ARTICLE
Journal of Cellular Biochemistry 116:1381–1390 (2015)

Journal of Cellular Biochemistry

Spatiotemporal Expression of EAPP Modulates Neuronal Apoptosis and Reactive Astrogliosis After Spinal Cord Injury

Minhao Chen,[1] Yingjie Ni,[2] Yonghua Liu,[3] Xiaopeng Xia,[1] Jianhua Cao,[1] Chengniu Wang,[4] Xingxing Mao,[1] Weidong Zhang,[1] Chen Chen,[1] Xinlei Chen,[1] and Youhua Wang[1]*

[1]*Department of Orthopaedics, Affiliated Hospital of Nantong University, Nantong, Jiangsu, PR China*

[2]*Department of Orthopaedics, Xishan People' Hospital, Wuxi, Jiangsu, PR China*

[3]*Jiangsu Province Key Laboratory for Inflammation and Molecular Drug Target, Medical College, Nantong University, Nantong, Jiangsu, PR China*

[4]*Basic Medical Research Centre, Medical School, Nantong University, Nantong, Jiangsu, PR China*

ABSTRACT

E2F-associated phosphoprotein (EAPP) is a novel E2F binding protein that interacts with the activating members of the E2F transcription factors family and involved in various biological processes. However, the expression and function of EAPP in central nervous system (CNS) are still unknown. In this study, we performed an acute spinal cord injury (SCI) model in adult rats, we found that EAPP protein levels were significantly increased and reached a peak at day 3, and then gradually returned to normal level at day 14 after spinal cord injury and we observed that the expression of EAPP is enhanced in the gray and white matter. Spatially, increased levels of EAPP were striking in neurons and astrocytes. Moreover, colocalization of EAPP/active caspase-3 was detected in neurons, and colocalization of EAPP/proliferating cell nuclear antigen (PCNA) was detected in astrocytes after spinal cord injury. These results indicated that EAPP might play an important role in neuronal apoptosis and reactive astrogliosis. Furthermore in vitro, EAPP depletion by siRNA inhibited astrocyte proliferation, migration and CDK4/cyclinD1 expression. Meanwhile, EAPP knockdown also reduce neuronal apoptosis and cell cycle related proteins. Which indicated that EAPP might integrate cell cycle progression and play a crucial role in cell proliferation and apoptosis. Taken together, we speculated that EAPP was involved in biochemical and physiological responses after SCI. J. Cell. Biochem. 116: 1381–1390, 2015.

KEY WORDS: SPINAL CORD INJURY; EAPP; NEURONAL APOPTOSIS; REACTIVE ASTROGLIOSIS; CELL CYCLE

Traumatic spinal cord injury (SCI) is a devastating event in humans, especially in young males [Wyndaele and Wyndaele, 2006]. It is universally acknowledged that acute spinal cord injury is a two-step process involving primary and secondary injury mechanisms, Primary injury is attributed to direct tissue damage and associated neurological dysfunction, which are the result of mechanical damage. Mechanisms of secondary injury include a complex cellular response through the activation or suppression of a large number of transcriptional pathways in different cells. These mechanisms include neuronal apoptosis, reactive astrogliosis and scar formation [Faden, 1993; Tator, 1996; Dumont et al., 2001].

Cell cycle activation occurs after central nervous system (CNS) trauma [Cernak et al., 2005], and is associated with proliferation of astrocytes, and apoptosis of post-mitotic cells, such as neurons [Becker and Bonni, 2004]. Generally, Cell cycle proteins are down-regulated in post-mitotic cells [Okano et al., 1993] and reenter the cell cycle can cause apoptosis rather than proliferation in such cells [Wartiovaara et al., 2002; Becker and Bonni, 2004]. Previous studies demonstrated that Up-regulation of cell cycle proteins results in neuronal apoptosis in multiple experimental models, including SCI [Di Giovanni et al., 2003; Tian et al., 2006; Byrnes et al., 2007], brain injury [Di Giovanni et al., 2005; Kabadi et al., 2012] and cerebral

Abbreviations: CDKIs, Cyclin-dependent kinase inhibitors; CDKs, Cyclin-dependent kinases; CNS, Central nervous system; EAPP, E2F-associated phosphoprotein; PCNA, Proliferating cell nuclear antigen; PRb, Phosphorylated retinoblastoma protein; SCI, Spinal cord injury.

Minhao Chen and Yingjie Ni contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81171140, 31300902; Grant sponsor: Colleges and Universities in Natural Science Research Project of Jiangsu Province; Grant number: 13KJB310009.

* Correspondence to: Youhua Wang, Department of Orthopaedics, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, PR China. E-mail: wangyouhua_nt@126.com

Manuscript Received: 7 November 2014; Manuscript Accepted: 23 January 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 21 February 2015

DOI 10.1002/jcb.25096 • © 2015 Wiley Periodicals, Inc.

ischemia [Zhu et al., 2007; Wang et al., 2008]. Mechanisms of cell cycle activation share common regulatory molecules including cyclins, cyclin-activated kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) [Draetta, 1994; Nguyen et al., 2002; Nishitani and Lygerou, 2002; Boonstra, 2003]. In response to SCI, activation of cell cycle to neuronal apoptosis involves formation of the cyclin D1-CDK4/6 complex, activation of CDK4/6, and then CDK4/6 phosphorylates the retinoblastoma protein (Rb), causing dissociation of Rb-E2F complex and activation of E2F transcription factor [Wu et al., 2011]. E2F can contribute to increased transcription of pro-apoptotic molecules such as caspases-3, 9 and 8 and pro-apoptotic Bcl-2 family members. Ultimately contributing to neuronal cell death [Nahle et al., 2002; Nguyen et al., 2003]. It is also demonstrated that SCI results in cell cycle activation in mitotic cells, such as astrocytes [Di Giovanni et al., 2003; Wu et al., 2011]. Which cause gliosis and formation of glial scar [Hoke and Silver, 1994; Raivich et al., 1999]. Thus, identification of the role of cell cycle proteins involved in both neuronal death and reactive gliosis, may help to clarify the pathobiology of CNS injury and provide a novel therapeutic targets.

E2F-associated phosphoprotein (EAPP) is a novel E2F binding protein that interacts with the activating members of the E2F transcription factors family and involved in various biological processes, including cell proliferation, apoptosis, cell cycle regulation and DNA damage response [Novy et al., 2005; Schwarzmayr et al., 2008; Andorfer and Rotheneder, 2011; Andorfer et al., 2011]. EAPP was discovered by yeast two-hybrid screen. Initial analysis of EAPP revealed a 1.4-kb mRNA transcript, encoding a 285-amino-acid protein [Novy et al., 2005]. EAPP binds only to E2F1-3, comprising the activator group of E2F proteins [Schwarzmayr et al., 2008]. Previous studies indicated that EAPP is present throughout the cell cycle but disappears during mitosis. EAPP depletion in U2OS cells reduced the fraction of cells in S-phase, whereas overexpression of EAPP resulted in a significant increase of cells in S-phase [Novy et al., 2005]. Furthermore, overexpression of EAPP also protected U2OS cells from apoptosis [Andorfer and Rotheneder, 2011]. Previous studies have examined the important role of EAPP in various tumor cells, such as U2OS, T98G, HELA and 293 cells [Novy et al., 2005; Andorfer and Rotheneder, 2013]. But its expression and possible roles in SCI remain a mystery. From these data, it seems that EAPP may involve in pathophysiological and biochemical progression after SCI.

In present study, we explored the relationship of EAPP expression with neuronal apoptosis and reactive astrogliosis after SCI. The change of temporal–spatial expression of EAPP and its colocalization with active caspase-3 and proliferating cell nuclear antigen (PCNA) after SCI were assessed in rat spinal cord contusion model in vivo. Furthermore, we studied the effect of EAPP-specific siRNA on the astrocyte proliferation and neuronal apoptosis in vitro to explore the potential value of EAPP. Our experiment is conducted to gain a deep insight into the functions of EAPP and its roles in the cellular and molecular mechanisms underlying SCI and repair.

MATERIALS AND METHODS

SPINAL CORD INJURY

Adult male Sprague Dawley rats (n = 48) weighing 275–325 g and aged 2 months were subjected to a contusive SCI. Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, I.P.), while a laminectomy was performed at the level of T9 to expose a circle of dura mater. Contusion injuries (n = 40) were performed by an NYU impactor [Gruner, 1992]. Rats were subjected to a sever spinal cord contusion injury by dropping a 10-g weight from a height of 5 cm onto an impounder positioned on the exposed dura. Sham-injured animals (n = 8) received a laminectomy without weight drop. After surgery, bladders of rats were manually expressed twice daily until bladder-emptying reflex was established. Animals were sacrificed at 6 h, 12 h, 1 day, 3 days, 5 days, 7 days and 14 days after injury. 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.

RAT PRIMARY ASTROCYTES

Spinal cord was taken from 3-day-old Sprague-Dawley rat and the tissues were gently minced by forceps, then dissociated in phosphate-buffered saline (Sigma) containing 0.1% collagenase A and 0.25% trypsin (Sigma) for 15 min at 37°C. After centrifugation at 1,200 rpm for 5 min, the tissues were removed, placed in Dulbecco′s modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and gently aspirated several times to inactivate the trypsin. The medium was decanted, replaced with DMEM supplemented with F12 (Sigma) containing 10% FBS, then cells were resuspended. The cells were plated in a tissue culture flask coated with poly-L-lysine (Sigma). Cells were grown for 8 days (37°C; 5% CO_2), with changes of the culture medium at days 3 and 6. At day 8, the flask were shaked at 200 rpm for 2 h at 37°C to remove oligodendrocytes and microglia which growing on top of the confluent astrocyte layer, then replacing the culture medium. Twenty-four hours later, the cells were trypsinized and plated at density (4×10^4 cells/mL) on 6-well culture plates. Before experimental treatments, cell culture medium was switched to serum-free DMEM/F12 culture medium.

RAT PRIMARY NEURONS

Primary neuronal cultures were prepared from the spinal cord of 3-day-old Sprague-Dawley rats, the procedures were carried out similarly to those previously described. After the medium had been decanted, cells were resuspended in Neurobasal medium (Gibco) supplemented with B27 (Gibco) containing 0.5 mM glutamine. Cells were plated at density (4×10^4 cells/mL) on poly-L-lysine-coated 6-well culture plates. Before experimental treatments, cells were grown for 8 days (37°C; 5% CO_2) to induce apoptosis.

ASTROCYTE MIGRATION ASSAY

Astrocytes transduced with either control or siRNA were plated in DMEM supplemented with F12 (Sigma) containing 10% FBS on 6-well plates and grown to confluence. The medium was then removed, and the monolayer was scratched with a sterile plastic pipette tip. The cells were washed twice with medium and incubated for 16 h before being counted under an inverted microscope.

WESTERN BLOT ANALYSIS

Spinal cord tissue (10 mm) centered on the injury site was obtained at 6 h, 12 h, 1 day, 3 days, 5 days, 7 days and 14 days after injury and snap-frozen at −80°C until use. For cultured cells, cells were washed in cold PBS buffer, Then the tissues and cell proteins were extracted by homogenizing the tissue in lysis buffer (1% NP-40, 5 mmol/L EDTA, 50 mmol/L Tris pH 7.5, 1% sodium deoxycholate, 1% SDS, 1% Triton X-100, 1 mmol/L PMSF, 1 mg/ml leupeptin, and 10 mg/ml aprotinin). Supernatants were collected after centrifugation at 20000*g* for 20 min at 4°C. The protein concentration was determined using Bradford assay (Bio-Rad), supernatant (50 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidine difluoridemembrane (Millipore) by a transfer apparatus at 300 mA for 2 h. The membrane was then blocked with 5% nonfat milk and probed with the following antibodies: against EAPP (1:500, Abcam), PCNA (1:1,000, Santa Cruz), active caspase-3 (1:500, Cell Signaling), CDK4 (1:1,000, Santa Cruz), cyclin D1 (1:1,000, Santa Cruz), β-actin (1:1,000, Santa Cruz). The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies; β-actin was used as a control. Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Pierce Company, USA). The gray value of each band was measured and data are presented as a ratio to β-actin.

IMMUNOHISTOCHEMISTRY

For immunohistochemistry, at specific times after injury, rats were anesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde. After perfusion, the spinal cords were removed and post-fixed in paraformaldehyde for 3 h, then replaced with 20% sucrose for 2 days, following 30% sucrose for 2 days. The tissues were embedded in O.T.C. compound after treatment with sucrose. Then, 8-μm frozen cross-sections at specified distances rostral and caudal to the injury epicenter were prepared and examined. 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. After washing, sections were incubated overnight at 4°C with anti-EAPP (1:500, Abcam). Staining was visualized with diaminobenzidine (Vector Laboratories). Cells with strong or moderate brown staining were counted as positive, while cells with no staining were counted as negative and cells with weak staining were scored separately. For immunofluorescence staining or double labeling, sections were firstly blocked with 10% normal serum blocking solution (3% BSA and 0.1% Triton X-100 and 0.05% Tween-20) 2 h at room temperature. Then sections were incubated overnight at 4°C with primary antibodies against EAPP (1:100, Abcam), active caspase-3 (1:100, Cell Signaling), PCNA (1:100, Santa Cruz). The cell-specific markers as follow: NeuN (neuron marker, 1:200, Millipore), GFAP (astrocytic marker, 1:100, Santa Cruz), followed by a mixture of FITC- and TRITC-conjugated secondary antibodies were incubated 2 h at room temperature. Immunofluorescence was visualized with a Leica fluorescence microscope (Germany).

siRNA VECTOR CONSTRUCTION AND TRANSFECTION

Double-stranded oligonucleotides corresponding to the target sequence for the human EAPP (Genbank accession no. NM_018453.3) gene was cloned into the pSilencer 4.1-CMV siRNA plasmid (Invitrogen). Astrocytes and neurons were transfected with the EAPP siRNA plasmids using lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Transfected cells were used for the subsequent experiments 48 h after transfection.

STATISTICAL ANALYSIS

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

RESULTS

CHANGES IN PROTEIN EXPRESSION FOR EAPP FOLLOWING SPINAL CORD INJURY

We investigated the temporal expression patterns of EAPP following spinal cord contusion injury by Western blot. EAPP protein level was low in the sham-operated spinal cords, but increased at 6 h after SCI and reached a peak at day 3, then gradually returned to normal level (Fig. 1a,b). These data suggested EAPP protein level might be up regulated after SCI.

EXPRESSION AND DISTRIBUTION CHANGES OF EAPP IN SPINAL CORD

To identify the distribution and expression changes of EAPP after SCI, immunohistochemistry was performed with anti-EAPP polyclonal antibody on transverse cryosections of the spinal cord. In the rostral spinal cord 2 mm to epicenter, EAPP was widely expressed in both gray and white matter including neurons and astrocytes, regardless of

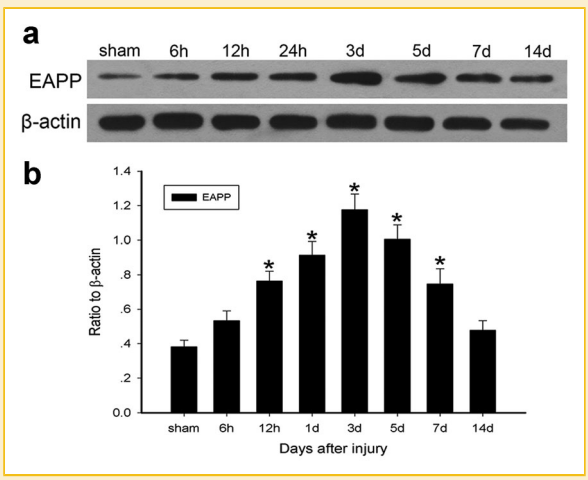

Fig. 1. Expression of EAPP changes over time following spinal cord injury. a: Spinal cord tissues from rats at various survival times after SCI were homogenized and subjected to immunoblot analysis. Sample immunoblots probed for EAPP and loading control (β-actin) are shown above. b: Expression levels of EAPP were normalized by β-actin, as estimated by optical density measurements. The data are means ± SEM (n = 3 rats per time point. $^{*}P < 0.05$, significantly different from the sham group).

sham or injury (Fig. 2a,b). Notably, in sham-operated controls, immunostaining of EAPP was observed at low levels in both gray and white matters (Fig. 2a,c,e), however, the staining was increased after SCI (Fig. 2b,d,f). Moreover, the number of EAPP-immunopositive cells was compared between sham-operated and injured spinal cord, and their quantitative changes were paralleled with Western blot results (Fig. 2g).

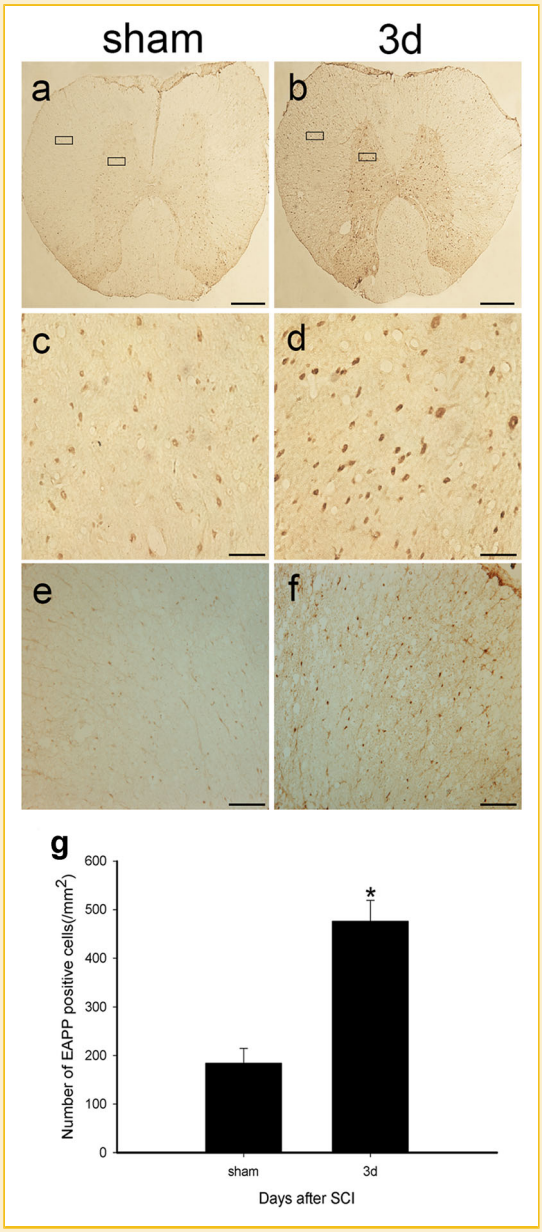

Fig. 2. Immunohistochemical expression of EAPP in adult rat spinal cord. Low-power views of transversal sections immunostained for EAPP in sham spinal cord (a) and 3 days after injury (b). Higher-power views in gray matter (c, d) and white matter (e, f). Immunostainings of EAPP demonstrates that EAPP expression is increased in gray matter (d) and white matter (f). Scale bars: 200 μm (a, b) and 20 μm (c–f). (g) Quantitative analysis of EAPP positive cells/mm^2 in sham and 3 days after SCI, indicating a significant increase comparison to sham. n = 2 in sham group, n = 3 in injured group. *$P < 0.05$ indicates significant difference compared with sham. Error bars represent SEM.

THE COLOCALIZATION OF EAPP WITH DIFFERENT PHENOTYPE-SPECIFIC MARKERS IN SPINAL CORD AFTER INJURY

To further characterize the cell types expressing EAPP, double immunofluorescent staining was performed with NeuN and GFAP (Fig. 3) in transverse cryosections of spinal cord within 2 mm distance from the epicenter. Notably, EAPP expression was widely in neurons (Fig. 3a–c) and astrocytes (Fig. 3g–i) and the expression were

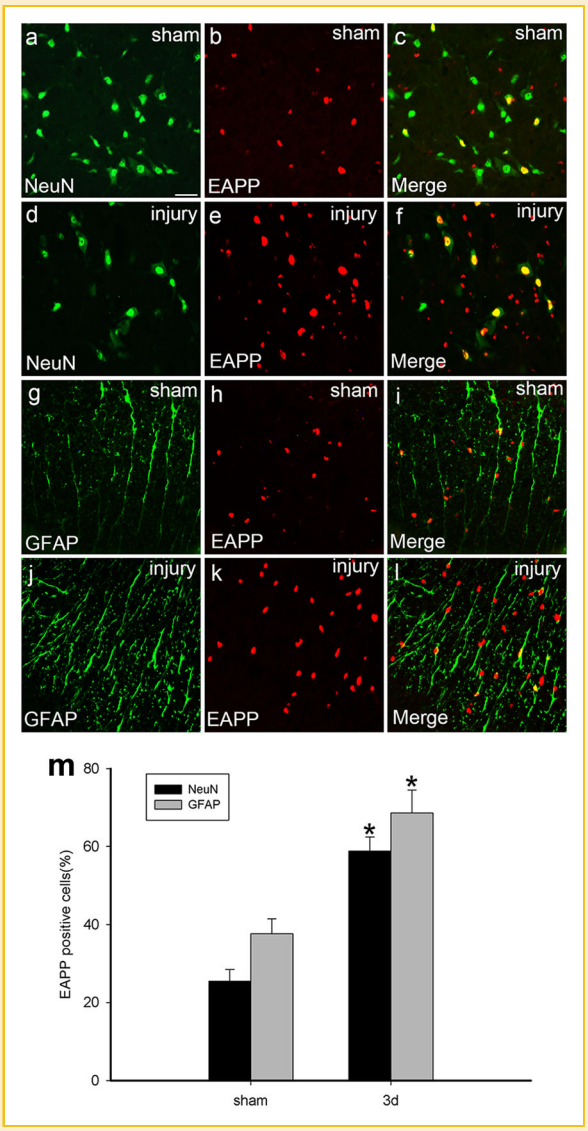

Fig. 3. Immunofluorescence staining for EAPP and different phenotype-specific markers in spinal cord. In the adult rats spinal cord 2 mm to epicenter at day 3 after injury, tissue sections labeled with EAPP (red) and different phenotype-specific markers (green), such as NeuN and GFAP. The colocalizations of EAPP and different phenotype-specific markers (yellow) are shown in the ventral horn (a–f) and white matter (g–l). (m) Quantitative analysis of different phenotype-specific markers positive cells expressing EAPP (%) in sham spinal cord and day 3 after SCI. The changes of EAPP expression after SCI were notable in neurons and astrocytes. n = 2 in sham group, n = 3 in injured group. * Indicates significant difference at $P < 0.05$ compared with sham. Error bars represent SEM. Scale bars: 20 μm (a–l).

significant increased in neurons (Fig. 3d–f) and astrocytes (Fig. 3j–l) at day 3 after SCI compared with sham-operated group ($P < 0.05$).

CELLULAR PROLIFERATION AFTER SPINAL CORD INJURY

To investigate whether EAPP is relevant to proliferation in injured spinal cord, we detected the expression of proliferating cell nuclear antigen (PCNA), a general marker of dividing cells [Morris and Mathews, 1989]. Western blot showed PCNA expression gradually increased and reached a peak at day 3, then gradually returned to normal level, they were in parallel with EAPP in a time-dependent manner (Fig. 4A a,b). Next, we performed double immunofluorescent staining of specific PCNA antibody with GFAP and EAPP at day 3 after SCI (Fig. 4B). The colocalization of PCNA/EAPP and GFAP/PCNA were observed at day 3 after SCI (Fig. 4B c,f). These results indicated that EAPP might be associated with glial reaction after SCI.

DETECTION OF APOPTOSIS AFTER SCI

Apoptosis of neurons has been reported as a crucial part of SCI, caspase-3 activation is a prominent characteristic of apoptosis after SCI, Therefore, we detected the expression of active caspase-3 by Western blot, the result showed that active caspase-3 expression was increased at 6 h and reached a peak at day 3 after SCI, then gradually

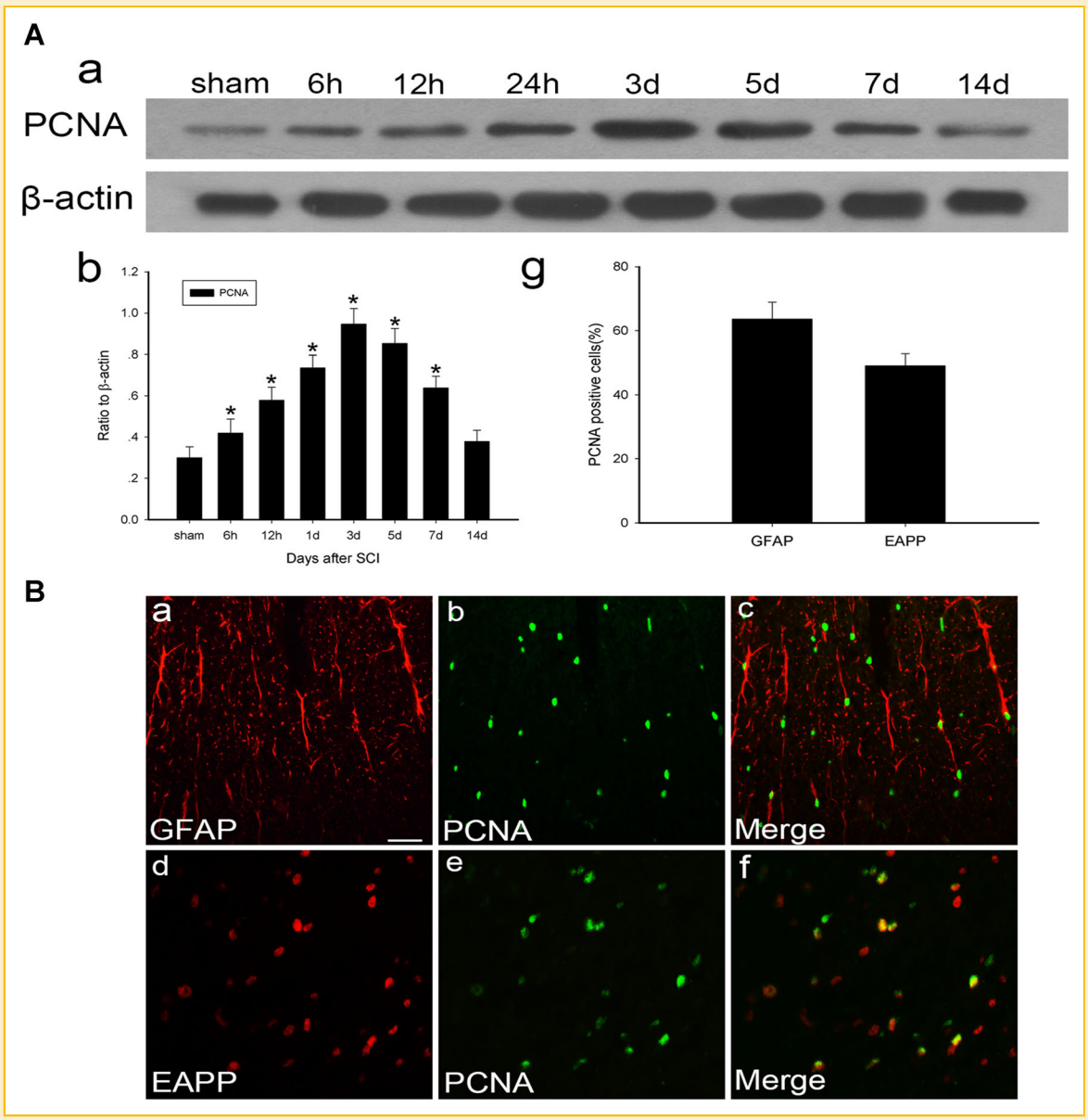

Fig. 4. Expression of EAPP in proliferation after SCI. A: Western blot analysis for markers of proliferation (PCNA) following SCI (a). The bar chart showed the ratio of PCNA to β-actin at each time point (b), the data are means ± SEM ($n = 3$, $^{*}P < 0.05$, significantly different from the sham group). B: Double immunofluorescence staining is for GFAP, EAPP and PCNA in spinal cord after injury. Tissue sections labeled with PCNA (green) and GFAP, or EAPP (red) are shown in the white matter (a–f). (g) Quantitative analysis of GFAP- and EAPP-positive cells expressing PCNA (%). $n = 2$ in sham group, $n = 3$–5 in injured group. Scale bars = 20 μm (a–f).

returned to normal level (Fig. 5A a,b). To confirm the distribution and colocalization of EAPP and activated caspase-3 after SCI, double immunofluorescent staining was performed, the colocalization of NeuN and active caspase-3 and EAPP and active caspase-3 were observed at day 3 after SCI (Fig. 5B c,f). These results indicated that EAPP might be associated with neuronal apoptosis after SCI.

EAPP KNOCKDOWN INHIBITS ASTROCYTE PROLIFERATION AND MIGRATION AND PROMOTES CELL CYCLE ARREST

To assess the role of EAPP during cell proliferation processes further, primary astrocytes were synchronized by serum starvation for 48 h. Subsequently, cells were incubated in medium containing 10% FBS, Western blot showed EAPP, PCNA, cyclinD1 and CDK4 expression were increased after serum addition 24 h (Fig. 6a). We then used EAPP-specific siRNA to knock down endogenous EAPP in primary astrocytes. The efficiency of the EAPP-siRNA was assessed by Western blot analysis. The result showed that EAPP-siRNA#1 significantly reduced the protein level of EAPP compared with negative control (Fig. 6c). Moreover, we measured PCNA protein level and the expression of PCNA was decreased in EAPP-specific siRNA (EAPP-siRNA#1) cells (Fig. 6c). These results show that the EAPP might be involved in cellular

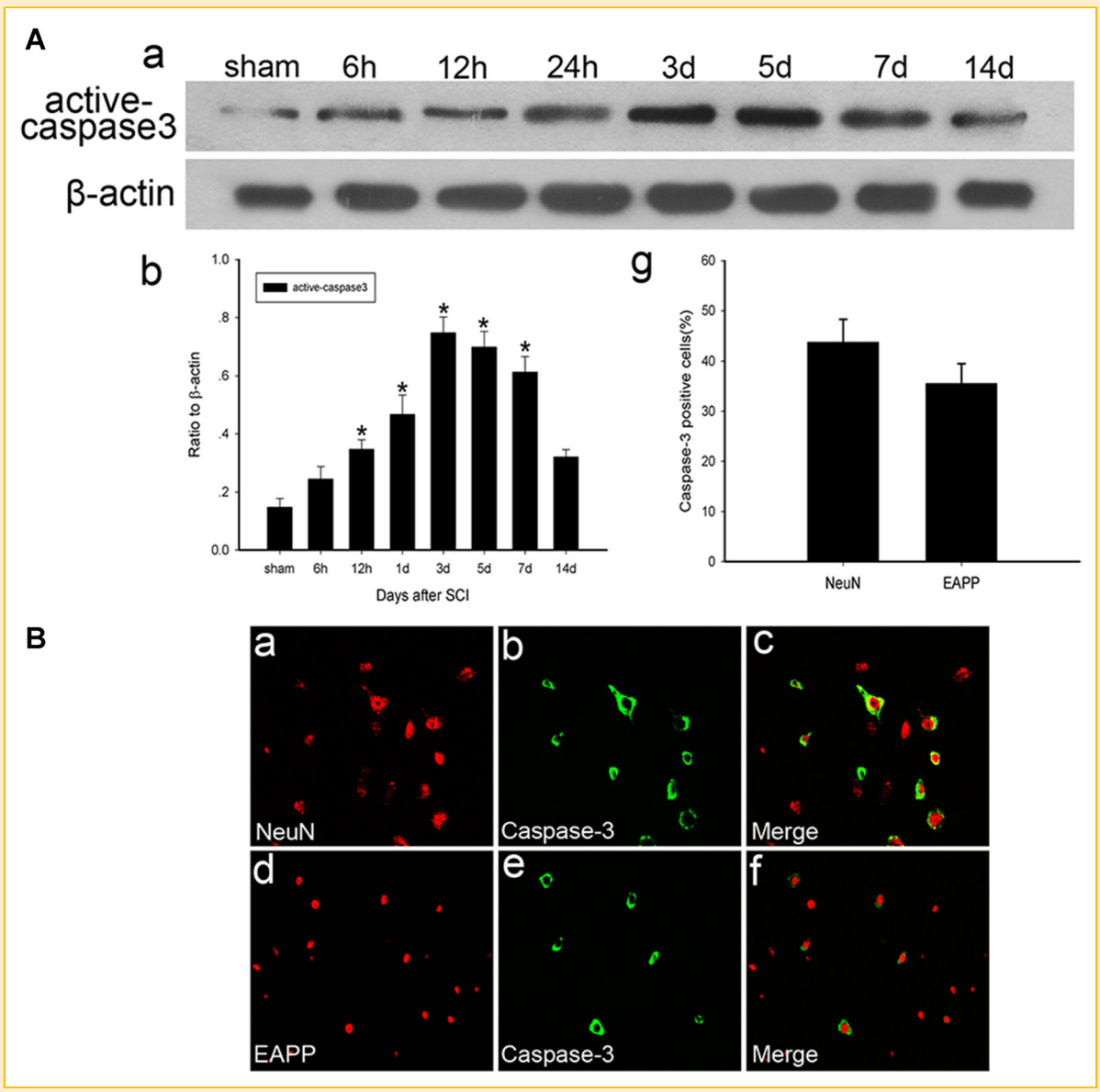

Fig. 5. Association of EAPP with apoptosis after SCI. A-a: Western blot analysis for markers of apoptosis (active caspase-3) following SCI. A-b: The bar chart showed the ratio of active caspase-3 to β-actin at each time point, the data are means ± SEM (n = 3, $^{*}P < 0.05$, significantly different from the sham group). B: Double immunofluorescence staining is for NeuN, EAPP and active caspase-3 in spinal cord at day 3 after injury after injury (a–f). Tissue sections labeled with active caspase-3 (green) and NeuN or EAPP (red) are shown in the ventral horn. (g) Quantitative analysis of NeuN- and EAPP-positive cells expressing active caspase-3 (%). n = 2 in sham group, n = 3–5 in injured group. Scale bars = 20 μm (a–f).

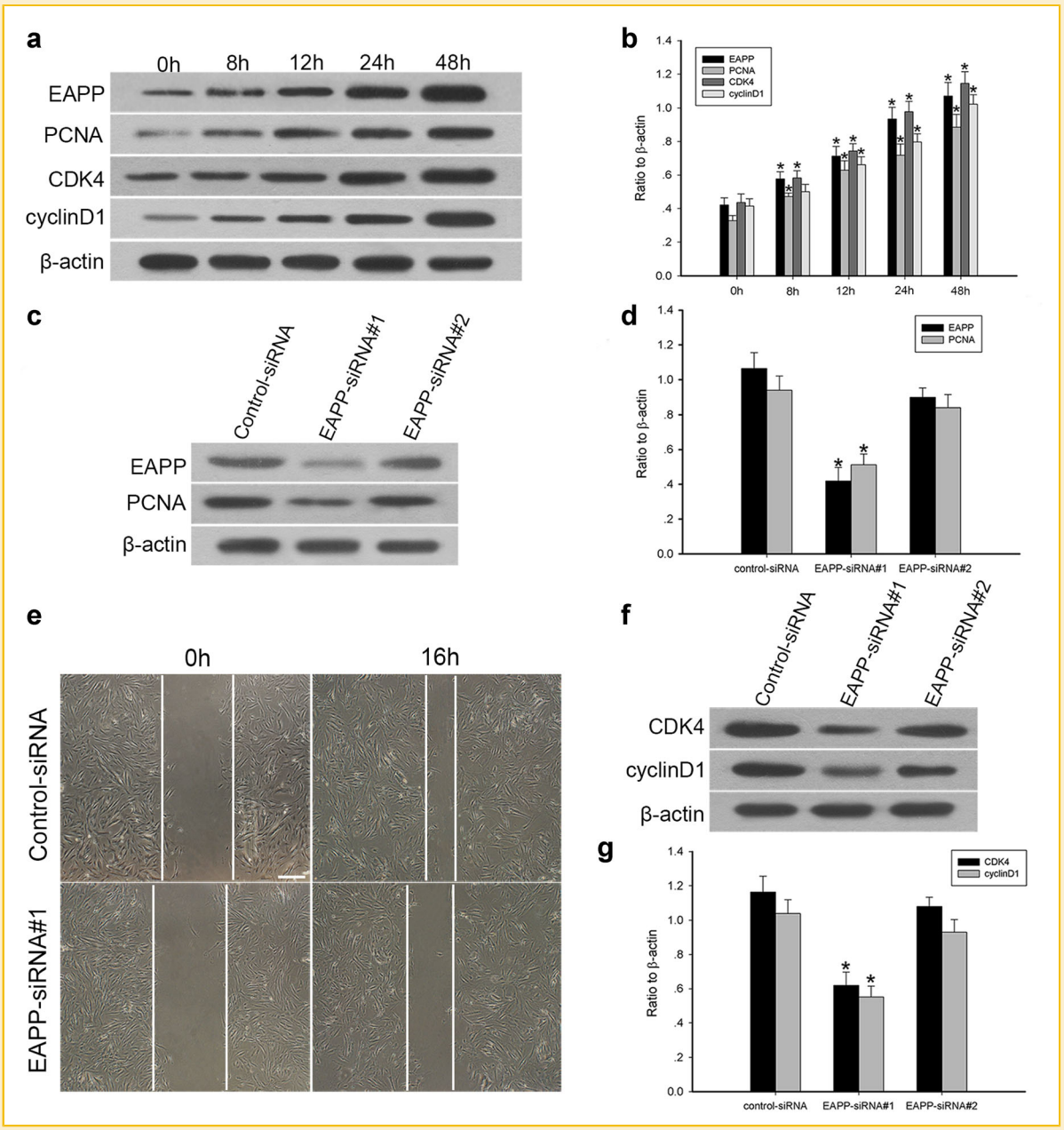

Fig. 6. Effects of EAPP-specific siRNA on cell Proliferation. (a) The expressions of EAPP, PCNA, cyclinD1 and CDK4 in proliferating primary astrocytes were evaluated by Western blotting. (b) The bar chart showed the ratio of EAPP, PCNA, CDK4 and cyclinD1 to β-actin. (c) Efficiency of EAPP-specific siRNA in primary astrocytes. Western blot analysis showed that siRNA treatment of EAPP markedly decreased EAPP and PCNA levels 48 h after siRNA transfection in primary astrocytes. (d) The bar chart showed the ratio of EAPP and PCNA to β-actin. (e) Migration of astrocytes processes into the denuded space in the scratch assay, 16 h after placing the scratch, more control-siRNA infected astrocytes have migrated into the denuded space than EAPP-siRNA#1 transduced astrocytes. Scale bars: 100 μm. (f) Western blot analysis of cell cycle related molecules in EAPP depletion primary astrocytes. (g) The bar chart below demonstrates the ratio of cyclinD1 and CDK4 protein to β-actin by densitometry. n = 9, * Indicates significant difference at $P < 0.05$ compared with sham. Error bars represent SEM.

proliferation. To investigate migration of astrocytes, we used an in vitro scratch assay in confluent siRNA transduced cultures. Sixteen hours later, the initially cell-free area was largely closed by the control-siRNA transduced astrocytes, while the space in the siRNA#1 transduced cultures remained empty (Fig. 6e). These observations show that EAPP knockdown reduces astrocyte migration in vitro.

Here, we considered the possibility that EAPP knockdown might inhibits cell proliferation by affecting the cell cycle processes. To investigate the mechanism by which knock down EAPP is related to cell cycle arrest, we measured the expression of cyclinD1 and CDK4 by western blot. Knocking down EAPP caused failure to accumulate cyclinD1 and CDK4 compared with control (Fig. 6f). In summary, these results suggested that the down-regulation of EAPP could

present a specific inhibitory effect on cell proliferation associated with cell cycle arrest in astrocytes.

EAPP KNOCKDOWN INHIBITS PRIMARY NEURONAL APOPTOSIS

To investigate the effect of EAPP on neuronal apoptosis further, neuronal apoptosis was induced by glutamate in primary cultured neurons, Western blot showed that EAPP, active Caspase-3, cyclinD1 and CDK4 expression were increased (Fig. 7a). Then, we used EAPP-specific siRNA to knock down EAPP, we determined the efficiency of the siRNA by Western blot and analysis showed that siRNA reduced the protein level of EAPP compared with control in primary neurons (Fig. 7c). We also measured the expression of active caspase-3 and showed that the protein level decreased after siRNA treatment (Fig. 7c). Moreover, we measured the expression of cyclinD1 and CDK4, the protein levels were decreased compared with negative control in EAPP depletion primary neurons (Fig. 7e). These data suggested that EAPP knockdown inhibits neuronal apoptosis associated with cell cycle arrest in primary neurons.

DISCUSSION

Traumatic SCI is a devastating disorder and a worldwide problem. The use of the rat spinal cord contusion model to recapitulate the clinical

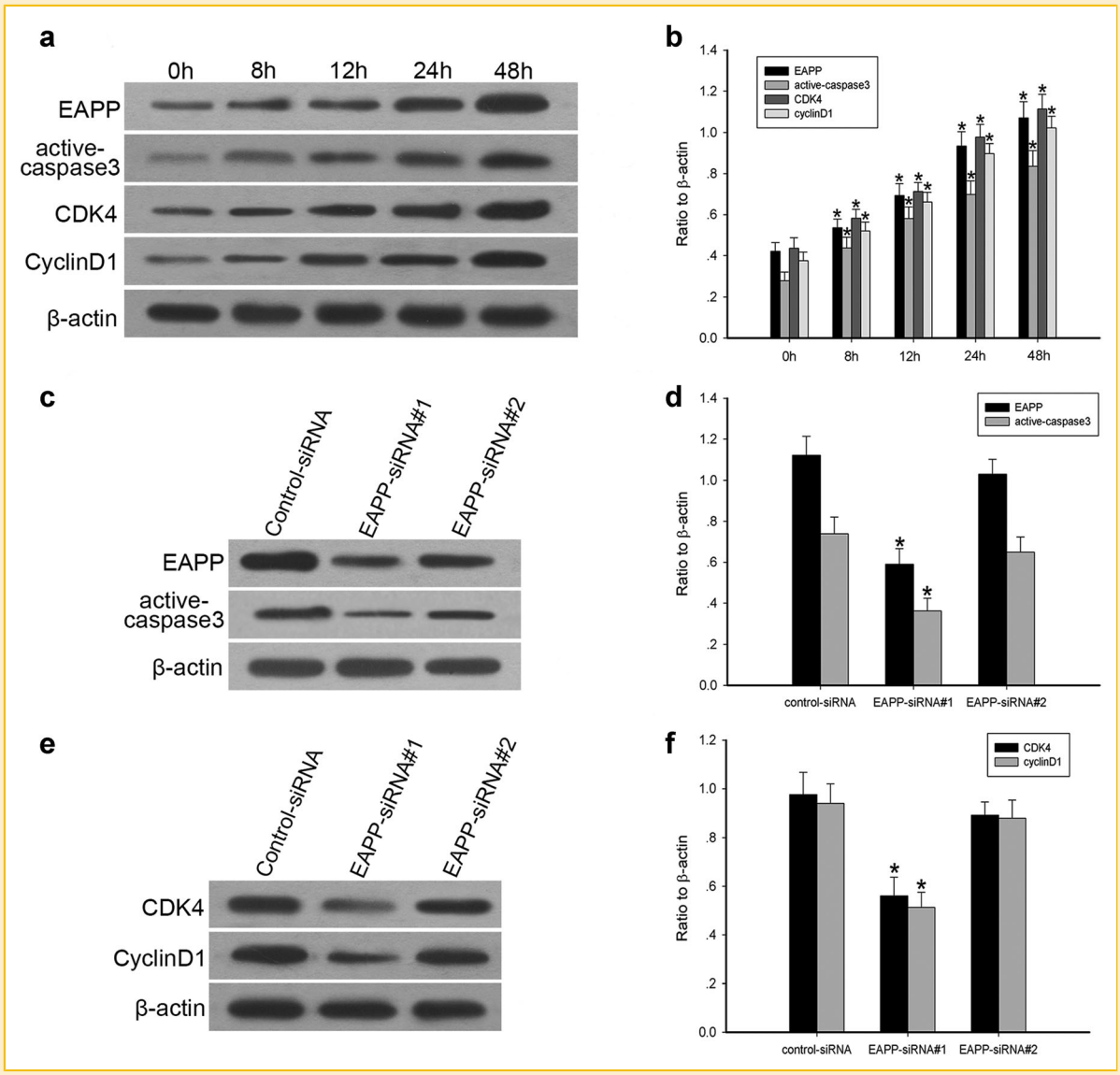

Fig. 7. Effects of EAPP-specific siRNA on glutamate induced primary neurons apoptosis. Cells were exposed to glutamate (200 μM) for 12 h at day 9, then, cells were transfected by siRNA for 48 h. (a) Western blot analysis for EAPP, active caspase-3, CDK4 and cyclinD1 in primary neurons. (b) The bar chart showed the ratio of EAPP, active caspase-3, cyclinD1 and CDK4. (c) Efficiency of EAPP-specific siRNA in primary neurons. Western blot analysis showed that siRNA treatment of EAPP markedly decreased EAPP and active caspase-3 levels 48 h after siRNA transfection in neurons. (d) The bar chart showed the ratio of EAPP and active caspase-3 to β-actin. (e) Western blot analysis of cyclinD1 and CDK4 in EAPP depletion primary neurons. (f) The bar chart below demonstrates the ratio of cyclinD1 and CDK4 protein to β-actin by densitometry. n = 9. * Indicates significant difference at $P < 0.05$ compared with sham. Error bars represent SEM.

trial is a favorable way to investigate the molecular and cellular mechanisms involved in the secondary injury. Spinal cord injury causes delayed secondary biochemical alterations, including a series of molecular and cellular events, such as neuronal apoptosis and reactive astrogliosis. These cause neuronal loss and poor outcome after SCI. Previous studies have suggested that activation of cell cycle plays an important role in mediating both neuronal apoptosis and reactive astrogliosis after SCI injury [Byrnes et al., 2007; Wu et al., 2011; Chen et al., 2014]. E2F is a family of transcription factors that integrate cell cycle progression and it plays a crucial role in cell proliferation and apoptosis. EAPP as a novel E2F binding protein and interacts with E2F1-3, comprising the activator group of E2F proteins [Novy et al., 2005]. Human EAPP is localized in the nucleus, in present study, we found the same localization of EAPP in rat spinal cord.

In the current study, for the first time, we provided some evidence that the change of EAPP protein expression after the traumatic spinal cord injury. Our results showed that the protein level of EAPP is significantly increased at day 3 after SCI, and a concomitant increase of PCNA and active caspase-3 has also been observed. Additionally, we have found the expression of EAPP is enhanced in the gray and white matter. Through double immunofluorescence staining, we found that the up-regulation of EAPP were predominant in neurons and astrocytes. Besides, the colocalization of EAPP and active caspase-3 has been detected in neurons. We have further demonstrated that EAPP had a predominant colocation with PCNA in astrocytes. These results indicated that EAPP might play an important role in neuronal apoptosis and reactive astrogliosis.

Mitotic cells, such as astrocytes and microglia, undergo cell cycle activation and proliferation after CNS injury [Kato et al., 2003]. Inhibition of cell cycle progression has been reported to inhibit proliferation of astrocytes [Wu et al., 2011]. Here, we have knocked down the expression of EAPP in primary astrocytes and the PCNA protein level has been significant inhibited by siRNA, indirectly suggested the role of EAPP in the cellular proliferation. To explore the possible mechanism relevant to down-regulation of EAPP in regulating cell proliferation of primary astrocytes, we have performed Western blot, which has revealed that siRNA reduces the cyclin D1 and CDK4 protein levels. This finding indicates that EAPP contributes to cell proliferation by affecting of cell cycle processes. Furthermore, EAPP knockdown also reduced migration of astrocytes, phenomenon that may be related to reduce GFAP expression. Reactive astrogliosis might serve either protective or destructive roles after SCI, the glial scar provides a physical barrier to regeneration and plasticity, and is a source of multiple inhibitory factors that may limit neuroplasticity [Davies et al., 1996; Silver and Miller, 2004]. Thus, we speculate that EAPP knockdown might facilitate endogenous restorative potential. The data provide preliminary evidence that EAPP plays a role in reactive astrogliosis after traumatic SCI.

Cell cycle activation has been associated with apoptotic cell death in non-proliferating cell types, such as neurons and oligodendrocytes [Di Giovanni et al., 2005], and both cell types undergo apoptotic cell death after SCI [Shuman et al., 1997; Grossman et al., 2001]. We have shown elevated levels of active caspase-3 in neurons following spinal cord injury. Caspase-3 plays an important role in caspase-dependent apoptosis pathway, and is involved in critical steps of the apoptosis [Wang et al., 2005]. Inhibition of cell cycle progression has also been reported to inhibit neuronal apoptosis [Wu et al., 2011]. Here, we induced neuronal apoptosis in primary cultured neurons by glutamate, Western blot showed that EAPP, active Caspase-3, cyclinD1 and CDK4 expression were increased. Then we knocked down EAPP in primary neurons by siRNA and observed that the active caspase-3 protein level was significant decrease. Meanwhile, siRNA also reduced the cyclin D1 and CDK4 protein levels. These results indicated that EAPP might promote neuronal apoptosis via regulate cell cycle processes.

In conclusion, the present studies have shown that EAPP expression changes after SCI, suggesting that EAPP might participate in regulating biochemical and physiological responses following this type of injury. Our results might provide a novel strategy for the treatment of spinal cord injury and the exact underlying mechanism of EAPP in regulating spinal cord injury remain for further investigation.

REFERENCES

Andorfer P, Rotheneder H. 2011. EAPP: Gatekeeper at the crossroad of apoptosis and p21-mediated cell-cycle arrest. Oncogene 30:2679–2690.

Andorfer P, Rotheneder H. 2013. Regulation of the MDR1 promoter by E2F1 and EAPP. FEBS Lett 587:1504–1509.

Andorfer P, Schwarzmayr L, Rotheneder H. 2011. EAPP modulates the activity of p21 and Chk2. Cell Cycle 10:2077–2082.

Becker EB, Bonni A. 2004. Cell cycle regulation of neuronal apoptosis in development and disease. Prog Neurobiol 72:1–25.

Boonstra J. 2003. Progression through the G1-phase of the on-going cell cycle. J Cell Biochem 90:244–252.

Byrnes KR, Stoica BA, Fricke S, Di Giovanni S, Faden AI. 2007. Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury. Brain 130:2977–2992.

Cernak I, Stoica B, Byrnes KR, Di Giovanni S, Faden AI. 2005. Role of the cell cycle in the pathobiology of central nervous system trauma. Cell Cycle 4:1286–1293.

Chen M, Xia X, Zhu X, Cao J, Xu D, Ni Y, Liu Y, Yan S, Cheng X, Liu Y, Wang Y. 2014. Expression of SGTA correlates with neuronal apoptosis and reactive gliosis after spinal cord injury. Cell Tissue Res 358:277–288.

Davies SJ, Field PM, Raisman G. 1996. Regeneration of cut adult axons fails even in the presence of continuous aligned glial pathways. Exp Neurol 142:203–216.

Di Giovanni S, Knoblach SM, Brandoli C, Aden SA, Hoffman EP, Faden AI. 2003. Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. Ann Neurol 53:454–468.

Di Giovanni S, Movsesyan V, Ahmed F, Cernak I, Schinelli S, Stoica B, Faden AI. 2005. Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc Natl Acad Sci U S A 102:8333–8338.

Draetta GF. 1994. Mammalian G1 cyclins. Curr Opin Cell Biol 6:842–846.

Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB, Dumont AS. 2001. Acute spinal cord injury, part I: Pathophysiologic mechanisms. Clin Neuropharmacol 24:254–264.

Faden AI. 1993. Experimental neurobiology of central nervous system trauma. Crit Rev Neurobiol 7:175–186.

Grossman SD, Rosenberg LJ, Wrathall JR. 2001. Temporal-spatial pattern of acute neuronal and glial loss after spinal cord contusion. Exp Neurol 168: 273–282.

Gruner JA. 1992. A monitored contusion model of spinal cord injury in the rat. J Neurotrauma 9:123–126(discussion 126–8).

Hoke A, Silver J. 1994. Heterogeneity among astrocytes in reactive gliosis. Perspect Dev Neurobiol 2:269–274.

Kabadi SV, Stoica BA, Byrnes KR, Hanscom M, Loane DJ, Faden AI. 2012. Selective CDK inhibitor limits neuroinflammation and progressive neurodegeneration after brain trauma. J Cereb Blood Flow Metab 32:137–149.

Kato H, Takahashi A, Itoyama Y. 2003. Cell cycle protein expression in proliferating microglia and astrocytes following transient global cerebral ischemia in the rat. Brain Res Bull 60:215–221.

Morris GF, Mathews MB. 1989. Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 264:13856–13864.

Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, Zhang MQ, Lazebnik Y, Bar-Sagi D, Lowe SW. 2002. Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 4:859–864.

Nguyen MD, Mushynski WE, Julien JP. 2002. Cycling at the interface between neurodevelopment and neurodegeneration. Cell Death Differ 9:1294–1306.

Nguyen MD, Boudreau M, Kriz J, Couillard-Despres S, Kaplan DR, Julien JP. 2003. Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. J Neurosci 23: 2131–2140.

Nishitani H, Lygerou Z. 2002. Control of DNA replication licensing in a cell cycle. Genes Cells 7:523–534.

Novy M, Pohn R, Andorfer P, Novy-Weiland T, Galos B, Schwarzmayr L, Rotheneder H. 2005. EAPP, a novel E2F binding protein that modulates E2F-dependent transcription. Mol Biol Cell 16:2181–2190.

Okano HJ, Pfaff DW, Gibbs RB. 1993. RB and Cdc2 expression in brain: Correlations with 3H-thymidine incorporation and neurogenesis. J Neurosci 13:2930–2938.

Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. 1999. Neuroglial activation repertoire in the injured brain: Graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev 30:77–105.

Schwarzmayr L, Andorfer P, Novy M, Rotheneder H. 2008. Regulation of the E2F-associated phosphoprotein promoter by GC-box binding proteins. Int J Biochem Cell Biol 40:2845–2853.

Shuman SL, Bresnahan JC, Beattie MS. 1997. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. J Neurosci Res 50:798–808.

Silver J, Miller JH. 2004. Regeneration beyond the glial scar. Nat Rev Neurosci 5:146–156.

Tator CH. 1996. Experimental and clinical studies of the pathophysiology and management of acute spinal cord injury. J Spinal Cord Med 19:206–214.

Tian DS, Yu ZY, Xie MJ, Bu BT, Witte OW, Wang W. 2006. Suppression of astroglial scar formation and enhanced axonal regeneration associated with functional recovery in a spinal cord injury rat model by the cell cycle inhibitor olomoucine. J Neurosci Res 84:1053–1063.

Wang ZB, Liu YQ, Cui YF. 2005. Pathways to caspase activation. Cell Biol Int 29:489–496.

Wang W, Redecker C, Yu ZY, Xie MJ, Tian DS, Zhang L, Bu BT, Witte OW. 2008. Rat focal cerebral ischemia induced astrocyte proliferation and delayed neuronal death are attenuated by cyclin-dependent kinase inhibition. J Clin Neurosci 15:278–285.

Wartiovaara K, Barnabe-Heider F, Miller FD, Kaplan DR. 2002. N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons. J Neurosci 22:815–824.

Wu J, Stoica BA, Faden AI. 2011. Cell cycle activation and spinal cord injury. Neurotherapeutics 8:221–228.

Wyndaele M, Wyndaele JJ. 2006. Incidence, prevalence and epidemiology of spinal cord injury: What learns a worldwide literature survey? Spinal Cord 44:523–529.

Zhu Z, Zhang Q, Yu Z, Zhang L, Tian D, Zhu S, Bu B, Xie M, Wang W. 2007. Inhibiting cell cycle progression reduces reactive astrogliosis initiated by scratch injury in vitro and by cerebral ischemia in vivo. Glia 55:546–558.