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RESEARCH ARTICLE

Correlation between receptor-interacting protein 140 expression and directed differentiation of human embryonic stem cells into neural stem cells

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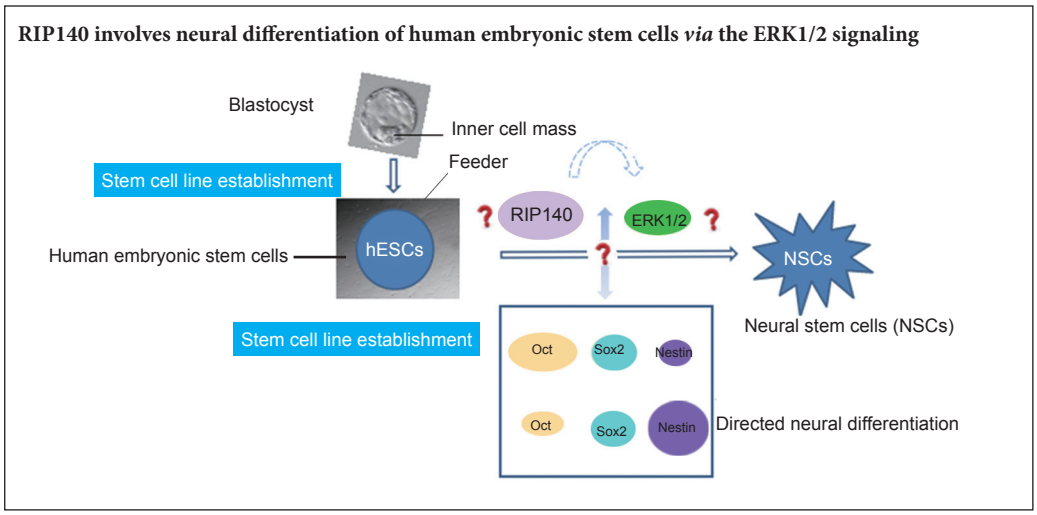
Graphical Abstract



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Abstract

Overexpression of receptor-interacting protein 140 (RIP140) promotes neuronal differentiation of N2a cells *via* extracellular regulated kinase 1/2 (ERK1/2) signaling. However, involvement of RIP140 in human neural differentiation remains unclear. We found both RIP140 and ERK1/2 expression increased during neural differentiation of H1 human embryonic stem cells. Moreover, RIP140 negatively correlated with stem cell markers Oct4 and Sox2 during early stages of neural differentiation, and positively correlated with the neural stem cell marker Nestin during later stages. Thus, ERK1/2 signaling may provide the molecular mechanism by which RIP140 takes part in neural differentiation to eventually affect the number of neurons produced.

Key Words: *nerve regeneration; receptor-interacting protein 140; neural stem cells; human embryonic stem cells; directed differentiation; Oct4; Sox2; Nestin; extracellular regulated kinase 1/2 signaling pathway; neural regeneration*

Introduction

During neurogenesis, neural differentiation is controlled by a selective spatiotemporal gene expression profile produced by switching expression of different genes on or off. Temporal expression of neural differentiation markers is the foundation of normal nervous system function. Abnormal neuronal differentiation, such as occurs with Down's syndrome or Alzheimer's disease, can lead to an exceptional number, density, morphology, and/or connection of neurons and synapses, as well as a low ratio of neural to glial cells and changes neural network building, resulting in a series of abnormal clinical manifestations involving the central nervous system (Greber et al., 1999; Martin et al., 2012; Moya-Alvarado et al., 2016). It has been demonstrated that Oct4, Sox2 and Nanog are core factors for stem cell maintenance and self-renewal (Niwa, 2007). As changes in the expression of these genes are

closely related to stem cell differentiation (Wu et al., 2014), their expression can be used to identify stages of neural differentiation. In addition, Notch, Wnt, repressor element-1-silencing transcription factor/neuron restrictive silence factor, sonic hedgehog, extracellular regulated kinase 1/2 (ERK1/2), and phosphatidyl inositol 3-kinase/protein kinase B pathways simultaneously play important roles in neural differentiation (Li et al., 2007, 2008; Canzonetta et al., 2008; Woo et al., 2009; Topol et al., 2015; Yan et al., 2015; Gao et al., 2016).

Receptor-interacting protein 140 (RIP140, also known as NRIP1) is a negative regulatory transcription factor located in the nucleus. RIP140 participates in the regulation of retinoic acid, thyroid hormones, glucocorticoids and other hormones (Subramaniam et al., 1999; Heim et al., 2009; Park et al., 2009). Our previous studies confirmed that RIP140 exhibits a dynamic spatiotemporal expression pattern during normal murine brain development (Li et al., 2007). Meanwhile, RIP140 overexpression promotes mouse neuroblastoma cell differentiation (Feng et al., 2015), while the RIP140/LSD1 complex fine-tunes the neural differentiation marker Pax6 during mouse neuronal differentiation (Wu et al., 2016). However, expression of RIP140 and its potential involved in directed differentiation of human embryonic stem cells (hESCs) to neural stem cells (NSCs) remains unclear. In this study, we investigated the spatiotemporal expression of RIP140 and its correlation with NSC markers during directed differentiation of hESCs.

Materials and Methods

Cell culture

The hESC line H1 was obtained from Peking University School of Life Sciences (Beijing, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Hyclone, Logan, UT, USA) supplemented with 20% KnockOut™ Serum Replacement (Invitrogen, Carlsbad, CA, USA), 2 mM GlutaMAX™ (Invitrogen), 100 U/mL non-essential amino acids, 100 U/mL penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), 0.55 mM β-mercaptoethanol, and 20 ng/mL basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA). hESCs were grown on a feeder cell layer of mouse embryonic fibroblasts, purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and treated with 0.01 mg/mL of mitomycin C (Roche, Basel, Switzerland), and maintained in a 5% CO_2 humidified incubator (Thermo) at 37°C. Mouse embryonic fibroblasts were maintained in DMEM/high glucose media supplemented with 10% fetal bovine serum (Gibco), 100 U/mL non-essential amino acids, and 100 U/mL penicillin/streptomycin.

In vitro directed neural differentiation

High-quality hESCs (with minimal or no differentiated colonies) were cultured on Matrigel® (Becton Dickinson, Franklin Lakes, NJ, USA). When hESCs reached 70–80% confluency, clones were dislodged with Dispase II (Roche) to generate cell clumps, which were then plated in Matrigel-coated 6-well plates at a density of 2.5–3 × 10^5 hESCs per well. One day after hESC plating, culture medium was replaced with 2.5 mL of pre-warmed complete PSC Neural Induction Medium (Gibco). Medium was refreshed every other day and the amount was doubled on days 4 and 6 of neural induction. Non-neural differentiated cells were removed with a Pasteur glass pipette (Corning Incorporated, Corning, NY, USA). Cells were collected on days 0, 3.5, and 7 of neural induction from both control (cultured in normal growth medium containing maintenance growth factors) and differentiation (cultured in neural induction medium containing directed induction factors) groups. Images of cell morphology were acquired using a Nikon Eclipse TE300 Inverted Microscope (Sendai, Japan).

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cell lines using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). cDNA was reverse transcribed from 0.5 μg of RNA using ReverTra ACE® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) in a Bio-Rad Opticon® 2 Real Time PCR System (Hercules, CA, USA). Cycling conditions were as follows: 94°C for 2 minutes; 40 cycles of 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds (Feng et al., 2015). Primer pairs were designed as described in **Supplementary Table 1** (Woo et al., 2009, Birket et al., 2011). Reaction specificity was confirmed *via* melting curve analysis. The housekeeping gene β-actin was used as an internal standard. Fold-changes between gene expression in control and differentiated cells were determined using the ΔΔCt method. All experiments were conducted at least in triplicate.

Immunofluorescence analysis

Cells were placed on glass coverslips (Fisher, Pittsburgh, PA, USA) and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cell membranes were permeabilized with 0.2% Triton X-100, washed with PBS and then blocked with 5% goat serum (Applygen, Beijing, China). Samples were then incubated with primary antibodies against Oct4 (1:200; Rabbit mAb; Sigma Aldrich, St. Louis, MO, USA), Sox2 (1:150; Mouse mAb; Sigma), Nestin (1:200; Rabbit mAb; Sigma), and/or RIP140 (1:50; Rabbit mAb; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Oct4 and Sox2 are markers for stem cells, while Nestin is a marker for NSCs. Following primary antibody incubation, samples were washed with PBS and incubated with rhodamine- or FITC-conjugated goat anti-rabbit IgG antibody (1:100; Millipore, Billerica, MA, USA) at 37°C for 2 hours protected from light. After additional washes, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Beyotime, Shanghai, China). Images were acquired with an Olympus IX70 Inverted Fluorescence Microscope (Tokyo, Japan). All experiments were conducted in triplicate and quantified in a blind manner. Corresponding quanti-

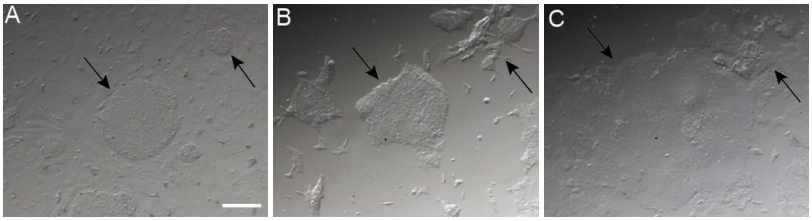

Figure 1 Morphologic changes of cell clones.
Changes in cell clone (arrows) morphology on day 0 (A), day 3.5 (B), and day 7 (C) of neural induction. The images were obtained by Nikon Eclipse TE300 Inverted microscope. Scale bar: 200 μm.

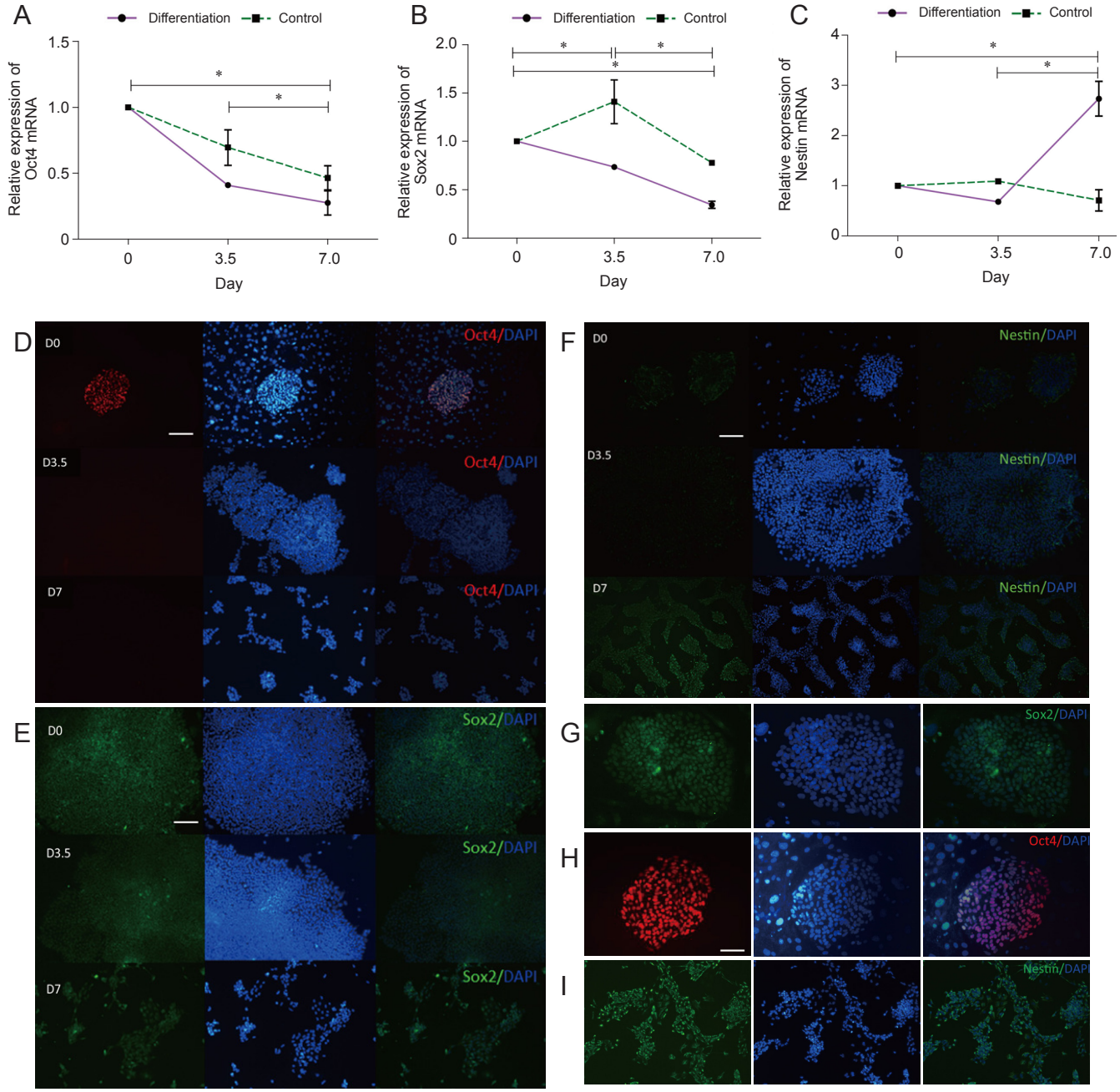

Figure 2 Expression of neural differentiation markers changed dynamically during neural induction from day 0 to day 7 of hESCs.
(A–C) Changing expression trends of Oct4 (A), Sox2 (B) and Nestin (C) mRNAs in differentiation and control groups, as detected by quantitative polymerase chain reaction. Data are expressed as the mean ± SD. Asterisks denote a statistically significant difference between different time points in the differentiation group ($P < 0.05$). All of these markers in the differentiation group had significant differences compared with the control group and on day 7 eventually. The Oct4 ($P = 0.003 < 0.05$) and Sox2 ($P = 0.021 < 0.05$) are lower and Nestin ($P = 0.001 < 0.05$) is higher in the differentiation group. One-way analysis of variance followed by a Dunnett's *post-hoc* test was used. All experiments were conducted at least in triplicate. (D–F) Immunofluorescent images (objective magnification 10×) of cells stained for Oct4 (D), Sox2 (E) and Nestin (F) on days 0, 3.5, and 7 in the differentiation group. Exposure time was about 590 ms; scale bar: 100 μm. (G–I) Immunofluorescent images (objective magnification 20×) of Oct4 (G) on day 0, Sox2 (H) on day 0 and Nestin (I) on day 7. Exposure time was approximately 910 ms. Scale bar: 40 μm. The marker expression changes in the control group seen in Supplementary Figure 1A and localization in Supplementary Figure 2A. hESCs: Human embryonic stem cells. D0, D3.5, D7: Day 0, day 3.5, day 7, respectively.

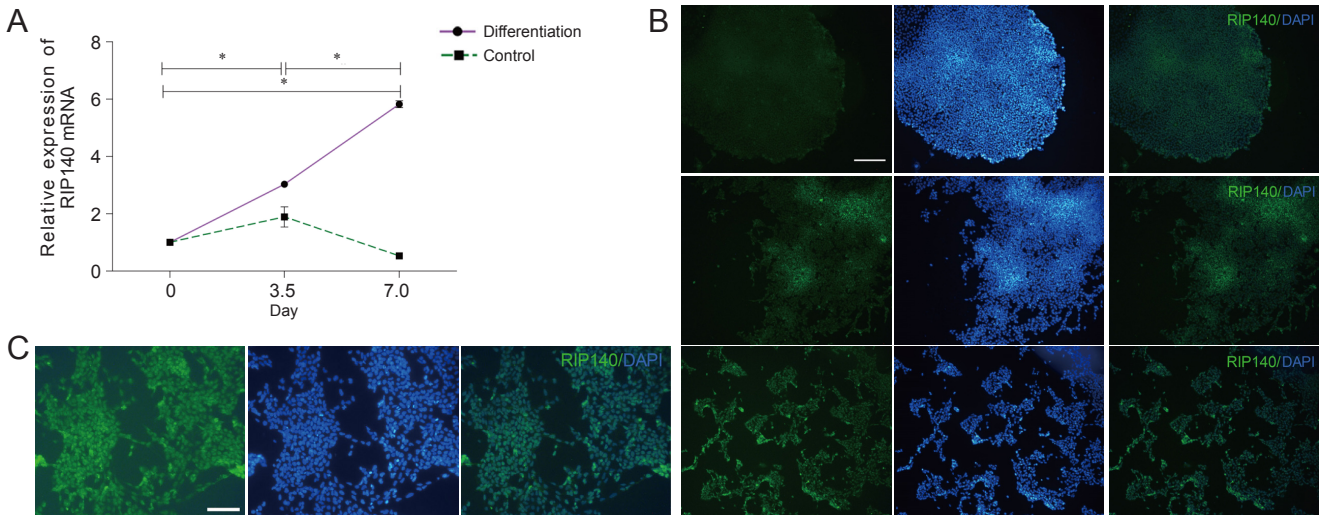

Figure 3 RIP140 expression increased during directed differentiation of hESCs.
(A) RIP140 expression trend as detected by quantitative polymerase chain reaction. Data are expressed as the mean ± SD. Asterisks denote a statistically significant difference between different time points in the differentiation group ($P < 0.05$; one-way analysis of variance followed by a Dunnett's *post-hoc* test). All experiments were conducted at least in triplicate. (B) Immunofluorescent images (objective magnification 10×) of cells stained with RIP140 at different differentiation time points. Exposure time was approximately 590 ms. Scale bar: 100 μm. (C) Immunofluorescent images (objective magnification 20×) of RIP140 localization on day 7. Exposure time was about 910 ms. Scale bar: 40 μm. RIP140 changes in the control group seen in Supplementary Figure 1B and localization on days 0 and 3.5 seen in Supplementary Figure 2B. RIP140: Receptor-interacting protein 140; hESCs: human embryonic stem cells.

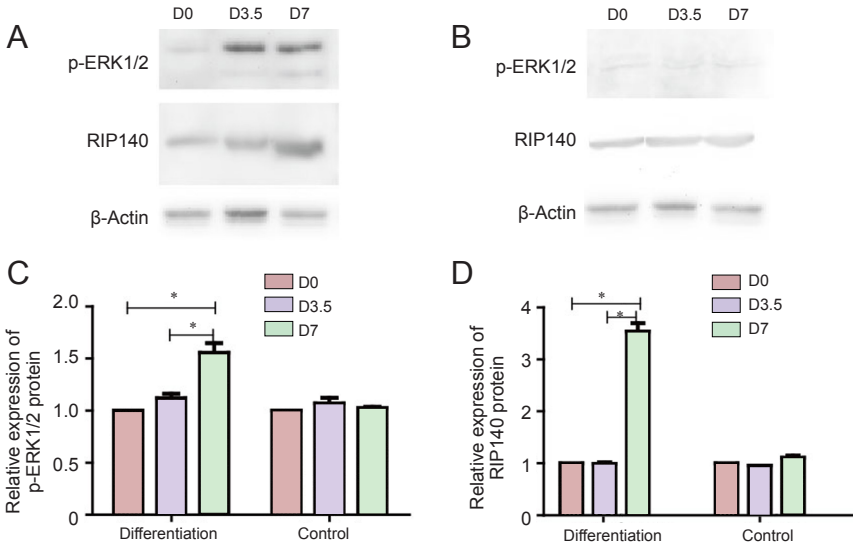

Figure 4 p-ERK1/2 expression increased during directed differentiation of hESCs.
(A) Western blot assay showing p-ERK1/2 and RIP140 protein levels on days 0, 3.5 and 7 in the differentiation group. Equal amounts of cell lysates (100 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting with anti-p-ERK1/2, anti-RIP140 or anti-β-actin antibodies. (B) Western blot assay showing p-ERK1/2 and RIP140 protein levels on days 0, 3.5 and 7 in the control group. (C, D) Bar graphs represent the relative expression levels, represented as gray value ratio of p-ERK1/2 (C) and RIP140 (D) on days 3.5 and 7 as quantified by ImageJ 2× (National Institutes of Health, Stapleton, NY, USA). The immunoblot shown is representative of three independent experiments, and bar graph data are represented as the mean ± SD. Asterisks denote a statistically significant difference ($P < 0.05$; one-way analysis of variance, followed by a Dunnett's *post-hoc* test). p-ERK1/2: Phosphorylated-extracellular regulated kinase 1/2; hESCs: human embryonic stem cells. D0, D3.5, D7: Day 0, day 3.5, day 7, respectively.

tative results are described in **Supplementary Table 2**.

Western blot assay

Protein lysates were obtained by scraping and subjecting cells to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma), and then transferring onto nitrocellulose membranes (Thermo). Membranes were blocked in a TBST solution consisting of 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0.05% TWEEN® 20, containing 5% non-fat milk powder for 1 hour at 25°C, then incubated with antibodies against RIP140 (1:500; Rabbit mAb; Santa Cruz Biotechnology) or phosphorylated ERK1/2 (p-ERK1/2, 1:500; Mouse

Table 1 Correlation between RIP140 and markers for neural differentiation

Time point	Gene	Correlation coefficient (r)	P
Days 0–7	Oct4	–0.836	0.005 < 0.05
Days 0–7	Sox2	–0.931	< 0.001
Days 0–7	Nestin	0.410	0.272
Days 0–3.5	Oct4	–0.833	0.039 < 0.05
Days 0–3.5	Sox2	–0.847	0.033 < 0.05
Days 0–3.5	Nestin	–0.480	0.336
Days 3.5–7	Oct4	–0.395	0.439
Days 3.5–7	Sox2	–0.902	0.014 < 0.05
Days 3.5–7	Nestin	0.984	< 0.001

RIP140: Receptor-interacting protein 140.

mAb; Cell Signaling Technology, Danvers, MA, USA) or β-actin (1:1,500; Mouse mAb; Cell Signaling Technology) overnight at 4°C. After TBST washes, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (1:2,000; Cell Signaling Technology) for 1 hour at 25°C. Protein expression was subsequently detected and visualized using an Enhanced Chemilumenescence substrate (Thermo) (Feng et al., 2015). Results were quantified by ImageJ 2× software (National Institutes of Health, Stapleton, NY, USA). All experiments were conducted in triplicate.

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA). Statistical significance of differences between groups was determined by one-way analysis of variance followed by a Dunnett's *post-hoc* test. Correlations were analyzed using Pearson correlation analysis. P values of less than 0.05 were considered statistically significant.

Results

Morphological changes of cell clones during directed neural differentiation of hESCs into NSCs

During directed differentiation, circular cell clones became irregular and reached confluency later. The edges of cell clones developed one or more branches. Scattered cells were observed to have oval, triangular, or stellate shapes, thus presenting NSC-like morphological characteristics (**Figure 1**).

Spatiotemporal expression of neural markers on days 0, 3.5, and 7 of neural induction

At the mRNA level, relative expression of Oct4 and Sox2 exhibited decreasing trends over days 0, 3.5, and 7 in the differentiated group (**Figure 2A**, **B**). Nestin expression decreased slightly from days 0 to 3.5, and then increased sharply in the differentiation group (**Figure 2C**). All three markers were significantly different in the differentiation group compared with the control group by day 7 and thereafter. Expression of Oct4 (P = 0.003) and Sox2 (P = 0.021) was lower, while Nestin (P = 0.001) was higher in the differentiation group. At the immunoreactive level, Oct4 showed high fluorescence intensity on day 0, but was barely visible on days 3.5 and 7, representing a decreasing trend (**Figure 2D**). However, the fluorescence intensity of Sox2 did not substantially change (**Figure 2E**). Nestin increased dramatically on day 7, coinciding with real-time PCR results (**Figure 2F**). While Oct4 and Sox2 localized to the nucleus, Nestin localized to the cytoplasm during neural induction (**Figure 2G–I**). Changes in marker expression in the control group are presented in **Supplementary Figure 1A**; whereas, localization patterns are outlined in **Supplementary Figure 2A**.

RIP140 increased during neural induction

RIP140 mRNA (**Figure 3A**) and fluorescence intensity (**Figure 3B**) gradually increased with prolonged induction time. Meanwhile, a significant increase in RIP140 mRNA expression was observed in the differentiation group on day 7 compared with the control group (P < 0.001). Levels of RIP140 protein, which localized to the nucleus throughout neural differentiation (**Figure 3C** and **Supplementary Figure 2**), also increased as neural differentiation progressed (**Figure 4A**, **C**).

Expression of RIP140 and neural differentiation markers were highly correlated at different phases of directed neural differentiation

Throughout neural differentiation, RIP140 expression showed a strongly negative correlation with that of Oct4 and Sox2 (P < 0.05 and P < 0.001, respectively), but had no significant correlation with Nestin expression (P > 0.05). During early differentiation (days 0–3.5), expression of RIP140 exhibited a strongly negative correlation with Oct4 and Sox2 (P < 0.05); moreover, during late differentiation (days 3.5–7), RIP140 exhibited a strongly negative correlation with Sox2 expression (P < 0.05) and strongly positive correlation with Nestin expression (P < 0.001; **Table 1**).

RIP140 may affect directed differentiation of hESCs into NSCs *via* the ERK1/2 pathway

Increasing evidence has pointed toward an important role for ERK1/2 activation during neuronal differentiation ((Li et al., 2006; Chan et al., 2013). Thus, we investigated levels of phosphorylated ERK1/2 (p-ERK1/2) expression during directed differentiation of hESCs into NSCs. Interestingly, an increasing trend in ERK1/2 phosphorylation was observed to mirror that of RIP140 (**Figure 4A**, **B**).

Discussion

Directed neural differentiation is essential during neurogenesis. Alterations in neural differentiation can lead to an aberrant number of neurons, resulting in the formation of abnormal cortical circuits that contribute to cognitive deficits such as those seen in Down Syndrome (Yabut et al., 2010; Haydar and Reeves, 2012).

Temporal expression of stem cell and neural markers, such as Oct4, Sox2, Nestin, Pax6, and Nanog, determines the phase of directed neural differentiation and serves as an established standard of induction *in vitro* (Wu et al., 2014). As such, induction models can be used to detect the expression and correlation between RIP140 and other genes in directed differentiation from hESCs into NSCs *in vitro*.

RIP140 is an auxiliary adjustment factor for nuclear receptor transcription. Initial studies of RIP140 primarily concentrated on aspects of energy metabolism and the female reproductive system (Nautiyal et al., 2013). However, our recent studies have suggested that RIP140 has a close relationship with neural differentiation induced by retinoic acid, as RIP140 promoted mouse neuroblastoma cell (N2a) differentiation *via* the ERK1/2 pathway, thereby leading to stem cell depletion, a reduction of nerve cells, and subsequent participation in a series of abnormal phenotypes during central nervous system development (Feng et al., 2014, 2015). A previous study also indicated that the RIP140/LSD1/Pit-1 complex could repress Pax6 expression, thereby participating in retinoic acid-induced neuronal differentiation of mouse embryonic stem cells (mESCs) (Wu et al., 2016). We established that increased RIP140 expression strongly correlated with neural differentiation markers during early neural induction. Thus, we hypothesized that RIP140 might also have an important role in directed neural differentiation of human cells. Use of human stem cells as a model of neural differentiation more faithfully recapitulates *in vivo* neurogenesis than use of mESCs or cancer cells. However, during different phases of induction, RIP140 might correlate with different markers via various pathways. ERK1/2 pathway has been demonstrated to play a significant role in neural differentiation (Lim et al., 2008; Hosseini Farahabadi et al., 2015, Hu et al., 2016). Meanwhile, we observed an increasing trend of ERK1/2, which may be a key pathway in N2a neural differentiation (Feng et al., 2015), and found similar variations in this study; namely, increased expression of p-ERK1/2 was also observed during differentiation. Thus, we concluded that ERK1/2 may also be a critical pathway in directed differentiation of hESCs.

Compared with animal cell models, hESCs are able to overcome species-specific differences and, thus, more closely reflect RIP140 expression trends during normal human embryonic development. However, to determine the exact functions and mechanisms involved in directed neural differentiation, hESC models of RIP140 overexpression, knockdown and/or knockout are needed. Moreover, improvement in the extremely low efficiency of establishing hESC lines is urgently needed.

Taken together, our data support the participation of RIP140 in directed differentiation of hESCs into NSCs. We hypothesize that this may be mediated through the ERK1/2 pathway. Establishing normal expression patterns is the goal of studying the function of RIP140 and other genes, as this information provides a theoretical basis for diagnosis of neurodevelopmental disorders that may also become potential targets for clinical intervention. Finally, the capacity of RIP140 to affect the number, structure, function, or proportion of neural and glial cells during NSC maturation into terminal neurons requires further investigation.

Author contributions: *ZRZ participated in study concept, definition of intellectual content, literature search, experimental studies, data acquisition, data analysis, statistical analysis and paper preparation. WDY participated in study concept and design, definition of intellectual content, literature search, data analysis, paper preparation, edition and review, and served as a guarantor. CS participated in study concept and design, and experimental studies. RL and XC participated in study concept and experimental studies. XF, XZ and QM participated in literature search. HS participated in paper review and served as a guarantor. JZG participated in study concept and design, definition of intellectual content, literature search, experimental studies, data acquisition, paper preparation, edition and review, and served as a guarantor. All authors approved the final version of this paper.*
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Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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Supplementary Table 1 Real time-polymerase chain reaction primer sequences

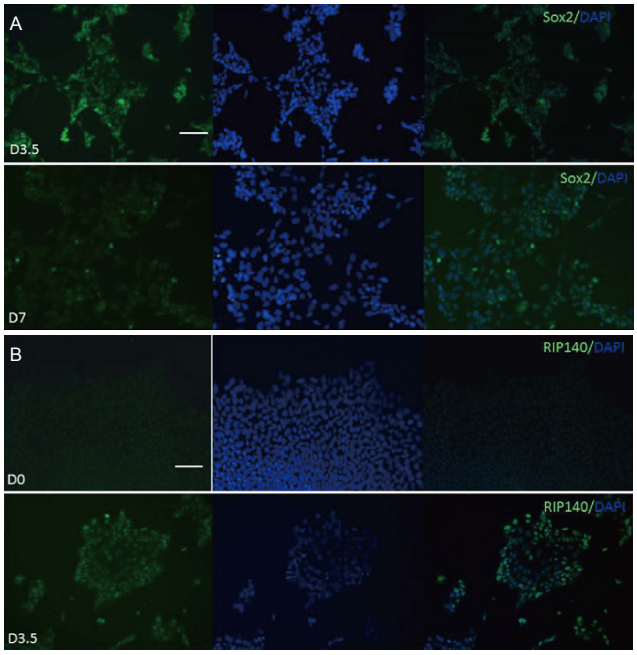
Gene	Sequence (5′–3′)		Size (bp)
Oct4	Forward	CTT GAA TCC CGA ATG GAA AGG G	164
	Reverse	GTG TAT ATC CCA GGG TGA TCC TC	
Sox2	Forward	GCC GAG TGG AAA CTT TTG TCG	157
	Reverse	GGC AGC GTG TAC TTA TCC TTC T	
Nestin	Forward	AAC AGC GAC GGA GGT CTC TA	220
	Reverse	TTC TCT TGT CCC GCA GAC TT	
RIP140	Forward	CCC ATT TGC AGC AGT ATT CTC	125
	Reverse	GTA ACT GCC AAC ATC CTT CTG	
β-Actin	Forward	CGC ACC ACT GGC ATT GTC AT	206
	Reverse	TTC TCC TTG ATG TCA CGC AC	

RIP140: Receptor-interacting protein 140.

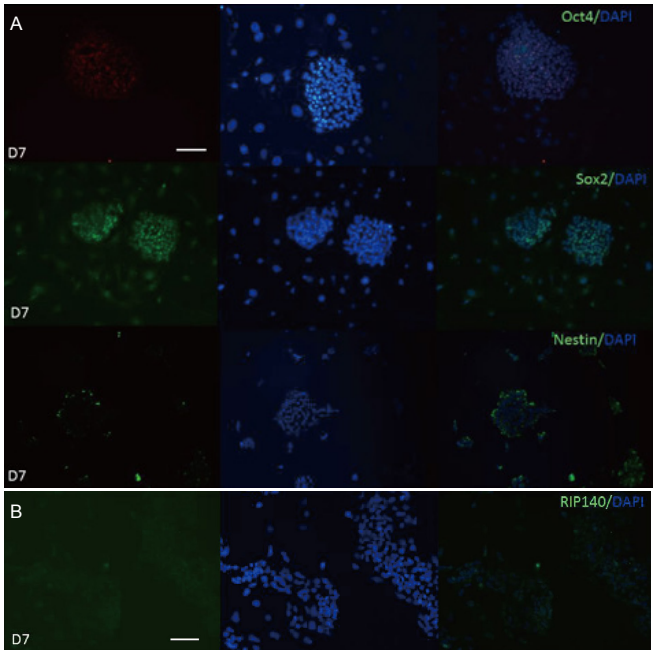
Supplementary Table 2 Relative gene expression at different time points

Gene	Group	Day 0	Day 3.5	Day 7
Oct4	Differentiation	1	0.42	0.21
	Control	1	0.79	0.53
Sox2	Differentiation	1	0.72	0.37
	Control	1	1.25	0.78
Nestin	Differentiation	1	0.66	0.49
	Control	1	1.09	0.86
RIP140	Differentiation	1	3.06	5.91
	Control	1	1.64	0.59

RIP140: Receptor-interacting protein 140.



Supplementary Figure 1 Neural differentiation by markers and RIP140 on day 7 in the control group.
Immunofluorescent images (objective magnification 10×) of Oct4, Sox2, Nestin (A) and RIP140 (B) on day 7. Exposure time was about 910 ms. Scale bars:100 μm. RIP140: Receptor-interacting protein 140.



Supplementary Figure 2 Localization of neural differentiated markers and RIP140 during directed differentiation of hESCs.
Immunofluorescent images (objective magnification 20×) of Sox2 (A) on days 3.5 and 7, RIP140 (B) on days 0 and 3.5. Exposure time was approximately 910 ms. Scale bars: 40 μm. RIP140: Receptor-interacting protein 140; hESCs: human embryonic stem cells.