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Research Paper

Visualization of migration of human cortical neurons generated from induced pluripotent stem cells

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HIGHLIGHTS

- We developed the new methods to observe the migratory behavior of the human cortical neuron derived from induced pluripotent stem cells *in vitro*.
- Cortical neurons derived from induced pluripotent stem cells showed two-stroke like movement.
- We successfully generated migrating cortical neurons from lissencephaly-patient derived induced pluripotent stem cells.

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ABSTRACT

Background: Neuronal migration is considered a key process in human brain development. However, direct observation of migrating human cortical neurons in the fetal brain is accompanied by ethical concerns and is a major obstacle in investigating human cortical neuronal migration.
New method: We established a novel system that enables direct visualization of migrating cortical neurons generated from human induced pluripotent stem cells (hiPSCs).
Results: We observed the migration of cortical neurons generated from hiPSCs derived from a control and from a patient with lissencephaly.
Methods: Our system needs no viable brain tissue, which is usually used in slice culture. Migratory behavior of human cortical neuron can be observed more easily and more vividly by its fluorescence and glial scaffold than that by earlier methods.
Conclusions: Our *in vitro* experimental system provides a new platform for investigating development of the human central nervous system and brain malformation.

1. Introduction

Brain development is characterized by complex events such as neuronal migration, which has been well characterized through morphological observations in animal brains (Rakic, 2006; Tabata and Nakajima, 2001). However, the mechanisms regulating a developing human cortex are still unclear owing to the ethical concerns associated with the use of human fetal-derived tissues. The use of human induced pluripotent stem cells (hiPSCs) derived from the cerebral cortex (Lancaster et al., 2013; Qian et al., 2016) is considered the most promising approach to overcome the problem of using fetal tissues. In the present study, we established a novel system that enables direct observation of the migration of human cortical neurons using brain-like structures generated from hiPSCs and have attempted to recapitulate the pathogenesis of a human neuronal migration disorder.

2. Materials and methods

2.1. Culture and maintenance of hiPSCs

hiPSCs 201B7 (Takahashi et al., 2007) and 409B2 (Okita et al., 2011) were obtained from RIKEN Cell bank (RIKEN Bioresource

Abbreviation: SFEB, serum free floating embryoid body like aggregates; RG, radial glia; hcNPC, human cortical neuronal progenitor cells; hiPSC, human induced pluripotent stem cell.

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Center, Tsukuba, Japan). Lissencephaly patient-derived hiPSC lines (TUBA1A-iPS-A#1, TUBA1A-iPS-A#3) were also used (Bamba et al., 2016). These cells were maintained as described previously (Shofuda et al., 2013).

2.2. Generation of SFEBs from hiPSCs

We generated brain-like structures in a floating culture using an SFEB (serum-free embryoid-body like aggregates) method as previously described (Kadoshima et al., 2013), with a slight modification. In brief, 9×10^3 hiPSCs were seeded into each well of a 96-well plate with spindle-shaped bottoms (Sumitomo Bakelite, Tokyo, Japan) with GMEM (Life Technologies, CA, USA) containing 20% Knockout Serum Replacement (KOSR; Life Technologies), 0.1 mM 2-mercaptoethanol (Life Technologies), 20 μM Y-27632 (Wako, Osaka, Japan), 5 μM SB431542 (Wako, Osaka, Japan), and 3 μM IWR1-endo (Merck Millipore, Darmstadt, Germany). In these spindle-shaped bottoms, cells could form embryoid bodies quickly, i.e., in a shorter time. On the next day, aggregates were transferred into a 75T ultra-low attachment flask (Corning, NY, USA) using a Gilson 1000P pipet, and full volumes of media were changed every three days thereafter. On day 18, the medium was changed to DMEM/F12 supplemented with 1% N2 supplement (Life Technologies), 1% chemically defined lipids concentrates (Life Technologies). On day 35, FBS [final 10% vol/vol], Matrigel-GFR [final 1% vol/vol] (BD Biosciences, NJ, USA), and heparin [final 5 μg/ml] were added to the medium. The aggregates were recovered and fixed for immunohistochemistry on day 42 and were used for generation of migrating neurons on day 44.

2.3. Generation of regionally patterned cerebral organoids from hiPSCs

Our induction methods of organoids were largely based on previous original protocols (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Undifferentiated pluripotent stem cells cultured on mouse embryonic fibroblast (MEF) feeder cells (KitayamaLABES, Kyoto, Japan) were dissociated and recovered using 1 mg/ml collagenase IV (Life Technologies) and 0.05% Trypsin-EDTA (Life Technologies). Next, 9×10^3 hiPSCs were seeded in V-bottomed 96-well plates (PrimeSurface, Sumitomo Bakelite, Tokyo, Japan) with GMEM containing 20% KOSR, 1 μM 2-mercaptoethanol, 5 μM SB431542, 3 μM IWR1-endo, 4 ng/ml bFGF (Wako), and 30 μM Y27632 (Wako). SB431542 inhibits SMAD signaling to promote neural induction, IWR1-endo attenuates Wnt signaling to promote region-patterning in the forebrain and Y-27632 improves the viability of iPSCs. On day 6, cell aggregates were transferred to ultra-low attachment flasks (Corning) with DMEM/F12/Glutamax (Life Technologies), 1% non-essential amino acids (Life Technologies), 1% N2 supplement (Life Technologies), 1 μg/ml heparin (Sigma-Aldrich), and 3 μM IWR1-endo. On day 9, cell aggregates with smooth and translucent surfaces were collected. Their appearances were vital indicators of successful neuroepithelial induction. If surfaces of aggregates were still rough, aggregates could be collected on day 10. Aggregates displaying uneven surfaces on day 10 indicated insufficient neural induction because of inappropriate conditions such as unhealthy condition of undifferentiated pluripotent stem cells. Collected aggregates were embedded in Matrigel-GFR (BD Biosciences, NJ, USA). Embedded aggregates were cultured in a low attachment flask with a 1:1 mixture of DMEM/F12 and neurobasal medium (Life Technologies) containing 0.5% N2 supplement, 1% B27 supplement minus vitamin A (Life Technologies), 1% Glutamax (Sigma-Aldrich), and 1 μg/ml insulin (Sigma-Aldrich), which is considered to have positive effects on neural stem cells. On day 15, when the neuroepithelial cells exhibited an elongated morphology, B27 supplement (Life Technologies) in the medium was replaced with B27 supplement plus vitamin A (Life Technologies) for differentiation of cortical neuron. IWR1-endo was added to the medium until day 18 for region patterning. During culture periods, flasks were placed on a rotary shaker at 40 rpm (TAITEC, Saitama, Japan) installed in a CO_2 incubator [5%CO_2, 37 °C].

2.4. Preparation of PiggyBac vectors

cDNA sequences of the fluorescent protein AcGFP and a Centrin2–Dsred fusion protein were cloned in pIRESAcGFP (Takara-Bio, Shiga, Japan) and dsRed-cent2, respectively [dsRed-cent2 was a gift from Dr. Joseph Gleeson (Addgene plasmid #29523) (Tanaka et al., 2004). The genes were cloned downstream of the CAG promoters of pPBCAGneo (Bamba et al., 2014) and pPBCAGhygro, which was derived from pPBCAGneo by replacing the expression cassette with that of the pEBmultihygro plasmid (Wako).

2.5. Generation of fluorescent pluripotent stem cell lines

The DNA for pPBCAG-EGFP-neo (Bamba et al., 2014) or both pPBCAG-Cent2DsRed-neo and pPBCAG-AcGFP-hygro were cotransfected with a PiggyBac transposase-expressing vector (System Biosciences, CA, USA) into an hiPSC line using a nucleofector I device and a human stem cell nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's protocols. After selection using 200 μg/ml geneticin (Life Technologies) and 50 μg/ml hygromycin B (Life Technologies), fluorescent-labeled hiPSCs were cultured and passaged more than three times to manually eliminate non-fluorescent colonies. hiPSCs with stable and homogenous fluorescence were used for experiments.

2.6. Time-lapse imaging

Live cell images were acquired using the Incucyte ZOOM HD cell imaging system (Essen BioScicences, Ann Arbor, MI, USA). Image acquisition intervals were 10 min for observation of neuronal cell movement.

2.7. Quantitative RT-PCR

Quantitative RT-PCR was performed and analyzed as described previously (Shofuda et al., 2013). Each primer sequence is shown in Supplementary Table 1 and in reference (Espuny-Camacho et al., 2013).

2.8. Immunocytochemistry

After fixation with 4% paraformaldehyde, cells were permeabilized with 0.3% Triton-X/PBS for 5 min and incubated with 10% goat-serum/PBS for blocking. Primary and secondary antibodies are listed in Supplementary Table 2, and nuclear staining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, JAPAN) or DRAQ5 (BioStatus, Leicestershire, UK). The images were acquired using the IX81 fluorescent inverted microscope (Olympus, Tokyo, Japan) and the confocal laser scanning microscopes LSM 710 and LSM 510 (Carl Zeiss, Hallbergmoos, Germany). Quantitative measurements were performed using Image J software.

Generation of migrating cortical neurons by radial-glia like cell-supported migration (RGM) system

Human cortical neuronal progenitor cells (hcNPCs) were isolated from SFEB on day 44 or cerebral organoids on day 30 using a nerve-cell culture system (Sumitomo Bakelite). For generation of the migrating neuron, we cocultured these cells with GDC90 (Bamba et al., 2014), which is a unique human glial cell with