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The simulated microgravity enhances the differentiation of mesenchymal stem cells into neurons

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ABSTRACT

Growing evidence shows that physical microenvironments and mechanical stresses, independent of soluble factors, help influence mesenchymal-stem-cell fate. rMSCs (rat mesenchymal stem cells) present spread, spindle shape when cultured in normal gravity (NG) while in simulated microgravity (SMG) they become unspread, round shape. Here we demonstrate that simulated microgravity can enhance the differentiation of mesenchymal stem cells into neurons, which might be a new strategy for the treatment of central nervous system diseases. rMSCs were cultured respectively in normal gravity and in a clinostat to simulate microgravity, followed with neuronal differentiated medium. The neuronal cells derived from rMSCs in SMG express higher microtubule-associated protein-2 (MAP-2), tyrosine hydroxylase (TH) and choline acetyltransferase (CHAT). Furthermore, as rMSCs are subjected to SMG, they excrete more neurotrophins like nerve growth factor (NGF), brain derived neurophic factor (BDNF) and ciliary neurotrophic factor (CNTF). Neuronal cells from SMG group generated more mature action potentials and displayed repetitive action potentials by comparison to cells from NG group. We conclude that simulated microgravity can enhance the differentiation of mesenchymal stem cells into neurons.

1. Introduction

The adult cerebral cortex has limited ability to regenerate lost neural tissue after brain damage, for the most part due to insufficient neurons produced to replenish neuronal loss after injury and restore cortical function [1,3,4]. To overcome the regenerative limitations of the lesioned brain, molecular reprogramming and soluble factors for MSCs have been applied to promote neuronal differentiation [5,9,19]. However, the gene transfer are commonly associated with a risk of activating cancerous genes, and the possible side effects of the large amount of cytokines and the potential malignancy of hyperdifferentiated grafted cells cause further concerns regarding the previous methods. Therefore, to increase their safety for clinical applications, it is necessary to obtain higher-quality stem cells with an improved differential potential.

Microgravity, as one of mechanical factors, has been confirmed to affect almost all human physiological systems, with bone loss, anaemia, muscle atrophy and immune alterations commonly seen [6,16,17]. Recently, numerous studies have demonstrated the effects of microgravity upon stem cells. hMSCs cultured in SMG possessed the strong proliferative characteristic of stem cells and retained their ability to differentiate into hyaline cartilage after transplantation [20]. Monticone M reported that MSCs in a space mission upregulated genes related to various processes of neural development, neuron morphogenesis, transmission of nerve impulse and synapse [14].

Our previous work observed that the shape of rMSCs became round from spindle under SMG, which never reported before. The morphological change reflected cytoskeletal reorganization, which could direct the differentiated fate of MSCs through RhoA/Rock signaling pathway [10,15].

In this study, we showed that simulated microgravity could shift the morphology of MSCs from spindle to round, which brings much stronger differential potential into neurons. This research might shed new light on the relationship between morphology and function of stem cells.

2. Materials and methods

2.1. Preparation and culture of adult rat mesenchymal stem cells (rMSCs) and nucleus basalis of Meynert (nBM) neurons

Animal experiments were approved by local committee review and conducted according to China Public Health Service Policy on Humane Care and Use of

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Laboratory Animal. rMSCs were collected as previously described by Azizi et al. [2], with modification. Briefly, adult rats (Wistar, two-week-old) were euthanized and both bilateral femurs and tibias were removed. Mesenchymal stem cell was cultured in DMEM (Gibco BRL) supplemented with 10% Fetal bovine serum (FBS, Gibco BRL), 100 U/ml penicillin G, 100 mg/ml streptomycin (Gibco BRL) at $2.5 \times 10^5/cm^2$. Fluorescence phase-contrast microscope (Axiocam MR R3, Carl Zeiss, Germany) was used to observe the rMSCs every 2 or 3 days. FACS analysis of CD 44, CD 90 and CD 45 were performed in rMSCs.

The dissociation technique of preparing the nBM neurons was used as described previously [11]. Briefly, the brain slices (400 mm thick) containing the nBM were enzymatically treated with 0.2 mg/ml Pronase for 40 min at 31 °C and subsequently with 0.2 mg/ml thermolysin for 20 min at 31 °C. Then, the nBM region was micropunched out with an electrolytically polished injection needle. The micropunched-out pieces were mechanically triturated in a culture dish with a fire-polished Pasteur pipette. The dissociated neurons were prepared as a control for differentiated rMSCs.

2.2. Clinorotation to modeled microgravity

The weightless environment resulting from a clinostat or rotating vessel is often regarded as "simulated microgravity". Conditions in which this requirement is met actually prevent the cell from feeling gravity; the gravity vector escapes its detection machinery. The machines described are based on the hypothesis that sensing no gravity would have similar effects as being weightless. The clinostat model system (clinorotation) used in this study is an equipment for providing a vector-averaged reduction in the apparent gravity on the cell culture (Fig. 1D). In the present study, stem cells were seeded at a density of 1×10^5 cells on 2.5 cm × 2.5 cm coverslips. The coverslips were inserted into the fixture of the chambers which were subsequently filled completely with DMEM with 10% FBS and aspirated to eliminate the presence of air bubbles. The chambers were divided into two groups randomly: NG controls without rotation and SMG groups with clinorotation (30 rpm). The rotation times were 3 days.

2.3. In vitro differentiation procedure

After the stimulation of SMG, rMSCs were differentiated as previously described, with modification [12]. Cells were maintained in pre-induction media consisting of DMEM, 0.1 mmol/l 2-mercaptoethanol (BME, Gibco BRL) and 2% dimethylsulfoxide (DMSO, Wak-Chemie Med Gmbh) for 5 h. Then, the pre-induction media were removed, and the cells were transferred to neuronal induction media composed of DMEM + 10% FBS, 10 μg/l basic fibroblast growth factor (b-FGF, R&D Systems, Inc.), 10 μg/l human epidermal growth factor (hEGF, R&D Systems, Inc.), 1 mmol dibutyryl cyclicn AMP (dbcAMP, Sigma, St. Louis, MO) and 0.5 mmol isobutylmethylxanthine (IBMX, Sigma, St. Louis, MO) for 7 days and 14 days.

2.4. Fluorescence immunocytochemistry

After SMG stimulations, cytoskeletons of the MSCs were observed. Briefly, cultured cells were fixed with 4% paraformaldehyde for 20 min and then immersed in PBS for about 10 min, followed by exposure to 0.01% Triton X-100 at room temperature for 10 min. rMSCs after rotating for 4 h, 72 h, 10 days were incubated with β-tubulin (dilution 1:200; Santa Cruz, CA). Likewise, neuronal-like phenotypes derived from rMSCs were incubated with one of the following primary antibodies: MAP-2 (dilution 1:200; Santa Cruz, CA), CHAT (dilution 1:200; Chemicon) and TH (dilution 1:200; Santa Cruz, CA); then, primary antibodies were washed and fluorescent secondary antibody fluorescein isothiocyanate (FITC) or TRITC (dilution 1:100; Santa Cruz, CA) was added respectively for 2 h. Subsequently, the cells were treated with Hoechst 33342 (dilution 1:100; Sigma) for 10 min. Images were obtained and processed using IPLab software (Scanalytics).

2.5. Fluorescence-activated cell sorting analysis (FACS)

After 7 days induction, neuronal-like phenotypes were analyzed using fluorescence-activated cell sorting analysis (FACS). Briefly, the harvested cells were incubated with antibodies including MAP-2, CHAT and TH after blocking with normal goat serum (10%). And then, the cells were resuspended in Dulbecco's PBS (DPBS) containing a working dilution of PE-labeled goat anti-mouse IgG (Ancell, Bayport, MN, USA) and incubated at 4 °C for 30 min. The cells were analyzed on a fluorescence activated cell sorter (FACScan) cytometer (Elite ESP, Miami, FL, USA).

2.6. Annexin V-FITC/propidium iodide assay

To explore if SMG induced the apoptosis of rMSCs, the Annexin V-FITC/propidium iodide (PI) assay was performed using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. rMSCs cultured in normal gravity and microgravity for 3 days were investigated. 10^6cells were treated with FITC-labeled antibodies against Annexin V and PI. About 1×10^4 events were acquired for each experimental point via a fluorescence activated cell sorter (FACScan) cytometer (Elite ESP, Miami, FL, USA) and analyzed with WinList software (Verity Software House, Topsham, ME).

2.7. Quantitative PCR

To detect the changes of neuronal gene expressions after SMG stimulation, total RNA was extracted from NG group and SMG group in different time points

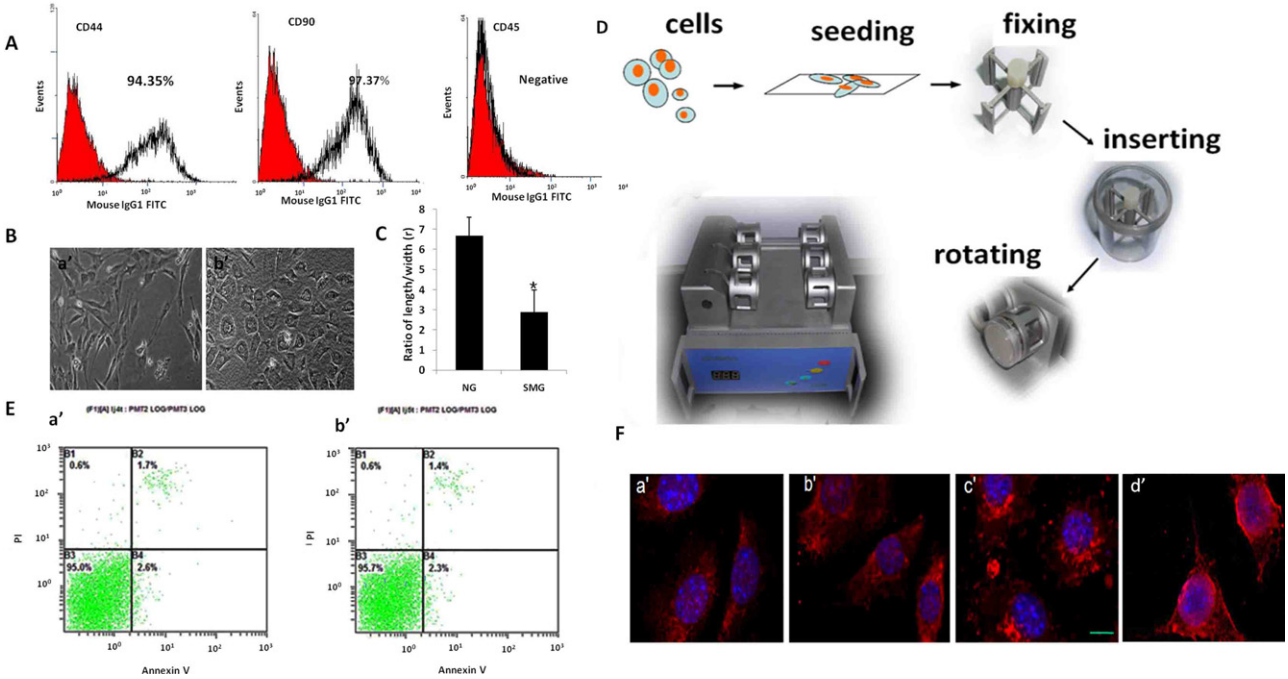

Fig. 1. (A) FACS analysis of CD 44, CD 90 and CD 45 in rMSCs. (B) Light microscopy analysis of the shape change of rMSCs under NG (a′) and SMG (b′) conditions (50×). (C) *r* value decreased significantly in rMSCs after SMG stimulation. * denotes $p < 0.05$, compared with NG group. (D) The SMG clinostat model system. (E) annexin V flow cytometry performed after SMG stimulation. (F) Microtubules formation was disrupted in SMG. Scale bar is 10 μm. Note that rMSCs showed modified microtubules at the initial 4 h (a′) and 72 h (b′) of SMG, and cells seemed to have reestablished microtubules after 10 days (c′) of SMG. rMSCs under normal gravity (d′) as a control.