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Short Report

Conversion of adult human peripheral blood mononuclear cells into induced neural stem cell by using episomal vectors

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ABSTRACT

Human neural stem cells (NSCs) hold great promise for research and therapy in neural diseases. Many studies have shown direct induction of NSCs from human fibroblasts, which require an invasive skin biopsy and a prolonged period of expansion in cell culture prior to use. Peripheral blood (PB) is routinely used in medical diagnoses, and represents a noninvasive and easily accessible source of cells. Here we show direct derivation of NSCs from adult human PB mononuclear cells (PB-MNCs) by employing episomal vectors for transgene delivery. These induced NSCs (iNSCs) can expand more than 60 passages, can exhibit NSC morphology, gene expression, differentiation potential, and self-renewing capability and can give rise to multiple functional neural subtypes and glial cells *in vitro*. Furthermore, the iNSCs carry a specific regional identity and have electrophysiological activity upon differentiation. Our findings provide an easily accessible approach for generating human iNSCs which will facilitate disease modeling, drug screening, and possibly regenerative medicine.

1. Introduction

Induction of pluripotent stem cells from somatic cells by defined transcription factors represents a major breakthrough in cellular reprogramming (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Transdifferentiation from one differentiated cell type into another by expression of lineage-related transcription factors has extended this concept (Caiazzo et al., 2011; Yoo et al., 2011; Ieda et al., 2010; Szabo et al., 2010; Sheng et al., 2012a). Those directly reprogrammed somatic cells are useful tools for understanding disease processes and discovering new therapeutics; however, they may have limited utilities as those induced cells always have little or no proliferation potential. Direct conversion of somatic cells to lineage-committed stem cells such as neural stem cells would allow production of sufficient cells for downstream research and clinical applications, and this approach meanwhile reduces the risk of tumor formation.

Recent methods of reprogramming human fibroblasts to iNSCs employed genome-integrating lentiviral or retroviral vectors (Kumar et al., 2012; Ring et al., 2012), which may produce insertional mutations and residual or reactivation of transgenes. We used *oriP/EBNA-1* episomal vectors to deliver reprogramming genes (OCT4, SOX2, NANOG, LIN28, c-MYC, KLF-4 and SV40LT) (Yu et al., 2009). This approach does not require viral packaging and the removal of episomal vectors from iNSCs may be accomplished by cell passaging due to the inherent instability of *oriP/EBNA-1* episomal state (Nanbo et al., 2007).

Up to now, the most common source from which to derive human and mouse iNSCs has been skin fibroblasts (Lu et al., 2013; Thier et al., 2012). However, the requirement for skin biopsies and the need to expand fibroblasts for several passages *in vitro* hurdle the broad applicability of this technology. Pei D et al. had reported using bladder-derived cells to generate iNSCs (Wang et al., 2013); however, bladder-derived cells can be easily contaminated by bacteria. Previous attempts to convert human hematopoietic cells toward NSCs were only restricted to the use of neonatal cord blood-derived CD34+ cells (Yu et al., 2015; Liao et al., 2015). In fact, inducing patients' somatic cells instead of fetal tissues into iNSCs seems to be a more practical approach. In the present study, we succeeded to generate iNSCs from the PB-MNCs of a 33-year-old male donor, using non-integrative episomal vectors. The iNSCs exhibited NSC morphology, gene expression and *in vitro* differentiation potentials.

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2. Materials and methods

2.1. Isolation and expansion of adult human peripheral blood mononuclear cells

The isolation and expansion of PB MNCs was carried out as previously described (Dowey et al., 2012). Six milliliters of donor's PB was collected with written informed consent, and from it around 5.6×10^6 mononuclear cells were isolated. After 2 weeks' culture, the numbers of MNCs increased to 6.9×10^6.

2.2. Reprogramming of PB-MNCs to iNSCs by episomal vector transfection

Two million MNCs were suspended in 100 μl nucleofector solution (Lonza, cat. no. VPA-1003, Basel, Switzerland) and then combined with the three episomal plasmids (2 μg each) (Yu et al., 2009) (Fig. S1). After that, the cell/DNA mixture was transferred to a sterile cuvette (Lonza), which was placed into the cuvette holder of the Amaxa Nucleofector II device. Following nucleotransfection using program 'T-016', the solution was transferred into 2 ml MNC medium in one well of a 12-well plate. After recovery at 37 °C, 5% CO_2 for 2 days, the cells were respectively plated on MEF-coated, Matrigel-coated or poly-D-lysine (PDL)/laminin-coated 12-well plates at a density of 2×10^5/well. The medium was changed to a chemical defined medium consisting of DMEM/F12: Neurobasal (1:1), 1 × N2, 1 × B27, 1% GlutaMAX (all from Life Technologies, Carlsbad, USA), 10 ng/ml recombinant human leukemia inhibitory factor (LIF, Millpore, Billerica, USA), 3 μM CHIR99021 and 2 μM SB431542 (both from Gene Operation, Michigan, USA). The medium was changed every 2 days. Eight to 10 days later, the epithelium-like colonies appeared on all of the three culture surfaces. On days 20 to 30, cell colonies were picked up and cultured further on PDL/laminin-coated 96-well plates for cell expansion. In the present study, three colonies derived from MEF-coated dishes were picked and expanded for subsequent experiments.

2.3. Neural differentiation of iNSC and regional patterning in vitro

For patterning and terminal differentiation of iNSCs, a modified protocol previously described by Zhang was used (Lu et al., 2013). Briefly, LIF, CHIR, and SB were removed from the medium, and small molecules, 1 μM retinoic acid (RA, Sigma-Aldrich, St. Louis, Missouri, USA) and 1 μM SAG1 (Enzo, New York, USA) were added to the medium for 2 weeks. For terminal differentiation, iNSCs were seeded at a density of 1×10^4 cells per 12 mm glass coverslip that had been coated with PDL/laminin in a neural differentiation medium (DMEM/F12 with 1 × N2, 1 × B27, 1 × NEAA supplemented with 100 ng/ml cAMP (Sigma-Aldrich), 10 ng/ml GDNF, 10 ng/ml BDNF, 10 ng/ml IGF-1 and 10 ng/ml neurotrophin 3 (NT3) (all from PeproTech, Rocky Hill, USA). To derive oligodendrocytes, iNSCs were seeded at a density of 20,000 cells per 12 mm glass coverslip coated with PDL /laminin and cultured in neural differentiation medium supplemented with RA (1 μM, Sigma-Aldrich), PDGF-AB (20 ng/ml, PeproTech), bFGF (10 ng/ml, PeproTech), SAG1 (1 μM, Enzo) for 2 weeks as described by HU (Hu et al., 2009). Afterwards, RA and bFGF were replaced with T3 (60 ng/ml), cAMP (1 mM) (both from Sigma-Aldrich), insulin-like growth factor 1 (IGF1), NT3 (all at 10 ng/ml from PeproTech) for another 6 weeks.

2.4. Immunocytochemistry

Cells were washed 3 times with PBS and fixed by 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min. For immunofluorescence staining, cells were blocked by 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) in 0.3% Triton-TBS for 1 h, and incubated with primary antibodies overnight at 4 °C followed by secondary antibody staining for 2 h at room temperature. All primary antibodies, sources, and dilutions were listed in Table S1. After nuclear staining with DAPI (Sigma-Aldrich), cell populations were counted using Image J software. At least nine fields of each coverslip were chosen randomly, and three coverslips in each group were counted. Data were expressed as mean ± SEM. Error bars represent SEM. Pictures were captured by using Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany).

2.5. Ethics statement and isolation of fetal NSCs

Human tissues were donated for research after written informed consent of the women seeking abortion. Tissue procurement was performed in accordance with the Declaration of Helsinki and the study protocol was approved by Institutional Review Committee of Xuanwu Hospital, Capital Medical University. The isolation of fetal brain NSCs was applied as described before (Wang et al., 2010). Briefly, cerebral cortical tissue was gently dissected into small pieces (about 1 mm^3), and the minced tissue was triturated gently with Pasteur pipet in 15 ml centrifuge tube (Corning, New York, USA) and was pelleted by gravity for 5 min. Then the supernatant was filtrated with 50-μm cell strainer and the cells were plated onto 10 cm well plates (Corning) in NSC medium consisting of DMEM-F12 (Life Technologies), 20 ng/ml bFGF (PeproTech), 20 ng/ml EGF (PeproTech), 2% B27 (Life Technologies) and 1% penicillin–streptomycin (Life Technologies) and incubated at 37 °C in 5% CO_2 atmosphere.

2.6. PCR, RNA isolation and RT-PCR

Genomic DNAs were extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) for PCR analysis using primers shown in (Table S2). RNA isolation was performed using the RNeasy plus Mini Kit (Qiagen, Hilden, Germany). Briefly, cells were pelleted, washed with PBS and resuspended in lysis buffer. RNA was eluted in 30 μl distilled water. The reverse transcription was carried out with the GoScript™ Reverse Transcription System (Promega, Madison, USA). RT-PCR analyses for detection of transgenes and regionalization markers were carried out using the primer pairs and annealing temperatures listed in Table S2. The PCR-program: 94 °C 2 min, 94 °C 30 s, X °C 30 s, 72 °C 1 min, 72 °C 7 min. Steps 2–4 were repeated 35 times.

2.7. Karyotype analysis

Karyotype analysis was carried out as described (Duinsbergen et al., 2008). Briefly, cycling cells were incubated with 100 ng/ml colcemid (Life Technologies) for 90 min, after which they were fixed with an ice cold methanol:glacial acetic (3:1) solution for 3 times. Chromosomes were stained with Hoechst (Life Technologies) and counted using a 630× magnification.

2.8. Electrophysiology

Whole cell patch clamp recording was performed on passage 20 iNSC–derived neurons after 7 weeks of differentiation in medium containing DMEM/F12 with 1 × N2, 1 × B27, 1 × NEAA supplemented with 100 ng/ml cAMP (Sigma-Aldrich), 10 ng/ml GDNF, 10 ng/ml BDNF, 10 ng/ml IGF-1 and 10 ng/ml neurotrophin 3 (NT3) (all from PeproTech). Cells were bathed in artificial cerebrospinal fluid (in mM: 126 NaCl, 2.5 KCl, 2 CaCl2, 1.25 NaH2PO4, 2 MgSO4, 25 glucose, and 26 NaHCO3). Internal solution consisted of (in mM: 143 KCl, 8 NaCl 10 HEPES, 1 MgCl2, 2 NaATP, and 0.4 NaGTP). Cells were visualized on an Olympus BX51WI system. Electrode resistance was 3–5 MΩ. Action potentials were induced in current clamp mode using a HEKA EPC-10 patch-clamp amplifier (HEKA Electronic Inc., Germany). Series resistance was constantly monitored and results were not included if significant variation (>20%) occurred during an experiment. Data acquisition and analysis were performed using PatchMaster and Igor software.