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Conversion of rat embryonic stem cells into neural precursors in chemical-defined medium

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ABSTRACT

Rat embryonic stem (ES) cells hold great interest for the research of neurodevelopment and neurodegenerative diseases. However, neural conversion of rat ES cells *in vitro* has proven to be a challenge owing to the proliferation arrest and apoptosis. Here we report that rat ES cells can commit efficiently to a neural fate in the presence of CHIR99021 and Y-27632 (CY medium). In addition, CHIR99021 is crucial for maintaining the metabolic activity of differentiated rat ES cells, while Y-27632 facilitates the neural differentiation of rat ES cells by inhibiting bone morphogenetic protein expression. The chemical-defined CY medium also provides a platform for exploring the mechanism of neural commitment and optimizing the production efficiency of neural precursor from rat ES cells.

1. Introduction

Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of blastocysts [1]. Derivation of rat ES cells was achieved in 2008 using a chemical-defined N2B27 medium supplied with CHIR99021 and PD0325901 [2,3]. The established rat ES cells have been an important experimental system for basic and applied neuroscience investigations since rats are more relevant to humans than mice. However, in contrast with mouse ES cells, rat ES cells die quickly after the removal of CHIR99021 and PD0325901 [2] instead of differentiating. In this study, we examined the effect of Y-27632 on the survival ability of the differentiated rat ES cells. Y-27632, an inhibitor of Rho associated coiled protein kinase (ROCK), is proven to promote mouse neural precursor survival ability [4] and rat functional recovery after spinal cord injury [5]. Researches also show that activation of ROCK signaling effects numerous intracellular pathways including apoptosis [6,7]. However, our results showed that Y-27632 was not sufficient to maintain the overall survivability of the differentiated rat ES cells. We then combined CHIR99021 and Y-27632 (CY medium) to differentiate the rat ES cells. CHIR99021 is a highly selective small molecule inhibitor of glycogen synthase kinase 3 (GSK3) [8]. Previous study showed that GSK3 signaling played important roles in neural development [9] and inhibition of GSK3 signaling regulated neuronal progenitor proliferation by modulating Wnt/β-catenin signaling [10]. Reports also showed that CHIR99021 facilitated long-term self-renewal of human ES cells-converted neural precursors [11]. Our results showed that rat ES cells differentiated and propagated in the CY medium. The differentiated cells uniformly expressed Nestin and Sox1, two early neural precursor markers [12,13]. The chemical-defined CY medium also provides a platform for exploring the mechanism of neural commitment and our results showed that Y-27632 facilitated neural conversion of rat ES cells by inhibiting expression of endogenous bone morphogenetic protein 4 (BMP4).

2. Material and methods

2.1. Cell culture and differentiation

2.1.1. Maintenance of rat ES cells

The rat ES cells were maintained in N2B27 supplied with CHIR99021 and PD0325901 (2i medium) as described elsewhere [2]. Briefly, the 2i medium was prepared with a 1:1 mixture of DMEM/F12 and Neurobasal medium supplied with 1% glutamax, 1 × N2, 1 × B27, 3 μM CHIR99021 (STEMGENT) and 0.4 μM PD0325901 (STEMGENT). The culture medium was changed by half every day. The rat ES cells were passaged by trypsinization with a dilution of 1:10.

2.1.2. Neural differentiation

The rat ES cells were dissociated into single cell and evenly plated onto laminin-coated plastic (Nunc) at a density of

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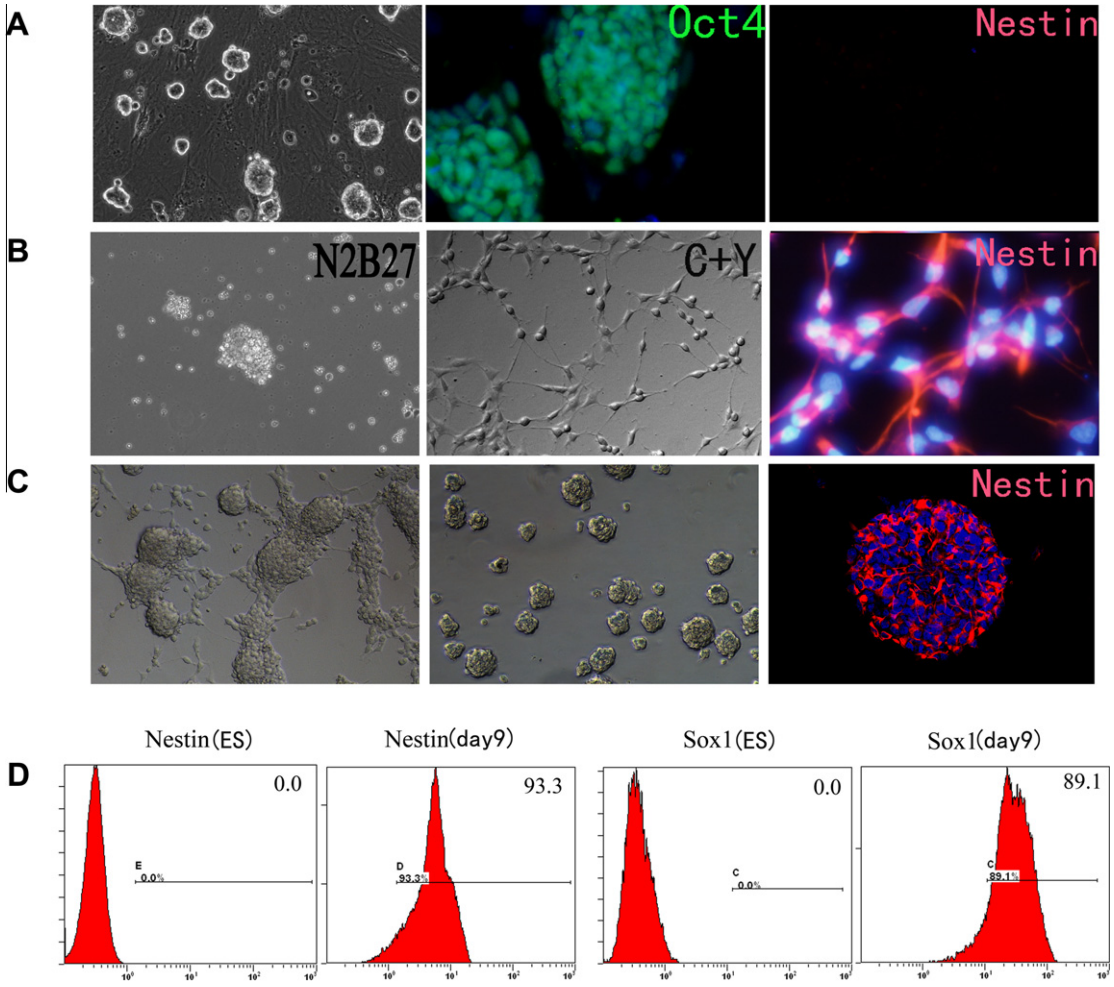

Fig. 1. Rat ES cells efficiently converted into neural precursors in CY medium. (A) Rat ES cells in 2i medium, immnostaining analysis of Oct4 and Nestin-expression. (B) Rat ES cells died in N2B27 medium, survived and showed Nestin-expression in CY medium. (C) The differentiated rat cells formed floating neural spheres on gelatin-coated plastics and showed Nestin-expression. DAPI (blue) staining of nuclei for total cell content. (D) Flow cytometry assay of Nestin-expression in rat ES cells and the converted neural precursors (left). Sox1-expression in rat ES cells and the converted neural precursors (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$0.5–1.0 \times 10^4/cm^2$ in N2B27 medium in the presence of 20 ng/ml Y-27632 (STEMGENT) and 0.5 μM CHIR99021. Three days later, the differentiated cells were dissociated and plated onto fresh laminin-coated plastic again. After 8 days, the cells were transferred onto gelatin-coated plastics to form floating neural spheres. The culture medium was changed to N2B27 medium supplied with 20 ng/ml Y-27632 and 10 ng/ml basic fibroblast growth factor (bFGF). The formed-neural spheres were maintained in floating for 3 days. In order to obtain subsequent differentiation, the floating neural spheres were transferred onto laminin-coated plastics again. Differentiation of TH-positive neurons was processed with Dopaminergic Neuron Differentiation Kit (R&D Systems, SC001B). GFAP-positive astrocytes and CNPase-positive oligodendrocytes were obtained by differentiating the neural spheres in N2B27 supplied with 5% fetal bovine serum.

2.2. Quantitative RT-PCR analysis

Total RNA was extracted from rat cells using an RNeasy Kit (TIANGEN). cDNA was synthesized from total RNA (1 μg) by using the Quantitect RT kit and transcript levels were determined via SYBR Premix Ex TaqTM II (TaKaRa) on StepOne equipment (ABI). Oct4 (a marker of pluripotency) and ZiC1 (a marker of neural cell) were used to document the extent of differentiation. For each experiment, samples were normalized to the housekeeping gene GAPDH. A complete primer was listed in Supplementary Table S1.

2.3. Semi-quantitative RT-PCR analysis

Total RNA was isolated with the Trizol reagent (Invitrogen). For each sample, total RNA (1 μg) was reverse transcribed (TIANGEN) according to the manufacturer's instructions. The following Neural markers were evaluated to analysis the extent of neural differentiation: Nestin, Sox1, Pax6, MAP2 and P75. Rat ES cells were used as negative control and neural stem cells were used as positive control. A complete primer was listed in Supplementary Table S1.

2.4. Immunostaining assay

For immunofluorescence assay, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT), washed 3 times with PBS, and then incubated with blocking buffer (BB) (0.2% Triton X-100, 1% BSA) in PBS for 1 h at RT. Then, the cells were incubated with the following primary antibody for 1 h at a dilution of 1:500: Oct3/4(Santa Cruz, sc-5279), Nestin (Santa Cruz, sc-20978), TH (Abcam, ab76442), CNPase (Abcam, ab6319), GFAP (Santa Cruze, sc-6170), β-|||-tubulin (Millipore, AB9354), Sox1 (Santa Cruz, sc-17318), Ki67 (Abcam, ab15580), Phh3 (Abcam,

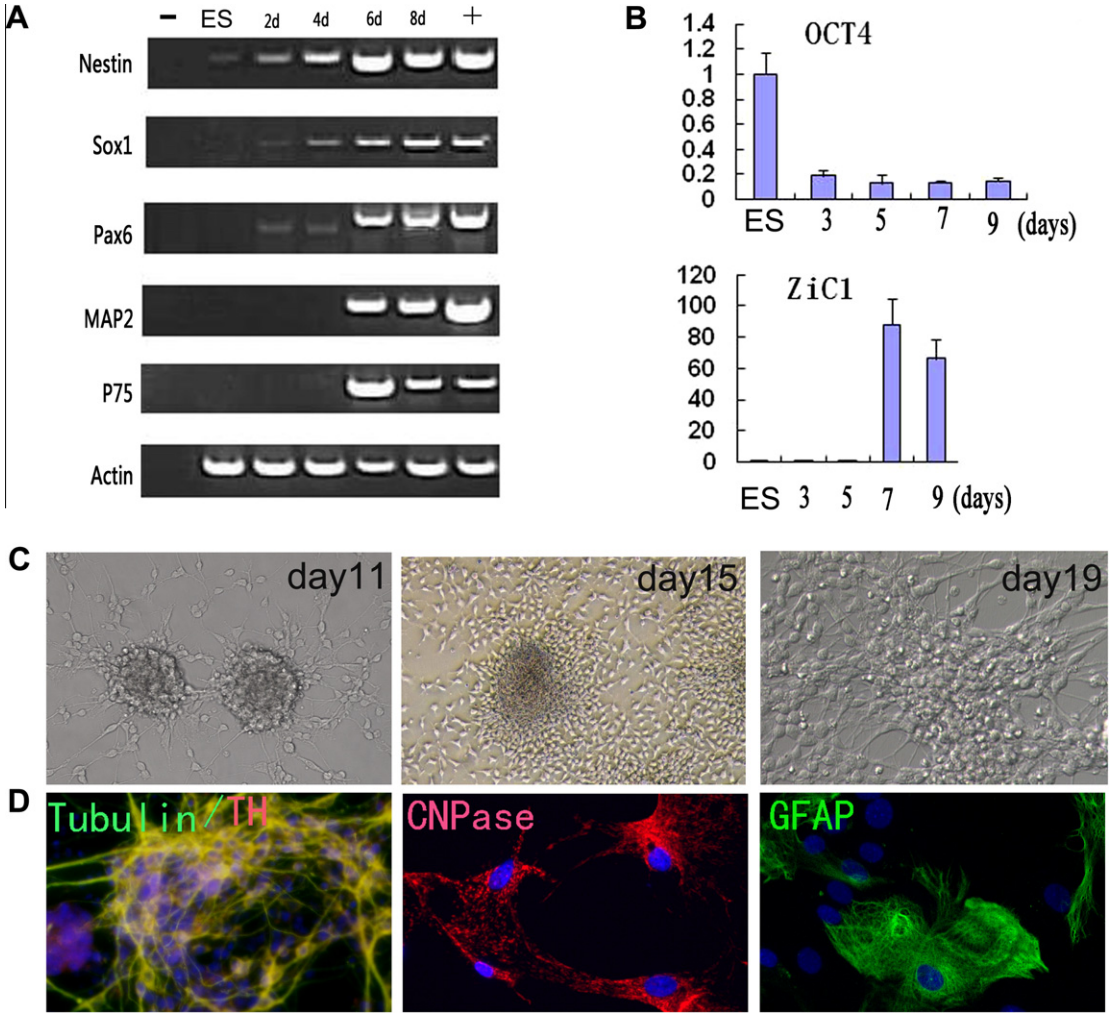

Fig. 2. The converted neural precursors maintained multipotential. (A) Analysis of Nestin, Sox1, Map2, Pax6 and P75–expression. (B) The amounts of Oct4 and ZiC1 expression were analyzed. (C) Sequential differentiation at day 11, day 15 and day 19. (D) β-|||-Tubulin and TH-positive neurons, CNPase-positive oligodendrocytes, GFAP-positive astrocytes.

ab47297). The cells were then washed with PBS and incubated with secondary antibody in BB for 1 h and then washed 3 times with PBS. Cell nuclei were counterstained with the nuclear dye DAPI. Fluorescent images were visualized using an inverted microscope (Olympus).

2.5. Western blot analysis

Total cell lysates (5×10^6) were collected and homogenized in radioimmunoprecipitation buffer (Pierce) supplied with protease inhibitor cocktail (Roche) for 30 min at 4 °C. Protein concentrations were determined by a Bicinchoninic Acid Protein Assay Kit (Thermo). Protein preparations were resolved on a 10% SDS–polyacrylamide gel and then transferred to nitrocellulose membranes (Protran). The membranes were rinsed and probed with the following antibodies at a dilution of 1:1000: BMP4 (Santa Cruz, sc-6896) and Tubulin (Sigma, T9026). Blots were visualized using enhanced chemiluminescence (Amersham) and quantified with a Kodak gel documentation system.

2.6. Flow cytometry assay

The cells were dissociated into single cell using 0.05% trypsin and fixed using BD cytofix (BD Biosciences). After that, the cells were washed 3 times with PBS and then incubated in BB for 30 min to block non-specific protein–protein interactions. The cells were then incubated with Phycoerythrin-conjugated anti-rat Nestin (R&D Systems, IC2736P) and anti-rat Sox1 (Santa Cruz, sc-17318) antibodies at a dilution of 1:100 for 1 h at 4 °C. Flow cytometry analysis was carried out with a FACScan flow cytometer (Becon Dickinson).

3. Result

3.1. Rat ES cells differentiated into neural precursors in CY medium

The rat ES cells were cultured in the 2i medium as previously described [2]. The undifferentiated rat ES cells were Oct4-possitive and Nestin-negative (Fig. 1A). After the removal of PD0325901 and CHIR99021, rat ES cells ceased to proliferate and died within 3 days in the N2B27 medium (Fig. 1B). In contrast, neural differentiation of rat ES cells can be induced by switching the 2i medium to the CY medium and the differentiated rat ES cells exhibited neural-like morphology (Fig. 1B). The immunostaining results indicated Nestin-expression for the differentiated cells on day 8 (Fig. 1B). After being transferred onto gelatin-coated plastics, the differentiated cells were able to aggregate and produce a large amount of floating

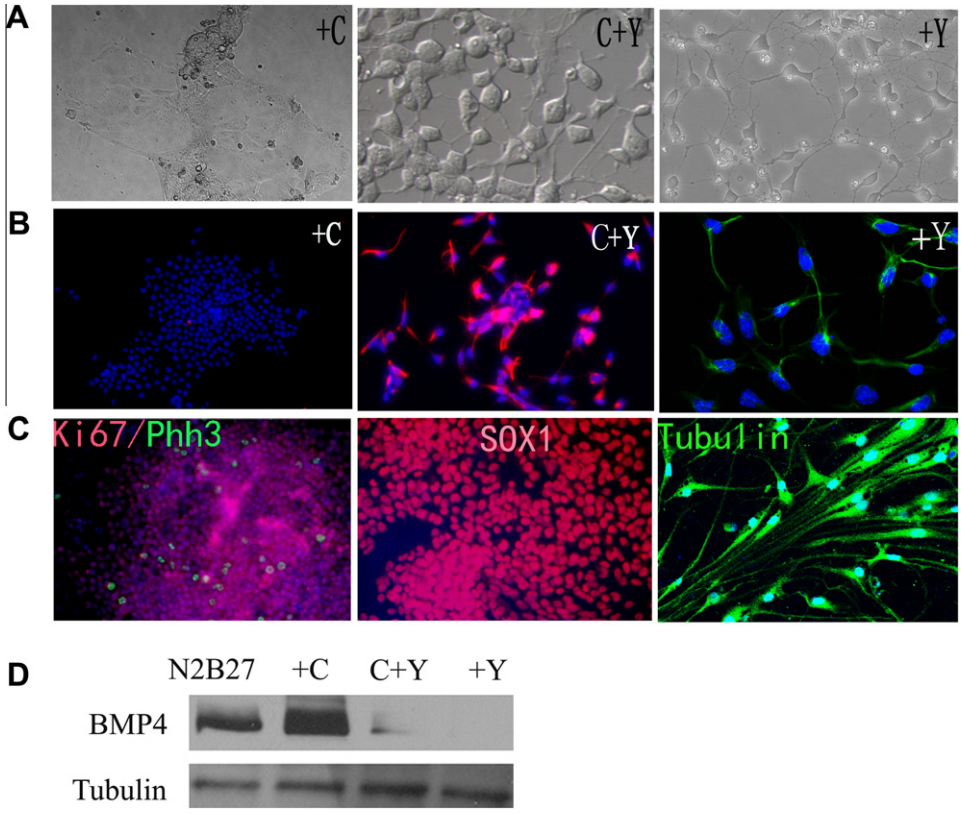

Fig. 3. Effects of Y-27632 and CHIR99021 on rat ES cells neural conversion. (A) Differentiation of rat ES cells in CHIR99021, CY and Y-27632. (B) Nestin-expression in CHIR99021, CY and Y-27632. (C) Expression of Ki67 and Phh3 in CY medium, Sox1 and Tubulin-expression of the converted neural precursors. (D) Western blot analysis of BMP4-expression in N2B27, CHIR99021, CY and Y-27632.

spheres (Fig. 1C). Immunostaining results showed that the floating spheres maintained Nestin-expression (Fig. 1C). To further quantify the efficiency of the neural induction, expression of Nestin and Sox1 in the spheres was analyzed by flow cytometry. Results showed that 93.3% of the differentiated cells showed expression of Nestin and 89.1% of the cells showed expression of Sox1 (Fig. 1D) on day 9. All these results indicate that rat ES cells can efficiently differentiate and produce a large amount of neural precursors in the CY medium.

3.2. Rat ES cells converted-neural precursors retained broad neural differentiation potentials

The character of neural precursor for the differentiated rat cells was also confirmed by the semi-quantitative PCR analysis. Results revealed that the cells showed Sox1 and Nestin-expression on day 4 and following examined time points (Fig. 2A). Moreover, various additional neural genes were also expressed by the cells, including Pax6, P75, and MAP2, strongly supporting their neural nature (Fig. 2A). Consistent with the observation, quantitative PCR results revealed that the cells showed a rapid reduction of Oct4-expression after being transferred into the CY medium (Fig. 2B). Along with the reduction of Oct4, from day 7, expression of ZiC1, a neural marker expressed in the dorsal neural tube [14] was significantly up-regulated (Fig. 2B). To further examine the multipotency of the neural precursors, cells were cultured in the differentiation medium for 2 weeks. Fig. 2C showed that the neural precursors could go on differentiating into neuron-like cells. The immunostaining assay confirmed that the neural precursors could subsequently differentiate into β-|||-tubulin-positive and TH-positive neurons (Fig. 2D). Moreover, the neural precursors could also convert into CNPase-positve oligodengrocytes and GFAP-positive astrocytes in the presence of serum (Fig. 2D). As a summary, these results indicated that the converted neural precursors retained broad neural differentiation potentials.

3.3. Y-27632 facilitated the neural differentiation by modulating BMP4 expression

To define the molecular mechanism underlying neural differentiation of rat ES cells, rat ES cells were plated in the N2B27 medium containing only CHIR99021, or only Y-27632, or in the CY medium. The results showed that CHIR99021 alone could maintain the overall survivability of differentiated rat ES cells but induce non-neural differentiation (Fig. 3A). The immunostaining results also showed that the differentiated cells were Nestin-negative (Fig. 3B). Interestingly, rat ES cells acquired neural-like morphology in the N2B27 medium containing only Y-27632 (Fig. 3A) and immunostaining results indicated Nestin-expression for the differentiated cells (Fig. 3B), suggesting that Y-27632 can facilitate the neural differentiation of rat ES cells. However, the rat cells ceased to proliferate in the N2B27 medium containing only Y-27632 (Fig. 3A). In contrast, the rat ES cells differentiated into neural-like cells and maintained highly proliferative capability in the CY medium (Fig. 3A). The immunostaining results confirmed that the differentiated cells expressed Nestin (Fig. 3B), Ki67, and Phh3 (Fig. 3C). Furthermore, the cells could form floating neural spheres and show Sox1 and β-|||-tubulin expression (Fig. 3C). The Western blot analysis showed that CHIR99021 facilitated the accumulation of endogenous BMP4 (Fig. 3D). Addition of Y-27632 antagonized the pleiotropic effects of CHIR99021 and inhibited BMP4-expression (Fig. 3D). Taken together, these results showed that CHIR99021

was crucial to maintain the overall survivability of differentiated rat ES cells, while Y-27632 facilitated neural differentiation of rat ES cells by antagonizing the BMP4-expression.

4. Discussion

Rat ES cells are maintained in the N2B27 medium supplied with two small molecular inhibitors CHIR99021 and PD0325901. PD0325901 is a small molecule inhibitor of mitogen-activated protein kinase (ERK) [15]. Report shows that CHIR99021 is important to maintain the overall survival ability of ES cells when ERK signaling is totally inhibited [16]. Our results showed that CHIR99021 was also crucial for maintaining the metabolic activity of newly differentiated rat ES cells (Fig. 1B). However, CHIR99021 promoted rat ES cells to differentiate into flattened non-neural cells (Fig. 3A) by facilitating the endogenous BMP4-expression (Fig. 3D). Previous study showed that endogenous expression of BMPs had negative effects on neural differentiation of ES cells [17] and BMP4 treatment increased the expression of the early mesodermal genes [18]. Our results showed that the addition of Y-27632 facilitated neural differentiation of rat ES cells, the cells attached separately and differentiated into neural-like cells in the presence of Y-27632 (Fig. 3A). Previous reports confirmed that Y-27632 inhibited cell–cell contact during neurodetermination of ES cells [19]. Smukler reported that individual ES cells had an intrinsic default program to become a neural precursor and ES cells could directly differentiate into neural cells in the absence of extrinsic influencing signaling (such as BMP) [20]. We propose that the addition of Y-27632 may provide an appropriate niche for neural differentiation of rat ES cells. The Western blot results also confirmed that Y-27632 facilitated the neural differentiation of rat ES cells by antagonizing the endogenous BMP4-expression (Fig. 3D). We found that rat ES cells efficiently differentiated into neural-like cells and maintained the overall survivability in the CY medium. The neural differentiation of rat ES cells was monitored by immunostaining, flow cytometry, and quantitative-PCR. The results showed that about 90% of the rat ES cells can convert into neural precursors (Fig. 1)and the neural precursors retained high neurogenic propensity and broad differentiation potential (Fig. 2).

In this study, we established an efficient protocol to differentiate rat ES cells into neural precursors in the chemical-defined condition, which provides a platform for defining the molecular machinery underlying neural commitment and optimizing the efficiency of neural precursor production from the rat ES cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.023.

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