Original Article

A method for gene transfer, single isolation and *in vitro* assay for neural stem cells

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The neurosphere method is widely used to examine self-renewal activity and the multipotency of neural stem cells (NSCs). We developed an efficient method for introducing a transgene into neurospheres using a retroviral vector and isolating a transgenic subpopulation of NSCs labeled with green fluorescent protein (GFP) using fluorescence-activated cell sorting, enabling these cells to be used in subsequent biological assays. This method is expected to be useful for studying the function of genes in NSCs.

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Introduction

Neural stem cells (NSCs), which exhibit self-renewal and multipotency, are generated in early embryonic brains and maintained throughout the lifespan¹). The neurosphere method is commonly used to culture and analyze NSCs *in vitro*²). The culture conditions used in this method allow the selective survival and proliferation of NSCs in floating cultures. A single NSC produces a single neurosphere under clonal culture conditions. The resulting neurosphere contains numerous NSCs, each of which forms the next generation of neurosphere. When the neurospheres are transferred onto an adherent substrate without growth factors, they start to differentiate and produce neurons, astrocytes and oligodendrocytes, the three major cell types of the central nervous system (CNS). These characteristics of neurospheres enable the self-renewal and differentiation of

NSCs to be studied *in vitro*.

Retroviral vectors are useful for introducing foreign genes into neurospheres for functional analyses³). However, the conventional protocol for preparing retroviral stocks includes the culture of a packaging cell line in a serum-containing medium that affects the capacity of NSCs to self-renew⁴). Moreover, retroviral vectors infect all the mitotic cells, including NSCs and more-committed progenitors, thereby reducing the reliability and efficiency of the studies.

Fluorescence-activated cell sorters (FACS) are widely used to isolate specific cell populations from tissues or cell cultures. Recently, a CD15-immunoreactive subpopulation of NSCs was successfully isolated using FACS and neurospheres derived from adult mouse brain cells⁵).

By combining retrovirus-mediated gene transfer and single

cell isolation technology, we have developed an efficient strategy for isolating NSCs that carry a gene of interest. This versatile method should become a useful strategy for studying the function of molecules in NSCs.

Materials and Methods

1) Neurosphere formation assay

For the neurosphere cultures, telencephalon cells were prepared from the forebrain of E14 CD-1 embryos and cultured as described previously⁶. Briefly, the cells were cultured at a density of 5×10^5 cells/ml in a basal medium with 20 ng/ml epidermal growth factor (EGF) (Genzyme) and 20 ng/ml fibroblast growth factor (FGF) (PeproTech) for 7 days. The basal medium was composed of Dulbecco Modified Eagle Medium (DMEM):F12 (1:1), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), HEPES (5 mM), insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (60 µM), B27 (1:100) and selenium chloride (30 nM) (all from Sigma except for B27, which was from GIBCO). Half of the culture medium was changed every 3 days.

2) FACS sorting

To isolate the NSCs *in vitro*, primary neurospheres were centrifuged and treated in Accumax (ICT) solution at 37 °C for 5 minutes. After washing with phosphate buffered saline (PBS), the neurospheres were mechanically dissociated and stained with anti-CD15 (1:100, clone MMA; Pharmingen) antibody (Ab) for 20 minutes followed by a PE-conjugated secondary Ab (1:500, Pharmingen). After washing, the cells were suspended in Hanks Balanced Salt Solution (HBSS) containing 10 mg/ml propidium iodide and filtered through a 30-µm mesh. Cell sorting and analyses were performed using an EPICS ALTRA system equipped with the Autoclone sorting option (Beckman Coulter). PI-negative viable cells were sorted at < 3000 events/s directly into 96-well, low-adhesion microtiter plates (Costar) containing basal medium supplemented with EGF and FGF. Seven days later, the number of neurospheres in each well was counted.

3) Differentiation assay

To test the self-renewal activity, single neurospheres were transferred into another well and dissociated individually. For differentiation, the neurospheres grown from the self-renewal experiments, i.e., all spheres that were derived from a single neurosphere were plated onto 10-mm poly-D-ornithine-coated glass cover slips (Matsunami) in basal medium without EGF or FGF for 4 days. The neurospheres grown on the cover slips

were then immersion-fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After three rinses in PBS, the neurospheres were incubated for 1 hour in TNB blocking solution (Vector), incubated with primary antibodies overnight, and then incubated for 60 minutes at room temperature with Alexa Fluor-conjugated secondary antibodies (1:200, Molecular Probes). The primary antibodies (final dilution and source) used in this study were as follows: rabbit polyclonal anti-GFAP (1:1000, Dako), mouse monoclonal anti-O4 IgM (1:20, Roche Diagnostics), chicken polyclonal anti-GFP (1:5000, BioChain Institute) and mouse monoclonal anti- α -tubulin IgG (1:1000, Sigma).

Retrovirus vector

pMX-IG retrovirus vector was produced as previously described⁷ but with several modifications, as follows. The Plat-E packaging cell line⁸ was maintained in a serum-free medium (Free Style 293 Expression Medium, GIBCO). For virus production, the Plat-E cells were transfected with the pMX-IG plasmid using the modified CaPO₄ transfection method, as previously described⁹, to maximize the gene transfer efficiency. Forty-eight hours later, the virus-containing medium was collected and filtered using a 0.45 µm syringe filter (Millipore). For infection, a 1/100 volume of the basal medium containing the virus particles was added to the neurosphere cultures at 1 day *in vitro* (DIV). Ten GFP-expressing cells were sorted into each well at 7 DIV. Seven days later, the number of neurospheres in each well was counted. CD15-immunoreactivity was defined as a signal with an intensity of at least 4 times greater than the control.

Results

To establish an efficient gene transfer system for NSCs *in vitro*, we utilized the retrovirus vector, pMX-IG⁷. This vector contains an internal ribosome entry site (IRES) that allows the simultaneous expression of an inserted gene and the GFP marker to be examined, allowing virus-transduced cells to be identified (Fig.5a). Serum contamination in the sphere culture results in a decreased self-renewal activity and multipotency of the NSCs⁴. Therefore, we used a modified serum-free medium (see Materials and Methods) to maintain and transfect the virus producer cell line, Plat-E⁸. The resulting culture medium containing the retrovirus vector was added to the neurosphere culture one day after the start of the culture. The virus stock was diluted appropriately to avoid multi-copy transduction¹⁰.

Fig.1 Neurospheres transfected by retrovirus vector
Neurospheres were grown in a retrovirus-containing medium for 1 week and GFP expression was visualized by fluorescent microscopy.

Fig.2 Isolation of GFP⁺ and CD15⁺ cells
Cultures containing GFP⁺ neurospheres were dissociated into single cells, stained by CD15 antibody, and sorted by FACS. A control neurosphere culture that was only stained with the PE-conjugated secondary antibody was used as a negative control (a). GFP⁺ and CD15⁺ cells were identified as NSCs carrying the transgene (b). The presence of a GFP⁺/CD15⁺ subpopulation suggests that more-differentiated progenitors also express the transgene.

Fig.3 Secondary neurosphere-forming activity of CD15⁺/GFP⁺ cells

The secondary neurosphere-formation efficiency (vertical axis) of the CD15⁺/GFP⁺ neurosphere cells group and GFP⁺ control group (horizontal axis) were compared. The CD15⁺ cells formed significantly more neurospheres than the CD15⁻ cells (** $p < 0.01$, unpaired-T). This result suggests that the NSCs were successfully transduced by the retrovirus vector in the neurosphere cultures and selectively isolated by FACS.

The multiplicity of infection (MOI), calculated as the percentage of GFP-positive cells among the total cultured cells, was 0.17, on average. Consequently, both GFP-positive and -negative neurospheres were identified in the same culture using fluorescent microscopy (Fig.1) after culturing for seven days. Under these culture conditions, most of the GFP-expressing cells were thought to carry only a single copy of the transgene, making the effects of the retrovirus-mediated gene transfer easier to interpret.

To isolate NSCs carrying the transgene, the neurospheres were dissociated to prepare single cell suspensions and immunostained with CD15 Ab. The CD15/GFP-double-positive cells were easily identified by FACS (Fig.2, CD15⁺/GFP⁺, 3.1%; CD15⁺/GFP⁻, 18.4%; CD15^{low/-}/GFP⁺, 13.6%; CD15^{low/-}/GFP⁻, 64.9%). The CD15/GFP-double-positive cells were sorted into 96-well plates at a density of 10 cells/well and cultured for seven days. The neurosphere-forming ability of the CD15⁺/GFP⁺ cells was significantly higher than that of the cells sorted only based on

Fig.4 Neurospheres derived from CD15⁺/GFP⁺ cells retain their multipotency

The differentiated cells from neurospheres derived from single CD15⁺ cells were immunostained for β III-tubulin (neuron, red), GFAP (astrocyte, blue), O4 (oligodendrocyte, magenta). The GFP-positive neuron (red arrow, yellow), astrocyte (blue arrow, blue green) and oligodendrocyte (magenta arrow) were shown.

GFP expression (Fig.3, GFP/CD15-double gated, 10.5%(2.67; GFP-single gated, 2.3% $\pm$ 1.04; $p < 0.01$, one-tail unpaired-T test, $n > 200$, 10 cells/well), suggesting that CD15-based cell-sorting yielded a population enriched in the NSCs. Indeed, the neurospheres generated from the double-positive sorted cells could be used to form the next generation of neurospheres, suggesting that they retained their self-renewal ability. The GFP-expression in the neurospheres could be observed even 6 month after infection. This result suggests that this virus vector should be able to express genes in NSCs without silencing. To confirm their multipotency, the secondary neurospheres were subjected to a differentiation assay (see Materials and Methods). Most of the neurospheres generated from the sorted CD15⁺ cells differentiated into β III-tubulin positive neurons, GFAP-positive astrocytes, or O4-positive oligodendrocytes (Fig.4). These data suggest that the method reported here is a powerful strategy for studying the function of specific genes of interest in NSCs *in vitro*.

Discussion

In this study, we used CD15 immunoreactivity and GFP fluorescence to develop a method for purifying NSCs that carry a

Fig.5 Schematic representation of the structure of the virus expression vector (a) and a summary of the protocol (b). LTR and IRES sequences enable this vector to express EGFP and another cDNA cloned into the MCS. LTR; long terminal repeat, PBS; primer binding site, ψ ; packaging signal, Δ Gag; mutated gag sequence, MCS; multi-cloning site, IRES; internal ribosomal entry site.

transgene (Fig.5b).

Serum contamination is a serious problem in the assessment of NSC activity. In the conventional method, ultracentrifugation is an essential step to concentrate the virus stock and remove the serum¹¹⁾. The VSV-G envelope must be used for this method, since other envelopes are not sufficiently stable to undergo ultracentrifugation. However, use of the VSV-G envelope is associated with a few technical disadvantages, such as a higher cytotoxicity and risk of infection in humans¹²⁾. In the present study, we used a serum-free medium for virus production, enabling the ultracentrifugation step to be omitted. Consequently, we were able to use the MuLV-derived virus envelope¹³⁾, which cannot withstand the mechanical stress of ultracentrifugation. The MuLV envelope has some advantages, compared to the VSV-G envelope, in that it is not infectious to humans and is only minimally toxic. Indeed, the viral gene transfer did not significantly affect the self-renewal ability and multipotency of the NSCs, enabling reliable functional studies to be performed.

We found that CD15 Ab was useful for isolating NSCs from neurospheres derived from mouse embryos. This result suggests that the molecule recognized by the CD15 Ab (the Le^x epitope)

is expressed on the surface of NSCs in the mouse embryo. Consistently, previous papers have reported that the Le^x epitope was expressed in the germinal zone of the mouse embryonic telencephalon, which contains a large number of NSCs¹⁴). The Le^x epitope is also expressed in human fetal neurospheres (M.S., unpublished results), suggesting that this method may be applicable to human NSCs.

In conclusion, we have developed a method for purifying NSCs that carry a transgene by monitoring the expression of GFP and Le^x. Recently, a variety of screening strategies have been used to identify numerous novel genes in NSCs and to implicate these genes in a variety of biological processes¹⁵). The *in vitro* assay system reported here would allow rapid, high-throughput, and precise functional assays of these genes in NSCs. This method would also be useful for screening for novel genes involved in the control of NSC activities. In the future, this method is expected to help improve our understanding of the molecular signature of NSCs.

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References

- 1) Okano H: Stem cell biology of the central nervous system. *J Neurosci Res*, 69: 698-707, 2002.
- 2) Reynolds BA, Weiss S: Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, 255: 1707-1710, 1992.
- 3) Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N and Gotoh Y: Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signaling. *Nat Cell Biol*, 6: 547-554, 2004.
- 4) Louis SA, Reynolds BA: Generation and differentiation of neurospheres from murine embryonic day 14 central nervous system tissue. *Methods Mol Biol*, 290: 265-280, 2004.
- 5) Capela A, Temple S: LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron*, 35: 865-875, 2002.
- 6) Shimazaki T, Shingo T, Weiss S: The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. *J Neurosci*, 21: 7642-7653, 2001.
- 7) Kitamura T, Koshino Y, Shibata F, Nakajima H, Nosaka T and Kumagai H: Retrovirus-mediated gene transfer and expression cloning, powerful tools in functional genomics. *Exp Hematol*, 31: 1007-1014, 2003.
- 8) Morita S, Kojima T, Kitamura T: Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther*, 7: 1063-1066, 2000.
- 9) Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol*, 7: 2745-2752, 1987.
- 10) Hargrove PW, Vanin EF, Kurtzman GJ, Nienhuis AW: High-level globin gene expression mediated by a recombinant adeno-associated virus genome that contains the 3'gamma globin gene regulatory element and integrates as tandem copies in erythroid cells. *Blood*, 89: 2167-2175, 1997.
- 11) Suzuki A, Obi K, Urabe T, Hayakawa H, Yamada M, Kaneko S, Onodera M, Mizuno Y and Mochizuki H: Feasibility of ex vivo gene therapy for neurological disorders using the new retroviral vector GCDNsap packaged in the vesicular stomatitis virus G protein. *J Neurochem*, 82: 953-960, 2002.
- 12) Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM and Trono D: In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, 272: 263-267, 1996.
- 13) Gomard E, Leclerc JC, Levy JP: Murine leukemia and sarcoma viruses: further studies on the antigens of the viral envelope. *J Natl Cancer Inst*, 50: 955-961, 1973.
- 14) Tole S, Kaprielian Z, Ou SK, Patterson PH: FORSE-1: a positionally regulated epitope in the developing rat central nervous system. *J Neurosci*, 15: 957-969, 1995.
- 15) Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA and Lemischka IR: A stem cell molecular signature. *Science*, 298: 601-604, 2002.