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Research article

Electric field stimulation induced neuronal differentiation of filum terminale derived neural progenitor cells

Zhi-yong Dong[a,1], Zhe Pei[b,1], Zhe Li[c], Yan-ling Wang[c], Amber Khan[d], Xiao-ting Meng[a,*]

[a] Department of Histology & Embryology, College of Basic Medical Sciences, Jilin University, Changchun 130021, PR China
[b] Department of Neuroscience and Pediatric, GSRB1 Duke University, Durham 27710, USA
[c] Laboratory Teaching Center of Basic Medicine, College of Basic Medical Sciences, Jilin University, Changchun 130021, PR China
[d] The Graduate Center and CUNY School of Medicine, CUNY, 85 St. Nicholas Terrace, New York, NY 10027, USA

HIGHLIGHTS

- We designed an EF setup and generated a protocol to help long-term differentiation induction.
- EFs significantly increase neuronal differentiation rate of FT derived NPCs.
- EFs align neurite outgrowth and promote the length of neurite processes.

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ABSTRACT

Adult filum terminale (FT) is an atypical region from where multipotent neural progenitor cells (NPCs) have been isolated. However, poor neuronal differentiation rate of FT-NPCs currently limits their clinical applications. Using custom-designed electric fields (EFs), this study sets up a method to significantly improve neuronal differentiation rate of rat FT-NPCs *in vitro*. We investigated the influence of EF strength on rat FT-NPCs differentiation. By adding reasonable strength of EF to FT-NPCs, our data shows a significant increase in neuronal differentiation rate. The present innovation provides a novel method of directional differentiation and efficient production of neurons from FT-NPCs *in vitro*. This improved approach for inducing neuronal differentiation can be applied to future research on autoplastic transplantation.

1. Introduction

The Filum Terminale (FT) is a vestigial structure located at the caudal end of the spinal cord [1]. As a source of autologous neural stem/progenitor cells (NSCs/NPCs), FT-NSCs/NPCs can provide great therapeutic potential [2–5]. However, poor survival and low neuronal differentiation rates are major obstacles holding back their clinical applications [6,7]. Neuronal differentiation rate of FT-NSCs/NPCs was low among most reported NSCs/NPCs' regeneration studies [4,5]. Thus, methodological improvement is urgently needed to efficiently and selectively induce neuronal differentiation of FT-NSCs/NPCs (which we designated FT-NPCs in this research).

Modifying either intrinsic transcription signals or extrinsic micro-environmental factors could induce neuronal differentiation of NSCs/NPCs [8–11]. Physiological electric fields (EFs) have been widely used to control neuronal activity. Neurons and embryonic NPCs both show directional migration towards the cathode in EFs [12,13]. Furthermore, electrical stimulation efficiently guides neuronal differentiation of human mesenchymal stem cells (hMSCs) and the embryoid bodies (EBs) [14,15]. However, detailed information about EF stimulated FT-NPCs differentiation is still lacking.

In this study, we designed a direct current EF setup to help long-term differentiation induction *in vitro*. We then evaluated the effectiveness of the developed setup and generated a protocol to induce neuronal differentiation of rat FT-NPCs. By adding EFs, our method significantly increases neuronal differentiation of FT-NPCs. Generally, our work improves the methodology of using young adult NPCs to obtain neurons for autoplastic transplantation.

* Corresponding author.
E-mail address: mengxt@jlu.edu.cn (X.-t. Meng).
[1] Both authors contributed equally to this work.

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2. Materials and methods

2.1. FT-NPCs preparation

All animals supplied by the Experimental Animal Center, Jilin University and all procedures followed the Institutional Animal Care and Use Committee guide lines.

FT-NPCs were prepared from 8 week old adult male Sprague-Dawley (SD) rats (n = 7). Following the published protocol [16,17], FT tissue was mechanically separated from the spinal cord and overlying meninges were removed. Isolated FT tissue was chopped into 1–2 mm^3 pieces in cold Dulbecco's Minimal Essential Media/Ham's F12 (DMEM/F12) (Hyclone, USA) medium and subsequently centrifuged at 500 rpm for 1 min to remove large tissue debris. Small pieces of FT tissue were then mechanically dissociated into a cell suspension and centrifuged at 800 rpm for 3 min to further remove the debris and cell membrane fragments. Isolated cells were re-suspended in growth medium composed of DMEM/F12 supplemented with B-27 (Invitrogen, USA), basic fibroblast growth factors (bFGF, 20 ng/ml, PeproTech, USA) and epidermal growth factors (EGF, 20 ng/ml, PeproTech, USA) and passed through a 75 μm cell strainer (BD Falcon, USA). Harvested single cell suspension (5000 cells/cm^2) was plated in Nunc T25 culture flasks, cultivated in 5% CO_2 at 37 °C with growth medium to form neurospheres.

2.2. Passaging

FT-NPCs were passaged at 1:3 every 3–5 days. FT-NPCs formed neurospheres were incubated with 1X Accutase (Invitrogen, USA) for 1–2 min at room temperature following trituration. To prepare single cell suspension, mechanically dissociated cells went through a 75 μm cell strainer, centrifuging and re-suspension in fresh growth medium. To reduce animal use and avoid repeating primary culture, we evaluated if cryopreservation would affect FT-NPCs properties. After 5 passages (P5), single FT-NPCs suspension were frozen within 24 h (Cryo Freezing Container, Nalgene, USA) with a cooling rate of 1 °C per minute. Serum-free expansion medium supplemented with 10% dimethyl sulfoxide (DMSO) was used for freezing medium [18]. Five days after freezing, post-thawed FT-NPCs were passaged continuously *in vitro* until 10 passages (P10) for further test.

Cells at different passage (P1, P5 and P10 of post-thawed cells) were used to confirm that cells we obtained from FT are NPCs. FT-NPCs at P5 and P10 were used to evaluate EF induced neuronal differentiation.

2.3. Characterization and differentiation

Two-week-old (P5) FT-NPCs or post-thawed FT-NPCs (P10) were plated at a density of 25 neurospheres/cm^2 into 35 mm petri-dishes coated with poly-D-lysine (0.01%, DingGuo, China) and laminin (20 mg/ml, Sigma, USA). Culture media contained DMEM/F12 (1:1) with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS, Gibco, USA). 7-day-culture of plating cells was processed for immunocytochemistry.

2.4. EF-stimulation

FT-NPCs (P5 and P10) were plated on poly-D-lysin/laminin-coated electrotactic chambers previously developed by our collaborators [12,19]. EF was applied to the chamber *via* agar salt bridges. To avoid Steinberg's solution stimulated cell growth, we used 5% FBS in agar bridge to maintain a stable voltage and current flow.

To evaluate the effectiveness of the developed setup, FT-NPCs were treated with a physiological strength direct current at 50 mV/mm, 100 mV/mm, 150 mV/mm, 200 mV/mm and 250 mV/mm respectively. Cells were exposed to EFs in DMEM/F12 medium containing 10% FBS with a 12-h-stimulation at the beginning day of experiment, followed by a 2-h treatment in EFs at the same time each day until the end of experiment. After 7 or 14 days of EF-stimulation, differentiation rate was detected by immunocytochemistry staining for further analysis.

2.5. RT-PCR and western blot

In this study, Nestin and Musashi1 were applied as NSC/NPC specific markers for RT-PCR and Western Blot analysis. FT-NPCs (P5) were harvested for total RNA extraction (RNeasy Kit, Qiagen, USA) followed by cDNA synthesis at 42 °C. Primers and PCR conditions are listed in Supplemental Table 1. 3 μl of final PCR product was separated in a 1% agrose gel, stained with ethidium bromide, and photographed.

FT-NPCs (P1, P5 and P10, ~2.5×10^6) were lysed in 200 μl SDS sample buffer for Western blot. Cell extracts were resolved in 4–12% Bis-Tris gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (wt/vol) skim milk in PBS-T (PBS, 0.1% [vol/vol] Tween 20) for 1 h at room temperature and subsequently incubated with desired primary antibodies (Supplemental Table 2) overnight at 4 °C. Membranes were rinsed with PBS-T and subsequently incubated with horseradish peroxidase coupled secondary antibodies for 1 h at room temperature. Chemiluminescence detection was performed using ECL reagents (Pierce ECL western blotting substrate, Thermo Fisher) and photographed by a CCD camera (GE ImageQuant LAS 4000, USA).

2.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% TritonX-100 for 10 min and blocking (5% BSA in PBS) for 30 min before incubating with primary antibodies at 4 °C overnight. After extensive washing with blocking solution, cells were incubated with secondary antibodies at 37 °C for 1 h, rinsed and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, UK). All antibodies (Supplemental Table 3) were diluted in blocking solution.

2.7. Imaging analysis

To determine the percentage of the positive cells, the occurrence of neurons (Tuj1 or MAP-2 positive cells) and astrocytes (GFAP positive cells) was counted and calculated as a percentage of total DAPI positive nuclei in 5 random visual fields (100–300 cells/field). Cultured cells were imaged under the fluorescence microscope (Olympus IX 71, Japan). Neurites were traced and measured by Image-J software and its plugin (National Institutes of Health, https://imagej.nih.gov/ij/; Simple Neurite Tracer) in three independent experiments.

2.8. Statistical analyses

The data was quantified and expressed as means ± standard deviations. Statistical analyses were performed using Student *t*-tests, Fisher's tests and One-way ANOVA by Origin Parametric software and Matlab. Probability values below 0.01 were considered statistically significant.