Original Article

Protective effects and molecular mechanisms of *Achyranthes bidentata* polypeptide k on Schwann cells

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Background: *Achyranthes bidentata* polypeptide k (ABPPk) is an active ingredient used in traditional Chinese medicine separated from *Achyranthes bidentata* polypeptides. So far, the role of ABPPk in peripheral nerve protection has not been comprehensively studied.

Methods: In this study, primary Schwann cells exposed to serum deprivation were treated with ABPPk or nerve growth factor (NGF) *in vitro*. Cell viability, cell apoptosis, apoptosis-related protein expression, and antioxidant enzyme activity were analyzed. To further explore the underlying molecular mechanisms and key regulatory molecules involved in the effects of ABPPk, integrative and dynamic bioinformatics analysis at different time points was carried out following RNA-seq of Schwann cells subjected to serum deprivation.

Results: We found that ABPPk could effectively reduce Schwann cell apoptosis caused by serum deprivation, which was comparable to NGF's anti-apoptotic effects. ABPPk had the largest number of upregulated and downregulated differential expression genes at the earliest 0.5 h time, while NGF had fewer differential expression genes at this early stage. The significant difference at this time point between the two groups was also displayed in heatmaps. The molecular regulation of diseases and functions and canonical pathways revealed that ABPPk had more participation and advantages in the vasculature and immune system areas, especially angiogenesis regulation. Also, ABPPk demonstrated an earlier start in these molecular regulations than NGF. Furthermore, the analysis of transcription factors also illustrated that ABPPk not only had more key initial regulatory factors participating in vascular-related processes, but these also remained for a longer period. There was no significant difference in neural-related molecular regulation between the two groups.

Conclusions: Using high-throughput sequencing technology, our work unveiled the protective effects of ABPPk on Schwann cells after serum deprivation in a more comprehensive manner. These results further enrich the positive functions and molecular mechanisms of ABPPk and traditional Chinese medicine and benefit the discovery of novel therapeutic targets for peripheral nerve regeneration.

Keywords: *Achyranthes bidentata* polypeptide k (ABPPk); Schwann cells (SCs); cell apoptosis; molecular mechanism; blood vessel

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Introduction

Achyranthes bidentata polypeptides (ABPP) are effective active substances independently extracted from the Achyranthes plant by our laboratory (patented). ABPP has been shown to promote blood circulation, remove blood stasis, and demonstrate protective effects against tumors, inflammation, and pain (1-4). Many studies have previously shown that ABPP protects hippocampal neurons from apoptosis in injury models (5,6) and promotes the repair of sciatic nerve injury (7-9). In recent years, *Achyranthes bidentata* polypeptide k (ABPPk), the more biologically active component, was further purified from ABPP by high-performance liquid chromatography (HPLC). Several studies have suggested that ABPPk can effectively protect neurons from apoptosis in various injury models, such as cortical neurons subjected to oxygen and glucose deprivation or transient middle cerebral artery occlusion, and dopaminergic neurons treated with the neurotoxic agent MPP^+ (1,10).

Schwann cells (SCs) are the predominant glial cells of the peripheral nervous system. They play an essential role in forming myelin sheaths around axons and are involved in the pathology of inflammatory, metabolic, and degenerative diseases (11,12). During serum deprivation, SCs cannot carry out their regular functioning, including the secretion of various neurotrophic factors to promote the repair of peripheral nerve damage (13-15). On the other hand, due to the various injuries of SCs, myelinopathy can occur due to the loss of support and protection from SCs, which causes severe damage to peripheral nerves (16,17). Therefore, exploring effective ways to protect SCs is of great importance for repairing peripheral nerve injury and may also provide new targets for peripheral nerve regeneration's clinical treatment.

Our previous research demonstrated that the ability of ABPPk to protect SCs from oxidative damage through the inhibition of SC apoptosis was mainly related to the PI3K/AKT and ERK1/2 signaling pathways (18). Based on this finding, we aim to study further the effects of ABPPk on injuries caused by serum deprivation of SCs, followed by an exploration of the underlying molecular mechanisms. It is ultimately hoped that these insights will help repair and remodel peripheral nerve damage and reveal ideal targets for developing new-generation neuroprotective agents.

This study investigated whether ABPPk could protect cultured SCs from cell death caused by serum deprivation, a different injury model from our previous research. It was observed that ABPPk protected SCs from the damage caused by serum deprivation, indicating the more potential for peripheral nerve injury repair. To investigate the molecular mechanisms and key regulatory molecules of the neuroprotective effects of ABPPk, bioinformatics approaches were carried out to analyze the transcriptome data of the ABPPk treated group and the nerve growth factor (NGF) treated group. We compared the differentially expressed genes, diseases and functions, signaling pathways, and transcription factors after treatment with these two protective factors. Our work demonstrated the correlations and differences at the molecular regulatory level between ABPPk and NGF's protective effects on SCs after serum deprivation injury. These results also contribute towards a comprehensive and accurate understanding of the molecular mechanisms of the protective effects of ABPPk on injured SCs, which may lead to more effective repair of peripheral nerve damage.

We present the following article following the MDAR checklist (available at http://dx.doi.org/10.21037/atm-20-2900).

Methods

Ethics statement

Experiments were performed under a project license (NO.: 20190303-15) granted by the Laboratory Animal Ethics Committee of Nantong University, in compliance with the Nantong University institutional guidelines for the care and use of animals.

Preparation of ABPPk

ABPPk was purified from ABPP by HPLC according to the previously described preparation protocol (1,7,18).

Cell treatment

Primary SCs were isolated from the sciatic nerves of 1-day-old Sprague-Dawley rats (19,20). The neonatal rats were acquired from the Experimental Animal Center of Nantong University [License No. SCXK (Su) 2014-0001 and SYXK (Su) 2012-0031, No. 20190225-004]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 ℃. Primary cultured SCs were identified by immunofluorescence staining (21,22).

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Primary cells were fixed in 4% paraformaldehyde for 30 min and incubated with the primary antibody overnight at 4 ℃, followed by the secondary antibody for 1 h at room temperature. The primary antibody was mouse anti-S100 antibody (1:1,000 dilution, Sigma). The secondary antibody was goat anti-mouse IgG-Alexa-488 (1:500 dilution, Abcam). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (SouthernBiotech). SCs were plated in 96- or 6-well plates previously treated with poly-L-lysine (PLL) (Sigma, USA). The next day, wells were washed twice with phosphate-buffered saline (PBS) (Corning, USA), and the cells were switched to serum-free medium and incubated for 4 h at 37 ℃. Then, the culture medium was replaced by DMEM with 10% FBS containing ABPPk (0.5 μg/mL, the optimal concentration selected) or NGF (0.1 μg/mL) for 24 h. All experiments were performed 3 times.

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan) in 96-well plates. In brief, cells were seeded and treated with ABPPk or NGF. Subsequently, the CCK8 reagent (10 μL/well) was added to each well, and cells were incubated for 2 h at 37 ℃. Cell viability was evaluated by measuring optical density (OD) values at 450 nm using a microplate reader (BioTek, USA).

Annexin V-FITC assay

Serum deprivation-induced SC apoptosis was determined by flow cytometry using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Briefly, cells were seeded in a 6-well plate for different treatments, followed the incubation with annexin V-FITC (5 μL) in binding buffer for 15 min at room temperature in the dark. Cell suspensions (1 mL) were extracted for propidium iodide (PI) (10 μL) staining with a 15 min incubation protected from light at 37 ℃; then cells were analyzed by calculating percentages of the total cells in each stage using flow cytometry (BD Biosciences) within 1 h. The assay was repeated 3 times for each group.

TUNEL assay

SCs exposed to different treatments were fixed with 4% paraformaldehyde and permeabilized with Triton X-100, and DNA fragmentation was determined using the DeadEnd™ fluorometric terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) System (Promega, Madison, WI, USA) according to the manufacturer's instructions. For quantitative results, the Hoechst positive cells and the TUNEL positive cells were counted in 5 random image areas for each sample under a fluorescence microscope.

Superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) assays

The enzyme activities of SOD, MDA, and GSH were determined in the lysates of SCs using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, China) (23). SOD activity was determined based on the inhibition of nitro-blue tetrazolium at 550 nm with an ELx-800 microplate reader. The absorbance of MDA, which was analyzed with 2-thiobarbituric acid, was detected at a wavelength of 532 nm. The GSH contents in cultured SCs were monitored using a microplate reader at 405 nm (BioTek, USA). All of the assays were performed according to the manufacturer's instructions. The results were calculated using the absorbance values of the SCs and standard equations (24).

Lactate dehydrogenase (LDH) release assay

The LDH assay determines the extent of cell membrane damage by measuring the amount of cytoplasmic LDH released into the culture medium. LDH, widely used to assess the presence of damage and toxicity in tissues and cells, was detected at 450 nm using a microplate reader with an LDH Detection Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions (18).

Western blot analysis

Briefly, cells were scraped and lysed, and proteins were measured with the BCA Protein Assay Kit (Beyotime, Shanghai, China). For each sample, 30 μg of protein was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were blocked with 5% non-fat milk at room temperature for 2 h. Membranes were then treated with primary antibodies overnight at 4 ℃. The following antibodies were used: anti-Bax (1:1,000 dilution; Abcam), anti-Bcl-2 (1:1,000 dilution; Abcam), anti-caspase-3

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Table 1 The primer sequences of genes used for qRT-PCR

Genes	Primer sequences	
	Forward	Reverse
EDN1	GGGAGTGTTCGTGTCTGACT	GTAGCGGAGGAGAGAGCGTA
EFNA3	ACTATCTGTGCCTGCCCTCTTTG	CATCTCCCATGATTCATTTGTTCT
EPHA2	AGTGTTAGCGGGAATTGGCT	AGGGGCTTTAGTTGTTCTGACTT
FGF8	CTCCAAGCCCAGCATGTGA	TCTTCTGCCATGGCGTTGAT
FHL2	GAGACCTGCTTCACCTGTCA	CCTCCTGTGGTAATTGGCTT
FOSL1	GTGAGCAGATCAGCCCGGAG	TCATCCTCCAACTTGTCGGTC
JAG1	ACTGCCTTTCAGTTTCGCCT	GCCGACAGAACTTGTTGCAG
MYC	ACCCAACATCAGCGGTCG	CGTGACTGTCGGGTTTTCCA
SERPINE1	CGTCTTCCTCCACAGCCATTC	CATCCCACTCTCAAGGCTCC
GAPDH	TGGAGTCTACTGGCGTCTT	TGTCATATTTCTCGTGGTTCA

(1:1,000 dilution; Abcam), anti-cleaved caspase-3 (1:1,000 dilution; Abcam), and anti-GAPDH (1:5,000 dilution; Abcam). The membranes were then washed with tris buffered saline with tween (TBST) 3 times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce) for 1 h at room temperature. Protein bands were visualized using the Odyssey densitometry program (LI-COR, Lincoln, NE, USA).

RNA-seq analysis

Triplicate samples at 0.5, 3, 9, 12, and 24 h from the four different experimental groups (ABPPk, NGF, serum deprivation, and normal SCs) were collected. According to the manufacturer's protocol, the 60 ribonucleic acid (RNA) samples were extracted using the mirVana miRNA Isolation Kit (Ambion). RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Strand-specific RNA-seq libraries were constructed, and the samples were sequenced on the Illumina Hiseq X Ten sequencing platform (OEbiotech, Shanghai, China) with 150 bp paired-end runs. All subsequent analyses were performed using clean reads. The sequencing data were uploaded to the sequence read archive (SRA) database (accession number: SRP253388, PRJNA613365).

Bioinformatics analysis

To study the possible mechanisms and signaling pathways of differentially expressed genes in serum deprivation-induced SC apoptosis, we performed bioinformatics analysis of mRNAs using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA). Genes with expression changes were uploaded to the IPA software for core analysis to construct molecular regulatory networks and obtain relevant information about diseases and functions, canonical pathways, and transcriptional regulators. The differentially expressed genes were in the threshold range of $2\leq$ fold change (FC) ≤ -2, except for the range of $1.2\leq$ FC ≤ -1.2 for transcription factor analysis. Principal component analysis (PCA) was performed with the "Population PCA" tool (http://cbdm.hms.harvard.edu/LabMembersPges/SD.html). Heatmap analysis, a technique where values are displayed in colors in a graphical representation of the data, was also performed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from SCs with TRIzol (Gibco, USA). A total of 1 μg of RNA served as the template for the synthesis of cDNA using the PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa, Japan), and qRT-PCR was performed using the TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Japan). The primer sequences of all genes investigated are listed in *Table 1*, and the gene expression levels were normalized to the mRNA expression of GAPDH.

Statistical analysis

All statistical analyses were carried out using one-way

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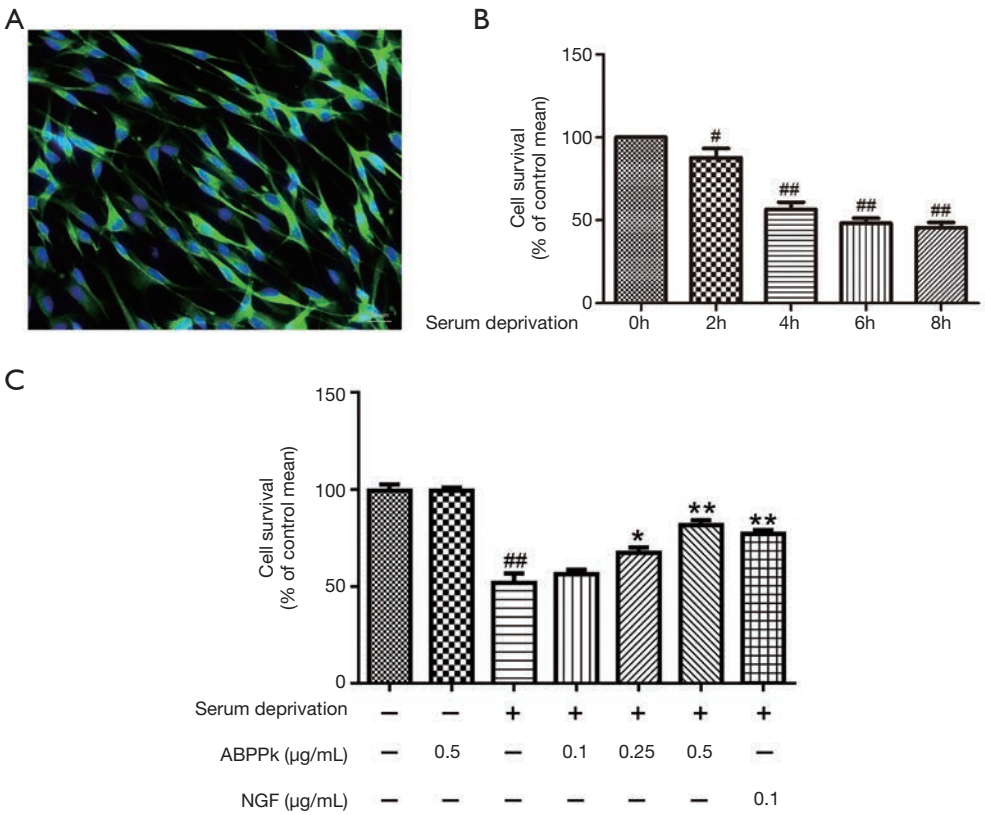

Figure 1 Effects of ABPPk on cell viability in serum deprivation-treated Schwann cells (SCs). (A) Immunocytochemistry identification of primary cultured SCs by S100 (green) staining combined with DAPI staining (blue). Scale bar =20 μm. (B) SCs were exposed to serum deprivation for the indicated times (2, 4, 6, and 8 h). (C) SCs were incubated with ABPPk (0.1, 0.25, and 0.5 μg/mL) or 0.1 μg/mL nerve growth factor (NGF) for 24 h following exposure to serum deprivation for 4 h. *, P<0.05; **, P<0.01 *vs.* the cells exposed to serum deprivation alone. #, P<0.05; ##, P<0.01 *vs.* the control cells. ABPPk, *achyranthes bidentata* polypeptide fraction k.

analysis of variance (ANOVA) with GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). The results were presented as mean ± standard error of the mean (SEM) calculated from at least 3 independent tests. P<0.05 was considered to be a statistically significant difference.

Results

ABPPk protected SCs against serum deprivation-induced cell cytotoxicity

Before the SCs were used for experimental treatments, immunocytochemistry with anti-S100 antibody was performed to test the purity of primary SCs (*Figure 1A*). The CCK8 assay showed that SCs had obvious time-dependent responses to serum deprivation for 2, 4, 6, and 8 h respectively, which was protected by restoring serum supply to SCs (*Figure 1B*). The activity of SCs decreased significantly with the prolongation of serum deprivation time. Exposure to serum deprivation for 4 h then serum protection for 24 h, which resulted in 56.2%±3.2% of the control value, was chosen to induce cytotoxic damage in subsequent experiments. To evaluate the potential cytoprotective effects of ABPPk against serum deprivation-induced SC death, the experimental group was exposed to ABPPk at various concentrations (0.1, 0.25, and 0.5 μg/mL) or 0.1 μg/mL NGF for 24 h, following serum deprivation for 4 h. Treatment with ABPPk attenuated the cell viability loss induced by serum deprivation alone, indicating that ABPPk protected cultured SCs against serum deprivation damage. However, the optimum protective effect of ABPPk at 0.5 μg/mL, which was selected for subsequent experiments, was similar to that of NGF (*Figure 1C*).

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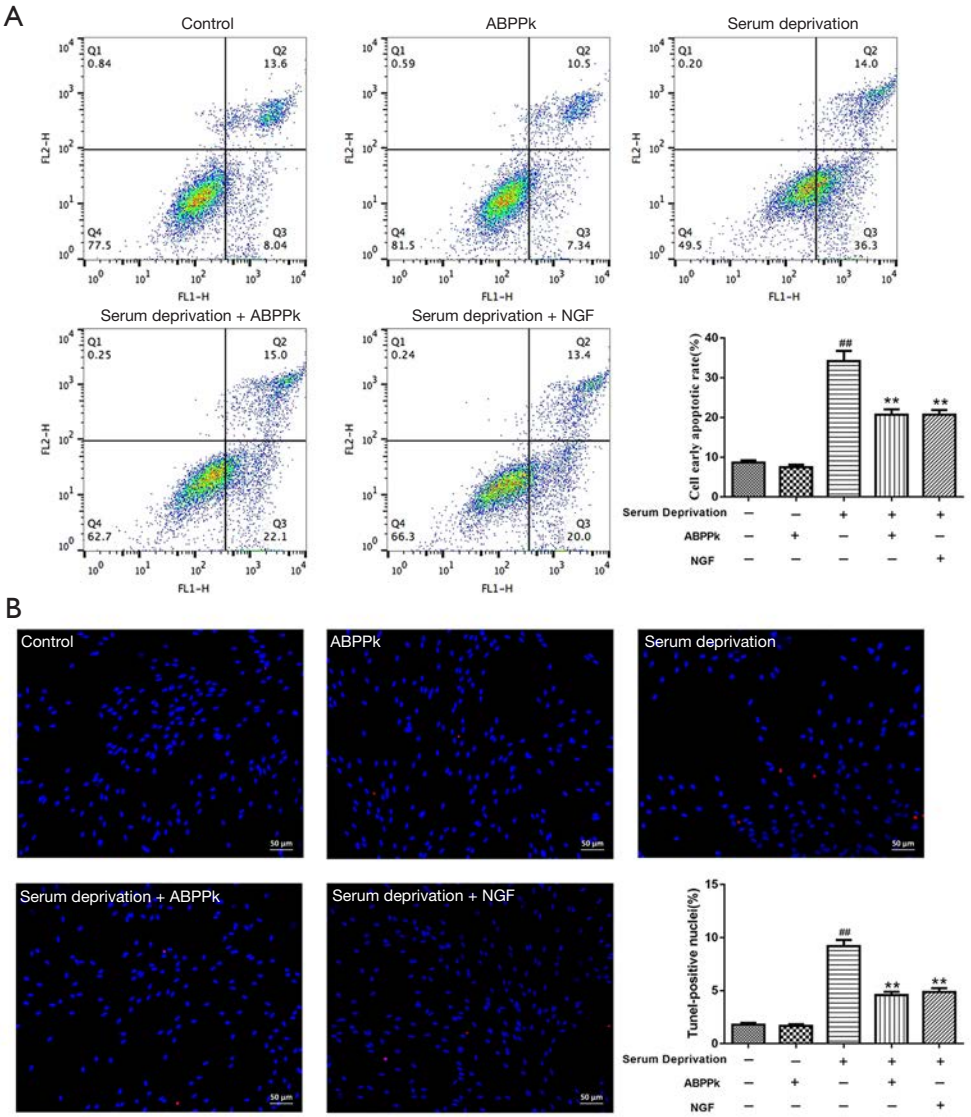

Figure 2 Effects of ABPPk on serum deprivation-induced cell apoptosis. Schwann cells (SCs) were incubated with 0.5 μg/mL ABPPk or 0.1 μg/mL nerve growth factor (NGF) for 24 h after exposure to serum deprivation for 4 h. (A) Apoptosis rates of SCs measured using flow cytometry with propidium iodide (PI)/annexin V-fluorescein isothiocyanate (FITC) staining. The lower right panel indicated the early apoptotic cells. (B) The images of TUNEL-positive cells (red) were captured using a fluorescence microscope, and the positive cells were counted. The results were expressed as the mean ± SEM, n=5. **, P<0.01 *vs.* the cells exposed to serum deprivation alone. ##, P<0.01 *vs.* the control cells. Scale bar =50 μm. ABPPk, *achyranthes bidentata* polypeptide fraction k.

ABPPk protected SCs against serum deprivation-induced cell apoptosis

To further confirm whether ABPPk had protective effects on serum deprivation-induced damage, the apoptosis and survival rates of SCs were then measured. Apoptotic cells were stained with annexin V/PI and detected with flow cytometry. ABPPk or NGF treatment induced significantly lower apoptosis or necrosis in SCs compared to serum deprivation alone (P<0.01) (*Figure 2A*). These results also indicated the efficiency of ABPPk to inhibit the early apoptosis induced by serum deprivation. Meanwhile, the TUNEL assay was performed in order to determine dead cells. There were few TUNEL-positive SCs in the control

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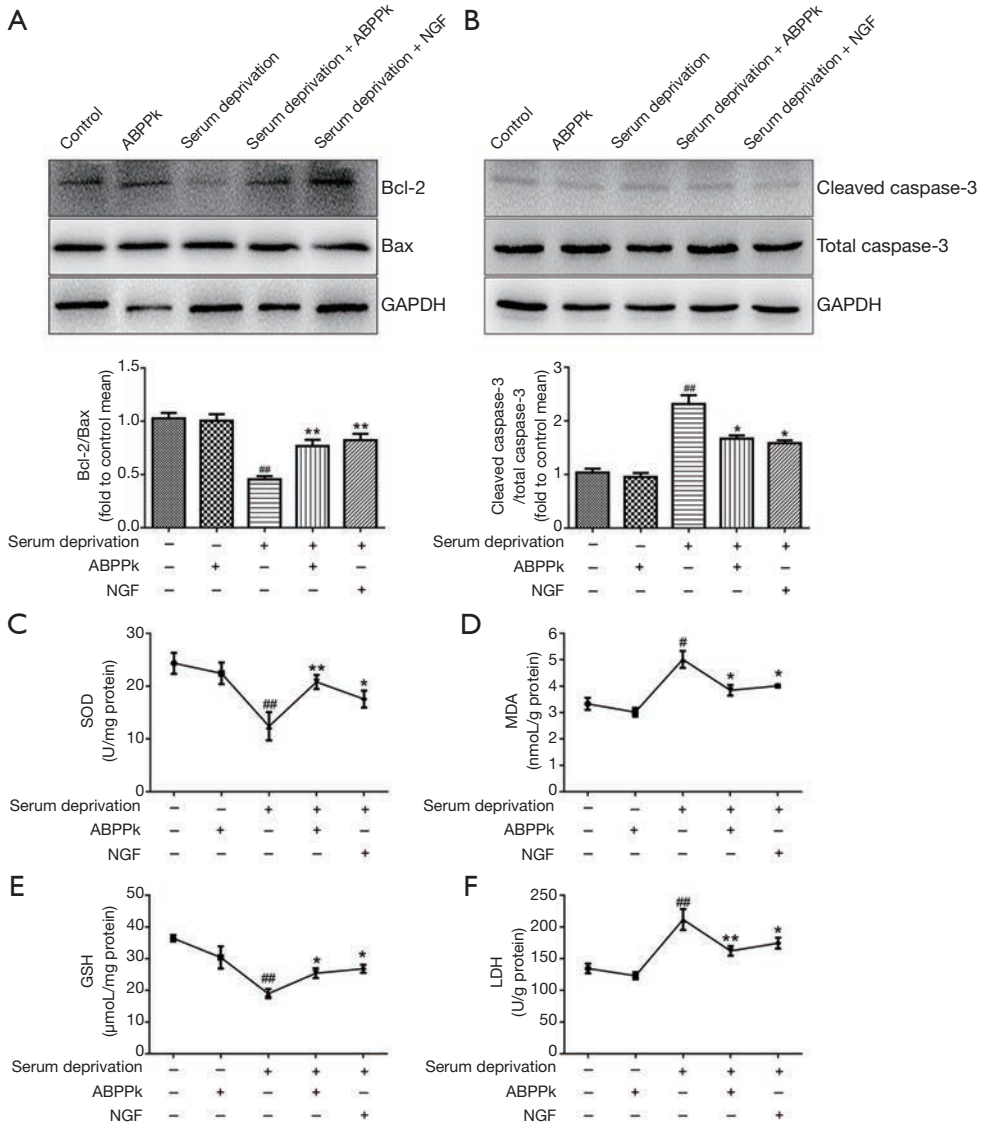

Figure 3 ABPPk inhibited apoptosis measured by protein levels and antioxidant enzyme activity in Schwann cells (SCs) after serum deprivation. The protein levels of (A) Bax, Bcl-2 and (B) cleaved caspase-3 were measured by Western blot. GAPDH was used as a loading control. (C,D,E,F) The intracellular superoxide dismutase (SOD) levels (C), malondialdehyde (MDA) levels (D), glutathione (GSH) levels (E), and the release of lactate dehydrogenase (LDH) (F) in SCs exposed to serum deprivation in the absence or presence of ABPPk or nerve growth factor (NGF). The results were expressed as the mean ± SEM, n=5. *, P<0.05; **, P<0.01 *vs.* the cells exposed to serum deprivation alone. #, P<0.05; ##, P<0.01 *vs.* the control cells. ABPPk, *achyranthes bidentata* polypeptide fraction k.

group (2.89%±1.21%), and exposure to serum deprivation increased the number of TUNEL-positive apoptotic cells (9.86%±3.59%). However, treatment with ABPPk or NGF appeared to protect SCs from apoptosis, with a decrease in TUNEL-positive cells down to 4.79%±1.47% or 4.92%±2.59% (P<0.01), respectively (*Figure 2B*). To further confirm the effects of ABPPk on cell apoptosis, protein levels of Bcl-2, Bax, and cleaved caspase-3 were examined by western blot. Treatment with ABPPk resulted in higher expression levels of Bcl-2 and lower expression levels of Bax and cleaved caspase-3 than serum deprivation treatment alone (*Figure 3A,B*). In summary, these data indicated that ABPPk could protect SCs against apoptosis induced by serum deprivation.

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Effects of ABPPk on antioxidant enzyme activity in SCs after serum deprivation

Compared with the group exposed to serum deprivation separately, the contents of SOD and GSH in the ABPPk and NGF groups significantly increased, while MDA decreased significantly ($P<0.01$). Additionally, LDH that leaked out after cell membrane disruption was significantly elevated after exposure to serum deprivation. When 0.5 μg/mL ABPPk was added to cells, the decrease in LDH was the most obvious compared to that of the serum deprivation group (*Figure 3C*,*D*,*E*,*F*). The results indicated that ABPPk or NGF significantly reduced serum deprivation-mediated cell membrane damage.

Overview of gene changes in SCs protected by ABPPk following serum deprivation

The differentially expressed genes of the three groups were screened, compared, and analyzed at each time point. The number of differentially expressed genes that were either up-or down-regulated in the ABPPk group reached the highest level at 0.5 h and was significantly higher than that of the NGF group, then the number decreased and stabilized. There were few differential genes at 0.5 h; however, up-regulated genes significantly increased at 9 h, and down-regulated genes significantly increased at 12 h for the NGF group. Moreover, the NGF group had numerous down-regulated differential genes distinct from the ABPPk group at 12 h (*Figure 4A*,*B*). PCA is a multivariate statistical analysis method that reduces variables in big data. The gene expression levels at each time point were comprehensively analyzed through the PCA method, and excellent repeatability of the sequencing was displayed. The gene expression values at 0.5 and 3 h, with a considerable distance between them, were relatively independent of those at other time points. However, the gene expression levels at 9, 12, and 24 h were concentrated as the third stage (*Figure 4C*). To display stage-specific gene expression, the heatmaps of the ABPPk group and the NGF group were generated using Cluster software (*Figure 4D*,*E*,*F*). There were more significant differences in gene expression levels between the ABPPk and NGF groups at the early 0.5 h time point and the later 12 h time point. Compared with the serum deprivation group, the ABPPk group had obvious different gene expression at 0.5 h, while the NGF group had a significant difference in gene expression at 12 h. ABPPk was revealed to play a protective role in SCs with significant gene regulations at an earlier stage compared to NGF.

Diseases and functions, and canonical pathways involved in the protective effects of ABPPk on SCs following serum deprivation

The top ten diseases and functions of significance analyzed by IPA were displayed (*Figure 5A*,*B*,*C*). At the earliest 0.5 h time point, the differentially expressed genes of the ABPPk group participated in the regulation of many aspects of cell protection, including "development of vasculature", "angiogenesis", "migration of cells", "cell movement", and "apoptosis". However, these genes and their processes were not observed in the NGF group. Furthermore, compared with NGF, ABPPk played a protective role in blood vessel regulation in SCs after serum deprivation at longer time points. Besides regulation related to blood vessels, the differentially expressed genes of the ABPPk group were also involved in immune system regulation at 12 h: "retention of B lymphocytes" and "migration of peripheral T lymphocyte", while these were not observed in the NGF group. A difference in T and B lymphocytes' regulation was also observed in the ABPPk group compared with the NGF group at 0.5 and 12 h. Meanwhile, the commonality between the ABPPk and NGF groups was seen in a few nervous system processes, such as "modification of neurons" and "differentiation of interneurons" for the ABPPk group, or "migration of Schwann cells" and "extension of neurites" for the NGF group both at 3 h.

The top ten canonical pathways of significance enriched by IPA were also demonstrated (*Figure 6A*,*B*,*C*). Regulation of signaling pathways at an earlier stage by the differentially expressed genes of the ABPPk group was also revealed. "PDGF signaling", "integrin signaling", and "TNFR2 signaling" related to blood vessels and immune reactions were found only in the ABPPk group at 0.5 h, and not in the NGF group, which were also reflected in the group of ABPPk compared with NGF involving "PDGF signaling", "TNFR2 signaling", and "TNFR1 signaling". At other time points, the "antigen presentation pathway" was found for the ABPPk group at 3 h, and a few signaling pathways related to the nervous system including "axonal guidance signaling" were also found for the ABPPk group at 3 h, while "agrin interactions at neuromuscular junction" was found for the NGF group at 3 h. Unlike diseases and functions, the differentially expressed genes of the NGF group at 9 h also participated in the signaling regulation of immune cells: "PKCθ signaling in T lymphocytes" and "B

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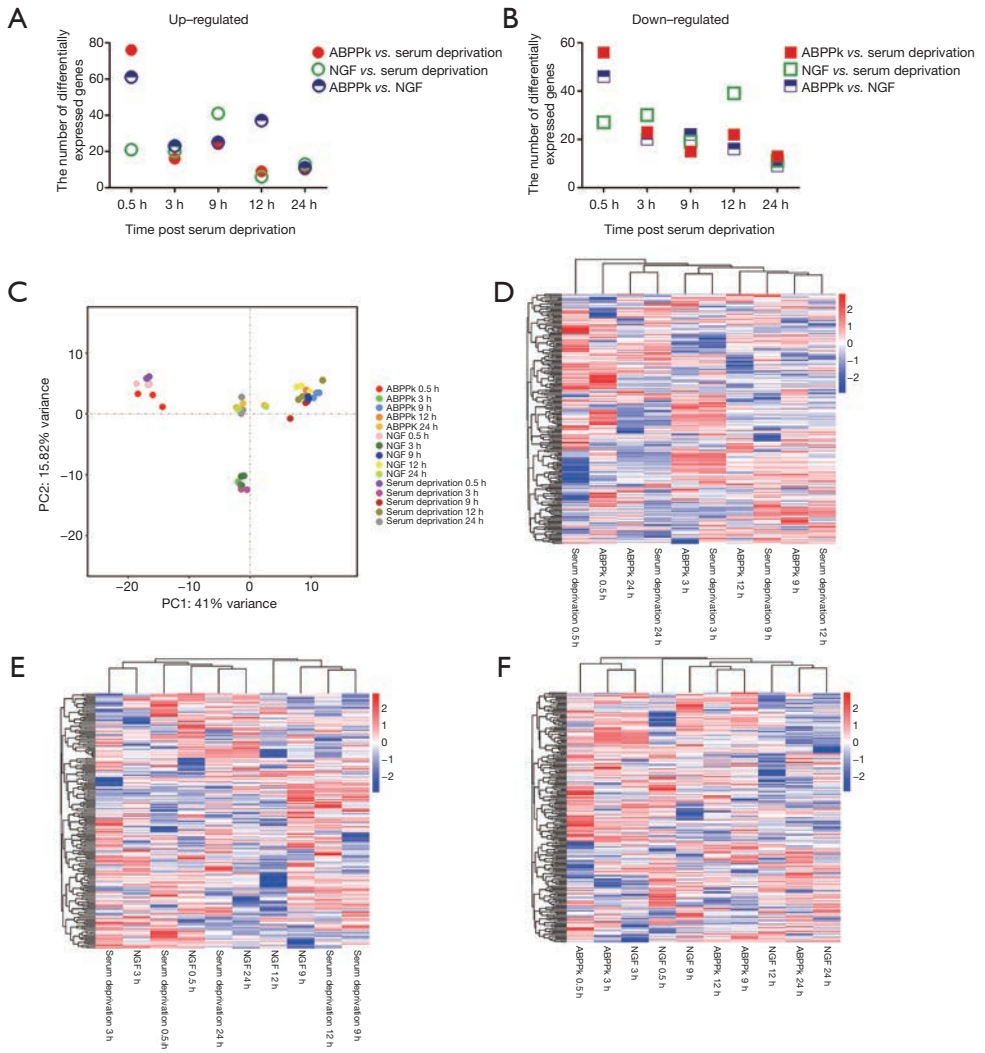

Figure 4 Outline of transcriptome changes in Schwann cells (SCs) protected by ABPPk following serum deprivation. (A,B) The up-regulated (A) and down-regulated (B) differentially expressed genes at each time point. (C) Principal component analysis of the dynamic gene expression levels at each time point. (D) Heatmaps of gene expression in the ABPPk group *vs*. the serum deprivation group. (E) The NGF group *vs*. the serum deprivation group. (F) The ABPPk group *vs*. the NGF group. Red means up-regulation, blue means down-regulation. ABPPk, *achyranthes bidentata* polypeptide fraction k.

cell receptor signaling".

Cascade regulations of transcription factors in SCs protected by ABPPk following serum deprivation

Transcription factors are upstream of regulatory molecular pathways, which play a key role in regulating molecular mechanisms. In SCs protected by ABPPk and NGF, transcription factors including diseases and functions directly related to the nervous system, vasculature, and immune system were described (*Figure 7A,B,C*). Firstly, the number of transcription factors was relatively large in the early stage at 0.5 h for all groups, then decreased. Compared to those of the ABPPk group, a few of the NGF group's transcription factors were still differentially expressed at 3 and 12 h. Moreover, the number of transcription factors directly involved in regulating neurological, vascular, and immune-related diseases and functions was not significantly different between the ABPPk and NGF groups. The regulatory advantages of the transcription factors of the ABPPk group

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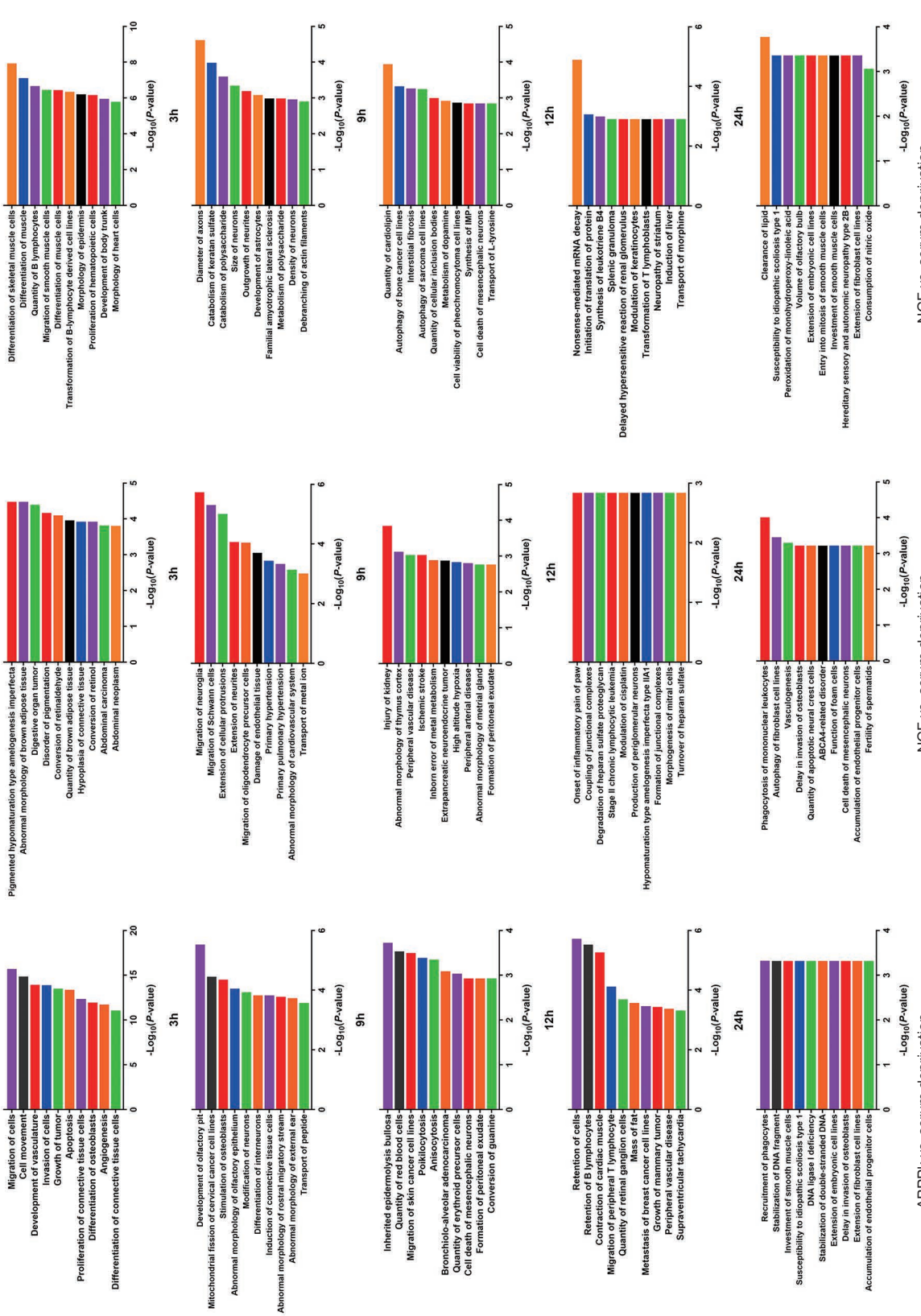

Figure 5 The top ten diseases and functions involved in the protective role of ABPPk at different time points calculated by Ingenuity Pathway Analysis (IPA). (A) Statistically significant diseases and functions were displayed for the ABPPk group compared with serum deprivation. (B) Statistically significant diseases and functions were displayed for the nerve growth factor (NGF) group compared with serum deprivation. (C) Statistically significant diseases and functions were displayed for the ABPPk group compared with the NGF group. ABPPk, *achyranthes hidentata* polypeptide fraction k.

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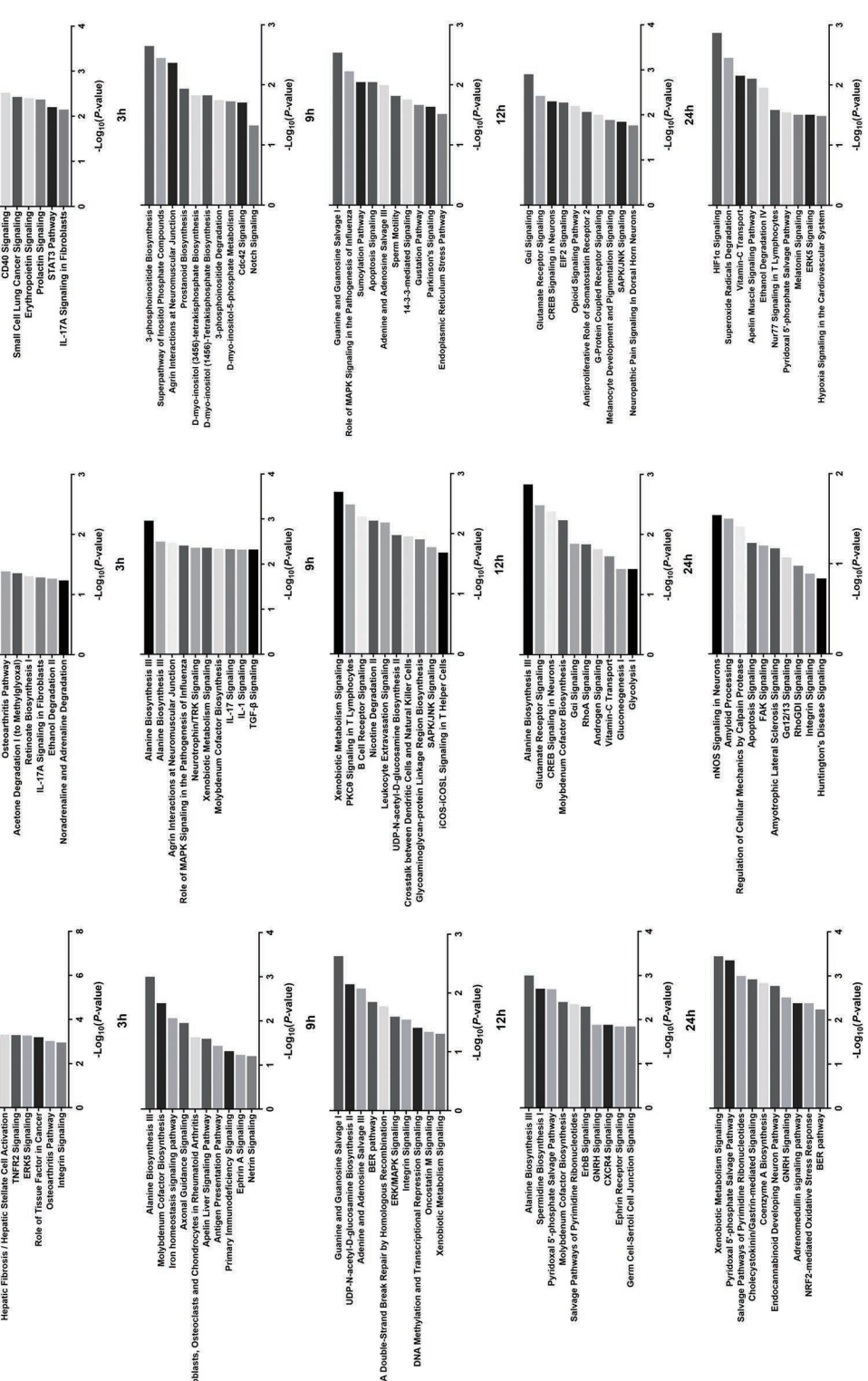

Figure 6 The top ten canonical pathways involved in the protective role of ABPPk at each time point analyzed by Ingenuity Pathway Analysis (IPA). (A) Significantly enriched canonical pathways were shown for the ABPPk group compared with serum deprivation. (B) Significantly enriched canonical pathways were shown for the nerve growth factor (NGF) group compared with serum deprivation. (C) Significantly enriched canonical pathways were shown for the ABPPk group compared with the NGF group.

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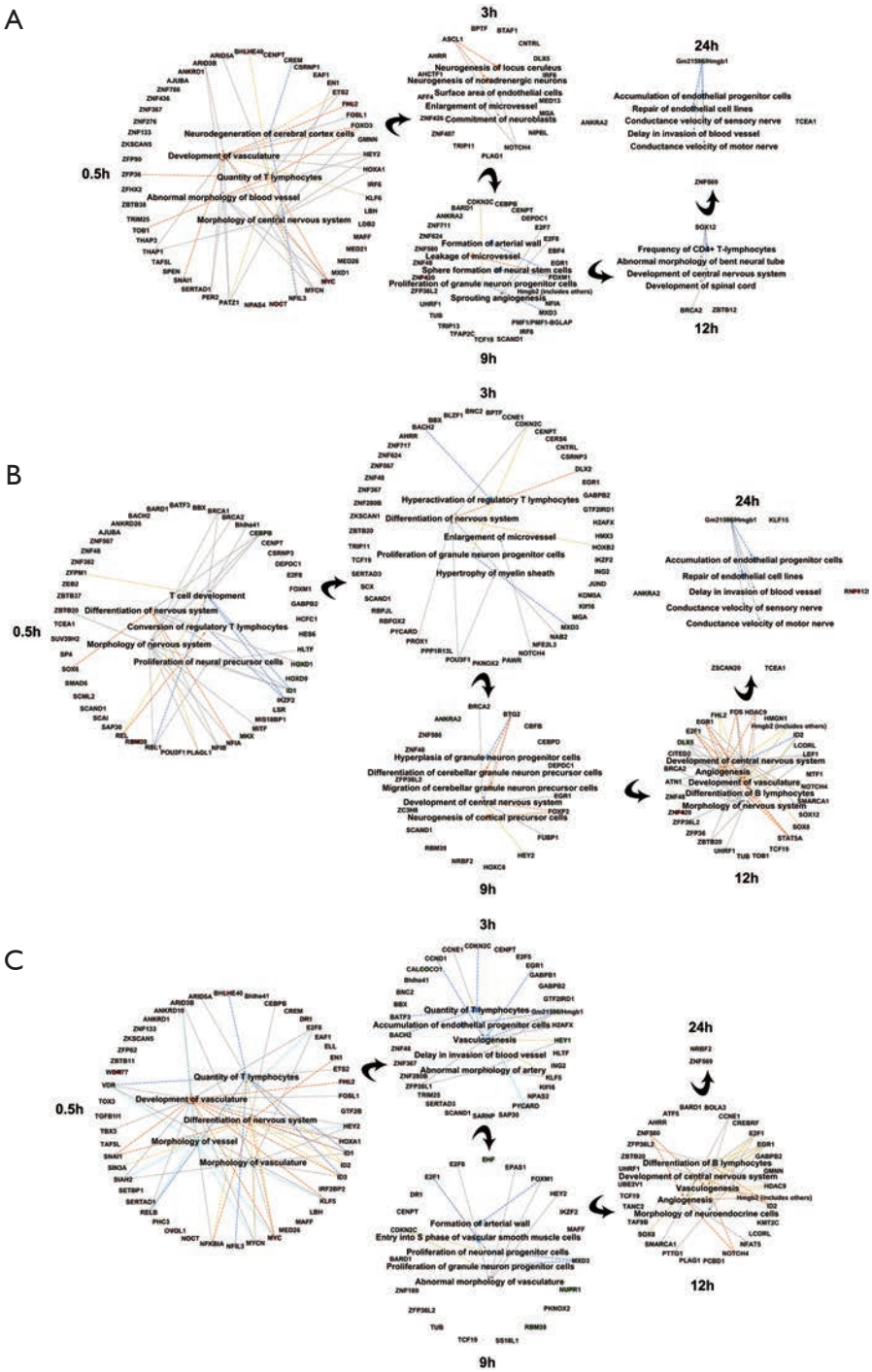

Figure 7 Cascade networks of differentially expressed transcriptional regulators (no more than 50) and diseases and functions (directly related to systems of nerve, blood vessel and immune) with ABPPk treatment in SCs after serum deprivation. The interaction networks of up-regulated (red) transcription factors and down-regulated (green) transcription factors of (A) the ABPPk group at each time point, (B) the nerve growth factor (NGF) group at each time point, and (C) the comparison between the 2 groups. More transcriptional regulators and longer time regulation of blood vessels of the ABPPk group compared to the NGF group were demonstrated. The brown of function stands for predicted activation. The blue of function stands for predicted inhibition. The brown line leads to activation. The blue line leads to inhibition. The yellow line means finding inconsistent with state of downstream molecular. The gray line means effect not predicted.

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in vascular-related aspects were more significantly reflected. They were enriched and displayed in both Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), diseases and functions, and canonical pathways. In addition to participating in regulating the nervous system and immune system, transcription factors directly regulated vascular diseases and functions at almost all time points, which were more numerous and comprehensive than those of the NGF group. At 0.5 h, a few of the transcription factors of the ABPPk group were directly involved in the regulation of vascular-related processes, while the only nervous system and immune regulations were demonstrated in the NGF group. For instance, transcription factors such as ARID3B, SNAI1, MYCN, FHL2, and FOXO3 were related to "development of vasculature" at 0.5 h, NOTCH4 was related to "enlargement of microvessel" and "surface area of endothelial cells" at 3 h, and FOXM1, E2F7, and E2F8 were involved in "formation of the arterial wall", "leakage of microvessel" and "sprouting angiogenesis" at 9 h.

qPCR validation of gene expression

The differential expression levels of 9 genes, including *EDN1*, *EFNA3*, *EPHA2*, *FGF8*, *FHL2*, *FOSL1*, *JAG1*, *MYC*, and *SERPINE1*, are related to blood vessels, immune responses, proliferation, and migration were verified (*Figure 8*). Correlation analysis suggested that qPCR data were highly correlated with the transcriptome data. *FGF8*, *FOSL1*, *MYC*, and *SERPINE1* in the ABPPk group and the NGF group were significantly expressed mainly in the middle and late stages, which differed from the expression levels after serum deprivation. *FHL2*, *FOSL1*, and *MYC*, which are transcription factors regulating vascular development, demonstrated higher expression levels at 0.5 h in the ABPPk group than the NGF group, which was consistent with ABPPk's early regulatory effects.

Discussion

The repair and functional reconstruction of peripheral nerve injury remains a challenging problem in the medical field and is also the focus of research in the field of neuroscience. Slow nerve regeneration, stump degeneration, tissue adhesion, muscle atrophy, and other factors can limit damaged nerves' functional recovery (25). Additionally, the repair and regeneration of peripheral nerve injury are very complicated biological processes, during which proliferating SCs can secrete a variety of neurotrophic factors to promote axonal growth and remyelination (18,26).

Previous studies on peripheral nerve regeneration have focused on constructing tissue-engineered grafts and the molecular regulatory mechanisms and signaling pathways in SCs (27,28). Additionally, drug treatments for peripheral nerve regeneration are another research focus. Even if some neurotrophic factors have been selected and used for peripheral nerve injury therapy, many shortcomings still exist, such as a lack of efficacy, cost, or lack of specifically targeted effects (29,30). Therefore, future studies should focus on finding a wide range of active ingredients with independent intellectual property rights, which can be developed into new drugs to protect SCs, promote axonal growth, and repair damage to peripheral nerves.

This study clearly showed that ABPPk could effectively protect SCs against serum deprivation-induced cell cytotoxicity and cell apoptosis while also increasing SOD and GSH and reducing MDA and LDH. Given these findings, we suspected that the protective effects of ABPPk on SCs should have complex regulatory mechanisms, which we further researched at the molecular level in this study. Full transcriptome sequencing technology and bioinformatics analysis involving the IPA database and other methods were combined to reveal the regulatory mechanisms. Through the overall description and analysis of differentially expressed genes, we found that the ABPPk group, compared with the NGF group, had a large amount of differential gene expression at the early 0.5 h time point, which highlighted the time-dependent effects of different regulatory factors.

The early protective effects of ABPPk on SCs, which had not yet been researched or reported on in the literature, were also demonstrated and confirmed in subsequent differential gene annotation analyses. In addition to the advantages of early protection, ABPPk also demonstrated a broad and unique scope of its protective effects on SCs. The diseases and functions and canonical pathways enriched by IPA analysis showed that the protective effects of ABPPk on SCs were significantly related to blood vessels, immune reactions, and cell apoptosis, which were distinct mechanisms from the NGF group. As its name implies, NGF's protective effects on SCs were more concentrated and directed towards neural regulations. However, besides regulating processes of the nervous system, ABPPk also participated in a blood vessel and immune regulations in more aspects and for a longer period, especially vascular-related regulations, which is consistent with its original traditional Chinese medicine applications and the results of previous studies on the central

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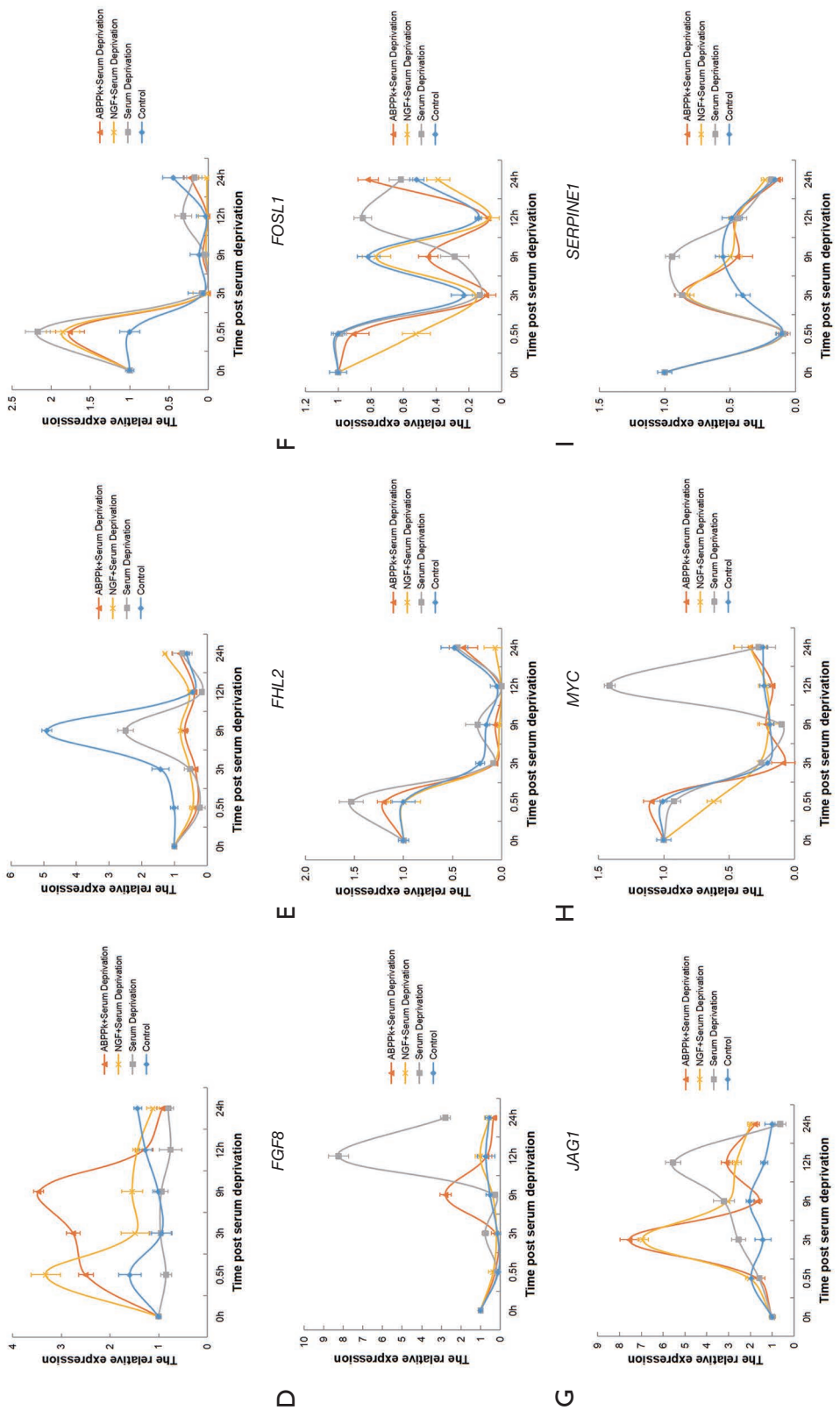

Figure 8 The qPCR validation of selected differentially expressed genes. Histograms showed the relative mRNA expression levels of *EDN1*, *EFNA3*, *EPHA2*, *FGF8*, *FHL2*, *FOSL1*, *JAG1*, *MYC*, and *SERPINE1*, which were normalized to GAPDH.

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nervous system (31-33). In other experiments from our laboratory, ABPPk demonstrated neuroprotective effects in a transient middle cerebral artery occlusion (tMCAO) rat model, promoting blood flow recovery, inhibiting secondary thrombosis, and improving microvascular patency (31).

To investigate the key regulatory factors, the dynamics of transcription factors during serum deprivation injury of ABPPk and NGF-protected SCs were further explored. The number of transcription factors in the ABPPk group was highest at 0.5 h, while in the NGF group, the number remained higher at the later time points besides the early stage, which reflects the advantages of ABPPk in early regulation. Furthermore, the characteristics of ABPPk in the regulation and control of blood vessels for SC protection were more clearly revealed through the analysis of transcription factors. In SCs protected by ABPPk, transcription factors were activated earlier and participated in vascular-related regulation with a longer regulation time and wider regulation range. Based on the above results, our research-validated the protective roles of ABPPk on SCs following serum deprivation and further determined the roles of ABPPk in the vasculature, which provides novel insights and a new direction for future research.

Currently, there have been a few studies addressing whether active plant peptides can promote axonal growth and peripheral nerve regeneration, and some new plant active peptide drugs have been used for peripheral nerve injury in clinical practice. *Achyranthes bidentata*, which is used as a medicinal plant, may be a promising specialty drug for treating neurological damage, which has sufficient sources and pharmacoeconomic advantages compared with currently known neurotrophic factor drugs. Based on our research, compared with the existing neurotrophic factor NGF, *Achyranthes bidentata* used in traditional Chinese medicine or the drugs developed by its active ingredient ABPPk hold great promise for the treatment of patients with peripheral nerve injury during the acute phase, and/or patients with severe vascular injury and inflammation due to trauma. Thus, SC survival can be maintained earlier, blood vessels and immunity can be better regulated, and peripheral nerve regeneration and quality of life can be improved. We also further focused on the correlations and differences between the molecular regulators of the protective mechanisms of ABPPk and NGF on SCs after serum deprivation injury based on the validation of their protective roles against cell apoptosis. High-throughput sequencing technology was used to study traditional Chinese medicine in a more comprehensive and in-depth manner, combining traditional Chinese medicine with modern technology. In summary, our work has contributed to an enhanced understanding of the functions and mechanisms of ABPPk and has facilitated the discovery of novel therapeutic targets, which provide an experimental and theoretical basis for clinical treatments of peripheral nerve injury to obtain better regeneration.

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