DUSP4 Regulates Neuronal Differentiation and Calcium Homeostasis by Modulating ERK1/2 Phosphorylation

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Protein tyrosine phosphatases have been recognized as critical components of multiple signaling regulators of fundamental cellular processes, including differentiation, cell death, and migration. In this study, we show that dual specificity phosphatase 4 (DUSP4) is crucial for neuronal differentiation and functions in the neurogenesis of embryonic stem cells (ESCs). The endogenous mRNA and protein expression levels of DUSP4 gradually increased during mouse development from ESCs to postnatal stages. Neurite outgrowth and the expression of neuron-specific markers were markedly reduced by genetic ablation of DUSP4 in differentiated neurons, and these effects were rescued by the reintroduction of DUSP4. In addition, DUSP4 knockdown dramatically enhanced extracellular signal-regulated kinase (ERK) activation during neuronal differentiation. Furthermore, the DUSP4–ERK pathway functioned to balance calcium signaling, not only by regulating Ca^{2+} /calmodulin-dependent kinase I phosphorylation, but also by facilitating Cav1.2 expression and plasma membrane localization. These data are the first to suggest a molecular link between the MAPK–ERK cascade and calcium signaling, which provides insight into the mechanism by which DUSP4 modulates neuronal differentiation.

Introduction

DURING DEVELOPMENT OF the nervous system, neural circuits are formed through several precise, step-wise processes, including the proliferation of neural precursor cells, migration, and differentiation into mature neurons with specific shapes and functional characteristics. Each stage of neuronal differentiation involves numerous cell types that respond to a large number of signaling cues, from surface receptors to intracellular signaling transduction pathways based on protein tyrosine phosphorylation and dephosphorylation [1,2].

Dual specificity phosphatases (DUSPs), also termed MAPK phosphatases (MKPs), constitute the largest family of protein tyrosine phosphatases (PTPs) capable of dephosphorylating both serine/threonine and tyrosine residues of MAPK substrates [3]. DUSP4 can be associated with the regulation of extracellular signal-regulated kinases (ERKs) [4,5], c-Jun N-terminal kinases (JNKs) [6], and p38 [7], depending on the cell type. Several studies have linked DUSP4 to the development of liver carcinoma [8], ovarian cancers [9], and acute myeloid leukemia [10]. Additionally, DUSP4 is considered to be a candidate tumor suppressor gene because its deletion has been implicated in breast cancer [11]. DUSP4 has an important role in endoderm

specification in zebrafish development through regulation of sox17 [12], and it has also been shown to function in cardiac specification from embryonic stem cells (ESCs) [13]. Moreover, cellular senescence increases DUSP4 protein levels by metabolic stabilization [14], and in cases of apoptosis induced by oxidative stress, DUSP4 has been shown to be a transcriptional target of p53 [15].

Intracellular calcium serves as a secondary signaling messenger that mediates a variety of neuronal functions, including motility, differentiation, synaptic plasticity, and memory formation [16]. The influx of calcium ions through voltage-selective calcium channels can produce biological signals and modulate the expression of genes involved in cell proliferation and neuronal differentiation [17,18]. Alterations in localized Ca^{2+} dynamics can induce ESCs to differentiate and undergo neuronal morphogenesis [19]. Ca^{2+} /calmodulin-dependent kinases (CaMKs) operate as potential downstream effectors of calcium elevation in neurons [20]. Recent studies have established critical roles for Ca^{2+} /calmodulin-dependent kinase I (CaMKI) activity alternation, such as growth cone motility [21], neurite outgrowth [22,23], and the activity-dependent growth of dendrites [24]. L-type voltage-dependent Cav1.2 channels have a key function in the maintenance of intracellular calcium homeostasis and are particularly effective at inducing

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changes in gene expression [25]. Blocking L-type Ca^{2+} channels inhibits neurogenesis both in vivo and in vitro [26]. Therefore, changes in Ca^{2+} channel expression could completely affect the generation of neuronal phenotypes in the developing nervous system.

Despite the considerable progress made by exploring in vitro substrate specificity and the expression profiles of DUSPs, we still do not fully understand their many intricate functions at the cellular level. DUSP4 remains an enigmatic protein, and none of the previous works has established a link between this protein and neuronal differentiation. In this study, we addressed the role and mechanistic action of DUSP4 in neural induction and differentiation using an in vitro mammalian ESC-derived neural lineage model.

Materials and Methods

Cell culture and neuronal differentiation

The mouse ESC (mESC) line, J1 ES [American Type Culture Collection (ATCC), Manassas, VA] derived from strain 129s4/Jae, was routinely maintained on γ -irradiated mouse embryonic fibroblasts in ESC medium [high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 15% ES tested fetal bovine serum (PAA, Colbe, Germany), 1% penicillin–streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Invitrogen), and 1×10^3 U/mL of leukemia inhibitory factors (LIF; Millipore, Billerica, MA)] at 37°C in a humidified atmosphere with 5% CO_2 .

For differentiation, the cells were cultured in a medium without LIF (Millipore), as described previously [27]. Embryoid bodies (EBs) were generated by plating 3×10^6 cells/cm² of ESCs onto 90-mm noncell culture-treated Petri dishes (SPL Lifesciences, Pocheon, Korea) in ES medium without LIF (Millipore). The medium was changed every 2 days (2EB, 4EB, 6EB, and 8EB). All trans-retinoic acid (RA; 10 μM) (Sigma-Aldrich, St. Louis, MO) was added at day 4 (4EB) and day 6 (6EB) of EB formation. At day 8, the EBs were trypsinized and the cells were plated at a density of 1.5×10^5 cells/cm² on poly-D-lysine-laminin (Sigma-Aldrich)-coated tissue culture dishes containing N2 medium (Invitrogen) with 20 ng/mL bFGF (Invitrogen).

Construction of lentiviral vectors and transduction

To construct J1 ESCs that stably expressed FLAG-tagged wild type or mutant DUSP4 protein, a lentiviral infection system was used. For the expression of DUSP4, DNA encoding the FLAG-tagged DUSP4 was transduced into J1 ESCs using the pLentiX-GFP-c1 vector (Clontech Laboratories, Mountain View, CA). For virus production, LentiX 293T cells were transfected with a vector containing the DUSP4 sequence and a MISSION Lentiviral Packaging Mix (Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the medium was collected as the first virus-containing supernatant and replaced with new medium. The medium was collected after 48 and 72 h. The supernatants were concentrated at 13,893 g for 90 min and used to infect cells in the presence of 8 $\mu\text{g}/\text{mL}$ of polybrene (Sigma-Aldrich). Infected cells were enriched using a FACS Aria cell sorter (BD Biosciences, San Jose,

CA) and were further maintained in growth medium. Ectopic expression of FLAG-tagged DUSP4 was confirmed by western blot analysis. The catalytically inactive mutant DUSP4, in which Cys-284 was replaced by Ser, was constructed by site-directed mutagenesis in plasmids. CaMKI mutants (T177A and T177D) were also generated by site-directed mutagenesis using primers for T177A (5'-CAG TGTGCTCTCCGCAGCCTGTGGGACTCC-3', 5'-GGAGT CCCACAGGCTGCGGAGAGCACACTG-3') and T177D (5'-CAGTGTGCTCTCCGCAGCCTGTGGGACTCC-3', 5'-GGAGTCCCACAGGCGTCGGAGAGCACACTG-3').

shRNA-mediated knockdown of DUSP4

Lentiviruses were generated using the MISSION Lentiviral Packaging Mix (Sigma-Aldrich). Lipofectamine 2000 (Invitrogen) was used to transfect LentiX 293T cells either with vectors containing shRNA sequences targeting DUSP4 (TRCN0000054348, TRCN0000054349, TRCN0000054350, TRCN0000054351, and TRCN0000054352) or a nontargeted shRNA control vector (scrambled-shRNA, SHC-002; Sigma-Aldrich). Stable knockdown J1 ESC lines were produced by lentiviral transduction with either DUSP4 targeting shRNA or scrambled shRNA in the presence of 8 $\mu\text{g}/\text{mL}$ polybrene (Sigma-Aldrich). Selection was performed in a medium containing 2 $\mu\text{g}/\text{mL}$ puromycin (Invitrogen) for 10 days.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from cultured cells using the TRIzol RNA reagent (Qiagen, Hilden, Germany). Cells in the TRIzol reagent were homogenized with a syringe. Following homogenization, the samples were centrifuged at 10,770 g for 10 min at 4°C. After removing the pellets, 0.2 mL of chloroform (Sigma-Aldrich) was added to the soluble fractions, which were then centrifuged at 10,770 g for 2 min at 4°C. RNA was precipitated by adding a 1 $\times$ volume of isopropanol (Sigma-Aldrich), incubating at room temperature for 10 min, and centrifuging at 10,770 g for 15 min at 4°C. The RNA pellet was washed with 0.5 mL of ice-cold 70% ethanol (Millipore), centrifuged at 10,770 g for 1 min at 4°C, and the supernatant was carefully removed. The RNA was resuspended in a small volume of RNase-free water.

First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA as a template, 500 ng of random primers (Promega, Madison, WI), 200 U of M-MLV Reverse transcriptase (Promega), and 25.2 U of Rnasin RNase inhibitor (Promega) in a total volume of 25 μL . Gene expression was analyzed in triplicate using 3 μL of the RT products (the cDNA was diluted 10 to 1 before use), 5 pmol of each primer (Table 1), and SYBR Premix Ex Taq TM (Takara Bio, Inc., Otsu, Japan) using a CFX96TM Real-Time System (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis

The cells were washed three times with ice-cold phosphate buffered saline (PBS) and harvested in ice-cold RIPA buffer [1% NP-40, 0.5% sodium-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM sodium chloride, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 50 mM sodium fluoride] or NP-40 buffer [1% NP-40, 150 mM NaCl, 10%

glycerol, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/mL leupeptin, and 10 μ g/mL aprotinin] containing a protease inhibitor and a phosphatase inhibitor cocktail (Roche, Penzberg, Germany). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories). Protein samples were separated on a SDS-polyacrylamide gel electrophoresis (PAGE) gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore) using standard procedures. The membrane was blocked with 5% (w/v) nonfat dry milk in Tris buffered saline (pH 7.5) and then incubated with primary antibodies for 12 h on a rocking platform at 4°C. The membrane was then washed three times with TBS-T buffer for 15 min and incubated with 1% skim milk in TBS-T buffer containing HRP-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA) for 1 h. The hybridized membrane was washed in TBS-T buffer and developed for visualization using an enhanced chemiluminescence detection kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The relative ratios of the band densities for each protein band were quantified using ImageJ software and expressed as the mean $\pm$ SD ($n=3$).

Brain protein extraction at distinctive developmental stages

To prepare protein samples from mouse embryos and pups, brain cortical regions were removed from C57BL/6J (KOATECH, Pyeongtaek, Korea) embryos at 10, 12, 15, and 18 days of gestation and pups postnatally at 1 and 4 days. After rapid dissection, the tissues were homogenized in buffer [20 mM HEPES, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100, supplemented with 1% mixture of protease and phosphatase inhibitors (Roche)]. The lysate was centrifuged at 10,770 g for 15 min at 4°C. The protein concentration in the lysate was determined by the Bradford assay (Bio-Rad Laboratories). Protein samples were incubated at $-70^\circ C$ until further experiments. All animal experiments were approved and conducted in accordance with the guidelines of the Institute of Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea).

Neurite outgrowth analysis

Parameters of neurite outgrowth were quantified from phase-contrast images of cells acquired using a microscope (Leica Microsystems, Wetzlar, Germany). A neurite was defined as a process that was equal to or greater than one cell body in length. The average neurite length was measured for individual cells using the MetaMorph 7.1 (Universal Imaging, Media, PA). Data were obtained for every cell with an identifiable neurite from three fields of view in each well (~ 25 cells per field). The data are presented as the mean $\pm$ SD from three wells ($n=3$).

Isolation of nuclear and cytoplasmic fractions from differentiated neurons

Western blot analysis was performed on cell homogenates after subcellular fractionation, as previously described [28]. At the end of each experiment, the cells were rinsed twice

with ice-cold PBS, scraped in PBS, and collected by centrifugation at 574 g for 20 min at 4°C. The supernatant was discarded, and the cells were resuspended in ice-cold fractionation buffer [250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.4), supplemented with a complete protease inhibitor tablet (Roche)] and incubated on ice for 10 min. Nuclei were pelleted at 500 g for 5 min and washed three times with a fractionation buffer containing 1% NP-40 on ice. The supernatant was further centrifuged at 20,000 g for 20 min to precipitate the membrane fraction. The resulting supernatant contained the cytoskeleton-containing fraction. Each sample was mixed with 5 $\times$ sample buffer containing SDS (Sigma-Aldrich) and β -mercaptoethanol (Invitrogen) and boiled at 95°C for 5 min. The samples were run on SDS-PAGE, and the expression levels were analyzed by western blotting. Anti-Histone H1 (Santa Cruz) and anti- α -tubulin (Sigma-Aldrich) antibodies were used as nuclear and cytosolic markers, respectively.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS and blocked with 5% goat serum (Invitrogen) for 1 h at room temperature. After blocking, the cells were incubated with primary antibodies, including anti-MAP2 (Abcam, Cambridge, UK), anti- β -tubulin III (Cell signaling, Danvers, MA), and anti-DUSP4 (Santa Cruz), diluted in 0.2% bovine serum albumin (BSA) in PBS. After washing with PBS-T (0.1% Tween-20 in PBS), the cells were incubated with Alexa-488- or -594-conjugated secondary antibodies (Invitrogen) in 0.2% BSA/PBS for 1 h at room temperature. After washing thoroughly, fluorescence images were captured on either a Leica microscope (Leica Microsystems) or an Axiovert 200 microscope (Carl Zeiss, Oberkochen, Germany).

ERK signaling phospho antibody array

The cells were lysed and proteins were extracted using the Protein Extraction Kit (Full Moon Biosystems, Sunnyvale, CA), as described previously [29]. The ERK signaling phospho antibody array contained 227 well-characterized phosphorylated antibodies against the ERK pathway and the corresponding total antibodies. To ensure reliability and consistency of the results, each array included six replicates of each antibody and phospho antibody. The antibody microarray was performed according to the manufacturer's protocol, and the slides were scanned using the Full Moon Biosystems Scanning Array Service. A ratio was calculated to measure the extent of protein phosphorylation. The results from quadruplicate samples were averaged.

Immunoprecipitation

For immunoprecipitation, anti-CaMKI kinase (CaMKK; Novus, Littleton, CO) or anti-microtubule affinity-regulating kinase 2 (MARK2; Abcam) antibodies were added to lysates containing equal amounts of protein. The mixture was incubated overnight at 4°C. Protein A/G plus agarose beads (Calbiochem, Billerica, MA) was added, followed by agitation for 2 h at 4°C. Immunoprecipitates were recovered by

brief centrifugation at 3,000 *g* and washed three times with NP-40 lysis buffer. Phosphorylation of CaMKK and MARK2 was detected by immunoblotting with antibodies against phosphorylated serine/threonine (ECM Biosciences, Versailles, KY).

Fluo-4/AM intracellular calcium staining and image analysis

To label the cytosolic free calcium, differentiated neuron cells were loaded with 5 μ M Fluo-4/AM dye (Invitrogen) in N2 media (Invitrogen) for 30 min at 37°C in an incubator with an atmosphere of 5% CO₂ and washed two times with N2 media (Invitrogen), according to the protocol provided by the manufacturer. The cells were then examined under a fluorescence microscope (Leica Microsystems). More than six fluorescence images were analyzed to compare the experimental groups, and the results were quantified using the MetaMorph 7.1 (Universal Imaging). Maximum intensity projections were obtained. For each portion, one 10×10 pixel region of interest (ROI) was drawn over (8–12 ROI/cell), and another ROI was drawn over nearby empty space. The fluorescence intensity of the captured image was then calculated by subtracting the fluorescence of the empty ROI from that of the ROI exhibiting staining.

Plasma membrane fractionation

The surface expression of Cav1.2 channels was assessed, as previously described [30] and according to the manufacturer's instructions (Thermo Fisher Scientific, Inc., Waltham, MA). Briefly, cell monolayers were washed three times with ice-cold PBS, incubated for 30 min at 4°C, and shaken gently in the presence of 0.5 mg/mL of the membrane-impermeable reagent Sulfo-NHS-SS-biotin in PBS. The cells were then washed three times with PBS supplemented with 10% BSA and incubated in the same solution, but without the biotin, for an additional 5 min to remove any unbound NHS-SS-biotin. After three additional cycles of PBS washing, the cells were scraped into 150 μ L of harvest buffer [20 mM Tris-HCl (pH 8.0), 137 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 0.5% Triton X-100, 50 mM sodium fluoride, 20 mM benzamide (Sigma-Aldrich), and protease inhibitor cocktail (Roche)].

The scraped cells were collected in Eppendorf tubes that were prechilled on ice, sonicated for 30 s, and centrifuged at 5,000 *g* for 10 min at 4°C. The supernatant was collected, and the protein content was determined using the Bio-Rad assay kit with BSA as a standard. The samples were adjusted to a similar protein concentration. Immobilized NeutrAvidin resin (Thermo Fisher Scientific, Inc.) was added to the samples, and the reaction mixture was gently rotated end over end at 4°C for 12 h. Thereafter, the resin was centrifuged at 6,000 *g* for 5 min and washed three times with ice-cold harvest buffer. The released proteins were retained and marked as the membrane fraction. Western blot analysis of the total protein and membrane fraction samples was performed using an anti-Cav1.2 antibody (Thermo Fisher Scientific, Inc.). Pan-cadherin was used to confirm equal protein loading, and pan-cadherin (Abcam) was used as a plasma membrane loading control.

Statistical analysis

Experimental differences were tested for statistical significance using analysis of variance and *t*-tests. *P* values less than 0.05 were regarded as statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). ANOVA was used for the statistical analysis of protein expression between groups, and the Tukey–Kramer method used for multiple comparisons.

Results

DUSP4 is continuously upregulated during neuronal differentiation

Neural progenitors derived from RA-treated mESCs can be propagated as neurospheres and further differentiated into mature neurons in vitro (Supplementary Fig. S1A–C; Supplementary Data are available online at www.liebertpub.com/scd). To identify novel candidates for PTPs associated with neuronal differentiation, the differences in gene frequency between undifferentiated ESCs and differentiated neurons were analyzed by a quantitative polymerase chain reaction (qPCR) array. Among the several PTPs that showed significant alterations in their mRNA levels, the *DUSP4* mRNA level was dramatically increased during neuronal differentiation (Fig. 1A). Additionally, western blot analysis clearly showed that DUSP4 was significantly upregulated during neuronal differentiation (Fig. 1B). To examine the subcellular localization of endogenous DUSP4, immunocytochemistry with an anti-DUSP4 antibody was conducted in both J1 ESCs and differentiated neurons. The data showed that DUSP4 was mainly localized to the nucleus, but was also detected in the cytosol (Fig. 1C). In particular, DUSP4 expression was dramatically increased during in vivo development of the brain (Fig. 1D). However, there have not been any direct reports on the exact roles of DUSP4 in neurogenesis. Thus, we chose to investigate whether the manipulation of DUSP4 affects neurogenesis in mESCs.

DUSP4 is functionally relevant for neuronal differentiation

To determine whether DUSP4 expression is required for neuronal differentiation, we generated J1 ESCs expressing shRNA against *DUSP4* (shDUSP4) using a lentiviral expression system and then induced them to differentiate into neurons (Supplementary Fig. S2A). The knockdown of DUSP4 did not affect the cell viability or survival in J1 ESCs or J1 ES-derived neuronal cells (Supplementary Fig. S2B, C). We directly compared the neural differentiation capacity of normal and DUSP4-depleted mESCs. In the absence of DUSP4, the differentiation efficiency at day 3 was significantly lowered compared with the scrambled negative control. The neurite outgrowth on MAP2-positive cells with DUSP4 depletion were significantly decreased (Fig. 2A). In the condition of neuronal differentiation that we used, almost all cells were stained with anti-MAP2 antibody. So, we measured the neurite outgrowth on cells visualized under phase contrast instead of on cells stained with anti-MAP2 antibody in the remaining experiments. To verify the negative effect of *DUSP4* knockdown on neuronal differentiation, the expression levels of neural progenitor or neuronal markers were analyzed by qPCR. A significant

FIG. 1. DUSP4 is continuously upregulated during neuronal differentiation. **(A)** Analysis of DUSP4 mRNA expression during neuronal differentiation of J1 ESCs (means $\pm$ SD; $n = 3$). **(B)** The level of DUSP4 protein was analyzed by western blotting at various differentiation time points. The results for DUSP4 expression were quantified by densitometric analysis (means $\pm$ SD; $n = 3$). **(C)** Representative confocal images of J1 ESCs (left) and differentiated neurons (right), fixed and stained with antibodies. DUSP4 was stained with Alexa Fluor-488 and β -tubulin III was stained with Alexa Fluor-594. DAPI-stained nuclei were identified by the blue color. Scale bars, 20 μ m. **(D)** Western blot analysis of DUSP4 protein levels during mouse neuronal development. E, embryonic day; PN, postnatal. DUSP4, dual specificity phosphatase 4; ESC, embryonic stem cell.

decrease in *nestin* gene expression was observed in DUSP4-knockdown cells compared with cells treated with the scrambled negative control vector. The expression levels of other neuronal specific genes, such as *MAP2*, *β -tubulin III*, and *synaptophysin*, were also significantly decreased in DUSP4-knockdown cells during differentiation (Fig. 2B). In addition, the protein levels of MAP2 and β -tubulin III were clearly downregulated (Fig. 2C, D). On the basis of these results, we propose that DUSP4 has important functions for neuronal differentiation.

Reintroducing DUSP4 into cells expressing shDUSP4 rescued the effects of DUSP4 depletion on neuronal differentiation

Next, we tested whether the suppression of neuronal differentiation by DUSP4 knockdown could be reversed by the reintroduction of *DUSP4*. We infected shDUSP4 J1 ESCs with a lentivirus containing the *DUSP4* gene to clarify the functional role of DUSP4 in neurogenesis.

A catalytically inactive mutant version of the *DUSP4* gene was used as a negative control. Overexpression of the wild type and catalytically inactive mutant DUSP4 was confirmed by western blot analysis (Supplementary Fig. S2B). As shown in Fig. 3B–D, the effects of DUSP4 gene knockdown on neuronal differentiation were significantly reversed by the reintroduction of wild-type DUSP4, but not catalytically inactive mutant DUSP4. DUSP4 reintroduction led to the restoration of a lower degree of neurite outgrowth than was noted with the scrambled negative control (Fig. 3A). The expression levels of the neural progenitor marker, *nestin*, and positive neuronal regulators, such as *MAP2*, *β -tubulin III*, and *synaptophysin*, were also consistently res-

cued and were similar to the levels observed in normal neuronal differentiation (Fig. 3B). However, the catalytically inactive mutant DUSP4 did not significantly affect neurite outgrowth or the expression of neurogenic markers compared with DUSP4-knockdown cells, indicating that the phosphatase activity of DUSP4 is critical for increasing the level of neuronal differentiation.

Hyperactivation of ERK was induced through DUSP4 depletion

DUSP4 inactivates its target kinases by dephosphorylating both phospho-serine/threonine and phosphotyrosine residues. DUSP4 can bind to and inactivate ERK, JNK, and p38 [31]. Therefore, we investigated the levels of active ERK, JNK, and p38 during neuronal differentiation in cells subjected to shRNA-mediated DUSP4 knockdown. As shown in Fig. 4A, the level of phospho-ERK was hyperactivated during neuronal differentiation after DUSP4 knockdown. The dephosphorylation of active ERK by DUSPs has been shown to play an important role in the regulation of ERK signaling in both time and space [32]. Although there was a clear increase in phospho-ERK induction in the absence of DUSP4, the magnitudes of JNK and p38 phosphorylation were not affected at several differentiation time points (data not shown). These results suggest that ERK activation is critical at an early stage of neuronal differentiation and that DUSP4 is involved in the regulation of ERK activity to achieve an appropriate magnitude of neuronal differentiation.

Lentiviral transduction of *DUSP4* into shDUSP4 J1 ESCs resulted in the phospho-ERK level being recovered when these cells were differentiated into neurons. Compared with

FIG. 2. DUSP4 knockdown inhibits neuronal differentiation. (A) DUSP4-knockdown cells (using the shDUSP4-I construct) were induced to differentiate into neuronal cells. After 3 days of differentiation, neurite outgrowth was measured on MAP2-positive cells and quantified using the MetaMorph software (means $\pm$ SD; $n = 3$; $**P < 0.01$). Scale bars, 100 μm . (B) The expression levels of neuronal specific markers were analyzed using quantitative polymerase chain reaction (qPCR) after DUSP4-knockdown J1 ESCs were induced to undergo neuronal differentiation for 3 days ($n = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (C) Immunocytochemistry analysis of differentiated neurons infected with either a scrambled or lentiviral shRNA construct against DUSP4. MAP2 was stained green and β -tubulin III was stained red. Scale bars, 100 μm . (D) J1 ESCs infected with scrambled (left) or shDUSP4 (right) and differentiated to neurons at the indicated time points. Representative western blots for neuronal specific markers are shown.

wild-type DUSP4, a marginal effect on ERK activation was detected in shDUSP4 J1 ESCs treated with vectors expressing the catalytically inactive DUSP4 mutant (Supplementary Fig. S3A). Next, we examined where hyperactive ERKs were located in DUSP4-knockdown cells. The depletion of

DUSP4 induced increased the ERK activity in both the cytosol and nucleus (Fig. 4C). The hyperactivation of ERK induced by shDUSP4 was diminished by importing a dominant negative ERK mutant whose phosphorylation sites, T202 and Y204, were replaced with A202 and F204

FIG. 3. Recovery from the effects of DUSP4 depletion by the introduction of a shRNA-resistant DUSP4 gene. **(A)** Analysis of morphological changes during neurogenic differentiation of DUSP4-knockdown cells after transduction with an empty vector, DUSP4, or a catalytically inactive DUSP4 mutant. After 3 days of differentiation, neurite outgrowth was evaluated and quantified using the MetaMorph software (means $\pm$ SD; $n=3$; *** $P<0.001$). Scale bars, 100 μ m. **(B)** The rescue experiment was performed by investigating the effects of reintroducing wild-type DUSP4 or a catalytically inactive DUSP4 mutant on the differentiation of DUSP4-knockdown J1 ESCs. Quantitative real-time PCR was performed using mRNA samples from neuronal differentiated cells, infected as in Supplementary Fig. S2B, for the neural progenitor gene, nestin, and neuronal specific markers ($n=3$; * $P<0.05$, ** $P<0.01$, *** $P<0.001$). **(C)** MAP2 and β -tubulin III levels were evaluated by immunocytochemistry analysis. Scale bars, 100 μ m. **(D)** J1 ESCs infected with shRNAs, as in Supplementary Fig. S2B, were plated at an equal density and differentiated into neurons. Whole-cell lysates were prepared on day 3 for western blot analysis of MAP2, β -tubulin III, and actin.

(Supplementary Fig. S3B). These results definitively indicate that DUSP4 regulates neuronal differentiation through modulation of ERK activation.

Taken together, we identified that the knockdown of DUSP4 induced the hyperactivation of ERK, and consequently reduced neuronal cell differentiation.

Effects of CaMKI on neuronal differentiation of shDUSP4 J1 ESCs

To gain additional insight regarding the effects of DUSP4 depletion on neuronal differentiation, we further examined changes in the phosphorylation status of signaling molecules

related to ERK signaling. For this purpose, a phospho-specific ERK antibody array experiment was performed.

To validate the antibody array data, western blot studies were also performed. Among the candidates, we focused on CaMKI because it has been reported that CaMKI has a role in neurite outgrowth [22,23]. The phosphorylation level of T177 of CaMKI was clearly reduced in shDUSP4-knockdown differentiated neurons. In the case of shDUSP4 neurons transduced with DN ERK, CaMKI phosphorylation and the expression of neural-specific markers, MAP2 and β -tubulin III, were surprisingly restored to a level similar to the control (Fig. 5A). These results highlighted the potential role of CaMKI in the mechanism of DUSP4–ERK signaling.

FIG. 4. DUSP4 depletion induces hyperactivation of ERK. (A) During neuronal differentiation, knockdown of DUSP4 increased the ERK activation level. Soluble fractions of differentiated neurons expressing scrambled control or shDUSP4 were assessed by western blot analysis (left). Densitometric measurements of the band intensities in the presented blots are shown (right) ($n=3$; $**P<0.01$, $***P<0.001$). (B) The phospho-ERK levels of DUSP4-knockdown neurons (day 3) were compared with the scrambled control by immunocytochemistry analysis. Scale bars, 20 μm . (C) Hyperactivation of ERK in the cytoplasm and nucleus was caused by DUSP4 inhibition. The effective fractionation states were checked using Histone H1 in the nucleus and α -tubulin in the cytoplasm. ERK, extracellular signal-regulated kinase.

To investigate this pathway, we studied the effects of DUSP4 knockdown on the neuronal differentiation of cells overexpressing CaMKI or the phosphomimetic mutant (CaMKI T177D). The neuronal differentiation behavior of shDUSP4 J1 ESCs was effectively reversed by CaMKI overexpression, as demonstrated by the increased neurite outgrowth and the expression of neuronal markers. Conversely, overexpression of a nonphosphorylatable CaMKI T177A mutant did not increase the expression of neuronal markers. By contrast, the overexpression of CaMKI T177D, a phosphomimetic form of CaMKI, led to elongated neurites (Fig. 5B, C).

CaMKI activities are strongly dependent on phosphorylation by a highly specific CaMKK at an equivalent threonine (Thr177 in CaMKI) in the activation loop of the kinase [33]. Upon activation by $\text{Ca}^{2+}/\text{CaM}$, the catalytic domain of CaMKI interacts with and phosphorylates multiple sites on MARK2. Therefore, MARK2 is a critical effector and is the immediate downstream effector of CaMKI signaling that promotes neuronal differentiation [34]. In DUSP4-knockdown cells, differentiated neuronal cells showed a reduced level of phosphorylation on CaMKK as well as on MARK2 (Fig. 5D, E). Thus, DUSP4 depletion induced increased ERK activity, thereby repressing neuronal differentiation, whereas dysregulation of neuronal differentiation was rescued by introducing DN ERK or CaMKI.

It was previously reported that neurite outgrowth and ERK activation are mediated by CaMKI activation through active CaMKK [23]. Notably, the inhibition of CaMKI influenced ERK activation during neuronal differentiation. Based on this result, we characterized the phosphorylation status of ERK in the presence and absence of a CaMKI inhibitor, KN93. As shown in Supplementary Fig. S4, application of the CaMKI inhibitor induced a reduction in the phospho-CaMKI level as well as active ERK and, consequently, the DUSP4 expression level after 3 days of neuronal differentiation. Taken together, these results support the presence of a molecular mechanism between the MAPK cascade and the CaMKI signaling pathway that regulates DUSP4-ERK pathway.

Effect of DUSP4 depletion on intracellular calcium levels in neuronal differentiation

In this study, we explored the functional properties of shDUSP4 mESC-derived neurons. To further determine the molecular mechanism by which the levels of phospho-CaMKK, phospho-CaMKI, and phospho-MARK2 were decreased in DUSP4-knockdown cells, we monitored the intracellular calcium levels. In differentiated neurons at day 3, intracellular calcium was brightly stained with Fluo-4/AM.

FIG. 5. Effects of CaMKI on the neuronal differentiation of shDUSP4 J1 ESCs. (A) Representative immunoblots of the indicated proteins from whole cell lysates of differentiated neurons stably infected with a scrambled control, shDUSP4, and shDUSP4 together with either an ERK vector or DN ERK (dominant-negative mutant of ERK). Neuronal specific markers, phospho-CaMKI and CaMKI were studied. (B) DUSP4-depleted J1 ESCs after reintroducing CaMKI or a CaMKI mutant (T177A, T177D) were induced into the neurogenic lineage for 3 days, according to standard procedures, and the length of neurites extending from the cells was analyzed. The data represent the mean length $\pm$ SD compared with the control vector ($n = 3$; $*P < 0.05$, $***P < 0.001$). (C) The protein levels of neuronal specific markers were examined after overexpression of CaMKI or the CaMKI mutant (T177A and T177D). (D, E) Total cell lysates containing equal amounts of protein were immunoprecipitated with anti-CaMKK and anti-MARK2 antibodies, and western blot analysis was performed with an anti-phospho-serine/threonine antibody. The levels of phospho-CaMKK and phospho-MARK2 were normalized to the total protein. CaMKI, Ca^{2+} /calmodulin-dependent kinase I; CaMKK, CaMKI kinase; MARK2, microtubule affinity regulating kinase 2.

However, when DUSP4 was depleted by shDUSP4, the intracellular calcium level was dramatically decreased. With the introduction of DN ERK into the cells, the calcium level was restored. In accordance with the recovery of the intracellular calcium level, the length of the neurite extensions was recovered (Fig. 6A). Our data revealed that neuronal cells derived from DUSP4-knockdown J1 ESCs lack the fundamental properties of the calcium signaling machinery.

Intracellular calcium release is a fundamental and conserved mechanism in neuronal development, indicating that

voltage-gated calcium channel (VGCC) function at this stage of differentiation [26]. Therefore, we assessed the effects of DUSP4 knockdown on the expression and surface trafficking of L-type calcium channels, as previous findings showed that the effects of GnRH-induced ERK activation and DUSP4 gene expression were dependent on calcium signaling from VGCC [35]. In the present study, we evaluated the effect of DUSP4 on the expression and surface localization of Cav1.2. Cellular expression of Cav1.2 was decreased in DUSP4-knockdown cells, which was corrected

by the overexpression of DN ERK. To assess the localization of Cav1.2 to the plasma membrane, purification of the plasma membranes was conducted using the Sulfo-NHS-SS-biotin and NeutrAvidin resin. Western blot data showed that the reduction in plasma membrane localization of Cav1.2 in the DUSP4 knockdown condition was much greater than the total expression of Cav1.2. Plasma membrane localization was also restored upon DN ERK expression (Fig. 6B, C). These findings clearly suggest the novel regulatory functions of DUSP4 and new mode for the regulation of Cav1.2 channels by the ERK signaling cascade.

Based on the obtained results, we hypothesized that DUSP4 knockdown led to defective cellular responses, such as calcium homeostasis, that are known to be linked to neuronal differentiation. Furthermore, DUSP4 plays a key role in effectively balancing the intracellular calcium level.

Discussion

Tyrosine phosphorylation is an important posttranslational modification that regulates fundamental biochemical processes. It is a dynamic process governed by the opposing activities of protein tyrosine kinases, which catalyze tyrosine phosphorylation, and PTPs, which are responsible for tyrosine dephosphorylation [36]. It is also possible that the functions of different phosphatases may be additive or antagonistic in different places and/or at different moments during development. In the case of neuronal differentiation, previous studies have demonstrated that the expression of neural cell markers and DUSPs were closely related to axon development in various types of neurons [37,38].

To assess the potential contribution of PTP signaling in the neural fate decisions of ESCs, we first surveyed the expression levels of PTPs during differentiation into neurons. The results of this array for PTPs demonstrated that DUSP4 expression was dramatically increased in neurons compared with ESCs. While significant progress has been made in understanding the expression profiles, functions, and targets of DUSP4 in carcinogenesis, relatively little is known about its roles in embryogenesis. Moreover, whether or not DUSP4 is involved in neuronal development is unknown. In the present study, the mechanism of the regulatory action of DUSP4 on neuronal differentiation was investigated using a neural differentiation model with mESCs. In situ hybridization analysis of DUSP4 previously indicated that it is highly expressed in many areas of the brain, as strong staining of the hippocampus, piriform cortex, and the suprachiasmatic nucleus was observed [39]. Thus, this report highlighted the potential roles of

DUSP4 during brain neurogenesis or in differentiated neurons.

To determine whether DUSP4 signaling is involved in the neural differentiation of ESCs, we next examined its expression pattern during ESC differentiation. Interestingly, quantitative qPCR and western blot analyses showed that the expression of DUSP4 gradually increased as the neuronal differentiation of ESCs progressed (Fig. 1A, B). The function of DUSP4 in the context of neurodevelopment was investigated by loss-of-function experiments using shRNA in vitro. Based on the data presented in this study, the most significant effect of inhibiting DUSP4 expression was found in the reduction of the neural precursor marker, nestin, and neuronal cell marker genes, such as MAP2, β -tubulin III, and synaptophysin, which work together to reduce neurite outgrowth (Fig. 2). These results demonstrate that DUSP4 has a role in both early neural commitment of mESCs and the differentiation of neural precursor cells into neuronal cells.

Intricate control over the phosphorylation level of MAPKs plays a key role in determining the magnitude and duration of kinase activation and, hence, in the physiological outcome of signaling [40]. DUSPs constitute the largest family of tyrosine phosphatases that are capable of regulating MAPKs in mammalian systems [3]. It has been reported that DUSP4 selectively dephosphorylates ERK1/2 [5] and that DUSP4 can be phosphorylated by ERK1/2 in MDA231 cells [41]. During neuronal differentiation, ERK activity gradually increased, and knockdown of DUSP4 specifically induced hyperactivation of ERK, but not p38 or JNK (Fig. 4). Treatment of differentiated neurons with U0126, a MEK inhibitor, induced degradation of DUSP4 through the proteasome system (data not shown). These results strongly suggest that DUSP4 may control neurogenesis by modulating ERK activity, specifically by regulating its phosphorylation and that DUSP4 and ERK regulate each other through feed-back control. It has been proposed that ERK signaling is integrated and can regulate crosstalk between the ERK cascade and other important signaling pathways. In other words, perturbation of the ERK signaling cascade may exert profound influences on many other pathways and their respective functions. Therefore, we searched for molecular targets that were influenced by disruption of the DUSP4–ERK balance. To understand how DUSP4 knockdown affected cellular signaling, we evaluated the profile of protein phosphorylation related to the MAPK–ERK pathway, and knockdown of DUSP4 in mESC-derived neuronal cells was found to decrease the phosphorylation status of CaMKI.

FIG. 6. DUSP4 regulates intracellular calcium levels in an ERK-dependent manner. (A) Cells were stained with Fluo-4/AM to visualize intracellular calcium at 3 days postneural induction. The data were obtained from two fields in each well using a Leica microscope, and the intensity was measured using the MetaMorph. To quantify the intracellular calcium level, the average intensity per $100\ \mu\text{m}^2$ area was determined by measuring the background-subtracted changes (the data represent the mean levels $\pm$ SD; $n=3$; *** $P<0.001$). Neurite outgrowth was also measured, and the quantification was performed using the MetaMorph software (means $\pm$ SD; $n=3$; *** $P<0.001$). Scale bars, $100\ \mu\text{m}$. (B) The depletion of DUSP4 decreased the surface expression of Cav1.2 channels. Representative blots from subcellular fractionation experiments are shown. The surface expression of Cav1.2 was also evaluated in differentiated neurons at day 3 with or without the DN ERK expression ($n=3$; * $P<0.05$, ** $P<0.01$, *** $P<0.001$). (C) The expression of Cav1.2 channels was detected by immunocytochemistry. Images were captured with an Axiovert 200 microscope, and the fluorescence intensity was measured in the MetaMorph. Scale bars, $20\ \mu\text{m}$. ($n=3$; * $P<0.05$, ** $P<0.01$).

FIG. 7. Proposed model for regulation of neuronal differentiation by DUSP4. This study is the first to demonstrate that DUSP4 regulates the ERK pathway through a negative feed-forward system for neuronal differentiation and is linked to Ca^{2+} signaling. Thus, DUSP4 knockdown seems to be responsible for the hyperactivation of ERK as well as the perturbation of calcium-related signaling.

To identify the mechanism of phospho-CaMKI reduction following DUSP4 knockdown, we investigated the intracellular calcium level in DUSP4-knockdown cells. Ca^{2+} imaging analysis revealed that the intracellular calcium level was elevated in differentiated neurons and was suppressed by DUSP4 knockdown. Changes in the intracellular calcium level can induce ESCs to differentiate into different neuronal phenotypes [42]. Generally, Ca^{2+} influx depends on the opening of voltage-gated Ca^{2+} channels. Ca^{2+} influx through VGCCs, such as long lasting Ca^{2+} channels (L-type, also known as Cav1), also affects the expression of genes involved in cell proliferation, programmed cell death, and neuronal differentiation [43–45]. Interestingly, the abnormalities induced by DUSP4 knockdown were almost completely reversed by the expression of constitutively active CaMKI (T177D) or DN ERK (Fig. 5). Consistently, we found that hyperactivation of ERK by DUSP4 knockdown decreased the trafficking of endogenous Cav1.2 channels to the plasma membrane. Notably, this manipulation did affect the total expression of Cav1.2 (Fig. 6). Moreover, the expression and surface localization of L-type calcium channels were also restored by DN ERK expression, indicating that these effects of DUSP4 knockdown were indeed dependent on ERK regulation by DUSP4. It has been reported that the expression of T-type calcium channels was enhanced through the activation of Ras–ERK signaling and neuronal Cav channels was regulated by growth factors through ERK-dependent signaling

[46,47]. Taken together, these results suggest that the coupling of DUSP4–ERK and calcium–Cav1.2–CaMKI signaling is the mechanism operating in neuronal differentiation. It was previously reported that, in addition to directly phosphorylating L-type calcium channels, ERK may indirectly regulate their surface expression by phosphorylating other molecules [48]. However, more detailed investigations are needed to reveal how ERK activity regulates the surface localization and gene expression of L-type calcium channels.

Neurodevelopmental disorders (NDs) are impairments that affect the development and growth of the central nervous system (CNS) during embryonic and early postnatal life [49]. At the cellular level, NDs are associated with abnormal neurogenesis and/or synaptogenesis [50]. Several studies have shown that upregulation of the Raf–ERK signaling pathway may contribute to the pathogenesis of autism by impairing cortical neuron development and causing neural circuit imbalances [51,52]. Postmortem studies have associated the expression of DUSP4 with a significant decrease in ERK activity and suggested a role for DUSP4 in depressive disorders [53]. In addition to its role in neural differentiation, it is possible that DUSP4 can modulate neural circuits or neurotransmitter release in the adult brain. Thus, disturbance of the ERK pathway by defects in DUSPs may contribute to diseases of the CNS, and adjustment of DUSP4 activity could potentially contribute to a solution for them.

TABLE 1. LIST OF PRIMERS USED IN THIS STUDY

Gene	Accession no.	Forward primer	Reverse primer
<i>DUSP4</i>	NM_176933.4	CTGGCCTACCTGATGATGAA	TGAGACTCAAACCTGCAGCAA
<i>Nestin</i>	NM_016701.3	GAGGTGACCCTTGGGTTAGA	TAGCCCTACCACTTCCTGCT
<i>β-tubulin III</i>	NM_023279.2	AGCCCTCTACGACATCTGCT	ATTGAGCTGACCAGGGAATC
<i>MAP2</i>	M21041.1	CACCTGGACATCAGCCTCACT	CACCCTGGTCCTTCATCTCT
<i>Synaptophysin</i>	NM_009305.2	GAGAACAACAAAGGGCCAAT	GCACATAGGCATCTCCTTGA
<i>Actin</i>	BC138614.1	CATCCGTAAAGACCTCTATGCCAA	ATGGAGCCACCGATCCACA
<i>GAPDH</i>	GU214026.1	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

DUSP4, dual specificity phosphatase 4.

DUSPs mediate their effects on gene expression through the dephosphorylation of the ERK, and DUSP6 (MKP3) has already been shown to reduce neurotransmitter release by downregulating the expression of Cav1.2 in PC12 cells [54]. Our data showed that DUSP4 expression also appears to have a strong effect on ERK-dependent regulation of Cav1.2 expression and activity. One of the most consistent findings from point mutations in the gene encoding the L-type voltage-gated Ca^{2+} channel Cav1.2 (*CACNA1C*) has been that it causes Timothy syndrome, a multisystem disorder that includes cardiac abnormalities and autism [55,56].

Taken together, the function of DUSP4 is most likely mediated by its effect on the balance of ERK signaling and intracellular calcium dynamics (Fig. 7). It is possible that the amount of DUSP4 present in neurons allows for fine-tuning of the transduction pathways to control neuronal differentiation. Additionally, it should be proceeded to perform the further study that DUSP4 has the role of neuronal differentiation in vivo and investigate whether DUSP4 is involved with neurodegenerative disorders related to Ca^{2+} , such as Alzheimer's disease, ischemic stroke, and amyotrophic lateral sclerosis or NDs, such as autism.

Acknowledgments

The authors would like to thank Professors Sung Goo Park and Sang J. Chung for their continuous encouragement and helpful advice. In addition, the authors thank Drs. Byoung Chul Park, Jeong-Ki Min, and Seung-Wook Chi for carefully reading the article and providing insightful comments. This work was supported by grants from KRIBB and the Korea National Research of Foundation (no. 2012M3A9C7050101, 2011-0030028, and 2013M3A9A7046301).

Author Disclosure Statement

The authors have nothing to disclose and no competing interests exist.

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Received for publication September 12, 2014

Accepted after revision November 14, 2014

Prepublished on Liebert Instant Online November 14, 2014