iTRAQ-based proteomics profiling of Schwann cells before and after peripheral nerve injury

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Abstract

Objective(s): Schwann cells (SCs) have a wide range of applications as seed cells in the treatment of nerve injury during transplantation. However, there has been no report yet on kinds of proteomics changes that occur in Schwann cells before and after peripheral nerve injury.

Materials and Methods: Activated Schwann cells (ASCs) and normal Schwann cells (NSCs) were obtained from adult Wistar rat sciatic nerves. After immunofluorescence identification, we identified differentially expressed proteins in the ASCs and NSCs using iTRAQ tags for relative and absolute quantitation (iTRAQ) combined with high-resolution Orbitrap liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). In addition, all the differentially expressed proteins were analyzed by Gene ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. Finally, several differently expressed proteins were selected for Western blot verification.

Results: A total of 122 differentially expressed proteins in ASCs and NSCs were screened. GO analysis suggested that these different proteins are likely to accumulate in the cytoplasm and are associated with single-multicellular organism processes. The KEGG pathway analysis suggested that proteins related to purine metabolism were significantly enriched. The expression of Transmembrane glycoprotein NMB (GPNMB), Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 (ENPP3), and other proteins were consistent with the proteomics data obtained by Western blot analysis.

Conclusion: GPNMB, ENPP3, GFPT2, and other proteins may play an important role in the repair of peripheral nerve injury. This study may provide new insights into changes in SCs after peripheral nerve injury.

Introduction

With the development of innovative technologies in the medical field worldwide, treatment of nerve injury tends to be diversified. In addition to the central nervous system (CNS) damage, peripheral nerve injury has become a common concern among scientists and doctors around the world (1-3). Since the previous drug and surgical treatments to the emergence of cell therapies today, treatment technology has been constantly updated, but there is also a corresponding emergence of some problems (4-6). In this study, we assess the differential protein expression of Schwann cells before and after peripheral nerve injury and proceed to explore some of the changes in protein expression occurring in the cell.

Schwann cells have gained increasing attention in the field of nerve regeneration owing to their ability to repair nerve injury and promote axonal regeneration and myelination (7-10). Recently, treatment with SCs combined with other stem cells, such as mesenchymal or neural stem cells, and other treatment strategies have also been more widely recognized (11-14). However, the role of SCs in repair of the peripheral nerves and the underlying specific pathophysiological mechanisms are still unknown. Furthermore, after peripheral nerve injury and Wallerian degeneration (15, 16), changes in the proteomics of SCs have not yet been clearly reported through specific studies.

Isobaric tags for relative and absolute quantitation (iTRAQ) is an equal weight labeling technique for relative and absolute quantification of protein (17-19). This technique allows comparison between proteins in varied samples, such as differences in protein expression levels in tissue samples under different pathological conditions or at different developmental stages (20). After iTRAQ labeling, high-precision mass spectrometer in series analysis can be performed and protein expression of up to 8 samples can be compared (21). Based on the above techniques, we selected SCs before and after peripheral nerve injury for iTRAQ labeling and mass spectrometry.

In summary, we isolated and purified SCs before and after peripheral nerve injury. After extracting the protein from SCs and labeling with iTRAQ, the samples were subjected to mass spectrometry to reveal a differentially...
expressed protein in the sample. The differentially expressed proteins were subjected to gene ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) analyses, and several proteins were identified by Western blotting. This study revealed the changes in SCs before and after peripheral nerve injury and formed a basis for subsequent cell therapy.

Materials and Methods

Animals and experimental groups

Nine Wistar rats (4-week-old, approx. 100±10 g, provided by Radiation Study Institute-Animal Center, Tianjin, China) were used in this study. Sciatic nerve injury surgeries were done as described previously (22). Rats were sacrificed and the sciatic nerves of each Wistar rat were isolated and the SCs were extracted. This study contained two major groups—Group A: ASCs from the ligation of the sciatic nerves and Group B: NSCs from the untreated sciatic nerves. All animal breeding experiments were performed according to the Guidelines for Laboratory Animal Safety and Care as issued by the United States National Institutes of Health. All procedures performed in the study involving animals were consistent with the ethical standards set by the above-mentioned institutions.

Isolation and culture of normal Schwann cells and activated Schwann cells

SCs were obtained from the 7-day pre-degenerated sciatic nerve of adult male Wistar rats (n = 9) according to a previous study (23). Briefly, nine adult Wistar rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g). After anesthesia satisfaction, the unilateral sciatic nerve was ligated in each rat. After one week, nine rats were sacrificed and the bilateral sciatic nerves of each rat were isolated. After removal of the epineurium, the nerve was washed three times with PBS and 2% antibiotic solution (penicillin, streptomycin) was added. Next, the remaining nerve tissue was cut into small pieces (0.5–1.0 mm²). The nerve tissue was digested in a 2-ml mixture for 10–15 min using an equal volume of medium containing 10% FBS was added and the cells were centrifuged (300 × g, 5 min) to remove the supernatant. An appropriate amount of DMEM/F-12, the tissue pieces were gently dispersed by pipetting and were centrifuged (300 × g, 5 min) to remove the supernatant. An appropriate amount of DMEM/F-12 medium containing 10% FBS was added and the cells were inoculated in a 25-cm² culture flask at 37 °C and 5% CO₂. After washing in DMEM/F-12, the tissue pieces were gently dispersed by pipetting and were centrifuged (300 × g, 5 min) to remove the supernatant. An appropriate amount of DMEM/F-12 medium containing 10% FBS was added and the cells were inoculated in a 25-cm² culture flask at 37 °C and 5% CO₂. After three weeks, the cells reached 90% confluency and were used in this experiment after three passages.

Immunofluorescence staining of Schwann cells

Cells were seeded at a density of 30,000 cells/well in a 24 Well Clear TC-Treated Multiple Well Plate. After 72 hr, they were fixed for 30 min in 4% (w/v) paraformaldehyde at room temperature. Then, the cells were washed in phosphate buffered saline before the addition of 5% (v/v) normal donkey serum together with 0.1% Triton X-100 (v/v) in PBS for a further 20 min at room temperature. After the blocking serum was removed, the primary antibodies, rabbit monoclonal anti-S100 (Gibco) at respective dilutions of 1:100 were added and the samples were incubated overnight at 4 °C. The cells were then washed in PBS, FITC conjugated donkey anti-rabbit IgG (1:200 dilution) was added and the samples were incubated for 2 hr at room temperature. After the reaction, the cells were washed three times with PBS, and the DAPI nuclear label (Sigma) was applied for 10 min. The Schwann cells were then examined under a fluorescence microscope (Leica DM2500, Germany).

Sample preparation and iTRAQ labeling

The medium was removed and the cells were washed three times with 1× phosphate buffered saline (PBS). 300 μl lysis buffer (10% SDS and TEAB) was added to the mixed sample and subjected to tissue homogenization and sonication on ice. After centrifugation at 17,000 × g for 10 min at 4 °C, the supernatant was collected and transferred to a new tube. The obtained protein extract was quantified by BCA assay (Transgene Biotech) according to the manufacturer’s protocol. To ensure that the data are available for technical and biological duplication, each group includes at least 3 repeated protein extracts. The final volume of the protein mixture was adjusted to 300 μl with 100 mM TEAB (triethylammonium bicarbonate, Santa Cruz, USA). The extracted protein was labeled with a lightly modified iTRAQ® reagent (AB Sciex Inc., MA, USA) according to the manufacturer’s instructions. Each sample was labeled with an isobaric tag for 3 hr at room temperature as follows: the proteins from ASCs were labeled with iTRAQ reagents 127, 129, and 131 and those from NSCs were labeled with iTRAQ reagents 126, 128, and 130. Finally, all samples were pooled before being subjected to separation techniques and analysis by tandem mass spectrometry.

Orbitrap LC-MS/MS analysis

For LC-MS/MS analysis, approximately 200 ng of each fraction was injected. Peptides were separated by LC-MS/MS coupled to an LTQ Orbitrap Velos mass spectrometer. At a resolution of 60,000, the MS spectra were acquired on Orbitraps in the range of 300–2000 m/z. The five most intense ions per survey were selected for collision-induced dissociation fragmentation to be analyzed in the linear trap.

Data analysis and quantitation

The masses of the peptide modifying the Tandem Mass Tags (TMT) zero, duplex, and sixplex reagents are present in the UNIMOD database (www.unimod.org Accessed 8 April 2017). Thermo Scientific Proteome Discoverer 1.1 and other software packages directly support the modification of the TMT reagent and the relative quantification of the reporter ions released from the labeled peptide. For data obtained using a combination of segmentation methods, the proteome discoverer may need to combine the spectra used for identification and quantification.

Bioinformatics analysis

Proteins/peptide sequences were imported into Cytoscape (version 3.4.0) for GO annotation. The screening of 122 differentially expressed proteins for GO annotation was performed from the biological process, molecular function, and cellular component. The KEGG-GENES corresponding to the differentially expressed
proteins were then analyzed by KEGG Orthology (KOs) and were mapped to KEGG pathways. Protein-protein networks that reveal significantly differentially expressed proteins were analyzed using the Cytoscape software.

**Western blotting**

Of the 122 differentially expressed proteins, several proteins were randomly selected for Western blot analysis validation. Briefly, the same amount of protein (20 μg) of each sample was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After blocking with 5% bovine serum albumin, the membrane was incubated with GPNMB (Anti-GPNMB antibody, Abcam, ab98856), ENPP3 (Anti-ENPP3 antibody, Abcam, ab190823), GFPT2 (Anti-GFPT2 antibody, Abcam, ab190966), and SDPR (Anti-SDPR antibody, Abcam, ab113876), and then incubated with the secondary antibody (1:5000 dilutions, Transgene Biotech). Detection of protein bands was performed using the ECL assay kit. Protein quantification was analyzed using the Image-Pro Plus (version 6.0) software.

**Statistical analysis**

Prism statistical software (Graph Pad v6.01, CA) was employed for data analysis. All data were reported as the mean±standard deviation (SD) in this study. The data were analyzed using one-way analysis of variance (ANOVA). *P*<0.05 was considered as statistically significant.

**Results**

**Culture and identification of Schwann cells**

At 10 days post-isolation of cells, the cells proliferated and covered the entire T75 bottom (Figure 1A). Both the ASCs and NSCs showed positive immunoreactivity for the S100 Schwann cell markers. Figure 1 (B, C, D) shows the expression of these mature markers in Schwann cells, whereas there were no significant differences between these two groups. The specific differences between these two groups can be found in our previous study (24). In conclusion, Schwann cells were prepared for protein extraction after immunofluorescence identification.

**Figure 1.** Immunofluorescence staining of Schwann cells. A. The shape of activated Schwann cells (ASCs) and normal Schwann cells (NSCs) under an optical microscope. Both of these SCs, long spindle cells, all were arranged in a fish shape and nuclei were ovoid or oblong. Scale bar: 200 μm. B. SCs were marked with S100 by immunofluorescence. C. The nucleus of SCs was marked with DAPI by immunofluorescence. D. SCs and nuclei of SCs were merged together by immunofluorescence. Scale bar: 100 μm

**Figure 2.** Differentially expressed proteins identified in Schwann cells before and after peripheral nerve injury. A. Proteins that showed increased levels in ASCs are shown in red and those that showed decreased levels are shown in green. B. The upregulated and down-regulated proteins were analyzed by functional clustering.
### Table 1. The differentially expressed proteins between normal Schwann cells and activated Schwann cells (up-regulated)

| Accession | Protein names | t-test P-value | Fold change |
|-----------|---------------|----------------|-------------|
| F1M3X5    | Maestro heat-like repeat family member 6 | 6.3315E-25 | 15.38461538 |
| CE162     | Centrosomal protein of 162 kDa | 9.0020E-28 | 10.98901999 |
| FETU1A    | Alpha-2-HS-glycoprotein | 1.7734E-19 | 4.69435681 |
| A0A0G2JSN5 | Serum albumin | 9.6575E-21 | 4.42477861 |
| GP4MB     | Transmembrane glycoprotein NMB | 4.8676E-20 | 4.16666667 |
| S3A8A     | Zinc transporter ZIP8 | 4.5425E-20 | 3.95256917 |
| ASM3A     | Acid sphingomyelinase-like phosphodiesterase 3a | 6.6147E-22 | 3.86100386 |
| MORBU0    | Cartilage oligomeric matrix protein | 7.4025E-19 | 3.63636363 |
| Q64599    | Hemiferrin | 1.6973E-19 | 3.46020761 |
| D3ZAD9    | NLR family, pyrin domain-containing 9 | 6.0388E-19 | 3.10539062 |
| D3ZAT4    | Serine (Or cysteine) peptidase inhibitor, clade A, member 9 | 5.6704E-20 | 3.06748466 |
| A0A0G2JST6 | Hexokinase 3, isofrom CRA_a, | 9.1401E-22 | 2.87356218 |
| CATC      | Dipeptidyl peptidase 1 | 6.2874E-16 | 2.74725747 |
| Q9QQW0    | Peroxisome proliferator activated receptor delta | 7.0965E-17 | 2.73972627 |
| Q5Z2R8    | Interferon activated gene 204 | 9.9933E-20 | 2.61780104 |
| D3ZFH5    | Uncharacterized protein | 8.8415E-19 | 2.48756218 |
| Q5MB60    | Rho GDP dissociation inhibitor beta | 1.9513E-13 | 2.42718466 |
| A0A0G2JXB1 | Galectin | 1.9674E-17 | 2.39234498 |
| D4A2Z6    | Thrombospondin 2 | 2.1905E-16 | 2.36406619 |
| Q5A857    | Phosphatase and tensin homolog | 6.1274E-17 | 2.20750519 |
| Q6I3G7    | GM2 ganglioside activator | 6.8476E-17 | 2.19780129 |
| A0A0H2UHE2 | Interleukin 1 receptor antagonist, isofrom CRA_c | 2.0353E-09 | 2.18340611 |
| G3VS2     | Neurofilament medium polypeptide | 1.3383E-11 | 2.08333333 |
| A0A0G2K3W2 | Coagulation factor V | 1.4240E-11 | 2.08333333 |
| A0A0G2JSV6 | Globin c2 | 4.1312E-19 | 2.04498977 |
| A0A0G2K014 | Lymphocyte cytosolic protein 1 | 5.4135E-17 | 2.02429498 |
| ENPP3     | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | 1.2369E-13 | 2.01612903 |
| E9PSM5    | 72 kDa type IV collagenase | 2.0859E-17 | 1.97628458 |
| A0A0G2K5B6 | Fatty acid-binding protein, adipocyte | 1.8102E-12 | 1.94931773 |
| DPP2      | Dipeptidyl peptidase 2 | 1.1589E-13 | 1.93798496 |
| D4AB2E3   | Ferric-chelate reductase 1 | 2.4754E-17 | 1.83150831 |
| A0A0G2JSB5 | Peroxiredoxin 5, isofrom CRA_c | 2.9507E-16 | 1.75438596 |
| A0A0G2K0T6 | Gamma-synuclein | 2.426E-10 | 1.74520069 |
| ILE3A     | Leukocyte elastase inhibitor A | 7.2965E-12 | 1.71526586 |
| Q9QD1     | Chemokine (C-X-C motif) ligand 12 | 4.4999E-10 | 1.71526586 |
| CATZ      | Cathepsin Z | 6.0410E-09 | 1.71232876 |
| OCA1      | Collagen alpha-1(XII) chain | 3.7569E-07 | 1.70357753 |
| A0A0G2JSW3 | Globin a4 | 9.8412E-16 | 1.68634064 |
| A0A0G2JSW2 | 3-hydroxybutyrate dehydrogenase, type 1, isofrom CRA_a | 8.2164E-16 | 1.67504187 |
| Q4QOY6    | Lymphoycte specific 1, isofrom CRA_a | 4.3649E-15 | 1.67224080 |
| A0A0G2K0T2 | Tetratspin | 2.2943E-10 | 1.66112958 |
| B4F7D5    | RGD1566403 protein | 2.3743E-14 | 1.65016501 |
| AMACR     | Alpha-methylacyl-CoA racemase | 6.3277E-12 | 1.63132137 |
| D4AB2O    | Cytochrome P450, family 2, subfamily s, polypeptide 1 | 6.6806E-10 | 1.61812297 |
| A0A0G2KAJ7 | Collagen alpha-1(XII) chain | 4.8802E-14 | 1.60771042 |
| G3V83     | Frizzled homolog 1 | 3.217E-09 | 1.60513643 |
| A0A0G2K4V4 | 3-ketohydrosphingosine reductase | 3.4489E-16 | 1.60256410 |
Continued Table 1.

| UniProt ID | Protein Name | Description |
|------------|--------------|-------------|
| Q60772     | Desmin       | Actin binding protein |
| A0A069MPJ9 | Sodium/potassium-transporting ATPase subunit beta | Transporter |
| A0A06GK8H6 | Pro-cathepsin H | Cathepsin |
| G3V636     | Scavenger receptor class B member 1 | Receptor |
| G3V624     | Insulin-like growth factor 2 receptor | Growth factor |
| G3V715     | Aldolase A, mitochondrial | Aldolase |
| G6Y4A8     | Poly (RC) binding protein 3 | RNA binding |
| NDRG1      | Serpin NDRG1 | Serpin |
| A0A06GK9H6 | Ceruloplasmin | Copper binding |
| RAC3D3     | BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation | Adapter |
| B5DF80     | Leprecan-like 2 (Predicted), isoform CRA_h | Leprecan |
| TIS8       | mRNA decay activator protein ZFP36L1 | mRNA decay |
| SERPH      | Serpin H1 | Serpin |
| A0A06G2Y66 | Aminopeptidase | Aminopeptidase |
| LAMP2      | Lysozyme-associated membrane glycoprotein 2 | Lysozyme |
| D32W38     | Exosome component 6 | Exosome |
| HXK2       | Hexokinase-2 | Hexokinase |
| NDC1       | Nucleoporin NDC1 | Nucleoporin |
| G6PD       | Glucose-6-phosphate 1-dehydrogenase | Glucose dehydrogenase |
| NAGAB      | Alpha-N-acetylgalactosaminidase | Galactosidase |
| Q60766     | Cathepsin D | Cathepsin |
| TMED3      | Transmembrane emp24 domain-containing protein 3 | Transmembrane |

UniProt accession numbers that can be found on www.uniprot.org. Accessed 8 April, 2017

Fold change: The quantity changes of protein abundance between the two groups

Integrated proteome information

The proteins expressed differentially between NSCs and ASCs were identified by the proteomics approach using iTRAQ. For each sample of SCs before and after peripheral nerve injury, the unique reporter in the low mass region of the MS/MS spectrum was used to measure the relative protein expression level during peptide fragmentation. Eventually, 4473 proteins were identified to be differentially expressed in groups A and B. The screening of differential protein and the selection of fold change were according to previous research (25). A total of 122 proteins were identified (fold ≥ 1.5, P-value ≤ 0.05) to be differentially regulated, of which 72 were upregulated (Table 1) and 50 were down-regulated (Table 2). Figure 2A shows the level of up-regulated and down-regulated proteins in ASCs. Moreover, we performed functional clustering analysis of up-regulated and down-regulated proteins (Figure 2B).

GO annotation of differentially expressed proteins

The above differential proteins were further analyzed by Cytoscape (version 3.4.0) software, divided into ‘Molecular function’, ‘Cellular component’, and ‘Biological process’ subcategories (Figure 3).

A biological process is a series of events resulting from an orderly combination of one or more molecules. Of the 122 differentially expressed proteins that were analyzed, most of the proteins were found to be enriched in the single-multicellular organism process (GO-ID:44707) and developmental process (GO-ID:32502). In addition, 57.14% differential expression proteins

Figure 3. Gene ontology annotation of differentially expressed proteins. Most of the proteins of differential abundance analyzed for the biological process, molecular function, and cellular component were single-multicellular organism process, protein binding, and cytoplasm, respectively.

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Table 2. The differentially expressed proteins between normal Schwann cells and activated Schwann cells (down-regulated)

| Accession | Protein names                                      | t-test P-value | Fold change |
|-----------|---------------------------------------------------|----------------|-------------|
| Q4V8N0    | Lipocalin 7, isoform CRA_a                        | 1.396E-07      | 0.664893617 |
| B0BMU8    | Musculo-skeletal, embryonic nuclear protein 1     | 2.81912E-05    | 0.663129973 |
| FH12      | Four and a half LIM domains protein 2             | 4.20985E-09    | 0.658761528 |
| A0A0G2K9W7| Zinc finger, MYND-type containing 8               | 0.001160328    | 0.657462196 |
| D4ADG9    | Collagen type V11 alpha 2 chain                  | 3.31488E-07    | 0.656598818 |
| ANX1A     | Annexin A3                                        | 0.000105632    | 0.654022237 |
| CEND      | Cell cycle exit and neuronal differentiation protein 1 | 4.91826E-06 | 0.651645798 |
| M0RAJE    | Proline-rich 14-1-like                            | 9.66231E-07    | 0.650195059 |
| D4A3ZB8   | Transmembrane and coiled-coil domain-containing protein 3 | 2.57001E-05 | 0.644329897 |
| B0VXR8    | JIP3 protein                                      | 2.01593E-05    | 0.643915003 |
| A0A096MK24| MORC family CW-type zinc finger 4                 | 1.60452E-06    | 0.643086817 |
| A2MG      | Alpha-2-macroglobulin                             | 0.000167324    | 0.642260758 |
| F1LPM3    | Sorbin and SH3 domain-containing protein 2        | 6.13675E-10    | 0.641025641 |
| HPRT      | Hypoxanthine-guanine phosphoribosyltransferase    | 3.01248E-12    | 0.639795266 |
| B6DY8    | Glutathione S-transferase                         | 0.000461515    | 0.636132316 |
| CYBR1     | Cytochrome b reductase 1                          | 0.000313257    | 0.635324015 |
| Q6HRK8    | Spectrin alpha chain, non-erythrocytic 1          | 1.54138E-07    | 0.631313131 |
| F1MOG3    | Ectonucleoside triphosphate diphosphohydrolase 1  | 1.90675E-09    | 0.630914826 |
| A0A0G2K9Y0| Uncharacterized protein                           | 2.70265E-05    | 0.62774639 |
| A0A146J2K6| Lasp-2                                            | 5.79706E-08    | 0.624219725 |
| FZD8      | Frizzled-8                                        | 3.28205E-06    | 0.623830318 |
| D3ZBS2    | Inter-alpha-trypsin inhibitor heavy chain H3      | 1.02664E-06    | 0.612369871 |
| PXDC1     | PX domain-containing protein 1                    | 4.33643E-06    | 0.612369871 |
| B9PTU4    | Myosin-11                                         | 9.78468E-13    | 0.610648717 |
| Q6HRK3    | Syndecan                                          | 2.71733E-06    | 0.597371565 |
| MUC18     | Gel surface glycoprotein MUC18                    | 3.79694E-09    | 0.586510264 |
| FBWFL6    | Protein FAM13B                                    | 1.09524E-15    | 0.582411182 |
| AKA12     | A-kinase anchor protein 12                        | 4.54799E-14    | 0.582072177 |
| AQP1      | Aquaporin-1                                       | 6.2957E-05     | 0.580383053 |
| D4A1D2    | Rho guanine nucleotide exchange factor 26         | 1.00711E-07    | 0.580046404 |
| A0A0GZ3K38| Four and a half LIM domains protein 1              | 1.79426E-05    | 0.574712644 |
| ANPRC     | Atrophin-1 like 3                                  | 3.79533E-08    | 0.565672328 |
| Q5FGS2    | Similar to tropomyosin 1, embryonic fibroblast rat isoform CRA_c | 6.48235E-10 | 0.564334086 |
| G3V831    | Maximerization protein 3                          | 1.43191E-10    | 0.563697858 |
| Q56A29    | Visinin-like 1                                    | 1.12729E-06    | 0.555555556 |
| M0RS2     | Apolipoprotein D                                  | 1.48461E-06    | 0.547345375 |
| A0A096MJ01| LIM domain-binding 3                              | 9.90342E-06    | 0.543478261 |
| Q6P92     | Four and a half LIM domains 1                     | 2.29744E-07    | 0.529661017 |
| NQ01      | NAD(P)H dehydrogenase [quinone] 1                 | 0.000698527    | 0.529661017 |
| A0A0G2JEB7| Neuron navigator 3                                | 1.46506E-08    | 0.518134715 |
| D3Z8US    | Metalloendopeptidase                              | 1.03836E-10    | 0.51875282 |
| A1M       | Alpha-1-macroglobulin                             | 8.78141E-06    | 0.44727191 |
| A0A096MN4 | Septin 4                                          | 4.76859E-12    | 0.44660333 |
| RLA2      | 60S acidic ribosomal protein P2                   | 1.25097E-08    | 0.430848772 |
| D3ZRD9    | Allograft inflammatory factor 1-like              | 3.31644E-06    | 0.40371417 |
| D4A9W1    | Glied-coil domain-containing 8C                   | 3.85007E-10    | 0.394477318 |
| GLDN      | Gliomedin [cleaved into: gliomedin sheddectodomain] | 1.71803E-09 | 0.37355248 |
| SDPR      | Serum deprivation-response protein                | 3.99342E-13    | 0.348109415 |
| GSTP      | Osteopontin                                      | 1.41268E-07    | 0.345781466 |
| A0A0G2K714| RSCD domain containing 1                          | 1.27673E-14    | 0.211292485 |

UniProt accession numbers that can be found on www.uniprot.org. Accessed 8 April, 2017

Fold change: The quantity changes of protein abundance between the two groups
were located in the cytoplasm (GO-ID:5737), followed by the extracellular space (GO-ID:5615). In the GO annotation analysis, ‘Molecular function’ can provide the function of the gene at the molecular level. In the differentially expressed proteins that were screened, it was found that most of the differential proteins were enriched in enzyme inhibitor activity (GO-ID:4857), followed by identical protein binding (GO-ID:42802), and peptidase regulator activity (GO-ID:61134). Of the 122 differentially expressed proteins, 53 most relevant proteins were screened out and a protein-protein interaction (PPI) network was prepared (Figure 5). In this PPI network, several proteins were further selected for analysis. Moreover, several of these proteins (Thbs2, Lgals3, Cathepsin D (Ctsd), and Sptan1) were chosen for analysis.

**KEGG analysis**

Protein expression data were mapped to KEGG Mapper-Search & Colour Pathway (http://www.kegg.jp/ Accessed 10 April 2017) to further analyze changes in biological processes. In addition, we counted the most meaningful 16 KEGG pathways, including purine metabolism, biosynthesis of antibiotics, amino sugar and nucleotide sugar metabolism, and Thiamine metabolism. (Figure 4A). In addition, one of the pathways, ‘Purine metabolism’, was chosen for analysis, in which different colors represent different enzymes (Figure 4B).

**Protein verification by Western blot analysis**

GPNMB, ENPP3, GFPT2, and SDPR were selected in ASC (Group A) and SC (Group B) samples by Western blot analysis. The change in protein abundance as detected in Western blot analysis and protein quantification was highly consistent with that in the proteomics data of SCs (Figures 6A and 6B).

**Figure 4.** Kyoto encyclopedia of genes and genomes pathway analysis of differentially expressed proteins A. The purine metabolism pathway was enriched in the majority of the differentially expressed proteins. The vertical bars represent the number of the differentially expressed proteins. B. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis maps of the Purine metabolism pathway. The proteins in different color frames are differentially expressed proteins identified in this study. The box represents proteins; the arrow represents activation.
Discussion

In this study, the proteomics of SCs before and after peripheral nerve injury were studied by using iTRAQ and high-resolution Orbitrap LC-MS/MS. Compared with that in NSCs, 122 differentially expressed proteins were identified in ASCs, of which 72 were upregulated and 50 were down-regulated. In addition, several proteins were randomly selected for Western blot analysis, and the results were consistent with those of proteomics analysis.

A total of 122 differentially expressed proteins were obtained from protein mass spectrometry. GO annotation analysis was performed using 'Molecular function,' 'Cellular component,' and 'Biological process.' GO annotation analysis results show that these different proteins are likely to accumulate in the cytoplasm and are associated with single-multicellular organism processes. Moreover, we found that pyruvate metabolism, biosynthesis of antibiotics, and amino sugar and nucleotide sugar metabolism pathways were significantly enriched in the KEGG pathway analysis. In the PPI network, Thbs2, Lgals3, Ctsd, and Sptan1 are four proteins related to the development of the CNS and peripheral nervous system (26-30). Previous research reported that Thbs2 can promote axonal regeneration and synaptic formation (31). Researchers found that Sprague-Dawley pregnant rats exposed to drinking water containing glycidol could show axonopathy and hippocampal nerve distortion (28). While in the hippocampal dentate gyrus, they found that Thbs2 could regulate the plasticity of neurons. Galectins control the important pathophysiological processes of the CNS. In addition, Lgals3 can promote the differentiation of oligodendrocyte, maintain the integrity of myelin, and promote the recovery of inflammatory demyelinating disease (32). Ctsd-knockout can lead to changes in the ultrastructure of myelin and metabolic disorders of cholesterol and the extreme absence of neurons in the brains of mice (33). In summary, the above proteins were found to be closely related to the pathophysiological processes of the nervous system, consistent with the results of the proteins we screened.

ENPP3 is a member of the ectonucleotide pyrophosphatase/phosphodiesterase family (E-NPPs). It has been reported that ENPP3 is present in almost all systems in the human body (34). Abnormal expression of ENPP3 can affect intracellular transduction pathways, leading to cellular dysfunction. A recent study has found the presence of ENPP1 and ENPP3 in rat podocytes and assessed their expression in rat podocytes cultured with 5 mM (normal glucose) or 30 mM glucose (high glucose) (35). In another study, the investigators examined the effect of endotoxin on nucleotide catabolism in the kidneys of mice by lipopolysaccharide (LPS) injection (36). The expression pattern of exogenous nucleotides showed that the level of Enpp3 mRNA was increased after LPS injection. Purine metabolic analysis by high-performance liquid chromatography assay confirmed this result. In our research, quantities of ENPP3 proteins were measured by Western blotting analysis, we found that the level of ENPP3 expression in SCs was significantly correlated with the peripheral nerve injury. Changes in protein abundance were consistent with proteomics data from SCs by Western blot analysis and protein quantification.

In dendritic cells (DC), the GPNMB is a transmembrane protein that acts as a coinhibitory molecule strongly inhibiting the responses of T cell (37). Major histocompatibility complex class II (MHCII) molecules similarly expressed in DC subsets. In addition, MHCII was upregulated in cultured SCs and degenerated nerve tissue (38). Therefore, we speculated whether GPNMB and MHCII co-controlled the antigen presentation of DC.
cells. However, the specific mechanism and the immune regulation need further exploration. In addition, many studies reported a number of new markers for Schwann cells as early as 2012 (39), for example, TUBB3, ATG5, and NEFM. A study on spinal muscular atrophy (SMA) showed that ubiquitin-like modification 1 (Uba1) and ubiquitin-dependent pathways play an important role in maintaining Schwann cell homeostasis and provide important additional experimental evidence (40). The above proteins were also detected in this research, but the objective of this study was determining what kind of proteomics changes have occurred in Schwann cells before and after peripheral nerve injury, thus validating only four related proteins. Similarly, Lgals3 was detected in relation to the pathophysiological processes of the CNS or the peripheral nerves (41, 42), while the other two proteins were screened (Ctsd and Sptan1), and there was no clear literature to support its role in the nervous system. Thus, studies on the function of the proteins in nerve injury are urgently needed for further exploration.

Similar to previous studies, our research is based on the in vitro culture of SCs to study the changes in the microenvironment of SCs after peripheral nerve injury (43-45). Additionally, we explored the application of sciatic nerve pre-injury model, which is generally recognized, in Wistar rats (46). However, we first used iTRAQ technology to label potential biomarkers in SCs to explore the possible changes in SCs after peripheral nerve injury. Although important discoveries were revealed in this study, there are also some limitations. First, the sample size of this study needs to be further expanded, and the selected peripheral nerves should be more diverse. Second, the pathways selected in this study need to be further validated. The changes in SCs before and after nerve injury require further exploratory mechanisms. Finally, we just explored SCs in vitro; the transplantation of SCs in conjunction with other cells into animals is the next major task.

Marking proteins based on iTRAQ technology is a popular topic in the current studies on protein labeling (47-49). We hope that this study further explored the changes in SCs in the peripheral environment after injury and provide a new approach for better clinical application of SCs.

**Conclusion**

We used iTRAQ-Orbitrap LC-MS/MS technique and bioinformatics analysis to conduct a proteomics study to identify proteins that were differentially expressed between ASCs and NSCs. Based on our findings, GPNMB, ENPP3, Thbs2, and Lgals3 may play a key role in repair of SCs after peripheral nerve injury. Here, we report a new finding on SCs after nerve injury and warrants further studies in the future.

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**Conflict of Interest**

The authors have no conflicts of interest to declare.

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