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FBP1 and p27kip1 Expression After Sciatic Nerve Injury: Implications for Schwann Cells Proliferation and Differentiation

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ABSTRACT

Far Upstream Element (FUSE) Binding Protein 1 (FBP1), first identified as a single-stranded DNA (ssDNA) binding protein that binds to the FUSE, could modulate c-myc mRNA levels and also has been shown to regulate tumor cell proliferation and replication of virus. Typically, FBP1 could active the translation of p27kip1 (p27) and participate in tumor growth. However, the expression and roles of FBP1 in peripheral system lesions and repair are still unknown. In our study, we found that FBP1 protein levels was relatively higher in the normal sciatic nerves, significantly decreased and reached a minimal level at Day 3, and then returned to the normal level at 4 weeks. Spatially, we observed that FBP1 had a major colocation in Schwann cells and FBP1 was connected with Ki-67 and Oct-6. In vitro, we detected the decreased level of FBP1 and p27 in the TNF-α-induced Schwann cells proliferation model, while increased expression in cAMP-induced Schwann cells differentiation system. Specially, FBP1-specific siRNA-transfected SCs did not show fine and longer morphological change after cAMP treatment and had a decreased motility compared with normal. At 3 days after cAMP treatment and SC/neuron co-cultures, p27 was transported to cytoplasm to form CDK4/6-p27 to participate in SCs differentiation. In conclusion, we speculated that FBP1 and p27 were involved in SCs proliferation and the following differentiation in the sciatic nerve after crush by transporting p27 from nucleus to cytoplasm. J. Cell. Biochem. 115: 130–140, 2014.

KEY WORDS: CIATIC NERVE INJURY; SCHWANN CELLS; PROLIFERATION; DIFFERENTIATION; FBP1; p27

Lesion of peripheral nerves may cause permanent denervation with paralysis and disability in humans and it also represents a challenging problem in neurosurgery. But unlike the central nervous system (CNS), the peripheral nerve system can regenerate towards its original target and recover functionally after injury. The injury of peripheral nerve induces a series of intrinsic cellular and molecular changes in the injured neurons and Schwann cells, which would all support axonal regeneration [Fawcett and Keynes, 1990; Hokfelt et al., 1994; Fu and Gordon, 1997; Han et al., 2007]. During the pathological process following sciatic nerve injury, Schwann cells experience the process of dedifferentiation, proliferation, and differentiation, which all play a predominant supportive role in the recovery of sciatic nerve injury. Thus, learning the molecular and cellular mechanisms that underlie peripheral nerve regeneration and identification of proteins involved in will contribute to our understanding of neuroregeneration significantly. Besides,

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understanding the mechanisms of axonal regeneration and improving recovery after nervous system injury also have been a significant aim of the neuroscience and medical community now.

During the recovery process following injury, the regulation of cell cycle may be indispensable. Cell cycle process is an important event and the ordered cell cycle progression is dependent on the interaction and association of cyclin-dependent kinases (CDKs), cyclin proteins, and CDK inhibitors (CDKIs) [Hunter and Pines, 1994; Morgan, 1995; Sherr and Roberts, 1999]. p27Kip1 (p27) is a member of Cip/Kip family (p21Cip1, p27Kip1, and p57Kip2) of CDK inhibitors and plays a critical role in eukaryotic cell cycle regulation [Sgambato et al., 2000]. p27 has been thought to be implicated in fate specification and differentiation of oligodendrocytes [Durand et al., 1997; Zezula et al., 2001]. Besides, p27 could work with p19Ink4d to maintain differentiated neurons in a non-mitotic state [Zindy et al., 1999]. p27 also regulates the division of transit amplifying progenitors in the adult subventricular zone [Doetsch et al., 2002]. According to Miyazawa and Carruthers, p27 participates in promoting cell cycle arrest of neural progenitors during embryogenesis [Miyazawa et al., 2000; Carruthers et al., 2003]. p27 promotes the exit of cell cycle by associating with specific Cdk/cylins complexes through its N-terminal binding domain and hence blocking their catalytic activity and preventing G1-S phase transition. Normally, p27 protein levels are high during G0 and G1 phases of the cell cycle, resulting in inhibition of cyclin/CDK. But when the inhibition of cyclin/CDK are relieved facing some proliferative cues, the level of p27 protein is reduced. Of course, p27 protein expression can be stimulated in other phases of the cell cycle by contact inhibition, mitogen withdrawal, differentiation signals, or other antiproliferative cues that result in G1 arrest [Koff et al., 1993; Polyak et al., 1994; Sherr, 1994; Toyoshima and Hunter, 1994; Durand et al., 1997; Baldassarre et al., 2000]. All these together confirmed the effect of p27 on CNS development. According to Youhua Wang, p27 protein level are decreased after the injury of peripheral nerve, and recover to normal at about 2 weeks [Wang et al., 2009]. But the role of p27 during the repair of peripheral nerve injury is little known and the mechanism how p27 regulates the repair of injured nerve is also unknown. It remains to be explored.

FBP1 is a member of FBP family including FBP1, FBP2, and FBP-interacting repressor (FIR) and it was first identified as a single-stranded DNA (ssDNA)-binding protein that binds to the FUSE, an A/T-rich element located in the c-myc promoter, and it modulates c-myc mRNA levels which indicating that FBP1 functions as a growth-dependent regulator of c-myc expression [Avigan et al., 1990; Duncan et al., 1994, 1996; Davis-Smyth et al., 1996; He et al., 2000]. Isolated from proliferating HL-60 cells, FBP1 (FBP), FBP2, and FBP3 comprise a family of ssDNA-binding proteins that specifically bind to FUSE elements. The FBP transcription factors share a conserved central DNA-binding domain and show significant homology in their carboxyl-terminal activation domains [Vindigni et al., 2001]. Expression of FBP1 is detected in undifferentiated cells and is substantially decreased following cellular differentiation. FBP1 was thought to interact with Untranslated Regions (UTRs) of Japanese encephalitis virus genome (JEV), a positive-sense RNA, and was involved in viral translation, the initiation of RNA synthesis, and the packaging of nascent virions and eventually Negatively regulated the replication of virus [Chien et al., 2011]. Besides, FBP1 could serve as a regulatory machinery that monitors the intensity of ongoing c-myc transcription and programming a pulse of c-myc expression required for cell cycle entry [Chung et al., 2006; Liu et al., 2006]. In human hepatocellular carcinoma (HCC), FBP1 could reduce the expression of its target gene p21 (a CDK inhibitors) to accelerate the cell cycle, proapoptotic genes tumor necrosis factor-α and TNF-related apoptosis-inducing ligand to inhibit the extrinsic apoptosis pathway, Noxa and Bik to suppress the mitochondrial apoptosis pathway, all together are required for tumor growth [Cao et al., 1997; Yamanaka et al., 2000; Sheikh and Huang, 2004; Ohira et al., 2006; Sturm et al., 2006; Rabenhorst et al., 2009]. FBP1 also regulate the cell proliferation by other pathway. According to Boyko, Ubiquitin-specific protease 22 (USP22) could altering the ubiquitination of FBP1 and affect the expression of p21 to regulate cell proliferation and tumorigenesis [Atanassov and Dent, 2011]. PGE2 could activate EP3 receptor, which leads the activated EP3 receptor to couple to Gs protein and activate cyclic AMP (cAMP)-PKA, downregulate the levels of JTV1 protein, consequently inhibit the ubiquitination of FBP1 and increase FBP1 protein expression and thus promote liver cancer cell growth [Ma et al., 2013]. Recently, FBP1 was thought to active the translation of p27 mRNA through its internal ribosomal entry site located in the 5′-untranslated region (5′-UTR) [Zheng and Miskimins, 2011]. The N-terminal domain and C-terminal domain of FBP1 have different and opposite consequences for the cell. The N-terminal domain of FBP1 play an important role in translational activation but repress transcription, but the C-terminal domain of FBP1 play an important role in transcriptional activation but repress translation [Duncan et al., 1996]. Now that FBP1 could stimulate the expression of c-myc and p27, the effect of p27 and c-myc maybe totally adversed in terms of cell cycle progression and tumorigenesis. So it is interesting that how FBP1 balance its functions on transcription and translation. Of course, we also take an interest in what is a role of FBP1 in lesion of peripheral nerves and what is the significance of regulation of p27 by FBP1 in the peripheral nerves injury and repair. In addition, our data provide some insights into the general mechanism underlying peripheral nerves regeneration after injury.

MATERIALS AND METHODS

ANIMALS AND SURGERY

Male Sprague Dawley rats weighing 250–300 g were used. The rats were anesthetized with pentobarbital (50 mg/kg). Using aseptic technique, the right sciatic nerve was exposed 1.0 cm distal to the sciatic notch by blunt dissection, then crushed by a small hemostat of 3-mm width at the mid-point for 10 s, and then unclamped for 10 s. This process was repeated three times as described before [2008a, b; Lou et al., 2012]. The wound was closed by suturing the muscles and skin. Sham-controlled rats were subjected to identical procedures as the experiment al rats, except for crushed. Experimental animals ($n = 4$ per time point) were anesthetized to extract the protein for Western blot analysis at 6 h, 12 h; 1, 3, 5 and 7 days; and 2 and 4 weeks after injury. Normal rats ($n = 4$) were anesthetized on the 3rd day. One-centimeter-long sciatic nerve segments centered on the lesion site from above time points, and corresponding segments from

the control group were excised and snap frozen at −70°C until use. Additional experimental animals (n = 3 per time point) for sections were anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde at each time point. All surgical intervention, sand postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals were approved by the Chinese National Committee to the Use of Experimental Animals for Medical Purposes, Jiangsu Branch. All efforts were made to minimize the number of animals used and their suffering.

WESTERN BLOT ANALYSIS

The samples were then homogenized in lysis buffer (1% NP-40, 50 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton-X100, 1 mmol/l PMSF, 10 mg/ml aprotinin, and 1 mg/ml leupeptin, Sigma, St. Louis, MO) and clarified by centrifuging for 20 min in a microcentrifuge at 4°C. After determination of its protein concentration with the Bradfordassay (Bio-Rad, Hercules, CA), the resulting supernatant (50 mg of protein) was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidine difluoride-membrane (Millipore Corporation, Bedford, MA) by a transfer apparatus at 350 mA for 2.5 h. The membrane was then blocked with 5% non-fat milk and incubated with primary antibody. After incubating with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody, protein was visualized using an enhanced chemiluminescence system (ECL, Pierce Company, Kocktord, IL).

IMMUNOHISTOCHEMISTRY

At the designated time points, the normal and injured rats were terminally anesthetized and perfused through the ascending aorta with physiological saline followed by 4% paraformaldehyde. After perfusion, the normal or crushed proximal and distal stumps of the sciatic nerve were removed and post-fixed in the same fixative for 3 h and then replaced with 20% sucrose for 2–3 days, followed by 30% sucrose for 2–3 days. Transverse sciatic nerve sections (8 μm) were cut using a cryostat microtome and processed for immunochemical analysis. All sections were blocked using 10% donkey serum with 0.3% Triton-X100 and 1% bovine serum albumin (BSA) for 2 h at room temperature (RT). After blocking, the membranes were incubated overnight at 4°C with an anti-FBP1 antibody (anti-mouse, 1:100; SantaCruz, CA) followed by incubating with a biotinylated secondary antibody. Staining was visualized using DAB. The stained sections were examined using a Leica fluorescence microscope (Germany).

IMMUNOFLUORESCENT STAINING

After air-dried for 1 h at RT, sections were blocked with 10% normal serum blocking solution-species the same as the secondary antibody, containing 3% (w/v) BSA and 0.1% Triton-X100 and 0.05% Tween-20 2 h at RT to avoid unspecific staining. Then, the sections were incubated with the primary antibodies for anti-FBP1 (anti-mouse, 1:100; SantaCruz), and different markers such as S100 (Schwann cell marker, anti-rabbit, 1:100; Sigma), NF200 (neuronal marker, anti-rabbit, 1:100; Sigma). Sections were incubated with both the primary antibodies overnight at 4°C, followed by a mixture of the FITC- and TRITC-conjugated secondary antibodies for 2 h at 4°C. The stained sections were examined with Leica fluorescence microscope (Germany).

IN VITRO MIGRATION ASSAYS

Cells (1×10^6) were subcultured on the Transwell filter. After incubation for 24 h, cells on the upper chamber were fixed with 4% paraformaldehyde and stained with toluidine blue before being counted under an inverted microscope. In all experiments, data were collected from triplicate chambers.

ISOLATION, PURIFICATION, AND CULTURE OF SCs

Both sciatic nerves were removed from 3-day-old Sprague-Dawley rat pups, shredded using fine forceps, and dissociated by incubating at 37°C for 30 min, with occasional mixing, in 10 ml of phosphate-buffered saline (Sigma) containing 0.1% collagenase A and 0.25% trypsin (Sigma). The dissociated cells were washed twice by resuspending in 10 ml of DMEM-HG containing 10% FCS followed by centrifugation (5 min at 190*g*). Finally, the cells were resuspended in 5 ml of fresh Schwann growth medium without the addition of heregulin. The cells were plated onto a poly-L-lysine-coated, 6-cm culture dish and allowed to adhere for 1 day at 37°C. Non-Schwann cells were then eliminated as follows. For purification, the cells were treated with cytosine arabinoside (10 μM; Sigma) twice for 24 h and subjected to immunopanning with an antibody against THY1.1 (Sigma). We obtained an SCs culture of >95% purity by these procedures. For proliferation experiments, primary Schwann cells with 90% confluency were treated, respectively, with a dosage of 1 ng/ml) of soluble recombinant rat TNF-α(Peprotech, endotoxin level <1 EU/μg) for different time. For differentiation experiments, the purified cells were cultured in DMEM supplemented with 10% FBS for 3 days then in serum-free, defined media, which consisted of a 1:1 mixture of DMEM supplemented with 10% FBS and Ham's F-12 with N2 supplement (Invitrogen, Carlsbad, CA), for another 2 days before treatment with cyclic adenosine monophosphate (cAMP; 10 μM, Sigma).

ISOLATION, PURIFICATION, CULTURE OF DRGs, AND DRG/SC CO-CULTURES

The procedures were carried out similarly to previously described methods [Deng et al., 2012].

FBP1 siRNA VECTOR CONSTRUCTION AND TRANSFECTION

Double-stranded oligonucleotides corresponding to the target sequence for the human FBP1 (Genbank accession no. NM_003902.3) gene were cloned into the pSilencer 4.1-CMV siRNA plasmid (Invitrogen). According to the manufacturer's instructions, Schwann cells were transfected with the FBP1 siRNA plasmids using Lipofectamine 2000 (Invitrogen). Then, the cells were further stimulated for differentiation for 1–5 days with 10 μM cAMP.

QUANTIFICATION AND STATISTICS

All data were expressed as means ± SEM of at least three experiments. Data were compared using the Student's *t*-test. *P*-values less than 0.05 were considered statistical significantly.

RESULTS

THE EXPRESSION AND DISTRIBUTION OF FBP1 FOLLOWING SCIATIC NERVE INJURY

To determine the total level of FBP1 after sciatic nerve crush (SNC), tissues′ extracts from sciatic nerves were separated by SDS–PAGE and analyzed by Western blotting. FBP1 protein levels were relatively higher in the normal sciatic nerves, significantly decreased and reached a minimal level at Day 3, and then nearly returned to the normal level at 4 weeks (Fig. 1A). Meanwhile, FBP1 was thought to active the translation of p27 mRNA [Zheng and Miskimins, 2011]. In order to investigate whether p27 protein levels changed during the peripheral nervous system injury and regeneration. We detected the expression of p27 during the SNC and found that there was a same tendency to change as the FBP1 protein levels. p27 protein levels were high in normal sciatic nerves at the expected molecular size and decreased at a low level after injury (Fig. 1A). These data indicated that FBP1 undergoes substantial alter after rat SNC. To further determine the distribution of FBP1 in normal or injured nerve tissue following SNC, we performed immunohistochemistry. Remarkably, FBP1 was expressed in the nucleus of cells. Compared to the injured nerve tissue (Fig. 1B c, d), FBP1 was abundantly expressed in normal sciatic nerve tissue (Fig. 1B a, b). The number of FBP1-positive cells expressed in normal or injured nerve tissue was counted (Fig. 1B f) and these results were consistent with Western blot results. Besides, this phenomenon demonstrated that the function of FBP1 is associated with the cell nucleus.

THE CO-LOCALIZATION OF FBP1 WITH CELLULAR OR HISTOLOGIC MARKER AND ASSOCIATION WITH SCHWANN CELLS PROLIFERATION MARKER KI-67

It was known that FBP1 was expressed within nucleus, but the cell type in which FBP1 expressed was unknown. Based on the results of immunohistochemistry staining, we doubly labeled FBP1 with two specific markers, NF200 (neuronal marker) and S100 (SCs marker) to further explore the cell types of FBP1 during the sciatic nerves injury. In the normal group (Fig. 2A a–c, g–i) and the crushed nerves (Fig. 2A d–f, j–l) for 3 days after crush, we could observed colocation of FBP1 together with S100 and decreased double immunoreactivity staining of FBP1 and S100 compared to normal nerves (Fig. 2A g–l). While there were few colocalizations observed in the axons with the NF200 immunofluorescence staining (Fig. 2A a–f). To identify the proportion

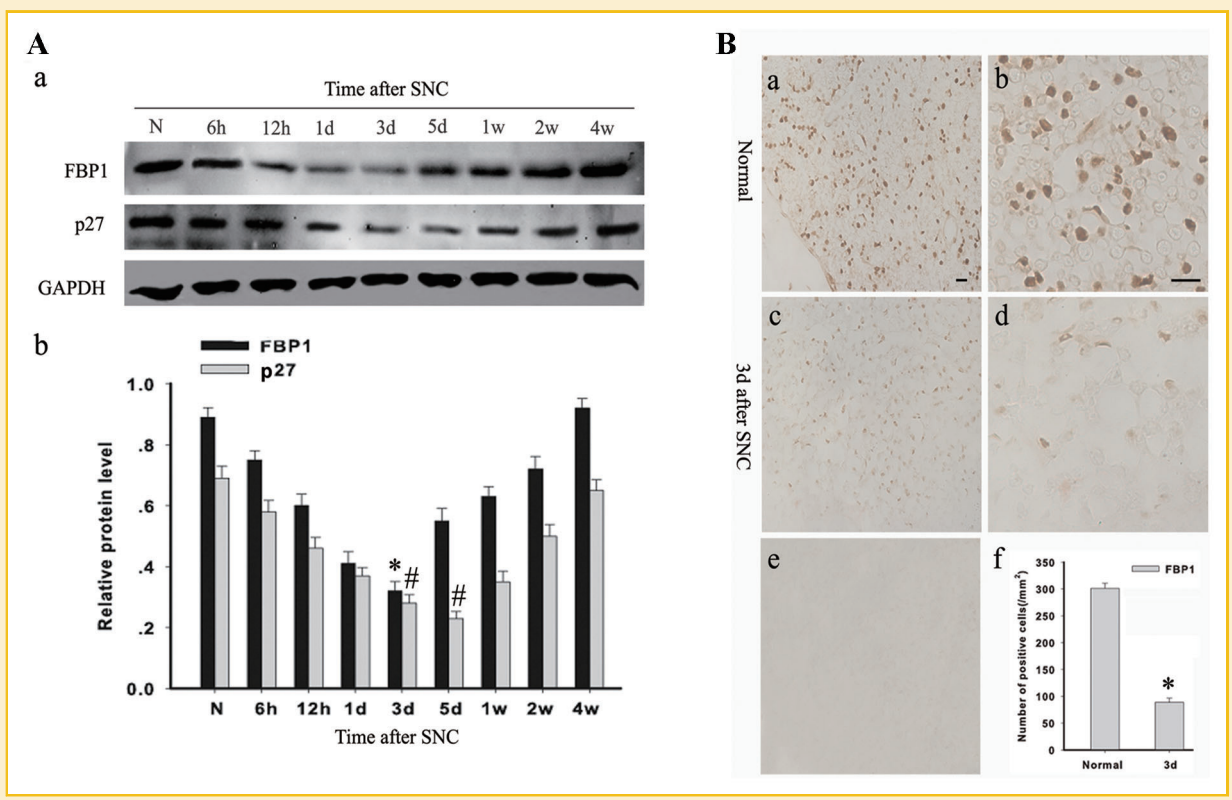

Fig. 1. The expression and distribution of FBP1 following sciatic nerve injury. A-a: Total protein were extracted from normal and injured sciatic nerves at various survival times after sciatic nerve injury, and then assessed by Western blot analysis. b: Bar chart represents the FBP1: GAPDH and p27: GAPDH intensity ratio. Results are the mean ± SEM of three independent sets of analyses. The asterisk (*, #) indicates significant difference at $P < 0.05$, compared with the normal group. B: Immunohistochemical expression and distribution of FBP1 in normal and injured nerve tissue. Low-magnification views of tissue cross-sections immunostained with an anti-FBP1 antibody in normal sciatic nerves (a) and 3 days after injury (c). Higher magnification views in normal sciatic nerve tissue (b) and 3 days after injury (d). e: Negative control of injured nerves in which the primary antibody to FBP1 was substituted by 1% normal goat serum. f: Quantitative analysis of FBP1-positive cells/mm^2 between the normal tissue and tissue 3 days after SNC. Asterisk $^*P < 0.05$ compared with normal tissue. The error bars represent the SEM. Scale bars 25 μm (a, c, e) and 40 μm (b, d).

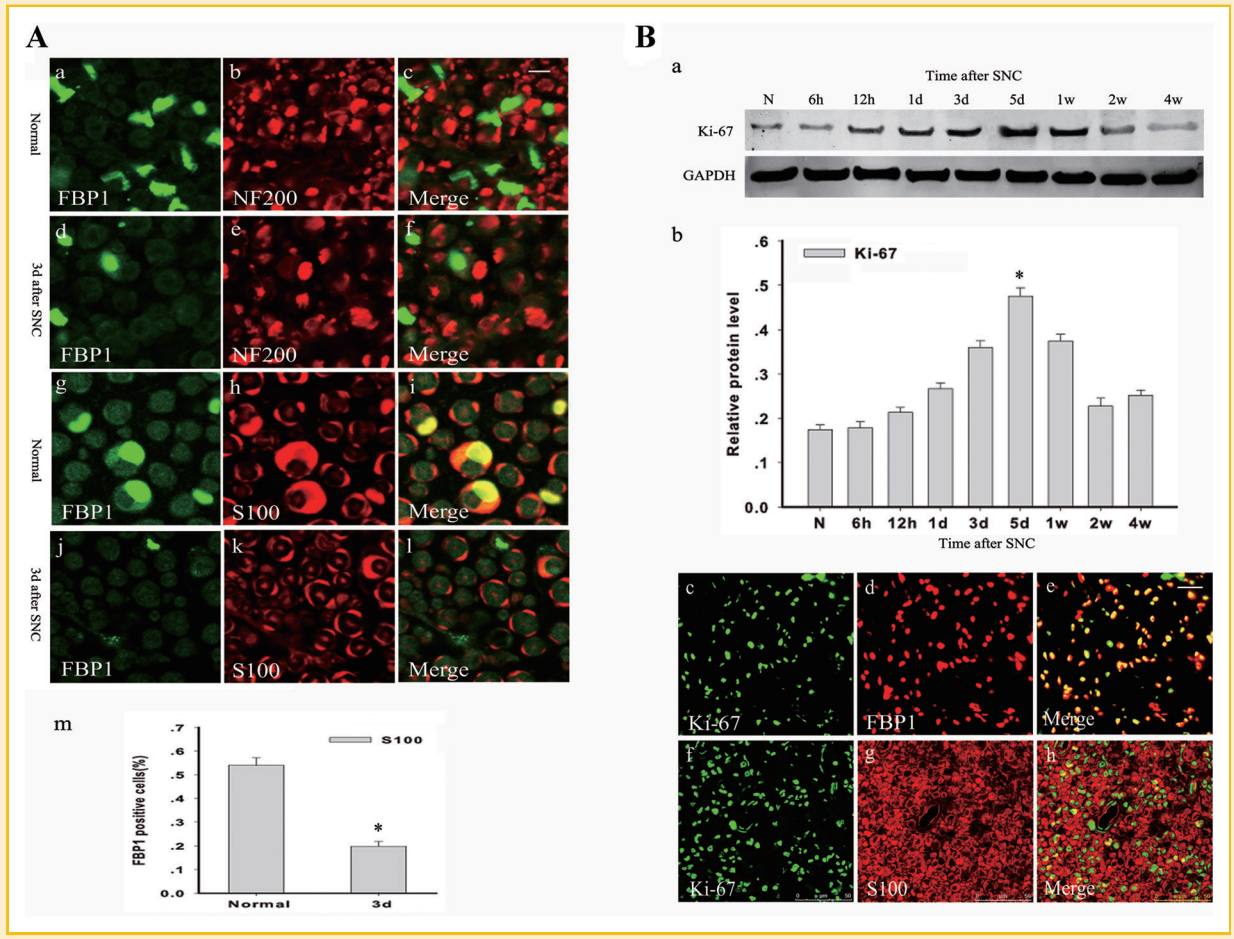

Fig. 2. The co-localization of FBP1 with cellular or histologic marker and association with Schwann cells proliferation marker Ki-67. A: Double-label IHC was performed on normal (a–c, g–i) and crushed (d–f, j–l) sections using antibodies specific for FBP1 (green, a, d, g, j), the Schwann cell marker S100 (red, h, k) and neurofilament-specific protein NF-200 (red, b, c). m: Quantitative analysis of S100 positive cells expressing FBP1 (%) in normal and crushed nerves. The asterisk "*" indicates significant difference at $P < 0.01$ compared with normal group. Error bars represent SEM. Scale bars 60 μm (a–l). B: Association of FBP1 with proliferation of SCs after SNC. a: Western blot showed that the expression of Ki-67 was increased after SNC and peaked at 5 days which was in parallel with the peak level of FBP1. b: Quantification graphs for Ki-67 ($P < 0.01$). Error bars represent SEM of the three independent experiments. Asterisk "*" indicates significant differences compared with the normal group by ANOVA followed by Tukey's post hoc analysis. c–h: Double immunofluorescence staining for FBP1 (red), Ki-67 (green), and S100 (red) in crushed sciatic nerves. Scale bar, 50 μm.

of S100-positive cells expressing FBP1, a minimum of 200 cells that were positive for S100 markers were counted between the normal group and 3 days after crush. FBP1 expression was significantly decreased in SCs 3 days after SNC ($P < 0.01$, Fig. 2A m). These data all showed typical nucleus-shaped immunoreactivity concentrations and FBP1 was associated with Schwann cells. But whether the function of FBP1 has a relationship with the precise one event including dedifferentiation, proliferation, and differentiation was not understood. In order to further investigate the possible function of FBP1 in the adult rat sciatic nerve after SNC, following studies were performed. Schwann cells proliferation has been reported as a significant part of the nerve injury [Verdu and Navarro, 1995; Shy et al., 1996]. FBP1 was thought to active the translation of p27 mRNA through its internal ribosomal entry site located in the 5′-untranslated region (5′-UTR) [Zheng and Miskimins, 2011]. p27 was thought to participate in promoting cell cycle arrest [Miyazawa et al., 2000; Carruthers et al., 2003]. From Figure 1A, we saw that p27 protein levels were low at 5 days after injury and then returned to the normal level. Thus, we wanted to know that when the proliferation of Schwann cells started. To get the message of Schwann cells proliferation after nerve injury, Western blot analysis was performed to determine the temporal changes of Ki-67 which has been used as proliferation marker. The expression of Ki-67 was lower in normal adult nerves, significantly increased from 3 days, reached the top at 5 days and kept a high level until about 1 week after SNC (Fig. 2B a, b). Interestingly, the protein expression of p27 was also at a low level at 5 days (Fig. 1A). Furthermore, we also performed double labeling of Ki-67, FBP1, and S100 at 5 days after injury and observed colocation between Ki-67 and FBP1 or S100 in injured nerve tissue (Fig. 2B c–h). Together, these findings suggested that the decrease of FBP1 after SNC might be associated with Schwann cells proliferation.

FBP1 LOCALIZATION IN SCHWANN CELLS AND ITS EXPRESSION PROFILE DURING SCs PROLIFERATION INDUCED BY TNF-α

In order to explore the function of FBP1 in the process of Schwann cell proliferation, we first performed double immunofluorescence

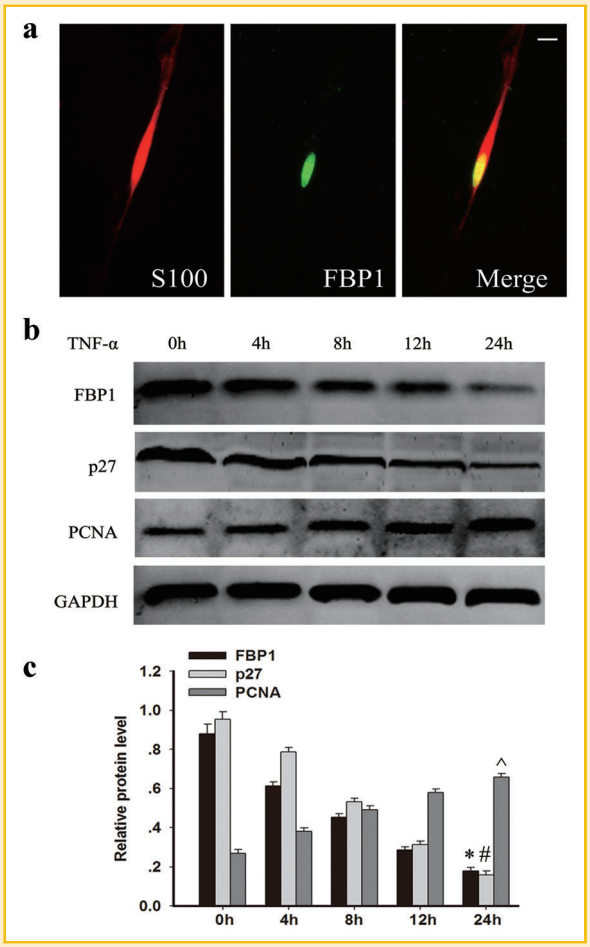

Fig. 3. FBP1 localization in Schwann cells and its expression profile during SCs proliferation induced by TNF-α. a: Immunofluorescent staining was performed with antibodies specific for FBP1 (green) and S100 (red). Scale bars 50 μm. b: Western blot analysis revealed the deregulation of FBP1 and p27 expression in Schwann cells and enhanced expression of PCNA after TNF-α treatment. c: The quantitative graphs demonstrated the ratio of FBP1, p27 and PCNA protein relative to GAPDH for each time point as measured using densitometry. These data are the means ± SEM (N = 3; the asterisk [*, #, ^] indicates a significant difference compared with the normal group, $P < 0.01$).

staining of cultural SCs in vitro and found FBP1 was located in the nucleus of Schwann cells (Fig. 3a). Following peripheral nerve injury, TNF-α can be released by Schwann cells as well as by macrophages [Wagner and Myers, 1996; Mulleman et al., 2006]. And in Hep3B cells, FBP1-knockdown also could induce mRNA levels of the death ligands TNFα [Rabenhorst et al., 2009]. When SCs were treated with TNF-α at a quite low dosage, SCs would proliferation significantly [Schafers et al., 2002; Pollheimer and Knofler, 2005; Wang et al., 2012]. Based on these, we stimulated SCs at a dosage of 1 ng/ml and collected cell extract to detect protein expression of FBP1 and PCNA. We found the increased expression of PCNA and decreased FBP1 and p27 protein levels (Fig. 3b, c). Together, all these provided a possibility that FBP1 might participate in Schwann cell proliferation after SNC through the regulation of p27. Besides, p27 was also shown to be involved in cell migration and differentiation. In our study, FBP1 and p27 returned to normal level gradually at 5 days after SNC which also suggested that FBP1 and p27 might have roles in SCs differentiation and we will further question it.

RECOVERED EXPRESSION OF FBP1 AND p27 WAS ASSOCIATED WITH SCHWANN CELLS DIFFERENTIATION

To get the message of Schwann cells differentiation after nerve injury, Western blot analysis was performed to determine the temporal changes of Oct-6 which has been used as promyelinating SC marker [Arroyo et al., 1998]. The expression of Oct-6 was lower in normal adult nerves, significantly increased from 3 days and kept a high level until about 2 weeks after SNC (Fig. 4A a, b). Furthermore, we performed double labeling of S100 with Oct-6 at 2 weeks after injury and observed a high level of colocation (Fig. 4A c–e). And we performed double labeling immunofluorescent staining of FBP1 together with Oct-6 (Fig. 4A f–k). We saw that the nucleus-expressed FBP1 almost coexisted with Oct-6-tagged Schwann cells. Together, these findings suggested that the recovered expression of FBP1 and p27 might be associated with Schwann cells differentiation. Further, we performed a cAMP-induced Schwann cell differentiation model system to explore the function of FBP1 during process of Schwann cell differentiation. At Day 3 after cAMP-induced Schwann cell differentiation, we saw that Schwann cells became polygonal, longer, fine, and tapering (Fig. 4B a). Besides, through Western blot we saw the increasing expression of FBP1 and p27 during the differentiation of Schwann cell induced by cAMP (Fig. 4B b, c). The increase of P0 expression also showed Schwann cell differentiation after the stimulation of cAMP (Fig. 4B b). Besides, immunofluorescent staining of p27 showed that p27 protein expression was transported from the nucleus to cytoplasm after cAMP stimulation for 3 days (Fig. 4B d–i). Thus, we conjectured that FBP1 may participated in the process of Schwann cell differentiation induced by cAMP through transporting p27 protein from the nucleus to cytoplasm.

EFFECTS OF FBP1-SPECIFIC siRNA ON CELL MOTILITY AND cAMP-INDUCED SCHWANN CELLS DIFFERENTIATION

To further confirm the role of FBP1 during Schwann cell differentiation processes, we knocked down FBP1 expression in SCs by the application FBP1-specific siRNA. First, we detected the siRNA effectiveness of FBP1 and analysis showed that siRNA-FBP1-3 significantly reduced the protein level of FBP1 compare with control in SCs (Fig. 5a). Besides, we used the specific antibody of p27 to measure p27 protein level and observed the decreased expression of p27 in FBP1-specific siRNA (siRNA-FBP1-3) cells (Fig. 5a). Through the immunofluorescent staining of transfected Schwann cell, we saw the morphological changes of Schwann cell after the stimulation of cAMP (Fig. 5b, c). In control, non-specific siRNA-transfected SCs started to change polygonal, longer and fine after 3 days treated with cAMP, but no morphology changes were observed in FBP1-specific siRNA-transfected SCs (Fig. 5b, c). Besides, we could also observe the unchanged P0 protein expression which showed Schwann cell differentiation in FBP1-specific siRNA-transfected SCs compared to the increased expression of P0 in non-specific siRNA-transfected SCs (Fig. 5d). Cell migration is a indispensable part in SCs differentiation. We observed the motility of SCs using transwell and wound-healing assay. We saw the decreased migration in FBP1-specific siRNA-

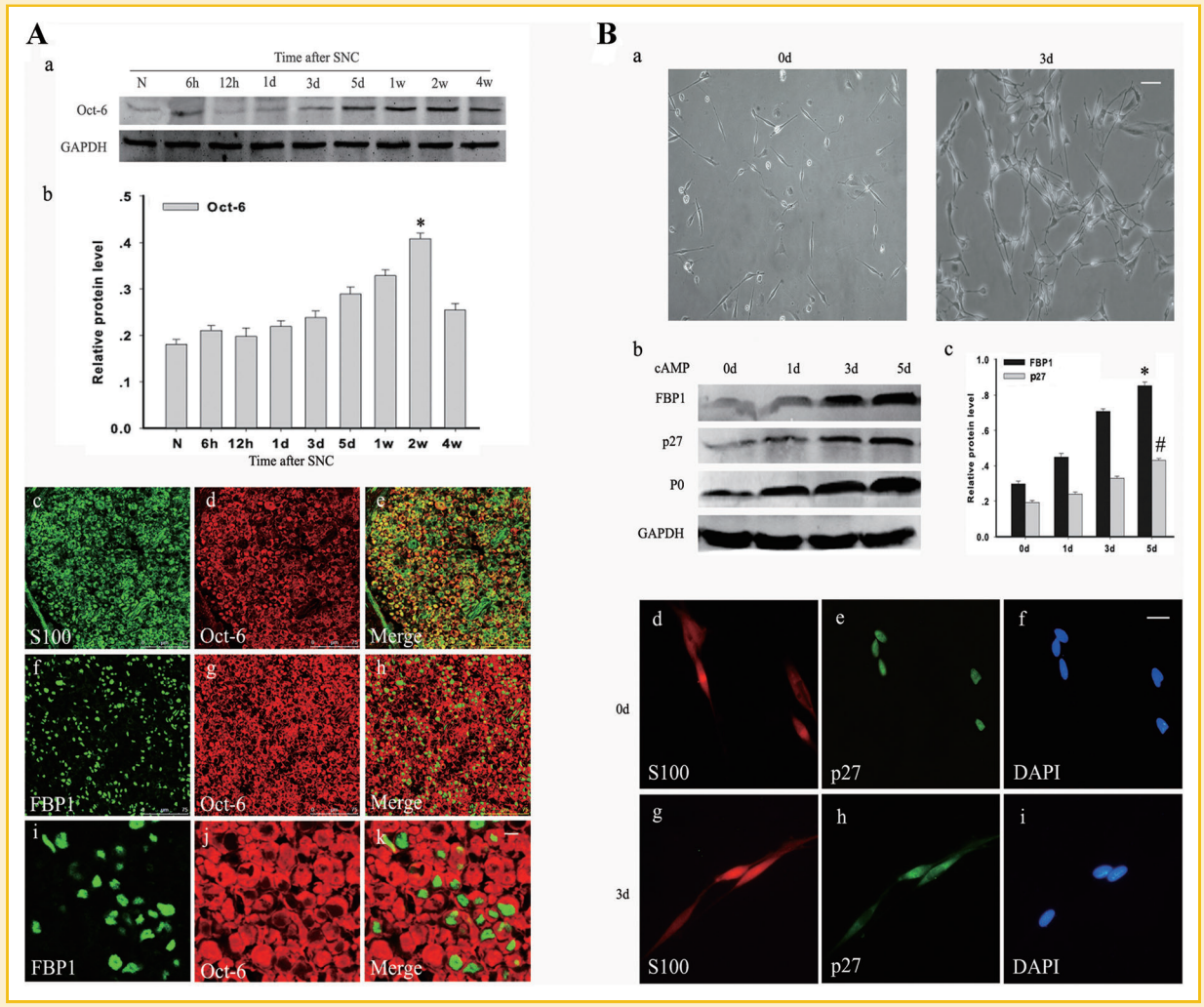

Fig. 4. Recovered expression of FBP1 and p27 was associated with Schwann cells differentiation. A–a: Western blot showed that the expression of Oct-6 was increased after SNC and peaked at 2 weeks. b: Quantification graphs for Oct-6 ($P < 0.01$). Error bars represent SEM of three independent experiments. Asterisk "*" indicates significant differences compared with the normal group by ANOVA followed by Tukey's post hoc analysis. c–k: Double immunofluorescence staining for FBP1 (green), S100 (red), and Oct-6 (red) in crushed sciatic nerves. Scale bars, 50 μm. Higher magnification views (i–k); Scale bars, 60 μm. B: FBP1 and p27 expression is upregulated in cAMP-induced SCs differentiation. a: Phase-contrast microscopy images showed that Schwann cells became polygonal, longer, fine, and tapering after treatment with 10 μM cAMP for 3 days. Scale bar, 50 μm. b: Western blot analysis revealed the upregulation of FBP1, p27, and P0 expression in Schwann cells after cAMP treatment. c: The quantitative graphs demonstrated the ratio of FBP1 and p27 protein relative to GAPDH for each time point as measured using densitometry. d–i: Immunofluorescent staining was performed with antibodies specific for p27 (green) and S100 (red) at 0 and 3 days after cAMP stimulation. These data are the means ± SEM (N = 3; the asterisk (*, #) indicates a significant difference compared with the normal group, $P < 0.01$).

transfected SCs when compared to normal and non-specific siRNA-transfected SCs (Fig. 5e, f). All together, these results demonstrated that FBP1 was involved in Schwann cell differentiation processes induced by cAMP and may be mediated by the regulation of p27 protein expression and transportation.

THE CYTOPLASMIC TRANSPORTATION OF p27 PROTEIN WAS INVOLVED IN SCHWANN CELLS DIFFERENTIATION IN THE SC/NEURON CO-CULTURES

To further illustrate the role of FBP1 and p27 during Schwann cells differentiation, DRG/SC co-cultures were performed, and total cellular protein was harvested for Western blot analyses. The expression of FBP1 and p27 increased sharply at 2 days and at the same time PCNA expression decreased acutely (Fig. 6a, b). At 5 days SCs differentiated completely and at 1 day, the early stage, it is also at the time that cells are preparing for and going to differentiation. To comprehend the meanings of cytoplasmic transportation of p27 protein and how p27 participate in SCs differentiation, nucleo/cytoplasmic separation at 1 and 5 days during SC/neuron co-cultures were performed. At 5 days, we observed that p27, CDK4 and CDK6 protein were apparently transportated from nucleus to cytoplasm compared to 1 day during SC/neuron co-cultures (Fig. 6c). We guessed that the forming of cytoplasmic CDK4/6-p27 contributed to SCs differentiation and we also confirmed it. Besides, we found the increased forming of CDK4/6-p27 at 5 days during co-cultures (Fig. 6d). Taken together, FBP1 might participate in SCs proliferation

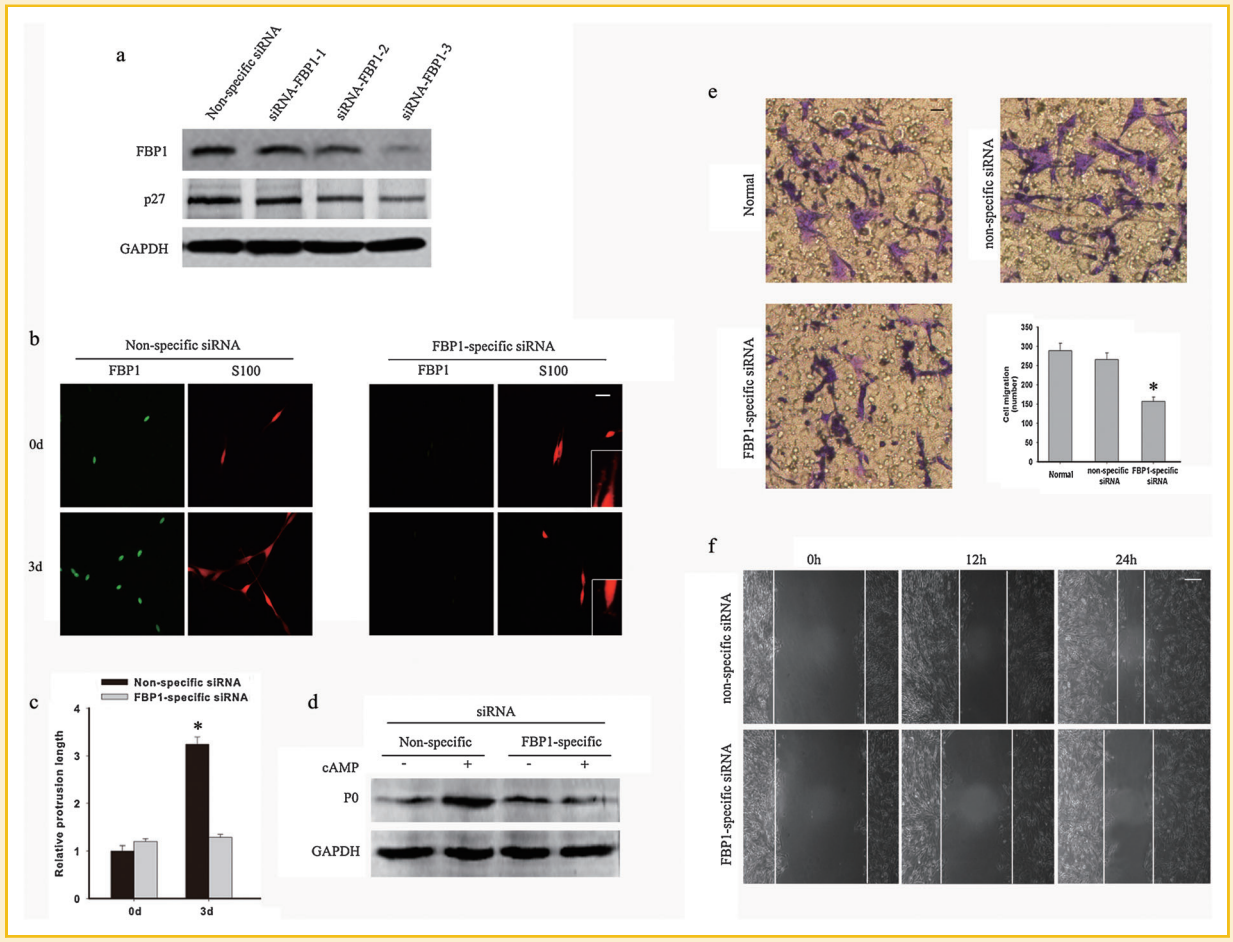

Fig. 5. Effects of FBP1-specific siRNA on cell motility and cAMP-induced SCs differentiation. a: Efficiency of FBP1-specific siRNA in Schwann cell. b: Suppression of FBP1 expression inhibited cAMP-induced Schwann cell morphological changes. c: Average protrusion lengths were quantified and data are mean values ± SEM. The asterisk indicates significant difference at $P < 0.05$ compared with control group. Scale bars, 50 μm. d: Expression changes of P0 after 3 days treated with cAMP in FBP1-specific siRNA-transfected Schwann cells. e: Tanswell assay of FBP1-specific siRNA-transfected SCs. These data are mean ± SEM (N = 3, * $P < 0.01$). f: Wound-healing assay. FBP1-specific siRNA-transfected SCs had a decreased migration.

and differentiation mediated by p27 protein expression regulation and cytoplasmic transportation.

DISCUSSION

Traumatic injury to the peripheral nerve is a worldwide problem [Robinson, 2000]. The injuries of peripheral nerve can contribute to major social and economic burdens for the reason that they generally happen in the most productive age group [Eser et al., 2009]. Thus, it is necessary and quite important to investigate the mechanism of nerve regeneration and repair after nerve injury. In our study, for the first time, we provided some evidence that the change of FBP1 protein expression after the crush of sciatic nerve. After crush, the FBP1 protein level significantly reduced, reached a minimum level at about 3 days, and then returned to the normal level at 4 weeks, and the expression of p27 was similar to FBP1. Through double immunofluorescence staining, we observed that FBP1 had a predominant colocation with S100, Schwann cell marker, while had few colocation with neuronal marker NF-200. Further, we observed FBP1 had a relationship with Ki-67 and Oct-6, which hinted that FBP1 might has an association with the proliferation and the following differentiation of Schwann cells.

Schwann cells are the principal glial cells of the peripheral nervous system. They are rapidly activated after peripheral nerve injury and differentiate into myelinating cells when facing the stimulation of a combination of signals. These signals induce Schwann cells to exit the cell cycle, reenter the cell cycle and then differentiate into myelinating cells [Akassoglou et al., 2002]. p27 is a member of Cip/Kip family of CDK inhibitors and plays a critical role in eukaryotic cell cycle regulation and fate specification and differentiation [Durand et al., 1997; Miyazawa et al., 2000; Sgambato et al., 2000; Zezula et al., 2001; Carruthers et al., 2003]. FBP1, a member of FBP family bound to the FUSE to modulate the c-myc mRNA levels [Avigan et al., 1990; Duncan et al., 1994, 1996; Davis-Smyth et al., 1996; He et al., 2000], was found to active p27 mRNA translation through its internal ribosomal entry site [Zheng and Miskimins, 2011]. During our study, we found that FBP1 and p27

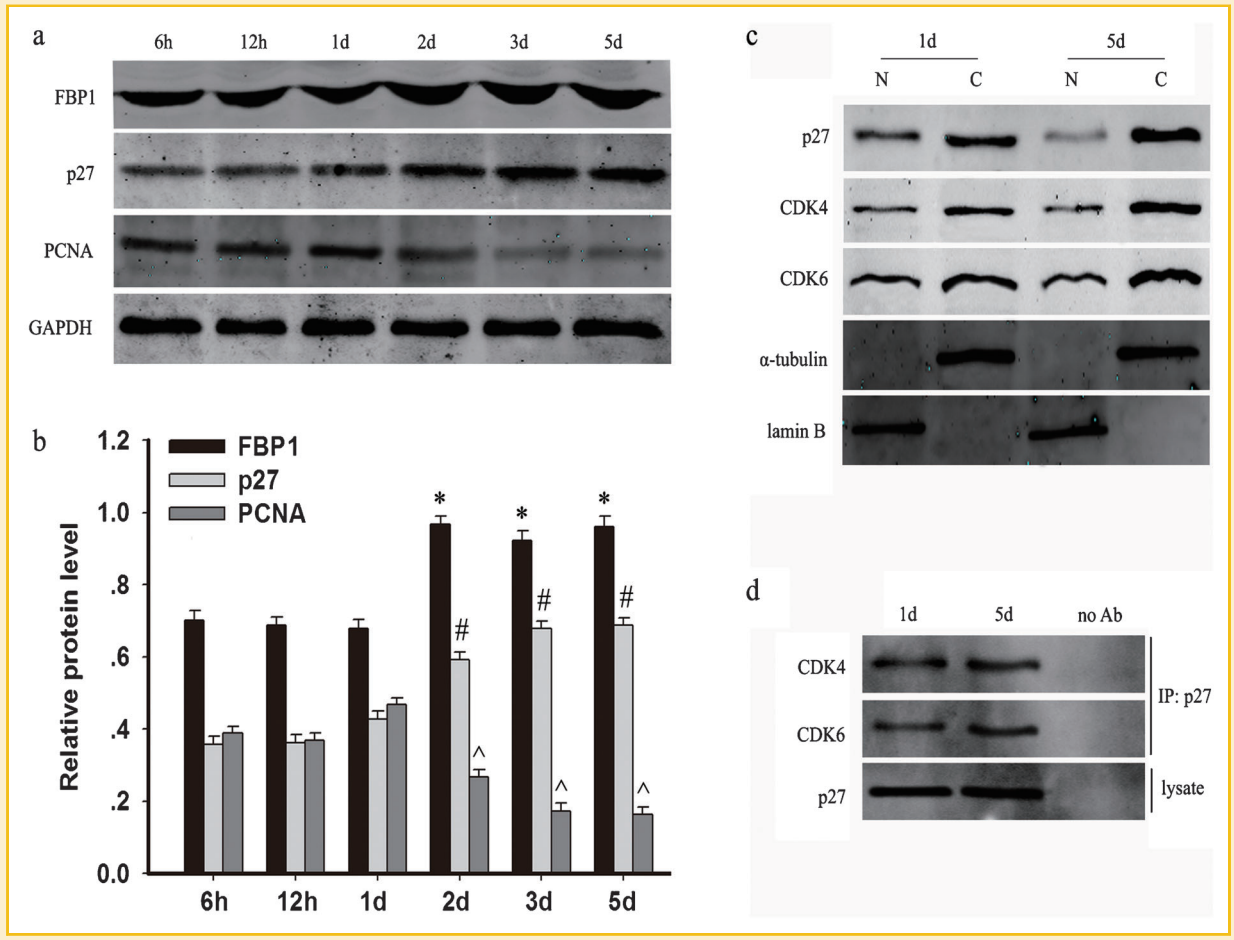

Fig. 6. The cytoplasmic transportation of p27 protein was involved in Schwann cells differentiation in the SC/neuron co-cultures. a: Western blot analysis revealed the deregulation of FBP1, PCNA, and p27 expression in SC/neuron co-cultures. b: The quantitative graphs demonstrated the ratio of FBP1, PCNA, and p27 protein relative to GAPDH for each time point as measured using densitometry. These data are the means ± SEM ($N = 3$; the asterisk (*, #) indicates a significant difference compared with the normal group, $P < 0.01$). c: Nucleo/cytoplasmic separation at 1 and 5 days during SC/neuron co-cultures. At 5 days, we observed that p27, CDK4, and CDK6 protein were apparently transportated from nucleus to cytoplasm compared to 1 day during SC/neuron co-cultures. d: Co-immunoprecipitation at 1 and 5 days during SC/neuron co-cultures. Enhanced interaction of CDK4 and CDK6 with p27 was observed at 5 days compared to one day during SC/neuron co-cultures.

protein expression level decreased during the process of TNF-α-induced SCs proliferation. All data indicated that FBP1 might participate in SCs proliferation mediated by regulation of p27 protein level. Besides, in Hep3B cells, FBP1-knockdown also could induce mRNA levels of the death ligands TNFα [Rabenhorst et al., 2009]. And following peripheral nerve injury, TNF-α can be released by Schwann cells as well as by macrophages [Wagner and Myers, 1996; Mulleman et al., 2006]. When SCs were treated with TNF-α at a low dosage, SCs would proliferation significantly [Schafers et al., 2002; Pollheimer and Knofler, 2005; Wang et al., 2012]. It was also the reason why we chose TNF-α to stimulate SCs proliferation. We also constructed forskolin (2 μM)-induced SCs proliferation model and observed the same expression profile of FBP1 and p27 (data not shown), which further raise the possibility that FBP1 might be involved in SCs proliferation. Of course, we also certified the conjecture that FBP1 and p27 also participate in SCs differentiation. During the process of cAMP-induced SCs differentiation, we observed intense expression of FBP1 and p27 and that p27 was transported to cytoplasm at Day 3. Besides, depletion of FBP1 inhibited Schwann cell morphological change induced by cAMP and cell migration. Taken together, we conjectured that the low expression of FBP1 and p27 made a possibility for SCs proliferation and their recovery laid a foundation for SCs differentiation mediated by p27 cytoplasmic transportion. To further ascertain the model of FBP1 and p27 protein expression, we made SCs/DRG neuron co-cultures and found the definite increase of FBP1 and p27. Through the analysis of nucleo/cytoplasmic separation of cells at Day 1 and 5, we not only found apparent p27 cytoplasmic transportion but also CDK4 and CDK6, which provide a presumption that p27 might coupled with CDK4 and/or CDK6 to induce SCs differentiation and we also proved it using co-immunoprecipitation. Cytoplasmic accumulation of CDK4/6 had a correlation with differentiation [Kohoutek et al., 2004; Kozar and Sicinski, 2005; Bryja et al., 2008]. In summary, we found that FBP1 and p27 were involved in SCs proliferation and the following differentiation after SNC by transporting p27 from nucleus to cytoplasm allowing for the conjunction of CDK4/6-p27.

Besides, FBP1 was identified as a regulatory mRNA-binding protein that could repress the translation of nucleophosmin (NPM/B23), a multifunctional oncoprotein whose protein expression levels dictate cellular growth and proliferation rates [Olanich et al., 2011]. FBP1 also was thought to interact with Untranslated Regions (UTRs) of JEV genome and involve in viral translation and eventually negatively regulated the replication of virus [Chien et al., 2011]. In HCC, FBP1 could reduce the expression of p21 to accelerate the cell cycle, inhibit the extrinsic apoptosis pathway, enhance the ability of tumor growth [Cao et al., 1997; Yamanaka et al., 2000; Sheikh and Huang, 2004; Ohira et al., 2006; Sturm et al., 2006; Rabenhorst et al., 2009; Atanassov and Dent, 2011]. Typically, FBP1 could serve as a regulatory machinery to regulate c-Myc expression required for cell cycle entry [Chung et al., 2006; Liu et al., 2006]. But the role of FBP1 and p27 in peripheral nerves injury and repair was little known. Here, for the first time, we found that the change expression of FBP1 during sciatic nerve injury and the some association of FBP1 with Schwann cells proliferation and differentiation. We also guessed that FBP1 could enhance p27 protein expression and the cytoplasmic transportation, which made it possible for the increasing cytoplasmic CDK4/6-p27 to promote SCs differentiation. However, the most important thing was that our data provided some insights into the general mechanism underlying peripheral nerves regeneration after injury. Of course, our study only investigated the primary correlation between FBP1 and peripheral nerves injury, further study have been planned to elucidate the other and concrete mechanism of FBP1 during peripheral nerves injury and regeneration.

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