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ORIGINAL RESEARCH

Regulatory Role of the JNK-STAT1/3 Signaling in Neuronal Differentiation of Cultured Mouse Embryonic Stem Cells

Zheng Zachory Wei · Shan Ping Yu · Jin Hwan Lee · Dongdong Chen · Tammi M. Taylor · Todd Carter Deveau · Albert Cheung Hoi Yu · Ling Wei

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Abstract Stem cell transplantation therapy has provided promising hope for the treatment of a variety of neurodegenerative disorders. Among challenges in developing disease-specific stem cell therapies, identification of key regulatory signals for neuronal differentiation is an essential and critical issue that remains to be resolved. Several lines of evidence suggest that JNK, also known as SAPK, is involved in neuronal differentiation and neural plasticity. It may also play a role in neurite outgrowth during neuronal development. In cultured mouse embryonic stem (ES) cells, we test the hypothesis that the JNK pathway is required for neuronal differentiation. After neural induction, the cells were plated and underwent differentiation for up to 5 days. Western blot analysis showed a dramatic increase in phosphorylated JNKs at 1–5 days after plating. The phosphorylation of JNK subsequently induced activation of STAT1 and STAT3 that lead to expressions of GAP-43, neurofilament, βIII-tubulin, and synaptophysin. NeuN-colabelled with DCX, a marker for neuroblast, was enhanced by JNK signaling. Neuronal differentiation of ES cells was attenuated by treatment with SP600125, which inhibited the JNK activation and decreased the activation of STAT1 and STAT3, and consequently suppressed the expressions of GAP-43, neurofilament, βIII-tubulin, and the secretion of VEGF. Data from immunocytochemistry indicated that the nuclear translocation of STAT3 was reduced, and neurites of ES-derived neurons were shorter after treatment with SP600125 compared with control cells. These results suggest that the JNK-STAT3 pathway is a key regulator required for early neuronal differentiation of mouse ES cells. Further investigation on expression of JNK isoforms showed that JNK-3 was significantly upregulated during the differentiation stage, while JNK-1 and JNK-2 levels decreased. Our study provided interesting information on JNK functions during ES cell neuronal differentiation.

Keywords Embryonic stem cell · JNK · STAT3 · Neurite outgrowth · Neuronal differentiation

Z. Z. Wei · S. P. Yu · J. H. Lee · D. Chen · T. M. Taylor · T. C. Deveau · L. Wei (✉)
Department of Anesthesiology, Emory University School of Medicine, 101 Woodruff Circle, Suite 617, Atlanta, GA 30322, USA
e-mail: lwei7@emory.edu

Z. Z. Wei · S. P. Yu · D. Chen
Center for Visual and Neurocognitive Rehabilitation, Atlanta VA Medical Center, Decatur, GA 30033, USA

A. C. H. Yu
Neuroscience Research Institute and Department of Neurobiology, Peking University School of Basic Medical Sciences, Beijing 100191, China

L. Wei
Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA

Introduction

Regenerative medicine based on transplantation of exogenous neural progenitor cells derived from pluripotent stem cells and stimulation of endogenous neural stem/progenitor cells has generated potential therapies for repair of damaged brain structures. Transplantation of stem cell-derived neural progenitor cells has been explored for treatment of neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), spinal cord injury, and

ischemic stroke (Liu et al. 2013; Yu et al. 2013). In stem cell-based therapy, neuronal differentiation is a key step for regeneration and replacement of neurons and neural networks. In vitro, embryonic stem (ES) cells could be induced to differentiate into neurons by retinoic acid (RA) at the embryonic stage. These ES-derived cells have the typical neuronal morphology and express neuronal associated proteins, including βIII-tubulin, neurofilament, synaptophysin, glutamate receptors, acetylcholine receptors, and acetylcholinesterase (Anjomshoa et al. 2009; Engberg et al. 2010). Compared with other cell lines, ES cell-derived neurons are better characterized for neuronal differentiation although the molecular mechanism of regulation is not well defined. Using ES cells as a stem cell model, we explored possible signaling pathways that might regulate neuronal differentiation and associated morphological alterations.

Neuronal differentiation involves changes in electrophysiological properties and morphological characteristics including growth of the dendrites and axons that directly affect the number of synaptic contacts through dendritic arbors and establish distinct functional outputs during development (He et al. 2005). Previous work has studied intracellular signaling components that control neurite outgrowth (Song et al. 2009; Ming et al. 2002). More recently, the c-Jun N-terminal kinase (JNK) signaling, also known as SAPK, has been implicated in playing an important role in neurite outgrowth (Qu et al. 2013). JNK is a sub-family member of mitogen-activated protein kinase (MAPK) and a multifunctional kinase involved in cell survival (Himes et al. 2006; Fraser et al. 2013), apoptosis (Ouyang and Shen 2006), proinflammatory cytokine production (Chang et al. 2006), and osmotic imbalance (Fanger et al. 1997). Recent studies indicated an important function of JNK signaling in embryogenesis and neuronal differentiation (Haeusgen et al. 2009). For instance, activation of JNK is required for neurite outgrowth of dopaminergic neurons (Pan et al. 2009), neural progenitors and glutamatergic neurons (Tiwari et al. 2012), the rat pheochromocytoma cell line PC12 cells (Waetzig and Herdegen 2003), and the human neuroblastoma cell line SH-SY5Y (Yu et al. 2003). JNK phosphorylates several transcription factors, including c-Jun, ATF-2, Elk-1, p53, Klf-4, and STAT3 (Weston and Davis 2007; Kook et al. 2013; Yao et al. 2014). STAT3 is involved in CB1-receptor–$G\alpha_{i/o}$-induced neurite outgrowth in the mouse neuroblastoma cell line Neuro-2A cells through the JNK pathway (He et al. 2005). How JNK signaling affects neuronal differentiation of cultured ES cells remains unknown. We tested the hypothesis that the activation of the JNK-STAT1/3 pathway may play an important role in neurite outgrowth for neuronal differentiation of ES cell-derived neurons.

Materials and Methods

Materials

JNK/SAPK, JNK-1, JNK-2, JNK-3, and phospho-SAPK/JNK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Stat1, Stat3, doublecortin (DCX), Bcl-2, VEGF, BDNF, and GDNF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). βIII-tubulin, synaptophysin, neuron-specific nuclear protein (NeuN), and neurofilament (NF) were purchased from Millipore (Billerica, MA, USA). Growth-associated protein 43 (GAP-43) was purchased from Boehringer Mannheim Company (Ingelheim, Germany). Retinoic acid (RA) was purchased from Sigma Aldrich (St Louis, MO, USA). SP600125, the specific inhibitor of JNKs, was purchased from A. G. Scientific. Inc. (Scientific. Inc, San Diego, CA, USA). VEGF-ELISA kit was purchased from RayBio (Norcross, GA, USA).

Embryonic Stem Cell Culture

The mouse ES D3 cell line was obtained from Dr. David Gottlieb (Washington University, St. Louis, MO, USA) and maintained in our laboratory. In our investigation, passages 19–32 of ES cells were used. Undifferentiated ES cells were maintained in ES cell growth media (ESGM) which consists of Dulbecco's Modified Eagle Media (DMEM) (Invitrogen, Calsbad, CA, USA) with 4.0 Mm L-glutamine, 4500 mg/l glucose, supplemented with 5 % fetal bovine serum (Lot No. 1379819; Invitrogen, Carlsbad, CA, USA), 5 % newborn calf serum (Lot No. 8092154; Invitrogen, Carlsbad, CA, USA), 8 μg/ml adenosine, 8.5 μg/ml guanosine, 7.3 μg/ml cytidine, 7.3 μg/ml uridine (Sigma Aldrich, St Louis, MO, USA), 2.4 μg/ml thymidine, leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA) at 1,000 units/ml, and 0.1 mM β-mercaptoethanol (Sigma Aldrich, St Louis, MO, USA). Undifferentiated ES cells were passaged every 2 days on gelatin-coated T25 flasks. The cell viability was assessed during culture process as previously described (Wei et al. 2005).

For neural precursor lineage induction, the cells were isolated from T25 flasks with 0.25 % trypsin–EDTA and placed onto a standard 100-mm bacterial Petri dish in ES cell induction media (ESIM, ESGM without adding LIF, and β-mercaptoethanol). Four days later, the culture media were replaced with ESIM containing 5×10^{-7} M retinoic acid (RA) for another 4 days. Upon termination of the "4−/4+" induction protocol, embryoid bodies (EB) were washed in a balanced salt solution and resuspended with 0.25 % trypsin–EDTA for 10 min at 37 °C. ESIM was added to stop trypsinization, and cells were digested into single-cell suspension and spun at 1,000×g for 5 min.

Then, cells were resuspended in modified Sato medium (van Inzen et al. 1996; Mohamad et al. 2014). Cells were then plated on poly-D-lysine (100 μg/ml) and murine laminin (4 μg/ml) (BD Biosciences, San Jose, CA, USA) coated 35-mm glass-bottom dishes for image analysis or onto 35-mm plastic dishes for protein isolation. In inhibition tests, SP600125 was resuspended in DMSO and added to the culture media at 10 μM after plating. At this concentration, SP600125 inhibited activation of all three JNK members (JNK-1, JNK-2, and JNK-3) (Bennett et al. 2001). Two days after plating, cytosine arabinoside (Ara-C) was added at a final concentration of 10 μM to halt cell division. All the experiments were performed in triplicate (at minimum) per sample.

Western Blotting Analysis and Protein Isolation

For Western blotting analysis, the proteins were isolated in undifferentiated ES cells, EB stage, and ES cell-derived neurons 1, 3, 5, and 7 days after plating. Cells were washed twice with ice-cold PBS and lysed in a medium containing 50 mM Tris–Hcl (pH 8.8), 150 mM NaCl, 2 mM EDTA, 1 % SDS, 2 mM sodium orthovanadate, 1 % NP-40, 1 % sodium deoxycholate, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were scraped from the dish, vortexed, and spun at 14,000×*g* for 25 min to remove cell debris. Supernatants were then used for determination of protein concentration using bicinchoninic acid assay (Pierce, Rockford, IL, USA). Cell lysates were separated by SDS-PAGE using gradient gels (6-15 %). Gels were blotted onto PVDF membranes (Amersham, UK). The blots were blocked with 5 % milk in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1 % Tween 20) and incubated with primary antibody overnight. For JNK family, JNK-1, JNK-2, JNK-3, and p-JNK were analyzed. In the second day, blots were washed 3 times with TBST buffer, and then incubated with AP-conjugated secondary antibody (1:5,000; Promega, Madison WI, USA) in TBST with 5 % milk. The blots were then washed 3 times with TBST buffer and three times with TBS buffer before developing membrane by BCIP/NBT substrate colorimetric method.

For separation of nuclear and cytoplasmic components, cells were washed with PBS three times, resuspended in hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF, and 0.5 mM Nonidet P-40 with protease and phosphatase inhibitors, and incubated on ice for 20 min. The nuclei were removed by centrifugation, and the supernatant (the cytoplasmic extract) was collected. The nuclear proteins were extracted in RIPA buffer containing 25 mM Tris–Hcl (pH 7.5), 150 mM NaCl, 0.1 % SDS, 1 % Triton, and 5 mM EDTA. The Image J program (Java 1.6.0; NIH, Bethesda, MD, USA) was used to compare the relative densities of the bands.

Immunocytochemistry

Immunocytochemical staining was used to detect the expressions of JNK/SAPKs, phospho-SAPK/JNKs, Stat1, Stat3, GAP-43, neurofilament, βIII-tubulin, and doublecortin, as well as NeuN. ES-derived cells were washed with phosphate-buffered saline (PBS), fixed with 10 % buffered formalin for 10 min, then permeabilized with ethanol:acetic acid (2:1) for 10 min and washed with PBS. Cells were blocked with 2 % fish gel in 0.2 % triton and then incubated in primary antibody in PBS overnight at 4 °C. Cells were washed in PBS and incubated with secondary antibody conjugated with Cy3 or Alexa488 (Jackson Laboratory, West Grove, PA, USA) in PBS. Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA) was used to stain nuclei. Results were visualized under an epifluorescence microscope (Olympus America Inc. New York, USA).

Measurement of Neurite Outgrowth in the Cultures of ES Cell-Derived Neurons

All photographs were taken from 8 to 11 randomly selected fields per slide, and same numbers of neurons were counted. Total neuritic extent and primary neurites of ES cell-derived neurons were measured among ES cell-derived βIII-tubulin and/or NF positive neurons using the Image-J system and NeuriteTracer plugin (Pool et al. 2008). A neurite was identified as any process longer than two cell diameters of the cell body in length. The primary neurite length was defined as the distance from the soma to the tip of the longest branch. Total neuritic extent was defined as the combined lengths of all neurites (Eom et al. 2005).

Electrophysiology of Whole-Cell Recordings

Whole-cell patch clamp recording was performed on mouse ES D3 cell-derived neurons at 10 days after the termination of RA induction. The membrane currents or action potentials were collected using an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany) at room temperature. The external solution contained 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose at a pH of 7.4. Recording electrodes were pulled from borosilicate glass pipettes (Sutter Instrument, USA). The tip resistance was between 5 and 7 MΩ when filling it with the internal solution consisted of 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES at a pH of 7.2. Series resistance was compensated by 75–85 %. Linear leak and

residual capacitance currents were subtracted online using a P/6 protocol. Action potentials were recorded under current-clamp mode using Pulse software (HEKA Elektronik). Data were filtered at 3 kHz and digitized at sampling rates of 20 kHz.

Statistical Analysis

All data were analyzed by Student's *t* test or one-way ANOVA. All values represent Mean ± SEM. Significance was assumed at a *P* value of 0.05 in all statistical analyses. Randomization was performed, and the sample size was determined using Power analysis (Power and Precision 4; Biostat, Inc, Englewood, NJ, USA).

Results

JNK/SAPK Expression and Its Effect on Neuronal Differentiation of ES Cells

Cultured mouse ES cells were treated with retinoic acid (RA) to generate neural progenitors using the 4−/4+ protocol (Theus et al. 2008). Western blot analysis on naive ES cells and neural progenitors after the RA induction

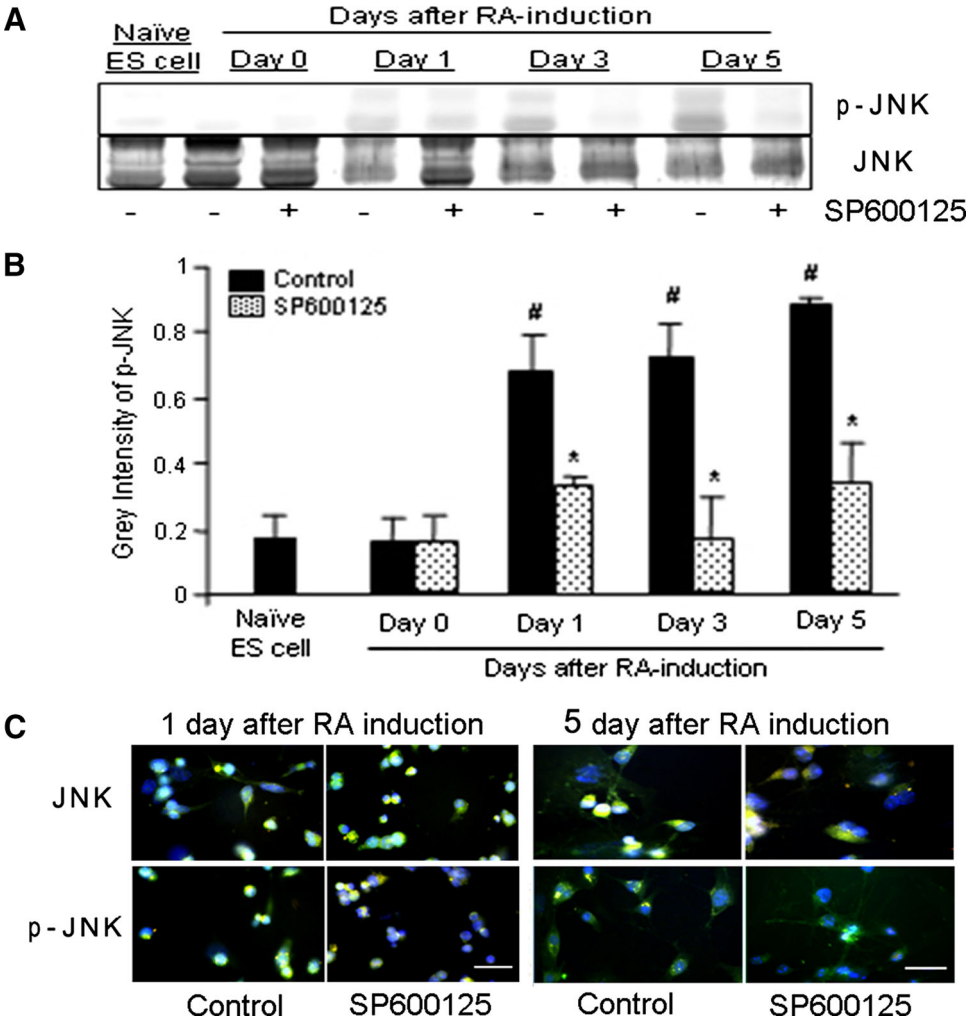

Fig. 1 Expressions of JNK and p-JNK during development of ES cell-derived neurons in vitro. **a**, **b** Western blot analysis of c-Jun N-terminal kinase (JNK) and, phosphorylated JNK (p-JNK) in cultured naïve ES cells and differentiating ES cells 0, 1, 3, and 5 days after RA treatment in the presence or absence of the specific JNK inhibitor, SP600125 (10 μM). Both JNK and p-JNK levels were increased in differentiating cells. The *bar graph* in B quantified the p-JNK level during neuronal differentiation of ES cells. It significantly increased from day 1 after RA induction. $^{\#}P < 0.05$ compared to naive ES cells. This increase was blocked when cells were grown in the presence of SP600125. *Error bars* represent SEM; $^{*}P < 0.05$ compared to control cells. $N = 5$. **c** Immunocytochemistry showing JNK and p-JNK (*green*) in NeuN (*red*) positive ES cells on 1 and 5 days after plating in the absence (Control) or presence of SP600125. Hoechst 33342 (*blue*) staining showed total cells. SP600125 incubation noticeably reduced expression of p-JNK, while less effect was seen on JNK staining. *Scale bar* 30 μm. Representative for 5–7 independent assays (Color figure online)

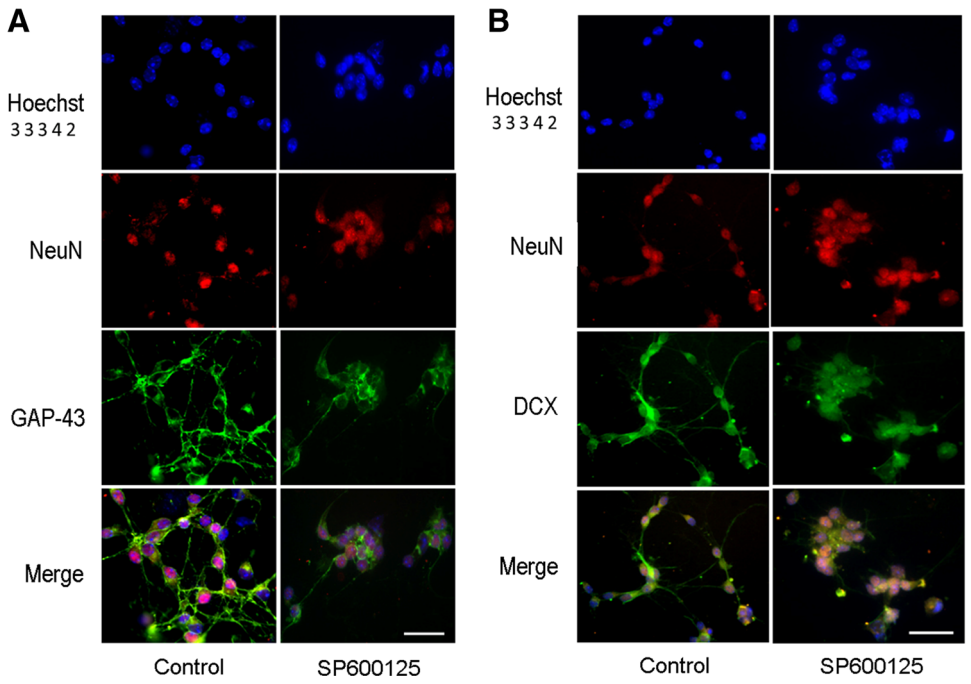

Fig. 2 Immunocytochemistry of GAP-43 and DCX in neurally differentiating ES cells. **a** Immunostaining showed positive staining of GAP-43 in neuronal differentiation of ES cells 3 day after RA induction. GAP-43 showed distribution in cell soma and neuritis of NeuN-positive cells in the control group. Treatment with 10 μM SP600125 substantially suppressed GAP-43 expression, and the neurites from the cell body were less and shorter in the presence of SP600125 compared with control cells. **b** NeuN-colabeled with DCX, a marker for neuroblast, was seen in control and attenuated after SP600125 treatment. *Scale bar* 50 μm

protocol showed lower levels of phospho-JNK (p-JNK). During the following 5 day differentiation period, p-JNK was dramatically increased in differentiating cells (Fig. 1). To verify the role of the JNK pathway in neuronal differentiation, the JNK specific inhibitor SP600125 (10 μM) was used to block activation/phosphorylation of JNK (p-JNK) (Fig. 1).

Differentiating cells showed positive staining with growth-associated protein 43 (GAP-43), a crucial growth gene associated with axonal growth during neuronal differentiation and maturation. The distribution of GAP-43 was in cell soma and neurites, which is consistent with its role in neurite outgrowth and synaptic connections. Addition of 10 μM SP600125 substantially suppressed GAP-43 expression. Localization of GAP-43 in the neurites almost disappeared (Fig. 2a). Doublecortin (DCX) has been used as a marker for neurogenesis. Previous studies demonstrated that DCX is phosphorylated by JNK in growth cones (Gdalyahu et al. 2004). To test the effect of JNK phosphorylation on DCX expression during neuronal differentiation, immunostaining was used to analyze the expression of DCX in the ES cell-derived neural progenitors and neuronal cells. We observed that the expression of DCX in ES cell-derived cells was inhibited in the presence of SP600125, suggesting that DCX may be involved in the JNK cascade signaling during neuronal differentiation of ES cells (Fig. 2b).

Neurofilament (NF) and βIII-tubulin were used to identify the cytoskeleton in ES cell-derived neurons. When grown in the presence of SP600125, the neurally differentiating cells had less expression levels of cytoskeletal proteins. Immunocytochemistry for NF and βIII-tubulin demonstrated that blocking JNK signaling reduced neurite outgrowth, including total neurite extents and primary neurite length, in ES cell-derived neurons (Fig. 3). The length of neurites in differentiating cells was significantly shorter with SP600125 treatment compared with control cells (Fig. 3a–d). Western blot analysis showed either no or low protein levels of NF and βIII-tubulin in these cells (Fig. 3e). During neuronal differentiation, the expressions of NF and βIII-tubulin in culture increased at 1 day after RA induction and remained high thereafter (Fig. 3e–g). Blocking p-JNK substantially reduced the expressions of these neuronal proteins (Fig. 3f–g). Consistent with decreased expressions of NF and βIII-tubulin, expression of the pre-synaptic vesicle protein synaptophysin was increased during the neuronal differentiation and was significantly decreased in the presence of SP600125 (Fig. 4).

Signaling Molecules in the JNK Mediated Pathway

In vitro, D3 mouse ES cells could differentiate into neurons with the formation and elongation of neurites. This differentiation is accompanied by the activation of ERK and JNK pathways as well as by an increased expression and phosphorylation of STAT1 and STAT3, downstream of the ERK and JNK cascade (Li et al. 2006). Coincident with increased p-JNK levels at 1–5 days after plating in RA-induced ES cell-derived neural progenitor cells, the increased phosphorylation of STAT1 and STAT3 in the JNK pathway was observed. This STAT1/STAT3 activation was attenuated in the presence of SP600125.

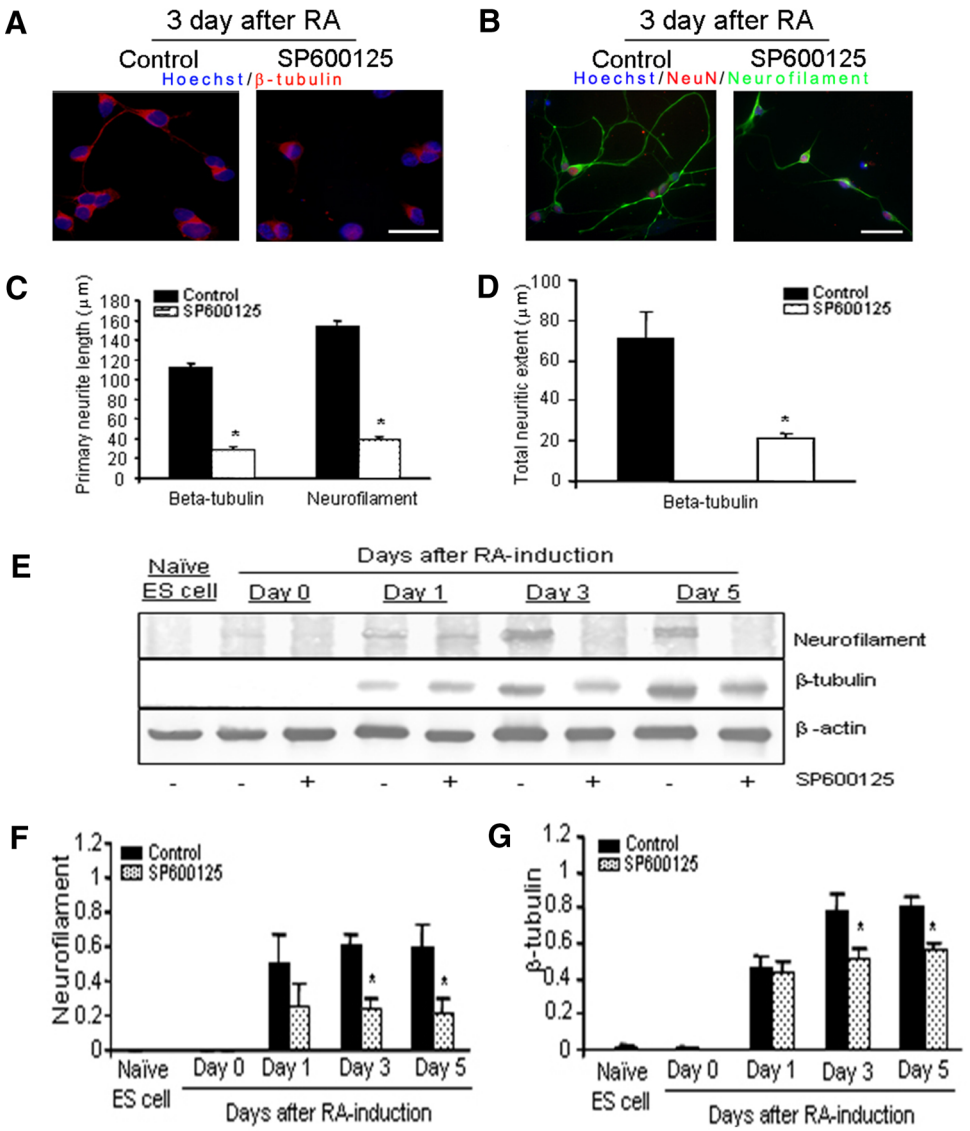

Fig. 3 Activation of c-Jun N-terminal kinase is required for neurite outgrowth of ES cell-derived neurons in vitro Immunohistochemical staining and Western blotting of *β*-tubulin and neurofilament (NF), two important neuronal cytoskeletal proteins, were performed to monitor neurite outgrowth and axonal development. **a**, **b** Immunostaining showed positive staining of βIII-tubulin (**a**) and neurofilament (**b**) in neurally differentiating ES cells. Addition of 10 μM SP600125 substantially decreased neuritic outgrowth of ES cell-derived neurons. *Scale bar* 50 μm. **c**, **d** Quantified analysis of primary neurite length (**c**) and total neurite extent (**d**), both were blocked by inhibition of JNK activation. $N = 5$. **e** Western blotting for expressions of NF and βIII-tubulin in differentiating ES cells in the presence or absence of SP600125. **f** and **g** Quantified summaries of protein levels of NF (**f**) and βIII-tubulin (**g**). Blocking JNK by SP600125 significantly attenuated expressions of these two cytoskeletal proteins in ES cell-derived neurons (1–5 days after RA induction). Mean ± SEM; $N = 3$, $^*P < 0.05$; $^*P < 0.05$ compared to non-SP600125 control cells

Moreover, SP600125 prevented phosphorylation of STAT1 at Tyr701 site and reduced the fluorescence of STAT3 colabelled with NeuN and p-STAT1 colabelled with β-tubulin in the nuclear compartment (Fig. 5). Western blot analysis confirmed that blocking p-JNK using SP600125 markedly reduced STAT3 localization in the nucleus and STAT1 phosphorylation (Fig. 6).

Bcl-2 has been widely used to study the apoptotic pathway. Moreover, it was shown that Bcl-2 could regulate neurite extension through the JNK signaling in MN9D dopaminergic neuronal cells (Eom et al. 2004). The function of Bcl-2 in ES-derived neural progenitors is unclear. Western blot showed that there was a decreased Bcl-2 expression in the presence of SP600125 compared with the untreated group (Fig. 7a, b). This supported that JNK signaling activates cell survival pathways in differentiating neurons.

JNK Signaling Contributes to the Maturation of ES Cell-Derived Neurons

In addition to surviving factors, neurotrophic factors including vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) were analyzed by Western blot. The data demonstrated that blocking JNK decreased VEGF but not GDNF and BDNF expression in ES cell-derived neural progenitors (Fig. 7c–e). To observe the secretions of VEGF from these cells, the enzyme-linked immunosorbent assay (ELISA) was performed. Compared with DMSO-treated group, JNK inhibition significantly decreased the level of VEGF in the culture medium (Fig. 7f). This strongly suggested that JNK signaling promotes functional VEGF secretions, which plays important roles in the maturation of ES neurons. At 10 days after the

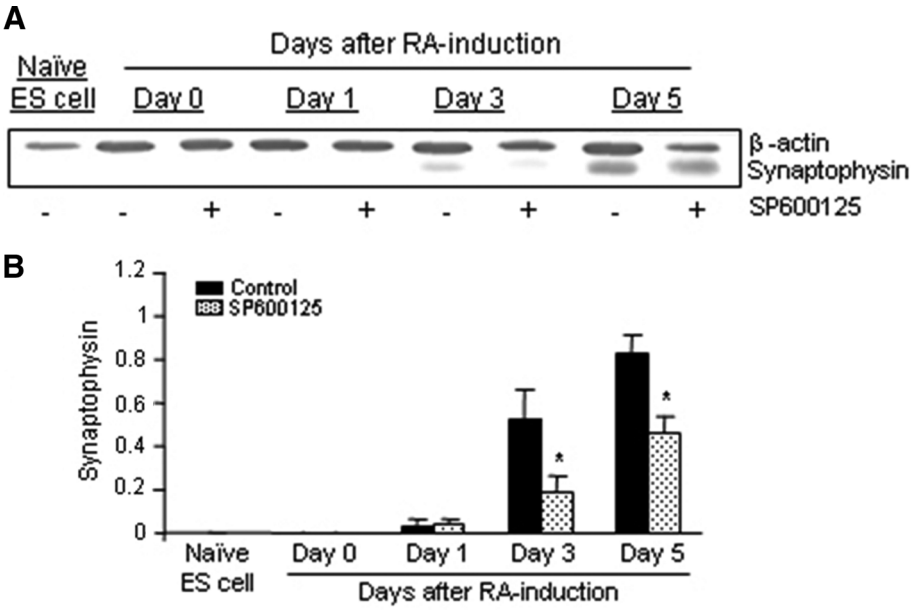

Fig. 4 Role of JNK pathway in synaptogenesis during ES cell neuronal differentiation. **a** Western blot analysis of the pre-synaptic vesicle protein synaptophysin. Synaptophysin expression markedly increased by day 3–5 after RA induction. Adding SP600125 during this period of time primarily decreased synaptophysin expression. **b** Summary of the result in **a**. Mean ± SEM; $N = 3$, $^*P < 0.05$ compared to controls

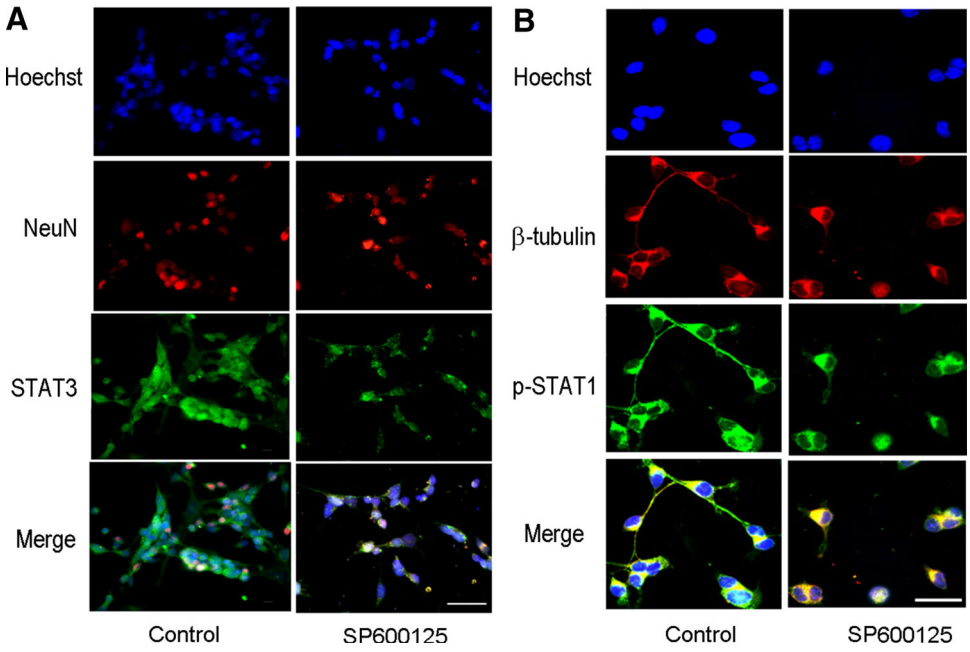

Fig. 5 Immunostaining of STATs proteins during ES cell neural differentiation Immunohistochemical staining of the STAT expression in ES cell-derived neuronal cells at the 3 days in vitro. The expressions of STAT3 and p-STAT1 in ES-derived neurons was markedly decreased by SP600125. The *merged image* shows decreased fluorescence of NeuN/STAT3, Hoechst 33342/STAT3 (**a**), and β-tubulin/p-STAT1 (**b**) colabelling in cells after JNK inhibition

termination of RA induction, we observed typical electrophysiology properties, including inward TTX-sensitive Na^+ currents, outward K^+ currents, and firing of repetitive action potentials, in ES cell-derived neurons (Fig. 8).

JNK Family is Selectively Upregulated in ES Neurons

We finally analyzed the expressions of JNK family members. JNK-3, which is uniquely expressed in neural cells, was shown to be upregulated in neutrally differentiating ES cells at all each inspected time points (1, 3, 5, and 7 days in vitro). JNK-1 and JNK-2, highly expressed in proliferating ES cell colonies, were lower in differentiating neurons compared with RA-induced neurospheres (Fig. 9a, b). Following RA 4−/4+ induction, expression levels of JNK-3 in cells after plating on dishes were higher than RA-treated ES neurospheres before plating (Fig. 9c).

Discussion

Our previous study showed that activation of the ERK1/2 cascade can induce neuronal differentiation of ES cell-derived neurons in vitro (Li et al. 2006). In the present

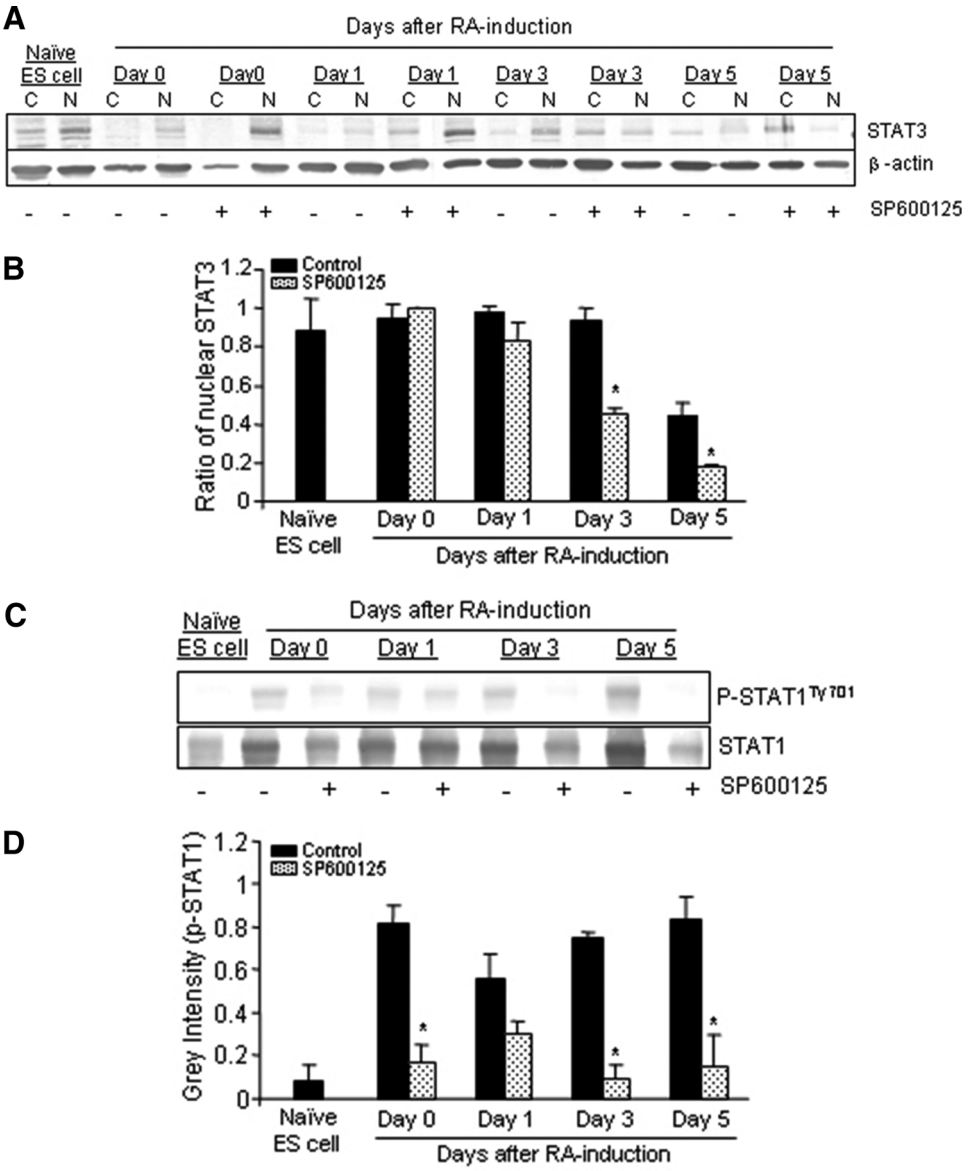

Fig. 6 Role of JNK signaling in STAT1 and STAT3 pathways during ES cell neural differentiation. **a** Western blotting was performed to examine protein levels of STAT3 in nuclear (N) and cytosolic (C) fractions of differentiating ES cells with and without SP600125. **b** Quantified summary from the assay in A. RA induction did not significantly increase STAT3 expression; on the other hand, the expression level decreased on day 5 into differentiation. Inhibition of JNK reduced the STAT3 levels in nuclear components of differentiating cells. **c** Western blot analysis of the expression of p-STAT1 in cultured naïve ES cells and ES-derived neurons. Phospho-STAT1 expression in ES-derived neurons was increased compared with the naïve ES cells. Adding SP600125 during this period of time primarily prevented the p-STAT1 increase. **d** Summary of the result in **c**. Mean ± SEM; $N = 3$–5; $^*P < 0.05$ compared to control

investigation, we explored the role for the JNK-STAT1/3 pathway in the neuronal differentiation of ES cells in vitro. Our results demonstrate that JNK signaling plays a pivotal role in promoting the neurite outgrowth, upregulating their associated cytoskeletal and synaptic proteins, and enhancing paracrine potential of ES cell-derived neurons. Furthermore, the JNK signaling cascade regulates nuclear translocation of transcriptional factors, STAT1, and STAT3, and expressions of several trophic factors in ES cell-derived neurons. ES cell-derived neurons were examined in this investigation 1–5 days after termination of the RA induction protocol. At this stage, voltage-gated ionic channels such as Ca^{2+} channels and K^+ channels are expressing in these cells. Neuron-specific Na^+ channels are emerging at this time, and neuronal receptors such as NMDA and AMPA receptors can be detected at lower levels (Zhou et al. 2011; Muth-Kohne et al. 2011). Therefore, the changes observed in the present investigation represent a relatively early stage after the neuronal commitment of neural progenitor cells.

The JNK family is consisted of JNK1, JNK2, and JNK3. The expressions of JNK1 and JNK2 are ubiquitous, but JNK3 is highly expressed in central nervous system (CNS) neurons. We detected the level of three JNKs and confirmed that JNK3 was upregulated in ES cell-derived neurons. Utilizing another cell type, the induced pluripotent stem (iPS) cells, we also confirm that JNK-3 was expressed after RA induction but not in the pluripotent state (data not shown). JNKs are activated by the upstream MAPK kinases and MAPK kinase kinases. Activated JNKs could phosphorylate its downstream transcription factors in the nucleus, such as STAT3, c-Jun, p53, ATF-2, and Elk-1,

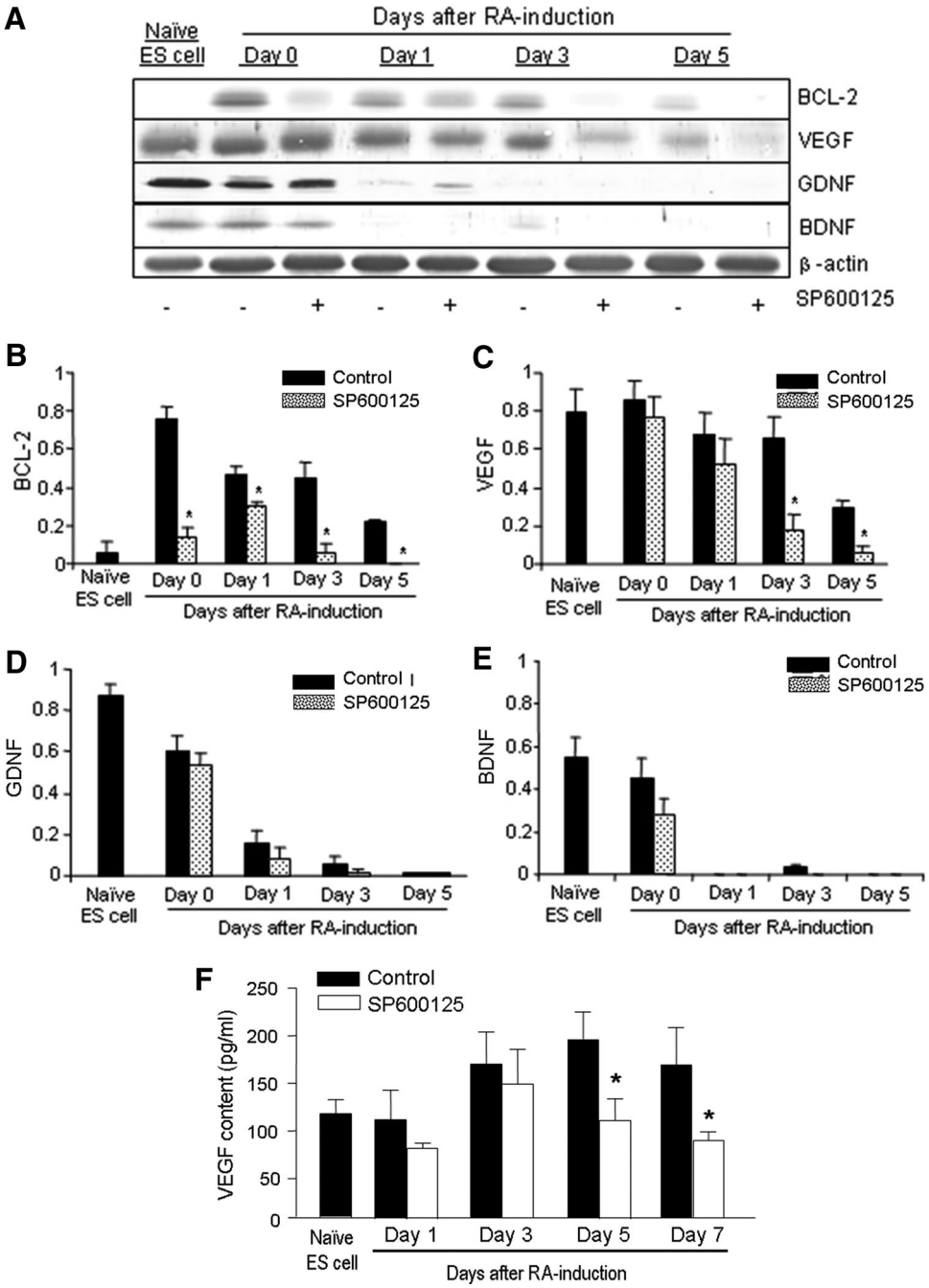

Fig. 7 Effects of blocking p-JNK signal on BCL-2 and VEGF, GDNF, and BDNF during ES cell neural differentiation. **a** Western blot analysis showing BCL-2, VEGF, GDNF, and BDNF in cultured naïve ES cells and differentiating ES cells 0–5 days after RA treatment in the presence or absence of SP600125. BCL-2 level increased after neural induction and was reduced when cells were grown in the presence of SP600125. There are high levels of VEGF, GDNF, and BDNF in undifferentiated ES cells. Neural differentiation of ES cells resulted in drastic decreases in VEGF, but not GDNF and BDNF after the SP600125 treatment. **b–e** Quantified data of changes in BCL-2 (**b**), VEGF (**c**), GDNF (**d**), and BDNF (**e**) levels 1–5 days after RA induction in the presence or absence of SP600125. Mean ± SEM; N = 3, $*P < 0.05$ compared to controls. **f** Quantified data of VEGF content released. Culture medium was collected at different days. Significant decrease was seen in VEGF secretion (pg/ml) after JNK inhibition. $*P < 0.05$ compared to controls

as well as activate some cytoskeleton molecules in cytoplasm, such as neurofilaments, microtubule-associated proteins, and tau protein (Rallis et al. 2010; Weston and Davis 2007). Recent study identified its transcriptional activities through direct binding to promoters as well (Tiwari et al. 2012). Activation of JNK signaling has been demonstrated during neuronal differentiation and extension of neurites in several cell lines (Waetzig and Herdegen 2003; Repici et al. 2009). JNK signaling plays an important role during RA-induced differentiation of MN9D dopaminergic neuronal cells and spontaneously occurring neurite outgrowth in primary cultures of dopaminergic neurons (Eom et al. 2005). In PC12-N1 cells, JNK is involved in the later stages of neurite generation when the cells form extensive networks and establish contacts (Xiao and Liu 2003). However, the downstream components of the JNK pathway, including the transcription factors responsible for neurite outgrowth are currently not fully understood. We explored the potential JNK-STAT1/3 signaling cascade during neuronal differentiation of ES cells.

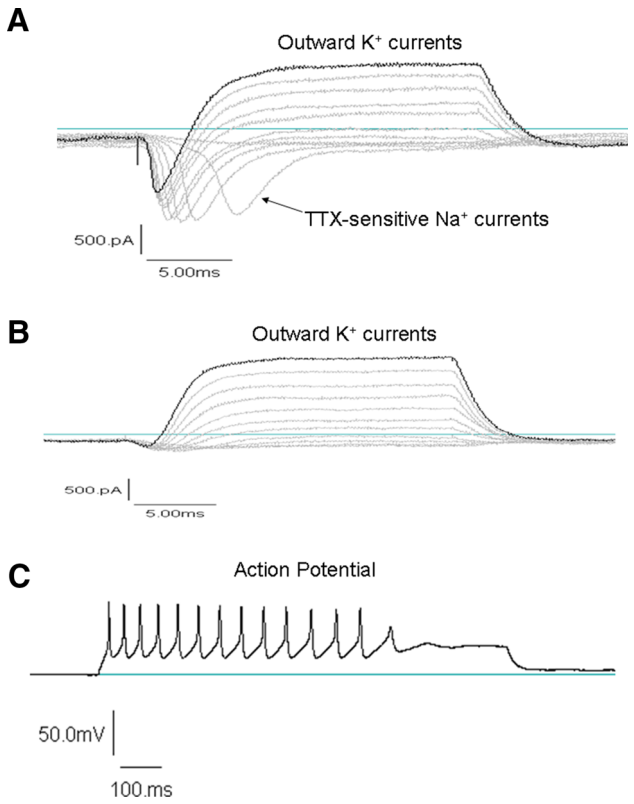

Fig. 8 Neuronal activities of ES cell-derived neuron-like cells. **a**, **b** After 10 days of differentiation, neuronal cells derived from ES cells exhibited electrophysiological activities of voltage-gated Na^+ and K^+ channels. **c** These cells also can fire action potentials upon membrane depolarization

We presented pharmacological evidence indicating that JNK activation is a key event for expressions of GAP-43, NF, and βIII-tubulin, which are essential elements for axonal development and neurite outgrowth during neuronal differentiation. Our data showed that the JNK pathway activity is required for the expression of synaptophysin during neuronal differentiation of ES cells.

The signaling pathways regulating neurite outgrowth in culture are likely to play a role in the terminal differentiation of neurons in vivo (O'Donnell et al. 2009). There are multiple pathways involved in regulating cell proliferation and differentiation (such as neurite outgrowth). For instance, the ERK1/2 pathway and STAT3 pathway coexist in the same cell, both of which can regulate cell proliferation (Li et al. 2006; Wu and Bradshaw 1996). Both the ERK1/2 and the STAT3 pathways can trigger proliferation of NIH-3T3 fibroblasts (Park et al. 2008). This could be true for neurite outgrowth as well. So far, most analyses identifying pathways regulating neuronal process formation have focused on the ERK1/2 pathway. Interestingly, our study indicates that inhibition of JNK signaling activity nearly abolished neurite outgrowth in cultured ES cell-derived neurons. Both STAT1 and STAT3 are affected by the JNK pathway activity. Phosphorylation of STAT1 and the nuclear translocation of STAT3 significantly decreased when JNK signaling was inhibited by SP600125. This observation indicates that both STAT1 and STAT3, as downstream factors of the JNK pathway, would be involved in regulating the neurite outgrowth during early neuronal differentiation of cultured ES cells. JNK can regulate STAT3 by mediating CB1 receptor-induced neurite outgrowth in Neuro-2A cells (He et al. 2005). In our study, the expression of STAT3 gradually decreased during the early neuronal differentiation, whereas p-STAT1^{Tyr701} sharply increased and maintained a high level in the same stage of neuronal differentiation. It was reported that STAT1 and STAT3 had opposing roles in cellular regulation in PC12 and PC12-E2 cells (Wu and Bradshaw 1996). Overexpression of STAT1 led to a partial inhibition of the neurite outgrowth by IL-6. The inhibition of neural proliferation correlates with an inhibition of the activation of endogenous STAT3 homodimers. Our data and other reports suggest that the JNK-STAT pathway has a crucial role in regulating neurite outgrowth.

DCX was originally identified as the gene responsible for X-linked lissencephaly in males and doublecortex in females (Guerrini and Marini 2006). More recently, DCX was identified as a JNK substrate in developing neurons. Inhibition of DCX activation or mutation of DCX decreases neurite outgrowth and neuronal migration (Gdalyahu et al. 2004). Here, we observed the relationship between DCX and neurite outgrowth during JNK cascade activation. The expression of DCX as well as neurite outgrowth decreased in differentiating neural progenitors after JNK phosphorylation was blocked. This is consistent with the report that nonphospho-forms of DCX reduced neurite length, while the phospho-form of DCX increased neurite length (Gdalyahu et al. 2004). Consistent with the idea that DCX is involved in the JNK regulation of neuronal differentiation, our results further confirmed that DCX is involved in neurite outgrowth.

To better understand potential downstream factors involved in JNK regulation of neurite outgrowth, we analyzed the expressions of several neurotrophins and growth factors and the survival factor Bcl-2 in neurally differentiating cells. Our data showed VEGF, but not BDNF and GDNF, playing a role in JNK-STAT3 signaling cascade in ES cell-derived neurons. Bone-marrow mesenchymal stem cells have been found to regulate VEGF secretion upon JNK activation recently (Cai et al. 2013). This investigation perhaps is the first evidence for the involvement of VEGF in the JNK regulation of neuronal differentiation of ES cells. Bcl-2 is best known for its anti-apoptotic function in different cell types. Previous studies showed that Bcl-2 is an effector in JNK-induced activation of the

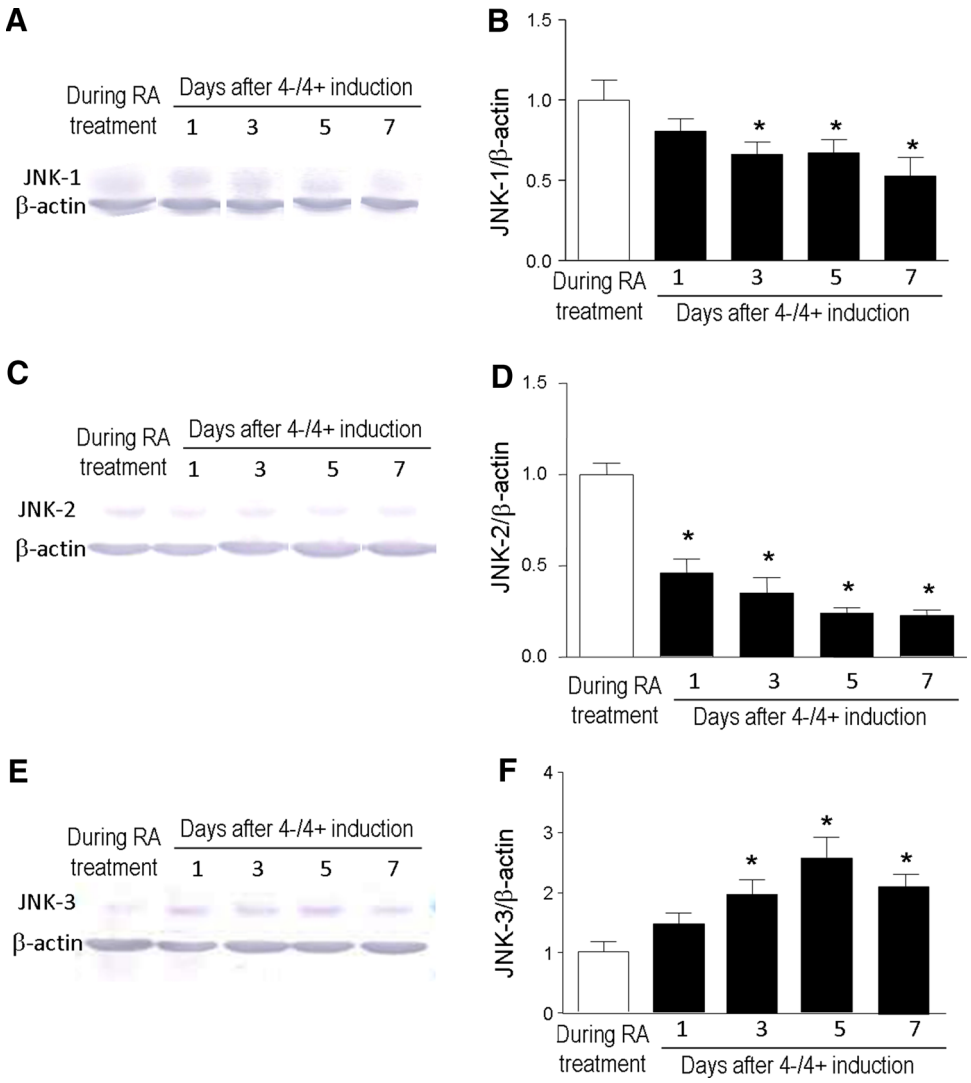

Fig. 9 Expressions of JNK family proteins during ES cell neural differentiation. **a**, **b** Quantified data of changes in JNK-1 (**a**) and JNK-2 (**b**) levels at 1–7 days analyzed by Western blotting. **c** Western blot analysis showing JNK-3 levels in neurally differentiating ES cells before and 1, 3, 5, 7 days after plating on poly-D-lysine and laminin-coated dishes. After RA 4−/4+ induction, JNK-3 was expressed in neurospheres and upregulated during neuronal differentiation

mitochondrial apoptotic pathway. Several Bcl-2-like proteins have been proposed to mediate the effects of JNK on cell death (Weston and Davis 2007). Bcl-2 can also promote the growth of neuronal processes and axonal regeneration in various models (Holm et al. 2001). We previously reported that transplantation of ES cell-derived neural progenitor cells overexpressing Bcl-2 could promote axonal growth and functional recovery after transient cerebral ischemia (Wei et al. 2005). In this investigation, we showed that Bcl-2 was involved in the effects of JNK signal cascade on neuronal differentiation of cultured ES cells. Further studies are required to determine whether VEGF and Bcl-2 can directly interact with the JNK signaling pathway.

The JNK pathway was involved in the heat shock factor (HSF)-induced escape from self-renewal program of human embryonic stem cells and in the suppression of Oct4 expression (Byun et al. 2013). Since only JNK-3 among three JNK isoforms was upregulated during the differentiation processes observed in this study, we concluded that JNK-3 plays the key roles during ES cell neuronal commitment and early neural development. Lowered levels of JNK-1 and/or JNK-2 in stem cells and many other cell types were reported to mediate functions for the self-renewal and cell proliferation (Hui et al. 2008; Alter et al. 2008; Cui et al. 2009; Haeusgen et al. 2011; Ribas et al. 2012). Our data showed decreases in both JNK-1 and JNK-2 levels during neuronal differentiation, which was consistent with an inhibitory effect on proliferation of ES cells. We then observed the maturation of these neurons at 10 days in vitro. The sodium and potassium current and action potentials were successfully recorded in these ES cell-differentiated neurons. SP600125 inhibiting the activation of all the three JNK isoforms did not induce a significant change in the total levels (data not shown). The neurite outgrowth during the differentiation and maturation stages was dramatically inhibited after SP600125 treatment in the current investigation. JNK-3 level has been shown to control the neurite outgrowth in

midbrain dopaminergic neurons (Tonges et al. 2011). We elucidated that JNK-3 upregulation and activation during these stages contribute to the neurite outgrowth and the maturation in ES cell-derived cortical neuronal cell type. However, in spiral ganglion and dorsal root ganglion neurons, the neurite growth may rely on JNK-1 and JNK-2 (Barnat et al. 2010; Atkinson et al. 2011). The mechanisms of the JNK isoforms signaling cascades in ES cells neural differentiation need to be investigated further.

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Conflict of interest All authors have no conflict of interest in this investigation.

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