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Reprogramming of somatic cells induced by fusion of embryonic stem cells using hemagglutinating virus of Japan envelope (HVJ-E)

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ABSTRACT

In this research, hemagglutinating virus of Japan envelope (HVJ-E) was used to reprogram somatic cells by fusion with mouse embryonic stem (ES) cells. Neomycin-resistant mouse embryonic fibroblasts (MEFs) were used as somatic cells. *Nanog*-overexpressing puromycin-resistant EB3 cells were used as mouse ES cells. These two cells were fused by exposing to HVJ-E and the generated fusion cells were selected by puromycin and G418 to get the stable fusion cell line. The fusion cells form colonies in feeder-free culture system. Microsatellite analysis of the fusion cells showed that they possessed genes from both ES cells and fibroblasts. The fusion cells were tetraploid, had alkali phosphatase activity, and expressed stem cell marker genes such as *Pou5f1*, *Nanog*, and *Sox2*, but not the fibroblast cell marker genes such as *Col1a1* and *Col1a2*. The pluripotency of fusion cells was confirmed by their expression of marker genes for all the three germ layers after differentiation induction, and by their ability to form teratoma which contained all the three primary layers. Our results show that HVJ-E can be used as a fusion reagent for reprogramming of somatic cells.

1. Introduction

Hemagglutinating virus of Japan (HVJ) was the first virus isolated in Japan in the early 1950s. As a mouse parainfluenza virus belonging to the *Paramyxoviridae* genus, HVJ is 150–600 nm in diameter and contains negative-strand RNA (15,383 bases) inside its viral envelope. Two glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN), are present on the viral envelope. HN binds to acetylated sialic acid as a receptor on cell membrane and induces cell fusion through F protein [1–3]. Based on these properties, inactivated HVJ-envelope (HVJ-E) was developed as a drug-delivery vector or a cell fusion agent [4,5].

New methods for reprogramming somatic cells have been developed in the field of regenerative medicine. One method is somatic cell nuclear transfer, in which a somatic nucleus is transplanted into an unfertilized egg whose nucleus is removed in advance [6,7]. In 2006, induced pluripotent stem (iPS) cells [8] were established by transfection with four genes, *Oct3/4* (*Pou5f1*), *Sox2*, *Klf4*, and *c-Myc*. In addition to these methods, a lot of researchers also tried to reprogram somatic cells by fusion with ES cells [9–12].

However, in previous studies, cell fusion was mostly induced by polyethylene glycol or with electrical stimuli. These methods normally could induce severe toxicity or require special electrical instruments. Since HVJ-E is considered less toxic and can be much more conveniently used without special requirements, in this study, we used HVJ-E for cell fusion and induced reprogramming of somatic cells.

2. Materials and methods

2.1. Cell culture

The mouse ES (mES) cell line, feeder-free EB3 cells (129/Ola-derived EB3 ES cells), which was kindly supplied by Dr. Niwa (RIKEN CBD), was cultured in GMEM (Glasgow minimum essential medium), supplemented with 15% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 1× nonessential amino acids, penicillin/streptomycin, and 1000 U/mL of leukemia inhibitory factor (LIF), in gelatin-coated dishes [13]. As a mouse somatic cell line, neomycin-resistant primary MEFs (Chemicon) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS and penicillin/streptomycin.

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2.2. Transfection

EB3 cells were transfected with pPyCAG-Nanog-IP (Addgene) [14] using Lipofectamine 2000 (Invitrogen). Twenty-four hours after treatment, the cells were cultured in the presence of 1–2 μg/mL puromycin for 2 months until a stable cell line was obtained. The cell line expressed Nanog and was puromycin-resistant.

2.3. Cell fusion and following culture

EB3 (1×10^6) and MEFs (1×10^6) were mixed and incubated with 10 μL of HVJ-E (Ishihara Sangyo Co.) in 100 μL of kit buffer on ice for 5 min. The cells were incubated for 15 min at 37 °C and then suspended in the ES cell culture medium. EB3 × MEF fusion cells were selected in the presence of 1 μg/mL puromycin, 400 μg/mL G418, and 2 mM of valproic acid (VPA) 24 h after the fusion experiment.

2.4. Microsatellite analysis by polymerase chain reaction (PCR)

Genomic DNA was extracted from the cultured cells by DNAzol (Molecular Research Center) and amplified with STR-primer (D11Mit71). The PCR product was analyzed by 12% polyacrylamide gel electrophoresis at 150 V for 90 min.

2.5. Karyotype

The cultured cells were treated with colcemid for 2 h and were then collected and incubated in 75 mM of KCl solution for 6 min. Then cells were fixed for 30 min with fixing solution (methanol:acetic acid = 3:1). The fixed cells were dropped onto a defatted slide glass and stained with Giemsa.

2.6. Alkaline phosphatase

The cultured cells were fixed with 3.7% formaldehyde and stained using an Alkaline Phosphatase Substrate Kit I (Vector).

2.7. Differentiation induction

To induce differentiation, 1000 cells/30 μL of fusion cells or EB3 cells were seeded to the lid of a 96-well plate to induce formation of embryoid bodies (EBs) using the hanging drop method. After 3 days of induction, the EBs were gathered for later experiments. Otherwise, cells were cultured in gelatin-coated dishes with LIF-free culture medium for 25 days. The medium was changed every 2 days.

2.8. Reverse transcription (RT)-PCR

Total RNA was extracted from cells using an RNeasy kit (QIAGEN). With a Superscript III reverse transcriptase kit (Invitrogen), cDNA was prepared and amplified by PCR [15,16] using the following primer pairs: *Nanog* (Nanog homeobox, forward: 5′-AGG GTC TGC TAC TGA GAT GCT CTG-3′, reverse: 5′-CAA CCA CTG GTT TTT CTG CCA CCG-3′); *Pou5f1* (POU class 5 homeobox 1, forward: 5′-CTG AGG GCC AGG CAG GAG CAC GAG-3′, reverse: 5′-CTG TAG GGA GGG CTT CGG GCA CTT-3′); *Sox2* (Sex determine region Y-Box 2, forward: 5′-TAG AGC TAG ACT CCG GGC GAT GA-3′, reverse: 5′-TTG CCT TAA ACA AGA CCA CGA AA-3′); *Col1a1* (Collagen type I alpha 1, forward: 5′-GCA GAC GGG AGT TTC TCC TC-3′, reverse: 5′-TCA AGC ATA CCT CGG GTT TC-3′); *Col1a2* (Collagen type I alpha 2, forward: 5′-CGA CTA AGT TGG AGG GAA CG-3′, reverse: 5′-CTT TGT CCA CGT GGT CCT CT-3′); *Nestin* (forward: 5′-GGA GTG TCG CTT AGA GGT GC-3′, reverse: 5′-TCC AGA AAG CCA AGA GAA GC-3′); *Pax6* (Paired box 6, forward: 5′-TGC CCT TCC ATC TTT GCT TG-3′, reverse: 5′-TCT GCC CGT TCA ACA TCC TTA G-3′); *T* (T brachyury homolog, forward: 5′-TGC TTC CCT GAG ACC CAG TT-3′, reverse: 5′-GAT CAC TTC TTT CCT TTG CAT CAA G-3′); *Gata4* (GATA binding protein 4, forward: 5′-CTG TCA TCT CAC TAT GGG CA-3′, reverse: 5′-CCA AGT CCG AGC AGG AAT TT-3′); *Ttr* (Transthyretin, forward: 5′-CCA AGT CCG AGC AGG AAT TT-3′, reverse: 5′-TTC CTG AGC TGC TAA CAC GG-3′); *Gfap* (Glial fibrillary acidic protein, forward: 5′-TCG AAT GAC TCC TCC ACT CCC T-3′, reverse: 5′-TGG CCT TCT GAC ACG GAT TTG G-3′); *Actc1* (Actin alpha cardiac muscle 1, forward: 5′-TGA CAT GGA GAA GAT CTG GC-3′, reverse: 5′-TAC GAT CGG CAA TAC CAG G-3′); *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase, forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′).

2.9. Teratoma formation assay

Cell suspension of 5.0×10^5 fusion cells or control ES cells (from the National Center for Child Health and Development) in 50 μL ES medium was subcutaneously implanted into the kidney capsule of immunodeficient SCID mice (CLEA). Teratomas surgically dissected out 4 weeks after implantation, were fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Sections with a thickness of 10 μm were stained with Hematoxylin–Eosin (HE). All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committees of the National Center for Child Health and Development, and confirmed to the NIH Guide for the Care and Use of Laboratory Animals.

3. Results and discussion

3.1. Cell fusion and reprogramming of MEFs

In 2001, using thymus cells as somatic cells, Tada et al. reported that reprogramming of somatic cells occurred by fusion with ES cells [9]. However, they also reported that the reprogramming efficiency of thymus cells is very low with the cell fusion method. Silva et al. then reported that overexpression of *Nanog* in ES cells might increase the efficiency of cell reprogramming [17]. Huangfu et al. reported recently that valproic acid (VPA), a histone deacetylase inhibitor, enhanced the reprogramming efficiency for generating iPS cells [18]. In this study, we used these methods to reprogram MEFs.

mES cells were transfected with *Nanog* expression vectors, and the cells were cultured in the present of puromycin for 2 months to get the stable puromycin-resistant *Nanog*-overexprssion mES cell line. Neomycin-resistant MEFs were used as the somatic cell line. These cells were fused in the presence of HVJ-E, and the cells were cultured in the presence of puromycin, G418, and VPA. Although no colonies were observed by fusing MEFs with normal mES cells (data not shown), several colonies were observed by fusing MEFs to *Nanog*-overexpression mES cells (Fig. 1A). Microsatellite analysis confirmed that the fusion cells had genes from both MEFs and mES cells (Fig. 1B) and karyotype analysis confirmed tetraploidy of the fusion cells (Fig. 1C). Furthermore, the fusion cells had alkali phosphatase activity, which is one indicator for the undifferentiated state of ES cells (Fig. 2A). Also, the expression of stem cell marker genes *Pou5f1*, *Nanog*, and *Sox2* were confirmed in the fusion cells (Fig. 2B). On the contrary, the marker genes for MEFs such as *Col1a1* and *Col1a2* disappeared from the fusion cells (Fig. 2B). These results indicated that HVJ-E can be used for fusing MEFs to mES cells and for MEFs reprogramming, and the reprogramming efficiency was promoted by overexpression of *Nanog*.