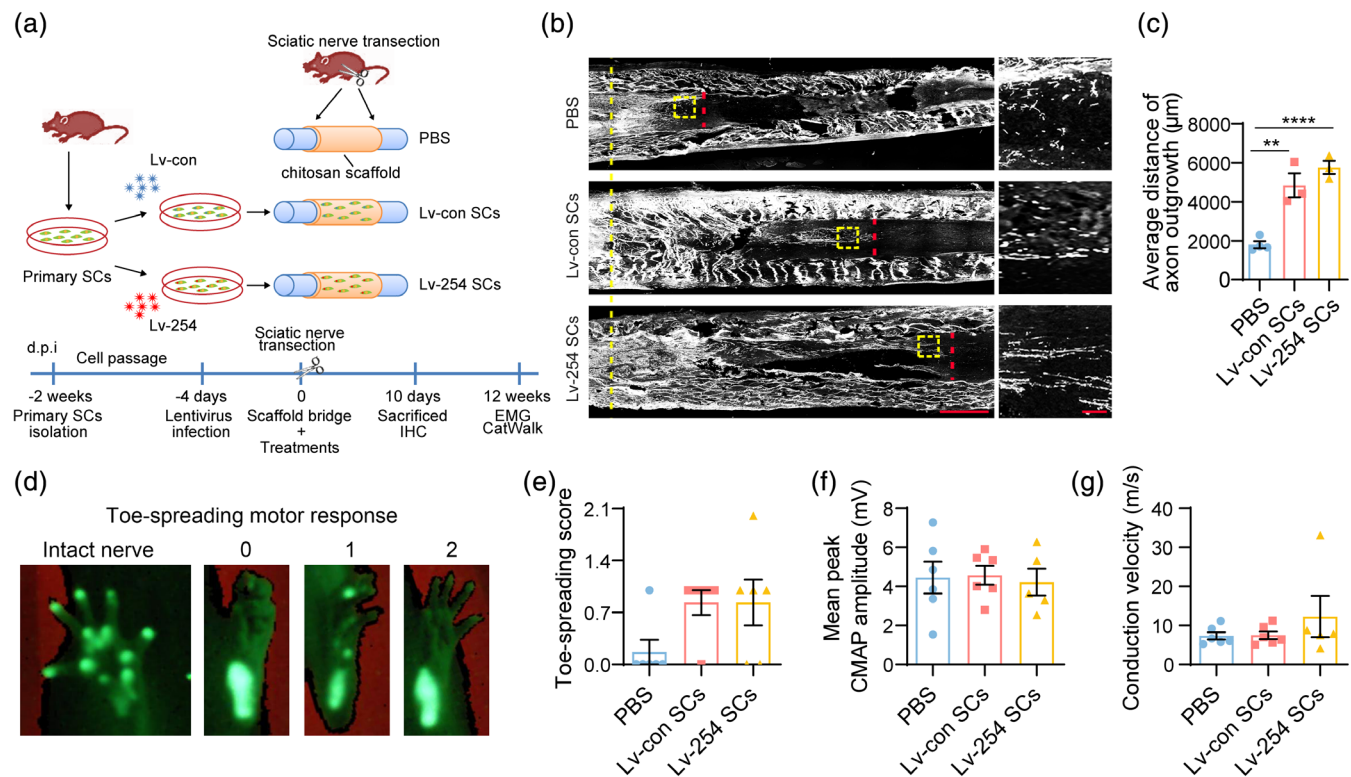
FIGURE 6 In vivo effect of loc680254-overexpressed Schwann cells after rat sciatic nerve transection and repair. (a) A diagram of treatments and time points for rats after sciatic nerve transection. Briefly, primary Schwann cells were transfected with loc680254-overexpressing lentivirus (Lv-254) or negative lentivirus control (Lv-con). The transfected Schwann cells (Lv-254 SCs and Lv-con SCs) or PBS were then injected into the chitosan scaffold that bridged the gap after sciatic nerve transection. (b) NF200 immunostaining (white) of regenerating nerves on day 10 after surgery in three groups. The yellow dotted line indicates the injury site. The right images show the enlarged yellow dotted square area from left. The red dotted line indicated the end of axon fibers. Scale bar: Left, 1000 μm; right, 100 μm. (c) Quantification of average axon outgrowth at day 10 after surgery. $N = 3$ for each group. ****$p < 0.0001$; **$p < 0.01$. (d) Typical toe-spreading images of different scores and intact nerve. (e) Toe-spreading reflex for motor function in loc680254-overexpressed Schwann cell group (Lv-con SCs), control Schwann cell group (Lv-con SCs), and PBS treated group at the 12th week after repair. $N = 6$ for each group. (f) The mean peak CMAP amplitude and (g) the conduction velocity detected in the injured side of loc680254-overexpressed Schwann cell group (Lv-con SCs), control Schwann cell group (Lv-con SCs), and PBS treated group at the 12th week after repair. $N = 6$ for each group [Color figure can be viewed at wileyonlinelibrary.com]

or Ska1 silencing reduced the proliferation rate of Schwann cells (Figure 5(b,c)). loc680254 overexpression in Schwann cells (Lv-254) increased Psrc1 and Ska1 mRNA expression (Figure 5(d)). Interfering Psrc1 (Psrc1-si2) or Ska1 (Ska1-si1) reduced the cell proliferation rate in loc680254-overexpressed Schwann cells. Ska1 siRNA had a more significant effect on Schwann cell proliferation compared with Psrc1 siRNA (Figure 5(e,f)). These data suggested that loc680254 might modulate Schwann cell proliferation through the regulation of Psrc1 and Ska1 expression.

3.6 | Effects of loc680254 in vivo after sciatic nerve transection in rats

To investigate the role of loc680254 in vivo, we constructed a rat sciatic nerve transection injury model. We then bridged the 10-mm-long sciatic nerve gap with a chitosan scaffold, which has been previously used by our group as a nerve guidance conduit to repair peripheral nerve gas (Gu et al., 2014; Hu et al., 2013). loc680254-overexpressed Schwann

cells (Lv-254 SCs), control Schwann cells (Lv-con SCs), or PBS was injected into the chitosan scaffolds (Figure 6(a)). After 10 days repair, we performed NF200 immunostaining to detect axon regeneration in each group. Figure 6(b,c) showed that both Lv-con SCs and Lv-254 SCs promoted axon outgrowth compared with PBS chitosan scaffolds. Although loc680254-overexpressed group had slightly faster axon outgrowth in the nerve gap, there was no significant difference between Lv-con SCs and Lv-254 SCs groups. We also evaluated functional recovery with the CatWalk system at 12 weeks post-surgery. The results showed that both Lv-con SCs and Lv-254 SCs groups had a higher toe-spreading score (Figure 6(d,e)). However, there was no significant difference in mean CMAP amplitude and conduction velocity by electromyography (EMG) analyses between each group (Figure 6(f,g)).

4 | DISCUSSION

As the principal glial cell in PNS, Schwann cells play a pivotal role in nerve regeneration after peripheral nerve injury. Numerous genes and
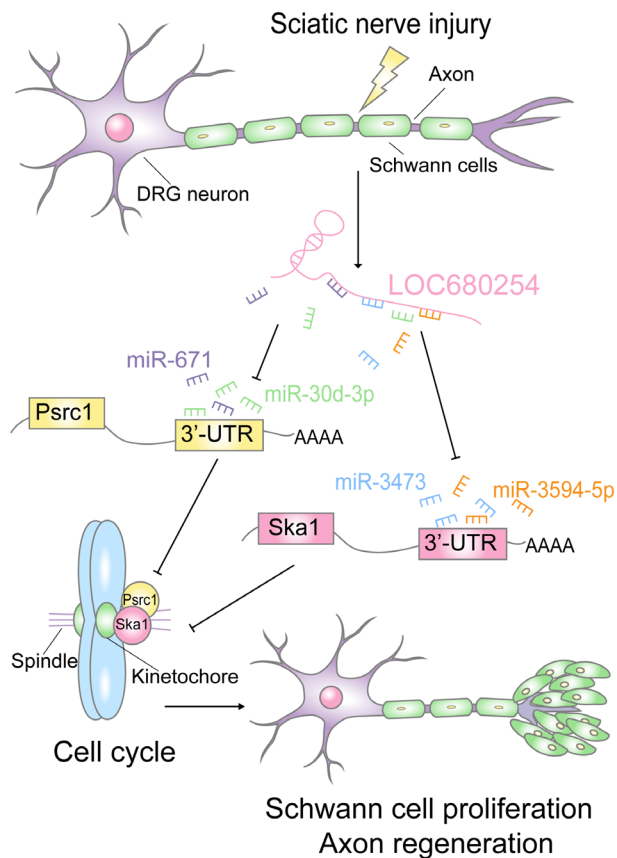
FIGURE 7 A brief schematic graph illustrates the possible regulatory mechanism of loc680254 after during peripheral nerve regeneration. Upon nerve injury, loc680254 was up-regulated and might bind free miR-671, miR-30d-3p, miR-3473, and miR-3594-5p in Schwann cell cytoplasm, leading to less miRNA interaction with Psrc1 and Ska1. As a result, the expressions of Psrc1 and Ska1, two components that participate in spindle attachment to kinetochore during cell cycle, were elevated. Schwann cell proliferation then increased to facilitate axon regeneration after peripheral nerve injury [Color figure can be viewed at wileyonlinelibrary.com]

pathways could regulate Schwann cell phenotypes, especially Schwann cell proliferation, such as ERK (Agthong et al., 2006; Monje et al., 2006), p38 (Roberts et al., 2017), and JNK signaling pathways (Arthur-Farraj et al., 2012; Fontana et al., 2012). Growth factors such as Neuregulin 1 (Shin et al., 2017) and Betacellulin (Vallieres et al., 2017) are up-regulated in the distal nerve stump and they promote Schwann cell proliferation. Besides these findings, many reports have suggested that miRNAs also participate in Schwann cell biological processes (Yu et al., 2015). For example, miR-221 and miR-222 promote Schwann cell proliferation, while miR-182 has a repressive effect (Yu, Qian, et al., 2012; Yu, Zhou, et al., 2012). Here, we proposed that lncRNAs, as another main subset of noncoding RNA, also regulate Schwann cell proliferation after peripheral nerve injury.

According to our previous research, we have identified a set of differentially expressed lncRNAs in the sciatic nerve after injury by microarray analysis and explored the role of lncRNAs in Schwann cell migration (Yao et al., 2018; Yao et al., 2020). Compared with microarray analysis, RNA-seq is a more advanced technology to study gene expression profile in healthy and injured tissues. For example, RNA-seq has a smaller sample requirement, lower background noise, and more precise expression measurements. Furthermore, RNA-seq facilitates detecting novel transcripts (Kogenaru et al., 2012). Thus, in this study, we performed RNA-seq analysis and identified a set of differentially expressed lncRNAs in sciatic nerve tissues after peripheral nerve injury. LncRNA loc680254, among the upregulated lncRNAs, was then characterized. We show that it regulates Schwann cell proliferation, apoptosis, and cell cycle progression.

As reported, lncRNAs regulate gene expression through nuclear and cytoplasmic mechanisms at transcriptional, post-transcriptional, and translational levels. Nuclear lncRNAs function through epigenetic modification or interaction with transcription factors and transcriptional co-regulators, while cytoplasmic lncRNAs exert their role by sponging miRNAs, affecting mRNA stability and inhibiting translation (Sun & Kraus, 2015). Since loc680254 mainly distributes in the cytoplasm of Schwann cells as detected by qRT-PCR and FISH studies, we speculated that loc680254 might work as a ceRNA to sponge miRNAs. By bioinformatics prediction and experiment validation, we found that loc680254 could affect the expression of Psrc1 and Ska1 by miR-30d-3p/miR-671 and miR3473/miR-3594-5p.

Psrc1, also named as DDA3, is a microtubule-associated protein. It is highly phosphorylated in mitotic cells and binds with EB3 to activate the β-catenin pathway (Hsieh et al., 2007). By recruiting MT depolymerase Kif2a, Psrc1 regulates spindle dynamics and chromosome movements (Jang et al., 2008). Ska1, spindle and kinetochore-associated complex subunit 1, is a newly discovered gene associated with mitosis (Hanisch et al., 2006). Ska1 can form a Ska complex and bind to microtubules, facilitating kinetochore-spindle microtubule attachment (Jeyaprakash et al., 2012; Sivakumar et al., 2016). Knockdown Ska1 inhibited cell proliferation and arrested cell cycle by decreasing the expression of Cyclin D1, Cyclin E1, and Cyclin B1 (L. J. Zhao, Yang, et al., 2017). Recently, researchers have reported that Ska1 can cooperate with Psrc1 in spindle dynamics and spindle attachment to the kinetochore (Park et al., 2016). These reports indicated that Psrc1 and Ska1 could take part in cell proliferation and cell cycle. Consistent with previous studies, our data showed that downregulation of Psrc1 and Ska1 in Schwann cells could decrease Schwann cell proliferation. Thus, loc680254 might regulate Schwann cell proliferation through the regulation of Psrc1 and Ska1 expression in a miRNA sponge manner.

Chitosan-based artificial nerve grafts, such as chitosan/PLGA-based, autologous marrow mesenchymal stem cells (MSCs)-containing tissue-engineered nerve grafts (TENGs), chitosan/silk fibroin-based, Schwann cell-derived extracellular matrix-modified scaffolds, are widely applied in peripheral nerve repair (Gu et al., 2014; Hu et al., 2013). To further explore the effect of loc680254 in nerve regeneration, we injected loc680254-overexpressing Schwann cells in the chitosan scaffolds to bridge never gaps after rat sciatic nerve transection. Although loc680254 overexpression slightly promoted axon outgrowth at 10 days after repair, its effect on functional recovery was not significant as detected at 12-week post-surgery. We considered that loc680254 might speed axon regeneration in a short time

after nerve injury. However, in the long period, all groups have a considerable axon regeneration. This phenomenon might own to the spontaneous regenerative ability of the peripheral nervous system and the promotion effects of chitosan scaffolds with their degradation products (Y. Zhao, Wang, et al., 2017).

In conclusion, our research uncovered a set of differentially expressed lncRNAs in sciatic nerve segments following peripheral nerve injury by RNA-seq analysis. Loc680254, one of the upregulated lncRNAs after nerve injury, could promote Schwann cell proliferation. Mechanism analysis showed that loc680254 might exert this regulation role by serving as a miRNA sponge to affect Psrc1 and Ska1 expression (Figure 7). Overall, our data provide further insights into the role of lncRNAs in peripheral nerve regeneration, and our findings might facilitate the development of new treatments for peripheral nerve repair.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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