Establishment and characterization of immortalized hippocampal neural precursor cell lines

Tomohiro Kaji, Naoko Yoshida, Kiyoshi Yamada, Tatsuhiko Hisatsune* & Shuichi Kaminogawa

Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

(* Author for correspondence; Tatsuhiko Hisatsune, Division of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo.)

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Abstract

In the mammalian central nervous system, a complex circuit of neurons contributes to higher behaviors. Each region of the brain has a unique function derived from various types of neurons. Several neural precursor cell lines have been established from basal ganglia of fetal brain. In this study, hippocampal neural precursor cell lines were established from the hippocampus of p53^{-/-} embryos. By means of integration of a MycER regulatable oncoprotein into p53^{-/-} neural precursor cells, several immortal lines were established from embryonic hippocampal primordium, with bFGF and estrogen continuously supplied for activation of the MycER protein. A dual luciferase study demonstrated that the MycER protein blocked the expression of a glial cell marker protein, GFAP, probably contributing to the persistent cell division of the immortalized neural precursor cells. These cell lines differentiate into neuronal and glial cell types after withdrawal of bFGF. The phenotype of the hippocampal cell lines differed from that of the basal ganglia cell lines as observed in a clonal density culture. This result implies that each region of the brain has a unique developmental program, that may be imprinted in each of the neural precursor cells.

Abbreviations: p53^{-/-}, p53 knock-out; CNS, central nervous system; bFGF, basic fibroblast growth factor; HBSS, Hank's balanced salt solution; E14.5, embryonic day 14.5; MycER, c-Myc-estrogen receptor fusion protein.

Introduction

The central nervous system (CNS) performs complex tasks depending on the activity of a large number of neurons and glial cells that are organized into numerous regions specialized for distinct functions. All of the cells of the CNS arise from dividing neural precursor cells in the ventricular zone of the developing brain (Jacobson, 1991). The proliferative potential of the neural precursor cells is strictly controlled in the context of developmental age and brain region (Finlay and Darlington, 1995; Caviness et al., 1995). Most murine neural precursor cells stop dividing before birth, except those in the basal ganglia and hippocam-

pus, and these adult-type neural precursor cells give rise to granule neurons throughout life (McKay, 1997).

The potential of neural precursor cells to divide can be prolonged by exogenous stimulation and endogenous cues. Basic fibroblast growth factor (bFGF) promotes the proliferation of embryonic as well as adult neural precursor cells (Cattaneo and McKay, 1990; Vicario-Abejon et al., 1995; Johe et al., 1996). Enforced expression of the *myc* gene promotes prolonged cell division of neural precursor cells, often leading to cell immortalization (Frederiksen et al., 1988; Snyder et al., 1992; Nakafuku and Nakamura, 1995; Hoshimaru et al., 1996). Eilers and his colleagues have reported that, using a Myc-estrogen receptor (MycER) chimeric oncoprotein, activation of the Myc-protein

leads to transformation of a rat fibroblast cell line (Eilers et al., 1989) and the Myc-protein induces expression of cyclin A and cyclin E which contribute to cell cycle acceleration (Jansen-Durr et al., 1993). The MycER fusion protein only works in the presence of estrogen, therefore the function of Myc protein is regulated by the culture condition.

p53 is a sequence-specific transcription factor that plays a central role in apoptosis and oncogenesis (Levine, 1997). In a previous study, we observed that p53 protein negatively affects the multiplication of neural precursor cells (Hisatsune et al., submitted for publication). We obtained several multipotent neural precursor cell lines from embryonic basal ganglia of p53-deficient embryos into which had been introduced the MycER regulatable oncoprotein. In this study, we established several hippocampal neural precursor cell lines, which differentiate into neuronal and glial cell types, and examined the difference in cellular phenotype between hippocampal and basal ganglial neural precursor cells.

Materials and methods

Cell culture

Transgenic mice carrying a null allele in the *p53* locus ($p53^{+/-}$ and $p53^{-/-}$) were obtained from Taconic, Inc. (Germantown, NY). $p53^{+/-}$ female mice were mated with $p53$ knock-out ($p53^{-/-}$) male mice. Plugged day was counted as E 0.5. Mouse telencephalon was dissected individually from embryonic day 14.5 (E14.5) embryos. Basal ganglia, hippocampus, and cortex were further dissected as described (Vicario-Abejon et al., 1995; Johe et al., 1996). Each tissue was then placed in ice-cold Hank's balanced salt solution (HBSS), and triturated using a P-1000 Pipetman pipette. After centrifugation at 4 °C, the cell pellet was resuspended in 5 ml of N2-DMEM/F12 medium, supplemented with 10 ng ml⁻¹ bFGF as described previously (Vicario-Abejon et al., 1995). Individual telencephalic cells were plated in 10 cm dishes coated with 15 µg ml⁻¹ poly-L-ornithine and 1 µg ml⁻¹ fibronectin and were cultured for 4 days.

During this initial period of cell expansion, the genotype of the embryos was checked by the polymerase chain reaction method as described using the following primers: for detection of wild type, gTg TTT CAT TAG TTC CCC ACC TTg AC (5W-primer), ATg ggA ggC TgC CAG TCC TAA CCC

(3W-primer); for detection of null-type, gTg ggA ggg ACA AAA gTT CgA ggC C (5N-primer), TTT ACg gAg CCC Tgg CgC TCg ATg T (3N-primer) (Timme and Thompson 1994). For passage, cells were incubated with 10 ml of HBSS for 15 min at 37 °C and gently harvested using a 10 ml pipette. The resulting cells were collected as $P53^{-/-}$ and $P53^{+/-}$ cells before centrifugation. The cells were counted, and the viability was assessed by trypan blue staining. Cells were plated in 10 cm dishes, previously coated with polyornithine and fibronectin.

Retrovirus infection

Proliferating telencephalic cells were infected with a retroviral vector harboring a Myc-Estrogen-Receptor (MycER) fusion gene. The activation of MycER protein requires the presence of the ligand of estrogen receptor, estradiol, however this mechanism has not been still clarified (Eiler et al., 1989). At 24 h after infection, the cells were placed under drug selection in N2-DMEM/F12 medium in the presence of bFGF (10 ng ml⁻¹) and estrogen. After cloning and long-term culture, colonies of proliferating cells were picked up, expanded, and MycER integration was analyzed by southern, northern, and western blotting. Full expansion of isolated cells often required the presence of fetal bovine serum.

To obtain differentiation of the immortal line, cells were harvested by trypsinization followed by the addition of a trypsin inhibitor, ovomucoid. The harvested cells were replated on pre-coated plastic chamber slides or in dishes containing N2-DMEM/F12 medium plus bFGF. After 4 days in the presence of bFGF, the cells were allowed to differentiate in N2-DMEM/F12 medium for 6–12 days. In the case of a low density culture, cells expanded initially in N2-DMEM/F12 medium plus bFGF for 4 days were harvested in HBSS as described before (Johe et al., 1996), and the harvested cells were seeded at a very low density and expanded for 10 days, then were allowed to differentiate for 9 days.

Luciferase assay

GFIL-pGL3 (a kind gift from Drs K. Nakashima and T. Taga) is a reporter plasmid containing the firefly luciferase gene driven by the GFAP promoter (Miura et al., 1990). R-Luc (a gift from Drs K. Shimozaki and S. Nagata) is a renilla luciferase expression plasmid, with expression driven by the EF-1α promoter (Mizushima

et al., 1990), and this plasmid was used as an internal control. Neural precursor cells were cotransfected with these plasmids using lipofectamine (Gibco BRL, Gaithersburg, MD). One day later, the cells were stimulated with 5% fetal calf serum in the absence or presence of an estrogen, 4-hydroxytamoxifen (100 nM; Sigma, St. Louis, MO), to activate the MycER-protein. The efficiency of transfection for the immortalized cells was around 10%, whereas the efficiency for primary-cultured cells was less than 0.1%, which is too low to assess the role of introduced gene. The cells were harvested 24 h later. Luciferase activity was determined using the Dual-luciferase Reporter Assay System (Promega, Madison, WI). The relative activity of the GFAP promoter as the firefly luciferin value was normalized with the EF-1 α activity as the renilla luciferin value. The relative GFAP-promoter activity in the absence of both 4-hydroxytamoxifen and fetal calf serum was defined as one arbitrary unit of GFAP-promoter activity.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 10 min. After permeabilization with 0.1% Triton X-100/3% normal goat serum/PBS, the cells were incubated overnight with primary antibodies against nestin (rabbit polyclonal antibody at 1/1000, kindly provided by Dr. R. McKay), MAP2 (clone HM-2 at 1/500, Sigma) or GFAP (clone G-A-5 at 1/100, Sigma). After washed with PBS 3 times, the cells were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase as described elsewhere (Yamada et al., 1999). The develop was performed using DAB (Sigma).

Results

Isolation of immortalized neural precursor cells from p53 knock-out brain carrying the integrated vector encoding the MycER regulatable oncoprotein

p53 is a sequence-specific transcription factor that plays a central role in apoptosis and oncogenesis (Levine, 1997). In a previous study, we observed that p53 directly induces apoptotic cell death in neural precursor cells, and isolated multipotent neural precursor cell lines derived from embryonic basal ganglia of p53-deficient embryos into which had been introduced the MycER regulatable oncoprotein (Hisatsune et al., submitted for publication).

Table 1. Plating efficiency of p53-deficient vs. p53-bearing neural precursor cells

	Cells	Number of colonies	
		p53 ^{-/-}	p53 ^{+/-}
Expanded E14.5	5000	25	0
Neural precursor cells	10000	34	0
	20000	144	2

Expanded neural precursor cells from p53^{-/-} or p53^{+/-} mice were pooled, and were seeded at graded cell densities in coated 10 cm dishes. The cells were cultured for 20 days and the number of colonies (up to 30 cells) was counted in each dish.

Table 2. Immortalized neural precursor cell lines

Protocol	Region	Day	Established lines
#1	Whole telencephalon	14.5	MTP-1~5
#2-1	Basal ganglia	15.5	MSP-1 ^a
#2-2	Hippocampus	15.5	MHP-2
#3-1	Basal ganglia	14.5	MSP-10~14
#3-2	Hippocampus	14.5	MHP-13

^a The MSP-1 line was isolated in a previous study (Hisatsune et al., submitted for publication).

To rule out the possibility that the p53-null mutation was responsible for the expansion of the neural precursor cells, both p53-deficient (p53^{-/-}) and p53-bearing (p53^{+/-}) neural precursor cells were seeded at graded cell densities from 5000–20 000/10 cm dish. As described in Table 1, the plating efficiency of the p53-deficient cells appeared to be superior to that of the control cells. The size of each colony was also larger in the case of the p53-deficient culture. Most clonally expanded cells gave rise to neuronal and glial cell types after withdrawal of bFGF (not shown). This result clearly demonstrates the enhanced cell proliferation activity of the p53-deficient neural precursor cells. Some p53-deficient clones were further cultivated to assess whether the p53 null-mutation leads to immortal cell division of neural precursor cells. However, spontaneous differentiation into both neuronal and glial cells in most expanded lines was observed during long-term expansion over a period of up to 6 weeks (not shown). These results clearly indicate that the p53-null mutation leads to enhanced cell expansion; however, this single mutation did not lead to immortal cell division in the neural precursor cells.

To block spontaneous differentiation and induce prolonged cell division, neural precursor cells from p53-deficient telencephalon were infected with the

Figure 1. Immortalized hippocampal neural precursor cell line, MHP-2, expressed nestin. Cells were expanded for 4 days in N2-DMEM/F12 medium containing 10 ng ml^{-1} bFGF for blocking cell differentiation. Note that the intermediate filament proteins were clearly stained. Scale bar is $25 \mu\text{m}$.

MycER regulatable oncogene (Eilers et al., 1989; Nakafuku and Nakamura, 1995). After infection, cloning, and long-term expansion, we established several proliferating cell lines (Table 2, protocol #1). These cell lines express nestin, an intermediate filament protein expressed in neural precursor cell (Figure 1), and proliferate exponentially in the growth media used. After withdrawal of the mitogen, all of these lines differentiated into neuronal or glial cell types (not shown). To assess whether the immortalization of neural precursor cells was dependent on a telencephalic subregion, proliferating precursor cells from developing hippocampus, neo-cortex, or basal ganglia of p53-deficient embryos were infected with the MycER oncogene. We succeeded in establishing cell lines from the hippocampus and basal ganglia, but not from cortex (Table 2). All of the established cell lines differentiated into neuronal or glial cell types under mass-differentiation conditions (not shown).

MycER protein blocks differentiation of neural precursor cells

To assess whether activation of the MycER protein contributes to blocking of cellular differentiation, the

GFAP-promoter activity in both MSP-1 and MHP-2 cells were evaluated in the absence or presence of Myc-protein function. GFAP is a glial cell specific marker protein (Miura et al., 1990). Glial differentiation is induced by a particular component in serum, and this is very problematic in immortalization of neural precursor cells, since prolonged division of neural precursor cells requires some mitogen present in serum. This putative mitogen has not been isolated yet.

Both MSP-1 and MHP-2 cells were transfected with the reporter gene and were stimulated with 5% fetal calf serum in the absence or presence of an activator of the MycER oncoprotein. As shown in Figure 2, glial cell specific GFAP-promoter activity induced by serum was greatly suppressed to the background level by activation of the MycER oncoprotein in both MSP-1 and MHP-2 cells. This clearly shows that the Myc-protein blocks glial differentiation of neural precursor cells.

Miura et al. have reported that promoter region required for cell specific expression was located within -256 bp from the transcription startpoint (Miura et al., 1990). In this region, there is no c-myc-responsive sequence CACGTG (Blackwell et al., 1990). This

Figure 2. GFAP-promoter activity is suppressed by MycER activation. MSP-1 and MHP-2 cells cotransfected with a GFAP promoter-driven firefly luciferase plasmid (GFIL-pGL3) and an EF-1 α -driven renilla luciferase plasmid (R-Luc). One arbitrary unit of GFAP promoter activity was calculated from the relative firefly luciferin value normalized by the renilla luciferin value, where MSP-1 or MHP-2 cells were transfected in the presence of neither fetal calf serum (FCS) nor 4-hydroxytamoxifen, an activator of MycER protein (c-Myc).

critical GFAP-promoter region possesses cAMP responsive element, nuclear factor I site, AP-1 and AP-2 sites. It is conceivable that c-Myc proteins would act as a negative regulator for one of the transcription activator sites, indirectly. This characteristic of the Myc-protein would contribute to the immortalization of neural precursor cells.

Cellular phenotype of immortalized hippocampal neural precursor cells

The phenotype of hippocampal neural precursor cells was fully analyzed in a culture undergoing cell differentiation and in a clonally expanded culture. As demonstrated in Figure 3A, neuronal differentiation of the hippocampal neural precursor cells was observed within two days after withdrawal of the mitogen. The hippocampal neural precursor cells also differentiated into astrocytic cells (Figure 3B). Oligodendrocytic differentiation did not occur under these conditions. We have previously reported that a basal ganglial cell line, MSP-1, needs 10 days for neuronal and glial differentiation (Yamada et al., 1999). To evaluate the clonal expansion potential, hippocampal precursor cells were plated at a density of $2\text{--}50 \times 10^4/10$ cm dish, but no clonally expanded cells were obtained from these cul-

tures (Figure 4A). In contrast, basal ganglial precursor cells formed colonies (Figure 4B), and clonally expanded cells give rise to both neuronal and glial lineages upon withdrawal of the mitogen (not shown).

These findings show that two types of cell lines derived from different telencephalic regions, basal ganglia and hippocampus, have different characteristics with respect to differentiation and clonal expansion. The hippocampal immortalized neural precursor cell lines, MHP-2 and MHP-13, are quite useful for analyzing the molecular basis of hippocampal development and function.

Discussion

Immortalization of neural precursor cells

Our strategy to isolate immortalized neural precursor cells was to transfer the gene encoding a MycER regulatable oncoprotein into a primary culture of p53-deficient neural precursor cells given a continuous supply of bFGF. This approach is basically the same as the method for immortalizing hematopoietic progenitor cells from p53 knockout mice using the myc and raf oncogenes (Metz et al., 1995), as the raf-signal

Figure 3. Multipotential differentiation of immortalized hippocampal neural precursor cell line, MHP-2. Both figures show MHP-2 cells that differentiated for 7 days. (A) Neuronal differentiation as detected with anti-MAP-2 antibody. (B) Glial differentiation as detected with anti-GFAP antibody. Scale bar is 25 μm .

Figure 4. Phenotype of the hippocampal neural precursor cell line MHP-2, compared with that of the basal ganglial cell line MSP-1. (A) MHP-2 cells were plated at a density of 5×10^5 cells per 10 cm dish and expanded for 7 days. (B) MSP-1 cells plated at a density of 3×10^4 cells per 10 cm dish and expanded for 11 days. Scale bar is 100 μm . Note that there is a clear difference in cell-cell interaction.

could be activated by bFGF in our system. This triple signal modulation (p53 null mutation, myc, and raf) would promote the proliferation of both hematopoietic and neural precursor cells.

Our study also demonstrates the role of the c-Myc-protein in promoting proliferation and differentiation of neural precursor cells. c-Myc is a sequence-specific transcription factor. We used a chimeric MycER protein (Eilers et al., 1989), and blocking of GFAP-promoter activity by this Myc-protein was clearly shown. Spontaneous glial differentiation during long-term cultivation of neural precursor cells maintained with serum components had been a serious problem; however, integration of the vector encoding the MycER protein into the neural precursor cells allowed us to overcome this issue. This strategy for immortalization of neural precursor cells (p53 null mutation, myc, and continuous supply of bFGF) allowed us the opportunity to study brain development using techniques of molecular and cellular biology.

Brain region and cell immortalization

We propose that immortalization of neural precursor cells requires a genetic cues, which involve gene products specifically expressed in the hippocampus and basal ganglia and which might be related to adult neurogenesis. It is conceivable that p53, raf, myc and other gene(s) may concertedly regulate the proliferation of neural precursor cells.

Neurogenesis continues throughout adulthood in discrete regions. Proliferative zones include the subependymal zone, from where progenitors migrate along the rostral migratory pathway to differentiate into neurons in the olfactory bulb, and the hippocampal subgranular zone, where they migrate and differentiate into granule neurons (Suhonen et al., 1996; McKay, 1997). Regarding the telencephalic region, we and others have isolated several cell lines from basal ganglia and hippocampus, but not from cortex (Martinez-Serrano and Bjorklund, 1997). These observations strongly suggest that prolonged division of neural precursor cells require putative genetic cues, possibly linked to adult neurogenesis. Recently, Monaghan et al. have demonstrated that the *tailless* gene plays an essential role in neurogenesis in the case of hippocampal granule neurons (Monaghan et al., 1997). Such a gene would be very useful for immortalization of neural precursor cell lines from a particular brain subregion.

A hope for brain repair

Neural grafting is currently limited by the lack of suitable donor material and failure in achieving full integration of the grafted cells. *In vitro* expansion of neural progenitor cells, including isolation of immortal neural precursor cells by regulatable oncogene transfer, will provide transplant-based strategies for treatment of neurodegenerative diseases (Lundberg et al., 1997; McKay, 1997). We hope human neural precursor lines can be obtained by a protocol similar to that described in this study. For clinical applications, cell culture offers an important opportunity to use sophisticated genetics in cell-based therapies for neural disease.

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References

- Blackwell TK, Kreztner L, Blackwood EM, Eisenman RN and Weintraub H (1990) Sequence-specific DNA binding by the c-MYC protein. *Science* 250: 1149–1151.
- Cattaneo E and McKay RDG (1990) Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* 347: 762–765.
- Caviness Jr VS, Takahashi T and Nowakowski RS (1995) Numbers, time and neocortical neuronogenesis: A general developmental and evolutionary model. *Trends Neurosci* 18: 379–383.
- Eilers M, Picard D, Yamamoto K and Bishop JM (1989) Chimeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* 340: 66–68.
- Finlay BL and Darlington RB (1995) Linked regularities in the development and evolution of mammalian brain. *Science* 268: 1578–1584.
- Frederiksen K, Jat PS, Valtz N, Levy D and McKay RDG (1988) Immortalization of precursor cells from the mammalian CNS. *Neuron* 1: 439–448.
- Hoshimaru M, Ray J, Sah DWY and Gage FH (1996) Differentiation of the immortalized adult neuronal progenitor cell line

- HC2S2 into neurons by regulatable suppression of the v-myc oncogene. *Proc Natl Acad Sci USA* 93: 1518–1523.
- Jacobson M (1991) *Developmental Neurobiology*. Plenum Press, New York.
- Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, Botz J, Wessbecher J, Draetta G and Eilers M (1993) Differential modulation of cyclin gene expression by MYC. *Proc Natl Acad Sci USA* 90: 3685–3689.
- Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM and McKay RDG (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* 10: 3129–3140.
- Levine AJ (1997) p53, the cellular gatekeeper for growth and division. *Cell* 88: 323–331.
- Lundberg C, Martinez-Serrano A, Cattaneo E, McKay RDG and Bjorklund A (1997) Survival, integration, and differentiation of neural stem cell lines after transplantation to adult rat striatum. *Exp Neurol* 145: 342–360.
- Martinez-Serrano A and Bjorklund A (1997) Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* 20: 530–538.
- McKay RDG (1997) Stem cells in the central nervous system. *Science* 276: 66–71.
- Metz T, Harris AW and Adams JM (1995) Absence of p53 allows direct immortalization of hematopoietic cells by the myc and raf oncogenes. *Cell* 82: 29–36.
- Miura M, Tamura T and Mikoshiba K (1990) Cell-specific expression of the mouse glial fibrillary acidic protein gene: identification of the cis- and trans-acting promoter elements for astrocyte-specific expression. *J Neurochem* 55: 1180–1188.
- Mizushima S and Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 18: 5322.
- Monaghan AP, Bock D, Gass P, Schwager A, Wolfer DP, Lipp H-P and Schutz G (1997) Defective limbic system in mice lacking the *tailless* gene. *Nature* 390: 515–517.
- Nakafuku M and Nakamura S (1995) Establishment and characterization of a multipotential neural cell line that can conditionally generate neurons, astrocytes, and oligodendrocytes *in vitro*. *J Neurosci Res* 41: 153–168.
- Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwig EA and Cepko CL (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* 68: 33–51.
- Suhonen JO, Peterson DA, Ray J and Gage FH (1996) Differentiation of adult hippocampus-derived progenitors into olfactory neurons *in vivo*. *Nature* 383: 624–627.
- Timme TL and Thompson TC (1994) Rapid allelotyping analysis of p53 knockout mice. *BioTechniques* 17: 461–463.
- Vicario-Abejon C, Johe KK, Hazel TG, Collazo D and McKay RDG (1995) Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* 15: 105–114.
- Yamada K, Hisatsune T, Uchino S, Nakamura T, Kudo Y and Kaminogawa S (1999) NMDA receptor-mediated Ca^{2+} responses in neurons differentiated from p53^{-/-} immortalized neural stem cells. *Neurosci Lett* 264: 165–167.