Original Article

Modulating Neurogenesis in Embryoid Body Using a Selective TGF beta1/ALK Inhibitor Affects Gene Expression of Embryonic Stem Cell-derived Motor Neurons

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Abstract

Mouse embryonic stem (ES) cells have been served as potential model for investigation of underlying mechanisms at cellular and molecular levels of neurological disorders. The improvement of motor neuron differentiation is prospected to gain more understanding aimed at overcoming several incurable motor neuron diseases. In this study, we examined the effects of selective inhibitor of TGF-β type I receptor on the efficacy of neuronal differentiation of mouse embryonic stem cells toward motor neuron. Pluripotent ES cells were induced to form as EB in suspension medium supplemented with retinoic acid using -4/+4 protocol. Thereafter, 8-day old EBs were further induced to differentiate into motor neurons on monolayer culture. Our result demonstrated that the aggregated ES cells differentiated into neuronal progenitor cells and neurons as examined with Pax-6 and Tuj-1. Quantitative RT-PCR analysis revealed that treatment of the EBs with selective TGF-β1 inhibitor up-regulated the motor neuron progenitor (Olig2) at higher levels than that obtained from the control (4.20±0.20 vs. 0.73±0.09, $p < 0.01$). In contrast, mRNA expression levels of motor neuron (Hoxc8) of the control group were significantly higher than the TGF-β1 inhibitor treated group (14.73±2.6 vs. 2.37±0.42, $p < 0.01$). Immunocytochemistry demonstrated that the differentiated cells expressed a neuronal marker (Tuj-1), motor neuron progenitor marker (Olig2) and developing motor neuron progenitor (Isl-1), all of which are essential for generation of spinal motor neurons during neural tube formation. Furthermore, a small proportion of differentiated cells were also positive for choline acetyltransferase (ChAT) a marker for functional motor neurons. We concluded that modifying TGF-β signaling affected the generation and differentiation fate of motor neuron progenitor cells.

Keywords: embryoid body, embryonic stem cells, motor neuron differentiation, TGF-β1 inhibitor

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บทคัดย่อ

การปรับเปลี่ยนการสร้างเซลล์ประสาทในเอ็มบริออยด์ บอดี โดยใช้สารยับยั้ง TGF beta1/ALK มีผลต่อการแสดงออกของจีนของเซลล์ประสาทนำสั่งที่เปลี่ยนแปลงมาจากเซลล์ต้นกำเนิดจากตัวอ่อน

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เซลล์ต้นกำเนิดจากตัวอ่อนหนูเม้าส์เป็นต้นแบบที่มีประสิทธิภาพสำหรับการวิเคราะห์กระบวนการทั้งระดับเซลล์และโมเลกุลของการเกิดโรคทางระบบประสาท การพัฒนาการเปลี่ยนแปลงเป็นเซลล์ประสาทนำสั่ง เพื่อเพิ่มความเข้าใจ สามารถรักษาโรคทางระบบประสาท การศึกษานี้วิเคราะห์ผลของสารยับยั้ง TGF-β ที่จำเพาะกับตัวรับแบบที่ 1 ต่อประสิทธิภาพการเปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทของเซลล์ต้นกำเนิดหนูเม้าส์ เพื่อให้เป็นเซลล์ประสาทนำสั่ง เซลล์ต้นกำเนิดจากตัวอ่อน ถูกกระตุ้นให้เกาะตัวเป็นกลุ่มเซลล์เอ็มบริออยด์บอดี ในน้ำยาเลี้ยงที่เสริมด้วยกรดเรติโนอิก (retinoic acid) โดยใช้วิธีการ -4/+4 จากนั้นเอ็มบริออยด์บอดีอายุ 8 วันจึงถูกเหนี่ยวนำ เพื่อให้เปลี่ยนแปลงไปเป็นเซลล์ประสาทนำสั่งด้วยการเลี้ยงในจานเพาะแบบเซลล์ชั้นเดียว ผลเซลล์ต้นกำเนิดที่เกาะกลุ่มกันเปลี่ยนแปลงไปเป็นเซลล์ต้นตอประสาทและเซลล์ประสาท จากการตรวจยืนยันด้วย Pax-6 และ Tuj-1 จากการวิเคราะห์ด้วย Quantitative RT-PCR พบว่าการเลี้ยงเอ็มบริออยด์บอดีด้วยสารยับยั้งจำเพาะ TGF-β1 เพิ่มระดับจีนที่จำเพาะต่อเซลล์ต้นตอประสาทนำสั่ง (Olig2) สูงกว่ากลุ่มควบคุม (4.20±0.20 เทียบกับ 0.73±0.09, $p < 0.01$) ในทางตรงกันข้ามระดับการแสดงออกของ mRNA ที่จำเพาะต่อเซลล์ประสาทนำสั่ง (Hoxc8) ของกลุ่มควบคุมสูงกว่ากลุ่มที่ใส่สารยับยั้ง TGF-β1 อย่างมีนัยสำคัญ (14.73±2.6 เทียบกับ 2.37±0.42, $p < 0.01$) อิมมูนโนไซโตเคมีแสดงให้เห็นว่าเซลล์ที่เปลี่ยนแปลงมีการแสดงออกของโปรตีน ตัวบ่งชี้เซลล์ประสาท (Tuj-1) เซลล์ต้นตอประสาทนำสั่ง (Olig2) และเซลล์ประสาทนำสั่งที่กำลังพัฒนา (Isl-1) ซึ่งทั้งหมดจำเป็นต่อการสร้างเซลล์ประสาทนำสั่งไขสันหลังในช่วงการก่อตัวของท่อประสาท ยิ่งไปกว่านั้นเซลล์ที่เปลี่ยนแปลงจำนวนหนึ่งยังแสดงผลบวกต่อ choline acetyltransferase (ChAT) ซึ่งบ่งชี้การทำงานของเซลล์ประสาทนำสั่ง จากการทดลองสรุปได้ว่าการปรับเปลี่ยนสัญญาณของ TGF-β ส่งผลต่อการสร้างและเปลี่ยนแปลงของเซลล์ต้นตอประสาทนำสั่ง

คำสำคัญ: เอ็มบริออยด์บอดี เซลล์ต้นกำเนิดจากตัวอ่อน การเปลี่ยนแปลงเป็นเซลล์ประสาทนำสั่ง สารยับยั้ง TGF-β1

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Introduction

During development of mammalian embryos, ectodermal cells are converted into rostral neural fate, and the neural inductive signals such as retinoic acid (RA) lead the caudalization of neural cells. The motor neurons are then generated via ventralization of the spinal motor progenitors which is directly dependent on the sonic hedgehog (SHH) activity (Jessell, 2000; Wilson and Edlund, 2001). Unlike the embryo development, most neurons of the mammalian central nervous system (CNS) of adulthood cannot be regenerated or repaired upon injury (Gage, 2002). Therefore, several motor neuron diseases (MNDs) including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) are classified as incurable neurological disorders due to the permanent loss of the motor neurons in the spinal cord/brain. To overcome this shortcoming, advanced technology for cell replacement via in vitro production of neuronal cells coupled with cell/tissue engineering has lightened a novel technique for treating these neurogenic disorders (Gowing and Svendsen, 2011). Pluripotent stem cells have capability to self-renew and to differentiate into all

types of the three germ layers including neuronal lineage (Keller, 1995). Thus, embryonic stem (ES) cells-derived functional mature motor neurons have increasingly become a potential model for studying the disease mechanism and ultimately for developing novel cell-based therapy for motor neuron diseases (Lunn et al., 2011).

Mouse ES cells manifest their ability to differentiate into several neuronal cell types of nervous system but specific signals for successive initiation of spinal motor neuron development are required. The successful establishment of motor neurons can be achieved through orchestrated stimuli of multiple signals such as RA and SHH that have been demonstrated to potentially differentiate cells into caudo-ventral pattern in nervous system and also have ability to advance the development of floor plate of neural tube and spinal cord (Marti and Bovolenta, 2002; Maden, 2006). Exposure to these morphogens prompted the differentiation fate of the ES into spinal motor neurons in a concentration-dependent manner. Furthermore, the presence of several transcription factors including Pax6, Nkx6.1, bHLH protein and Olig2 in neuronal population hinted at the potential role in specific position and stage of motor neuron differentiation and development (Pituello et al., 1995; Muller et al., 2003; Lee et al., 2005).

To differentiate the ES cells toward neuronal lineage, several strategies have been examined but the most convenient- and cost-effective technique is the neuronal differentiation by allowing aggregated ES cells to differentiate into 3D structure resembling to embryo development, so-called embryoid body (EB) formation (Li et al., 2005; Karumbayaram et al., 2009). Although this technique allows spontaneous differentiation of the ES cells, specific cell types, for example, Pax 6-positive neuronal cells, could directly be driven when culturing the EBs with specific growth factors (Bibel et al., 2007). Among growth factors affecting the neuronal differentiation, signaling via transforming growth factor family plays a crucial role in determining the cell fate in neurogenesis. Over expression of TGF-β superfamily member, Nodal, in mouse ES cells up-regulated mesodermal and endodermal cell gene expressions but the neuroepithelial markers were down regulated (Pfendler et al., 2005). The inhibition of the TGF-β members via activin/nodal and BMP pathways increased a functional neuron in culture (Chambers et al., 2009). In the current study, we examined the effects of a selective inhibitor of TGF-β type I receptor on the efficacy of neuronal differentiation of mouse embryonic stem cells toward motor neuron.

Materials and Methods

Chemicals and Cell culture condition: All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37^0C in a humidified atmosphere containing 5% CO_2. Medium was replaced daily for mouse ES cell culture and on every 2-3 days during differentiation.

Mouse pluripotent cell culture: Mouse (129S2/SvPas) D3 ES cells (ATCC, Manassas, VA, USA) were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) as previously described (Magin et al., 1992). The pluripotent cells were maintained in ES medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum, 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 1% (v/v) Antibiotic-Antimycotic (Anti-Anti) and 1,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon Int, Temecula, USA). The cells were subcultured prior to reaching 70% confluency (approximately every 1-2 days). Mouse ES cells were cultured on gelatin-coated dishes in the presence of LIF (2,000 U/ml) in ES medium for at least one passage prior to differentiation in order to discard MEFs from the system.

Differentiation of mouse ES cells into motor neurons: Mouse D3 ES cells were initially induced to differentiate into the neuronal lineage via embryoid body formation as recently described (Klincumhom et al., 2012). Mouse ES cell colonies were disaggregated into single cells using 0.05% (w/v) Trypsin-EDTA, then seeded at a density of $3x10^5$ cells/ml in differentiation medium (ES medium without LIF) onto bacteriological dishes pre-coated with Poly 2-hydroxyethyl methacrylate (Poly-HEMA) to prevent cell attachment. Pluripotent cells were allowed to aggregate in suspension and form the EBs for 4 days. All-trans RA (5 μM) was then added to the medium and the EBs were then cultured for a further 4 days. The EBs were either cultured with or without TGF-β1 inhibitor (A83-01, Biovision, Milpitas, USA). Thereafter, 8-day old EBs were further induced to differentiate into motor neurons as previously described with some modifications (Li et al., 2005). In brief, EBs were dissociated and plated onto Poly-L-ornithine and laminin (Roche, CA, USA) coated dish at a density of $2x10^5$ cells/cm^2 in Neurobasal medium containing N2 supplement, 1 μM cAMP, 100 ng/ml sonic hedgehog (SHH) and 0.1 μM retinoic acid (RA) for 7 days. Then, Brain-derived neurotrophic factors (BDNF, 10 ng/ml), Gial cell line-derived neurotrophic factor (GDNF, 10 ng/ml) and Insulin-like growth factor (IGF-1, 10 ng/ml) were added into the medium. The concentration of SHH was reduced to 50 ng/ml onwards. The medium was renewed every 2 days. The experiments were replicated three times.

Analysis of alkaline phosphatase (ALP) activity: ES cells were prepared for characterization following 2-3 days of culture or until reaching approximately 70% confluency on MEF-coated coverslips. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4^0C until analysis. Alkaline phosphatase staining technique was performed using an ALP histochemistry kit (Sigma, St. Louis, USA) according to the manufacturer's protocol. The ALP-positive ES colonies demonstrated

in bright pink/red color under an inverted light microscope (Olympus CKX41, Japan).

Immunocytochemistry: Undifferentiated mouse ES cells and differentiated cells on day 18 were fixed with 4% (w/v) PFA for 15 min at room temperature. Permeabilization was performed using 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 3% (w/v) bovine serum albumin containing 0.5% (v/v) Tween20 in PBS for 30 min. The cells were then sequentially incubated with the following primary antibodies diluted in a blocking solution overnight at 4^0C: Oct4 (SC-9081, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; mouse) and Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit) for pluripotency analysis. The neuronal markers including: β-III Tubulin (Tuj1, PRB-435P, dilution 1:2,000; Covance; rabbit), motor neuron progenitors: Olig2 (MABN50, dilution 1:200; Chemicon; mouse) and motor neuron markers: Isl-1 (40.2D6, dilution 1:50; DSHB; mouse) and choline acetyltransferase (ChAT, AB143, dilution 1:100; Chemicon; rabbit) were used. The cultures were washed 3 times with PBS and then incubated with fluorescent-labeled secondary antibodies (Alexa fluor® 488, Alexa fluor®594 and Alexa fluor® 647-labeled goat IgG; dilution 1:2,000; Gibco) for 1 hour at RT. After washing, the cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) for 10-20 min. Vectashield (Vector Lab, Temecula, CA) was used as a mounting medium to prevent photobleaching during fluorescent examination. The images were recorded using fluorescent microscope equipped with a DP72 camera and DP2-BSW software (BX51 Olympus, Tokyo, Japan).

Histological analysis: Seven micrometers thickness cross-sections of 8 day-old EBs were rinsed in PBS and incubated for 30 min in a blocking solution containing 10% (v/v) serum and 0.1% (v/v) Triton X-100 in PBS. Sections were then incubated with neuroepithelial marker (Pax 6, dilution 1:200, DSHB; Monoclonal mouse antibody) and early neuronal marker β-III Tubulin (Tuj1, dilution 1: 2,000; Covance, PRB-435P; Polyclonal rabbit antibody) as primary antibodies in blocking solution for 12 hours at 4^0C. After washing with PBS, primary antibodies were detected by using anti- rabbit FITC secondary antibody (ab6717, dilution 1:250, Abcam) for 1 hour at 37^0C. The sections were counterstained with DAPI and then were analyzed using a fluorescent microscope (BX51 Olympus, Tokyo, Japan).

Quantitative RT-PCR analysis: Total RNA was extracted from differentiated cells on day 18 of differentiation using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was reversely transcribed using the First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. After reverse transcription, the final concentration of cDNA samples was diluted to 2.5 ng/µl and moved to one-time-use aliquots. Quantitative RT-PCR was performed on the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK). For cDNA amplification, the cycling parameters consisted of 1 cycle at 50^0C for 2 min and 95^0C for 10 min, followed by 45 cycles at 95^0C for 15 sec and 60^0C for 60 sec. Gene expression of individual transcripts was detected as an amplification exceeded threshold. RT-PCR analysis for pluripotent gene expression of ES cells was performed on automated thermal cycler (Nyx Technik A6, Ramsey, MN, USA) using Go Tag® Green Master Mix (Promega, WI, USA). Our amplification protocol consisted of 1 cycle at 95^0C for 10 min, followed by 45 cycles at 95^0C for 15 sec, 55^0C for 30 sec and 68^0C for 60 sec. The PCR products were electrophoresed on 2% (w/v) agarose gel (Bio-Rad, CA, USA) stained with 0.4 mg/ml ethidium bromide (Promega, WI, USA) and were finally visualized under UV light (Syngene, CB, UK). The negative controls were performed using mouse embryonic fibroblast cells and sterile water (H2O). The primer sequences are shown in Table 1. The glyceraldehydes-3-phosphatedehydrogenase (GAPDH) was used as a housekeeping gene for endogenous normalization.

Statistical analysis: Data were expressed as a mean±standard error of the mean (SEM), for at least three independent replicates. Student's t-test was used to compare means between control and treatment groups. Statistical analysis was performed using SPSS software (version 17.0 SPSS Inc., Chicago, IL, USA). Results were considered significant difference when *p*-values were less than 0.01 (*).

Table 1 Primers used for determination of mRNA expression levels by RT-PCR and quantitative RT-PCR

Gene name	Forward primer	Reverse primer	Product size (bp)	References
Oct4	GTCTGGAGACCATGTTTCTGAAGT	AGAACCATACTCGAACCACATCCT	105	Otsu et al., 2011
Sox2	ACCAGCTCGCAGACCTACAT	CCTCGGACTTGACCACAGAG	111	Keramari et al., 2010
Nanog	AGTGGAGTATCCCAGCATCCATT	CTGCCCCACATGGAAAGG	81	Mitsui et al., 2003
Olig2	CTGCTGGCGCGAAACTACAT	CGCTCACCAGTCGCTTCAT	66	Samanta et al., 2007
HoxC8	CGAAGGACAAGG CCACTTAAAT	AGGTCTGATACCGGCTGTAAGTTT	115	Kruger and Kappen, 2010
GAPDH	CTGCACCACCAACTGCTTAGC	CAGTCTTCTGGGTGGCAGTGA	110	Otsu et al., 2011

Abbreviations: GAPDH: glyceraldehyde-3-phosphate dehydrogenase, Oct4: octamer-binding transcription factor 4, Sox2: Sex determining region Y-box 2, Olig2: Oligodendrocyte transcription factor 2, HoxC8: Homeobox C8 protein

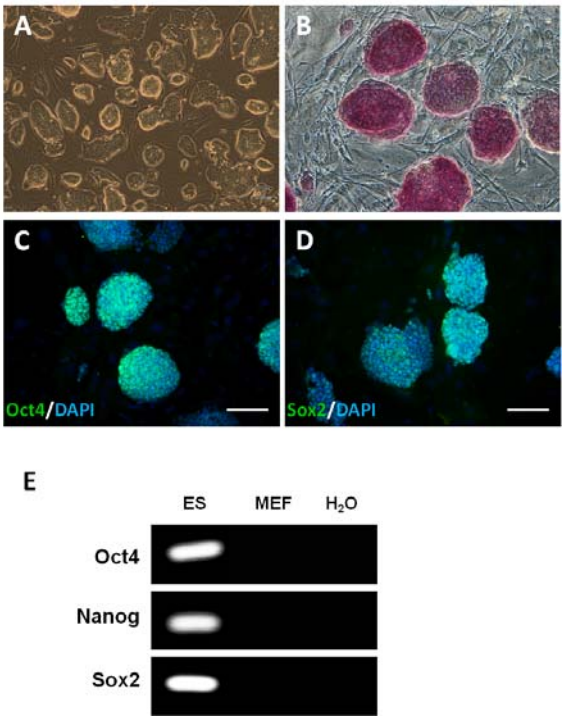

Figure 1 Characterization of pluripotent ES cells. (A) Mouse ES cells were cultured on MEF cells in ES medium supplemented with LIF. Identified by the shiny and round shape of mouse ES colonies (scale bar= 100 µm). (B) ALP-positive pluripotent cells were presented in bright pink/red colonies (scale bar= 50 µm). (C-D) Immunostaining demonstrates undifferentiated ES cells detected by pluripotent markers: Oct4 and Sox2 (green color). DAPI was used for co-staining as nuclei marker (blue color) (scale bar= 100 µm). (E) RT-PCR analysis reveals expressions of Oct4, Nanog and Sox2.

Results

Mouse embryonic cell line used in this study demonstrated a typical ES morphology as ES colonies were round to oval shape with shiny appearance. The ES cells strongly expressed pluripotency markers including ALP activity (bright pink/red colored colonies, Figure 1A-1B), Oct4 and Sox2 (Fig 1C-1D). Moreover, RT-PCR analysis revealed that these ES cells also expressed pluripotency associated genes (Oct4, Nanog and Sox2) (Fig 1E).

These undifferentiated ES cells were aggregated into spherical EBs with heterogeneous size in differentiation medium (Fig 2). Further examination on neuronal characteristics of cross-sectioned the 8 day-old EBs using immunocytochemistry found that the aggregated ES cells differentiated into neuronal progenitor cells, irrespective to the treatment with TGF-β1 inhibitor, as they were positively stained with Pax-6 and Tuj-1 neuronal markers. To generate motor neurons, the EBs were disaggregated, and the monolayer cultured for additional 10 days. Our study demonstrated that this technique efficiently differentiated the ES cells into motor neurons. Treating the EB with a selective TGF-β1 inhibitor up-regulated the motor neuron progenitor (Olig2) at higher levels than that obtained from the control (4.20±0.20 vs. 0.73±0.09, Fig 4, $p < 0.01$). In contrast, mRNA expression levels of motor neuron Hoxc8 of the control group were significantly higher than those of the TGF-β1 inhibitor treated group (14.73±2.6 vs. 2.37±0.42, Fig 4, $p < 0.01$).

In all cases, immunocytochemical analysis demonstrated that the differentiated cells expressed a neuronal marker (Tuj-1), motor neuron progenitor marker (Olig2) and developing motor neuron (Isl-1),

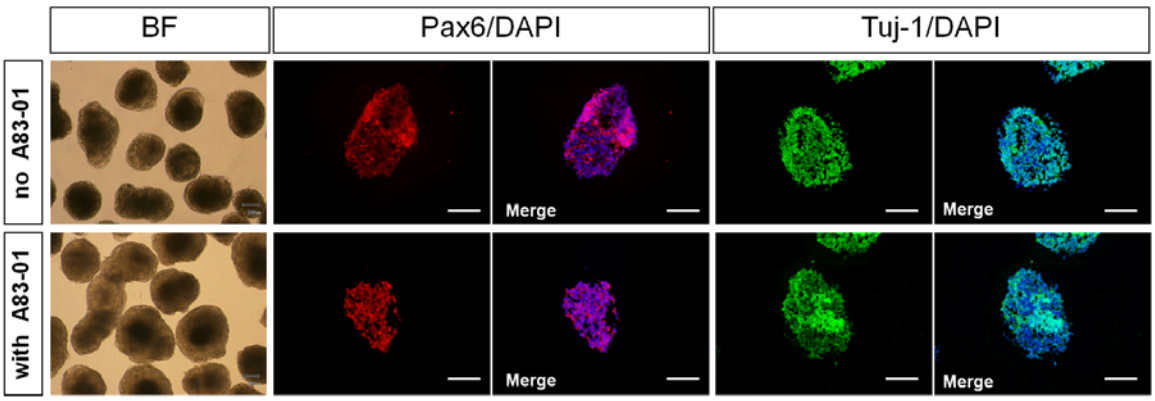

Figure 2 Morphological and histological analysis of EBs during suspension culture. Heterogeneous sizes and multiple shapes of EBs derived from mouse ES cells can be observed during 8 days of suspension period. Cross-sections of A83-01 treated EBs (lower panel) and the control group (upper panel) expressing neuroepithelial marker Pax6 (red) and neuronal marker Tuj-1 (green) by immunohistochemical analysis. Cell nuclei were stained using DAPI (blue). Scale bar: 100 µm.

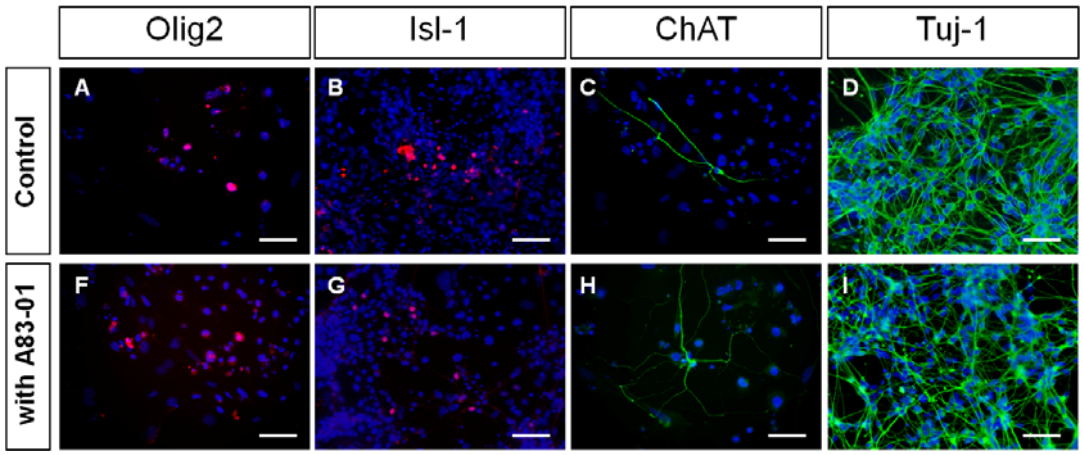

Figure 3 Motor neuron differentiation from mouse ES cells. After EB dissociation, differentiated cells were cultured on substrate-coated plate for motor neuron differentiation. By day 18 of differentiation, differentiated cells derived from A83-01 treated EBs and the control group were assessed for motor neuron progenitor marker (Olig2), motor neuron-specific marker (Isl-1 and ChAT), and neuronal marker (Tuj-1). Nuclei were labeled with DAPI (blue). Scale bar: 100 µm.

all of which are essential for generation of spinal motor neurons during neural tube formation. Furthermore, a small proportion of differentiated cells was also positive for choline acetyltransferase (ChAT), a marker for functional motor neurons (Fig 3).

Discussion

In this study, we demonstrated the effects of a selective TGF-β1 inhibitor on differentiation of undifferentiated ES cells toward motor neuron lineages through EB formation. Several techniques have been used to generate the motor neurons from embryonic and induced pluripotent stem cells such as stromal cell co-culturing and adherent culture with a defined medium (Barberi et al., 2003; Shin et al., 2005). Here, we reported that modifying neurogenic stimuli during embryoid body culture influenced the derivation of neuronal subtypes. These techniques allowed the generation of motor neuron subtypes that share some phenotypic and genotypic similarity to that of mature motor neurons (Miles et al., 2004; Karumbayaram et al., 2009; Patani et al., 2011). However, type and time of neurogenic stimuli have marked influence on cell fate and patterning of motor neurons (Kirkeby et al., 2012).

In the current study, we differentiated the embryonic stem cells to form 3D structure of embryoid body that allows spontaneous differentiation of embryonic stem cells. This technique is quite robust and also cost- and time-effective (Keller, 1995). However, genes associated with the pluripotency of the ES cells within the EB slowly down-regulated (Bibel et al., 2004). Recently, dual inhibition of SMAD signaling (SB431542 and noggin) efficiently improved differentiation of embryonic stem cells toward neurogenic cells with a more synchronized manner (Chambers et al., 2009; Fasano et al., 2010). These TGF inhibitors completely down-regulated the pluripotency associated genes of embryonic stem cells, while neuronal gene expression was simultaneously up-regulated (Kirkeby et al., 2012). This finding is in agreement with our previous study, which found that the selective TGF-β1/ALK inhibitor down regulated the pluripotency-associated genes at a faster rate than that of the control (Klincumkhom et al., unpublished data).

As a result, although we did not observe a marked difference between EB treated with TGF-β1 inhibitor and the control, in terms of Pax-6 and Tuj-1 positive cells, these types of cells have been shown to be capable of further differentiation toward motor neurons (Briscoe et al., 2000; Li et al., 2005). Following disaggregating the day-8 EB into single cells, we further cultured these cells in the presence of sonic hedgehog (SHH) and RA. These two factors function to ventralize and caudalize MN progenitors, respectively (Wichterle et al., 2002). However, this process is somewhat ineffective in terms of prolonged differentiation process (Hester et al., 2011).

In the current study, although protein expressions (Pax-6, and Tuj) were independent to the presence of TGF-β1 inhibitor during the EB culture, the expression of Olig2 in neuronal differentiated cells from TGF-β1 inhibitor-treated EBs was significantly higher than that of the control and vice versa for Hoxc8-specific caudal motor neurons. A previous report indicated that the TGF-β1 negatively affected the proliferation of the hippocampal and neuronal precursor cells by a mechanism related to cell cycle (G0/G1 phase) arrest (Wachs et al., 2006), while inhibition of TGF-β1 restored the limited proliferation ability of retinal progenitors (Close et al., 2005). Therefore, it is possible that applying the TGF-β1 inhibitor during EB may help the produced neuronal progenitors to be maintained and proliferated in their progenitor fate. In addition, since several transcriptional and growth factors involve the organization of spinal motor neuron identities along the axis including Isl-1, Lim3 and HB9 (Pfaff et al., 1996; Arber et al., 1999; Thor et al., 1999), modulating the TGF-β1 signaling pathway by its inhibitor, therefore, probably drives the neuronal progenitor to other motor neuron identities rather than Hoxc8-specific caudal motor column subtype. The identification of the type of motor neurons and also its function of neuronal cells derived from EB treated

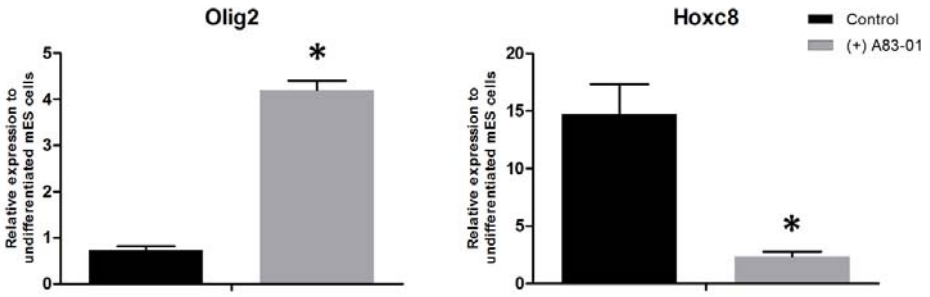

Figure 4 The effect of A83-01 on motor neuron differentiation. Differentiated cells were investigated for gene expression level of Olig2 and Hoxc8 after 18 days of differentiation using quantitative RT-PCR analysis. Gene expression was normalized to housekeeping gene, GAPDH. Results demonstrate as means±SEM of three independent experiments and represent the relative expression compared with mRNA levels of undifferentiated ES cells (arbitrarily set at 1). Statistical analyses on the relative mRNA level of differentiated cells in each gene between A83-01-treated group versus control group are showed, $p < 0.01$ (*).

with TGF-β1 inhibitor remains to be further studied. Furthermore, a small number of these differentiated cells exhibiting ChAT and Isl-1 were only observed. It is, therefore, also of importance to determine other factors that can improve the efficacy of motor neuron differentiation from embryonic stem cells.

In conclusion, our study illustrate that motor neurons can be generated from mouse ES cells after in vitro differentiation. Modifying TGF-β signaling positively affects neuronal differentiation toward motor neuron progenitors.

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