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ORIGINAL PAPER

Dysregulated Transcription Factor TFAP2A After Peripheral Nerve Injury Modulated Schwann Cell Phenotype

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Abstract

Transcription factors regulate the transcriptions and expressions of numerous target genes and direct a variety of physiological and pathological activities. To obtain a better understanding of the involvement of transcription factors during peripheral nerve repair and regeneration, significantly differentially expressed genes coding for transcription factors in rat sciatic nerves after sciatic nerve crush injury were identified. A total of 9 transcription factor genes, including GBX2, HIF3A, IRF8, LRRC63, SNAI3, SPIB, TBX21, TFAP2A, and ZBTB16 were identified to be commonly differentially expressed at 1, 4, 7, and 14 days after nerve injury. TFAP2A, a gene encoding transcription factor activating enhancer binding protein 2 alpha, was found to be critical in the regulatory network. PCR validation and immunohistochemistry staining of injured rat sciatic nerves showed that TFAP2A expression was significantly up-regulated in the Schwann cells after nerve injury for at least 2 weeks. Schwann cells transfected with TFAP2A-siRNA exhibited elevated proliferation rate and migration ability, suggesting that TFAP2A suppressed Schwann cell proliferation and migration. Collectively, our study provided a global overview of the dynamic changes of transcription factors after sciatic nerve injury, discovered key transcription factors for the regeneration process, and deepened the understanding of the molecular mechanisms underlying peripheral nerve repair and regeneration.

Keywords Transcription factor · TFAP2A · Peripheral nerve injury · Schwann cells · Proliferation · Migration

Introduction

Transcription factors, also named sequence-specific DNA-binding factors, are key regulatory proteins that control the transcriptions and expressions of target genes by recognizing and binding to specific sequences of DNA adjacent to their target genes [1–3]. Transcription factors function as "activators" or "repressors", turn on or turn off target genes, and direct various important biological processes including development, cellular processes, metabolism, responses to stimulus, immune system processes, reproduction, and localizations [1, 4]. Besides physiological conditions, transcription factors are involved in many pathological conditions [5–7]. In the nervous system, transcription factors are associated with the cellular fate of injured neurons, the regeneration of axons, and the repair of injured peripheral nerves [8]. A large number of transcription factor genes were identified to be differentially expressed in the L4–L6 dorsal root ganglia (DRG) tissues after rat sciatic nerve injury, indicating that transcription factors might play central roles in neurons during peripheral nerve repair and regeneration [9, 10].

Peripheral nerve injury is a serious clinical issue that affects approximately 2.8% of trauma patients [11]. Peripheral nerve injury could cause severe disability and greatly compromise patients' quality of life [12, 13]. Understanding the underlying cellular and molecular mechanisms and identifying critical factors in the regeneration of injured

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peripheral nerves would provide new potential targets for the clinical treatment of peripheral nerve injury.

Emerging studies demonstrated that the successful regeneration of injured peripheral nerves requires two critical elements: the survival and the activation of the intrinsic growth capacity of neurons and the construction of a favorable microenvironment [14, 15]. A permissive microenvironment in the injured peripheral nerves encourages the regrowth of axons toward their target organs and contributes to the functional recovery of injured nerves. Therefore, in the current study, we analyzed previously achieved sequencing data of the sciatic nerves at different time points after rat sciatic nerve crush injury [16, 17], explored the expression patterns of transcription factor genes, and screened significantly involved ones. Moreover, considering that a favorable microenvironment in peripheral nerves is largely constructed by the de-differentiation, proliferation, migration, re-differentiation, and myelination of Schwann cells [18, 19], we further investigated the biological functions of TFAP2A, a gene encoding transcription factor activating enhancer binding protein 2 alpha, on Schwann cell phenotype.

Materials and Methods

Animal Surgery

Rat sciatic nerve crush injury was conducted as previously described [16]. Briefly, male adult Sprague-Dawley (SD) rats weighting 180–220 g were anaesthetized by an injection of mixed narcotics containing 85 mg/kg trichloroacetaldehyde monohydrate, 42 mg/kg magnesium sulfate, and 17 mg/kg sodium pentobarbital. Rat sciatic nerves at 10 mm above the bifurcation into the tibial and common fibular nerves were crushed with a serrated forceps at a force of 54 N for 3 times with 10 seconds for each time. Rats were sacrificed and the sciatic nerve segments of 5 mm in length at the crush site were collected at 0, 1, 4, 7, and 14 days after crush injury.

SD rats were purchased from the Experimental Animal Center of Nantong University, China. Animal surgery was conducted in accordance with the Institutional Animal Care Guideline of Nantong Province, Jiangsu, China and ethically approved by the Administration Committee of Experimental Animals, Jiangsu, China.

Bioinformatic Analysis

Gene expression levels in rat sciatic nerves at 0, 1, 4, 7, and 14 days after crush injury were obtained from previously conducted RNA deep sequencing (NCBI database; accession number PRJNA394957; SRP113121) [16] and calculated using the Reads per kilobase transcriptome per million mapped reads (RPKM) method. Genes with an absolute fold change > 10 and a false discover rate (FDR) < 0.001 at 1, 4, 7, or 14 days as compared with at 0 day were screened as significantly differentially expressed genes [17]. Significantly differentially expressed transcription factors were identified using Ingenuity pathway analysis (IPA; Ingenuity Systems Inc., Redwood City, CA, USA). Differentially expressed transcription factors at 1, 4, 7, or 14 days after crush injury were illustrated using the Venny 2.1.0 online software (http://bioinfogp.cnb.csic.es/tools/venny/index.html) [20] and displayed in a heatmap.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RNA samples were extracted from sciatic nerves collected at 0, 1, 4, 7, and 14 days after crush injury using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed to cDNA using a Prime-Script reagent Kit (TaKaRa, Dalian, Liaoning, China). qRT-PCR was performed for gene expression quantification using a SYBR Green Premix Ex Taq (TaKaRa) on a StepOne real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences of the target gene TFAP2A were 5′-CTC CGGATTGCTCTCTCGAC-3′ (forward) and 5′-CTGGCT TCACGACCTGTTCT-3′ (reverse). Primer sequences of the reference gene GAPDH were 5′-AACGACCCCTTC ATTGAC-3′ (forward) and 5′-TCCACGACATACTCA GCAC-3′ (reverse). Relative abundances of TFAP2A mRNA was normalized to GAPDH and calculated using the comparative $2^{-\Delta\Delta Ct}$ method [21].

Immunohistochemistry Staining

Rat sciatic nerve slices were fixed with 4% paraformaldehyde (Xilong Scientific, Guangzhou, Guangdong, China) in phosphate-buffered saline (PBS; Gibco) for 30 min at room temperature, blocked with Immunol Staining Blocking Buffer (Beyotime, Shanghai, China) and incubated with primary antibodies mouse-anti-TFAP2A (1:50; sc-12726, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit-anti-S100β (1:100; ab52642, Abcam, Cambridge, MA, USA) overnight at 4 °C. Sciatic nerve slices were then incubated with secondary antibodies Cy3-conjugated anti-mouse IgG(H+L) (1:400; 00009-1, Proteintech Group, Rosemont, IL, USA) or Alexa Fluor 488-labeled anti-rabbit donkey IgG(H+L) (1:1000; A21206, Thermo Scientific, Rockford, IL, USA) for 2 h at room temperature and visualized using fluorescent signals. Images were taken using an optical and epifluorescence microscope (Axio Imager M2, Carl Zeiss Microscopy GmbH, Jena, Germany).

Schwann Cell Culture and Transfection

Primary Schwann cells were collected from the sciatic nerves of 1-day-old neonatal SD rats and subjected to purification using anti-Thy1.1 antibody (Sigma, St. Louis, MO, USA) and rabbit complement (Sigma). Isolated Schwann cells achieved a purity of > 98% as determined by immunostaining with anti-S100β. Schwann cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 incubator at 37 °C. Schwann cells were transfected with siRNA segments against TFAP2A (TFAP2A-siRNA-1 and TFAP2A-siRNA-2) or a non-targeting negative control (NC-siRNA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Synthesized siRNAs were obtained from Ribobio (Guangzhou, China).

Western Blotting

Western blotting was carried out using standard protocols. The following primary antibodies were used: mouse anti-TFAP2A (1:200; sc-12726, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-actin (1:1000, Proteintech, Rosemont, IL, USA).

Cell Proliferation Assay

Schwann cells were seeded onto 96-well plates pre-coated with poly-L-lysine at a density of 2×10^5 cells/ml. A total of 100 μM EdU was added to the cell culture medium and cells were cultured for additional 24 h. Schwann cells were fixed in 4% paraformaldehyde in PBS for 30 min and then stained with Hoechst 33342. Cell proliferation rate was determined using the Cell-Light™ EdU DNA Cell Proliferation Kit (Ribobio). Images were taken using a DMR fluorescence microscope (Leica Microsystems, Bensheim, Germany).

Cell Migration Assay

Schwann cells suspended in 100 μl DMEM medium were seeded onto the upper chamber of a 6.5 mm transwell chamber with 8 μm pores (Costar, Cambridge, MA, USA) at a density of 3×10^5 cells/ml. A total of 500 μl cell culture medium containing DMEM and 10% FBS was added to the bottom chamber. Schwann cells were cultured in a humidified 5% CO_2 incubator at 37 °C for 24 h. Schwann cells left on the upper surface of the upper chamber were wiped using a cotton swab and Schwann cells migrated to bottom surface were stained with 0.1% crystal violet (Beyotime) for 15 min at room temperature. Crystal violet was dissolved using 33% acetic acid and the absorbance of crystal violet was measured at 570 nm using a Bio-tek (BioTek Instruments, Inc., Winooski, VT, USA). Images were taken using a DMR inverted microscope (Leica Microsystems).

Statistical Analysis

Results were summarized from 3 experiments and expressed as mean ± SEM. Statistical differences were determined using one-way analysis of variance and Dunnett's multiple comparisons test or Student's *t* test. A p-value less than 0.05 was considered as significantly different. Statistical test and graphs were made using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Identification of Significantly Differentially Expressed Transcription Factor Genes After Peripheral Nerve Injury

Significantly differentially expressed genes with an absolute fold change > 10 and a false discover rate (FDR) < 0.001 at 1, 4, 7, or 14 days as compared with at 0 day were selected in our previous study [17]. Considering the importance of transcription factors, significantly differentially expressed transcription factor genes were further screened (Table S1). A total of 39, 27, 19, and 14 transcription factor genes were identified to be significantly differentially expressed at 1, 4, 7, and 14 days after rat sciatic nerve crush injury (Fig. 1a). The percentage of significantly differentially expressed transcription factor genes among total genes ranged from 6.05 to 4.38% and peaked at 1 day (Fig. 1a).

The expression patterns of these significantly differentially expressed transcription factor genes at different time points were displayed in a heatmap (Fig. 1b). Consistent with the expression trend of total genes, the majority of these significantly differentially expressed transcription factor genes were up-regulated while about 1/5 of them were down-regulated after sciatic nerve crush injury.

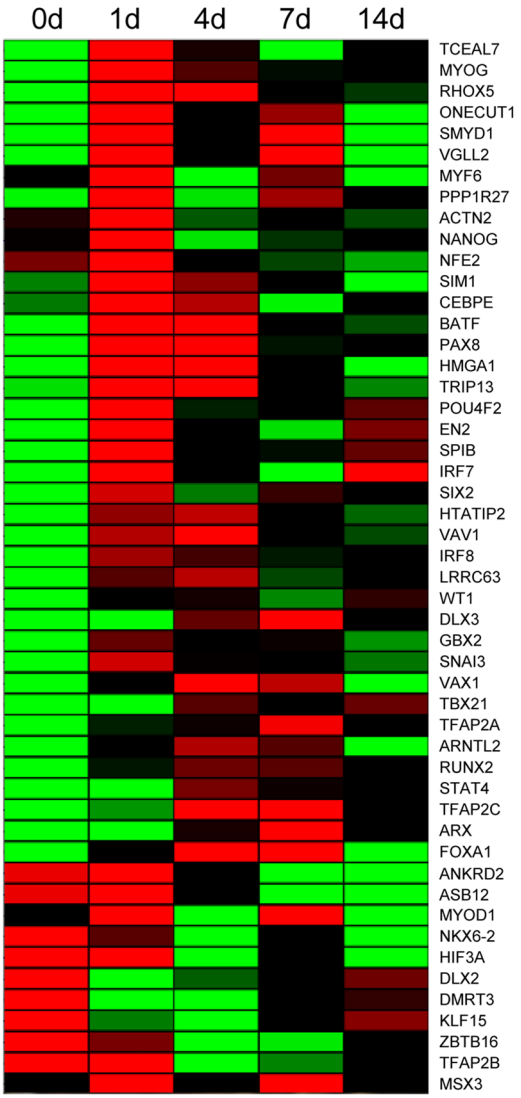
The intersections of these significantly differentially expressed transcription factor genes were obtained using a Venn diagram (Fig. 1c). A total of 9 transcription factor genes, including GBX2, HIF3A, IRF8, LRRC63, SNAI3, SPIB, TBX21, TFAP2A and ZBTB16, were overlapped at 1, 4, 7, and 14 days (Fig. 1c, d).

Furthermore, a regulatory network of significantly differentially expressed transcription factor genes was further constructed to illuminate a gene cascade and to examine the interactions of transcription factor genes (Fig. 2).

A

Numbers	1d	4d	7d	14d
TFs	39	27	19	14
Genes	645	602	360	320
Ratio	6.05%	4.49%	5.28%	4.38%

B



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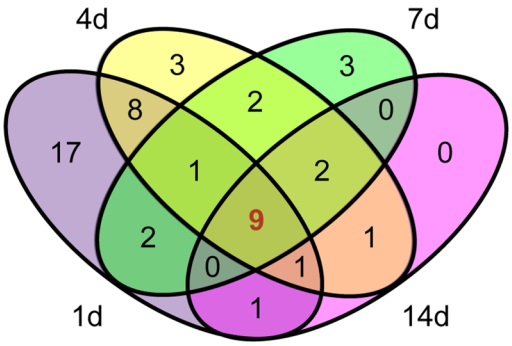

D

Symbol	Description
GBX2	Gastrulation Brain Homeobox 2
HIF3A	Hypoxia Inducible Factor 3 Subunit Alpha
IRF8	Interferon Regulatory Factor 8
LRRC63	Leucine Rich Repeat Containing 63
SNAI3	Snail Family Transcriptional Repressor 3
SPIB	Spi-B Transcription Factor
TBX21	TBX21
TFAP2A	Transcription Factor AP-2 Alpha
ZBTB16	Zinc Finger And BTB Domain Containing 16

Fig. 1 Overview of significantly differentially expressed transcriptional factor genes. **a** Numbers of significantly differentially expressed transcriptional factor genes in rat sciatic nerve stumps at 1, 4, 7, and 14 days after sciatic nerve crush injury. **b** Heatmap of the expression patterns of significantly differentially expressed transcriptional factor genes. Red color indicated up-regulation and green color indicated down-regulation. **c** Venn diagram of significantly differentially expressed transcriptional factor genes at 1, 4, 7, and 14 days after sciatic nerve crush injury. **d** The description of 9 commonly significantly differentially expressed transcriptional factor genes at 1, 4, 7, and 14 days (Color figure online)

Expression of TFAP2A in Rat Sciatic Nerve Stumps After Peripheral Nerve Injury

TFAP2A was found to be differentially expressed in sciatic nerves at all tested time points after rat nerve crush injury. The dynamic expression level of TFAP2A mRNA revealed by RNA deep sequencing (Fig. 3a) was further validated using different sets of RNA samples for sequencing. Consistent with the sequencing data, qRT-PCR results showed that as compared with TFAP2A abundance in uninjured rat sciatic nerves, the amount of TFAP2A mRNA elevated to more than 6 folds at 1 day after nerve injury and increased to even higher levels at later time points (Fig. 3b).

Immunohistochemistry staining was further performed to detect the localization and abundance of immunoreactive TFAP2A protein in vivo. In the intact sciatic nerve, the expression level of TFAP2A was very low (Fig. 3c, d). After crush injury, the fluorescence intensity of TFAP2A in the sciatic nerve increased at all of the examined time points (1, 4, 7, and 14 days after nerve injury), especially at the crush site (Fig. 3c, d). Analysis of the co-localization of TFAP2A and S100β signals showed that while the percentage of

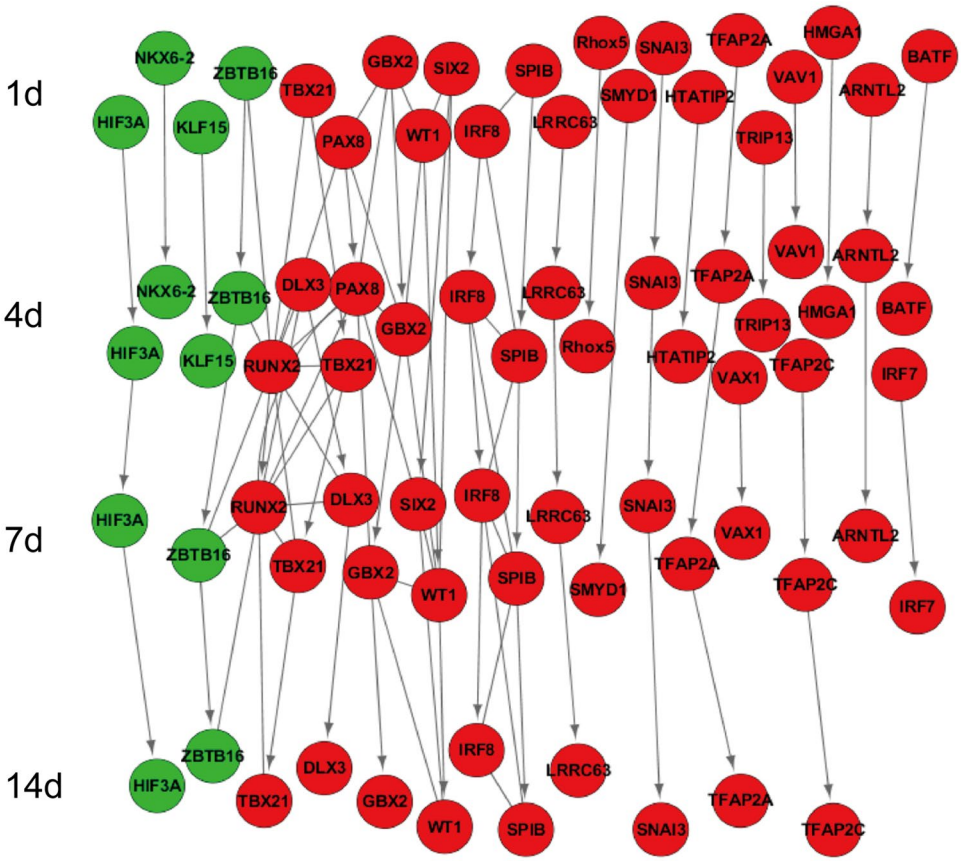

Fig. 2 Cascades of significantly differentially expressed transcriptional factor genes. The cascade network of significantly differentially expressed transcriptional factor genes in rat sciatic nerve stumps at 1, 4, 7, and 14 days after sciatic nerve crush injury was constructed. Red color indicated up-regulation and green color indicated down-regulation (Color figure online)

S100β+ cells expressing TFAP2A increased in all three regions after injury, it was most obvious at the crush site (Fig. 3c–e). In addition, most of the TFAP2A+ cells were also S100β+ (Fig. 3d, f), suggesting TFAP2A was mainly induced in the Schwann cells at sciatic nerve injury.

We further examined cell proliferation by co-immunostaining of Ki67 and TFAP2A in the injured nerve at 7 days post injury when TFAP2A expression reached its highest level. Immunofluorescence showed that 24.9 ± 1.5% of the TFAP2A+ cells were also Ki67+ (Fig. 3g, h) at this time point. Importantly, 55.3 ± 3.7% of the proliferating cells were TFAP2A+ (Fig. 3g, h), suggesting TFAP2A induction in the Schwann cells was associated with cell proliferation.

Effects of TFAP2A on Schwann Cell Phenotype

The biological functions of TFAP2A in Schwann cells were evaluated by culturing primary Schwann cells and transfection with siRNA segments against TFAP2A. The efficiency of siRNA transfection was examined by qRT-PCR and western blotting (Fig. 4a, b). An siRNA segment with high efficiency (siRNA-2) was selected for subsequent functional study (Fig. 4a, b).

Schwann cells transfected with non-targeting negative control or TFAP2A siRNA were treated with EdU to determine the effects of TFAP2A knockdown on Schwann cell proliferation. Compared with Schwann cells transfected with non-targeting negative control, there was a larger number of proliferating Schwann cells in the TFAP2A siRNA group (Fig. 4c). The ratio of proliferating cells to total cells was also higher in Schwann cells transfected with TFAP2A siRNA, suggesting that TFAP2A inhibited the proliferation rate of Schwann cells (Fig. 4c).

Schwann cells transfected with non-targeting negative control or TFAP2A siRNA were also seeded onto transwell chambers to determine the effects of TFAP2A knockdown on Schwann cell migration. Notably more cells migrated to the bottom surface of the transwell chamber after TFAP2A siRNA transfection. Summarized results of the absorbance of dissolved crystal violet suggested that TFAP2A suppressed the migration ability of Schwann cells (Fig. 4d). Taken together, our results indicate TFAP2A is an inhibitor of Schwann cell proliferation and migration after peripheral nerve injury.

Discussion

The development of high-throughput and bioinformatic analysis benefits the systematic determination of expression profiles of genes and proteins after peripheral nerve

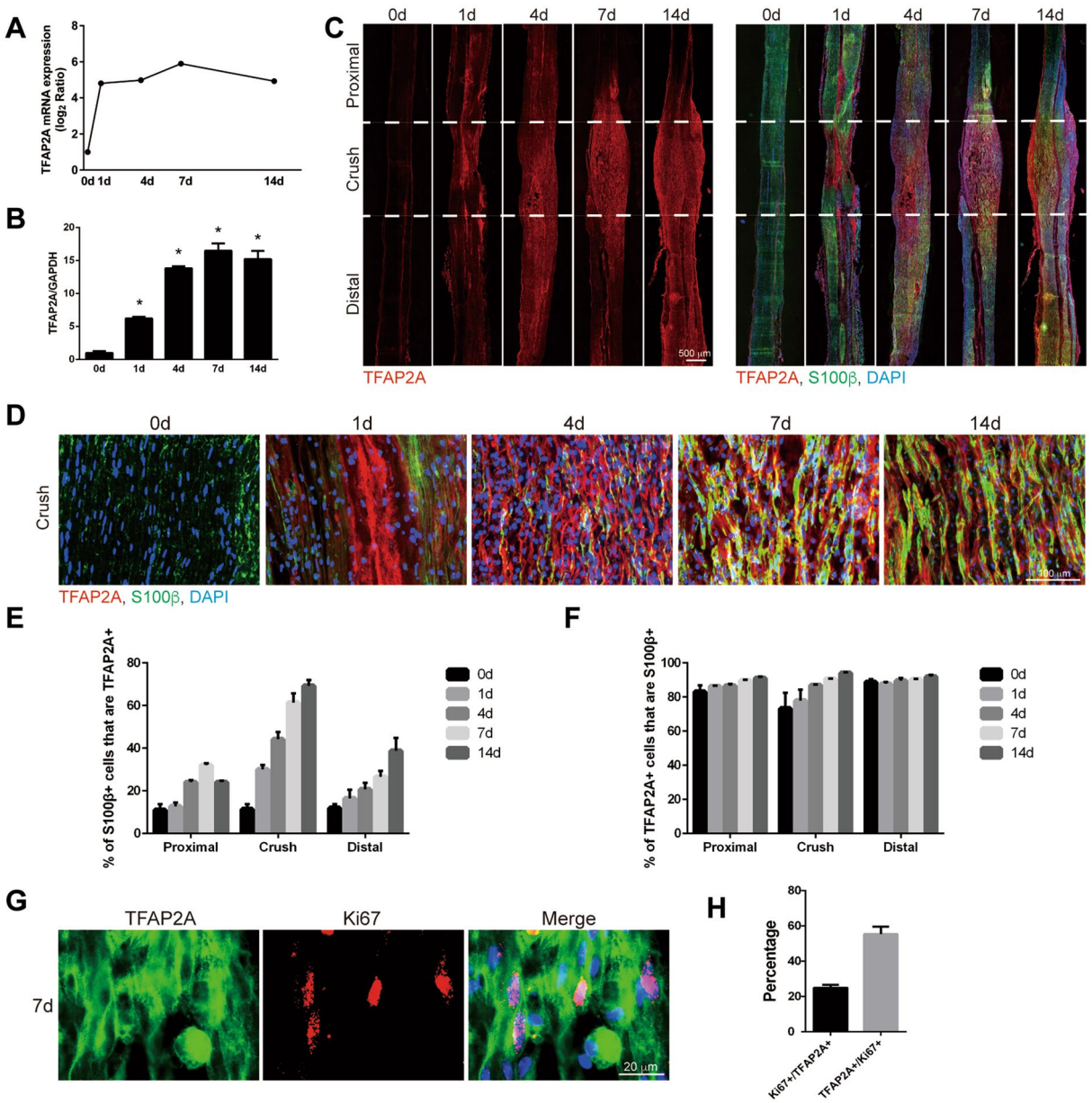

Fig. 3 The expression patterns of TFAP2A. **a** The fold changes of TFAP2A at 1, 4, 7, and 14 days after sciatic nerve crush injury identified by RNA deep sequencing. **b** The relative mRNA abundances of TFAP2A at 1, 4, 7, and 14 days after sciatic nerve crush injury. The asterisk indicated significant difference. **c** Immunostaining of TFAP2A protein at 1, 4, 7, and 14 days after sciatic nerve crush injury. TFAP2A was labeled in red color and S100β was labeled in green color. **d** TFAP2A and S100β immunofluorescence images showing their co-localization in the crush region at indicated time points. **e**, **f** Quantification of TFAP2A and S100β co-localization in the sciatic nerve before and after injury. **g** TFAP2A and Ki67 immunofluorescence showing their co-localization in the crush region at 7 days post injury. **h** Quantification of TFAP2A and Ki67 co-localization in (**g**). Ki67+/TFAP2A+, the percentage of TFAP2A+ cells that are also Ki67+; TFAP2A+/Ki67+, the percentage of Ki67+ cells that are also TFAP2A+ (Color figure online)

injury, and the identification of critical factors for peripheral nerve regeneration [22–24, 16]. Many transcription factors have been identified to be important for the regeneration process. For example, axonal injury induced the activations of transcription factors c-JUN, CCAAT/enhancer binding proteins (C/EBPs), activating transcription factor 3 (ATF3), and signal transducer and activator of transcription 3 (STAT3) in neurons and led to increased or suppressed transcriptions of target genes [8]. In one study, it was shown that up-regulated transcription factor genes ATF3, JUN, SMAD1, and STAT4 in DRG tissues functioned as neuronal regeneration-enhancing factors and modulated the switch of injured neuron from "a pre-regeneration phase" to "a regeneration phase" [10]. In another

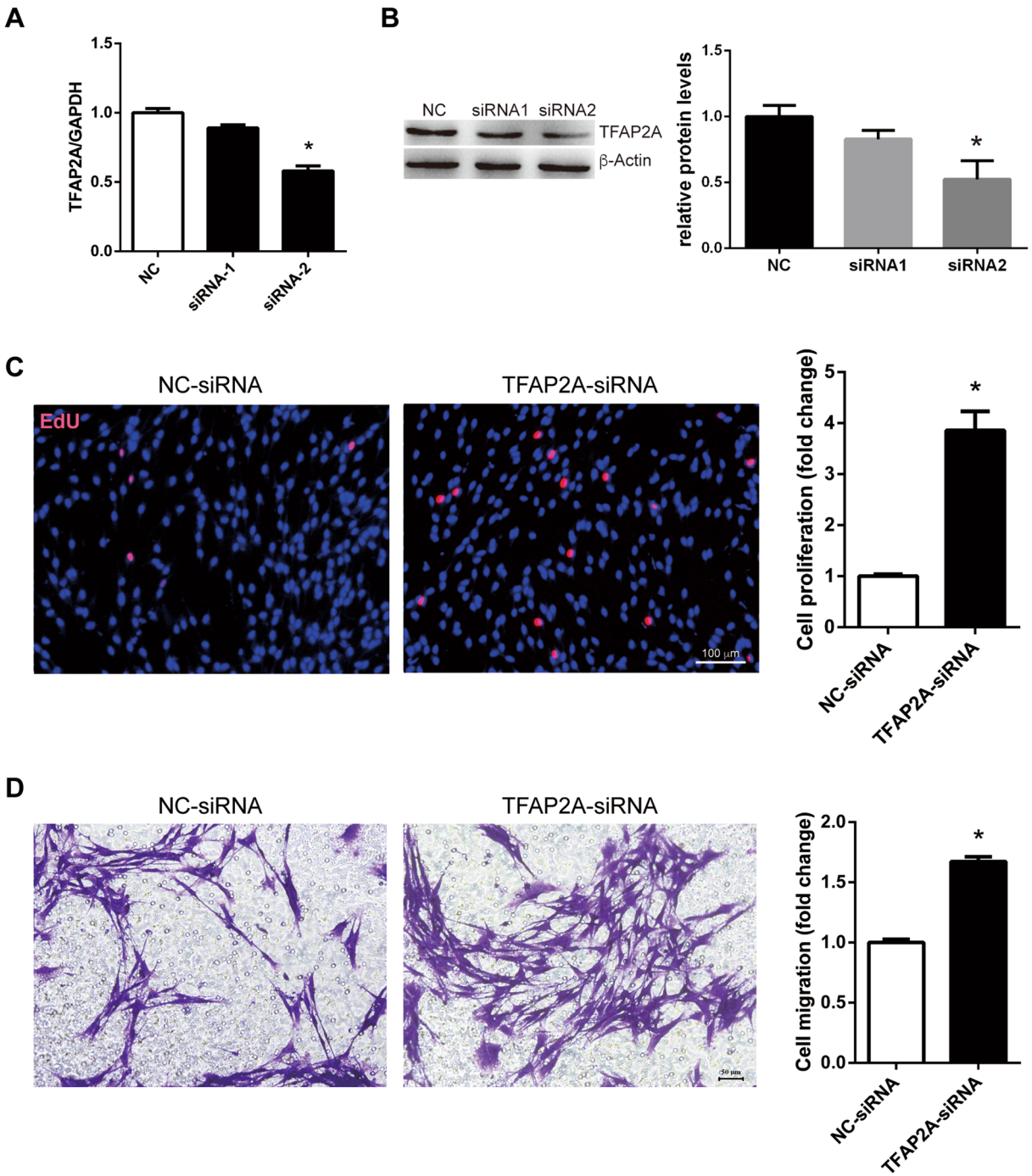

Fig. 4 The biological effects of TFAP2A on Schwann cell proliferation and migration. **a** Transfection efficiency of TFAP2A-siRNA. **b** Western blotting and quantification showing the efficacy of the two TFAP2A siRNAs. The asterisk indicated significant difference. **c** Representative images and summarized results of the proliferation rates of Schwann cells transfected with a non-targeting negative control (NC-siRNA) or TFAP2A-siRNA. **d** Representative images and summarized results of the migration rates of Schwann cells transfected with a non-targeting negative control (NC-siRNA) or TFAP2A-siRNA. The asterisk indicated significant difference

study, up-regulated ATF3 and JUN and down-regulated STAT1 and IRF7 in DRG tissues could also function as hub genes and regulated cell death and survival [9].

Despite the deepening recognisation of transcription factors in DRG tissues after peripheral nerve injury, our knowledge of the involvement of transcription factors in the

injured peripheral nerves was still limited. In the current study, we analyzed the global changes of transcription factor genes after peripheral nerve injury by screening previously obtained sequencing data and constructed a cascade network of significantly differentially expressed transcription factor genes. Our analysis identified TFAP2A, a transcription factor that was continuously dysregulated during peripheral nerve regeneration, and its function was further investigated.

TFAP2A has been shown to exhibit physical interactions with APC (Adenomatous polyposis coli) [25], EP300 (Histone acetyltransferase p300) [26], CITED2 (Cbp/p300-interacting transactivator 2) [26, 27], DEK (DEK proto-oncogene) [28], MYC [29], and TP53 [30]. Therefore, direct associations between TFAP2A, APC, EP300, CITED2, DEK, MYC, and TP53 were built using IPA connect function based on Ingenuity Pathways Knowledge Base (IPKB) (Fig. 5a). IPKB revealed that there were protein–protein interactions, activations, modifications, and other relationships among these proteins. Moreover, the expression patterns of genes in the TFAP2A-centered network during peripheral nerve regeneration were obtained from a sequencing data [16] and displayed in a heatmap (Fig. 5b). The changes of mRNAs coding for these TFAP2A-associated proteins were not as robust as that of TFAP2A, suggesting that other TFAP2A-associated or modified targets may exist during peripheral nerve regeneration. Nevertheless, as these TFAP2A-associated proteins are important regulators of cell proliferation, migration and apoptosis, it will be worthwhile to further investigate whether they mediated the function of TFAP2A in Schwann cell proliferation and migration after sciatic nerve injury.

TFAP2A was previously found to be present in Schwann cell precursors in peripheral nerves as a potential regulator of Schwann cell generation and development [31]. A later study showed that TFAP2A was also expressed in adult peripheral nerves and mature Schwann cells and was expressed at even higher levels in Schwann cell-derived tumors, indicating that up-regulated TFAP2A might be associated with the undifferentiated state of Schwann cells [32]. This was consistent with our observation that TFAP2A was localized in Schwann cells in rat sciatic nerves and was up-regulated in sciatic nerves when Schwann cells underwent dedifferentiation after nerve injury.

Our functional study showed that inhibiting TFAP2A expression led to elevated proliferation and migration of Schwann cells, suggesting that up-regulated TFAP2A hindered the proliferation and migration of Schwann cells after sciatic nerve injury. Therefore, to create a more favorable microenvironment after nerve injury, one could utilize the strategy to knockdown TFAP2A in Schwann cells and this may improve the regeneration of peripheral nerves. It has been shown that TFAP2A exhibited either oncogenic or suppressive features in different types of cells and tissues [33]. For example, TFAP2A could suppress the proliferation of skeletal myoblast [34] but promote the growth and proliferation of acute myeloid leukemia cells [35]. A recent study showed that besides affecting cellular behaviors, TFAP2A was also able to regulate lipid droplet biogenesis

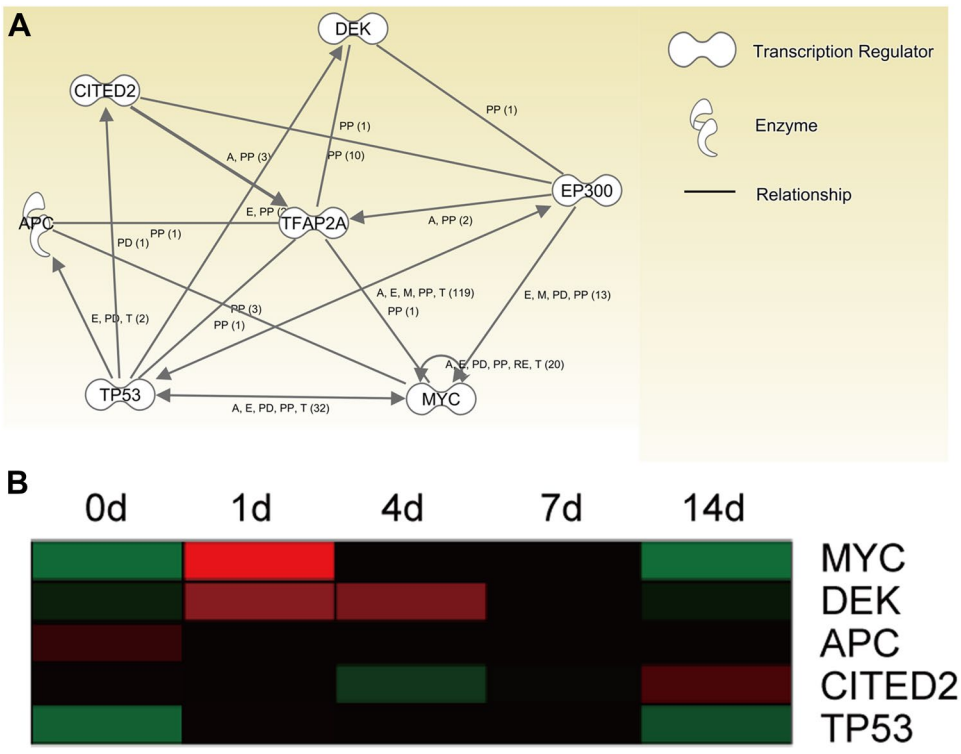

Fig. 5 The connection of TFAP2A with interacted proteins. **a** The interaction network of TFAP2A and associated proteins APC, CITED2, DEK, EP300, MYC, and TP53. PP indicated protein–protein interactions, A indicated activation, E indicated expression, M indicated modification, PD indicated protein–DNA interactions, T indicated transcription, and RE indicated reaction. **b** Heatmap of the expression patterns of interacted genes. Red color indicated up-regulation and green color indicated down-regulation (Color figure online)

and mediate energy supply [36]. Therefore, up-regulated TFAP2A after nerve injury might be linked with energy storage and metabolism. The specific functions of TFAP2A on other cellular behaviors, including Schwann cell differentiation and myelination, also needed to be further investigated in future studies.

Being key modulators of gene expression, transcription factors play critical roles in converting injury-induced signals to protein alternations and phenotype changes. Our study provided an overall view of the changes of transcription factors and may help the understanding of the molecular mechanisms underlying peripheral nerve repair and regeneration.

Author Contributions Conceived and designed the experiments: SY HX. Performed the experiments: FZ XG. Analyzed the data: FZ XG SY. Contributed reagents/materials/analysis tools: SY. Wrote the manuscript: SY HX.

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Data Availability Sequencing data were uploaded to NCBI database (Accession Number PRJNA394957; SRP113121).

Compliance with Ethical Standards

Ethics Approval and Consent to Participate All the experimental procedures involving animals were conducted in accordance with Institutional Animal Care guidelines of Nantong University and approved ethically by the Administration Committee of Experimental Animals, Jiangsu, China.

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