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EXPRESSION OF SKI AND ITS ROLE IN ASTROCYTE PROLIFERATION AND MIGRATION

X. ZHAO,[a,b] X.-W. WANG,[a,b] K.-S. ZHOU,[a,b] W. NAN,[a,b] Y.-Q. GUO,[a,b] J.-L. KOU,[a,b] J. WANG,[b] Y.-Y. XIA[a] AND H.-H. ZHANG[a]*

[a] *The Second Clinical Medical College of Lanzhou University, 82 Cuiying Men, Lanzhou 730030, PR China*

[b] *Orthopaedics Key laboratory of Gansu Province, Lanzhou 730000, PR China*

Abstract—Ski, as an evolutionarily conserved protein, is a versatile transcriptional regulator which widely distributes in various tissues and species. Recently, we have demonstrated for the first time that Ski was strikingly up-regulated in reactive astrocytes after spinal cord injury (SCI) *in vivo*, which indicates that maybe Ski is a new molecule that controls astrocytes' biological properties after SCI. However, the accurate distributions and functions of Ski in astrocytes after central nervous system (CNS) injury are still unclear. Astrocytes were collected from rats' cerebral cortex. To elucidate the expression and role of Ski in reactive astrocytes, we performed an activated astrocytes model induced by LPS and scratch injury *in vitro*. Our results showed that Ski gradually increased and reached a peak at 4 days, then declined at 6 days after induction by LPS. Up-regulation of Ski was accompanied with the increase in proliferation-related proteins including PCNA, CDK4 and CyclinD1. Furthermore, immunofluorescent staining analysis also demonstrated a highly positive relationship between Ski and GFAP, PCNA in astrocytes. These results indicated that Ski might play an important role in astrocyte proliferation. To further explore the role of Ski, astrocytes were transfected with Ski-specific small interfering RNA (siRNA). We found that the primary activated astrocytes' proliferation decreased significantly after transfection with Ski-specific siRNA. Surprisingly, Ski knockdown also weakened the primary astrocyte migration. Based on the above, we could conclude that Ski might play a crucial role in astrocyte proliferation and migration. This discovery might contribute to a promising therapeutic intervention in CNS injury.

Key words: central nervous system, spinal cord injury, Ski, astrocyte, glial fibrillary acidic protein.

INTRODUCTION

Spinal cord injury (SCI), as one of the most important worldwide health problem, is an enormous challenge in both clinical medicine and basic studies, and brings a heavy burden to patients, families and the society (Dumont et al., 2001; Zhou et al., 2017). Although plenty of studies have focused on the molecular and cellular mechanisms of SCI, the accurate pathophysiology mechanism of SCI is still unclear (Kim et al., 2017). As a consensus of recognition, there are two major mechanisms of injury after SCI: primary injury and secondary injury (Alonso-Calviño et al., 2016). The former involves tissue fracture and necrosis directly caused by external mechanical forces. The latter follows the initial impact, which may result from hemorrhage, edema, ischemia, inflammatory reactions, free radical damage, electrolyte imbalance, excitotoxicity or axonal stretch injury (Kwon et al., 2004; Kim et al., 2017). It is known that all these processes involve reactive astrocytes or astrogliosis, resulting in the formation of a dense glial scar (McGraw et al., 2001; Sofroniew, 2009). However, reactive astrocytes play an important role in the pathophysiological processes after SCI and maybe a double-edged sword (Sofroniew, 2009; Pekny et al., 2014; Lukovic et al., 2015). It's well-known that reactive astrocytes exert a detrimental effect that contributes to the formation of glial scar as a mechanical barrier to inhibit the axonal regeneration, and then, affects the functional recovery after SCI (Silver and Miller, 2004; Busch and Silver, 2007). On the contrary, plenty of evidence from diverse animal models has indicated that under normal conditions reactive astrocytes exert a protective role in the central nervous system (CNS) in many ways, such as neuroprotection and secreting growth-promoting neurotrophic factors (Anderson et al., 2003; Faulkner et al., 2004; Okada et al., 2006; Z. Liu et al., 2014). Therefore, early intervention of excessive reactive astrocytes' proliferation and migration may inhibit glial scar formation, which could provide a favorable environment for neuronal regeneration and enhance CNS injury recovery.

*Corresponding author. Address: The Orthopedics Department of the Second Hospital of Lanzhou University, 82 Cuiying Men, Lanzhou 730000, Gansu Province, PR China.
E-mail address: zhanghaih1968@163.com (H.-H. Zhang).

Abbreviations: CDK4, cyclin-dependent kinase4; CNS, central nervous system; DMEM, Dulbecco's modified essential medium; EDTA, ethylenediaminetetraacetic acid; EdU, 5-erhynyl-2′-deoxyuridine; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; IFs, intermediate filaments; LPS,, lipopolysaccharide; PBS, phosphate-buffered saline; PCNA, proliferation cell nuclear antigen; SCI, spinal cord injury; SDS–PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; SiRNA, small interfering RNA; TBS, Tris-buffered saline; TBST, TBS-containing Tween20.

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Glial fibrillary acidic protein (GFAP) is a primary intermediate filament protein and constitutes the astrocytes' cytoskeleton (Middeldorp and Hol, 2011). It has been reported that GFAP is up-regulated in reactive astrocytes after injury (Sofroniew, 2014; Sticozzi et al., 2013). Moreover, GFAP is related to astrocyte activation, motility and proliferation (Hol and Pekny, 2015; Zhan et al., 2017). All the above data demonstrates that GFAP takes part in the pathophysiological and biochemical progression after CNS injury.

Ski belongs to evolutionarily conserved protein. It is a versatile transcriptional regulator and widely distributes in various species tissues (Bonnon and Atanasoski, 2012). Ski presents various roles by interfering the proliferation and differentiation of many types of cells. Previous studies have shown that Ski plays a significant role in some physiological and pathological processes, such as proliferation of vascular smooth muscle cells (Li et al., 2013), liver regeneration (Macias-Silva et al., 2002), wound healing (Li et al., 2011) and muscle differentiation (Zhang and Stavnezer, 2009). Additionally, as a proto-oncogene, Ski participates in many types of tumors' progression via promoting cell proliferation and maintaining transcriptional program stability during development (Bonnon and Atanasoski, 2012), while the study on Ski in the nervous system is relatively rare (Baranek et al., 2012). Recently, we have demonstrated for the first time that Ski is strikingly up-regulated in reactive astrocytes after SCI *in vivo* (Zhou et al., 2017), and the expression of Ski is positively correlated with the expression of GFAP, which indicates that maybe Ski is a new molecule that controls astrocytes' biologic properties after CNS injury. Both proliferation and migration are indispensable for the recondition process after CNS injury (Becker and Bonni, 2004; Di et al., 2005). However, the expression of Ski, the relationship between Ski and astrocyte biological properties and the possible clinical significance of Ski after CNS injury have not been revealed.

In this research, the changes of temporal–spatial expression of Ski and GFAP were assessed in primary astrocyte proliferation models *in vitro*. Besides, we have observed its colocalization with proliferation cell nuclear antigen (PCNA) using a reactive astrocyte model *in vitro*. Furthermore, we have explored the potential value of Ski on astrocyte proliferation and migration by performing Ski specific small interfering RNA (siRNA) transfection. Our results showed that Ski could affect the biological properties of astrocyte, such as activation, proliferation and migration. These above data indicate that Ski takes part in pathophysiological and biochemical progression of astrocytes after CNS injury. The object of this study is to achieve better insight into the physiologic function of Ski and molecular mechanisms underlying CNS injury and recondition.

EXPERIMENTAL PROCEDURES

Isolation, purification and culture of astrocytes

Astrocytes were obtained from the cortices of neonatal Sprague–Dawley rats (1–3 days old). Briefly, cerebral cortices of neonatal rats were removed from the brain, dissected with fine forceps, transferred to petri dish which filled with Dulbecco's modified essential medium/F12 (DMEM/F12) (Life Technologies, USA), and then treated with trypsinase (Life Technologies) for digestion in CO_2 cell incubator (37 °C, 5% CO_2). Then 3 min later, specimens were transferred to a 15-mL sterile centrifuge tube, and appropriate amount of complete medium (DMEM/F12 mixture 10% fetal bovine serum (FBS) (PAN-Biotech GmbH, Germany)) was added, then centrifuged at 1500 rpm for 5 min. Then the suspension was discarded, complete medium was added to reach 4.5 ml, and it was cultured in CO_2 cell incubator (37 °C, 5% CO_2). The medium was changed with complete culture after 3 days. About 7 days later, the point of cells reached confluence. Oligodendrocytes and microglia were removed through mechanically shaking the flask at 210 rpm for 6 h at 37 °C and then the cells were trypsinized and plated for further experiment.

The purity of astrocytes was identified by double-labeling with GFAP antibody (1:400, Sigma–Aldrich) and 4′6-diamidino-2-phenylindole (DAPI) (Solarbio, Beijing, China), and the proportion of GFAP-positive cells in total cells was counted in every visual field.

Lipopolysaccharide (LPS) treatment

After the astrocytes' density reached 80% confluence, the cells were synchronized at the G0/G1 phase by incubating in serum-free DMEM/F12 culture medium for 24 h, then incubated with different concentrations (0, 0.001, 0.01, 0.1, 1, 10, 100 μg/ml) of LPS containing low concentration of serum (2%). Total proteins were collected and analyzed by Western blot. Then we choose the stimulation concentration of LPS which influences GFAP expression most obviously for the following experiments. Astrocytes were incubated for 1, 2, 3, 4, 5 and 6 days to observe the expression of Ski and GFAP protein-level change. Non-treatment cells were included as controls in all experiments.

Scratch injury model

Monolayer confluent astrocytes were scratched with sterile pipette tips (200 μl) longitudinally and latitudinally every 3 mm at right angles to each other in 35 mm dishes. This process had been described to establish a reproducible model of 30–40% damage by the Lowry's method (Liu et al., 2011). Immediately, the detached cells and debris were washed away with phosphate-buffered saline (PBS). Scratched models were continuously cultured for 1, 2, 4, 6 and 8 days for the following experiments.

Western blot analysis

Total proteins of each group were isolated from astrocytes using ice-cold RIPA buffer, and the protein concentrations were measured by the BCA Protein Assay Kit (Pierce, USA). Protein samples (20ug per lane) were separated on 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)