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Promotion of mouse embryonic stem cell differentiation by Rho kinase inhibitor Y-27632

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HIGHLIGHTS

- ROCK inhibitor promoted the differentiation of ES cells into neurons (motor and sensory neurons).
- ROCK inhibitor promoted the differentiation of ES cells into muscle cells.
- ES cells primarily differentiated into neurons rather than muscle cells.
- ROCK inhibitor may promote the neuronal differentiation of ES cells by activating the ERK pathway.

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ABSTRACT

Rho kinase (ROCK) is one of the major downstream mediators of Rho. Rho plays crucial regulatory roles in the cellular proliferation and differentiation. Because a ROCK inhibitor, Y-27632, is known to inhibit the dissociation-induced cell death in human embryonic stem (ES) cells, we investigated the effects of this ROCK inhibitor on the differentiation of the mouse ES cells. The ROCK inhibitor promoted the differentiation of the ES cells into neurons, particularly motor and sensory neurons. The addition of both ROCK inhibitor and nerve growth factor (NGF) strongly stimulated the differentiation of the ES cells into neurons. Moreover, the ROCK inhibitor promoted the differentiation of the ES cells into muscle cells. The ES cells primarily differentiated into neurons rather than muscle cells. We found that the ROCK inhibitor may promote the neuronal differentiation of the ES cells by activating the extracellular signal-regulated kinase (ERK) signaling pathway. These results suggest that the ROCK inhibitor has a significant potential to regulate the differentiation of the ES cells.

1. Introduction

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of 3.5-day-old blastocysts of preimplantation mouse embryos and have a pluripotent ability to differentiate *in vitro* into various cell lineages, including neurons [1,2]. Research efforts have been focused on ways of controlling the differentiation of the ES cells into neurons to understand the potential applications of the ES cells in neuroscience and regenerative medicine [3].

Watanabe et al. reported that incubation with a selective Rho kinase (ROCK) inhibitor, Y-27632, permits the survival of the human ES cells in clonal culture by inhibiting the dissociation-induced cell death [4]. Therefore, we presumed that a ROCK inhibitor may promote the ES cell differentiation into neurons by inhibiting apoptosis. In addition, ROCKs play key roles in mediating the control of the actin cytoskeleton through the Rho family of GTPases in response to extracellular signals. Such signaling pathways contribute to diverse neuronal functions [5,6]. Because ROCK regulates the function of several target proteins through its kinase activity, the inhibition of ROCK activity may provide new possibilities of controlling the *in vitro* differentiation of the ES cells into neurons.

In this study, to achieve the efficient differentiation of the ES cells into neurons, we investigated the effects of the ROCK inhibitor,

Abbreviations: BSA, bovine serum albumin; DRG, dorsal root ganglion; ERK, extracellular signal-regulated kinase; ES, embryonic stem; NGF, nerve growth factor; PBS, phosphate-buffered saline; ROCK, Rho kinase.

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Y-27632, and nerve growth factor (NGF) on the differentiation of the ES cells.

2. Materials and methods

2.1. The differentiation of the mouse ES cells

We used the mouse ES cells (129SV; Dainippon Pharmaceutical, Osaka, Japan) after 16–20 passages. The colony formation of the mouse ES cells was performed as described previously [3]. To evaluate the effects of the ROCK inhibitor Y-27632 (253-00513; Wako Pure Chemical Industries, Osaka, Japan) on the ES cell differentiation, we detached the undifferentiated colonies (approximately 200 μm in diameter) from the nonadhesive 100-mm plastic dishes (AU2010; Eikenkizai, Tokyo, Japan). One colony per well was plated with the DMEM/F-12K medium in a gelatin-coated 96-well assay plate (353948; Becton Dickinson, Franklin Lakes, NJ, USA). The DMEM/F-12K medium consisted of 49% DMEM (SLM-220-B; Millipore, Temecula, CA, USA) and 49% F-12 nutrient mixture (21127-022; Gibco BRL, Grand Island, NY, USA), which contained 1% N-2 supplement (17502-048; Gibco BRL) instead of serum and 1% penicillin/streptomycin (15140-122; Gibco BRL). After the ROCK inhibitor or the ROCK inhibitor along with NGF (10 ng/ml; 2256X; Techne, Minneapolis, MN, USA) was added to the culture medium, the colonies were cultured for 12 days at 37 °C in the humidified atmosphere of 5% CO_2. Half of the medium was replaced with a fresh batch containing the ROCK inhibitor or combination of the ROCK inhibitor and NGF every 3 days.

2.2. Immunofluorescence analysis

The mouse ES cell colonies were cultivated in a gelatin-coated 96-well assay plate, washed thrice with cold phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde phosphate buffer solution for 45 min at room temperature. After washing thrice, the cells were incubated with cold 99.8% methanol for 15 min at −80 °C. The cells were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used for labeling: anti-βIII-tubulin (MAB1637; Millipore), anti-Lim-3 (AB3202; Millipore), anti-Brn-3 (SC6026; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α-actinin (sc-7453; Santa Cruz Biotechnology) antibodies. After washing thrice with cold PBS, we incubated the cells for 30 min at room temperature using an Alexa Fluor 488-labeled secondary antibody (A11055 or A11008; Molecular Probes, Eugene, OR, USA). After washing thrice with cold PBS, we measured the fluorescence intensity of the cells using a fluoroimage analyzer (FLA-3000R; Fujifilm, Tokyo, Japan).

2.3. Flow cytometry

The differentiated ES cell colonies were incubated with a trypsin/EDTA solution (SM-2003-C; Millipore) for 2 min at room temperature and then we added DMEM to the solution. After centrifugation, the supernatant was discarded and the colonies were washed with cold PBS. The cells were dispersed by pipetting, and approximately 10^6 cells were transferred to a 1.5-ml tube. After a wash in cold PBS, the cells were incubated with 4% paraformaldehyde phosphate buffer solution for 30 min at room temperature. After centrifugation, the supernatant was discarded and the cells were washed with cold PBS. The cells were incubated with cold 99.8% methanol for 15 min at −80 °C. After a wash in cold PBS, the cells were incubated with primary antibodies overnight at 4 °C. After three washes with cold PBS, the cells were incubated with the secondary antibody for 30 min at room temperature. After three washes with cold PBS, we measured the number of fluorescence-activated cells using a flow cytometer (JSAN; Bay Bioscience, Kobe, Japan).

2.4. Western blotting of extracellular signal-regulated kinase (ERK)

The ES cell colonies (10 colonies/dish) were cultured for 30 min at 37 °C in the humidified atmosphere of 5% CO_2 in a 35-mm culture dish (153066; Nalge Nunc International, Roskilde, Denmark) with 2 ml of the DMEM/F-12K medium containing 20 μM ROCK inhibitor and 10 ng/ml NGF. The colonies were collected and centrifuged for 5 min at 1500 rpm. The cell pellets were washed with 500 μl of cold PBS. The cell pellets were lysed in 50 μl of Pathscan sandwich ELIZA lysis buffer (7018; Cell Signaling Technology, Danvers, MA, USA). The electrophoresis was conducted under reducing conditions by standard procedures.

For western blotting, the proteins were transferred to a PVDF membrane using an electrophoretic transfer apparatus (AE6675; Atto, Tokyo, Japan). After washing, the membrane was incubated with ERK primary antibodies [an anti-p44/42 MAP kinase antibody (4695; Cell Signaling Technology) or an anti-phospho-p44/42 MAP kinase antibody (9010; Cell Signaling Technology)] overnight at 4 °C. An ECL anti-rabbit IgG, horseradish peroxidase-conjugated, species-specific whole antibody (NA934; GE Healthcare, Buckinghamshire, UK) was used as the secondary antibody. ERK and phospho-ERK (p-ERK) were detected using an ECL Plus western blotting detection system (RPN2132; GE Healthcare). ERK and p-ERK were visualized using an ECL minicamera (RPN2069; GE Healthcare) and scanned by a GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA, USA) for quantification of protein signals on the immunoblot images.

2.5. Statistical analysis

Data were calculated as mean ± SEM (standard error of the mean). Statistical comparisons were made using one-way ANOVA and the Tukey–Kramer multiple-comparison *post hoc* test. Values of P less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of the ROCK inhibitor on the differentiation of the ES cells into neurons

We tested whether the ROCK inhibitor promoted the differentiation of the ES cells into neurons. Fig. 1A reveals the results. The differentiated ES cells were labeled with an antibody against βIII-tubulin (a marker of postmitotic neurons). The ES cells effectively differentiated into neurons as a result of the addition of the ROCK inhibitor. Furthermore, the addition of the ROCK inhibitor and NGF strongly stimulated the differentiation of the ES cells into neurons, compared with control. Fig. 1B presents the number of neurons differentiated from the ES cells. The percentage of neurons differentiated as a result of the addition of 20 μM ROCK inhibitor alone or both 20 μM ROCK inhibitor and 10 ng/ml NGF were 61% or 71%, respectively. Fig. 1C presents the fluorescence micrographs of neurons differentiated from the ES cells. The ES cell colonies clearly exhibited neurite outgrowth after incubation with the ROCK inhibitor. These results are in line with results of the immunofluorescence analysis.

We characterized neurons that differentiated from the ES cells by immunofluorescence analysis. Fig. 2 presents the different types of neurons. We used the antibodies against Lim-3 (a marker of cranial motor neurons) and Brn-3 (a marker of sensory neurons) because the ES cells predominantly differentiated into motor and