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Characterization of Oct4-GFP Spermatogonial Stem Cell Line and Its Application in the Reprogramming Studies

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ABSTRACT

Establishment of mouse spermatogonial stem cell (SSC) culture systems offers a useful stem cell model for studies of proliferation and self-renewal of mammalian germline stem cells. In addition, spontaneous development of pluripotent stem cells from cultured SSCs emphasizes their possible applications in regenerative medicine as a substitute for embryonic stem cells (ESCs). These pluripotent stem cells termed multipotent germline stem cells (mGSCs) or germline-derived pluripotent stem cells (gPSCs) exhibit almost identical properties in terms of morphology and gene expression patterns to mouse ESCs (mESCs). In this study, to help understand mechanisms underlying reprogramming of SSCs into pluripotent stem cells, we established a culture system of SSCs derived from mice harboring green fluorescence protein (GFP) transgene whose expression is modulated by Oct4 regulatory sequences. Our results indicated that GFP intensity faithfully reflected cellular states upon reprogramming of SSCs or treatment with a selective extracellular signal-regulated kinase (ERK) inhibitor PD0325901. Moreover, in contrast to mESCs, regulation of Nanog expression did not appear to couple to the Oct4 level in SSCs. Further analysis of Oct4-GFP SSCs demonstrated that a posttranscriptional control of pluripotency marker genes such as Oct4 and Sox2 might play an important role as an inhibitory mechanism preventing the acquisition of pluripotency. J. Cell. Biochem. 114: 920–928, 2013.

KEY WORDS: SPERMATOGONIAL STEM CELLS; MULTIPOTENT GERMLINE STEM CELLS; Oct4-GFP; REPROGRAMMING

Biologically active spermatogonial stem cells (SSCs) first appear from gonocytes 3–4 days after birth in mouse testes and persist throughout life of the male [McLean et al., 2003]. SSCs have the unique potential for both self-renewal and the production of unlimited number of sperm through multiple differentiation steps referred to as spermatogenesis [Kubota and Brinster, 2006; Yoshida, 2010; Lee and Shinohara, 2011; Reijo Pera et al., 2011; Singh et al., 2011]. Therefore, SSCs are one of the valuable cell models in the field of reproduction.

Niches are supportive microenvironments and crucial for the symmetric and asymmetric division of stem cells. The major contributor to the SSC niche is Sertoli cells although Leydig and myoid cells are also known to participate in the maintenance of SSCs [Yoshida et al., 2007]. Sertoli cells reside in the seminiferous tubules and closely contact with spermatogonia while producing glial cell-derived neurotrophic factor (GDNF), an essential growth factor for SSC maintenance [Meng et al., 2000]. Kubota et al. [2004] reported the long-term culture of SSCs for the first time using GDNF-containing medium, which prompted subsequent mechanistic studies regarding self-renewal and proliferation of the stem cells. The first gene discovered as an intrinsic regulator of SSC self-renewal was the transcriptional repressor promyelocytic leukemia zinc finger protein (*Plzf*) [Buaas et al., 2004; Costoya et al., 2004]. Expression of *Plzf* is restricted to the undifferentiated spermatogonia in the mouse testes and its genetic disruption induced the progressive loss of germ cells. *Nanos2*, *Bcl6b*, *Etv5*, and *Lhx1* are known to play roles in the maintenance of SSCs while pluripotency marker genes such as *Oct4*, *Sox2*, and *Lin28* are also expressed in germline stem cells [Dann et al., 2008; Oatley and Brinster, 2008; Sada et al., 2009; Zheng et al., 2009].

Kanatsu-Shinohara et al. [2004] reported derivation of ES-like cells from the cells of neonatal mouse testes during in vitro cultivation without any genetic modifications. These pluripotent stem cells termed multipotent germline stem cells termed mGSCs showed very similar morphology and gene expression patterns to mESCs, successfully developed into all three germ layers both in vitro and in vivo and contributed to chimera formation. Ko et al. [2009] subsequently reported a robust protocol for clonal derivation

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of gPSCs from adult unipotent germline stem cells. Extensive gene expression profiling of gPSCs, mESCs and induced pluripotent stem cells (iPSCs) revealed that the gene expression patterns of gPSCs or mGSCs are much closer to those of mESCs than those of iPSCs, implying that germline stem cell-derived pluripotent stem cells might be a better substitute for mESCs for therapeutic applications [Ko et al., 2009]. Although what causes the reprogramming event in SSCs is currently unknown, it has been hypothesized that the dedifferentiation of SSCs would be relatively simple compared to that of other cell types, due to the close similarity in the gene expression profiles between mESCs and SSCs. In fact, *Oct4*, *Sox2*, and *Nanog*, a trinity of transcription factors maintaining pluripotency in mESCs, were also shown to be expressed in SSCs although there exist considerable discrepancies regarding gene expression patterns among different studies [Kanatsu-Shinohara et al., 2004; Guan et al., 2006; Conrad et al., 2008; Ko et al., 2009].

In humans, very limited information about SSCs is available and the identity of the true SSCs is still unknown. However, several groups reported the derivation of pluripotent stem cells from adult human testicular cells although their gene expression patterns and characteristic morphologies were quite distinct from those of typical human ESCs (hESCs) [Conrad et al., 2008; Geijsen and Hochedlinger, 2009; Ko et al., 2010]. Nevertheless, these results shed light on the possibility that SSCs could be used as a practical source of human pluripotent stem cells.

Oct4-GFP reporter construct is composed of regulatory sequences of *Pou5f1* (*Oct4*) gene and has been widely used to visualize the pluripotent state of mESCs [Nichols and Smith, 2009; Li et al., 2011]. It is very well known that the intensity of green fluorescence protein (GFP) signal is stably maintained in mESCs but significantly decreases upon differentiation [Cantz et al., 2008]. In this study, we attempted to use Oct4-GFP transgenic mice to isolate SSCs and establish a culture system. Our results indicated that the established Oct4-GFP SSCs were able to successfully modulate the intensity of GFP signal upon the cell fate change and respond to small molecule inhibitors of extracellular signal-regulated kinase (ERK). Therefore, Oct4-GFP SSCs can be used as a useful tool in helping better understand mechanisms underlying the reprogramming. Furthermore, we analyzed gene expression profiles both in RNA and protein levels to better characterize SSCs. Our results suggest that, in contrast to the gene regulatory network in mESCs, low translational activities of *Oct4* and *Sox2* and transcriptionally inactive *Nanog* may restrict these genes not to cooperate in the regulatory circuitry in SSCs and may be responsible for their inability to gain pluripotency in vivo.

MATERIALS AND METHODS

ANIMALS

DBA/2 mice were purchased from Harlan (USA). B6;CBA-Tg(Pou5f1-EGFP) 2Mnn/J mice (stock number 004654) were purchased from the Jackson Laboratory (USA) and these transgenic mice express enhanced GFP under the control of the POU domain, class 5, transcription factor 1, promoter, and distal enhancer. Nanog-GFP knock-in mice carry a GFP-IRES-puro cassette in the endogenous Nanog locus as previously described [Hatano et al., 2005; Maherali et al., 2007]. All mice used in the current study were housed in a specific pathogen-free environment at Korea University. The experimental protocols of this study were approved by the Laboratory Animal Care and Use Committee of Korea University.

MEF PREPARATION AND IRRADIATION

Mouse embryonic fibroblasts (MEFs) were isolated from wild-type ICR mice. Briefly, embryos were collected at 12.5–13.5 days post coitum (dpc), after removal of head and internal organs, minced and trypsinized. Cell suspension was filtered through a 40 μm cell strainer and then plated on culture dishes containing DMEM (Sigma, St. Louis, USA), 10% fetal bovine serum (FBS) (Hyclone, USA), 1× nonessential amino acids (Invitrogen, USA), 1× Glutamax (Invitrogen), 1× penicillin–streptomycin solution (Invitrogen), and incubated in a 5% CO_2 incubator. When the cells were confluent, they were trypsinized and seeded into 100 mm cell culture plates at 2×10^6 cells. To mitotically inactivate MEFs, cells when confluent were detached from the plates and irradiated with 4,000 rads from a γ-radiation source.

ISOLATION AND CULTURE OF SSCS

Testes from 7 to 10 days postpartum (dpp) males were collected and digested with a two-step enzymatic digestion method as previously described [Kanatsu-Shinohara et al., 2003]. Briefly, testes were digested with 1 mg/ml collagenase type IV at 37°C for 20–30 min. Next the dispersed testis tubules were centrifuged at 1,200 rpm for 5 min, followed by 0.25% trypsin/1 mM EDTA (Invitrogen) digestion at 37°C for 10 min. The trypsin was neutralized with 1 ml of FBS and cells were collected and filtered through a 40 μm cell strainer to obtain single cell suspension. The cell suspension was then washed three times with DMEM with 2% FBS. Collected cells were resuspended in SSC culture medium and transferred to 0.1% (w/v) gelatin-coated 12-well plates at a density of 2×10^5 cells/well. The composition of the SSCs medium is as follows: StemPro-34 (Invitrogen) supplemented with 1× β-mercaptoethanol (Invitrogen), 1× d(L)-lactic acid (Invitrogen), 1× L-glutamine (Invitrogen), 1× pyruvic acid (Invitrogen), 1× penicillin/streptomycin, 25 μg/ml insulin, 1× MEM non-essential amino acids (Invitrogen), 6 mg/ml D(+)-glucose (Sigma), 60 ng/ml progesterone (Sigma), 100 μg/ml transferrin (Sigma), 0.1 mM ascorbic acid (Sigma), 30 nM sodium selenite (Sigma), 5 mg/ml bovine serum albumin (Sigma), 30 ng/ml β-estradiol (Sigma), 10 μg/ml D-biotin (Sigma), 60 μM putrescine (Fluka, Switzerland), 1× MEM vitamin solution (Cellgro, USA), 20 ng/ml mouse epidermal growth factor (EGF) (R&D systems, USA), 10 ng/ml human GDNF (R&D systems), 10 ng/ml human basic fibroblast growth factor (bFGF) (Stemgent, USA), and 1% FBS (Hyclone). For routine maintenance, SSCs were thoroughly dispersed by 0.25% trypsin/EDTA, replated to new plates with iMEF feeders, and passaged at 1:5–1:10 ratios. After the passage, the isolated SSCs started forming clumps in a day, which usually consisted of 5–10 cells. These SSC clumps continuously grew and rapidly formed typical germ cell colonies. Most of these germ cell colonies consisted of 10–50 cells with tight intercellular contacts.

MOUSE EMBRYONIC STEM CELL CULTURE AND DIFFERENTIATION

J1, Oct4-GFP, and Nanog-GFP mESCs were maintained on 0.1% gelatin-coated plates with irradiated MEFs and in standard mES culture media (DMEM supplemented with 15% FBS, nonessential amino acids, L-glutamine, penicillin–streptomycin, β-mercaptoethanol, and with 1,000 U/ml leukemia inhibitory factor (LIF)). For in vitro differentiation, hanging drop method was used as previously described [Wang and Yang, 2008].

ISOLATION OF TOTAL RNA

Collected cells were resuspended in Trizol (Invitrogen) for RNA isolation. Following chloroform extraction, total RNA was precipitated in 2-propanol, washed with 70% ethanol, resuspended in nuclease-free water and used for cDNA synthesis. The absorbance of total RNA for each sample was evaluated at 260 and 280 nm to determine its concentration and purity using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

CDNA SYNTHESIS AND GENE EXPRESSION ANALYSIS BY REAL-TIME PCR

Relative mRNA expression levels were quantified by semi-quantitative reverse transcription polymer chain reaction (qRT-PCR). One microgram of total RNA was reverse-transcribed into cDNA using Superscript III RNase H-Reverse Transcriptase kit (Invitrogen). qPCR primer sequences were selected for each cDNA with the aid of PRIMER EXPRESS software (Applied Biosystems, USA) and are listed in Table I. Quantitative measurements were performed with the SYBR-Green PCR-Master Mix (Applied Biosystems) using CFX 96 (Bio-Rad, USA). The results were normalized to the relative amounts of β-actin.

SMALL MOLECULE INHIBITORS

PD0325901 (an ERK inhibitor), CHIR99021 (a glycogen synthase kinase 3 beta (GSK-3β) inhibitor), SC1 (pluripotin; dual inhibitor of RasGAP and ERK1), and Y27632 (a Rho-associated kinase (ROCK) inhibitor) were purchased from Stemgent. Inhibitors were used at the following concentrations unless otherwise specified; PD0325901; 0.4 μM, CHIR99021; 1 μM, SC1; 1 μM, and Y27632; 10 μM.

IMMUNOCYTOCHEMISTRY AND ALKINALINE PHOSPHATASE STAINING

The immunocytochemical determination of specific markers in the pluripotent stem cells and SSCs was performed as follows: Cells were fixed for 15 min with 4% paraformaldehyde (Sigma) at room temperature, and blocked for 20 min with Triton-X 100 (Sigma), which included 10% BSA in PBS. Primary antibodies used in this study are as follows; α-OCT4 (1:200), α-SOX2 (1:300), α-NANOG (1:300), α-DAZL (1:300), α-VASA (1:300), α-PLZF (1:300), α-TUBB3 (1:500), α-MYOG (1:250), α-TNNI2 (1:250), and α-MYOSIN (1:250). Primary antibodies were incubated overnight at 4°C. Cells were washed three times in PBS and then the staining was visualized using secondary antibodies conjugated to FITC, Cy3, or Cy5 (Jackson ImmunoResearch, USA). Alkaline phosphatase activity was measured using AP staining kit (Sigma) according to manufacturer's recommendation.

WESTERN BLOT ANALYSIS

Cells were briefly washed with cold PBS and the pellets were lysed with 100 μl of PIPA buffer for 30 min on ice and then centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were transferred into new microfuge tubes. Protein concentrations were then measured by Bradford assay (Sigma). 15 μg of whole cell extracts were mixed with 5× loading dye and electrophoresis was run on 10% SDS–PAGE gels. Following transfer, PDVF membranes were incubated overnight with 5% skim milk in PBST containing a primary antibody (1:1,000). The following primary antibodies were used: Oct4 (Santacruz, USA), Sox2 (Abcam, USA), Nanog (Calbiochem, USA), and tubulin (Abcam). The next day membranes were washed three times with PBST for 10 min each and then incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell signaling, USA, 1:2,000), washed again, and exposed with ECL reagents (Abcam).

FLOW CYTOMETRY ANALYSIS

For analysis of GFP signals, cells were dissociated with accutase and centrifuged for 5 min at 1,000 rpm to remove cell debris. After removal of supernatant, cell pellets were resuspended in 300 ml of Hank's buffered salt solution (HBSS) containing 4% serum and kept on ice. GFP signal was acquired and analyzed with BD FACS Canto II Flow Cytometry System (BD Biosciences, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed by Student's *t*-test with significant differences determined as $P < 0.05$.

TABLE I. List of Mouse Primers Used for qRT-PCR Analysis

Gene	Forward primer	Reverse primer
Nanog	AAGCCAGGTTCCTTCCTTCTTCCA	AGGTCAGGAGTTCAAATCCCAGCA
Sox2	AAAGGAGAGAAGTTTGGAGCCCGA	GGGCGAAGTGCAATTGGGATGAAA
Oct4	AGCTGCTGAAGCAGAAGAGGATCA	TCTCATTGTTGTCGGCTTCTTCCA
Klf4	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT
c-Myc	ATGCCCCTCAACGTGAACTTC	CGCAACATAGGAGAGCA
MDM2	TGCTAAAGAGCCATGTGCTGAGGA	ACTCTTTCACGCTTTCTTGGCTGC
Blimp1	TTCTCTTGGAAAAACGTGTGGG	GGAGCCGGAGCTAGACTTG
Vasa	GCTTCATCAGATATTGGCGAGT	GCTTGGAAAACCCTCTGCTT
Dazl	ATGTCTGCCACAACTTCTGAG	CTGATTTCGGTTTCATCCATCCT
Nanos	TTGTAACTTCAGACTGCCTCGGGT	TGGGACACACACTGACGTAACACA
Nanos3	TTCTGCAAACACAATGGCGAGTCC	AATTCCGGGTGGTGTAGCAGTAGA

RESULTS

DERIVATION OF A SSC LINE FROM B6;CBA-TG(POU5F1-EGFP) 2MNN/J (OCT4-GFP) MICE

Since the derivation efficiency of SSCs from DBA/2 strain was shown to be high compared with other strains [Kanatsu-Shinohara et al., 2010], this strain was chosen to validate our protocol for the establishment of SSC lines (Fig. 1A). Testes from 7 to 10 dpp or adult males were collected and processed by a two-step enzymatic digestion method as previously described in the experimental section. Several lines of SSCs either from neonates or adults were successfully established and these cells have been cultured more than 2 years.

To take advantage of the Oct4-GFP system for reprogramming study, we attempted to establish SSC lines from B6;CBA-Tg(Pou5f1-EGFP) 2Mnn/J mice. These transgenic mice harbor enhanced GFP (EGFP) coding sequences controlled by mouse Oct4 regulatory sequences. To validate the Oct4-GFP system, GFP expression in the mouse testes isolated from various developmental stages has been measured. Fluorescence images indicated that GFP expression is exclusively found in the cells of testes. Strong GFP signal was detected in the seminiferous tubules at 13.5 dpc (Fig. 1B) and 3 dpp (Fig. 1C). The GFP signal was also exclusively found in the undifferentiated spermatogonia in the vicinity of blood vessels of adult mouse testis as previously reported (Fig. 1D) [Sada et al., 2009]. For the derivation of SSCs from Oct4-GFP mice, isolated testicular cells were seeded on the gelatin-coated cell culture plate. The next day, the majority of the testicular cells attached to the plate but the germ cells remained floating or semi-adherent in the culture in the form of single cells or clumps. At this point, about half of the floating germ cells exhibited GFP signal. The floating population of cells was then transferred to secondary culture plates after vigorous pipetting. Over the first week of cultivation, adherent cells gradually stopped proliferating and were outnumbered by the cells with germ cell morphology. After a week, germ cell colonies were transferred by gentle pipetting to new plates with irradiated MEF (iMEF). After the transfer to the iMEF plate, germ cell colonies began active proliferation. Thereafter, cells were maintained on the iMEF plate with medium changed every 2–3 days. The morphology of established Oct4-GFP SSCs resembled that of SSCs from DBA/2 strain but GFP signal intensity seemed relatively low compared with that of GFP-positive cells in the testes (Fig. 1E).

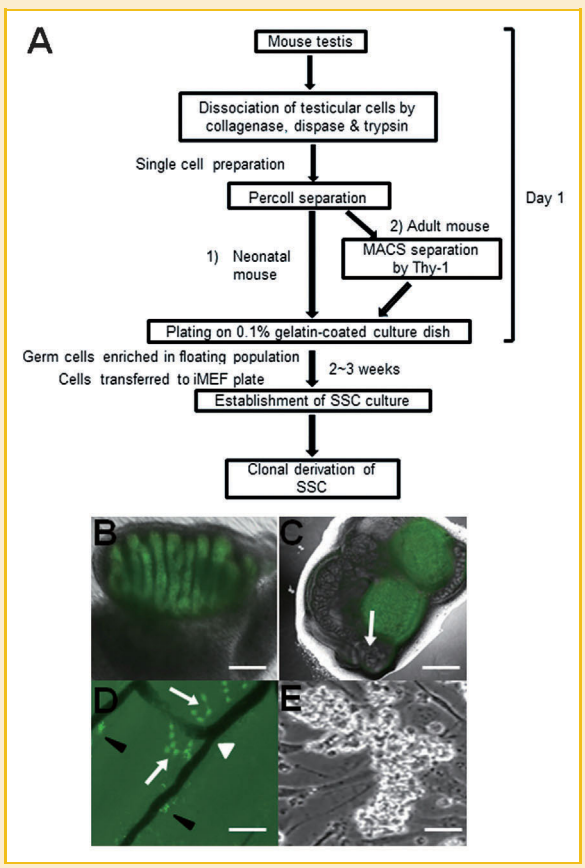

Fig. 1. General SSC derivation procedure and images of cells from Oct4-GFP mice. A: Schematic diagram of summarized SSCs derivation process. B: An Oct4-GFP mouse testis at 13.5 dpc. Mesonephros structure is indicated by an arrow. C: A pair of Oct4-GFP mouse testes at 3 dpp. Mesonephros structure is indicated by an arrow. D: A 5-week-old Oct4-GFP mouse testis. Undifferentiated spermatogonia expressing GFP (arrows) were found to be preferentially present along blood vessels (white arrowhead), exclusively in places where the vessels branch. It is of note that auto-fluorescence signal was also detected along the blood vessels (black arrowheads). E: Cultured SSCs from Oct4-GFP mice. Note that GFP signal of cultured SSCs is markedly weak compared with that of the cells in the testis. Scale bars: 200 μm in B and C; 50 μm in D and E.

DEVELOPMENT OF ES-LIKE COLONIES FROM OCT4-GFP SSCS

When Oct4-GFP SSCs were maintained in culture, the majority of the colonies had the typical appearance of germ cells (Fig. 2A).

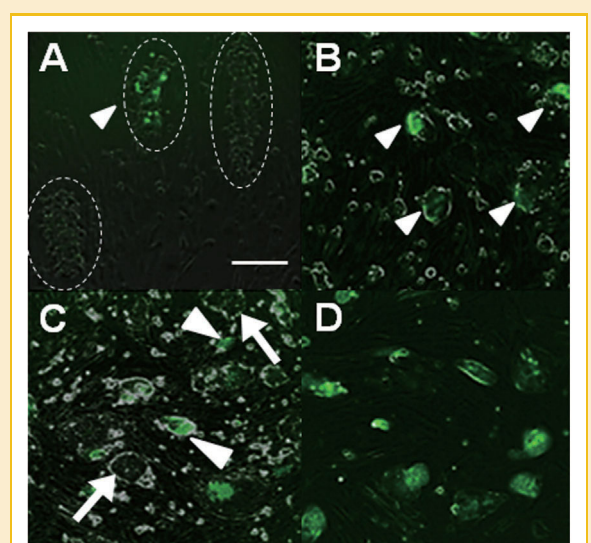

Fig. 2. ES-like cells derived from Oct4-GFP SSCs. A: The morphology of cultured Oct4-GFP SSCs after 1 month of derivation. GFP expression pattern in SSCs was highly heterogeneous. Some SSCs exhibited higher GFP expression (arrowhead) while the expression in most cells remained very weak. SSC colonies are indicated in white dashed lines. B: ES-like colonies (arrowheads) derived from Oct4-GFP SSCs, under SSC culture condition on the first day of their appearance. C: GFP-positive ES-like colonies (arrowheads) and GFP-negative epiblast-like colonies (arrows) on the second day. D: Typical ES-like colonies after first passage. Note that all the ES-like cells are GFP-positive. Scale bar 100 μm.

However, we also found a few rare colonies spontaneously arising from the culture that remarkably resembled mESCs in terms of morphology (Fig. 2B). Notably, GFP signal of the cells in the colonies was significantly stronger than that of SSCs, suggesting that dedifferentiation of SSCs might have occurred. On the 2nd day of their appearance, these colonies were more tightly packed with smooth boundaries. At this stage, GFP-negative epiblast stem cell (EpiSC)-like cells with flattened morphology also appeared (Fig. 2C), which may imply that some SSCs did not complete reprogramming into ES-like state. After the first subculture, most colonies exhibited typical ES morphology and growth rate in the standard ES medium (Fig. 2D). In contrast, SSCs disappeared from the culture in this condition since they could not be propagated due to the absence of GDNF, an essential growth factor for the self-renewing division of SSCs. FACS analysis displayed clear distinction in GFP intensity between SSCs and ES-like cells, validating the use of Oct4-GFP system in reprogramming studies (Supplementary Fig.). The morphology of the ES-like cells did not change as long as the cells were maintained in the standard ES cell culture condition and these cells continuously propagated in vitro for a long time while maintaining an undifferentiated state.

MOLECULAR CHARACTERIZATION OF CULTURED OCT4-GFP SSCS AND THE ES-LIKE CELLS

To examine if the SSC-derived cells with ES-like morphology and stronger GFP expression have the characteristics of pluripotent stem cells, we measured the expression levels of pluripotency markers as well as germ cell specific genes in MEF, Oct4-GFP SSCs, Oct-GFP SSC-derived ES-like cells, and J1 mESCs by qRT-PCR (Fig. 3A). The data showed that the germline specific markers such as *Vasa*, *Dazl*, *Blimp1*, *Nanos*, and *Nanos3* were strongly expressed while pluripotency markers such as *Nanog* and *Oct4* were expressed at lower levels in SSCs. we also confirmed that the expression levels of pluripotency markers in the ES-like cells are similar to those in J1 mESCs. Immunocytochemical analysis clearly indicated that the ES-like cells express crucial regulators of pluripotency, OCT4, SOX2, and NANOG while SSCs are strongly positive for germ cell markers, PLZF, VASA, and DAZL (Fig. 3B). Interestingly, we could not detect OCT4 and SOX2 in the immunostaining, while they were clearly expressed in mRNA levels (Fig. 3A and data not shown). The ES-like cells were positive for AP staining and successfully formed cell lineages of the three germ layers upon differentiation (Fig. 3C and D). These results demonstrated that the ES-like cells with stronger

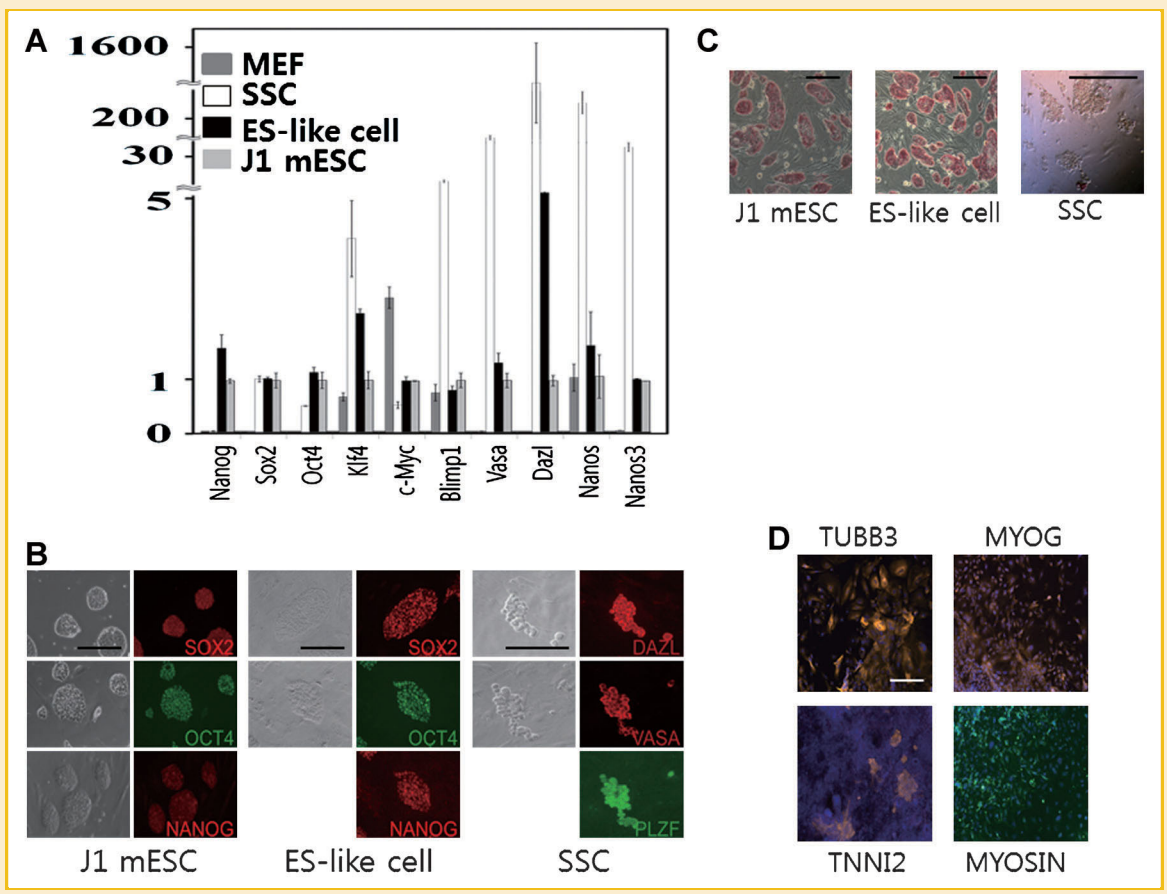

Fig. 3. Molecular characterization of SSCs and the ES-like cells. A: Measurement of gene expression levels by qRT-PCR. QRT-PCR analysis of several pluripotency markers and germline specific genes in MEF, Oct4-GFP SSCs, Oct4-GFP SSC-derived ES-like cells and J1 mESCs was performed. Expression levels of each gene were normalized to the expression levels of β-actin. The J1 mESC gene expression levels were set to unity. Data are shown as mean ± SEM. B: Immunocytochemistry of SSCs and ES-like cells. Note that OCT4, SOX2, and NANOG were not detected in SSCs. C: Alkaline phosphatase staining. Scale bars 100 μm. D: In vitro differentiation assay. Tubb3 (neuronal marker), myogenin (muscle marker), troponin I (skeletal muscle marker) and heavy chain cardiac myosin antibodies were used with Hoechst (blue). Scale bar 200 μm.

GFP signal are indeed pluripotent stem cells originated from cultured SSCs. Importantly, the results also indicated that the reprogramming of SSCs into the ES-like cells could be predicted by the increment of GFP intensity.

EFFECTS OF SMALL MOLECULE INHIBITORS IN OCT4-GFP AND NANOG-GFP MESCS

Small molecule inhibitors have recently been paid great attention in stem cell biology for their applications in the maintenance of undifferentiated states, reprogramming and directed differentiation into a variety of lineage restricted cells [Zhu et al., 2011; Li et al., 2012]. We reasoned that compounds effective for the maintenance of pluripotent stem cells or for dedifferentiation of non-pluripotent cells may also exert an effect on the reprogramming of SSCs into ES-like cells and any influence on reprogramming mechanisms may appear as a change in GFP signal intensity. We first tested a variety of small molecule inhibitors in Oct4-GFP mESCs and analyzed the transcriptional activity of *Oct4* according to the intensity of GFP signal under the microscope. When Oct4-GFP mESCs were maintained with indicated chemicals for 2–3 passages, ERK inhibitors, PD0325901, and SC1, markedly increased GFP signal (Fig. 4A). Combination of PD0325901 and GSK-3β inhibitor CHIR99021 (2*i*) did not seem to further increase the level of GFP although GSK-3β inhibition was shown to be required to optimally maintain mESCs [Ying et al., 2008]. Moreover, PD0325901 appeared to increase GFP intensity in Nanog-GFP mESCs as well (Fig. 4B). Although some inhibitors induced morphological changes of mESCs they did not seem to significantly affect their viability.

We further analyzed the changes of GFP intensity in flow cytometry. We confirmed that in both serum-containing and serum-free culture conditions, PD0325901 but not CHIR99021 intensified GFP signal in Oct4-GFP mESCs (Fig. 4C). In addition, similar patterns were observed in Nanog-GFP mESCs (Fig. 4D), indicating that the inhibition of ERK signaling pathway simultaneously enhances transcriptional level of both *Oct4* and *Nanog* in mESCs. This result implies that Oct4 and Nanog are closely linked in the core regulatory network in mESCs.

MODULATION OF GFP SIGNAL IN OCT4-GFP SSCS BY AN ERK INHIBITOR PD0325901

Pluripotency marker genes such as *Oct4*, *Sox2*, and *Lin28* are expressed in SSCs [Zheng et al., 2009] and the SSC reprogramming

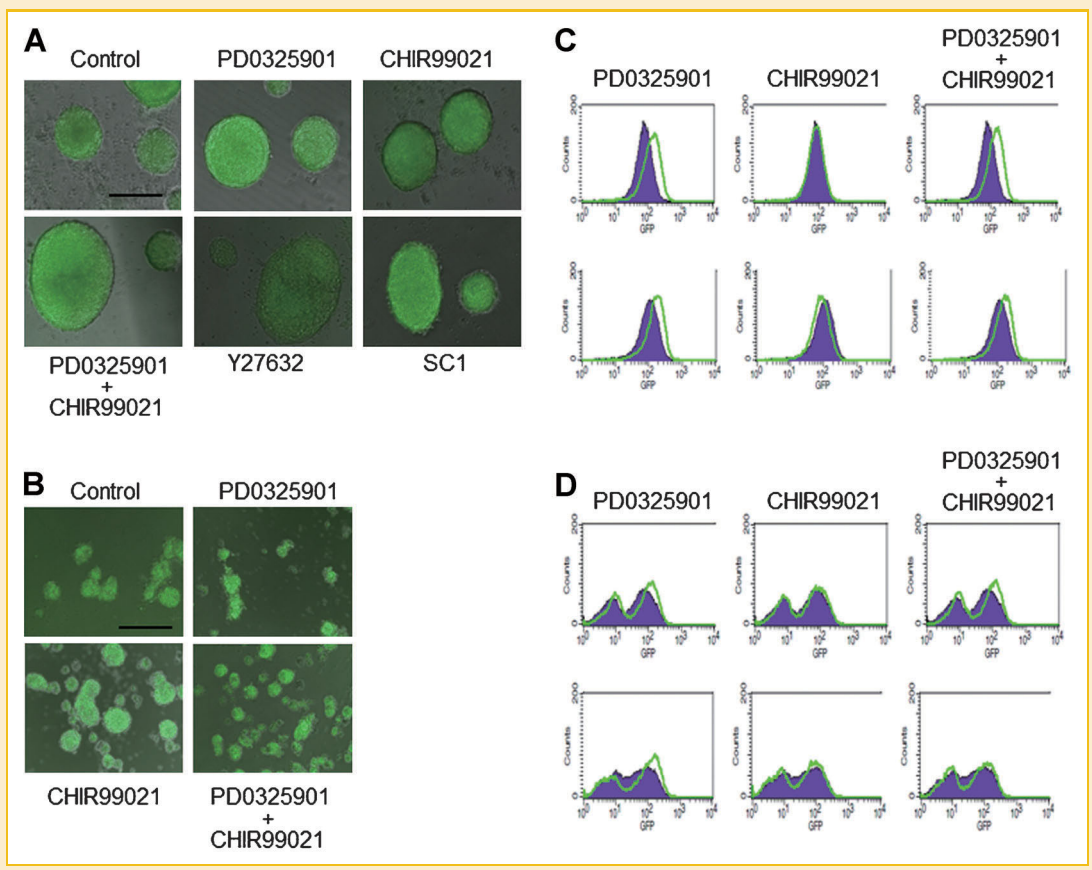

Fig. 4. Effects of chemical inhibitors in mESCs. A: Representative images of Oct4-GFP mESCs cultured with PD0325901 (0.4 μM), CHIR99021 (1 μM), PD0325901 (0.4 μM) + CHIR99021 (1 μM) (2*i*), Y27632 (10 μM) or SC1 (1 μM) in standard ES culture condition. B: Fluorescent images of Nanog-GFP mESCs cultured with PD0325901 (0.4 μM), CHIR99021 (1 μM), PD0325901 (0.4 μM) + CHIR99021 (1 μM) (2*i*). Note that the average size of ES colonies was smaller than those of Oct4-GFP mESC probably due to the presence of a single Nanog allele. C: Flow cytometry analysis of Oct4-GFP mESCs in the presence of indicated small molecule inhibitors. Upper panel: standard mES medium with 15% FBS. Lower panel: the condition where FBS was replaced by Knockout Serum Replacement (KSR) and N2B27 supplement. D: Flow cytometry analysis of Nanog-GFP mESCs in the presence of indicated small molecule inhibitors. Upper panel: standard mES medium with 15% FBS. Lower panel: the condition with KSR and N2B27 supplement. Scale bars 200 μm.

into ES-like cells does not require artificial genetic modifications. Therefore, we hypothesized that even a small change in gene regulatory network may facilitate the initiation of the reprogramming. We speculated that selective small molecule inhibitors can be effectively used for this purpose. To explore the possibility that Oct4-GFP SSCs can be used to screen small molecules effective in reprogramming, we tested several compounds including CHIR99021, Y27632, several selective inhibitors of histone deacetylases (HDACs), histone methyltransferases (HMTs) and p53, which are known to function in mESC maintenance and/or in promoting reprogramming events. Most examined chemicals failed to alter the intensity of GFP signal (data not shown). Notably, PD0325901 dose-dependently increased GFP intensity although the increased GFP expression level seemed insufficient to induce dedifferentiation of the SSCs (Fig. 5A). In qRT-PCR analysis, PD0325901 increased endogenous levels of *Oct4* approximately up to twofold but failed to increase the expression level of *Nanog* (Fig. 5B). This result indicated that activation of ERK functions to downregulate *Oct4* both in mESCs and mouse SSCs. SC1 appeared to have a moderate effect on GFP intensity. However, 1 μM of SC1 seems highly toxic to the SSCs and the cells did not survive the condition in 3–5 days of the treatment (data not shown). In mESCs, *Oct4*, *Sox2*, and *Nanog* govern pluripotency and self-renewal as master regulators [Loh et al., 2006]. The products of these genes function together to sustain the properties of ES cells by auto- and feedforward-regulations. However, it is not clear if these genes play similar functions in the gene regulatory network in SSCs. Therefore, to explain why the increase in *Oct4* is not coupled with the concurrent upregulation of *Nanog* (Fig. 5B), we attempted to measure protein levels of these genes in SSCs. Surprisingly, consistent with the results in immunocytochemistry, the protein levels of OCT4 and SOX2 were extremely low if any and NANOG protein was not detectable in Western blot analysis (Fig. 5C). Furthermore, complete absence of GFP-positive cells in Nanog-GFP mouse testis strongly suggests that *Nanog* is transcriptionally silent in mouse SSCs (Fig. 5D). These data may imply that the Oct4-Sox2-Nanog regulatory triad that controls the intrinsic properties of mESCs does not comprise the same functional regulatory circuitry in SSCs.

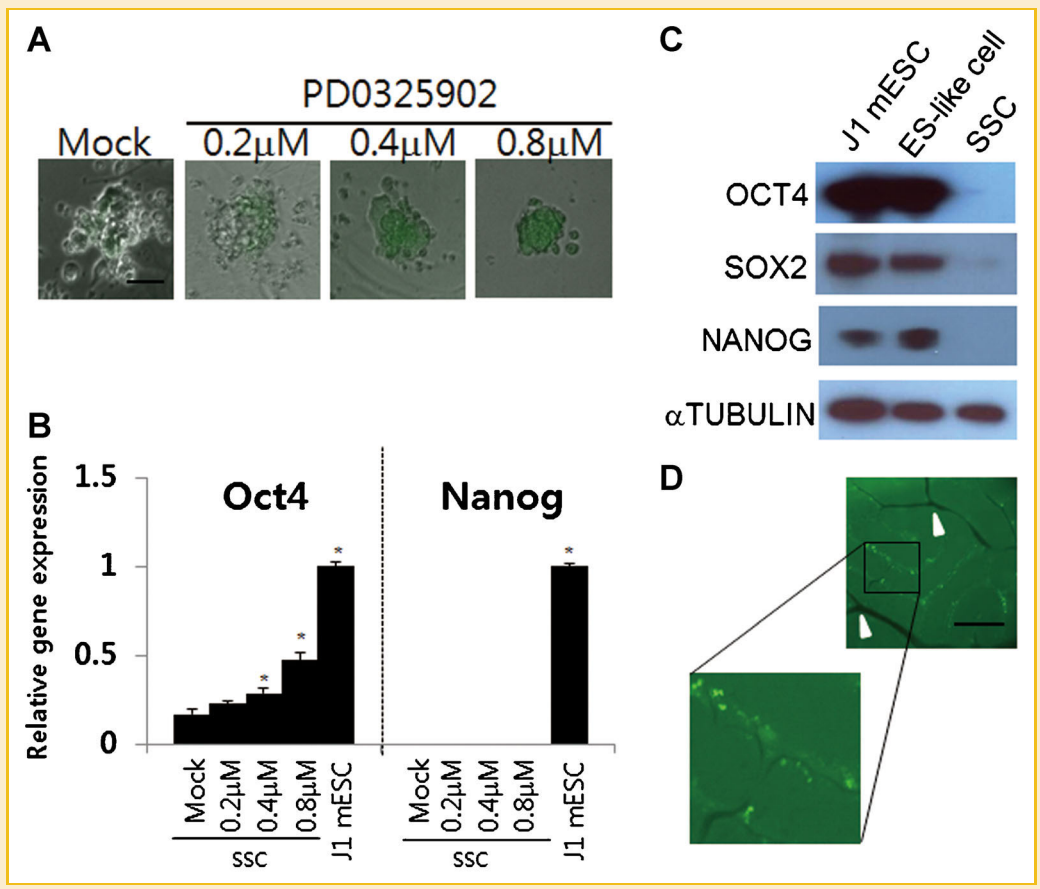

Fig. 5. Effects of an ERK inhibitor PD0325901 and analysis of gene expression in SSCs. A: PD0325901 dose-dependently increased GFP signal in Oct4-GFP SSCs. B: Oct4-GFP SSCs were treated with indicated concentrations of PD0325901 and subjected to qRT-PCR analysis. Expression levels of Oct4 and Nanog were normalized to the expression levels of β-actin. The mESC gene expression levels were set to unity. Data are shown as mean ± SEM. Nanog expression level in the SSCs was approximately a thousand fold less compared with that in J1 ESCs and was not significantly altered by the treatment with PD0325901. $^{*}P < 0.05$ versus mock control. C: Western blot analysis of J1 mESCs, Oct4-GFP SSC-derived ES-like cells and Oct4-GFP SSCs using 50 μg of whole cell extracts. D: A fluorescent image of Nanog-GFP mouse testis from a 35 dpp male. No GFP-positive cells were detected in the testes. Major vasculatures are indicated by white arrowheads. Note that small vasculature structures often generate auto-fluorescence signal. Scale bars: 50 μm in A; 100 μm in D.

DISCUSSION

SSCs are unipotent adult stem cells and give rise to functional spermatozoa through a series of multiple steps of differentiation. Recently, apart from their unique roles in the testes, spontaneous dedifferentiation of the cells into a pluripotent state while they are in culture has been paid special attention due to their possible applications in human regenerative medicine. However, practical uses of ES-like cells derived from SSCs are hardly considered at present since there is a lack of information regarding mechanisms underlying the reprogramming.

Here we report successful derivation and culture of SSCs isolated from mice expressing GFP under the control of *Oct4* regulatory sequences. Although *Oct4* regulatory sequence in SSCs is highly methylated compared to that in embryonic stem cells (ESCs) [Kanatsu-Shinohara et al., 2004], these cells express GFP as they function in vivo throughout all stages of testis development. Relatively weak GFP signal was detected in cultured SSCs, which fortunately allowed better contrast in GFP intensity between SSCs and SSC-derived ES-like cells. Occurrence of spontaneous reprogramming was clearly identified by significantly stronger GFP signal under the fluorescence microscope in real-time in a non-invasive manner. PD0325901 is a small molecule inhibitor targeting mitogen-activated protein kinase (MAPK/ERK kinase or MEK) with potential antineoplastic activity and has also been shown to increase the efficiency of reprogramming human primary fibroblasts into iPSCs [Lin et al., 2009]. In our study, PD0325901 was shown to effectively increase GFP intensity of Oct4-GFP SSCs and the endogenous transcription level of *Oct4*, indicating ERK signaling pathway plays an important role in regulating the transcriptional level of *Oct4* in SSCs. It is of note that a recent study revealed that FGF2 plays an important role for self-renewal of SSCs by activating ERK signaling pathway [Ishii et al., 2012]. In our study, high concentration of another ERK inhibitor, SC1 induced apoptosis of most SSCs while much lower concentrations of PD0325901 did not seem to exert an effect on cell viability, indicating that the amount of inhibitors should be adjusted depending on the purpose of experiments.

In mESCs, the *Oct4–Sox2–Nanog* triad consists of master regulators of pluripotency and self-renewal. Therefore, even moderate modulation of any of these factors may affect the whole gene regulatory network by influencing downstream effectors and other key cofactors connected by regulatory loop. As shown in our study, in mESCs the upregulation of *Oct4* coincided with the simultaneous induction of *Nanog* expression. On the contrary, the expression level of *Oct4* was successfully altered by PD0325901 in SSCs while the level of *Nanog* expression was not affected, indicating that the regulation of Oct4 and Nanog is not coupled in SSCs. Regulatory uncoupling between *Oct4* and *Nanog* may be required to keep SSCs from acquiring pluripotency while maintaining unipotency as germline stem cells. *Oct4* is such a unique gene that the expression of the gene is mandatory in all cases of cellular reprogramming into a pluripotent state whereas other pluripotency marker genes such as *Sox2* and *Nanog* can be omitted in the experimental setup. It is of crucial note that *Oct4* is transcriptionally expressed in SSCs whereas its protein level remains quite low, probably due to posttranscriptional controls of the transcript. Likewise, SOX2 protein expression appears to be strongly suppressed although the transcript level is comparable to that in mESCs. It is extremely intriguing that a small population of mESCs termed 2C cells with endogenous retrovirus activity also exhibits transcriptional activities of these genes without translational output [Macfarlan et al., 2012]. It is speculated that there may exist common posttranscriptional regulatory mechanisms in SSCs and 2C cells that actively suppress translation of OCT4 and SOX2. It is also possible that transcripts of *Oct4* and *Sox2* are expressed in different contexts for unknown roles other than safeguarding pluripotency. In addition, inefficient translation of these genes may explain why *Nanog*, a target gene of OCT4 and SOX2, is transcriptionally silenced in SSCs. Therefore, a common strategy to transcriptionally activate key pluripotency marker genes may not be applicable to induce dedifferentiation of SSCs into ES-like cells. Accordingly, the Oct4-GFP system has a limitation in that it only shows the transcriptional activity of *Oct4*.

However, as shown in the reprogramming event of SSCs, the Oct4-GFP system functions as an excellent indicator of the acquisition of pluripotency. Furthermore, compared with the Nanog-GFP system, which gives fluorescence signal upon the complete gaining of pluripotency, Oct4-GFP can be considered a better reporter system in that it allows visualization of subtle changes in the transcriptional activity of *Oct4* even before the acquisition of pluripotency. Future studies will still be required to elucidate the mechanisms involved in the reprogramming of SSCs. It will be particularly interesting to evaluate the importance of posttranscriptional controls of *Oct4*, *Sox2* and possibly other pluripotency markers in SSCs. For these purposes, Oct4-GFP can be used as an excellent reporter system for real-time visualization of changes in cellular state.

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