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Nanog interact with CDK6 to regulates astrocyte cells proliferation following spinal cord injury

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ABSTRACT

Previous research had reported transcription factors Nanog expressed in pluripotent embryonic stem cells (ESCS) that played an important role in regulating the cell proliferation. Nanog levels are frequently elevated in ESCS, but the role in the spinal cord was not clear. To examine the biological relevance of Nanog, we studied its properties in spinal cord injury model. The expression of Nanog and PCNA was gradually increased and reached a peak at 3 day by western blot analysis. The expression of Nanog was further analyzed by immunohistochemistry. Double immunofluorescent staining uncovered that Nanog can co-labeled with PCNA and GFAP in the spinal cord tissue. In vitro, Nanog can promote the proliferation of astrocyte cell by Fluorescence Activating Cell Sorter (FACS) and CCK8. Meanwhile, the cell-cycle protein CDK6 could interact with Nanog in the spinal cord tissue. Taken together, these data suggested that both Nanog may play important roles in spinal cord pathophysiology via interact with CDK6.

1. Introduction

Spinal cord injury (SCI) can lead to tissue loss and associated neurological dysfunction by both physical injuries and secondary responses [1]. Secondary injury mechanisms include neuronal apoptosis, reactive astrogliosis, scar formation and so on [2]. Glial reaction is typical feature after SCI, which may lead to toxic damage on neurons [3]. The formation of glial scar offers a physical and biochemical barrier to plasticity and regeneration and thus affects functional recovery following SCI by prompting the cell proliferation [4]. Previous studies have reported that the up-regulation of cell cycle proteins leads to reactive gliosis in many experimental animal models, including SCI [5,6], brain injury [7] and cerebral ischemia [8,9]. Some molecules including cyclins and cyclin-activated kinases can control the mechanisms of cell cycle activation through same ways [10,11]. Thus, to find the mechanism of SCI become more important for the diagnosis and treatment.

Nanog is one of the critical transcription factors expressed in pluripotent embryonic stem cells (ESCs). Since the discovery of Nanog in 2003, it was shown that Nanog, Oct4 and Sox2 are all essential for maintaining the pluripotency and self-renewal of ESCs [12–14]. Previous studies suggested that Nanog express in germline stem cells but not be found in adult tissues [15]. Recently, Nanog has drawn great attention because it was detected in multiple types of cancers, including breast cancer [16–19]. Nanog can also regulate ovarian cell migration and invasion [18]. These results indicate that Nanog has oncogenic characteristics and might be a potential target for cancer therapy. However, the biological function of Nanog in SCI is poorly understood.

CDK6 is a member of a family of serine–threonine kinases and CDK6 is a key regulator during the G1/S cell cycle transition [20]. Aberrant CDK6 expression has been reported in pancreatic cancer [21], T-cell lymphoma [22], malignant glioma [23] and medulloblastoma [24], suggesting the involvement of CDK6 in cancer. Furthermore, significant evidence indicates that overexpression of CDK6 in patients with bladder cancer is associated with a worse prognosis [25].

In this study, we first investigated temporal-spatial patterns of Nanog at protein level and its co-localization with PCNA using an acute SCI model on adult rats, compared to previous studies on tumors. And, we found the Nanog can interact with cell-cycled protein CDK6 in the SCI model. These results were conducted to gain greater insight into the functions of Nanog and its roles in the cellular and molecular mechanisms underlying spinal cord injury and repair.

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2. Materials and methods

2.1. Animals and surgery

All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch. Male Sprague–Dawley rats (n = 60) with an average body weight of 250 g (220–275 g) were used in our study. Dorsal laminectomies at the level of the ninth thoracic vertebra (T9) were performed under anesthesia with pentobarbital (50 mg/kg i. p.). Contusion injuries (n = 50) were performed using the NYU impactor [26]; [27], the exposed spinal cord was contused by dropping a 2.0 mm in diameter and 10 g in weight rod from a height of 100 mm. Sham-operated animals (n = 10) were anesthetized and surgically prepared but did not undergo spinal injury. After SCI, the overlying muscles and skin were closed in layers with 4–0 silk sutures and staples, and the animals were returned to their home cages. 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 and postoperative infections. Bladders were manually expressed twice daily until reflex bladder emptying returned. All animals were then housed under a 12-h light/dark cycle in a pathogen-free area, and the room temperature was kept at 37 ± 0.5 °C with free access to water and food. SCI animals were sacrificed at 6 h, 12 h, 1 day, 3 days, 5 days, 7 days, and 14 days after injury. The sham groups were used as non-injured controls. Moreover, two rats were lost in the SCI groups. All efforts were made to minimize the number of animals used and their suffering.

2.2. Western blotting analysis

To extract the protein for western blotting analysis, the sham or injured spinal cords were excised (n = 3 for each time point). A 10-mm-length spinal cord rostral and caudal to the injury epicenter was immediately dissected out and snap frozen at−80 °C until use. In order to prepare lysates, frozen spinal cord samples were minced with eye scissors on ice. The samples were then homogenized in lysis buffer [1% NP-40, 50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 1% sodium dodecyl sulfate (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 (100 μg of protein) was subjected to SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, Massachusetts, USA) by a transfer apparatus at 300 mA for 2.0 h. The membrane was then blocked with 5% nonfat milk and incubated with primary antibodies against Nanog (anti-mouse, 1:500; Santa Cruz), PCNA (anti-mouse, 1:1000; Santa Cruz), cyclinD1 (anti-mouse, 1:1000; Santa Cruz), cyclinA (anti-mouse,1:1000; Santa Cruz), CDK6(anti-rabbit, 1:1000; Santa Cruz), GAPDH (anti-rabbit,1:1000; Santa Cruz) and β-actin (anti-mouse, 1:1000; Santa Cruz) at 4 °C overnight. After incubating with horseradish peroxidaseconjugated (1:2000; Southern-Biotech) secondary antibody, protein was visualized using an enhanced chemiluminescence system (ECL, Pierce Company, USA).

3. Cell culture

3.1. Isolation, purification and culture of astrocytes

Spinal cord was taken from 3-day-old Sprague–Dawley rats and the tissues were gently minced by forceps and then dissociated in phosphate-buffered saline (PBS; Sigma) containing 0.1% collagenase A and 0.25% trypsin (Sigma) for 15 min at 37 °C. After centrifugation at 1200 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 and replaced with DMEM supplemented with F12 (Sigma) containing 10% FBS and then the cells were resuspended and plated in tissue culture flasks 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 was shaked at 200 rpm for 2 h at 37 °C to remove oligodendrocytes and microglia that were growing on top of the confluent astrocyte layer and then the culture medium was replaced. After 24 h, the cells were trypsinized and plated at a density of 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 [28,29].

3.2. SiRNA and transfection

The rat Nanog-specific RNAi duplex with 3′-dTdT overhang was synthesized in Invitrogen. Target: 1# GCAATGGTGTGACGCAGAA; 2# CCAGACCTGGAACAATTCA; 3# CGTGTGAAGAGTGAAA and scrambled shRNA control plasmid (shanghai) for transient transfection, the Nanog siRNA vector or the non-specific vector was transfected using lipofectamine 2000 (Invitrogen). After 6 h of transfection, the medium was replaced with DMEM medium supplemented with 10% FBS. Approximately 30 h after transfection, cells were harvested for analysis.

3.3. Immunohistochemistry

After defined survival times, sham-operated and injured rats were terminally anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde. After perfusion, the sham-operated and injured spinal cords were removed and post-fixed in the same fixative solution for 6 h and then replaced with 20% sucrose for 1–2 days, followed by 30% sucrose for 2–3 days. After treatment with sucrose solutions, the tissues were embedded in O.C.T. compounds. 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% donkey 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 anti-Nanog antibody (anti-rabbit, 1:100; Santa Cruz), followed by incubation in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). Staining was visualized with DAB (Diaminobenzidine, 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.

3.4. Double immunofluorescent staining

The frozen cross-sections (8-μm) were prepared and examined. All sections were first blocked with 10% normal donkey serum blocking solution from the same species as the secondary antibody, containing 3% (w/v) BSA and 0.1% Triton X-100 and 0.05% Tween-20, for 2 h at RT to avoid nonspecific staining. Then, the sections were incubated with both primary antibodies for anti-Nanog (anti-mouse, 1:100; Santa Cruz) and different cell markers as follows: NeuN (neuron marker, anti-rabbit, 1:100;