

Neuronal Differentiation of a Human Induced Pluripotent Stem Cell Line (FS-1) Derived from Newborn Foreskin Fibroblasts

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Isolation of induced pluripotent stem cells (iPSCs) from fully differentiated somatic cells has revolutionized existing concepts of cell differentiation and stem cells. Importantly, iPSCs generated from somatic cells of patients can be used to model different types of human diseases. They may also serve as autologous cell sources that can be used in transplantation therapy. In this study, we investigated the neuronal properties of an iPSC line that is derived from human neonatal foreskin fibroblasts (FS-1). We initially examined the morphology and marker expression of FS-1 cells at undifferentiated stage. We then spontaneously differentiated FS-1 cells in suspension culture and examined the expression of markers representing three germ layers. We finally differentiated FS-1 cells into neuronal lineages by co-culturing them with PA6 stromal cells, and found that, under the conditions we used, they have a tendency to differentiate into more forebrain-type neurons, suggesting that FS-1 iPSC-derived neural cells will be useful to be used in cell therapy of stroke or Huntington's disease, among others. Taken together, FS-1 cells derived from human neonatal fibroblasts exhibit very similar properties with human ES cells, and can provide useful sources for cell therapy and various other applications.

Keywords: Induced pluripotent stem cells (iPSCs), Foreskin, Spontaneous differentiation, Neuronal differentiation, Cell therapy, Human ES cells

Introduction

Reprogramming of fully differentiated human somatic cells into the pluripotent cell state using defined four transcription factors (Oct4, Sox2, c-Myc and Klf4) has completely changed our existing concept of hierarchical organization of cell lineage and/or fate determination (1). Moreover, this would allow the generation of patient-specific pluripotent cell lines that can provide immune rejection-free autologous sources for novel cell therapies, as

well as patient-derived, customized materials for disease modeling and drug screening (2). Over the past six years since the original discovery of iPSCs by Yamanaka and colleagues (1), there has been a great expansion of technologies to establish iPSC lines using more safe and efficient methods (3).

In 2007, Thomson group in Wisconsin established multiple iPSC lines using lentiviral systems overexpressing Oct4, Sox2, Nanog and Lin28 (4), without using c-Myc, which is known to cause death and differentiation of human ES cells (5). Interestingly, in this study, they used newborn foreskin fibroblasts, in addition to IMR90 fetal fibroblasts, for the generation of iPSCs. While fetal fibroblasts are derived from aborted human fetuses, newborn foreskin is easy to obtain; otherwise, it will be discarded after circumcision at birth. Since foreskin can provide non-invasive cell sources for iPSC generation and its cell age is very young, it will be likely that the resulting iPSCs

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would have more proliferative activity and differentiation potentials, compared with other iPSCs generated from aged skin fibroblasts.

In their original paper, they established four different types of iPSCs from foreskin fibroblasts (FS-1, FS-2, FS-3 and FS-4) and reported the initial characterization results (4). However, they didn't report the detailed analysis on the marker expression and differentiation potentials of FS-iPSC lines. On the other hand, since FS-iPSC lines were generated by lentiviral integration, it is less likely that they can be used for therapeutic purposes. Neverthe-

less, considering the practical usefulness of sample collection and the expected developmental potentials, we decided to focus on FS-1 line for our initial analysis. In this study, we initially examined the morphology and marker expression of FS-1 cells at undifferentiated stage, and then examined their differentiation potentials in suspension culture. Finally, we differentiated FS-1 cells into neuronal lineages and their properties. Our results indicate that FS-1 cells exhibit very similar properties with human ES cells and can serve as useful sources for cell therapy and various other applications.

Table 1. RT-PCR primers used in this study

Gene name	Forward (F) and reverse (R) primer sequences	Product size (bp)	Annealing temp. (°C)	No. cycles	GenBank accession no.
Oct4	F: 5'-CTGAAGCAGAAGAGGATCAC-3' R: 5'-GACCACATCCTTCTCGAGCC-3'	366	60	30	NM_002701.4
Nanog	F: 5'-TTCTTGACCGGGACCTTGTC-3' R: 5'-GCTTGCCCTTGCTTTGAAGCA-3'	256	60	30	NM_024865
Nestin	F: 5'-TCCAGAACTCAAGCACCA-3' R: 5'-AAATTCTCCAGGTTCCATGC-3'	183	59	30	NM_006617
Beta III tubulin	F: 5'-ATGAGGGAGATCGTGACAT-3' R: 5'-GCCCCTGAGCGGACACTGT-3'	239	59	30	BC000748.2
Bf-1	F: 5'-CTCAGAACTCGCTGGGCAAC-3' R: 5'-CGTGGGGGAAAAAGTAACTGG-3'	225	59	30	NM_005249
En-1	F: 5'-CTAGCCAAACCGCTTACGAC-3' R: 5'-GCAGAACAGACAGACCGACA-3'	358	59	30	NM_001426.3
Darpp-32	F: 5'-CCTGAAGGTCATCAGGCAGT-3' R: 5'-GGTCTTCCACTTGGTCCTCA-3'	131	50	30	AF464196.1
Calbindin	F: 5'-GCAACAAGACTGTTGATGACAC-3' R: 5'-AAGAGCAAGATCCGTTCCGGTAC-3'	363	58	30	NM_004929.2
GFAP	F: 5'-GCAGAGATGATGGAGCTCAATGACC-3' R: 5'-GTTTCATCCTGGAGCTTCTGCCTCA-3'	266	59	30	BC041765.1
GAPDH	F: 5'-GTCATACCAGGAAATGAGCT-3' R: 5'-TGACCACAGTCCATGCCATC-3'	422	60	30	BC083511.1

Table 2. Real-time qPCR primers used in this study

Gene name	Forward (F) and reverse (R) primer sequences	Product size (bp)	Annealing temp. (°C)	No. cycles	GenBank accession no.
GATA4	F: 5'-CTGCTGTCTGTCTGCTCCTC-3' R: 5'-TGTGTTGTGTTTGTGCTTCG-3'	150	59	40	NM_002052.3
Sox17	F: 5'-TGTTCCAGAGATTTGTTCCCATAG-3' R: 5'-ACACACCCAGGACAACATTTTC-3'	141	59	40	NM_022454.3
Brachyury	F: 5'-CCAGTGGCAGTCTCAGGTTAAG-3' R: 5'-GCAGGTGTGAGCAAGGGATG-3'	116	59	40	NM_003181.2
Nkx2.5	F: 5'-CGGTTGGAAGTGGGACTGAG-3' R: 5'-CGACGGCGAGATAGCAAAGG-3'	102	59	40	NM_004387.3
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTACCTAATTTGCGT-3'	94	59	30	NR_004394

Materials and Methods

Culture and neuronal differentiation of FS-1 hiPSC

We cultured and maintained human iPS (Foreskin)-1 (briefly called FS-1 thereafter; obtained from WiCell, Wisconsin, USA) according to the method described previously (4). FS-1 line was originally established under feeder-free condition, but we changed the culture condition so that the cells were maintained in the presence of embryonic fibroblasts, which provided more stable culture conditions. As controls, H9 human ES cells (obtained from WiCell) and two human iPSC lines (551-8 and F5), which were generated by retroviral integration of Oct4, Sox2, c-Myc and Klf-4 (6-8), were used. Neuronal differentiation of iPSCs was induced by co-culturing the cells with PA6 stromal cells (obtained from RIKEN Cell Bank) as described previously (9). Using 5-stage differentiation protocol, we differentiated FS-1 cells into mature neurons (7, 8). To do this, we plated neurospheres directly onto PLO/FN-coated dishes in DM medium supplemented with 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems) in the absence of bFGF.

Immunocytochemical analysis

To analyze the marker expression of cells from various stages, we carried out immunocytochemical analyses using the following primary antibodies: OCT4 (1:250, Santa Cruz), SSEA-4 (1:100, Developmental Studies Hybridoma Bank), TRA-1-81 (1:250, Chemicon), type III β -tubulin (Tuj1) (1:500, Chemicon), Myogenin (1:200, Developmental Studies Hybridoma Bank), SOX17 (1:200, Santa Cruz), OTX2 (1:500, Chemicon), MAP2 (1:200, Chemicon), NeuN (1:500, Chemicon), Gamma-aminobutyric acid (GABA) (1:5,000, Sigma), and TH (1:1,000, Pel-Freez). Secondary antibodies used were goat anti-mouse IgG-conjugated Alexa-555 (1:200, Molecular Probes), goat anti-rabbit IgG-conjugated Alexa-488 (1:200, Molecular Probes) and goat anti-mouse IgM-conjugated Alexa-555 (1:200, Molecular Probes). DAPI was used to counter-stain the cells. Staining patterns were examined and photographed using a confocal laser-scanning microscope imaging system (LSM510, Carl Zeiss Inc.).

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) analysis

We isolated total RNA from cells using Trizol RNA extraction method (Gibco). cDNA was synthesized using M-MLV reverse transcriptase (Promega) at 42°C for 1 hr. PCR amplification was performed using Taq polymerase according to the manufacturer's instructions (Intron, Korea).

Primer sequences, number of cycles, annealing temperatures for semi-quantitative RT-PCR and real-time qPCR are described in Table 1 and Table 2, respectively. The house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 were used as internal loading controls.

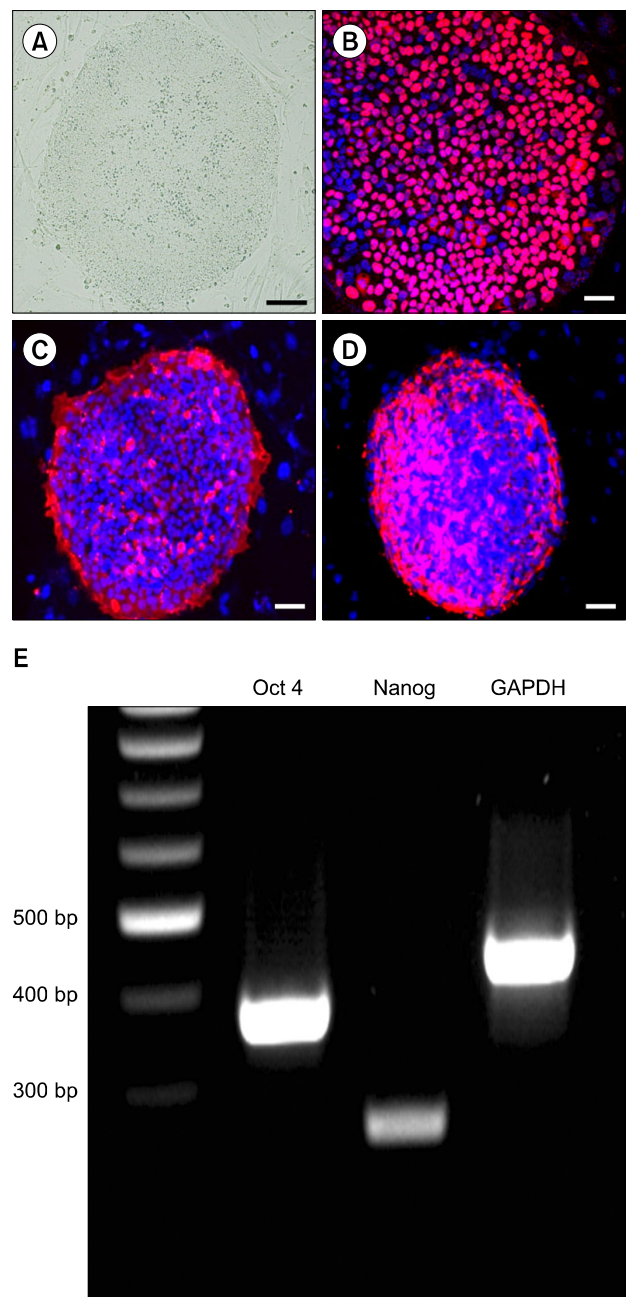


Fig. 1. Morphology and marker expression of FS-1 cells at undifferentiated stage. (A) A colony showing undifferentiated FS-1 cells. Immunocytochemical staining showing the expression of OCT4 (B), (C) SSEA-4 and (D) TRA-1-81. (E) Semi-quantitative RT-PCR showing high levels of *Oct4* and *Nanog* mRNA expression. Scale bar: 100 μ m.

Results

Morphology and marker expression of FS-1 cells at undifferentiated stage and after spontaneous differentiation

As shown in Fig. 1, we found that morphological characteristics and marker expression patterns of FS-1 cells at undifferentiated stage were very similar to those of other pluripotent stem cells, including human ES cells (H9, data not shown) and two other human iPSC lines (551-8 and F5, data not shown). Since the original report didn't provide these information (4), the current study adds a new information on FS-1 cells at undifferentiated stage. While the morphology and marker expression in FS-1 cells are

very similar to those in H9 human ES cells, there is a slight difference in the methylation status of *Oct4* promoter (4), implying that their epigenetic status are not entirely identical.

We also examined the differentiation potential of FS-1 cells after spontaneous differentiation in suspension culture. As shown in Fig. 2, FS-1 cells form embryoid bodies (EBs) (Fig. 2A) and express markers for endoderm (SOX17), ectoderm (Tuj1) and mesoderm (Myogenin) (Fig. 2B). Real-time qPCR analysis further revealed that they express mRNAs for endoderm (GATA4 and Sox17), mesoderm (brachyury and Nkx2.5) and ectoderm (type III β -tubulin and Nestin) (Fig. 2C). However, comparative analysis of each mRNA expression shows some differences

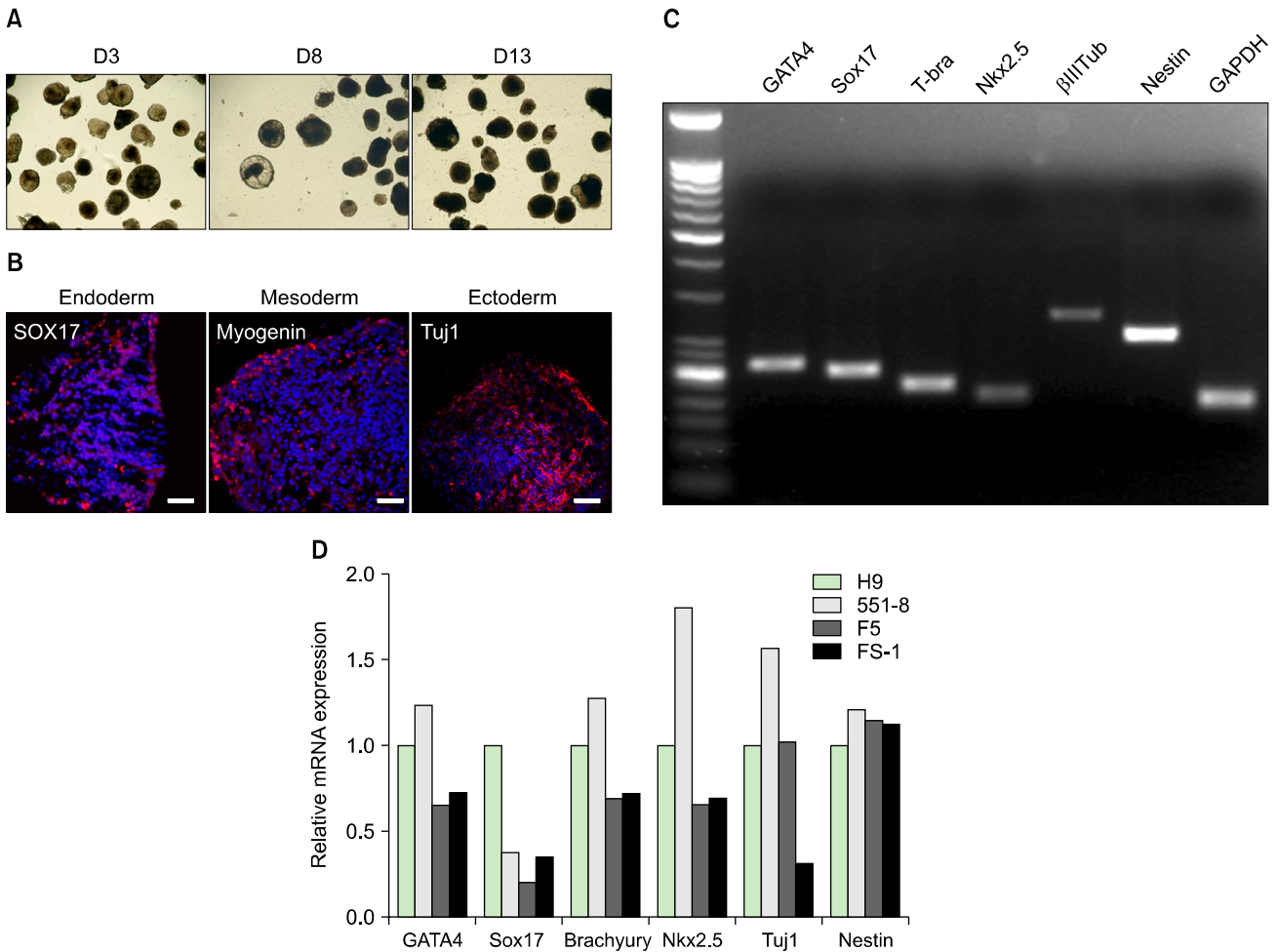


Fig. 2. Morphology and marker expression of FS-1 cells after spontaneous differentiation. (A) Formation of embryoid bodies (EBs) at 3, 8 and 13 days (D3, D8 and D13, respectively) in suspension culture. (B) Immunocytochemical staining showing germ layer-specific markers: SOX17 (endoderm), Tuj1 (ectoderm) and Myogenin (mesoderm). (C) Real-time qPCR results showing expression of markers for endoderm (GATA4 and Sox17), mesoderm (Brachyury and Nkx2.5) and ectoderm (β III Tub [Tuj1] and Nestin). (D) Relative expression of each marker in FS-1, compared with human ES cells (H9) and two different iPSC lines (551-8 and F5). Expression levels in H9 were set as "1." Scale bar: 50 μ m.

in each cell line (Fig. 2D). Interestingly, we observed that FS-1 cells are more inclined to differentiate into ectodermal lineage, judged by relatively high expression of Tuj1 and Nestin (Fig. 2D). Taken together, these results indicate that, although gross morphology and immunocytochemical marker expression in FS-1 cells are very similar to other pluripotent stem cells, their detailed expression levels for specific markers are quite different.

Neuronal differentiation of FS-1 cells

We have recently shown that our 5-stage differentiation protocol is efficient to generate mature neurons (7, 8). Using this protocol, we first co-cultured undifferentiated FS-1 cells (Stage 1) with PA6 stromal cells (Stage 2) and isolated neural rosettes (Stage 3). We then induced neurosphere formation in suspension culture (Stage 4). Afterwards, we further differentiated the cells into mature neurons (MN) (Stage 5-MN). Although it is well known that PA6 co-culture method drives the fate of neuronal differentiation into more midbrain-type neurons (9), we found that isolation of neuroepithelial cell types at early stage, and treatment with BDNF (brain-derived neurotrophic factor) renders the nature of neuronal populations to be maintained as forebrain-type or GABAergic neurons (8). Importantly, with our differentiation protocol we efficiently generated early and mature neurons (i.e., type III β -tubulin and Map2). Semi-quantitative RT-PCR results showed that the neuronal cells derived from FS-1 cells express high levels of forebrain (i.e., Bf-1), striatal (i.e., Calbindin) and MSN (i.e., Darpp-32) markers (Fig. 3A). There are some neurons expressing a marker for mid/hindbrain (i.e., En-1). However, expression of markers for dopaminergic neurons (i.e., TH) or glial cells (i.e., GFAP, data not shown) were either very low or absent. Immunocytochemical staining further confirmed that markers for mature neurons (i.e., NeuN and MAP2) and early neurons (i.e., Tuj1) are highly expressed, whereas TH-positive dopaminergic neurons are detected only at low levels. Expression of markers for fore/midbrain (i.e., OTX2) and GABAergic neurons (i.e., GABA) are frequently detectable. Taken together, these results indicate that, under the conditions we used, FS-1 cells have a tendency to differentiate into more forebrain-type neurons, suggesting that FS-1 iPSC-derived neural cells will be useful to be used in cell therapy of stroke or Huntington's disease, among others.

Discussion

In this study, we have extensively analyzed the mor-

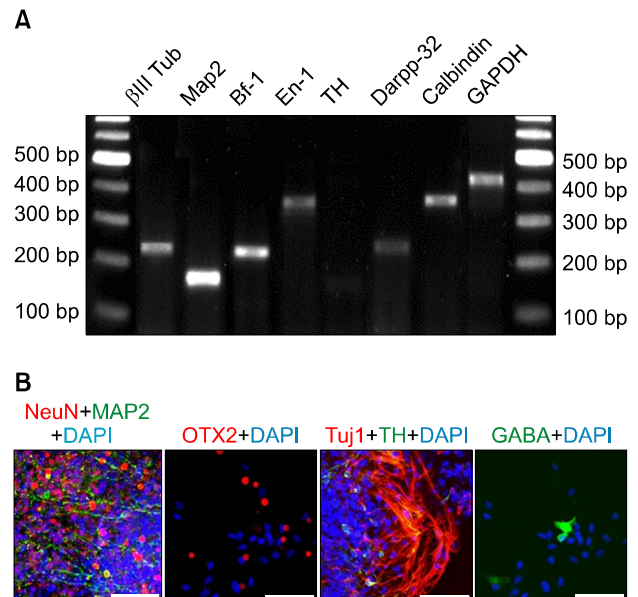


Fig. 3. Expression of neuronal markers of FS-1 cells at differentiated neuronal stage. (A) Semi-quantitative RT-PCR showing expression of markers for early neuron (β III Tub, type III β -tubulin), mature neuron (Map2), forebrain (Bf-1), mid/hindbrain (En-1), dopaminergic neuron (TH), medium spiny neuron (Darpp-32) and striatal neurons (Calbindin). (B) Immunocytochemical staining showing the expression of mature neurons (NeuN and MAP2), forebrain/midbrain (OTX2), early neurons (Tuj1), dopaminergic neurons (TH) and GABAergic neurons (GABA). Scale bar: 50 μ m.

phology and marker expression of FS-1 cells at undifferentiated, after spontaneous differentiation and at differentiated neuronal stages. Although the gross morphology and immunocytochemical marker expression patterns of FS-1 cells are very similar to those of human ES cells (H9) and other two different human iPSC lines (551-8 and F5), their detailed gene expression patterns and/or levels are shown to be somewhat different. Nevertheless, from this study and the original report, it is evident that FS-1 cells meet the general criteria of human pluripotent stem cells. Moreover, since foreskin can provide non-invasive cell sources for iPSC generation and its cell age is very young, it has strong advantages over aged skin fibroblasts in many respects. However, even in this case, it will be essential to generate iPSC using non-integrating methods, such as episomal system (10), from foreskin fibroblasts under good manufacturing protocol (GMP) conditions in order to be used for therapeutic purposes. More recently, several groups have published elegant methods to convert fibroblasts into neurons directly (iN cells) (11, 12), so it will be interesting to apply these technologies to make neurons directly from foreskin fibroblasts in the future. Taken together, our results indicate that FS-1 cells derived

from human neonatal fibroblasts exhibit very similar properties with human ES cells, and can provide useful sources for cell therapy and various other applications, including drug screening.

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Potential conflict of interest

The authors have no conflicting financial interest.

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