Generation of Neuronal Progenitor Cells and Neurons from Mouse Sleeping Beauty Transposon–Generated Induced Pluripotent Stem Cells

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Abstract

Mouse embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells can be used as models of neuronal differentiation for the investigation of mammalian neurogenesis, pharmacological testing, and development of cell-based therapies. Recently, mouse iPS cell lines have been generated by Sleeping Beauty (SB) transposon-mediated transgenesis (SB-iPS). In this study, we determined for the first time the differentiation potential of mouse SB-iPS cells to form neuronal progenitor cells (NPCs) and neurons. Undifferentiated SB-iPS and ES cells were aggregated into embryoid bodies (EBs) and cultured in neuronal differentiation medium supplemented with 5 μM all-trans retinoic acid. Thereafter, EBs were dissociated and plated to observe further neuronal differentiation. Samples were fixed on days 10 and 14 for immunocytochemistry staining using the NPC markers Pax6 and Nestin and the neuron marker βIII-tubulin/Tuj1. Nestin-labeled cells were analyzed further by flow cytometry. Our results demonstrated that SB-iPS cells can generate NPCs and differentiate further into neurons in culture, although SB-iPS cells produced less nestin-positive cells than ESCs (6.12 ± 1.61 vs. 74.36 ± 1.65, respectively). In conclusion, the efficiency of generating SB-iPS cells–derived NPCs needs to be improved. However, given the considerable potential of SB-iPS cells for drug testing and as therapeutic models in neurological disorders, continuing investigation of their neuronal differentiation ability is required.

Introduction

Pluripotent stem cell research holds great promise for revolutionizing the future of medicine, especially for the regeneration of damaged and diseased organs. Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of preimplantation blastocyst-stage embryos that can differentiate in vivo and in vitro into all cell types of an adult animal (Evans and Kaufman, 1981). Mouse ESCs are used as model systems for neurological diseases and investigation of mammalian neurogenesis. In particular, they can be used in the generation of neurons for pharmacological testing and development of models for cell therapy applications, which may help to overcome incurable neurological diseases, such as stroke, spinal cord injuries, Alzheimer’s disease, and Parkinson’s disease (Langston, 2005; Taupin, 2006).

Recently, a novel alternative method has been developed to establish induced pluripotent stem (iPS) cells. Mouse and human iPS cells have been directly reprogrammed from adult cells (e.g., fibroblasts) by the introduction of pluripotency transcription, initially by Oct3/4, Sox2, c-Myc, and Klf4, known as Yamanaka factors (Takahashi and Yamanaka, 2006), or complementing/replacing them partially with other factors, such as Nanog and Lin28 (Liao et al., 2008). These iPS cells resemble ESCs with respect to morphology, proliferation, gene expression, teratoma formation, their ability to differentiate into all three germ layers, and, in the case of mouse iPS cells, to also form mouse chimeras (Maherali et al., 2007; Takahashi and Yamanaka et al., 2006). The iPS cells might also be useful for patient- and disease-specific cell transplantation through their differentiation potential into several different cell lineages, including cardiac...
cells (Zwi et al., 2009), hepatic cells (Iwamaru et al., 2010), hematopoietic cells (Tolar et al., 2011), and neurons (Hu et al., 2010).

To date, iPS cells have been established by several methods, such as viral transduction (Takahashi et al., 2007), recombinant cell-penetrating proteins (Zhou et al., 2009), administration of synthetic modified mRNA (Warren et al., 2010), and recently by transposon-transposase-mediated transgenesis using the piggyBac transposon (Kaji et al., 2009; Nagy et al., 2011; Wolften et al., 2009). Transposons are sequences of DNA that have the capability to change their positions within the genome by use of a transposition mechanism. Besides PiggyBack, another transposon, Sleeping Beauty (SB), was engineered by the molecular reconstruction of the inactive Tc1/mariner element found in the salmonid fish genome (Ivics et al., 1997). This transposon has been used as a powerful tool to introduce genes into various cell types (Essner et al., 2005; Izsavak et al., 2009). Very recently, SB has been reported by us to be a suitable tool for mouse iPS cell line generation (Muenthaisong et al., 2012) and as offering an alternative method for the efficient generation of iPS cells.

Neuroepithelial (NEP) cells are multipotent cells in the neural tube that have the capability to self-renew and give rise to neurons in the central nervous system (CNS) and in the peripheral nervous system (PNS), such as glial cells and ependymal cells (Pevny and Rao, 2003). Differentiation of NEP cells occurs via the generation of two major types of progenitor cells, including neuroblasts or neuronal progenitor cells (NPCs) that can generate into multiple kinds of neurons (Kalyani et al., 1998; Mayer-Proschel et al., 1997). The developmental conversion of the undifferentiated inner cell mass in the early embryo into committed neurons has been partially emulated by in vitro differentiation of ESCs (Okabe et al., 1996). It has been reported that ESCs are able to form neurons, astrocytes, and oligodendrocytes (Bain et al., 1995). Early development and neuronal differentiation of mouse ESCs has been extensively studied in vitro. For the initial steps of neuronal induction, most strategies include use of aggregates of a few hundred stem cells, so called embryoid bodies (EBs). Although EBs consist of several cell types of many lineages, it has been shown that supplementation with retinoic acid can induce the formation of a relatively uniform glutamatergic neuronal population (Bibel et al., 2007).

Retinoic acid (RA) is a biologically active form of retinol (vitamin A) and has been demonstrated to have a significant role during embryogenesis and CNS development (Maden, 2001; Ross et al., 2000). RA first appears in the mouse embryo at E7.5 (Ulven et al., 2000). It has been reported that ESCs are able to differentiate into various types of many lineages, it has been shown that supplementation with retinoic acid can induce the formation of a relatively uniform glutamatergic neuronal population (Bibel et al., 2007).

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basic fibroblast growth factor (bFGF). Two days later, the medium was changed to DMEM/F12:neurobasal medium (1:1), 1 mM glutamax, 3 mg/mL AlbuMaxI, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.5% (vol/vol) N2 Supplement, and 1% (vol/vol) B27 supplement. The medium was renewed every second day until day 14. The cells were harvested for further analyses on days 10 and 14 after the start of the EB formation. The experiments were repeated three times.

**Immunocytochemistry**

ESCs and SB-iPS cells were prepared for characterization by 2-day culture (until reaching 70% confluency) on gelatin-coated coverslips. Differentiating cells, following dissociation of EBs, were plated onto poly-L-ornithine/laminin–coated coverslips for 2 and 6 days (referred as day 10 and 14, respectively). Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% (vol/vol) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4°C until analysis. Permeabilization was performed using 0.2% (vol/vol) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 5% (wt/vol) bovine serum albumin containing 0.5% (vol/vol) Tween 20 in PBS for 30 min. The cells were then incubated sequentially with the following primary antibodies diluted in blocking solution overnight at 4°C: pluripotent marker Oct4 (sc9081, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse), Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse), neuroectodermal marker Pax6 (Pax6, dilution 1:200, DSHB; mouse), neuronal progenitor marker Nestin (Rat-401, dilution 1:200, DSHB; mouse), and neuron marker βIII-tubulin (TuJ1, dilution 1:2,000; Covance, PRB-435P; rabbit). The cultures were washed three times with PBS and then incubated with fluorescently labeled secondary antibodies [Alexa Fluor® 488, Alexa Fluor® 594, and Alexa Fluor® 647-labeled goat immunoglobulin G (IgG); dilution 1:2000; Gibco] for 1 h at RT. After three washes with PBS, the cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector Laboratory, Temecula, CA, USA). The images were taken on a Zeiss AxiosImager fluorescent microscope using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany).

**Flow cytometric analysis**

On day10 of the neuronal differentiation process, cells were trypsinized into single cells, washed with PBS, and centrifuged at 1000 rpm for 5 min. Cells were fixed in 4% PFA for 15 min at RT. Following washing with PBS, cells were stained with the primary antibody Nestin (Rat-401, dilution: 1:50, DSHB; mouse) in 0.1% (vol/vol) Triton X-100 in PBS for 1 h at RT. Cells were washed once with 0.05% Tween-20 in PBS, then incubated with Alexa Fluor® 647–labeled secondary antibody (goat IgG; dilution 1:500; Gibco) for 1 h at RT. The cells were washed and resuspended in PBS. Flow cytometry was performed using a Becton-Dickson (Palo Alto, Temecula, CA, USA) FACSCalibur flow cytometer.

**Statistical analysis**

Data concerning flow cytometry analysis are expressed as mean±standard error of the mean (SEM) and include at least three independent experiments. Statistical analyses for comparison between nestin-positive cells derived from ESCs and iPS cells were conducted using the Student’s t-test. A p<0.05 was considered statistically significant.

**Results**

**Immunocytochemistry analysis of pluripotent cells**

The SB-iPS cell lines exhibited characteristics typical for pluripotent stem cells, including ESC-like morphology, strong alkaline phosphatase (ALP) positivity, and pluripotency marker gene expression patterns (verified by quantitative real-time PCR) as recently described by us (Muenthaisong et al., 2012). In this study, iPS cells were examined for immunofluorescent staining patterns as shown in Figure 1. The results showed that SB-iPS cell lines expressed the pluripotency markers Oct4, Sox2, and Nanog when cultured in an undifferentiated state.

**Characterization of neuronal phenotype**

To investigate the ability of neuronal differentiation of mouse ESCs and SB-iPS cells, these pluripotent cells were induced to differentiate into NPCs and neurons through EB formation and supplementation of RA (Rungarunlert et al., 2011). The morphology of 8-day-old EBs derived from ESCs and SB-iPS cells are shown in Figure 2, A and B. We found that the 8-day-old EBs derived from ESCs and SB-iPS cells show the spherical structures with various sizes. SB-iPS

**FIG. 1.** Immunocytochemistry analysis of iPS cell line. Mouse SB-mediated iPS cells were maintained in an undifferentiated stage before neuronal differentiation. The cells were stained with the pluripotent markers Oct4, Sox2, and Nanog. Scale bars, 100 µm.
cells–derived EBs formed smaller aggregates when compared with those from the ESC line.

Two days after plating the cells onto culture dishes (day 10 of differentiation), the cells exhibited a neuron-like appearance with neurite processes organized in a network (Fig. 2C, D).

**Immunocytochemistry analysis of differentiating cells**

The results of the immunostaining showed that undifferentiated ESCs and SB-iPS cells expressed the pluripotency markers Oct4, Sox2, and Nanog (Fig. 1). After performing the neuronal differentiation procedure (see Materials and Methods), ESCs and SB-iPS cells subsequently expressed neuronal markers as well. Within 2 weeks in culture, ESCs and SB-iPS cells were able to differentiate into cells expressing Pax6, a neuroectodermal marker (Suter et al., 2009) (Fig. 3A, B), and nestin, a specific antibody against the intermediate filament protein of NPCs (Lin et al., 1995) (Fig. 3C, D). In particular, SB-iPS cells started to generate few neurons indicated by the early postmitotic neuronal marker Tuj-1 (Lee and Pixley, 1994) (Fig. 3F). Then the neuronal population was gradually increased by day 14 (Fig. 3H), although approximately 2–3 times lower in number when compared to ESCs (Fig. 3G, H). Our results demonstrated that mouse ESCs and SB-iPS cells have the ability to generate NPCs and differentiate further into neurons through EB formation in culture.

A quantitative analysis was performed by flow cytometry on both ESC- and SB-iPS–derived NPC populations. Two days after EB dissociation and plating, 76.68% of the ESC-derived NPCs were labeled by the nestin-specific antibody, whereas the amount of nestin-positive SB-iPS cells–derived NPCs was only 6.2% (Fig. 4). However, we showed that SB-iPS cells have the potential to differentiate further into neurons, although SB-iPS cells exhibited the unfavorable potential to generate a NPC population.

**Discussion**

We have investigated neuronal differentiation of mouse ESCs and SB-iPS cells using EB formation to initially induce the cells in the presence of RA followed by plating and culture in defined media. Our method allowed SB-iPS cells to aggregate and differentiate in suspension culture and form EBs. EBs recapitulate many aspects of cell differentiation during early mammalian embryogenesis, and the cells can be terminally differentiated into cell types belonging to the three germ layers (Keller, 1995). The lack of structural organization and positional information within EBs during pluripotent cell differentiation results in heterogeneity both within and between EBs. However, a high yield of neuronal population can be generated from ESC-derived EBs by using RA to commit cell fate to the neuronal lineage (Bain et al., 1995).

This is the first report describing the capability of mouse SB-iPS cells, reprogrammed by the Sleeping Beauty transposon, to differentiate into NPCs and neurons. This is also the first in vitro study where the efficiency of neuronal induction of SB-iPS cells was compared with the efficiency of that in ESC lines. The neuronal phenotypes were observed through phase-contrast microscope (Fig. 1C–D) and by immunofluorescence staining (Fig. 3). SB-iPS–derived neuronal cells expressed Pax6, an essential transcription factor in neurogenesis involved in controlling neural stem cell proliferation and multipotency (Sansom et al., 2009). Nestin is a type IV intermediate neurofilament expressed specifically in NEP stem cells or NPCs. In the developing embryos, nestin is expressed in both the ventricular and subventricular zones of the CNS and is also expressed in radial glial cells (Hockfield and McKay, 1985; Lendahl et al., 1990).

We found that differentiated cells derived from SB-iPS cells had a significantly lower level of Pax6- and nestin-positive cells than those originating from ESCs. However, SB-iPS cells did show a potential to differentiate further into neurons.
neurons expressing Tuj-1, which is a marker of early post-mitotic neural cell types. There are many specific-neuronal markers, apart from Pax6 and nestin, that have been used for determination of NPC fate, including SOX1 (Aubert et al., 2003), SOX2 (Ellis et al., 2004), Musashi-1 (MacNicol et al., 2008), and Cx43 (Duval et al., 2002).

To determine a more specific cell fate for our NPCs, further investigation is required. In the future, by applying these markers (Sox1, Cx43, or Musashi), the cell types will be classified further, and perhaps differences between ESC- and SB-iPS–derived NPCs can be revealed. It has been reported that mouse iPS cells possess morphological, molecular and developmental features closely resembling those of ESCs (Takahashi and Yamanaka, 2006). A recent study showed that different pluripotent cell lines or even subclones of the same cell line can display different potentials to form EBs or to generate NPCs. The reasons behind these differences are not yet clear. We can only speculate whether these differences are related to different epigenetic modifications or perhaps cell cycle–related gene expression differences (Martinez et al., 2011). One of probable reasons is that the reprogramming cassette remains in our SB-iPS cells. The transposase system is now addressing this and is being developed further to render practical and more promising iPS cell lines for efficient and safety application issues. However, these SB-iPS cells have been differentiated into the neuronal lineage even when containing the transposon construct.

We hypothesized that perhaps the silencing of transgenes in SB-iPS cells occurred during the neuronal differentiation process. A previous report revealed that repression of the exogeneous pluripotent factors is necessary for allowing efficient cell differentiation toward lineages (Chamberlain et al., 2008). Moreover, incomplete promoter DNA methylation has been reported, which results in the retention of transcriptional memory and may predispose somatic cell–derived iPS cells to differentiate more readily into the

![FIG. 3. Differentiation potential of HM1 ESC and B5 iPS cell lines into neuronal lineage. The neuroectoderm (radial glia) marker Pax6, the neuronal progenitor cell marker Nestin, and the neuronal tubulin marker βIII-tubulin (Tuj1) are expressed in differentiated cells on day 10 (A–F) and day 14 (G–H). Scale bars, 200 μm.](image-url)
sought after iPS cell lines. The neuronal differentiation approach may be a suitable tool for obtaining these much needed cell lines. Our results are encouraging and show that both ESCs and SB-iPS cells show a difference in their capacity to differentiate toward the neuronal lineage. Even though the neuronal differentiation rates of iPS cells in their capacity to differentiate toward the neuronal lineage need to be improved, our results are encouraging and show that SB-iPS cells are capable of forming neurons. Thus, the Sleeping Beauty transposon-mediated reprogramming approach may be a suitable tool for obtaining these much sought after iPS cell lines.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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