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FOXO3a/p27^{kip1} Expression and Essential Role After Acute Spinal Cord Injury in Adult Rat

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ABSTRACT

FOXO3a (Forkhead Class box O3a), as an important direct target of the phosphatidylinositol 3-kinase (PI3K)/protein B (Akt) pathway, which regulates the cell survival and the cell-cycle progression. Recent reports showed that FOXO3a could inhibit cell-cycle progression at the G1/S transition by controlling transcription of the cyclin-dependent kinase inhibitor p27^{kip1}, which is also a key regulator of the mammalian neurogenesis. To elucidate the expression and role of FOXO3a in nervous system lesion and repair, we performed an acute spinal cord contusion injury (SCI) model in adult rats, which showed a temporal–spatial expression pattern of FOXO3a. Temporally, FOXO3a protein level significantly reduced Day 3 after injury, and following FOXO3a down-regulation, p27^{kip1} protein and mRNA levels were also decreased after injury. Spatially, decreased levels of FOXO3a and p27^{kip1} were predominant in astrocytes, which were regenerating axons and largely proliferated after injury. Furthermore in vitro, Western blot analysis, RT-PCR, and immunofluorescence staining analysis demonstrated the relationship between FOXO3a and p27^{kip1} in primary astrocytes. FOXO3a modulated the cell cycle by transcriptional regulation of p27^{kip1} in astrocytes. Administration of the PI3K pharmacological inhibitor LY294002 abrogated this effect by regulating FOXO3a and p27^{kip1} expression and subcellular localization. These results suggest that decreased levels of FOXO3a and p27^{kip1} in spinal cord are involved in axonal regeneration and the proliferation of glial cells after SCI. J. Cell. Biochem. 114: 354–365, 2013.

KEY WORDS: SPINAL CORD INJURY (SCI); PROLIFERATION; GLIAL CELLS; FOXO3a; p27^{kip1}

Traumatic spinal cord injury (SCI) involves primary and secondary injury mechanisms [Dumont et al., 2001]. Primary injury is due to direct tissue detritions, which are the result of external mechanical forces. Secondary injury follows the initial impact, which may result from spinal cord edema, ischemia, free radical damage, electrolyte imbalance, excitotoxicity, inflammatory injury, proliferation, or apoptosis [McDonald and Sadowsky, 2002; Kwon et al., 2004]. These factors cause microglia activation and astrocyte proliferation, resulting in the formation of a dense astrocytic scar [McGraw et al., 2001]. This glial scar provides a physical and biochemical barrier to regeneration and plasticity, and it is a source of multiple inhibitory factors that affect functional recovery from SCI [Davies et al., 1996; Silver and Miller, 2004]. During the repair process, cell cycle regulation may be indispens-

Abbreviations: SCI, spinal cord injury; FOXO, forkhead box class O; FOXO3a, forkhead class box O3a; CNS, central nervous system; PI3-K, phosphatidylinositol 3-kinase; PAGE, polyacrylamide gel electrophoresis; NeuN, neuronal nuclei; GFAP, glial fibrillary acidic protein; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BSA, bovine serum albumin; ECL, enhanced chemiluminescence system; RT-PCR, reverse transcriptase PCR.

Shuangwei Zhang and Weipeng Huan contributed equally to this work.

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able. Cell cycle inhibition has been shown to reduce glial proliferation and scar formation after traumatic injury [Di Giovanni et al., 2005]. Additionally, cell cycle activation has been demonstrated to contribute to post-mitotic cell death and glial cell activation and proliferation after SCI [Becker and Bonni, 2004; Byrnes et al., 2007].

$p27^{kip1}$ is a key regulator of the cell cycle in mammalian cells. It negatively regulates cell cycle progression by directly inhibiting the cyclinE/Cdk2 complexes in mammalian cells [Fillies et al., 2007]. $p27^{kip1}$ has been implicated in promoting cell cycle arrest of neural progenitors during embryogenesis [Miyazawa et al., 2000; Carruthers et al., 2003], in regulating the division of transit amplifying progenitors in the adult subventricular zone [Doetsch et al., 2002]. These data confirmed the effect of $p27^{kip1}$ on central nervous system (CNS) development, and we recently reported its expression in SCI [Shen et al., 2008]. The activity of $p27^{kip1}$ is controlled by its concentration, distribution among different cellular complexes, and its cellular location [Burgering and Kops, 2002; Shen et al., 2008].

The FOXO transcription factors play an evolutionarily conserved role in the control of metabolism, proliferation, survival, stress resistance, and longevity [Burgering and Kops, 2002; Tran et al., 2003; Accili and Arden, 2004]. Expression of FOXO proteins can induce cell-cycle arrest in many cell types. Forkhead Class box O3a (FOXO3a previously termed FKHRL1), is a member of forkhead transcription factor of the forkhead box, class O (FOXO) subfamily, which is a target of phosphatidylinositol 3-kinase (PI3-K) signaling. When PI3K and Akt are active, FOXO3a is directly phosphorylated by Akt [Burgering and Kops, 2002; Van Der Heide et al., 2004]. By binding to the nuclear importer, active FOXO3a translocates to the nucleus, binds to DNA, and promotes the transcription of its target genes, for example, $p27^{kip1}$ and Bim [Burgering and Kops, 2002]. However, activated Akt regulates the transcription of FOXO3a target genes by modulation of FOXO3a activity by phosphorylating its three conserved serine/threonine residues (Thr-32, Ser-253, and Ser-315), leading to FOXO3a release from DNA and translocate to the cytoplasm, which reduces transcription levels of $p27^{kip1}$ [Brunet et al., 1999; Burgering and Kops, 2002]. FOXO3a controls cell cycle, cell death, and oxidative stress response through trans-activating different sets of genes [Tran et al., 2003]. Recent data showed FOXO3a directly regulated the transcription of the $p27^{kip1}$ gene [Dijkers et al., 2000; Medema et al., 2000; Stahl et al., 2002], suggesting that a proliferative stimulus that results in a decrease in $p27^{kip1}$ levels may be associated with the inhibition of FOXO-induced transcription. In addition, ectopic expression of activated FOXO3a led to cell cycle arrest in which induction of $p27^{kip1}$ through FOXO3a appeared to play an important role [Medema et al., 2000]. Injury to spinal cord induced a series of changes, including neuronal cells apoptosis as well as glial cells proliferation. During the repair process, cell cycle regulation may be indispensable. However, the status of FOXO3a expression in SCI, including its possible clinical significance and its correlation with $p27^{kip1}$ in astrocyte proliferation after SCI has not been examined.

In this study, the changes of temporal–spatial expression of FOXO3a and $p27^{kip1}$ after SCI were assessed in rat spinal cord contusion model in vivo and primary astrocyte proliferation model in vitro. Furthermore, we studied the effect of PI3K inhibitor LY294002 on the astrocyte proliferation in vitro to explore the potential value of FOXO3a. The sub-cellular localization of FOXO3a changed and thereby prevented its nuclear translocation during SCI. Our specific aim is to achieve better insight into the physiologic function of FOXO3a and molecular mechanisms underlying CNS lesion and repair.

MATERIALS AND METHODS

ANIMALS AND SURGERY

Male Sprague–Dawley rats (n = 56) with an average body weight of 250 g (220–275 g) were used in this study. Rats were deeply anesthetized with chloral hydrate (10% solution) and surgery was performed under aseptic conditions. Dorsal laminectomies at the level of the ninth thoracic vertebra (T9) were carried out under anesthesia with pentobarbital (50 mg/kg i.p.). Contusion injuries (n = 48) were performed using the NYU impactor [Gruner, 1992]. The exposed spinal cord was contused by dropping a rod 2.0 mm in diameter and 10 g in weight from a height of 10 cm. Sham-operated animals (n = 8) were anesthetized and surgically prepared but did not receive spinal injury. After SCI, the overlying muscles and skin were closed in layers with 4-0 silk sutures and staples, respectively, and the animals were allowed to recover on a 30°C heating pad. Postoperative treatments included saline (2.0 cc, s.c.) for rehydration and Baytril (0.3 cc, 22.7 mg/ml, s.c., twice daily) to prevent urinary tract infection. Bladders were manually expressed twice daily until reflex bladder emptying returned. Animals were sacrificed at 6 h, 12 h, 1 day, 3 days, 5 days, 7 days, and 14 days after injury. Eight naïve animals were used as non-injured controls. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals [National Research Council, 1996, USA] and 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

To obtain samples for Western blot analysis, the naïve or injured spinal cords were excised. The portion of spinal cord extending 5 mm rostral and 5 mm caudal to the injury epicenter was dissected out and immediately frozen at −70°C until use. To prepare lysates, frozen spinal cord samples were minced with eye scissors in ice. 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 X-100, 1 mmol/L PMSF, 10 mg/ml aprotinin, and 1 mg/ml leupeptin) and clarified by centrifuging for 20 min in a microcentrifuge at 4°C. After determination of its protein concentration with the Bradford assay (Bio-Rad), the resulting supernatant (50 μg of protein) was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidene difluoridemembrane (Millipore) by a transfer apparatus at 350 mA for 2.5 h. The membrane was then blocked with 5% nonfat milk and incubated with primary antibodies against $p27^{kip1}$ (rabbit, 1:500; Santa Cruz), FOXO3a (rabbit, 1:500; Cell Signaling), CDK2, cyclin E (rabbit, 1:1,000; Santa Cruz), PCNA (mouse, 1:1,000; Santa Cruz), GAPDH (mouse, 1:1,000;

Sigma). After incubating with the appropriate horseradish peroxidase-conjugated secondary antibody, protein was visualized using an enhanced chemiluminescence system (ECL; Pierce Company).

IMMUNOHISTOCHEMISTRY

After defined survival times, naïve and injured rats were terminally anesthetized and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. After perfusion, the naïve and injured spinal cords were removed and post-fixed in the same fixative solution for 3 h and cryoprotected with 20% sucrose for 2–3 days, followed by 30% sucrose for 2–3 days. After treatment with sucrose solutions, the tissues were embedded in O.C.T compound. Then 8-μm frozen cross-sections at two spinal cord levels (2 mm rostral and caudal to the epicenter of injury) were prepared and examined. All of the sections were blocked with 10% goat serum with 0.3% Triton X-100 and 1% (w/v) bovine serum albumin (BSA) for 2 h at room temperature (RT) and incubated overnight at 4°C with an anti-FOXO3a antibody (anti-rabbit, 1:500; Cell Signaling) followed by incubation in biotinylated secondary antibody (Vector Laboratories, Burlin-game, CA). Staining was visualized with DAB (Vector Laboratories). Cells with strong or moderate brown staining were counted as positive, cells with no staining were counted as negative, and cells with weak staining were scored separately.

DOUBLE IMMUNOFLUORESCENT STAINING

Slide-mounted sections were removed from the freezer and kept in an oven at 37°C for 30 min. Sections were blocked with 10% normal serum blocking solution, using normal serum from the same species as the secondary antibody, containing 3% BSA, 0.1% Triton X-100, and 0.05% Tween-20 for 2 h at RT to prevent nonspecific staining. Then the sections were incubated with anti-FOXO3a (anti-rabbit, 1:500; Cell Signaling), anti-PCNA (anti-rabbit, 1:100; Merck), or anti-PCNA (anti-mouse, 1:100; Santa Cruz) antibodies. The cell-specific markers NeuN (neuronal marker, 1:600; Chemicon) and GFAP (astrocytic marker, 1:200; Sigma) were applied simultaneously. Sections were incubated with both primary antibodies overnight at 4°C, followed by a mixture of CY2- and CY3-conjugated secondary antibodies for 2 h at 4°C. The stained sections were examined with a Leica fluorescence microscope (Germany).

QUANTITATIVE ANALYSIS

The numbers of FOXO3a-positive cells in the spinal cord 2 mm rostral to the epicenter were counted in a 500 μm × 500 μm measuring frame. For each animal, a measure was taken in a section through the dorsal horn, the lateral funiculus and the ventral horn. Cells double labeled for FOXO3a, $p27^{kip1}$, and the other phenotypic markers (NeuN, GFAP, and PCNA) used in the experiment were quantified. To avoid counting the same cell in more than one section, we counted every fifth section (50 μm apart). The cell counts were then used to determine the total number of FOXO3a, $p27^{kip1}$-positive cells per square millimeter. GFAP-positive cells were counted in the gray and white matter, whereas NeuN-positive cells were counted only in the gray matter. To identify the proportion of neurons and astrocytes expressing FOXO3a, $p27^{kip1}$ a minimum of 200 cells expressing a cell-specific marker were counted.

ASTROCYTE CULTURES

Astrocyte cultures were prepared from spinal cords of adult male Sprague–Dawley rats (P65–P70, weighing 220–275 g) using a previously described method with some modifications [Tawfik et al., 2006; Codeluppi et al., 2009]. The spinal cords were ejected from the vertebral column using a saline-filled syringe. The tissue was chemically dissociated with 0.25% trypsin–EDTA for 10 min followed by mechanical trituration in Dulbecco's modified essential medium (DMEM). After centrifugation at 1,200 rpm for 5 min, the cells were suspended in DMEM with nutrient mixture F12 (1:1 v/v; Gibco, Grand Island, NY), containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, and plated in a flask coated with poly-L-lysine (Sigma). The cultures were maintained in a atmosphere of 95% O_2/5% CO_2 at 37°C for 10 days, with changes of the culture medium at Days 4 and 7. Approximately on Days 10 and 11, oligoden-drocytes andmicroglial cells growing on top of the confluent astrocyte layer were removed by shaking at 200 rpm for 2 h at 37°C and replacing the culture medium. The next day, the cells were trypsinized and replanted in six-well plates (40,000 cells per well). Prior to experimental treatments, cultures of astrocytes were passaged twice. Cell culture medium was switched to serum-free DMEM/F12 culture medium.

CELL PROLIFERATION STUDIES AND CELL CYCLE S PHASE PROGRESSION ANALYSIS

Astrocyte cells were synchronized at the G0/G1 phase by serum deprivation 48 h. Upon serum addition, cells were released from G1 to S phase. For cell growth measurement, Western blot analysis was performed to examine the expression profile of the widely used marker of dividing cells, PCNA (anti-mouse, 1:1,000; Santa Cruz) and the the cell cycle markers of S phase progression, cyclin A (anti-rabbit, 1:1,000; Santa Cruz).

CELL FRACTIONATION, IMMUNOBLOT ANALYSIS

Cell pellets from a culture were incubated in a hypotonic buffer (10 mM HEPES pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 20 mM NaF, 100 μM Na_3VO_4, and protease inhibitor mixture) for 30 min at 4°C on a rocking platform. Cells were homogenized (Dounce, 30 strokes), and their nuclei were pelleted by centrifugation (10 min × 14,000 rcf, 4°C). The supernatant was saved as the cytosolic fraction, and nuclear pellets were incubated in nuclear lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) for 1 h at 4°C on a rocking platform. The nuclear fraction was collected by centrifugation (10 min × 14,000 rcf, 4°C). Prior to immunoblotting, cells were washed twice with ice-cold PBS, resuspensed in 2× lysis buffer (50 mM Tris–HCl, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na_3VO_4, and protease inhibitor mixture), and incubated for 20 min at 4°C while rocking. Lysates were cleared by centrifugation (10 min × 12,000 rpm, 4°C) and 50 μg total proteins was resolved by SDS–PAGE and transferred on to a poly (vinylidene difluoride) membrane filter (Immbilon; Millipore). The membranes were firstly blocked and then incubated with the primary antibody described above for 2 h at RT. After washing three times, filters were incubated with horseradish peroxidase-conjugated human anti-mouse or anti-rabbit antibodies

(Pierce) for 1 h at RT. Immuno-complexes were detected with an ECL (NEN Life Science Products, Boston, MA).

RNA ISOLATION AND REVERSE TRANSCRIPTASE PCR (RT-PCR) ANALYSIS

Total RNA of astrocyte cells were extracted using a trizol extraction kit according to the manufacturer's procedure. Total RNA was reverse-transcribed using the RT-PCR system (Invitrogen). Primer pairs for $p27^{kip1}$ were sense, 5′-CAGAATCACAAACCCCTA-3′ and antisense, 5′-TGTTTTGAGTAGAAGAAT-3′. Cycling conditions were: 94°C for 45 s, 47.5°C for 45 s, 72°C for 30 s, and a total of 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control and was detected using the primers sense, 5′-TGATGACATCAAGAAGGTGGTGAA G-3′ and antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. Cycling conditions were: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a total of 28 cycles. After amplification, the products were separated on an agarose (1.5%) gel (cast in the presence of ethidium bromide) and visualized under UV light.

STATISTICAL ANALYSIS

All data were analyzed with Stata 7.0 statistical software. All values were expressed as the mean ± SEM. One-way ANOVA followed by the Turkey's post hoc multiple comparison tests was used for statistical analysis. *P*-values <0.05 were considered statistically significant. Each experiment consisted of at least three replicates per condition.

RESULTS

THE EXPRESSION PROFILES OF FOXO3a/$p27^{kip1}$ AND ASSOCIATED CELL CYCLE GENES FOLLOWING SCI BY WESTERN BLOT ANALYSIS

Western blot analysis was performed to investigate the temporal patterns of FOXO3a and $p27^{kip1}$ expression after SCI (Fig. 1A). FOXO3a protein levels were high in naïve group, gradually reduced at Day 1 after injury and reached a valley at Day 3 ($P < 0.05$). After 14 days, FOXO3a expression gradually returned to the level of naïve spinal cords. At the same time, the expression of $p27^{kip1}$ was also high in naïve group, reduced at Day 1 after injury and reached a valley at day 3. Therefore, SCI induced a decrease of FOXO3a and $p27^{kip1}$ expressions. $p27^{kip1}$ is a key regulator of the cell cycle in mammalian cells. It negatively regulates cell cycle progression by directly inhibiting the cyclin E/CDK2 complexes in mammalian cells [Fillies et al., 2007]. The temporal patterns of cyclin E and CDK2 expression were performed by Western blot (Fig. 1B). Cyclin E and CDK2 expression were all obviously up-regulated which negatively correlated with FOXO3a and $p27^{kip1}$ expression at days 3–7 after SCI.

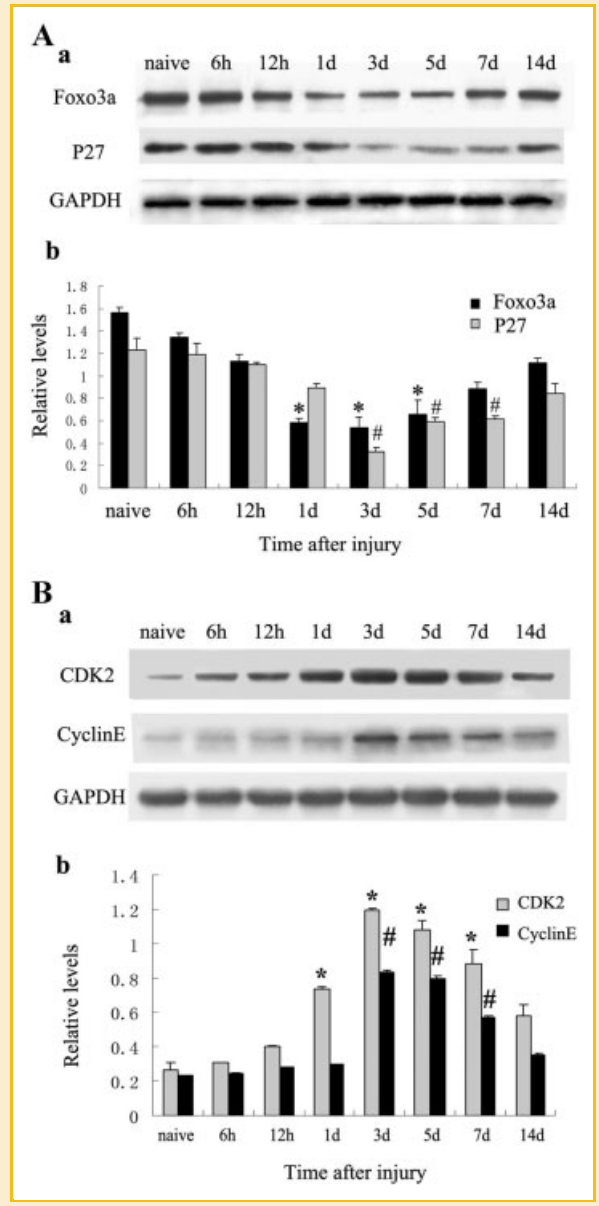

Fig. 1. Western Blot analysis the expression profiles of FOXO3a/$p27^{kip1}$ and their associated cell cycle genes following SCI. Spinal cord tissues from rats at various survival times after SCI were homogenized and subjected to immunoblot analysis. Samples immunoblots probed for FOXO3a, $p27^{kip1}$, and GAPDH are shown (A-a). The bar chart below demonstrates the ratio of FOXO3a, $p27^{kip1}$ to GAPDH for each time point (A-b). Samples immunoblots probed for CDK2, cyclinE and GAPDH are shown (B-a). The bar chart below demonstrates the ratio of CDK2, cyclin E to GAPDH for each time point (B-b). Cyclin E and Cdk2 expression were negatively correlated with FOXO3a and $p27^{kip1}$ expression following SCI. The data are means ± SEM (n = 3, #,*$P < 0.05$, significantly different from the naïve group).

THE DISTRIBUTION OF FOXO3a AND $p27^{kip1}$ IN THE SPINAL CORD

To identify the distribution of FOXO3a and $p27^{kip1}$ after SCI, we performed immunohistochemistry with anti-FOXO3a and anti-$p27^{kip1}$ rabbit monoclonal antibodies. As Western blot analysis shown, FOXO3a and $p27^{kip1}$ all have the lowest protein expression at Day 3 after SCI, so we chose Day 3 as the time point of immunohistochemistry. In the rostral spinal cord 2 mm from the epicenter, FOXO3a and $p27^{kip1}$ were extensively expressed in both the ventral horn and white matter of spinal cord whether the animals were naïve or injured (Fig. 2A-a and b). Notably, in naïve group FOXO3a was primarily expressed in the nucleus (Fig. 2A-c and e). However, after injury, FOXO3a was transported out of the nucleus and largely sequestered in the cytoplasm (Fig. 2A-d and f).

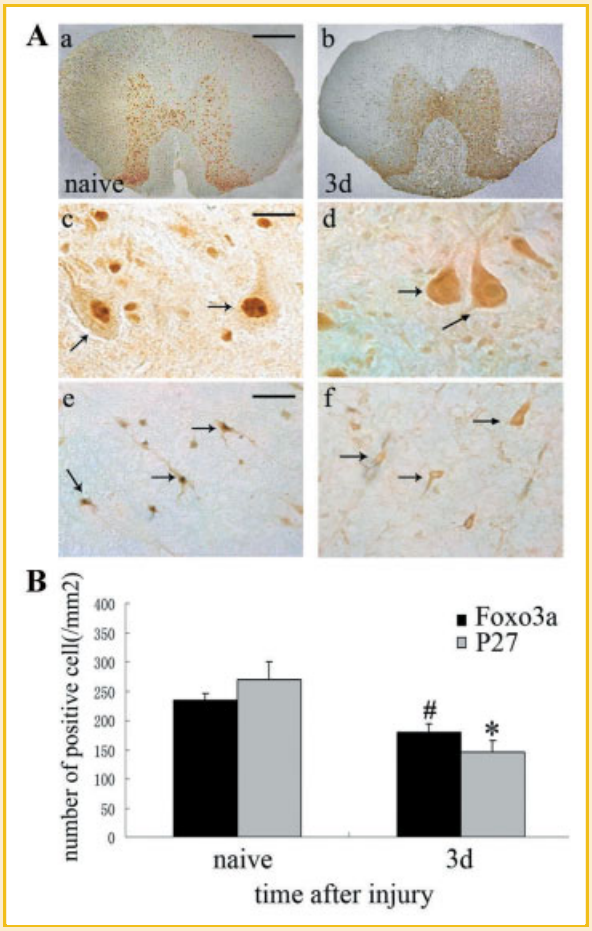

Fig. 2. Immunohistochemical analysis the distribution of FOXO3a in the adult rat spinal cord. A: Low-power views of cross-sections immunostained with antibody specific for FOXO3a in naïve spinal cord (a) and day 3 after injury (b). Higher-power views in the ventral horn (c) and white matter (e) of naïve spinal cord. FOXO3a staining was localized to the nucleus, while day 3 after injury FOXO3a was transported out of the nucleus and sequestered in the cytosol, nucleus staining was decreased in the ventral horn (d) and white matter (f). Scale bars: 200 μm (a,b) and 20 μm (c–f). B: The quantitative analysis of FOXO3a/27^{kip1} positive cells/mm^2 between naïve and day 3 after SCI. #,*Significant difference at $P < 0.05$ compared with naïve. Error bars represent SEM.

Additionally, the immunohistological changes of $p27^{kip1}$ after SCI were consistent with our previous study [Shen et al., 2008] (data not shown here). Subsequently, the profiles of spatial distribution of FOXO3a and $p27^{kip1}$ were measured between naïve group and Day 3 after injury group, the data were consistent with Western blot results (Fig. 2B).

THE CO-LOCALIZATION OF FOXO3a AND $p27^{kip1}$ WITH DIFFERENT PHENOTYPE-SPECIFIC MARKERS BY DOUBLE IMMUNOFLUORESCENT STAINING

To further characterize the cell types expressing FOXO3a, immunofluorescent staining was performed with the following cell-specific markers: NeuN (neuron marker; Fig. 3A-a, b, and c) and GFAP (astrocyte marker; Fig. 3A-d, e, and f). FOXO3a was expressed in NeuN-positive cells and GFAP-positive cells. To identify the proportion of neurons and astrocytes expressing FOXO3a, a minimum of 200 neurons and astrocytes were counted between naïve spinal cords and spinal cords 3 days after SCI (as described in Materials and Methods Section). As showed in Figure 3B, injury significantly decreased FOXO3a expression in astrocytes compared with sham spinal cord ($P < 0.05$). Concomitantly, there was a similar change with $p27^{kip1}$ staining positive cells after SCI (Fig. 4).

ASSOCIATION OF FOXO3a WITH PROLIFERATION AFTER SCI

It has been demonstrated decreased $p27^{kip1}$ levels through inhibition of FOXO transcription was associated with cell proliferation [Dijkers et al., 2000; Medema et al., 2000]. To study the endogenous repair after SCI, we examined proliferating cell nuclear antigen (PCNA), which has been used as a general marker of dividing cells [Morris and Mathews, 1989], is involved in both DNA replication and DNA repair, and is up-regulated during the S phase of the cell cycle. Western blot data showed PCNA were obviously up-regulated at Day 3 after injury and peaked at Day 7 (Fig. 5A), after day 14, PCNA levels declined. To identify the proliferative cell types in spinal cord after SCI, we performed double immunofluorescent staining with specific PCNA antibody and different phenotype-specific markers. Many GFAP-positive cells expressed PCNA (Fig. 5B-a, b, and c). Moreover, there was also p-FOXO3a co-localization with PCNA showed (Fig. 5B-g, h, and i), on contrary there was only a few co-localizations between PCNA and FOXO3a (Fig. 5B-d, e, and f). Collectively, the down-regulation of FOXO3a and $p27^{kip1}$ expressions in spinal cord was concomitantly with glial cells proliferation after SCI.

FOXO3a/$p27^{kip1}$ EXPRESSION AND SUBCELLULAR LOCALIZATION IN PRIMARY SPINAL ASTROCYTES IN VITRO

To verify the accuracy of the results in vivo studies, we used the model of proliferation of astrocyte cultured in vitro. Astrocytes were synchronized at the G0/G1 phase by serum deprivation 48 h. Upon serum addition, cells were released from G1 to S phase. First, Western blots showed a considerable increase in PCNA (the marker of proliferating cell) after on serum addition (Fig. 6A), and at the same time cyclinA (the markers of S phase progression) expression was significantly increased after serum addition 24 h (Fig. 6A). These results showed that the model is successful. Previous studies showed that ectopic expressions of activated FOXO3a lead to cell cycle arrest in which regulation of $p27^{kip1}$ by FOXO3a might be play an important role [Medema et al., 2000]. We examined whether the expression kinetics of p-FOXO3a, FOXO3a and $p27^{kip1}$ were regulated in a cell cycle-dependent manner in vitro. Western blot showed that p-FOXO3a protein expression was low but FOXO3a and $p27^{kip1}$ protein levels were high in the G0/G1 phase. Upon serum addition, cells were released from G1, the abundance of FOXO3a and $p27^{kip1}$ declined with similar kinetics and were markedly reduced 24 h after serum stimulation, which negative correlation with p-FOXO3a expression after serum stimulate(Fig. 6B). Using RT-PCR analysis, we determined whether changes of $p27^{kip1}$ protein could account for the effect of FOXO3a transcriptional regulation. In agreement with the findings of $p27^{kip1}$ protein expression, $p27^{kip1}$ mRNA expression declined after serum stimulation (Fig. 6C). In

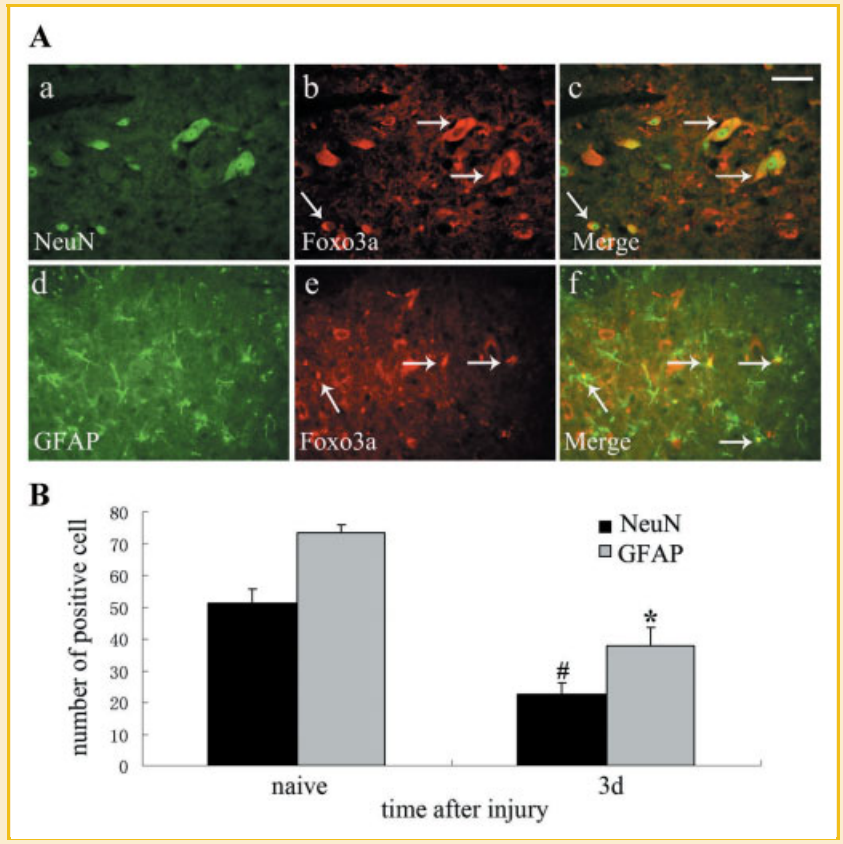

Fig. 3. Double immunofluorescence staining for FOXO3a with NeuN, GFAP in spinal cord. In the adult rat spinal cord 2 mm to epicenter at Day 3 after SCI, horizontal sections labeled with FOXO3a (red) with different phenotype-specific markers (green), including NeuN (neuronal marker, A-a, b, and c), GFAP (astrocytic marker, A-d, e, and f) and the co-localization (yellow, arrows). B: The quantitative analysis of different phenotype-specific markers positive cells expressing FOXO3a (%) in the naïve spinal cord and Day 3 after SCI. The changes of FOXO3a positive expression cells after SCI were prominent in the astrocytes. *Significant difference at $P < 0.05$ compared with naïve animals. The error bars represent SEM. Scale bars: 20 μm.

addition to the tight control of $p27^{kip1}$ abundance, there is also important regulation at the level of $p27^{kip1}$ subcellular localization. Following serum addition, FOXO3a and $p27^{kip1}$ significantly decreased in the nuclear fraction in a time-dependent manner (Fig. 6D). These data suggested that proliferating of astrocyte cells in vitro also induced down-regulation of FOXO3a and $p27^{kip1}$ in a cell cycle-dependent manner.

PI3K/Akt PATHWAY REGULATED FOXO3a/$p27^{kip1}$ EXPRESSION AND SUBCELLULAR LOCALIZATION IN PROLIFERATING PRIMARY ASTROCYTES

Recent studies suggest that FOXO3a is a major molecule in the PI3K/Akt signaling pathway which plays an important role in promoting cell survival and proliferation [Kennedy et al., 1997; Songyang et al., 1997; Downward, 1998]. To further elucidate the mechanism of FOXO3a/$p27^{kip1}$ in the regulation of astrocyte cells proliferation, we used the PI3K pharmacologic inhibitor LY294002. After synchronization by serum starvation for 48 h, astrocyte cells were incubated in medium containing 10% FBS for the indicated times. On serum addition, the cells were transformed from the G1 phase to the S phase, while exposure to 50 μM LY294002 for 48-h blocked proliferation and caused an accumulation in the G1 phase of the cell cycle, as assessed by flow cytometric analysis (date not shown). First, we were sought to study whether PI3K/Akt pathway was involved in astrocyte cells proliferation or not. The Western blot results showed that the phosphorylation of Akt Ser473 was decreased in astrocytes exposured to LY294002 (Fig. 7A). Second, by Western blot analysis, we observed that threonine32-phosphorylation of FOXO3a was down-regulated after treatment with LY294002, but FOXO3a and $p27^{kip1}$ were up-regulated (Fig. 7A). Third, due to LY294002 is solubled in DMSO and diluted into aqueous buffers to yield the desired concentrations. To look at the specificity of the LY294002, control dishes received the same volume of the solvent DMSO and Western blot results showed that the phosphorylation of Akt Ser473 was decreased exposure to LY294002 instead of MODS (Fig. 7B). Since there is broad consensus that activated FOXO3a could function to promote the transcription of cell-cycle inhibitors [Brunet et al., 1999; Nakamura et al., 2000], our results suggest that LY294002 could help to retain a greater amount of FOXO3a in the activated form to inhibit astrocyte cells growth (Fig. 7A). We further investigated the location of FOXO3a by LY294002 treatment, which is related to its activation state. In response to LY294002 treatment

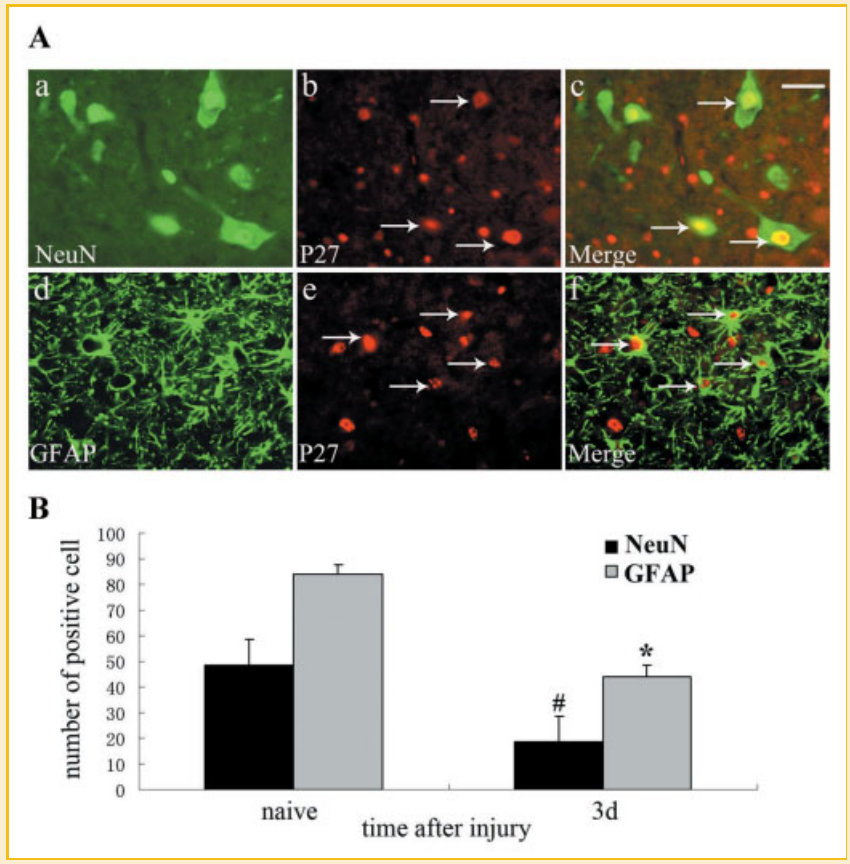

Fig. 4. Double immunofluorescence staining for $p27^{kip1}$ with NeuN, GFAP in spinal cord. In the adult rat spinal cord 2 mm to epicenter at Day 3 after SCI, horizontal sections labeled with $p27^{kip1}$ (red) and different phenotype-specific markers (green), including NeuN (A- a, b, and c), GFAP (A-d, e, and f) and the co-localization of FOXO3a with different phenotype-specific markers (yellow, arrows). B: The quantitative analysis of the positive cells expressing $p27^{kip1}$ (%) with the different phenotype-specific markers in the sham-operated spinal cord and Day 3 after SCI. The changes of $p27^{kip1}$ positive expression cells after SCI were also prominent in the astrocytes (*) Indicates significant difference at $P < 0.05$ compared with naïve. The error bars represent SEM. Scale bars: 20 μm.

for 24 h, subcellular fractionation was performed for immunoblot analysis, FOXO3a was largely expression in the nucleus and the amount in the cytoplasm was declined (Fig. 8B). To confirm these results, we also tested the subcellular distribution of FOXO3a by immunofluorescence analysis in astrocyte cells. Similarly, FOXO3a seemed to be mainly in the nucleus after administration of LY294002 for 24 h (Fig. 8A). As expected, it also caused a parallel change in the distribution of $p27^{kip1}$ (Fig. 8A). In agreement with $p27^{kip1}$ protein expression, $p27^{kip1}$ mRNA expression was also up-regulated after administration of LY294002 (Fig. 7B). These findings suggested that the PI3K/Akt pathway controlled astrocyte cells proliferation by regulating FOXO3a/$p27^{kip1}$ expression and subcellular localization.

DISCUSSION

Traumatic SCI is a devastating disorder and a great financial burden on society. The use of the rat spinal cord contusion injury model to recapitulate the clinical scenario is a favorable way to investigate the molecular and cellular mechanisms involved in the secondary insult. In vivo study, we demonstrated FOXO3a expression was positively correlated with $p27^{kip1}$ expression, but inversely associated with cell proliferation. Additionally, Double immunofluorescence staining shown the down-regulation of FOXO3a and $p27^{kip1}$ were predominant in glial cells, which were regenerating axons or largely proliferated. Glial cells proliferation expresses PCNA after injury. Moreover, there was also p-FOXO3a co-localization with PCNA showed, on contrary there was only a few co-localizations between PCNA and FOXO3a. FOXO3a is an inhibitor of cell proliferation, so the FOXO positive cells with no PCNA activity may show active FOXO3a translocates to the nucleus, binds to DNA and promotes the transcription of $p27^{kip1}$, which regulation of glial cells over-proliferation, and at this very moment glial cells may be stop proliferation or cell death and this require further investigation. These data provided an preliminary evidence of FOXO3a underlying CNS injury and repair. Secondary SCI includes complex physiological and biochemical mechanisms [Young, 1993; Kwon et al., 2004]. A critical mechanism in the pathophysiology of SCI is the activation of astrocyte proliferation, characterized by increased expression of the astrocyte-specific marker GFAP. Although astrocytes secrete important growth factors

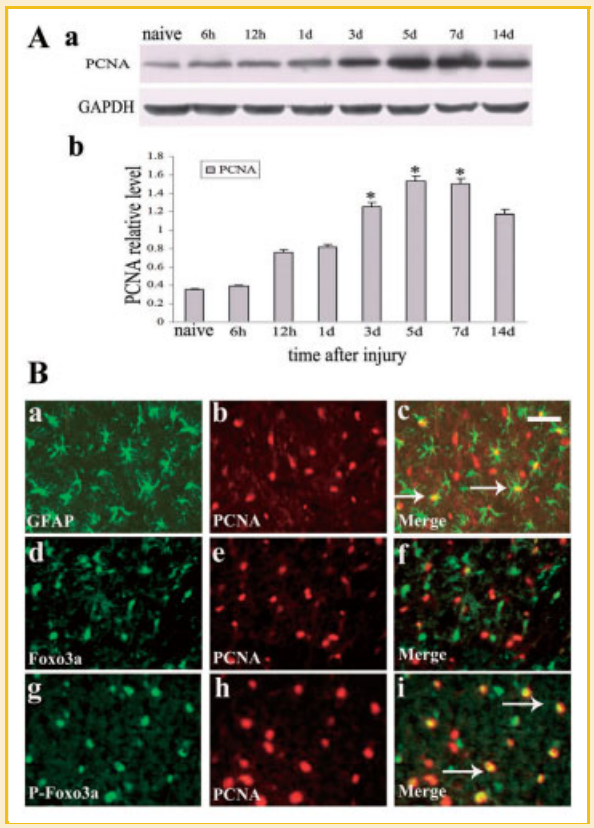

Fig. 5. Association of FOXO3a with proliferation after SCI. A: Sample immunoblots probed for PCNA in the spinal cord after injury and GAPDH were shown above. PCNA expression was obviously increased at days 3–7 after SCI and was maintained for 2 weeks. B: Double immunofluorescence staining for PCNA (red), GFAP (green), FOXO3a (green), and p-FOXO3a (green) after SCI. The majority of reactive astrocytes were PCNA-positive at Day 3 after SCI (B-a, b, and c). There were only a few co-localizations between PCNA and FOXO3a (B-f). On contrary there was p-FOXO3a co-localization with PCNA showed (B-g, h, and i). Scale bars: 20 μm (a–i). The co-localizations of PCNA with different phenotype-specific markers were shown (arrows).

for neurons, the glial scar presents a physical barrier to regeneration and plasticity. It is a source of multiple inhibitory factors that may limit neuroplasticity [Davies et al., 1996; Silver and Miller, 2004]. The role of astrocytes after CNS injury therefore seems detrimental for neuronal survival and regeneration [Fawcett, 1997; Ridet et al., 1997; Nieto-Sampedro, 1999]. Thus, a better control of astrocytes proliferation after SCI and identify of proteins involved will be helpful in aiding the regeneration of the spinal cord following injury.

During the repair process, cell cycle regulation may be indispensable. $p27^{kip1}$, as a member of Cip/Kip family of cyclin-dependent kinase inhibitors, plays an important role in cell cycle regulation and neurogenesis in the developing CNS. Over-expression of $p27^{kip1}$ arrests cells in G1 [Coats et al., 1996]. While the loss of $p27^{kip1}$ leads to an increase in cell proliferation, this has been demonstrated by studies showing that mice lacking the $p27^{kip1}$ gene have increased body and organ weights and develop pituitary adenomas [Fero et al., 1996; Nakayama et al., 1996]. We recently reported $p27^{kip1}$ was down-regulated in the injured thoracic spinal cord [Shirane et al., 1999; Zhao et al., 2011] and the lumbar spinal cord after sciatic nerve injury [Shi et al., 2007], and the down-regulation of $p27^{kip1}$ was very likely to be involved in the nervous system injury and repair process after SCI. Thus, the amount of $p27^{kip1}$ expression is very important for the injury and repair of spinal cord. Recent data show FOXO3a directly regulates $p27^{kip1}$ transcription [Medema et al., 2000; Nakamura et al., 2000; Stahl et al., 2002], suggesting that reduced $p27^{kip1}$ levels after a proliferative stimulus may also be associated with FOXO3a in the nervous system injury and repair process after SCI.

FOXO3a is a member of a large family of forkhead transcription factors. About 40 different forkhead transcription factors have been identified to date in mammalian cells. Forkhead proteins have been assigned to 17 sub-families ranging from FOXA to FOXQ [Kops and Burgering, 1999]. Of these, the FOXO factors are the only ones known to date to be regulated by the PKB/Akt pathway. FOXO transcription factors have been implicated in a remarkable number of diverse cellular processes such as development and metabolism, stress and aging, proliferation, and programmed cell death [Carlsson and Mahlapuu, 2002; Lehmann et al., 2003].

Previous studies showed down-regulated $p27^{kip1}$ through FOXO transcriptional inhibition was associated with multitude cell type's proliferation [Dijkers et al., 2000; Stahl et al., 2002; Potente et al., 2003]. In the current study, we revealed that down-regulation of $p27^{kip1}$ followed the inhibition of FOXO3a after SCI, and decreased levels of FOXO3a and $p27^{kip1}$ were predominant in glial cells, which were largely proliferated after injury. Therefore, we hypothesized that down-regulation of FOXO3a and $p27^{kip1}$ expressions after SCI were implicated in the proliferation of glial cells. To further validate this hypothesis, primary astrocyte cultures were prepared from spinal cords. Astrocyte cells were synchronized at the G0/G1 phase by serum deprivation for 48 h. Following serum administration, the expression of the widely used marker of proliferation cells, PCNA, gradually increase; at the same time, the Markers of S phase progression, cyclin A, was significantly increased after serum addition 24 h. FOXO3a and $p27^{kip1}$ significantly decreased in the nuclear fraction in a time-dependent manner. In agreement with the findings of protein expression, $p27^{kip1}$ mRNA expression also declined after serum stimulation. These data suggested that FOXO3a also regulated transcriptional expression of $p27^{kip1}$ in proliferating astrocyte cells in vitro.

There is broad consensus that Akt-dependent phosphorylation is crucial to preventing FOXO3a displacement to the nucleus where they would function to promote the transcription of cell cycle inhibitors $p27^{kip1}$ [Brunet et al., 1999; Nakamura et al., 2000]. We observed how PI3K/Akt pathway controlled astrocyte cell proliferation in vitro by regulating FOXO3a. From our series of studies, we found that LY294002 significantly inhibited the phosphorylation and activation of Akt, resulting in lower levels of P-FOXO3a. More important, LY294002-treatment caused FOXO3a and $p27^{kip1}$ nuclear accumulation compared with control groups. These results suggested that LY294002 regulated the activity of FOXO3a by regulating its phosphorylation and subcellular location in astrocytes. It is known that FOXO3a regulates the transcription of $p27^{kip1}$ by binding to its promoter [Nam et al., 2001; Yang et al., 2005].

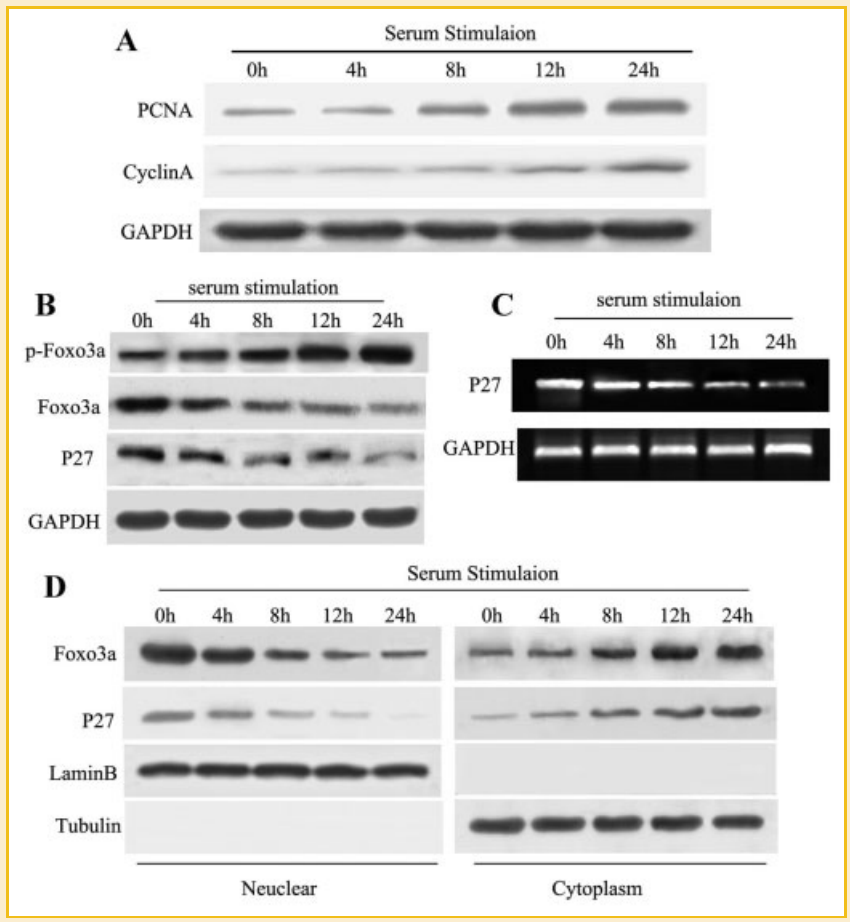

Fig. 6. Analysis of FOXO3a/p27^{kip1} expression during the proliferation of primary astrocyte cells. A: Primary astrocyte cells were synchronized by serum starvation for 48 h. After that, primary astrocyte cells were incubated in medium containing 10% FBS for the indicated times. Upon serum addition, Western blots showed gradually increase in PCNA, and the markers of S phase cyclin A expression was significantly increased after serum addition 24 h. B: p-FOXO3a, FOXO3a, p27^{kip1} protein and (C) p27^{kip1} mRNA expressions were evaluated by Western blotting (B) and RT-PCR (C), respectively. D: Nuclear and cytosolic proteins were immunoblotted for FOXO3a and p27^{kip1}. The levels of lamin B and tubulin in the nuclear and cytosolic fractions, respectively, were also immunoblotted to confirm the purity of the subcellular fractions. Each immunoblot shown was representative of three experiments with similar results. Reduced expression of FOXO3a in the nucleus was observed 4 h after serum addition; in contrast, expression of FOXO3a was increased in cytoplasm. Similar results were observed for the expression of p27^{kip1}.

Indeed, we found that p27^{kip1} expression was also increased by LY294002 treatment in astrocytes, whereas alteration of the level of p27^{kip1} protein by LY294002 treatment could also be because of the altered activity of protease as observed in other agent treatments and this require further investigation.

Studies in mammalian cells have shown that the over production of FOXO3a induced either cell cycle arrest or apoptosis via transcriptional modulations in a variety of genes, including p27^{kip1} [Lim et al., 2002]. Evidence showed FOXO3a induced spinal cord Moto-neurons and cerebellar granule neurons death through the Fas pathway [Brunet et al., 1999; Barthelemy et al., 2004]. Besides, FOXO3a up-regulated Bim expression and enhanced sympathetic neurons death of after NGF deprivation [Brunet et al., 1999]. Furthermore, previous data also suggested p27^{kip1} could have both pro-apoptotic and anti-apoptotic roles, dependent on the cell types and stress types [Coqueret, 2003]. In the current study, we observed FOXO3a and p27^{kip1} were also expressed in neurons, and decreased after injury too. These data may provide insight into a novel molecular pathway that decreased levels of FOXO3a and p27^{kip1} are important for post-traumatic neuronal cell death after SCI. However, the molecular mechanisms by FOXO3a resist to neurons apoptosis after SCI need our further investigate.

In conclusion, we have shown that FOXO3a and p27^{kip1} expression were negative associated with proliferation index of PCNA in spinal cord of adult rat in vivo and we have shown large variations of FOXO3a expression and a close correlation with p27^{kip1} and cell proliferation in primary glial cells in vitro, changes of both FOXO3a and p27^{kip1} expression might contribute to the dysregulation of cell cycle and might precede the progression of glial cells proliferation. Although the detailed biological functions of FOXO3a in glial cells proliferation have not been identified, our results demonstrated that FOXO3a as a positive regulator of p27^{kip1} in astrocytes proliferation, which was associated with CNS injury and repair.

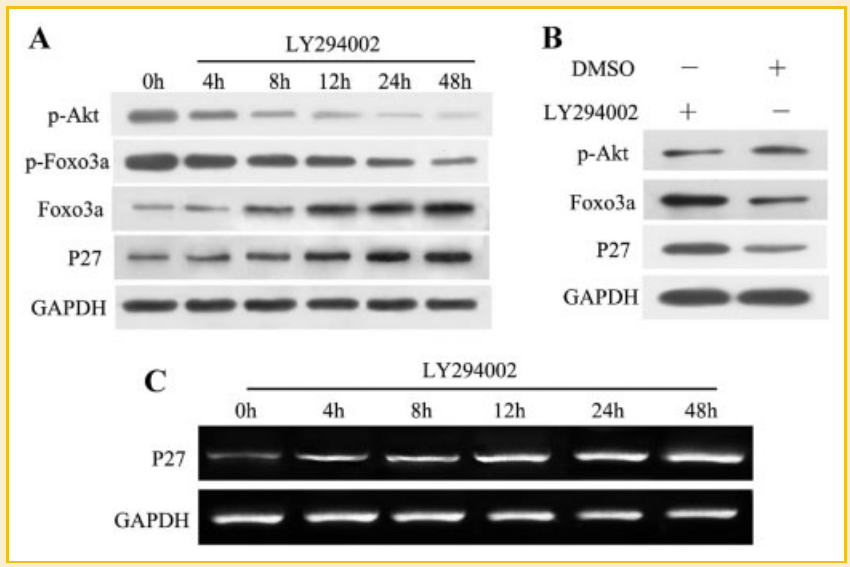

Fig. 7. The Akt activation and the expression of LY294002 on FOXO3a expression during primary astrocyte cells proliferating. Inhibition of PI3K induced FOXO3a activation and translocation in astrocyte cells. After synchronization by serum starvation for 48 h, then astrocyte cells were incubated in medium containing 10% FBS for the indicated times. A: On serum addition, the cells were transformed from the G1 phase to the S phase, shown above cyclin A (markers of S phase) expression was increased, whereas astrocyte cells were incubated in the absence and presence of LY294002, then cells were collected at the times indicated. P-Akt, p-FOXO3a, FOXO3a, and $p27^{kip1}$ protein and (C) $p27^{kip1}$ mRNA expression were evaluated by Western blotting (A) and RT-PCR (C), respectively. FOXO3a and $p27^{kip1}$ protein expression and mRNA expression were all obviously increased after incubated 48 h in the presence of LY294002. B: Astrocyte cells were incubated with or without low concentrations LY294002 (10 μM) for 48 h in the absence and presence of the same volume of the solvent DMSO. Cell lysates isolated at the times indicated, were subjected to immunoblot analysis for p-Akt, FOXO3a, and $p27^{kip1}$. These differences in protein expressions, suggesting that the specificity role of the LY294002 on Akt. C: RT-PCR showed $p27^{kip1}$ mRNA expression was also increased after incubated 48 h in the presence of LY294002, which in agreement with $p27^{kip1}$ protein expression after administration of LY294002. Each experiment was performed in triplicate and representative results were shown.

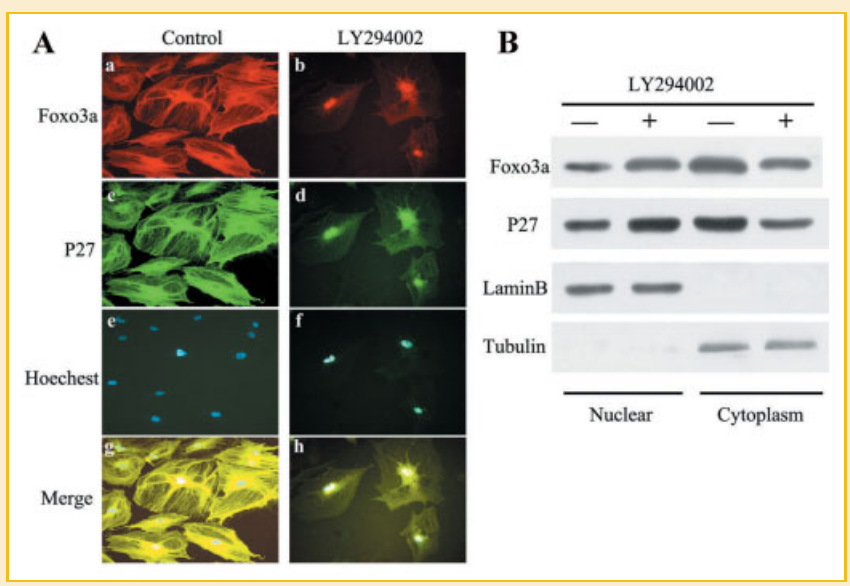

Fig. 8. LY294002 regulated the localization of FOXO3a/$p27^{kip1}$ in primary rat astrocyte cells. A: Astrocyte cells were incubated in the absence and presence of LY294002 for 48 h. In response to LY294002 treatment for 48 h, FOXO3a was largely redistributed to the nucleus and the amount in the cytoplasm declined. B: Samples of cytoplasmic or nuclear extracts, isolated at the times indicated, were subjected to immunoblot analysis for FOXO3a and $p27^{kip1}$. The levels of lamin B and a-tubulin in the nuclear and cytosolic fractions, respectively, were immunoblotted to confirm the purity of the subcellular fractions. Each experiment was performed in triplicate and representative results were shown. Immunofluorescence gave a result similar to that from Western blot.

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