Received: 2 June 2019 | Accepted: 7 October 2019

DOI: 10.1002/jcp.29393

ORIGINAL RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

Signatures of altered DNA methylation gene expression after central and peripheral nerve injury

Guidong Shi[1,2,3,4*] | Xianhu Zhou[3,4*] | Xu Wang[3,4*] | Xiaolei Zhang[3,4] | Ping Zhang[1,2] | Shiqing Feng[3,4]

[1]Department of Anatomy and Histology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

[2]Key Laboratory of Hormones and Development (Ministry of Health), Tianjin Key Laboratory of Metabolic Diseases, Tianjin Medical University, Tianjin, China

[3]Department of Orthopedics, Tianjin Medical University General Hospital, Tianjin, China

[4]International Science and Technology Cooperation Base of Spinal Cord Injury, Tianjin Key Laboratory of Spine and Spinal Cord Injury, Department of Orthopedics, Tianjin Medical University General Hospital, Tianjin, China

Correspondence
Ping Zhang, Department of Anatomy and Histology, School of Basic Medical Sciences, Tianjin Medical University, 22 Qixiangtai Rd., 300070 Tianjin, China.
Email: pizhang2008@163.com

Shiqing Feng, Department of othopaedics, Tianjin Medical University General Hospital, 154 Anshan Rd., Heping, 300052 Tianjin, China.
Email: sqfeng@tmu.edu.cn

Funding information
State Key Program of National Natural Science Foundation of China, Grant/Award Number: 81930070; State General Program National Natural Science Foundation of China, Grant/Award Numbers: 81371957, 81572100, 81772405; International Cooperation Program of National Natural Science Foundation of China, Grant/Award Number: 81620108018; Special Program for Sino-Russian Joint Research Sponsored by the Ministry of Science and Technology, Grant/Award Number: 2014DFR31210; Key Program Sponsored by the Tianjin Science and Technology Committee, China, Grant/Award Numbers: 13RCGFSY19000, 14ZCZDSY00044; Tianjin key research and development plan, key projects for science and technology support, Grant/Award Number: 19YFZCSY0060

Abstract

Nerve damage can lead to movement and sensory dysfunction, with high morbidity and disability rates causing severe burdens on patients, families, and society. DNA methylation is a kind of epigenetics, and a great number of previous studies have demonstrated that DNA methylation plays an important role in the process of nerve regeneration and remodeling. However, compared with the central nervous system, the peripheral nervous system shows stronger recovery after injury, which is related to the complex microenvironment and epigenetic changes occurring at the site of injury. Therefore, what common epigenetic changes between the central and peripheral nervous systems remain to be elucidated. We first screened differential methylation genes after spinal cord injury and sciatic nerve injury using whole-genome bisulfite sequencing and methylated DNA immunoprecipitation sequencing, respectively. Subsequently, a total of 16 genes had the same epigenetic changes after spinal cord injury and sciatic nerve injury. The Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes enrichment analysis were performed to identify the critical biological processes and pathways. Furthermore, a protein–protein interaction network analysis indicated that *Dnm3*, *Ntrk3*, *Smurf1*, *Dpysl2*, *Kalrn*, *Shank1*, *Dlg2*, *Arsb*, *Reln*, *Bmp5*, *Numbl*, *Prickle2*, *Map6*, and *Htr7* were the core genes. These outcomes may provide novel insights into the molecular mechanism of the subacute phase of nerve injury. These verified genes can offer potential diagnostic and therapeutic targets for nerve injury.

KEYWORDS
bioinformatic analysis, DNA methylation, peripheral nerve injury, spinal cord injury

Abbreviations: Arsb, arylsulfatase B; Bmp5, bone morphogenetic protein 5; BP, biological processes; CC, cellular component; CNS, central nervous system; DAVID, the Database for Annotation, Visualization and Integrated Discovery; Dlg2, discs large MAGUK scaffold protein 2; DMGs, differential methylation genes; Dnm3, dynamin 3; Dpysl2, dihydropyrimidinase-like 2; GDNF, glial cell line-derived neurotrophic factor; GO, Gene Ontology; Htr7, 5-hydroxytryptamine (serotonin) receptor 7; Kalrn, kalirin, RhoGEF kinase; KEGG, Kyoto Encyclopedia of Genes and Genomes; Lmx1a, LIM homeobox transcription factor 1α; Map6, microtubule-associated protein 6; MeDIP-seq, methylated DNA immunoprecipitation sequencing; MF, molecular function; mRNA, messenger RNA; NGF, nerve growth factor; NT3, neurotrophin-3; Ntrk3, neurotrophic receptor tyrosine kinase 3; Numbl, NUMB-like, endocytic adaptor protein; PNI, peripheral nerve injury; PPI, protein–protein interaction; Prickle2, prickle planar cell polarity protein 2; qRT-PCR, quantitative real-time polymerase chain reaction; Reln, reelin; SCI, spinal cord injury; Shank1, SH3 and multiple ankyrin repeat domains 1; Smurf1, SMAD specific E3 ubiquitin protein ligase 1; Tenm2, teneurin transmembrane protein 2; WGBS, whole-genome bisulfite sequencing.

*Guidong Shi, Xianhu Zhou and Xu Wang contributed equally to this study.

1 | BACKGROUND

The central nervous system (CNS) is a comprehensive, dynamic system. Examples of neural plasticity, whether at the level of the tissue, the cell, or at the genetic level, can be found during development, throughout the progression of the disease, or after injury (Kempermann et al., 2018; Kim, Kumar, Jo, & Kim, 2018; Zholudeva et al., 2018) However, nerve regeneration is still faced with a variety of problems, including (a) lack of neurotrophic factors, (b) primary and secondary apoptosis of nerve cells, and (c) microenvironment at the site of injury not conducive to axon regeneration (Dyck, Kataria, Akbari-Kelachayeh, Silver, & Karimi-Abdolrezaee, 2019; Huang, Mao, Chen, & Liu, 2015; Y. Wang et al., 2018). In recent years, exosomes, drugs and pharmacology, surgical repair of nerve defects, neurotrophic factors, tissue engineering, and genetic engineering have become mainstream research methods in the field of spinal cord injury (SCI; Bellver-Landete et al., 2019; Cheng et al., 2018; Zhou et al., 2018). The advances in conditional gene targeting and genetic fate mapping have allowed research of the specific biology of neurons in various experimental contexts, including central nervous injury (Chen et al., 2017). Previous studies have reported that paclitaxel-liposome-based collagen microchannel scaffolds induce neural stem cells to differentiate into neurons via the Wnt/β-catenin signaling pathway (X. Li et al., 2018). For the microenvironment of SCI, neurons seem to be more beneficial for nerve regeneration than a variety of glial cells (astrocytes, oligodendrocytes or microglia; Ben Haim & Rowitch, 2017).

The microenvironment of peripheral nerve regeneration consists of a variety of adhesion molecules, extracellular matrix molecules, and neurotrophic factors, which may be easily regenerated relative to the spinal cord (Haggerty, Bening, Pherribo, Dauer, & Oudega, 2019; Zhu et al., 2018). A sequential response triggered after peripheral nerve injury (PNI) is known as Wallerian degeneration (Jang et al., 2017). Subsequently, Schwann cells remove axonal and myelin debris, and secrete cytokines, thereby accelerating axonal regeneration (Ma, Duong, Moran, Junaidi, & Svaren, 2018; Stierli et al., 2018). In addition, the peripheral nerve microenvironment contains a variety of neurotrophic factors or growth factors, which play an irreplaceable role in nerve regeneration. In previous studies, different scaffolds combined with various neurotrophic factors or growth factors, such as glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3), achieved good nerve repair effects (Ma et al., 2018b; Zeng et al., 2015; Zhang et al., 2017).

DNA methylation is a common epigenetic modification in gene expression (Berson, Nativio, Berger, & Bonini, 2018; Chomyk et al., 2017). Other common epigenetic changes include histone modifications, genomic imprinting, gene silencing, chromatin remodeling, and noncoding RNA regulation (Chomyk et al., 2017). DNA methylation occurs primarily in the specific genomic region of the CpG island, which contains a large number of cytosine and guanine nucleotides (Lao & Grady, 2011). Therefore, a large amount of DNA methylation or demethylation changes in the promoter region are caused by nerve injury. Our previous study compared DNA methylation changes in spinal cord tissue after SCI and explored changes in genes and proteins in Schwann cells after sciatic nerve injury (Shi et al., 2018; Zhou et al., 2017). Although the therapeutic effect after PNI is significantly better than that after central nerve injury, no research has been conducted to explore whether there is an epigenetic link between the CNS and the peripheral nervous system.

Therefore, the purpose of this study was to evaluate the role of DNA methylation in nerve regeneration. We first performed DNA methylation sequencing before and after SCI and sciatic nerve injury in rats and analyzed their common hypermethylation and demethylation genes with bioinformatic analysis. Furthermore, the related genes were verified by quantitative real-time polymerase chain reaction (qRT-PCR). Our study contributes to a comprehensive understanding of the roles of DNA methylation in SCI and PNI, which may guide subsequently experimental studies on epigenetic treatment in nerve repair.

2 | MATERIALS AND METHODS

2.1 | Study design

All animals handling experimental protocols and procedures were approved by the Use Committee and Animal Care of Tianjin Medical University General Hospital. All procedures involving animals were consistent with the ethical standards set by the above-mentioned institutions. Wistar rats (230–280 g) were provided by the Laboratory Animal Center of Chinese People's Liberation Army General Hospital (Beijing, China, Approval Number: SCXK2012-0068). All Wistar rats were kept on a 12-hr light/dark cycle and maintained at a constant temperature of 25°C for 2 weeks before surgery.

2.2 | SCI and PNI

Thirty-six Wistar rats were used in this study. Four experimental groups were established for spinal cord surgery or peripheral nerve surgery: Group 1: Laminectomy group ($n = 9$); Group 2: SCI group ($n = 9$); Group 3: Sciatic nerve exposure group ($n = 9$); Group 4: Sciatic nerve injury group ($n = 9$). The surgery of spinal cord transection and sciatic nerve injury was performed according to previous studies (Du et al., 2015; Lai, Wang, Ling, Wu, & Zeng, 2014; Woodhoo et al., 2009). Briefly, all Wistar rats were deeply anesthetized with isoflurane (1–2%) to minimize suffering before surgery. Regarding SCI, following laminectomy at the T10–11 vertebral level of Group 2, the spinal cord was transected and a 2-mm segment of spinal cord tissue including visible spinal roots was completely removed at the T10–11 spinal cord level. Group 1 rats underwent laminectomy while without SCI. For the rats of Group 3, the bilateral sciatic nerves were exposed and ligated with silk at the proximal end of the sciatic nerve. Rats of Group 4 also underwent sciatic nerve exposure surgery while without PNI.

After the surgical incisions were closed, all rats received an intramuscular injection of penicillin (50,000 $U \cdot kg^{-1} \cdot day^{-1}$) for 7 days to prevent infection. For postoperative care, the bladder was emptied

TABLE 1 Information on primer sequences

Gene	Forward primer 5′ to 3′	Reverse primer 5′ to 3′	Annealing temperature (℃)
Kalrn	AGAAGGAGGTGCTGGAGGATGTC	GGTCTGCTGCTGCTGGAACTG	58.6
Dnm3	GTCACACCAGCCAACACCGATC	GGTGATAACGCCAATGGTCCTCAG	58.5
Bmp5	CCTCTTGCCAGTCTACACGATACC	GCTGCCGTCACTGCTTCTCC	57.5
Ntrk3	CACTTGTAATGGCTCTGGCTCTCC	TGTCTTCGCTCGTCACATTCACC	58.2
Smurf1	ACAGCAACATCGTCAGGTGGTTC	GCAGAGCCTTGAAGCCTTGGAG	58.4
Htr7	TTCTGTCGGTCTGGCTGCTCTC	ACCGCAGTGGAGTAGATCGTGTAG	58.2

manually twice a day until its function returned. After 2 weeks, all rats of Groups 1 and 2 were killed and the spinal cord tissue was harvested for whole-genome bisulfite sequencing (WGBS) and quantitative real-time PCR analysis, all rats of Groups 3 and 4 were killed and the bilateral sciatic nerves were isolated and harvested for methylated DNA immunoprecipitation sequencing (MeDIP-seq).

2.3 | Methylated DNA immunoprecipitation sequencing

MeDIP-seq were used to detect each sample according to the protocol of a previous study (N. Li et al., 2010). Briefly, 8 μg DNA from each group was sonicated to produce DNA fragments (100–500 bp) and purified using the a PCR purification kit (Takara, Japan). 5 μg of adapter-ligated DNA was immunoprecipitated using anti-5-methylcytosine monoclonal antibody (Abcam). Subsequently, qRT-PCR analysis was performed to verify the specificity of the immunoprecipitated fragments. A ~200 bp DNA fragment was purified using the DNA Clean & Concentrator-5 column. The amplicon quality and quantity were assessed using a 2100 analyzer DNA 1000 chip. The MeDIP library was sequenced on an Illumina HiSeq 2000 Sequencing System.

2.4 | Whole-genome bisulfite sequencing

Genomic DNA was extracted from the tissue of the spinal cord according to the DNA extraction kit instructions (TIANamp Genomic DNA Kit, China). In brief, 1 μg DNA from each group was sonicated to produce DNA fragments (300 bp) and analyzed by Illumina Infinium Human Methylation 450 BeadChip array (Illumina, China). Raw data analysis and preliminary data quality control were performed with GenomeStudio Software (v2.0, Illumina, China). Differentially methylated genes were identified (mean methylation difference ≥ 20, $p < .05$), as described earlier (S. Li et al., 2013).

2.5 | Protein–protein interaction (PPI) network analysis

The information of gene fusions, neighborhood, interaction of proteins, and gene fusions were provided by the String (Search Tool for the Retrieval of Interacting Genes Database, http://string-db.org/ 2019.02.06); Szklarczyk et al., 2015). The input gene sets were gene modules and species were *Rattus Norvegicus* in this study. To further explore the potential relevance of the differential expression genes in the peripheral nerve, an evidence threshold > 0.900 (highest confidence) was set as the cutoff value. Regarding the differential expression genes in the spinal cord, evidence threshold > 0.400 (medium confidence) was set as the cutoff value.

2.6 | Bioinformatic analysis

Afterwards, all differential methylation genes (DMGs) in the peripheral nerve genome and spinal cord genome were identified. For further statistical and functional analysis of gene expression, all DMGs were imported into GraphPad Prism Software (GraphPad v8.0, CA) and Cytoscape Software (v3.7.1; Shannon et al., 2003). All genes containing DMGs were used for subsequent functional enrichment analysis of Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using the DAVID (the Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/list.jsp 2019.02.06).

2.7 | Quantitative real-time PCR

For the quality and quantity analysis of the RNA in spinal cord tissues, the total RNA was extracted with a TRI® Reagent (Solarbio, China). RNA (1 μg) from each sample was reverse-transcribed into complementary DNA with a Primer Script RT Master Mixt (Takara, Japan). qRT-PCR was performed on a CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad) using SYBR-Green Premix Ex Taq (Takara). β-Actin acted as the internal control. All samples were analyzed in duplicate. Then, the average value of the duplicates was used for quantification. All primers are shown in Table 1.

2.8 | Statistical analysis

All data was presented as mean ± *SEM*. $p < .05$ was considered a statistically significant difference. Prism statistical software (GraphPad v8.0, CA) was employed for data analysis. Statistical differences between two groups were analyzed using Student's *t* test.

3 | RESULTS

3.1 | Identification of the common DMGs between SCI and PNI

After PNI, DMGs were crudely classified into six major groups according to the genomic architecture, including distal intergenic (58.17%), intron (31.0%), exon (4.03%), promoter (2.76%), 3′-utr (2.55%) and down stream (1.49%). The classification of DMGs based on their genomic architecture is shown in Figure 1a. The chromosomal distribution of these DMGs in both SCI and PNI ise shown in Figure 1b. In addition, the Venn diagram revealed some common DMGs between SCI or PNI (Figure 1c). 16 of these genes changed in common after both nervous system injuries, including: dynamin 3 (*Dnm3*), teneurin transmembrane protein 2 (*Tenm2*), reelin (*Reln*), neurotrophic receptor tyrosine kinase 3 (*Ntrk3*), bone morphogenetic protein 5 (*Bmp5*), LIM homeobox transcription factor 1α (*Lmx1a*), NUMB-like, endocytic adaptor protein (*Numbl*), discs large MAGUK scaffold protein 2 (*Dlg2*), kalirin, RhoGEF kinase (*Kalrn*), prickle planar cell polarity protein 2 (*Prickle2*), dihydropyrimidinase-like 2 (*Dpysl2*), 5-hydroxytryptamine (serotonin) receptor 7 (*Htr7*), arylsulfatase B (*Arsb*), microtubule-associated protein 6 (*Map6*), SMAD specific E3 ubiquitin protein ligase 1 (*Smurf1*), and SH3 and multiple ankyrin repeat domains 1 (*Shank1*) (Table 2). Among them, six genes were hypermethylated and 10 genes were hypomethylated. All of these aberrantly expressed genes are shown in a heat map (Figure 1d).

3.2 | GO and PPI networks analysis

Protein–protein interaction network analysis was performed by String. The PPI network of DMGs of the peripheral nerve is shown in Figure 2a. The red region indicate that these genes appear in the Hippo signaling pathway, and the yellow region shows these genes appear in the Endocytosis signaling pathway. A total of 473 nodes and 621 interaction pairs were included in this network. We used the genes that appeared both in the PPI map and the above heat map as the core genes, a total of 12 core DMGs were chosen: *Dnm3*, *Ntrk3*, *Smurf1*, *Dpysl2*, *Kalrn*, *Shank1*, *Dlg2*, *Arsb*, *Reln*, *Bmp5*, *Numbl*, and *Prickle2*. These genes and the number of gene cords are shown in Figure 2b.

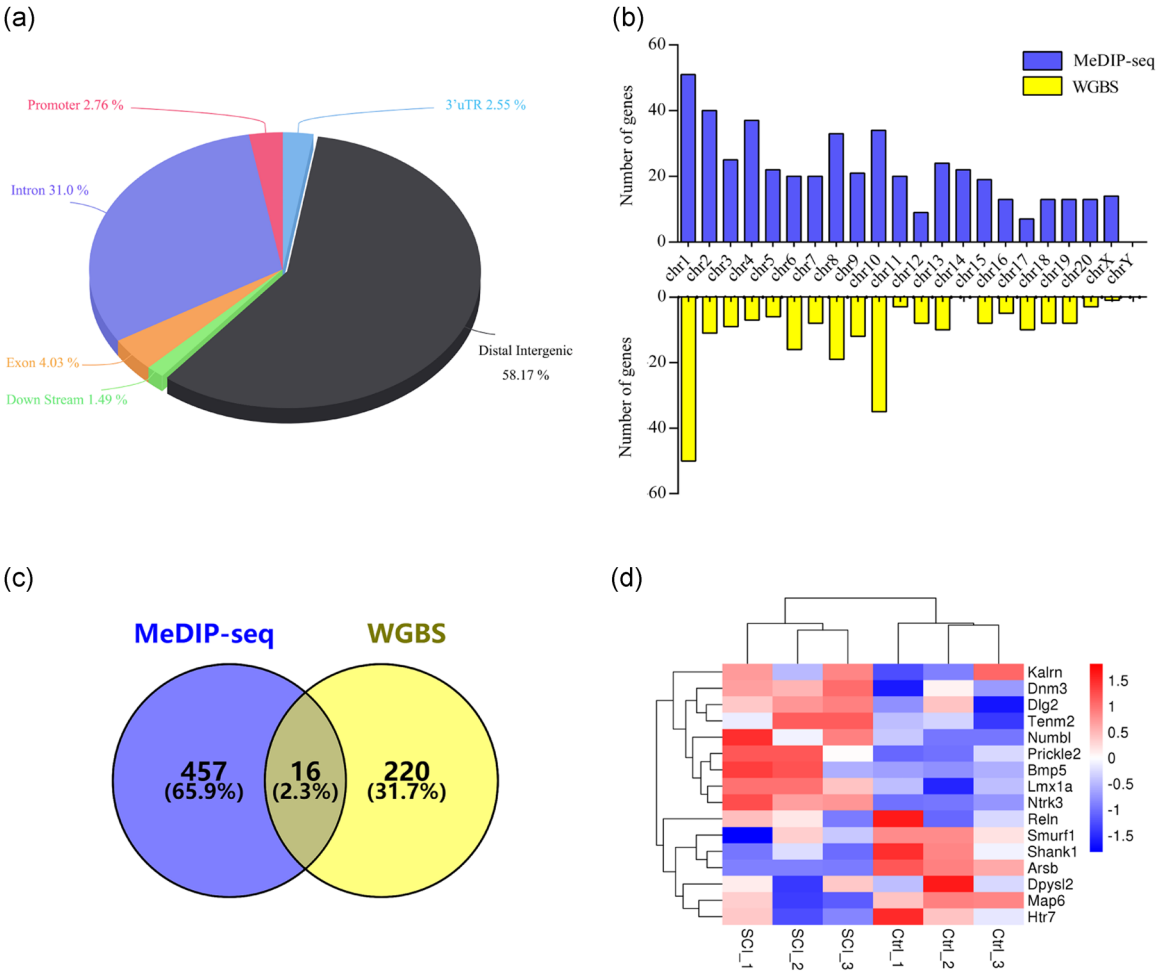


FIGURE 1 Expression signatures of differential methylation genes in SCI and PNI. (a) Differential methylation genes were classified according their genomic architecture in PNI. (b) Chromosome distribution showed the numbers of upregulated and downregulated genes located at different chromosomes. (c) The venn diagram shows the differential methylation genes both in SCI and PNI. (d) Representative heat map of the 16 common DMGs. DMGs, differential methylation gene; PNI, peripheral nerve injury; SCI, spinal cord injury

TABLE 2 Complete list of the 16 common genes

Symbol	Hyper/Hypo	mean.meth.diff	*p* Value	Q value
Shank1	Hyper	28.85705862	.00205133	0.005137091
Smurf1	Hyper	27.24660072	.023759601	0.03991613
Map6	Hyper	24.32450858	.005989683	0.011545902
Arsb	Hyper	22.42210761	5.69E − 14	2.65E − 12
Htr7	Hyper	21.33722148	.002715503	0.006281086
Dpysl2	Hyper	20.0708061	.006622656	0.009169831
Prickle2	Hypo	−20.45460701	.000537784	0.001861562
Kalrn	Hypo	−21.01056268	.035328719	0.049153
Dlg2	Hypo	−21.01963082	.033857952	0.047239905
Numbl	Hypo	−23.81366961	.006823299	0.012653334
Lmx1a	Hypo	−24.64511588	.007914547	0.014030334
Bmp5	Hypo	−26.02653512	.007933711	0.021016356
Ntrk3	Hypo	−26.34513213	.000915163	0.00273615
Reln	Hypo	−26.43260288	.006377447	0.013044777
Tenm2	Hypo	−28.79523122	.006225554	0.015732143
Dnm3	Hypo	−30.86117565	.004282423	0.009028545

The results of GO enrichment analysis were presented in Figure 2c. In biological processes (BP), the differentially methylated genes of PNI were significantly enriched in generation of neurons (GO: 0048699), neurogenesis (GO: 0022008), nervous system development (GO: 0007399), neuron differentiation (GO:0030182), and cell differentiation (GO: 0030182). Regarding molecular function (MF), the differentially methylated genes of PNI were significantly enriched in binding (GO: 0005488), protein binding (GO: 0005515), enzyme binding (GO: 0019889), signaling receptor binding (GO: 0005102), and ion binding (GO: 0043167). In the cellular component (CC), the differentially methylated genes of PNI were significantly enriched in plasma membrane bounded cell projection (GO: 0120025), neuron part (GO: 0097458), neuron projection (GO: 0043005), plasma membrane bounded cell projection part (GO: 0120038), and somatodendritic compartment (GO: 0036477).

The PPI network of DMGs of spinal injury cord is shown in Figure 3a. A total of 233 nodes and 178 interaction pairs were included in this network. We used the genes that appeared both in the PPI map and the above heat map as the core genes, a total of 13 core DMGs after SCI were chosen: *Dlg2*, *Dnm3*, *Arsb*, *Smurf1*, *Dpysl2*, *Kalrn*, *Numbl*, *Ntrk3*, *Reln*, *Prickle2*, *Shank1*, *Map6*, and *Htr7*. These genes and the number of gene cords are shown in Figure 3b. The results of GO enrichment analysis were presented as DMGs of SCI in Figure 3c. In biological processes, the differentially methylated genes of SCI were significantly enriched in biological regulation (GO: 0065007), regulation of multicellular organism development (GO: 2000026), regulation of intracellular signal transduction (GO: 1902531), regulation of plasma membrane bounded cell projection organization (GO:0120035), and regulation of biological quality (GO: 0065008). Regarding the molecular function, the differentially methylated genes of SCI were significantly enriched in binding (GO: 0005488), protein domain specific binding (GO: 0019904), protein binding (GO: 0005515), metal ion binding (GO: 0046872), and cation binding (GO: 0043169). In the cellular component, the differentially methylated genes of SCI were significantly enriched in the membrane (GO: 0016020), plasma membrane (GO: 0005886), synapse (GO: 0045202), cell part (GO: 0044464), and plasma membrane part (GO: 0044459).

3.3 | KEGG analysis

Regarding the KEGG enrichment analysis, the results of KEGG enrichment analysis showed that the DMGs of both SCI and PNI were mainly enriched in Hippo signaling pathway and Endocytosis pathway (Figure 4a,b). Among these pathways, the Hippo signaling pathway caught our attention. Our previous studies have demonstrated that after PNI, the Hippo signaling pathway is activated and accompanied by a large number of gene demethylation changes (Zhou et al., 2017). In the Hippo signaling pathway, genes with blue background indicated the DMGs after PNI, genes with yellow background indicated the DMGs after SCI, while the genes with green background indicated the common DMGs after SCI and PNI. *Id1*, *Id2*, *Gli2*, and *CTGF* are genes related to antiapoptosis, proapoptosis, and pro-proliferation. This suggests that the Hippo signaling pathway may be an important pathway relate to apoptosis and proliferate during the neuroregeneration (Figure 4c).

3.4 | Genes expression validation by qRT-PCR

In addition to validating the analysis results, qRT-PCR was used to quantify parts of mRNAs (messenger RNAs) of the corresponding methylated genes in the SCI group compared with the Sham group. Among these core genes, there were two differentially hypermethylated genes (*Htr7* and *Smurf1*) and four differentially hypomethylated genes (*Dnm3*, *Bmp5*, Ntrk3, and *Kalrn*). A search of PubMed revealed that all of these genes are involved in CNS repair. Figure 5 shows that two mRNAs of differentially hypermethylated genes were downregulated in the SCI group compared with the sham group ($p < .05$), and four mRNAs of differentially hypomethylated genes were upregulated in the SCI group compared with the sham group ($p < .05$).

4 | DISCUSSION

Induction of effective neuron and axonal regeneration are the basis for improved neurological function, whether in central or PNI. It has been proved that whether neurons and axons can regenerate after nerve injury not only depends on their inherent growth ability but also depends on the microenvironment and epigenetic changes of the injured nerve (Kameda, Imamura, & Nakashima, 2018; Loh et al., 2017). After nervous system injury, the ability of nerve cells to express axon regeneration-related genes and the ability to form cytoskeletal materials are the main intrinsic factors that determine

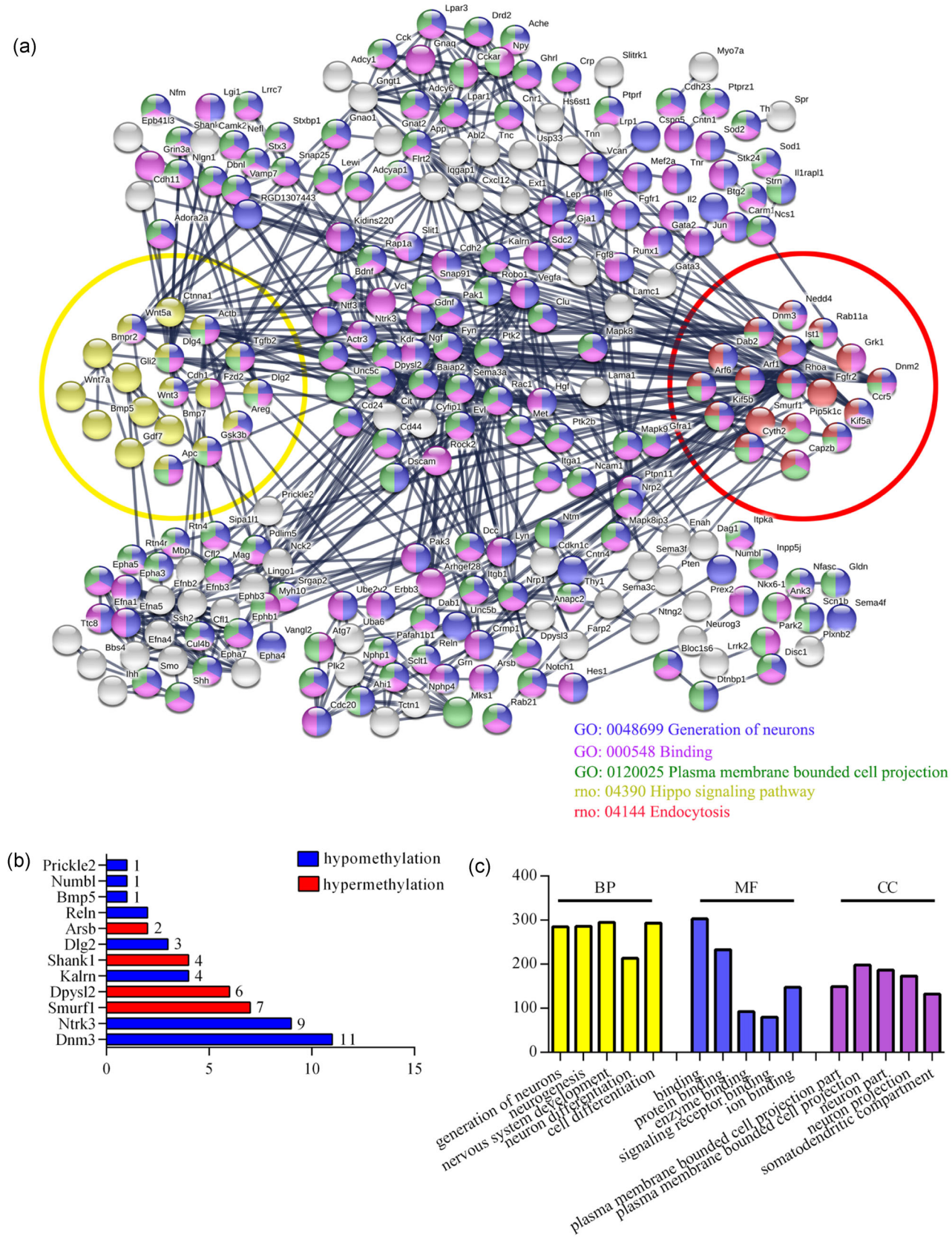


FIGURE 2 PPI network and the Core genes of the differential methylation genes in PNI. (a) PPI network of the differential methylation genes in PNI. Red region indicates that these genes appear in the Hippo signaling pathway, and the yellow region shows these genes appear in the Endocytosis signaling pathway. (b) Core genes of hypermethylated genes and hypomethylated genes in PNI. (c) GO analysis of differential methylation genes in PNI. BP, biological processes; CC, cellular component; GO, gene ontology; MF, molecular function; PNI, peripheral nerve injury; PPI, protein–protein interaction

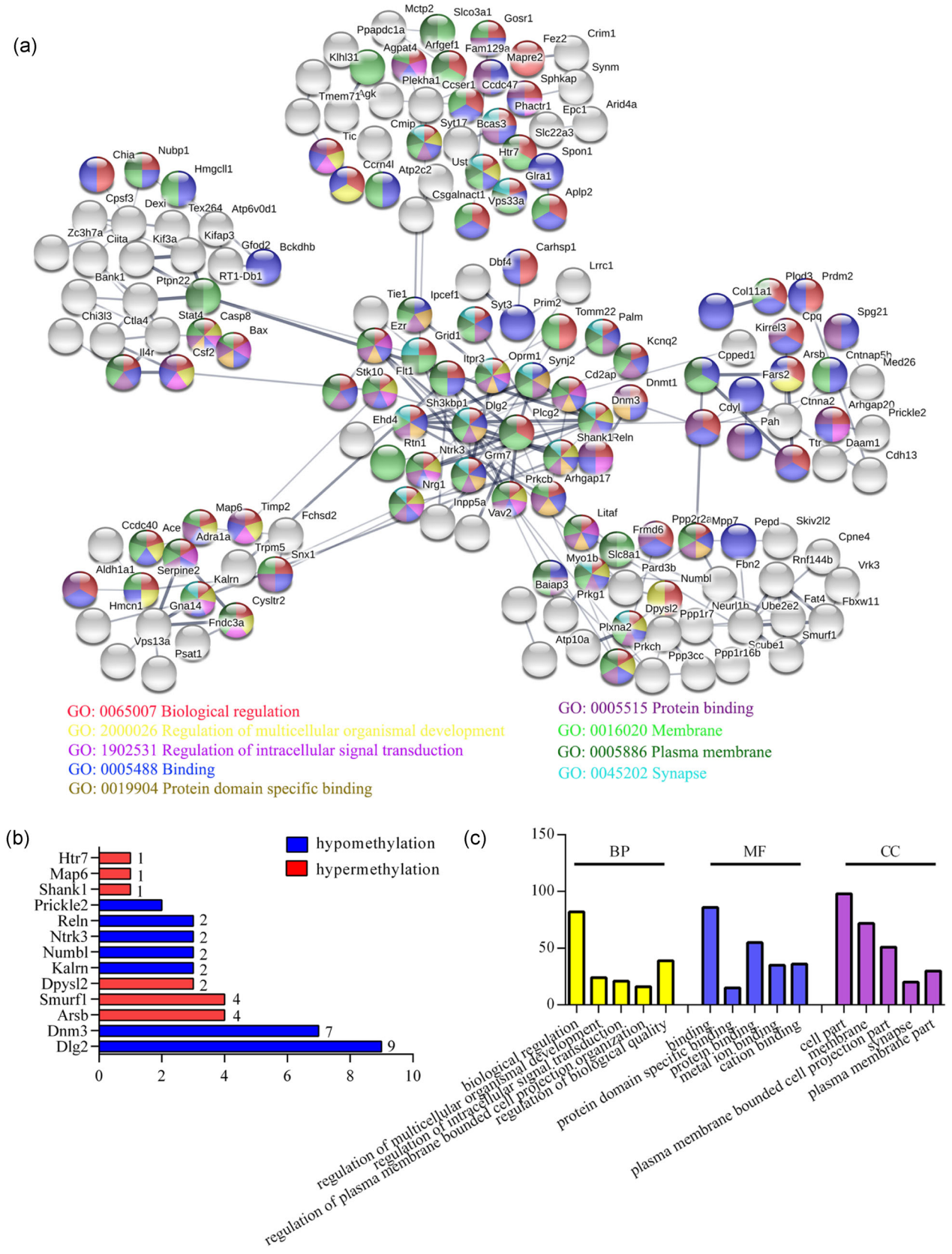
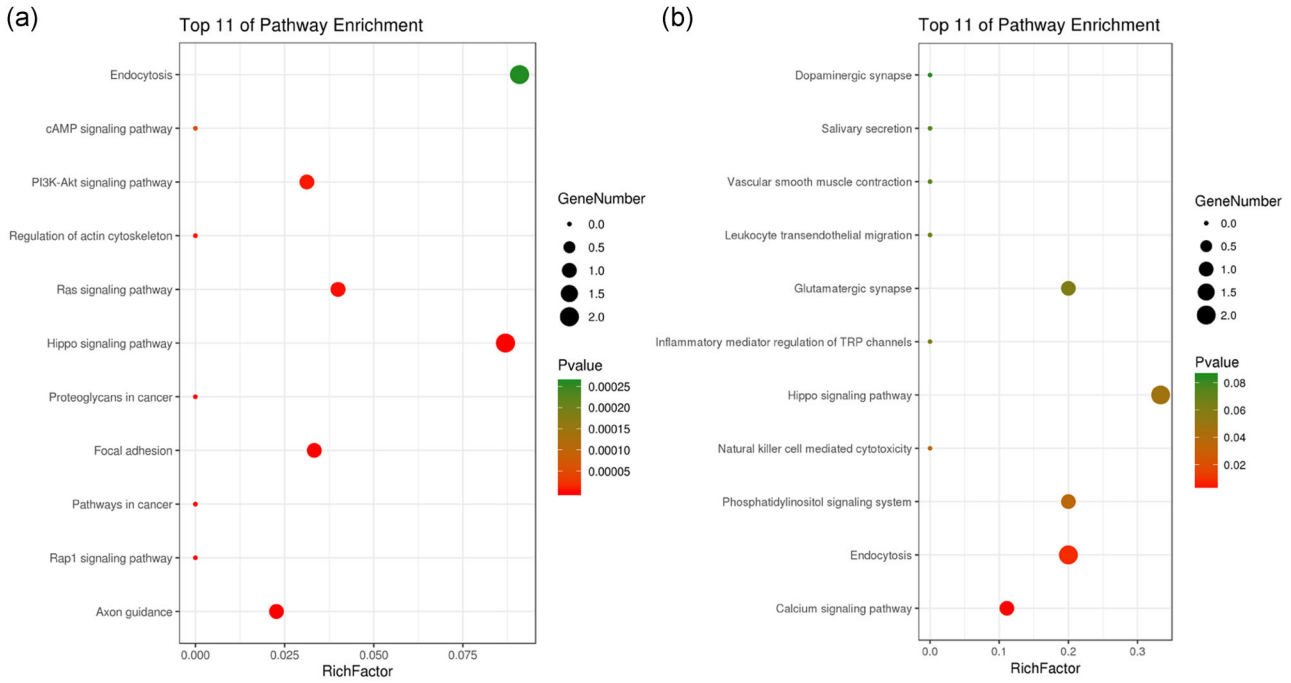


FIGURE 3 PPI network and the Core genes of the differential methylation genes in SCI. (a) PPI network of the differential methylation genes in SCI. (b) Core genes of hypermethylated genes and hypomethylated genes in SCI. (c) GO analysis of differential methylation genes in SCI. BP, biological processes; CC, cellular component; GO, gene ontology; MF, molecular function; PPI, protein–protein interaction; SCI, spinal cord injury



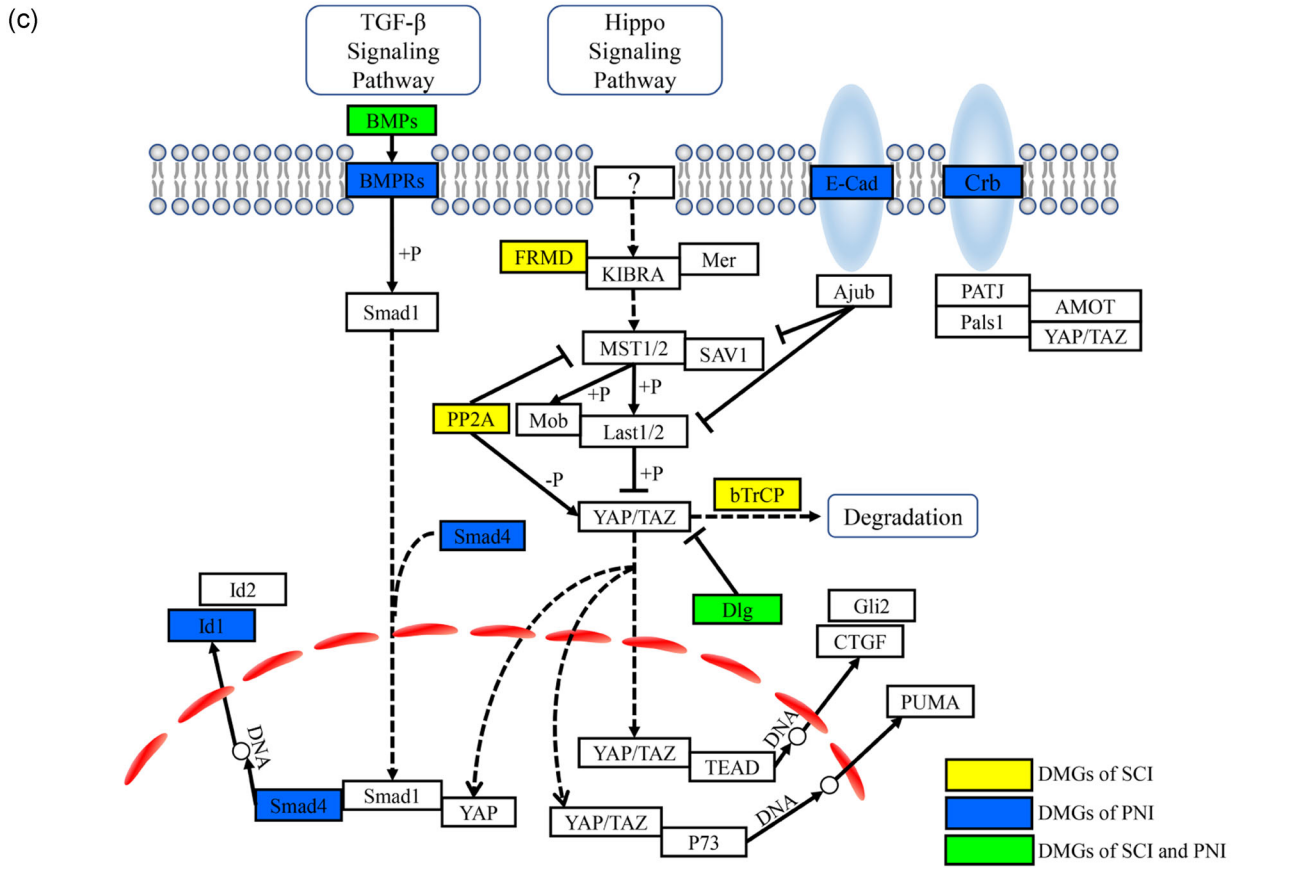


FIGURE 4 KEGG pathway analysis of mRNAs in the subacute phase of SCI and PNI. (a) Top 11 of KEGG pathway analysis of differential methylation genes in PNI. The circles represent biological processes; The color represents *P* value; The size of the circles indicate the number of genes in one pathway; (b) Top 11 of KEGG pathway analysis of differential methylation genes in SCI. (C) Both *BMP* and *Dlg* may exert an antiapoptotic and proliferative role through the Hippo signaling pathway. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; PNI, peripheral nerve injury; SCI, spinal cord injury

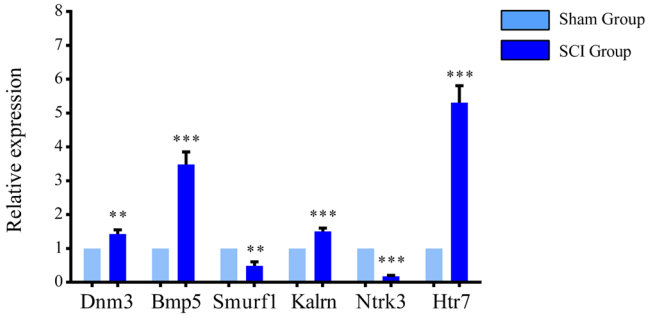


FIGURE 5 Validation of the differential expression of 6 mRNAs of the corresponding genes identified in the SCI group compared with the sham group by qRT-PCR. Values are means ± *SE* (*$p < .05$, **$p < .01$, ***$p < .001$). mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; SCI, spinal cord injury

the intrinsic growth ability of axons (Fu, 2014). In addition, as the external cause of axon regeneration, the inhibitory or promoting mediators in the lesion microenvironment also play an important role (York, Petit, & Roskams, 2013). In recent years, with the optimization and development of techniques such as gene sequencing and chromatin immunoprecipitation, epigenetic targets closely related to neuronal and axonal regeneration have been discovered, which is expected to provide powerful clinical treatment for neurological damage. In this study, we first established a subacute model of SCI and sciatic nerve injury. Then, whole-genome bisulfite sequencing and methylated DNA immunoprecipitation sequencing were used to detect differential DNA methylation genes after central and PNI. Approximately 273 DMGs were screened out after SCI and 473 DMGs were identified after PNI. We further detected 16 common genes between these two DMGs. After GO analysis, KEGG signaling pathway analysis, and PPI network analysis of these common DMGs, several core genes were screened out, such as *Kalrn*, *Dnm3*, *Ntrk3*, *Bmp5*, *Dpysl2*, *Htr7*, *Smurf1*, and *Shank1*. Finally, qRT-PCR was used to verify the expression of these genes.

The GO enrichment analysis and KEGG enrichment analysis were carried out on the DMGs. The GO analysis showed that generation of neurons, neurogenesis, binding, protein binding, plasma membrane bounded cell projection, and the neuron part were enriched in the biological processes of PNI. In addition, the biological regulation, regulation of multicellular organism development, binding, protein domain specific binding, membrane, and plasma membrane bounded cell projection part were enriched in the biological processes of SCI. There is increasing evidence that epigenetic components are present in the regulation of changes in gene expression necessary for neural regeneration (VandenBosch & Reh, 2019). Epigenetics is mainly embodied in DNA methylation and histone acetylation during the development and regeneration of neural tissue. The role of DNA methylation in nervous system development is an intense area of research. Previous studies have supported the fact that DNA methylation affects many aspects of neural stem cell maintenance and proliferation, neuronal differentiation and maturation, and synaptogenesis (Jobe & Zhao, 2017; Stricker & Gotz, 2018). This is consistent with our results in the GO enrichment analysis.

From the results of the KEGG pathway analysis, the most significant pathways were involved in the Hippo signaling pathway, Endocytosis pathway, Ras signaling pathway, Glutamatergic synapse pathway and Phosphatidylinositol signaling pathway. It has been reported that BMPs were involved in protection and recovery after injury as well as in neurogenesis. Previous studies showed that BMP/Smad signaling molecules were upregulated on dedifferentiated Schwann cells after PNI (Kokubu, Tsujii, Akeda, Iino, & Sudo, 2018). BMP ligand and its receptor units are also highly expressed in the neurogenesis process of the embryonic CNS, and help to regulate cell proliferation, survival, differentiation, apoptosis and lineage commitment. In addition, BMPs can be re-expressed after injury and participate in the functional recovery of the CNS, they also can antagonize the oligodendrocyte apoptosis caused by tumor necrosis factor (X. Wang, Xu, Wang, Yang, & Li, 2016; Zhang, Mehler, Song, & Kessler, 1998). These facts are consistent with our results that BMP is highly expressed after nerve injury to inhibit apoptosis and promote cell proliferation.

Furthermore, the PPI network suggested that *Dnm3*, *Ntrk3*, *Smurf1*, *Dpysl2*, *Kalrn*, *Shank1*, *Dlg2*, *Arsb*, *Reln*, *Bmp5*, *Numbl*, and *Prickle2* were the top12 common DMGs, which may be involved in the pathological process after PNI. And *Dlg2*, *Dnm3*, *Arsb*, *Smurf1*, *Dpysl2*, *Kalrn*, *Numbl*, *Ntrk3*, *Reln*, *Prickle2*, *Shank1*, *Map6*, and *Htr7* were the top13 common DMGs after SCI. Among the above genes, *Smurf1* attracted our attention. Smad ubiquitin regulatory factors belong to the HECT-family of E3 ubiquitin ligases and comprise two members: Smurf1 and Smurf2. Through regulation of a lot of proteins in multiple cellular compartments, Smurfs regulate diverse cellular processes, including cell proliferation, cell-cycle progression, and differentiation (David, Nair, & Pillai, 2013). After acute SCI, Smurf1 is upregulated by pro-inflammatory cytokines and associated with oligodendrocyte apoptosis, these suggest that Smurf1 might promote neuronal necroptosis after neuroinflammation (D. Li et al., 2013). This is consistent with the results of methylation and upregulation of Smurf1 after nerve injury in our study. In addition, previous studies have shown that inhibition of Smurf1 in rat mesenchymal stem cells can significantly promote bone formation and accelerate bone mineralization (Sun et al., 2017). Some genes that are affected only after PCI but not after SCI also caused concern (*Rhoa*, *Rac1*, *Fyn*, *Ephb1*, *Efnb3*, *Gngt1*, and *App*). Among them, *Rhoa* is an important inhibitory factor in neural regeneration. The previous study indicates that ascorbic acid facilitates neural regeneration after sciatic nerve crush injury. That maybe related to the fact that ascorbic acid could promote neurite outgrowth and alleviates *Rhoa* expression in cultured dorsal root ganglia neurons (L. Li et al., 2019). This is consistent with the height change of Rhoa gene detected after PNI in this project.

Although there are important discoveries related to these studies, there are also limitations. First, only 36 Wistar rats were studied in this study, with a small sample size, and the experimental results of rodents could not represent all the animals. In future research, a larger sample of primates can be used as experimental subjects, and the results obtained may provide a strong theoretical basis for clinical nerve repair. Second, the peripheral nerve of this project is the sciatic nerve, while the sciatic nerves cannot represent

all the peripheral nerves. We expect to see more work on different peripheral nerve regeneration in future studies. Third, two different epigenetic sequencing methods, WGBS and MeDIP, were used in this study. Compared with WGBS, although MeDIP-seq requires less time and cost, it requires a large sample size and the obtained sequencing data are not comprehensive enough to be used for single-base level sequencing. Finally, this study did not evaluate the central and peripheral nerves histologically and behaviorally, and we will add the corresponding experiments in our future work.

5 | CONCLUSION

In conclusion, the present study reveals the common changes of DNA methylation after SCI and sciatic nerve injury. We used bioinformatics and qRT-PCR to identify and verify DMGs after nervous system injury. Epigenetic changes of some common genes after central and PNI are considered to be candidate targets or biomarkers for nerve regeneration and remodeling. These outcomes may provide novel insights into the molecular mechanism of the subacute phase of nerve injury. Further investigations are required to validate the function and relationships among these epigenetic changes.

ACKNOWLEDGMENTS

This study was supported by grants from the State General Program National Natural Science Foundation of China (81772405 and 81572100 to PZ, 81371957 to XZ); the State Key Program of National Natural Science Foundation of China (81330042 to SF); the Special Program for Sino-Russian Joint Research Sponsored by the Ministry of Science and Technology (2014DFR31210 to SF); the International Cooperation Program of National Natural Science Foundation of China (81620108018 to SF); and the Key Program Sponsored by the Tianjin Science and Technology Committee, China (14ZCZDSY00044 and13RCGFSY19000 to SF).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

G. S. wrote the manuscript. X. Z. and X. W. conceived the study and designed the experiments. X. Z. contributed to the data collection, performed the data analysis and interpreted the results. P. Z. and S. F. contributed to the critical revision of article. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Shiqing Feng http://orcid.org/0000-0001-9437-7674

REFERENCES

Bellver-Landete, V., Bretheau, F., Mailhot, B., Vallieres, N., Lessard, M., Janelle, M. E., … Lacroix, S. (2019). Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury. *Nature Communications*, *10*(1), 518.

Ben Haim, L., & Rowitch, D. H. (2017). Functional diversity of astrocytes in neural circuit regulation. *Nature Reviews Neuroscience*, *18*(1), 31–41.

Berson, A., Nativio, R., Berger, S. L., & Bonini, N. M. (2018). Epigenetic regulation in neurodegenerative diseases. *Trends in Neurosciences*, *41*(9), 587–598.

Chen, J., Wang, Z., Zheng, Z., Chen, Y., Khor, S., Shi, K., … Xiao, J. (2017). Neuron and microglia/macrophage-derived FGF10 activate neuronal FGFR2/PI3K/Akt signaling and inhibit microglia/macrophages TLR4/NF-kappaB-dependent neuroinflammation to improve functional recovery after spinal cord injury. *Cell Death & Disease*, *8*(10):e3090.

Cheng, J., Korte, N., Nortley, R., Sethi, H., Tang, Y., & Attwell, D. (2018). Targeting pericytes for therapeutic approaches to neurological disorders. *Acta Neuropathologica*, *136*(4), 507–523.

Chomyk, A. M., Volsko, C., Tripathi, A., Deckard, S. A., Trapp, B. D., Fox, R. J., & Dutta, R. (2017). DNA methylation in demyelinated multiple sclerosis hippocampus. *Scientific Reports*, *7*(1), 8696.

David, D., Nair, S. A., & Pillai, M. R. (2013). Smurf E3 ubiquitin ligases at the cross roads of oncogenesis and tumor suppression. *Biochimica et Biophysica Acta*, *1835*(1), 119–128.

Du, B. L., Zeng, X., Ma, Y. H., Lai, B. Q., Wang, J. M., Ling, E. A., … Zeng, Y. S. (2015). Graft of the gelatin sponge scaffold containing genetically-modified neural stem cells promotes cell differentiation, axon regeneration, and functional recovery in rat with spinal cord transection. *Journal of Biomedical Materials Research. Part A*, *103*(4), 1533–1545.

Dyck, S., Kataria, H., Akbari-Kelachayeh, K., Silver, J., & Karimi-Abdolrezaee, S. (2019). LAR and PTPsigma receptors are negative regulators of oligodendrogenesis and oligodendrocyte integrity in spinal cord injury. *Gila*, *67*(1), 125–145.

Fu, X. (2014). The immunogenicity of cells derived from induced pluripotent stem cells. *Cellular & Molecular Immunology*, *11*(1), 14–16.

Haggerty, A. E., Bening, M. R., Pherribo, G., Dauer, E. A., & Oudega, M. (2019). Laminin polymer treatment accelerates repair of the crushed peripheral nerve in adult rats. *Acta Biomaterialia*, *86*, 185–193.

Huang, H., Mao, G., Chen, L., & Liu, A. (2015). Progress and challenges with clinical cell therapy in neurorestoratology. *Journal of Neurorestoratology*, *3*, 91–95.

Jang, S. Y., Yoon, B. A., Shin, Y. K., Yun, S. H., Jo, Y. R., Choi, Y. Y., … Park, H. T. (2017). Schwann cell dedifferentiation-associated demyelination leads to exocytotic myelin clearance in inflammatory segmental demyelination. *Glia*, *65*(11), 1848–1862.

Jobe, E. M., & Zhao, X. (2017). DNA Methylation and Adult Neurogenesis. *Brain Plasticity*, *3*(1), 5–26.

Kameda, T., Imamura, T., & Nakashima, K. (2018). Epigenetic regulation of neural stem cell differentiation towards spinal cord regeneration. *Cell and Tissue Research*, *371*(1), 189–199.

Kempermann, G., Gage, F. H., Aigner, L., Song, H., Curtis, M. A., Thuret, S., … Frisen, J. (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell*, *23*(1), 25–30.

Kim, H. Y., Kumar, H., Jo, M. J., & Kim, J. (2018). Therapeutic efficacy-potentiated and diseased organ-targeting nanovesicles derived from mesenchymal stem cells for spinal cord injury treatment. *Nano Letters*, *18*(8), 4965–4975.

Kokubu, N., Tsujii, M., Akeda, K., Iino, T., & Sudo, A. (2018). BMP-7/Smad expression in dedifferentiated Schwann cells during axonal regeneration and upregulation of endogenous BMP-7 following administration of PTH (1-34). *Journal of Orthopaedic Surgery*, *26*(3), 2309499018812953.

Lai, B. Q., Wang, J. M., Ling, E. A., Wu, J. L., & Zeng, Y. S. (2014). Graft of a tissue-engineered neural scaffold serves as a promising strategy to restore myelination after rat spinal cord transection. *Stem Cells and Development*, *23*(8), 910–921.

Lao, V. V., & Grady, W. M. (2011). Epigenetics and colorectal cancer. *Nature Reviews Gastroenterology & Hepatology*, *8*(12), 686–700.

Li, D., Zhang, J., Huang, W., Jin, H., Shen, A., Yang, L., … Cui, Z. (2013). Upregulation of Smurf1 after spinal cord injury in adult rats. *Journal of Molecular Histology*, *44*(4), 381–390.

Li, L., Li, Y., Fan, Z., Wang, X., Li, Z., Wen, J., … Guo, J. (2019). Ascorbic acid facilitates neural regeneration after sciatic nerve crush injury. *Frontiers in Cellular Neuroscience*, *13*(108), 1–17.

Li, N., Ye, M., Li, Y., Yan, Z., Butcher, L. M., Sun, J., … Wang, J. (2010). Whole genome DNA methylation analysis based on high throughput sequencing technology. *Methods*, *52*(3), 203–212.

Li, S., Garrett-Bakelman, F. E., Akalin, A., Zumbo, P., Levine, R., To, B. L., … Mason, C. E. (2013). An optimized algorithm for detecting and annotating regional differential methylation. *BMC Bioinformatics*, *14*(Suppl. 5), S10.

Li, X., Fan, C., Xiao, Z., Zhao, Y., Zhang, H., Sun, J., … Dai, J. (2018). A collagen microchannel scaffold carrying paclitaxel-liposomes induces neuronal differentiation of neural stem cells through Wnt/beta-catenin signaling for spinal cord injury repair. *Biomaterials*, *183*, 114–127.

Loh, Y. E., Koemeter-Cox, A., Finelli, M. J., Shen, L., Friedel, R. H., & Zou, H. (2017). Comprehensive mapping of 5-hydroxymethylcytosine epigenetic dynamics in axon regeneration. *Epigenetics*, *12*(2), 77–92.

Ma, K. H., Duong, P., Moran, J. J., Junaidi, N., & Svaren, J. (2018). Polycomb repression regulates Schwann cell proliferation and axon regeneration after nerve injury. *Glia*, *66*(11), 2487–2502.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., … Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research*, *13*(11), 2498–2504.

Shi, G. D., Zhang, X. L., Cheng, X., Wang, X., Fan, B. Y., Liu, S., … Feng, S. Q. (2018). Abnormal DNA methylation in thoracic spinal cord tissue following transection injury. *Medical Science Monitor*, *24*, 8878–8890.

Stierli, S., Napoli, I., White, I. J., Cattin, A. L., Monteza Cabrejos, A., Garcia Calavia, N., … Lloyd, A. C. (2018). The regulation of the homeostasis and regeneration of peripheral nerve is distinct from the CNS and independent of a stem cell population. *Development*, *145*(24), 1–12.

Stricker, S. H., & Gotz, M. (2018). DNA-methylation: Master or slave of neural fate decisions? *Frontiers in Neuroscience*, *12*, 5.

Sun, Y., Xu, J., Xu, L., Zhang, J., Chan, K., Pan, X., & Li, G. (2017). MiR-503 promotes bone formation in distraction osteogenesis through suppressing Smurf1 expression. *Scientific Reports*, *7*(1), 409.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., … von Mering, C. (2015). STRING v10: Protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, *43*(Database issue), D447–D452.

VandenBosch, L. S., & Reh, T. A. (2019). Epigenetics in neuronal regeneration. *Seminars in Cell & Developmental Biology*, *S1084-9521*(18), 30335–30335.

Wang, X., Xu, J. M., Wang, Y. P., Yang, L., & Li, Z. J. (2016). Protective effects of BMP-7 against tumor necrosis factor alpha-induced oligodendrocyte apoptosis. *International Journal of Developmental Neuroscience*, *53*, 10–17.

Wang, Y., Wu, W., Wu, X., Sun, Y., Zhang, Y. P., Deng, L. X., … Chen, C. (2018). Remodeling of lumbar motor circuitry remote to a thoracic spinal cord injury promotes locomotor recovery. *eLife*, *7*, 1–29. e39016.

Woodhoo, A., Alonso, M. B., Droggiti, A., Turmaine, M., D'Antonio, M., Parkinson, D. B., … Jessen, K. R. (2009). Notch controls embryonic Schwann cell differentiation, postnatal myelination and adult plasticity. *Nature Neuroscience*, *12*(7), 839–847.

York, E. M., Petit, A., & Roskams, A. J. (2013). Epigenetics of neural repair following spinal cord injury. *Neurotherapeutics*, *10*(4), 757–770.

Zeng, X., Qiu, X. C., Ma, Y. H., Duan, J. J., Chen, Y. F., Gu, H. Y., … Zeng, Y. S. (2015). Integration of donor mesenchymal stem cell-derived neuron-like cells into host neural network after rat spinal cord transection. *Biomaterials*, *53*, 184–201.

Zhang, D., Mehler, M. F., Song, Q., & Kessler, J. A. (1998). Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating trkC expression. *The Journal of Neuroscience*, *18*(9), 3314–3326.

Zhang, L., Yang, W., Tao, K., Song, Y., Xie, H., Wang, J., … Wang, L. (2017). Sustained local release of NGF from a Chitosan-Sericin composite scaffold for treating chronic nerve compression. *ACS Applied Materials and Interfaces*, *9*(4), 3432–3444.

Zholudeva, L. V., Qiang, L., Marchenko, V., Dougherty, K. J., Sakiyama-Elbert, S. E., & Lane, M. A. (2018). The neuroplastic and therapeutic potential of spinal interneurons in the injured spinal cord. *Trends in Neurosciences*, *41*(9), 625–639.

Zhou, X., Shi, G., Fan, B., Cheng, X., Zhang, X., Wang, X., … Feng, S. (2018). Polycaprolactone electrospun fiber scaffold loaded with iPSCs-NSCs and ASCs as a novel tissue engineering scaffold for the treatment of spinal cord injury. *International Journal of Nanomedicine*, *13*, 6265–6277.

Zhou, X. H., Lin, W., Ren, Y. M., Liu, S., Fan, B. Y., Wei, Z. J., … Feng, S. Q. (2017). Comparison of DNA methylation in Schwann cells before and after peripheral nerve injury in rats. *BioMed Research International*, *8*. 5393268.

Zhu, H., Xue, C., Yao, M., Wang, H., Zhang, P., Qian, T., … Gu, X. (2018). miR-129 controls axonal regeneration via regulating insulin-like growth factor-1 in peripheral nerve injury. *Cell Death and Disease*, *9*(7), 720.

How to cite this article: Shi G, Zhou X, Wang X, Zhang X, Zhang P, Feng S. Signatures of altered DNA methylation gene expression after central and peripheral nerve injury. *J Cell Physiol*. 2019;1–11. https://doi.org/10.1002/jcp.29393