Cell Transplantation, Vol. 26, pp. 271–281, 2017
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0963-6897/17 $90.00 + .00
DOI: https://doi.org/10.3727/096368916X692852
E-ISSN 1555-3892
www.cognizantcommunication.com

Establishment and Characterization of Immortalized Minipig Neural Stem Cell Line

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Despite the increasing importance of minipigs in biomedical research, there has been relatively little research concerning minipig-derived adult stem cells as a promising research tool that could be used to develop stem cell-based therapies. We first generated immortalized neural stem cells (iNSCs) from primary minipig olfactory bulb cells (pmpOBCs) and defined the characteristics of the cell line. Primary neural cells were prepared from minipig neonate olfactory bulbs and immortalized by infection with retrovirus carrying the *v-myc* gene. The minipig iNSCs (mpiNSCs) had normal karyotypes and expressed NSC-specific markers, including nestin, vimentin, Musashi1, and SOX2, suggesting a similarity to human NSCs. On the basis of the global gene expression profiles from the microarray analysis, neurogenesis-associated transcript levels were predominantly altered in mpiNSCs compared with pmpOBCs. These findings increase our understanding of minipig stem cells and contribute to the utility of mpiNSCs as resources for immortalized stem cell experiments.

Key words: Minipig; Olfactory bulb; Neural stem cells (NSCs); *v-myc*; Immortalization

INTRODUCTION

Neural stem cells (NSCs) are multipotent cells that originate from the nervous system and are capable of self-renewal[1]. In particular, NSCs are promising for stem cell therapies for a variety of human neurological disorders, such as spinal cord injury, stroke, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), as well as for basic research in areas including neural development[2]. Although the importance of NSCs in biomedical research is increasing, their limited proliferation capacity in vitro is a major obstacle to expanding their usability. Therefore, several researchers have attempted to generate immortalized NSCs (iNSCs) with high proliferation potentials and without the loss of neural differentiation[3–5]. However, research is necessary on iNSCs derived from a variety of species to improve their feasibility as resources for basic research and stem cell therapy.

Pigs have been used widely in preclinical studies because of their high anatomical, physiological, genetic, and biochemical similarities to humans[6]. The most common experimental data using mammalian models are produced from rodents and dogs[7], and preclinical data from rodents are often necessary to support the use of larger animals in clinical applications[8]. However, minipig models are becoming increasingly promising based on their close phylogenic distance to humans and their large litter size, as well as the relatively few ethical issues related to this species[6]; thus, these models could increase the ability to produce more reliable data for the discovery of new drugs[9]. Many research groups have tried to isolate resources from a variety of minipig tissues[8,10] as well as to generate disease models and bio-organ donors via transgenesis methods[11]. Despite the increasing importance of minipigs as a larger higher animal model for human diseases, there has been little or no research concerning the derivation and use of minipig iNSCs (mpiNSCs).

Received February 04, 2016; final acceptance October 27, 2016. Online prepub date: August 12, 2016.
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In this study, we generated mpiNSCs via stable transfection of primary minipig olfactory bulb cells (pmpOBCs) with a retroviral vector encoding the *v-myc* gene. The mpiNSCs successfully expressed NSC-specific markers, including nestin, vimentin, and (sex-determining region Y)-box 2 (SOX2). Moreover, the microarray analysis demonstrated that mpiNSCs highly expressed neurogenesis markers, particularly genes related to the Wnt signaling pathway. The results support that mpiNSCs can be used as efficient models in studies on neural differentiation and stem cell therapy.

MATERIALS AND METHODS

Culture of pmpOBCs

Minipig cells (Micro-pig®; Medi Kinetics, Gyeonggi-do, Republic of Korea) were isolated from 7-day-old neonatal olfactory bulb tissue, dissociated using papain (Sigma-Aldrich, St. Louis, MO, USA), and plated as primary cultures. Use of minipig cells was approved by the Chung-Ang University Ethics Committee (Certificate #09-0041). The pmpOBCs were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Welgene, Kyungsangbuk-do, Republic of Korea) and F12 medium (1:1; Welgene), supplemented with 10% fetal bovine serum (FBS; GenDEPOT, Katy, TX, USA), 20 mg/ml gentamicin, 10 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich), and epidermal growth factor (EGF; Gibco, Carlsbad, CA, USA). The cells were incubated at 37°C with 5% CO_2 in a humidified incubator.

Immortalization of Minipig Cells Using v-myc *Gene*

pLNCX2.*v-myc* plasmid containing full-length avian *v-myc* cDNA derived from *Gallus gallus* was used in this study. The *v-myc* gene was inserted into a pLNCX2 retroviral vector using *Bgl*II and *Hin*dIII restriction. Retroviral vectors were transfected into a packaging cell line to produce *v-myc* retrovirus using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). These viruses were transduced into minipig cells and selected for by supplementing the medium with 1,000 μg/ml neomycin for 4 weeks. Neomycin-resistant minipig colonies were isolated and used for further study.

Karyotypic Analysis

The cell lines were plated at a density of 0.5×10^6 cells in 60-mm culture dishes (BD Falcon, Tewksbury, MA, USA) coated with 1% gelatin. After 24 h, the growth medium was replaced with medium containing 0.1 μg/ml colcemid (KaryoMAX™ Colcemid; Gibco) and incubated for 4 h. After dissociation, cells were incubated with 0.075 M KCl for 15 min in a 37°C water bath. Then three to five drops of freshly prepared fixative (3:1 methanol/glacial acetic acid) were added to stop the reaction, and cells were fixed in fixative at 4°C. One drop of cell suspension was placed on glass slides, and the slides were dried at 60°C for a minimum of 18 h and incubated in a trypsin solution for 30 s. Slides were stained with KaryoMAX™ Giemsa stain (Gibco) for 5 min. After drying, the slides were mounted and observed under a microscope.

RT-PCR Analysis

Total RNA was isolated from pmpOBCs and minipig NSCs using an miRNA Isolation Kit (Qiagen, Hilden, Germany). First, 1 μg of total RNA was reverse transcribed into first-strand cDNA using oligo-dT primer. Reverse transcription was performed with TOPscript™ RT DryMIX (Enzynomics, Daejeon, Republic of Korea) for 1 h at 42°C, inactivated for 5 min at 95°C, and cooled to 4°C. The cDNA was amplified using 30 polymerase chain reaction (PCR) cycles, and real-time (RT)-PCR products were separated in 1.5% agarose gels and visualized under UV light. The primers used for RT-PCR are listed in Table 1.

Immunocytochemistry

mpiNSCs were plated on poly-L-lysine-coated 9-mm-wide ACLAR plastic coverslips (SPL Life Sciences, Seoul, Republic of Korea) and incubated in growth medium for 2 days. Then the mpiNSCs were rinsed in phosphate-buffered saline (PBS) and fixed in cold acidic alcohol (5% glacial acetic acid in 95% ethanol) for 10 min at −20°C. After washing, the fixed cells were incubated in blocking solution (10% normal goat serum) at room temperature (RT) for 1 h; this was followed by primary antibodies specific for nestin (1:200; sc-21248; Santa Cruz Biotechnology, Dallas, TX, USA), βIII-tubulin (1:200; MAB1637; Merck Millipore, Darmstadt, Germany), neurofilament [1:200; NF-L (AB9568), -M (AB1987), and –H (AB1989); Merck Millipore], A2B4 (1:200; MAB312R; Merck Millipore), glial fibrillary acidic protein (GFAP; 1:1,000; Z0334; Dako, Glostrup, Denmark), 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase; 1:200; MAB326; Merck Millipore), Nav 1.1 (1:200; ad106514; Abcam, Cambridge, UK), and Nav 1.6 (1:200; ab83764; Abcam) overnight at 4°C and then labeling with Alexa Fluor 488- or 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h at RT. Cells were mounted using antifading solution containing 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) and viewed under a microscope (IX71; Olympus, Tokyo, Japan).

Microarray Hybridization and Data Analysis

To analyze the global gene expression profiles of pmpOBCs and mpiNSCs, Agilent Porcine Gene Expression

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Table 1. The List of Primers for RT-PCR

Genes	Primer Sequences	GenbankAccession No.	Product Size (bp)
Nestin	F: 5′-GGC AGT GGT TCC AAG GCT-3′ R: 5′-GGC TGG CAT AGG TGT GTC AA-3′	XM_005663265.2	162
Sox2	F: 5′GAA CAG CCC AGA CCG AGT TA-3′ R: 5′-TAA TCC GGG TGC TCC TTC AT-3′	NM_001123197.1	219
Vimentin	F: 5′-GTG ATG TCC GCC AGC AGT-3′ R: 5′-GCG TTC CAG AGA CTC GTT-3′	XM_005668107.2	218
NF	F: 5′-CCT TGC TGT GGC TGT TGA CT-3′ R: 5′-GAA TTG CCT CCA AGA AAG CC-3′	NM_001244331.1	309
βII-tubulin	F: 5′-TCC TAT CCC ACC TGC TCA CA-3′ R: 5′-CCT TTG CAT AGA GCA GAG CG-3′	NM_001113696.1	405
GFAP	F: 5′-AGA AGG CCA CCT CAA AAG GA-3′ R: 5′-CCC AAC TTC TTT GCT CCC TC-3′	NM_001244397.1	397
MBP	F: 5′-ATC CTT CCC GGC TTC TAA CA-3′ R: 5′-GGA GGC ACG AAA GAA AC-3′	NM_001001546.2	286
GDNF	F: 5′-CTG AAG AGG TCC CCC GAT AA-3′ R: 5′-TAC ACA GCG GTC TCT GGA GC-3′	XM_005672424.2	439
BDNF	F: 5′-TGT TGG ACG AGG ACC AGA AA-3′ R: 5′-TTT CGA GAC GGG GAC TTT TT-3′	XM_013987278.1	314
c-kit	F: 5′-ACC AAT TAC TCC CTC ACG GG-3′ R: 5′-TGA TCG TCA GCT TTT CCT GC-3′	NM_001044525.1	403
bFGF	F: 5′-GCA GTC CTC CTG TTC AAG CA-3′ R: 5′-AAA CTC TCC ACT GCA TCC CC-3′	XM_003129213.3	274
IGF1	F: 5′-GCA CAT CAC ATC CTC TTC GC-3′ R: 5′-TCC AGC CTC AGA TCA CA-3′	NM_214256.1	246
NGF	F: 5′-ATT CCC TTG ACA CAG CCC TC-3′ R: 5′-CAA CAC CAT CAC CTC CTT GC-3′	XM_005674272.2	356
NT3	F: 5′-GGA AGA CTC GCT CAA TTC CC-3′ R: 5′-AGA CTC TCG CTG TCG CAC AC-3′	NM_001123152.1	390
VEGF	F: 5′-GAA GGA GAC CAG AAA CCC CA-3′ R: 5′-GAT CCG CAT AAT CTG CAT GG-3′	NM_214084.1	234
Nav1.1	F: 5′-AGT TAA GGG AGA CTG CGG GA-3′ R: 5′-GAT GTC AAG GCA GTG GAT GC-3′	XM_013984216.1	349
Nav1.2	F: 5′-AGT TTG GAG GTC AAG ACA TT-3′ R: 5′-CAC ATT CTC CAG TGA ACA GA-3′	XM_013984209.1	291
Nav1.4	F: 5′-GAA GTT TGG AGG GA-3′ R:5′-TTG ATG GTG TAG AGG ATG TC-3′	XM_003131284.3	264
Nav1.5	F: 5′-ACA TCT TCA TGA CAG AGG AG-3′ R: 5′-CAA GAT GTT GAC CTT CTC AG-3′	XM_013981470.1	230
Nav1.6	F: 5′-GCA TCC ACT GCT TGG ACA TC-3′ R: 5′-CTC TCC TTT TTC TCC CGG TG-3′	XM_003481581.3	289
v-myc	F: 5′-AAG CGG TGT CAC GTC AAC AT-3′ R: 5′-GCA AAG AAA CTC AGC TTC AG-3′	NM_001030952.1	236
GAPDH	F: 5′-TCG GAG TGA ACG GAT TTG-3′ R: 5′-CCT GGA AGA TGG TGA TGG-3′	NM_001206359.1	219

(V2) 44K GeneChips (Agilent Technologies, Santa Clara, CA, USA) were used for the microarray analyses and processed by eBiogen Inc. (Seoul, Republic of Korea). Double-stranded cDNA was synthesized, amplified, and labeled with the Agilent Low RNA Input Linear Amplification Kit PLUS (Agilent Technologies) from total RNA, and the probes were hybridized using the Agilent Gene Expression Hybridization Kit (Agilent Technologies), followed by washing with the Agilent Gene Expression Wash Buffer Kit (Agilent Technologies). Images were processed using an Agilent DNA microarray scanner and Feature Extraction software (Agilent Technologies). Raw data were normalized using the GeneSpring GX software (ver. 7.0; Agilent Technologies) for analysis.

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Statistical Analysis

Differences between pmpOBCs and mpiNSCs were calculated using *t*-tests as appropriate. All tests were two sided, and statistical significance was set at a threshold of $p<0.05$. Statistical analyses were performed using the R program (R foundation, Vienna, Austria). Data are presented as means ± standard error of the mean (SEM).

RESULTS

mpiNSCs Generation

To generate the mpiNSCs, primary neural cells were prepared from minipig olfactory bulb tissue and transfected with a retroviral vector carrying the *v-myc* oncogene (Fig. 1). Among several stable transfectants isolated after completion of antibiotic selection, a representative clone was used for the subsequent analyses. The two cell types differed slightly in cell morphology. Whereas pmpOBCs were triangular, mpiNSCs exhibited a bipolar morphology with relatively thin, long body shapes (Fig. 2A and B). The RT-PCR analysis showed that *v-myc* was highly expressed in mpiNSCs (Fig. 2C). In addition, the mpiNSC clone had a normal karyotype (38, XY) (Fig. 2D).

mpiNSCs Gene Expression

From the RT-PCR analysis of several cell-specific markers, we found that mpiNSCs expressed markers for NSCs (i.e., *Nestin*, *SOX2*, and *Vimentin*), neurons (i.e., *NF* and *βIII-tubulin*), and glia [i.e., *GFAP*, myelin basic protein (*MBP*), and *CNPase*], similar to the pmpOBCs. However, the marker expression levels differed slightly between the pmpOBCs and mpiNSCs. *Nestin*, *SOX2*, *NF*, *MBP*, receptor of stem cell factor (*c-Kit*), and stromal cell-derived factor 1 (SDF-1) receptor (*CXCR4*) were relatively higher, and *βIII-tubulin* and *GFAP* were lower in the mpiNSCs. There were no changes in growth factor transcript levels between the two cell types (Fig. 3). In immunocytochemistry analysis using pmpOBCs, specific markers for NSC (nestin and A2B5), neuron (βIII-tubulin and NF), and oligodendrocyte (CNPase) were detected in a large part of cell population, whereas the astrocyte marker (GFAP) was rarely stained (Fig. 4A–F). In addition, we further found that immunoreactivities against NSC and neuronal markers were increased in mpiNSCs compared to those in pmpOBCs (Fig. 4G–L). In contrast, immunoreactivities against astrocyte (GFAP)

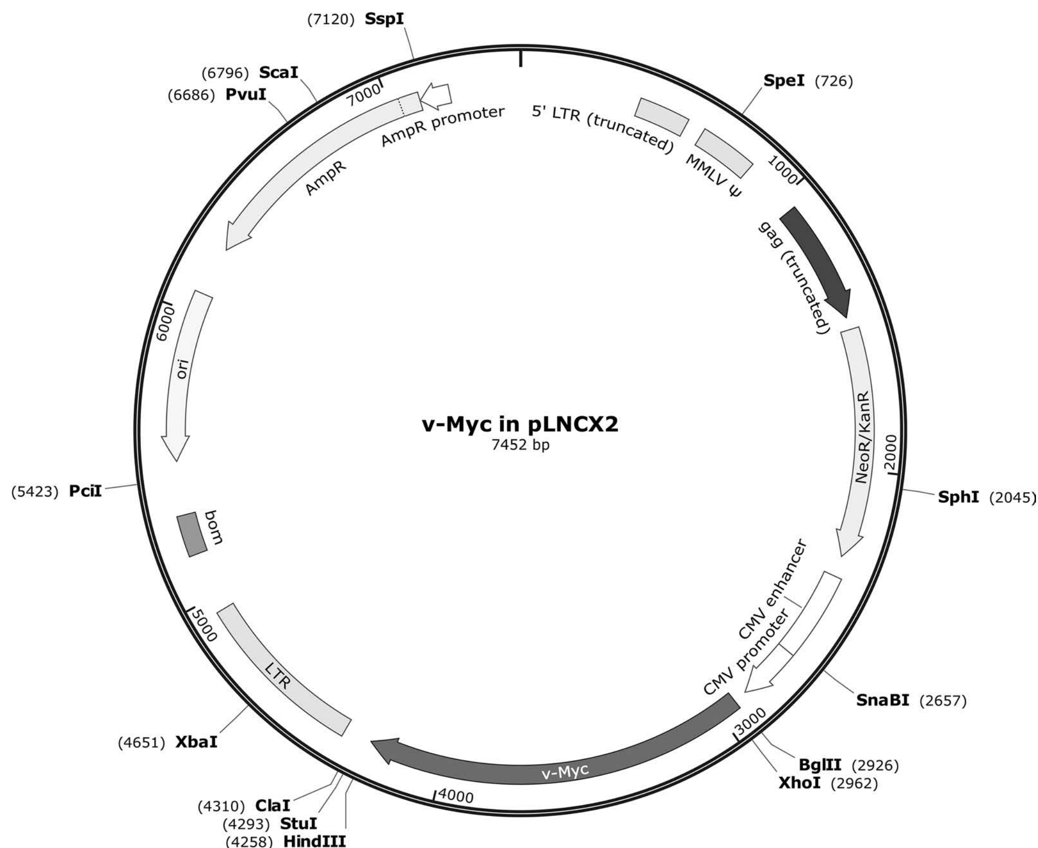

Figure 1. Diagram of *v-myc* retroviral construction.

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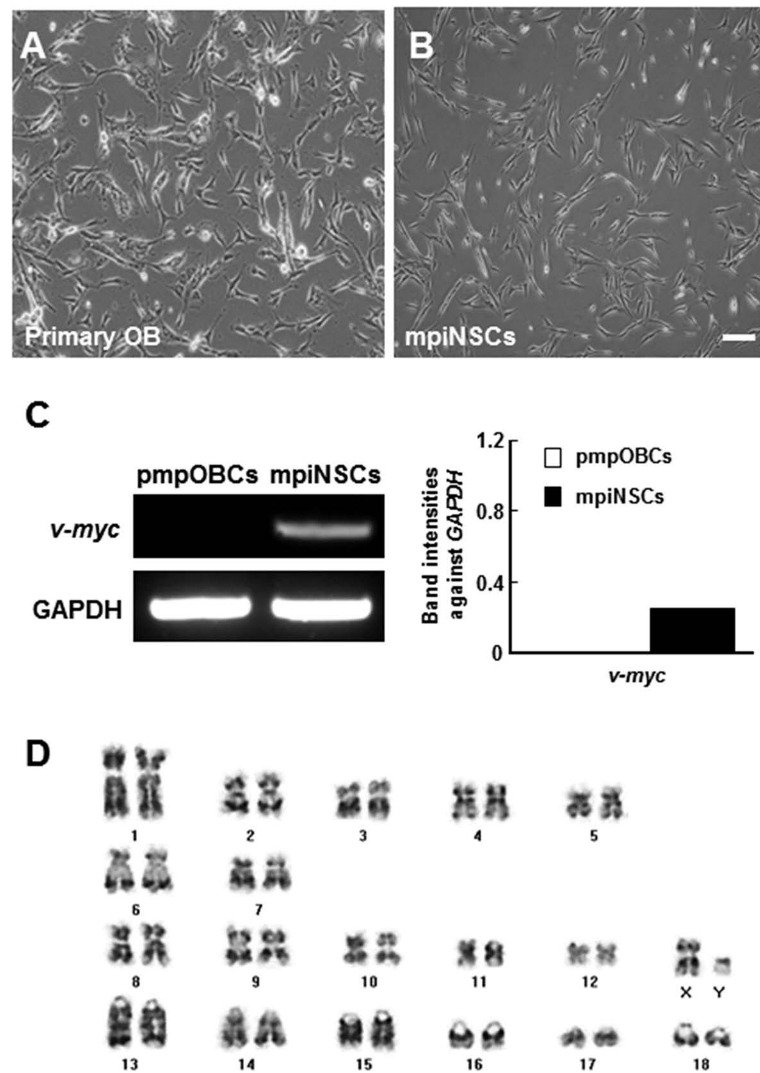

Figure 2. Generation of immortalized minipig neural stem cell (NSC) line (mpiNSCs). (A, B) mpiNSCs had bipolar morphology. Scale bar: 100 µm. (C) RT-PCR analyses indicate that the *v-myc* gene was overexpressed in mpiNSCs after infection with *v-myc* retrovirus, but not in primary NSCs. (D) mpiNSCs expressed normal karyotype (38,XY).

and oligodendrocyte (CNPase) markers were decreased in mpiNSCs.

Further analysis using RT-PCR and immunocytochemistry revealed that neuron-specific sodium ion channel isotypes were differently expressed in pmpOBCs and mpiNSCs. *Nav 1.5* was preferentially expressed in pmpOBCs, whereas *Nav 1.2*, *1.4*, and *1.6* were detected only in mpiNSCs. However, high expression levels of *Nav 1.1* were detected in both cell types (Fig. 5).

Global Gene Profiling in mpiNSCs

To examine changes in global gene expression during immortalization, total RNA was isolated from pmpOBCs and mpiNSCs in triplicate and subjected to a microarray analysis using the Agilent Porcine Gene Expression cDNA Chip containing 43,604 genes. On the basis of hierarchical clustering data from Agilent and eBiogen Inc., overall transcript levels of mpiNSCs were downregulated compared with those of pmpOBCs (Fig. 6). Among 7,781 genes that more than doubled their gene expression levels, 515 were upregulated, whereas 7,266 were downregulated.

On the basis of the gene ontology analysis (eBiogen Inc., http://www.e-biogen.com), the altered global gene expression profiles were classified into several functional clusters, including neurogenesis (26.1%, 158 of 606 genes), regulation of neurogenesis (27.3%, 72 of 264 genes), stem cell differentiation (28.4%, 61 of 215 genes), stem cell development (31.4%, 54 of 172 genes), Wnt receptor signaling pathway (27.1%, 48 of 177 genes), stem cell maintenance (38.1%, 40 of 105 genes), regulation of Wnt

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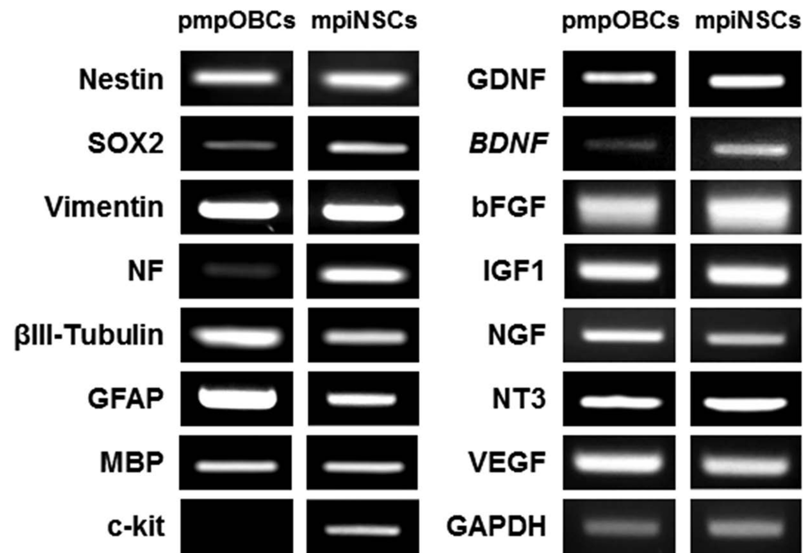

Figure 3. RT-PCR of primary OB cells and mpiNSCs. Minipig primary OB and mpiNSCs expressed neural cell markers including nestin, βIII-tubulin, neurofilament (NF), glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP).

receptor signaling (28.6%, 32 of 112 genes), stem cell proliferation (25.9%, 29 of 112 genes), negative regulation of Wnt receptor signaling pathway (27.3%, 24 of 88 genes), and negative regulation of Wnt receptor signaling pathway (47.1%, 16 of 34 genes) (Table 2). The most up- and downregulated genes are listed in Tables 3 and 4.

From further analysis of the global gene profile, we found that NSC stemness-associated transcript levels were relatively high in mpiNSCs compared to pmpOBCs. Indeed, NSC markers, nestin (5-fold) and Sox2 (1.6-fold) transcripts were upregulated in mpiNSCs, which is greatly similar to the results from the RT-PCR analyses (Fig. 3). In contrast, no changes between both cell types were found in the transcript levels of markers for astrocyte (GFAP; 1.007-fold) and oligodendrocyte (MBP; 1.072-fold). In addition, neurofilament heavy chain was upregulated (1.6-fold) in mpiNSCs, whereas neurofilament light chain was decreased (3.725-fold) in mpiNSCs.

DISCUSSION

In this study, we established a stable immortalized minipig NSC line for the first time via transfection using a *v-myc*-expressing retroviral vector. The mpiNSCs expressed both NSC and neural markers. Thus, these

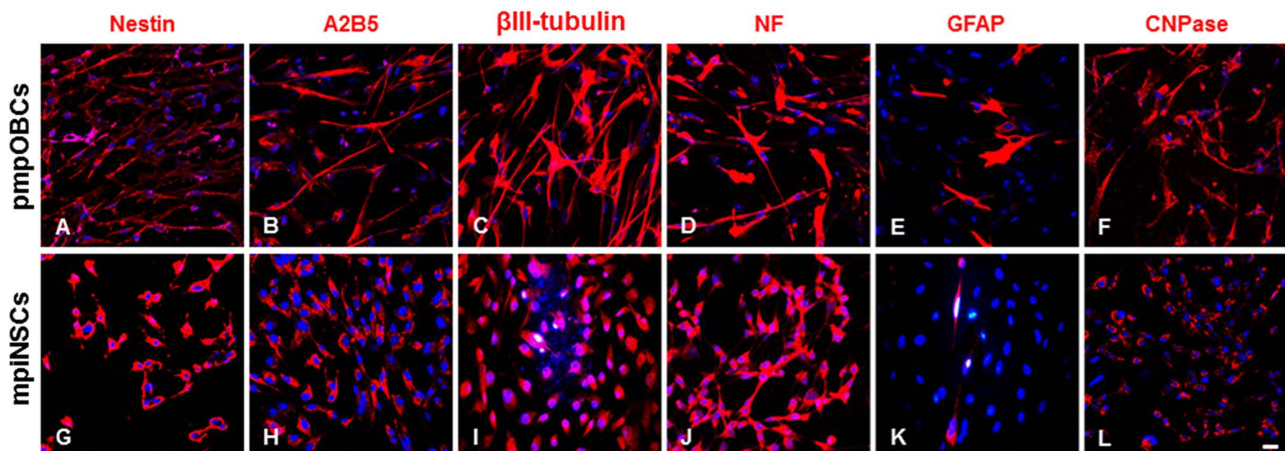

Figure 4. Immunocytochemical characterization of pmpOBCs and mpiNSCs. Immunocytochemistry analysis of markers for NSCs (nestin and A2B5), neurons (β-III tubulin and NF), and glia (GFAP and CNPase) was carried out using pmpOBCs and mpiNSCs. Abbreviations: NF, neurofilament; GFAP, glial fibrillary acidic protein; CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase. Scale bar: 20 μm.

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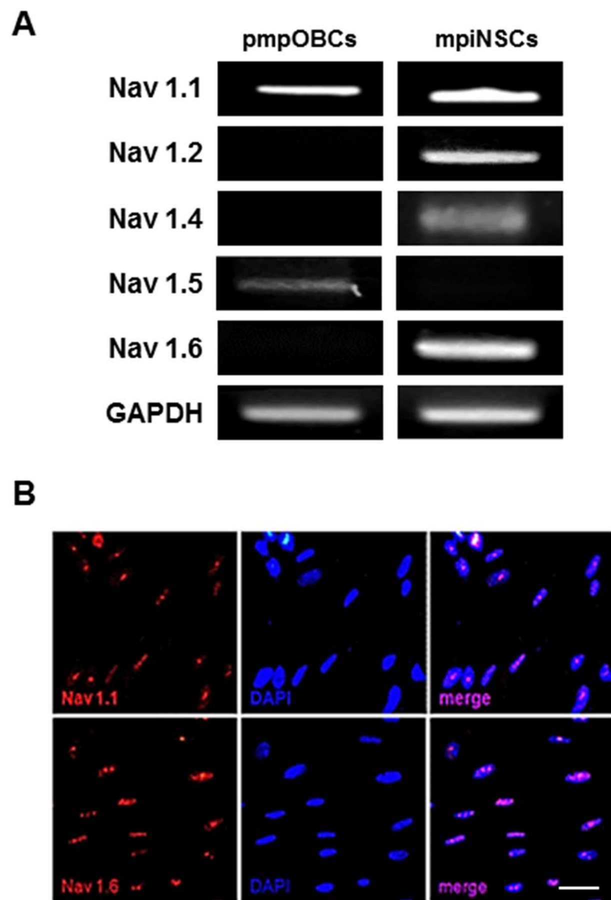

Figure 5. Expression of sodium ion channel in mpiNSCs. To identify the activity of ion channels, Na^{+} channel activity was measured by RT-PCR and immunocytochemistry. Nav 1.1 and 1.6 were expressed and localized in the nucleus in mpiNSCs. Scale bars: 50 µm.

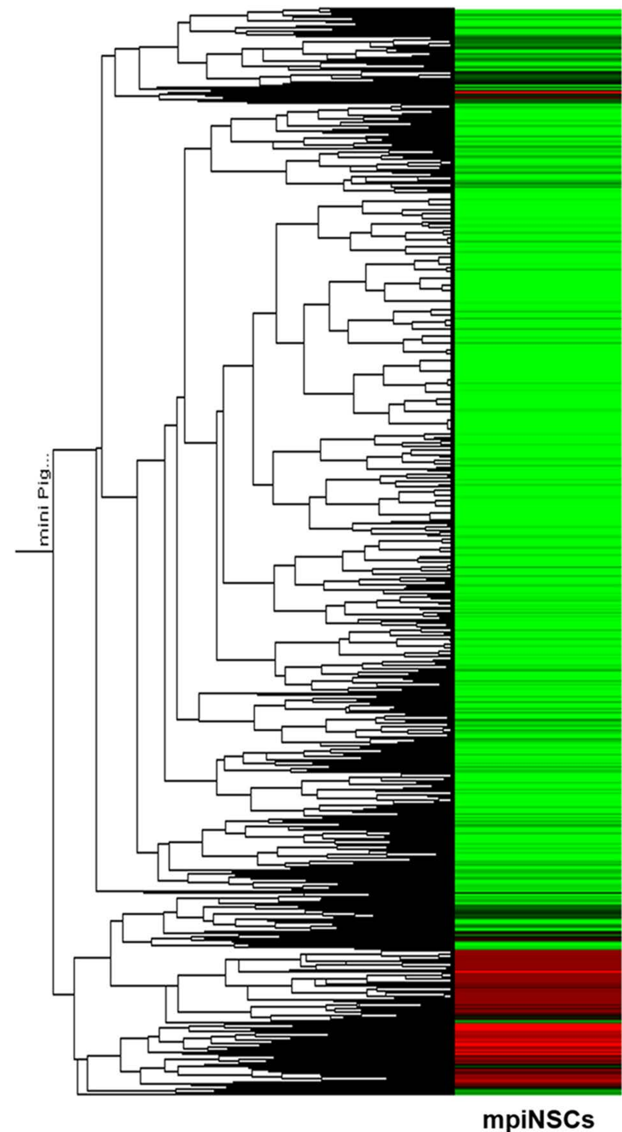

Figure 6. Gene chip-based clustering data for candidate genes activated in the initial phase of mpiNSCs compared to primary OB cells. To determine the expression level of microarray data, selected twofold changed genes were processed using cluster (version 2.11) and TreeView (ver. 1.6) programs. The figure shows a heatmap of the top up- and downregulated genes of over twofold responded genes of mpiNSCs compared to primary OB cells. The cluster shows the utility of this approach in studying persistent alterations in gene expression. Red and green intensities indicate higher and lower expressions, respectively, relative to median, and black intensity indicates expression near median.

results suggested that mpiNSCs may be pluripotent and able to self-renew. In addition, we characterized the expression profiles of neurotrophic factors and sodium ion channels. The results indicate that the cell lines can be effectively used for a variety of biomedical studies associated with stem cell therapy.

Minipigs have become increasingly important and useful as large animal models in preclinical studies because of their many similarities to humans in terms of anatomy and physiology[6,12]. Therefore, there are many studies that have used minipigs in various biomedical applications, including stem cell therapies. For example, Raore and colleagues used a minipig model to assess the long-term risks of multiple intraspinal cell injections[13]. Furthermore, Usvald et al. used an immunosuppressed minipig model to analyze the reproducible therapeutic effects of human spinal stem cells via intraspinal grafting into immunosuppressed minipig models[14]. Although NSCs might be a valuable source of cells for stem cell-based therapies for neurological diseases[15], there have been relatively few studies on the derivation of immortalized minipig stem cells for preclinical studies[12].

NSCs can be used to repair damaged brain or spinal cord by replacing degenerated neurons or producing neuroprotective molecules[16,17]. Several researchers have reported

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Table 2. Functional Classification of Gene Differential Expression of the Minipig iNSCs

Category	Gene Number	Significant Change	% of Total Significance
Neurogenesis	244	110	2.1951706
Stem cell differentiation	97	67	1.3370585
Stem cell development	87	61	1.2173219
Regulation of neurogenesis	106	55	1.0975853
Stem cell maintenance	56	43	0.8581122
Wnt receptor signaling pathway	67	40	0.7982439
Stem cell proliferation	50	33	0.6585512
Regulation of Wnt receptor signaling pathway	32	18	0.3592097
Negative regulation of Wnt receptor signaling pathway	27	14	0.2793854
Positive regulation of neurogenesis	27	14	0.2793854
Negative regulation of neurogenesis	30	13	0.2594293
Positive regulation of Wnt receptor signaling pathway	13	6	0.1197366
Total	43,604	5,011	100

that transplanted NSCs are recruited by signal molecules, such as cytokines, from injured regions[18–25]. In addition, various cytokines and their receptors were expressed in human F3 NSCs, which were involved in NSC migration toward injury sites. Cytokines are important in increasing the injury-induced proliferation and migration of neural progenitor cells[26–28]. Moreover, secreted cytokines have a neuroprotective effect in damaged brains[15]. Furthermore, they can provide stable and homogeneous populations with which to investigate neuronal development and

Table 3. Top 20 Upregulated Genes of the Minipig iNSCs

Gene	Fold	Genbank Accession	Description
CRSP-2	23.631256	NM_213747	Calcitonin receptor-stimulating peptide-2
CRSP3	21.406818	NM_213746	Mediator complex subunit 23
TM6SF1	15.271544	XM_001929144	Transmembrane 6 superfamily member 1
UGPP	13.469201	AJ539380	UDP-sugar diphosphatase
TM6SF1	11.692888	XM_001929144	Transmembrane 6 superfamily member 1
LOC100152544	9.963879	XM_001929522	Helix–loop–helix protein 2-like
ARG1	9.067524	NM_214048	Arginase, liver
FADS6	6.925721	NM_001244792	Fatty acid desaturase domain family, member 6
LOC100154508	5.9371886	XM_001928639	Histone H2A type 1-F-like
5-HT3R	5.5377345	AY513690	5-Hydroxytryptamine receptor 3
ND6	5.4611387	AK399664	NADH dehydrogenase subunit 6
ZNF467	5.3164725	AK347604	Zinc finger protein 467
DDIT4	5.035703	NM_001243452	DNA-damage-inducible transcript 4
SPARCL1	5.0085344	NM_001097422	SPARC-like 1 (hevin)
NES	5.001399	XM_001925549	Nestin
VNN1	4.961567	AK235021	Vanin 1
CYP2A19	4.8363047	NM_214417	Cytochrome P450 2A19
ARHGAP27	4.7432137	AK231220	Rho GTPase-activating protein 27
LOC100524425	4.4203496	XM_003123664	THO complex subunit 3-like
PRDM1	4.4150257	XM_005659341.2	PR domain-containing 1, with ZNF domain
CELA2A	4.331014	NM_214109	Chymotrypsin-like elastase family, member 2A
LOC100521597	4.121977	XM_003121761	Membrane progestin receptor γ-like
LOC595122	4.024677	NM_001031792	Histone H1.3-like protein
LOC396781	3.902964	NM_213828	IgG heavy chain
SREBF1	3.8902533	NM_214157	Sterol regulatory element-binding transcription factor 1
LOC100626321	3.8321953	BI185513	Alkylated DNA repair protein alkB homolog 8-like
RPS23	3.8133929	NM_213764	Ribosomal protein S23
Sox2	1.6031394	NM_001123197	SRY (sex-determining region Y)-box 2

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Table 4. Top 20 Downregulated Genes of the Minipig iNSCs

Gene	Fold	Genbank Accession	Description
DCN	–100	NM_213920	Decorin
STC1	–98.98651	AK234059	Stanniocalcin 1
DCN	–74.60359	NM_213920	Decorin
MMP1	–73.5821	NM_001166229	Matrix metallopeptidase 1 (interstitial collagenase)
TIMP-3	–71.35979	AF156031	Tissue inhibitor of metalloproteinase-3
LOC733635	–70.14049	NM_001044570	Aldo-keto reductase family 1 member C2-like
DCN	–55.91256	AK397441	Decorin
RNASE4	–49.28044	AK389332	Ribonuclease, RNase A family, 4
DCN	–49.26517	NM_213920	Decorin
ANGPT1	–47.00666	NM_213959	Angiopoietin 1
LOC100624590	–46.31113	AK344932	ATP-dependent RNA helicase DDX3X-like
ZNF618	–43.85709	AK350510	Zinc finger protein 618
CBLN4	–40.58437	NM_001123092	Cerebellin 4 precursor
LRRK2	–39.33661	NM_001113437.1	Leucine-rich repeat kinase 2
RNASE4	–37.21619	AK389332	Ribonuclease, RNase A family, 4
LOC100156514	–36.43986	AK343670	Transmembrane protein 68-like
SDC2	–32.91191	AY609407	Syndecan 2
CD96	–30.4517	NM_001243480	CD96 molecule
SF3B1	–29.95057	XM_001928794	Splicing factor 3b, subunit 1, 155kDa
ATP8B1	–29.81465	AK232415	ATPase, aminophospholipid transporter, class I, type 8B, member 1
LOC100512762	–29.78434	XR_130371	Uncharacterized LOC100512762
TTC14	–29.61947	XM_001927185	Tetratricopeptide repeat domain 14
FLRT3	–28.67724	XM_001926449	Fibronectin leucine-rich transmembrane protein 3
TMEM59	–28.30705	AK234544	Transmembrane protein 59
SDC2	–27.80549	AY609407	Syndecan 2
PDE4B	–27.42477	NM_001130019	Phosphodiesterase 4B, cAMP-specific

neurological diseases[29]. To expand the usability of NSCs, we generated iNSCs based on ease of maintenance and proliferation in vitro.

We found that mpiNSCs expressed several neurotrophic factors, such as brain-derived neurotrophic factor (*BDNF*), glial cell line-derived neurotrophic factor (*GDNF*), *bFGF*, vascular endothelial growth factor (*VEGF*), nerve growth factor (*NGF*), neurotrophin-3 (*NT3*), and insulin-like growth factor 1 (*IGF1*). Further studies are needed to determine whether grafted mpiNSCs can be integrated into host brain networks to support the generation of new synaptic connections and replace damaged neurons. Currently, we are working to further define the characteristics of the mpiNSCs using an in vivo transplantation study.

On the basis of the microarray results, 5,011 of 43,604 genes had significantly different expression levels after immortalization. These genes were mainly classified into three categories: neurogenesis, neuronal physiology, and stem cell-related ontology, which comprised ~9.5% of the total significantly modulated genes. In addition, the top 20 up- and downregulated genes were closely associated with transmembrane receptors, transcription factors, extracellular matrices, physiological enzymes, and histones. These results suggest that the physiological activity and properties of mpiNSCs might differ. Immunocytochemistry analysis showed that most of pmpOBCs were intensively stained with NSC, neuron, and oligodendrocyte markers, whereas astrocyte marker was rarely stained, indicating NSC and neuronal characteristics of pmpOBCs. In addition, we further found that immunoreactivities against NSC and neuronal markers were increased in mpiNSCs compared to those in pmpOBCs. On the contrary, immunoreactivities against glia markers were decreased (Fig. 4). Together with similar results from the RT-PCR analysis (Fig. 3), immunocytochemistry data demonstrated that the *v-myc*-mediated immortalization process may contribute to enhancement of NSC stemness and neuronal differentiation potential.

mpiNSCs were transduced with *v-myc* for immortalization. However, recent reports have already revealed that most of the *v-myc*-transfected cell lines including F3, F3.ChAT, and F3.BDNF cells were successfully differentiated into neurons after transplantation in rodent[30–32] as well as nonhuman primate brains[22]. Safety of oncogene has also been debated for a long time. In previous reports, *v-myc* gene has shown that no tumorigenic phenotypes were proved by transplanting *v-myc* dependently immortalized human stem cell lines into rodent animal

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models[30–32]. Especially, human NSCs can survive for a long time (24 months) in the brain of cynomolgus monkey without tumorigenesis[22]. These results indicated that *v-myc* can be used as a useful and safe gene to immortalize primary cells.

In this study, we successful generated mpiNSCs with characteristics similar to those of human NSCs, which expressed NSC- and neuron-specific markers, as well as several neuroprotective factors. These results could increase the usability of minipig adult stem cells. Furthermore, the mpiNSCs could be useful in examining the applicability of the immortalization technique to stem cell therapy.

ACKNOWLEDGMENTS: This study was supported by grants from the KRIBB Research Initiative Program (KGM4251723 and KGM4611714), Republic of Korea, and the Next-Generation BioGreen 21 Program (No. PJ0099592016), Rural Development Administration, Republic of Korea. The authors declare no conflicts of interest.

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