Biochemical and Biophysical Research Communications xxx (2017) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PAX3 inhibits β-Tubulin-III expression and neuronal differentiation of neural stem cell

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ARTICLE INFO

Article history:
Received 25 January 2017
Accepted 17 February 2017
Available online xxx

Keywords:
β-Tubulin-III
Paired box 3 protein
Neural stem cells
Neuronal differentiation

ABSTRACT

PAX3 functions at the nodal point in neural stem cell maintenance and differentiation. Using bioinformatics methods, we identified PAX3 as a potential regulator of β-Tubulin-III (TUBB3) gene transcription, and the results indicated that PAX3 might be involved in neural stem cell (NSC) differentiation by orchestrating the expression of cytoskeletal proteins. In the present study, we reported that PAX3 could inhibit the differentiation of NSCs and the expression of TUBB3. Further, using luciferase and electrophoretic mobility shift assays, we demonstrated that PAX3 could bind to the promoter region of *TUBB3* and inhibit *TUBB3* transcription. Finally, we confirmed that PAX3 could bind to the promoter region of endogenous *TUBB3* in the native chromatin of NSCs. These findings indicated that PAX3 is a pivotal factor targeting various molecules during differentiation of NSCs *in vitro*.

1. Introduction

Members of the *PAX* family are reported to be key regulators of processes as diverse as migration, differentiation, and survival during organogenesis in embryonic development [1–3]. A mutation in *PAX* was responsible for development of splotch (sp) phenotype in mice and waardenburg syndrome type 1 (WS1) in humans [4]. In the nervous system, PAX3 is involved in the neural tube closure, neural crest development, and peripheral neuron differentiation [1]. PAX3 functions at the nodal point in stem cell maintenance and differentiation by regulating different transcription factors crucial for self-renewal or neurogenesis in neural stem/progenitor cells. For example, PAX3 regulates Hairy and Enhancer of split homolog-1 (HES-1) and Neurogenin 2 (Ngn2) by directly binding to their promoters, which may couple neural stem cell maintenance and neurogenesis [5,6]. In addition, PAX3 could directly regulate the expression of cytoskeletal proteins, which are spatially restricted in different neural cells. We have previously reported that PAX3 negatively regulates glial fibrillary acidic protein (GFAP) during differentiation of NSCs into astrocytes [7]. Thus, we further investigated whether it could affect the neuron-specific cytoskeletal proteins during differentiation of NSCs to neurons.

To initiate differentiation of neural cells upon growth and guidance cues, dynamic populations of microtubules are required [8]. Microtubules are polarized structures composed of α/β tubulin dimers. β-tubulin III (TUBB3), a β-tubulin with expression primarily limited to neurons, is crucial for neurite outgrowth because microtubules enriched in TUBB3 are considerably more dynamic than those enriched in other β-tubulin isotypes [9,10]. Thus, the spatiotemporal expression pattern of TUBB3 might be accurately regulated for neurogenesis and axon maintenance. However, the regulatory mechanism of TUBB3 expression is still unknown. The direct way is to retrieve the transcription factors that could bind to the promoter region of this gene. In our preliminary study, the promoter region of *TUBB3* was analyzed with Genomatix software to identify new transcription factors that may regulate *TUBB3* expression. Two potential PAX3-binding sites were identified that contain well-recognized DNA motifs. In the present study, we investigated the expression pattern of PAX3 and TUBB3 in an *in vitro* model of NSC differentiation, and we demonstrated that PAX3 directly binds to the *cis*-regulatory elements on the *TUBB3* promoters and inhibits neurite outgrowth.

2. Materials and methods

2.1. Culture and differentiation of NSCs

NSCs were prepared from the cerebral cortex of E14 Sprague-Dawley (SD) rats as described previously [11]. The cells were

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http://dx.doi.org/10.1016/j.bbrc.2017.02.086
0006-291X/

maintained in DMEM/F12 medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% N2 (Invitrogen), 20 ng/mL recombinant bFGF, and 20 ng/mL EGF (Sigma). To induce neuronal differentiation, bFGF and EGF were withdrawn from the medium, and the NSCs were seeded onto either dishes or coverslips coated with poly-l-lysine (Sigma) and fibronectin (Invitrogen).

2.2. Bioinformatics analysis and plasmid construction

The promoter region, about 2 kb upstream of transcription start site of rat *TUBB3,* was analyzed by Genomatix software (http://www.genomatix.de) to retrieve the potential binding sites of transcription factors. The promoter sequence spanning from −2028−+35 bp of rat *TUBB3* was cloned into pGL3-Basic luciferase reporter vector (Promega) and termed as pTUBB3-2000. The primers designed for polymerase chain reaction (PCR) analysis are as follows: sense, 5′-*cg*acgcgtTTCAGGCTCCTTCCAGTG-3′; antisense, 5′-*ccg*ctcgagCTCCCTCATGCTGACTTCAC-3′. The pCI-PAX3 plasmid was also constructed. The primers designed for PCR analysis are as follows: pCI-neo-PAX3-sense, 5′-GGAATTCAT-GACCACGCTGGCCGG-3′; pCI-neo-PAX3-antisense, 5′-ACGCGTC-GACGAACGTCCACGGCTTAC-3′. All of these constructs were confirmed by sequencing.

2.3. PAX3 knock down, overexpression, and infection with adenovirus

The target sequences used to knock down PAX3 by shRNA interference were as follows: sense, 5′-AGCTGGGAAATCAGAGA-CAAA-3′; antisense, 5′-TTTGTCTCTGATTTCCCAGCT-3′. The *hU6* promoter and shRNA sequence were subcloned into pYrbio-hU6-EGFP-shRNA vector. The control and PAX3 overexpression adenoviral constructs were established as described previously [7]. The generated adenoviral construct was used to produce recombinant viruses. Then, the NSCs were transferred onto glass coverslips in 24-well dishes for virus infection. After culturing for an additional indicated time, the NSCs were fixed and processed for immunocytochemistry analysis followed by microscopic observation.

2.4. Plasmid transfections and luciferase assays

HEK 293T cells were purchased from American Type Culture Collection (ATCC) and cultured in the recommended medium and conditions. Luciferase reporter assay was performed by co-transfecting *TUBB3* promoter reporter plasmid, PRL (Promega), and pCI-PAX3 or pCI-neo into HEK 293T cells in triplicate by Lipofectamine 2000 (Invitrogen). The cell lysates were processed for luciferase activity using the Dual Luciferase Assay Kit according to the manufacturer's specifications (Promega, http://www.promega.com).

2.5. Electrophoretic mobility shift assay

Nuclear extracts from HEK 293T cells transfected with pCI-PAX3 were prepared and Electrophoretic mobility shift assay (EMSA) was performed as described previously [12]. The oligonucleotide sequences used in this assay are given below and the mutated bases are shown in italics and bold. P1 probe-sense, 5′-TTCGTCCAGCTTGCACACC-3′; P1 probe-antisense, 5′-GGTGTGCAAGCTGGACGAA-3′; P2 probe-sense, 5′-TTCGA-CATCCTCCGCTGAG-3′; P2 probe-antisense, 5′-CTCAGCGGAG-GATGTCGAA-3′; MP1 probe-sense, 5′-T***GATG***CCAGCTTGCACACC-3′; MP1 probe-antisense, 5′-GGTGTGCAAGCTGG***CATC***A-3′; MP2 probe-sense, 5′-T***GATC***CATCCTCCGCTGAG-3′; MP2 probe-antisense, 5′-CTCAGCGGAGGATG***GATC***A-3′. For supershift assay, nuclear extracts were pre-incubated with anti-PAX3 polyclonal antibodies (Santa Cruz) before addition of the biotin-labeled probes.

2.6. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using a commercially available kit (Upstate Biotechnology) according to the manufacturer's instructions. The oligonucleotide primers for PCR applied to detect the fragment of the *TUBB3* promoter region are as follows: TUBB3-sense1, 5′- GACCCATTTC-TAAACTCGGCTAAC-3′; TUBB3-antisense1, 5′-ACATCCATTTCACAAGAGCCG-3′; TUBB3-sense2, 5′-TCCA-TACCCCCTCTTTGCC-3′; TUBB3-antisense2, 5′-TGAGTTTGCT-GAGTCGCTTGA-3′. For immunoprecipitation, anti-PAX3 (sc-34918, Santa Cruz) or mouse IgG (negative control) antibodies were added and incubated overnight at 4 °C.

2.7. Immunocytochemistry

The cultured cells were fixed in 4% paraformaldehyde for 20 min and were incubated with PAX3 polyclonal antibody (1:300, Abbexa), TUBB3 antibody (1:600, Abcam; and 1:2000, BioLegend), and GFAP polyclonal antibody (1:400, Cell Signaling Technology), respectively. Fluorescence (Cy3 or FITC)-labeled secondary antibodies were added and further incubated, followed by visualization by microscopy.

2.8. Western blot

Western blot analysis was performed as described previously [13]. Briefly, protein samples were separated by SDS-PAGE, and then transferred onto PVDF membranes, which were blocked with 5% nonfat dry milk in Tris-buffer saline (TBS), followed by incubation with primary and secondary antibodies, then finally the signal was developed using enhanced chemiluminescence method. The antibodies used were GAPDH polyclonal antibody (1:800, Santa Cruz), PAX3 polyclonal antibody (1:200, Abbexa), nestin (1:300, Santa Cruz Biotechnology), beta-actin (1:2000, Proteintech), Cy3-AffiniPure Goat Anti-Rabbit IgG (1:400, Jackson), Alexa Fluor® 488-AffiniPure Goat Anti-Mouse IgG (1:400, Jackson), and FITC-conjugated AffiniPure Donkey Anti-Goat IgG (1:400, Jackson).

3. Results

3.1. PAX3 is expressed in NSCs and is downregulated during differentiation of NSCs

PAX3 is a key regulator in development of multiple tissues and organs, including those in the nervous system [14]. The effect of PAX3 on the neural tissue was usually focused on the cells derived from neural crest [1,15], and its effect on the differentiation of NSCs into glial cells has been previously reported [7]. In this study, we found that PAX3 was expressed in NSCs derived from rat embryonic brain, and its expression was decreased after induction of neuronal differentiation by withdrawing bFGF and EGF from the medium. Upon induction treatment, the number of differentiated cells increased, while the PAX3 level decreased continually. After 96 h of induction, the number of TUBB3-, GFAP-, and O4-positive cells was 70%, 22%, and 6%, respectively (Fig. 1). The results of western blotting further confirmed that PAX3 expression was negatively correlated to TUBB3 expression, which indicated that *TUBB3* might be a target candidate for PAX3 regulation.

Please cite this article in press as: S. Cao, et al., PAX3 inhibits β-Tubulin-III expression and neuronal differentiation of neural stem cell, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.02.086