Receptor-interacting Protein 140 Overexpression Promotes Neuro-2a Neuronal Differentiation by ERK1/2 Signaling

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Abstract

Background: Abnormal neuronal differentiation plays an important role in central nervous system (CNS) development abnormalities such as Down syndrome (DS), a disorder that results directly from overexpression of genes in trisomic cells. Receptor-interacting protein 140 (RIP140) is significantly upregulated in DS brains, suggesting its involvement in DS CNS development abnormalities. However, the role of RIP140 in neuronal differentiation is still not clear. The current study aimed to investigate the effect of RIP140 overexpression on the differentiation of neuro-2a (N2a) neuroblastoma cells, in vitro.

Methods: Stably RIP140-overexpressing N2a (N2a-RIP140) cells were used as a neurodevelopmental model, and were constructed by lipofection and overexpression validated by real-time polymerase chain reaction and Western blot. Retinoic acid (RA) was used to stimulate N2a differentiation. Combining the expression of Tuj1 at the mRNA and protein levels, the percentage of cells baring neurites, and the number of neurites per cell body was semi-quantified to determine the effect of RIP140 on differentiation of N2a cells. Furthermore, western blot and the ERK1/2 inhibitor U0126 were used to identify the specific signaling pathway by which RIP140 induces differentiation of N2a cells. Statistical significance of the differences between groups was determined by one-way analysis of variance followed by the Dunnett test.

Results: Compared to untransfected N2a cells RIP140 expression in N2a-RIP140 cells was remarkably upregulated at both the mRNA and protein levels. N2a-RIP140 cells had a significantly increased percentage of cells baring neurites, and numbers of neurites per cell, as compared to N2a cells, in the absence and presence of RA (P < 0.05). In addition, Tuj1, a neuronal biomarker, was strongly upregulated in N2a-RIP140 cells (P < 0.05) and phosphorylated ERK1/2 (p-ERK1/2) levels in N2a-RIP140 cells were dramatically increased, while differentiation was inhibited by the ERK1/2-specific inhibitor U0126.

Conclusions: RIP140 overexpression promotes N2a cell neuronal differentiation by activating the ERK1/2 pathway.

Key words: Differentiation; Down Syndrome; ERK1/2; Neuro-2a Cells; Neurodevelopment; Overexpression; Receptor-interacting Protein 140

Introduction

The central nervous system (CNS) is a complex neuronal network consisting of a diverse array of cellular subtypes that arise from a relatively uniform population of neural progenitors, in a precise spatial and temporal pattern throughout development.[1] The differentiation of neural cells is an important process during the development of the CNS, determining the development of new neurons, their specialization into concrete subtypes, and their cell-to-cell interactions.[2]

Previous studies of Down syndrome (DS) showed that the abnormal differentiation could cause CNS development abnormalities and functional damage due to gene copy imbalance.[3-5] We previously demonstrated that receptor-interacting protein 140 (RIP of 140 kDa) was significantly upregulated in the brains of DS fetuses, compared to their normal counterparts, implying that RIP140 may be involved in DS CNS developmental abnormalities. At present, however, the role of RIP140 in neuronal differentiation is not clear.

Neuro-2a (N2a), a mouse neuroblastoma cell line, can be induced to differentiate into a neuron-like phenotype by all-trans-retinoic acid, which has been extensively used to study neuronal differentiation, neurite growth, and signaling pathways.[2,6] In this study, we used N2a cells as a cell model to investigate the effects of RIP140 and ERK1/2 inhibition on neuronal differentiation.
Methods

Reagents
The mouse neuroblastoma cell line N2a was obtained from the National Platform of Experimental Cell Resource for Sci-Tech (Beijing, China), Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Hyclone (Logan, UT, USA), 0.05% trypsin/ethylenediaminetetraacetic acid, fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin was purchased from Gibco (Grand Island, NY, USA), and puromycin and G418 were purchased from Amresco (Solon, OH, USA).

Cell culture and differentiation
Neuro-2a cells were cultured in DMEM and 10% FBS supplemented with 1% penicillin/streptomycin (growth medium [GM]), and maintained in a 5% CO₂ humidified incubator at 37°C. To induce differentiation, cells were first cultured in GM for 24 hours after seeding, and the medium then changed to DMEM plus 2% FBS containing up to 20 μM retinoic acid (RA), according to published protocols.[7,8]

Generation of stable receptor-interacting protein 140-overexpressing cell lines
For the generation of stable cell lines, 2 μg of pCMV6-RIP140-GFP vectors (Origene, Rockville, MD, USA) were transfected into 2 × 10⁵ undifferentiated N2a cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Controls were transfected with empty vector (Origene). In parallel, cells were plated and mock-transfected in the absence of DNA. 48 hour after transfection, transfected cells were selected by treating with puromycin (2 μg/ml) or G418 (500 μg/ml). Cells remained under antibiotic selection, and by 15–20 days (with fresh media and antibiotic replenishment every 48 hours), the mock-transfected cells all died. Individual clones were then selected to be identified by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting.

Cell morphology analysis and evaluation of cellular differentiation
To evaluate differences in cell morphology, cultures were observed under an inverted microscope (IX-70, Olympus, Japan) and images were captured using a digital camera connected to the microscope. Cells are bearing at least one neurite equal to or longer than the soma diameter were considered to be differentiated. Cell bodies and neurites present in five randomly selected fields were counted. The percent of cells bearing neurites (i.e. the ratio of differentiated cells divided by total cell number), and the average number of neurites per cell (ratio of neurites divided by cell bodies), were counted in each field. The ratios were expressed as means ± standard deviation (SD).

Real-time quantitative polymerase chain reaction
Total RNA was isolated from all cell lines using RNeasy Mini Kits (Qiagen, Hilden, Germany). cDNA was synthesized from 0.5 μg total RNA by using ReverTra ACE® qPCR RT Master Mix gDNA remover (Toyobo, Osaka, Japan). RT-PCR was performed using a SYBR Green PCR master mix kit (Applied Biosystems, Grand Island, NY, USA) in a Bio-Rad Opticon2 RT-PCR System (Bio-Rad, Hercules, CA, USA). The cycling conditions were as follows: 94°C for 2 minutes; 40 cycles of 94°C for 20 seconds and 60°C for 20 seconds and 30 seconds at 72°C. Primer pairs were designed as described in Table 1. Reaction specificity was confirmed by melting curve analysis. The housekeeping gene β-actin was used as an internal standard. Fold-changes between expression in control and treated cells were determined by the delta Ct (ΔΔCt) method.

Western blotting analysis
Protein lysates were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Membranes were blocked with tris-buffered saline Tween 20 (tris-buffered saline with Tween [TBST]); 20 mmol/L tris-HCl, pH 7.5, 137 mmol/L NaCl, 0.05% Tween 20) containing 5% nonfat milk powder, for 1 hour at room temperature, and incubated with antibodies against RIP140 (1:200; Santa Cruz, CA, USA), Tuj1 (1:500; Sigma, St. Louis, MO, USA), ERK1/2 (1:1000; Cell Signaling Technology, Bilerica, MA, USA), p-ERK1/2 (1:500; Cell Signaling Technology) and β-actin (1:1500; Proteintech Group, Chicago, IL, USA) overnight at 4°C. After TBST washing, blots were incubated with horseradish peroxidase-linked goat anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies (1:2000; Cell Signaling Technology, USA) for 1 hour at room temperature. Bands were then detected and visualized using epichemiluminescence kits.

Immunofluorescence analysis
Cells were plated on glass coverslips (Fisher, Pittsburgh, PA, USA) and treated with RA for 24 hours, followed by fixation in 4% paraformaldehyde in phosphate buffered saline (PBS). Cell membranes were permeabilized with 0.2% Triton X-100, and then washed with PBS and blocked with 5% goat serum (Applygen, Beijing, China). Samples were then incubated with primary antibodies against Tuj1 (1:100; Sigma) at 4°C overnight. Following primary antibody incubation, samples were washed with PBS and incubated with rhodamine-conjugated goat anti-rabbit IgG antibody (1:100 dilution; Millipore, Billerica, MA, USA) at 37°C for 2 hours in darkness. After additional washing, nuclei were counterstained with DAPI (Beyotime, Shanghai, China).

Table 1: RT-PCR primers

| Genes  | Forward (5’→3’)                      | Reverse (5’→3’)                  |
|--------|--------------------------------------|----------------------------------|
| RIP140 | GAACCTGGGCTTCTT                      | GGTGTTGGCTAGCTTT                |
|        | GAATGG                               | GGGAGACCTTT                    |
| Tuj1   | ACCCCGTGGGCTC                       | CCGGAACATGGCT                  |
|        | CAATAT                               | GTGAACT                        |
| β-actin| CTGTCCCTTGATG                        | ATGTACCGACCGA                  |
|        | GCCCTCCTG                            | TTCCC                           |

RT: Real time; PCR: Polymerase chain reaction; RIP140: Receptor-interacting protein 140.
Inhibitor treatment
A 20 mmol/L stock solution of the mitogen-activated protein kinases (MEK1/2) inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA) was diluted with media before use. For the assay, cells were pretreated with or without U0126 for 30 minutes, and then stimulated with RA for 24 hours.

Statistical analysis
Data were expressed as mean ± SD, obtained from at least three independent experiments. The statistical significance of the differences between groups was determined by one-way analysis of variance, followed by the Dunnett test. P < 0.05 were considered statistically significant. The analyses were performed using Statistical Package for the Social Sciences 20.0 software (IBM, Inc., Armonk, NY, USA).

RESULTS
Retinoic acid induces neuronal differentiation of neuro-2a cells and expression of receptor-interacting protein 140
Retinoic acid has been shown to promote neurite outgrowth from a variety of neuronal cell types in culture. Therefore, we chose RA to induce N2a cells differentiation. As shown in Figure 1a, after exposure to 20 μmol/L RA for 24 hours, N2a cells assumed a neuronal-like phenotype by activating neurite production, concurrent with upregulation of endogenous RIP140 [Figure 1b].

The formation of cell clones and the identification of cell models
To determine whether RIP140 played a role in neuronal differentiation, we created a stable RIP140-overexpressing N2a cell line. Cells were maintained under antibiotic selection, and by 15–20 days, transfected cells formed individual clones, while untransfected cells all died. RT-qPCR and western blot were used to quantify the mRNA and protein levels of RIP140 expressions. RT-qPCR showed that relative to the parental N2a cells, RIP140 mRNA expression of cells transfected with empty vector was 1.28 ± 0.07 (P = 0.081)-fold higher, while cells transfected with RIP140 overexpression plasmid were 2.38 ± 0.07 (P < 0.01)-fold higher. Western blots further indicated that RIP140 protein expression also increased in cells transfected with RIP140 overexpression plasmid, as compared to the other two control groups [Figure 2]. A single clone from a plate of RIP140-overexpressing cells was selected for further investigation. We named this cell line N2a-RIP140, and the cell line transfected with empty vector was named N2a-M.

Receptor-interacting protein 140 overexpression promotes neuro-2a differentiation along with neurite outgrowth
We observed no significant difference in cell morphology between N2a cells and N2a-M cells in the absence of RA, with scarcely 4% of the cells developing neurites. However, nearly 26% of the N2A-RIP140 cells had already differentiated even prior to the addition of RA [Figure 3a and b]. We also quantified the number of neurites per cell body in each case. N2a-RIP140 showed an increased number of neurites per cell compared to N2A cells and N2A-M cells [Figure 3c]. At 24 hours after treatment with RA, in contrast to N2a cells and N2a-M cells, the percentage of N2a-RIP140 cells baring neurites soared to 94%, 6.7 times that of the other two cell lines [Figures 3a and b]. In addition, N2a-RIP140 cells had a significant increase in the number of neurites per cell in comparison with N2a cells and N2a-M cells [Figure 3c].

The neuronal biomarker, Tuj1 was upregulated when receptor-interacting protein 140 overexpressed in neuro-2a cells
Because Tuj1 is one of the earliest cytoskeletal proteins specifically associated with neuronal development, it is widely used as a specific differentiation marker. To further confirm the stimulatory effect of RIP140 overexpression on neuronal differentiation, we measured Tuj1 expression levels by RT-qPCR, western blot, and immunofluorescence staining. Figure 4a and b shows that both the mRNA and protein levels of Tuj1 were enhanced significantly (P < 0.05) in N2a-RIP140 cells, as compared to N2a cells and N2a-M cells, regardless of the absence or presence of RA.

![Figure 1: Exposure of neuro-2a (N2a) cells to retinoic acid (RA) leads to neurite outgrowth and Receptor-interacting protein 140 (RIP140) upregulation. (a) Phase-contrast micrographs of N2a cells treated with RA (+RA) or without RA (−RA). (b) Western blotting analysis shows RIP140 expression in the absence (−RA) and presence of RA (+RA).](image)

![Figure 2: Western blotting analysis shows receptor-interacting protein 140 (RIP140) protein levels in each cell line. Equal amounts of cell lysates (100 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with anti-RIP140 or anti-β-actin antibodies. Immunoblot shown is representative of three independent experiments.](image)
Immunofluorescence images further showed that Tuj1 was detectable in N2a cells and N2a-M cells after RA treatment. Interestingly high intensity Tuj1 immunofluorescence staining was detected in N2a-RIP140 cells even in the absence of RA [Figure 4c]. These results strongly suggest that RIP140 overexpression promotes neuronal differentiation.

Receptor-interacting protein 140 overexpression promotes activation of ERK1/2

Increasing evidence suggests that the activation of ERK1/2 plays an important role in neuronal differentiation.[11,12] We thus investigated whether RIP140 overexpression promoted N2a cell differentiation via ERK1/2 activation. Western blot

Figure 3: Receptor-interacting protein 140 (RIP140) overexpression promotes neurite outgrowth. (a) Cell morphology of neuro-2a (N2a), N2a-M, and N2a-RIP140 cells in the absence (retinoic acid [RA]) and presence of RA (+RA). (b) Bar graphs represent the percentage of neurite-bearing cells for each cell line grown in the absence (−RA) and presence of RA (+RA). (c) Bar graphs represent the numbers of neurites per cell for each cell line grown in the absence (−RA) and presence of RA (+RA). All data are representative of three independent experiments and are shown as mean ± standard deviation (*P < 0.05).

Figure 4: Receptor-interacting protein 140 (RIP140) overexpression promotes neuro-2a (N2a) differentiation into Tuj1-positive neurons. (a) Bar graphs showing mRNA levels of Tuj1 expressed in N2a-M and N2a-RIP140 cells, relative to N2a parental cells, in the absence (retinoic acid [RA]) and presence of RA (+RA). Data were normalized to β-actin mRNA, and differences were considered significant when P < 0.05. (b) Western blotting analysis showing Tuj1 protein levels under each condition for each cell line. Samples were collected after 24 hours RA treatment. Equal amounts of cell lysates (100 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with anti-Tuj1 or anti-β-actin antibodies. Immunoblot shown is representative of three independent experiments. (c) Detection of Tuj1 (red) by immunocytochemistry in N2a, N2a-M and N2a-RIP140 cells treated with (+RA) or without (−RA) RA. Blue color corresponds to DAPI (*P < 0.05).
analysis showed that in the absence of RA, compared to N2a and N2a-M cells, N2a-RIP140 cells possessed high levels of p-ERK1/2. Following 24 hours of RA addition, p-ERK1/2 levels of N2a-RIP140 dramatically increased. However, p-ERK1/2 levels in N2a and N2a-M cells following RA treatment were still undetected [Figure 5a].

To further illustrate whether RIP140 involvement in N2a cells differentiation was mediated by ERK1/2, the MEK inhibitor U0126 (10 μmol/L) was used to block phosphorylation of ERK1/2. As shown in Figure 5b-d, U0126 significantly reduced the number of differentiated N2a-RIP140 cells both in the absence and presence of RA. Taken together, these data indicate that RIP140 overexpression promotes N2a cell differentiation by ERK1/2 signaling.

**Discussion**

Mental retardation in DS is mainly a consequence of developmental and functional brain alterations caused by gene dosage imbalance.\(^{13,14}\) Alterations in the number of neurons, as well as in the timing of differentiation, likely lead to the formation of abnormal cortical circuits contributing to the cognitive deficits observed in transgenic mice and DS patients.\(^{3,4}\)

While cortical neuronal density in DS appears normal in early gestation, there are fewer neurons than normal in late gestation (>23 weeks), and this paucity continues throughout early life. It is well known that the major period of generation of cortical neurons occurs from 10 to 25 weeks of gestation. Thus, the timing of the cortical neuron deficiency observed in DS coincides with the maturation phase of human cortical development.\(^{15}\) We previously showed that RIP140 was significantly upregulated in 20 weeks DS fetal brain tissues, compared to matched normal ones.\(^{16}\) Since 20 weeks of gestation is a critical period of differentiation of neural precursor cells, RIP140 overexpression may have disturbed that process. Here, using a gain-of-function approach in a model neuroblastoma cell line, we showed that RIP140 could induce N2a cell differentiation and promote neurite outgrowth and elongation as an independent agent.

It is known that the balance between self-renewal and differentiation of stem/precursor cells is a key factor that maintains a normal number of lineage-committed progeny. To prevent stem cell exhaustion, stem cells must undergo self-renewing cell divisions by preventing commitment to differentiation.\(^{17}\) Premature differentiation of progenitor cells likely affects the size of the progenitor pool, thereby resulting in a reduced number of neurons. Because RIP140 is overexpressed in DS patients, these observations suggest the possibility that RIP140 overexpression may result in an inadequate number of neurons in cortical structures of the brain. In addition to differentiation of neural stem/precursor cells, suitable quantity and types of neural cells depend on the

![Figure 5: Receptor-interacting protein 140 (RIP140) overexpression promotes neuro-2a (N2a) cell differentiation by activation of ERK1/2. (a) Levels of p-ERK1/2 were detected by western blot in N2a, N2a-M and N2a-RIP140 cells with (+retinoic acid [+RA]) or without (−RA) RA treatment. (b) Cell morphology of N2a-RIP140 cells cultured in growth or differentiating media (containing 20 μmol/L RA) in the presence or absence of U0126 (10 μmol/L) for 24 hours. (c) Bar graphs representing the percentage (%) of neurite-bearing cells of N2a-RIP140 cells grown in growth or differentiating media (containing 20 μmol/L RA) in the presence or absence of U0126 (10 μmol/L) for 24 hours. (d) Bar graphs represent the numbers of neurites per cell of N2a-RIP140 cells grown in growth or differentiating media (containing 20 μmol/L RA) in the presence or absence of U0126 (10 μmol/L) for 24 hours. All data are representative of three independent experiments and are shown as mean ± SD.](image-url)
proliferation, apoptosis, and migration of neural cells. The effect of RIP140 overexpression on these cell behaviors in N2a cells requires further rigorous study. To that end, studies of the human neuroblastoma cell line SH-SY5Y cells revealed a number of genes that were rapidly induced by RA, and were highly expressed in the developing nervous system, and a number of these genes have been shown to play a role in neurite outgrowth. In our study, RIP140 mRNA was significantly upregulated in N2a cells by 20 μmol/L RA. However, regardless of the absence or presence of RA, ectopic overexpression of RIP140 promoted neurite outgrowth, suggesting RIP140 to be a RA-responsive gene facilitating this process. This conclusion is supported by our findings that RIP140 overexpression enhances the ability of RA to induce N2a cell differentiation. The ERK1/2 signaling cascade is a central pathway regulated during brain development and is involved in RA to induce N2a cell differentiation.

The ERK1/2 signaling cascade is a central pathway regulated during brain development and is involved in the regulation of several cellular events central to brain development, including proliferation, differentiation, cell survival, and synaptic plasticity. Since other studies revealed that activated ERK1/2 was required for neuronal differentiation, we evaluated the levels of p-ERK1/2 in N2a cells, showing sustained ERK1/2 activation in RIP140-overexpressing cells (even in the absence of RA), while the parental and empty vector-transfected N2a cells had undetectable RIP140 levels. However, neuronal differentiation was negatively affected only by the ERK1/2 pathway inhibitor U0126. Taken together, RIP140 acts to indirectly phosphorylate ERK1/2, a key signaling molecule involved in the rate-limiting step of differentiation of proliferative neuroepithelial cells into neurons.

Though N2a cells present a convenient model of neuronal differentiation, there are differences between cell lines and primary neural stem/precursor cells. Moreover, RIP140-modulated neuronal differentiation may be cell-context-dependent. Moreover, since the mechanism of neuronal differentiation from DS neural stem/precursor cells is unknown, further work should investigate the detailed effects of RIP140 on DS neural stem/precursor cells. Nevertheless, we believe this is the first study to identify a role for RIP140 overexpression in neuronal differentiation, and our study provides a framework for the investigation of other genes that affect brain development in DS.

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