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Identification of an Intermediate State as Spermatogonial Stem Cells Reprogram to Multipotent Cells

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The aim of this study was to understand the mechanisms that allow mSSC lines to be established from SSCs. Small, multilayer clumps of SSCs formed during two to four weeks of *in vitro* culture and were then transferred to MEF feeders. Small, round, monolayer colonies containing cells destined to convert to mSSCs, designated as intermediate state SSCs (iSSCs), first appeared after two to three passages. During an additional nine passages (47-54 days) under the same culture conditions, iSSCs slowly proliferated and maintained their morphology. Ultimately, a cell type with an ES-like morphology (mSSC) appeared from the iSSC colonies, and two mSSC cell lines were established. The mSSCs had a high proliferative potential in serum-free ES culture medium and have been successfully maintained since their first establishment (> 12 months). We also compared the specific characteristics of iSSCs with those of SSCs and mSSCs using immunocytochemistry, FACS, RT-PCR, DNA methylation, and miRNA analyses. The results suggest that iSSCs represent a morphologically distinct intermediate state with characteristic expression patterns of pluripotency-related genes and miRNAs that arise during the conversion of SSCs into mSSCs. Our results suggest that iSSCs could be a useful model for evaluating and understanding the initiation mechanisms of cell reprogramming.

INTRODUCTION

Pluripotent stem cells that are derived from embryos, fetuses, and adult tissues are increasingly being used as sources for cell therapy-based regenerative medicine. Among these cell types, embryonic stem (ES) cells (Thomson et al., 1998) used in conjunction with somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997) have drawn particular attention and may open an era of patient-specific cell transplantation therapies for incurable diseases (Egli et al., 2007; Wakayama et al., 2001). However, significant concerns about the use of these techniques remain due to ethical and technical problems associated with the use of human oocytes and embryos. As ES cells are derived from embryos using long-term culture with specific conditions, embryonic germ (EG) cells and multipotent adult progenitor cells (MAPCs) have also been established spontaneously from primordial germ cells (PGCs) and bone marrow-derived cells, respectively (Jiang et al., 2002; Matsui et al., 1992; Shamblott et al., 1998). Somatic cells have also been successfully reprogrammed by fusion with ES cells, and it has been suggested that some factors in ES cells are capable of reprogramming somatic cells into pluripotent stem cells (Cowan et al., 2005; Yu et al., 2006). Takahashi and Yamanaka (2006) have recently identified *Oct-3/4, Sox2, c-Myc,* and *Klf4* as pluripotency-inducing factors from among 24 candidates, and they showed that induced pluripotent stem (iPS) cells could be generated from mouse embryonic or adult fibroblasts by introducing the corresponding pluripotency genes into the cell's genome by retrovirus-mediated gene transfer.

The conversion of SSCs from neonatal hybrid (Kanatsu-Shinohara et al., 2004), adult inbred (Oh et al., 2009), and adult transgenic mice (Guan et al., 2006; Izadyar et al., 2008; Seandel et al., 2007) into ES-like cells (multipotent spermatogonial stem cells, mSSCs), another reprogramming event, has been reported. Interestingly, SSCs and mSSCs, which share an identical genetic origin, exhibit significant differences in gene expression and epigenetic properties (Kanatsu-Shinohara et al., 2008). In particular, mSSCs have lost the expression of germ stem cell-specific genes and show enhanced expression of pluripotent genes that are capable of inducing ES-like cells from fibroblasts (Takahashi and Yamanaka, 2006). In addition, the pluripotent genes *Oct3/4*, *Sox2*, *Myc,* and *Klf4* are known to be expressed at some level in SSCs (Kanatsu-Shinohara et al., 2008).

The type of reprogramming involved in converting SSCs, PGCs, and bone marrow cells into pluripotent stem cells differs from that in the iPS study, and the reprogramming of these

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cells requires only long-term culture under specific culture conditions. However, the process of reprogramming SSCs into a multipotent state and the characteristics of converting SSCs are not yet known. In this study, we attempted to reveal the initiation mechanism for the conversion of SSCs from an outbred mouse strain into mSSCs using long-term culture with specific conditions. The spontaneous reprogramming of SSCs was mediated by a different mechanism than that of iPS.

MATERIALS AND METHODS

Animals

All mice were housed under a 12 h:12 h light:dark cycle in a temperature- and humidity-controlled room. The mice were provided food and water *ad libitum*. Protocols for the use of animals in these experiments were approved by the Institutional Animal Care and Use Committee of CHA University. Testis cells used for this study were obtained from 3-5-day-old neonatal outbred (wild-type) ICR male mice (IcrTacSam, Samtaco Co., Ltd., Korea).

Cell culture for reprogramming

Seminiferous tubules were collected from decapsulated testes and dissociated enzymatically in a buffer containing 5 mg/ml collagenase (Type IV; Sigma-Aldrich, USA), 10 μg/ml DNase I, and 1 mg/ml hyaluronidase (Sigma-Aldrich) for 5 min with agitation at 37°C. The dissociated testicular cell suspension was plated on 0.1% gelatin-coated dishes (1-1.5 × 10^4 cells/cm^2) in serum-free germ-stem cell (GS) medium with modifications as previously described (Kanatsu-Shinohara et al., 2003). The composition of the GS medium was as follows: StemPro-34 SFM (Invitrogen, USA) supplemented with 6 mg/ml D(+)-glucose, 5 × 10^{-5} M β-mercaptoethanol, 1 μM d(L)-lactic acid, 2 mM L-glutamine, 30 μM pyruvic acid, 10^{-4} M ascorbic acid, 60 ng/ml progesterone, 30 ng/ml β-estradiol, (Sigma-Aldrich), 0.2% BSA (ICN Chemicals, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1× Insulin-Transferrin-Se-lenium supplement, 1× MEM vitamin solution, 1× MEM non-essential amino acids, 20 ng/ml mouse epidermal growth factor (EGF), 10 ng/ml human basic fibroblast growth factor (bFGF), 1% Knockout™ serum replacement (KSR, Invitrogen), 10 ng/ml rat glial cell-derived neurotrophic factor (GDNF, R&D systems, Inc., USA) and 10^3 U/ml ESGRO (Chemicon, USA). During culture, somatic testicular cells grew attached to the bottom of the dish. Testicular cell-derived SSCs proliferated slowly and then formed clump-like structures on the somatic cells two to four weeks after seeding. Cultured cells were dissociated by trypsinization and re-plated every five to seven days. SSC clumps obtained from early (0-1) and middle (9-10) passages were transferred onto mitomycin-treated mouse embryonic fibroblast (MEF) feeder layers and passaged every five to six days. When colonies with a flattened or ES-like shape appeared, they were either continuously cultured in GS medium or transferred into serum-free KSR-mES medium, which is composed of Dulbecco's modified Eagle's medium (DMEM) containing 20% KSR, 50 μM β-mercaptoethanol, 1× non-essential amino acids (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and 10^3 U/ml ESGRO (Chemicon), and then they were passaged onto new MEFs every three to five days. All cultured cells were maintained at 37°C in 5% CO_2 and fed two to three times per week.

Analysis of marker genes by RT-PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen), and first-strand cDNA was synthesized using Superscript™ II reverse transcriptase (Invitrogen). The PCR conditions used were 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 40 s. Specific primers used for PCR reactions are shown in Supplementary Table S1.

Immunocytochemistry and FACS analysis

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 10 min at 4°C. Fixed cells were then stained with specific antibodies and analyzed by immunofluorescence microscopy. Immunocytochemistry was performed using specific antibodies (Supplementary Table S2), and nuclei were counterstained with DAPI. Cells were also stained for alkaline phosphatase (AP), as described by the manufacturer (Sigma-Aldrich).

For FACS analysis, dissociated cells were stained with specific antibodies (Supplementary Table S2) and then analyzed with a FACSVantage SE (BD Biosciences, USA).

DNA methylation profiles of Oct4 and Nanog in SSCs, iSSCs and mSSCs

Genomic DNA was purified by phenol/chloroform/isoamyl alcohol extraction. Bisulfite genomic sequencing was carried out using the EZ DNA Methylation-Gold Kit (Zymo Research, USA) using specific primers (Supplementary Table S3), as described previously (Cavaleri et al., 2008; Blelloch et al., 2006). Cycling conditions for first-round PCR were as follows: 94°C for 5 min; 5 cycles at 94°C for 30 s, 56°C for 1 min (-1°C per cycle) and 72°C for 1 min, followed by 30 cycles at 94°C for 30 s, 51°C for 45 s and 72°C for 1 min 20 s. Second-round PCR conditions were 94°C for 5 min, 30 cycles of 94°C for 30 s, 53.5°C for 1 min, and 72°C for 1 min 20 s. The resulting amplified products were gel-purified (NucleoSpin Extract II, MACHEREY-NAGEL Inc., USA), subcloned into the pGEM-T EASY vector (Promega, USA) and sequenced using M13R primers.

microRNAs expression analysis in SSCs, iSSCs and mSSCs

Preparation of small RNAs and Hybridization: Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). To prepare the cellular miRNAs, small-sized RNA containing miRNAs was isolated from total RNA using the RNeasy MinElute Cleanup kit (Qiagen, USA) and then labeled with a fluorescent dye using the Platinum Bright 647 Infrared nucleic acid labeling kit (KREATECH, Netherlands), according to the manufacturer's instructions. Labeled miRNA was hybridized to Genopal®-MICH DNA chips (Mitsubishi Rayon, Japan), and hybridization signals were examined and analyzed using a DNA chip image analyzer, following the manufacturer's instructions. The microarray analysis was repeated at least twice, and hybridized signal intensities were analyzed as described previously (Hohjoh and Fukushima, 2007a; 2007b).

miRNA quantitative stem-loop real-time PCR: Single-tube TaqMan miRNA assays were used as previously described (Chen et al., 2005). Comparative real-time PCR reactions, including no template controls, were performed in triplicate using specific primers for mmu-miR-290, mmu-miR-293, mmu-miR-295 and mmu-let-7f. The reactions were incubated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reagents and protocols were from Applied Biosystems (USA), and all data were analyzed using Cluster 2.11 and Treeview 1.60, available from Michael Eisen's laboratory (http://rana.lbl.gov/EisenSoftware.htm) (Eisen et al., 1998).

Characterization of iSSC and mSSC using *in vitro*/*vivo* differentiation

For *in vitro* differentiation through embryoid body (EB) formation,

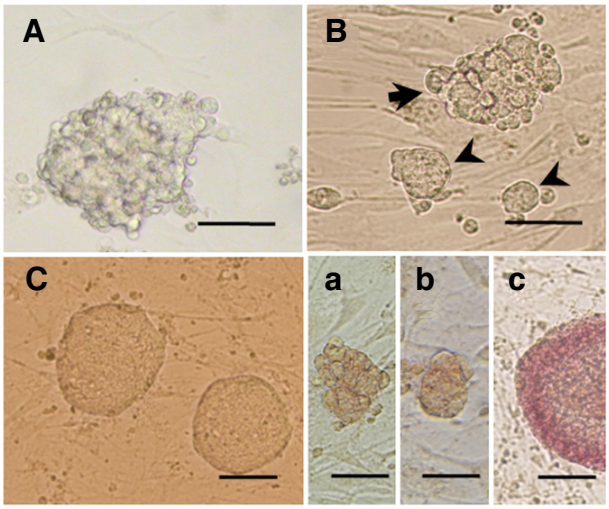

Fig. 1. Appearance of two types of SSCs and multipotent SSCs. (A) Spermatogonial stem cells (SSCs) formed a clump morphology and continuously expand in serum-free GS medium. (B) After being transferred onto a MEF feeder layer, two shapes of SSCs appeared; the typical shape of clumps (SSC, arrow) and a rare shape of small, round colonies (intermediate state SSCs (iSSCs), arrowhead) could be observed in the same culture conditions. (C) Finally, multipotent spermatogonial stem cells (mSSCs), which derive from intermediate state SSCs, were established in KSR-ES medium. (a-c) All three types of cell showed AP activity. Bars = 100 μm (A and B) and 50 μm (a-c).

iSSC and mSSC colonies were picked and dissociated. Isolated cells were then transferred into DMEM medium containing 15% FBS (Hyclone, USA) and aggregated in 50 μl medium using the hanging-drop method. EBs were cultured for five to ten days and collected for RT-PCR and immunocytochemistry analyses.

For the *in vivo* differentiation, 1-2 × 10^5 putative iSSCs and mSSCs were harvested and injected into testicular capsules of 4-8-week-old severe combined immunodeficient (SCID) mice (CB 17 strain; Jackson Laboratory, USA) using a sterile 26G needle. After five to six weeks, the resulting tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined histologically.

RESULTS

Morphological conversion of SSCs and appearance of ES-like colonies from neonatal SSCs

To develop a more efficient and practical protocol for the establishment of mSSC, we modified Shinohara's culture method into a three-step procedure. In the first step, testicular cells containing SSCs obtained from neonatal ICR mice (unmodified outbred strain) were incubated in GS medium without any specific antibody-mediated cell sorting. SSCs formed small multilayer clumps and proliferated on feeder cells derived from their own testicular somatic cells (Fig. 1A). In the second step, clumps obtained from cells cultured for 0-1 and 9-10 passages were recovered and transferred onto MEFs. During cultivation, SSC clumps not only continued to actively divide (Fig. 1B, arrow), but some SSC colonies began to change their morphology slightly to a round, monolayer shape; the cells in these colonies were designated as intermediate state SSCs (iSSCs) (Fig. 1B, arrowhead). Thereafter, colonies began to appear with a full ES-like morphology from iSSCs within six to nine passages (40-54 days) after being transferred onto MEFs, and these cells were designated as multipotent SSCs (mSSCs). As the two monolayer types of cell (iSSCs and mSSCs) appeared, cultured cells were divided into two groups and transferred to different culture media; in this third step, one group was maintained in GS medium as a control and the other group was transferred into mouse ES medium. Cells from round, flat-shaped colonies (iSSCs) slowly proliferated in GS medium and were maintained for more than ten additional passages (20-30 passages total) before finally disappearing or retracting into clumps. Consequently, mSSCs were not supported in GS medium and eventually disappeared. Conversely, mSSC colonies quickly proliferated in KSR-mES medium and became the dominant population within ten additional passages; these cells have been maintained continuously since then (> 12 months) for a total of more than 100 passages (Fig. 1C). Colonies of mSSCs were passaged every three to five days onto new MEFs using 1:15 to 1:20 dilutions. iSSC colonies were not supported in KSR-mES culture medium and disappeared within five to seven passages. Ultimately, two cell lines showing ES-like morphology, designated as CHA-mSSC-1 and -2, were established from outbred, wild-type mice and were cultured in KSR-mES culture medium without any antibody-mediated cell sorting. We first examined stem cell activity in all three types of cell (SSS, iSSC and mSSC) using alkaline-phosphatase (AP) activity staining. Positive staining was more prominent during the transition from SSC to mSSC (Figs. 1a-1c). Strong AP activity signals were especially dominant for both mSSCs and mouse ES cells maintained in KSR-mES medium. However, mSSCs cultured in GS medium exhibited a reduction in the AP signal as compared to those mSSCs cultured in ES medium, possibly reflecting cellular differentiation (data not shown).

Immunocytochemistry of stem cell markers

To analyze the characteristics of cell clumps and the two types of colony derived from iSSCs and mSSCs, immunocytochemical analysis was used with germ cell and embryonic stem cell-specific antibodies. Cultured cells (9 passages) on MEFs were co-stained with anti-Oct4 (green color) and anti-SSEA1 (red color) antibodies (ESC-specific markers) (Solter and Knowles, 1978). The SSC clumps grown with testicular cells stained positively with several germ cell markers and an anti-Oct4 antibody (Supplementary Fig. S1). Likewise, clumps of SSCs grown on MEFs only stained positively with the Oct4 antibody. However, as expected, iSSC colonies (white arrow) were co-stained with both antibodies (Oct4 and SSEA1) (Fig. 2A). Surprisingly, iSSC colonies clearly showed the expression of pluripotent-specific markers (Nanog, Sox2, Klf4 and c-Myc), which were not expressed in the SSC clumps during cultivation (Fig. 2B). mSSCs were also immnunostained and showed strong signals for pluripotent stem cell markers (Oct4, Nanog, Sox2, and SSEA1) (Supplementary Fig. S2).

FACS analysis

To quantify the characteristic changes associated with conversion from SSC to mSSC, we collected the three types of cultured cell, SSCs, iSSCs and mSSCs, and analyzed each for reactivity to specific antibodies by FACS. The SSCs obtained from primary cultured cells showed a relatively high percentage of Thy-1^+ germ cells (16.27%), but SSEA1 was essentially undetectable (0.66%) (Fig. 3A). The percentage of germ cells (Thy-1^+) gradually decreased in the iSSC and mSSC groups to 9.42% and 2.21%, respectively (Figs. 3B and 3C). By contrast, the population of c-kit^+ cells increased from the SSC group

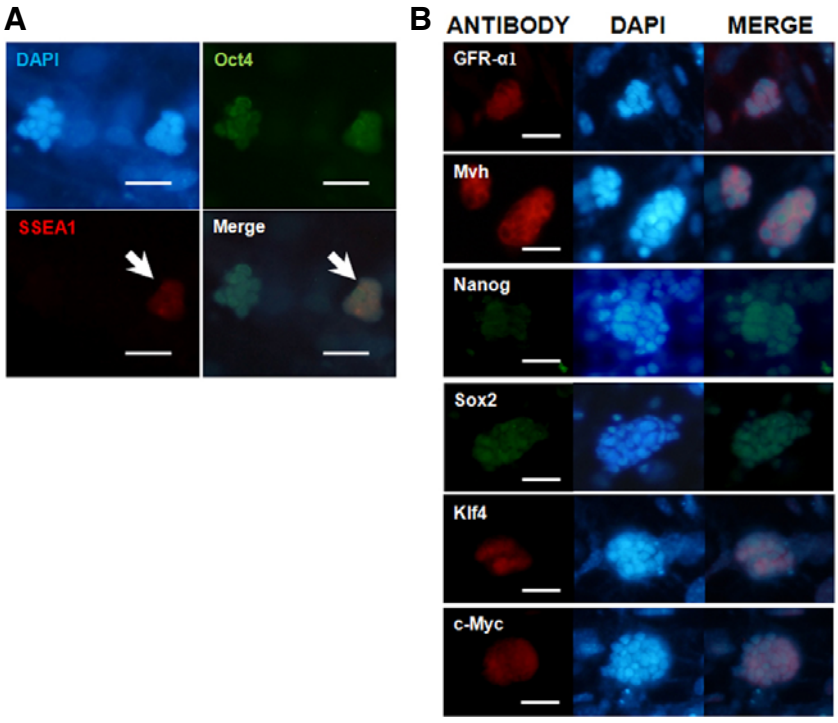

Fig. 2. Immunocytochemical analysis of intermediate state SSCs. (A) The clump type of SSCs and the small, round type of intermediate state SSCs on MEFs were double-immunostained with the ES-specific antibodies, anti-Oct4 (green color) and anti-SSEA1 (red color). SSCs stained positively with anti-Oct4 antibody only, but iSSCs co-stained with the anti-Oct4 and anti-SSEA1 antibodies (white arrow). (B) iSSC colonies also stained positively with germ cell-(GFR-α1 and Mvh) and other ES-specific antibodies (Nanog, Sox2, Klf4, and c-Myc). Nuclei were counterstained with DAPI. All bars = 50 μm.

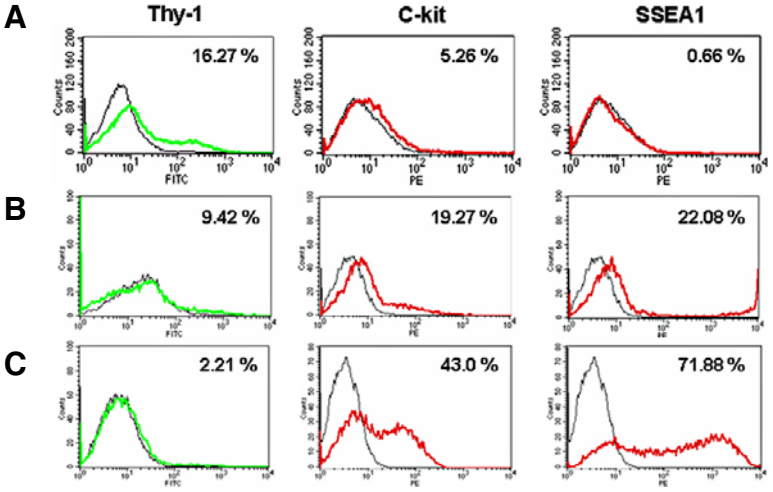

Fig. 3. FACS analysis of iSSCs and mSSCs. (A) SSCs grown on testicular cells, (B) iSSCs (ten passage-converted SSCs on MEFs), and (C) mSSCs were stained with antibodies against specific germ cell (Thy-1), stem cell (C-kit) and mouse ES cell (SSEA1) markers and analyzed by FACS. Black line, control immunoglobulin; green/red line, specific antibodies.

(5.26%) to the iSSC (19.27%) and the mSSC (43.0%) groups, respectively. The number of SSEA1$^+$ cells similarly increased during SSC reprogramming; there were few SSEA1$^+$ cells among the SSCs (0.66%), and their number increased in the iSSC (22.08%) and mSSC (71.88%) groups (Figs. 3B and 3C). These FACS data also support the idea that SSCs can convert to mSSCs by passing through an intermediate state during spontaneous reprogramming.

Gene expression analysis during conversion to mSSCs

The expression profiles of stem cell and germ cell-specific genes were analyzed during the transformation of SSCs to mSSCs by RT-PCR (Fig. 4). The SSC clumps obtained from our first culture system expressed high levels of mRNA for the SSC-specific markers, *Piwil2, Mvh* and *Ngn3*, and showed relatively low levels of mRNA for the ES cell-specific markers, *Intβ1* and *Oct4*. However, these cells expressed some of the pluripotent stem cell-specific markers, *Klf4* and *c-Myc*, but they did not express the other pluripotent marker genes, *Nanog* and *Sox2*. During long-term culture, iSSCs and mSSCs expressed all markers of SSCs with reduced expression levels of *Piwil2 and Mvh.* Interestingly, *Nanog* and *Sox2,* which were not expressed in SSCs, were upregulated during the transition from iSSCs to mSSCs. In addition, *Intβ1* and *Oct4* expression were enhanced to a greater extent in mSSCs than in iSSCs. The expression levels of *Ngn3*, *Stat3, Klf4,* and *c-Myc* did not change during cell conversion.

DNA methylation analysis

To analyze the contribution of epigenetic modifications to the

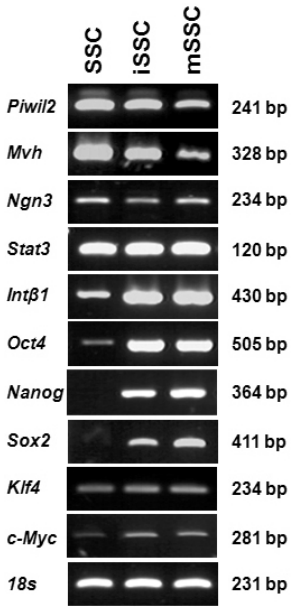

Fig. 4. Comparison of gene expression profiles during dedifferentiation. (A) For comparison of the gene expression profiles, three different RNA groups of cells derived from spermatogonial stem cells (SSC, iSSC, and mSSC) were analyzed by RT-PCR. Germ cell-marker genes: *Piwil2*, *Mvh*, *Ngn3*, *Stat3*, and *Intβ1*; and Pluripotent cell-marker genes: *Oct4*, *Nanog*, *Sox2, Klf4, and c-Myc*.

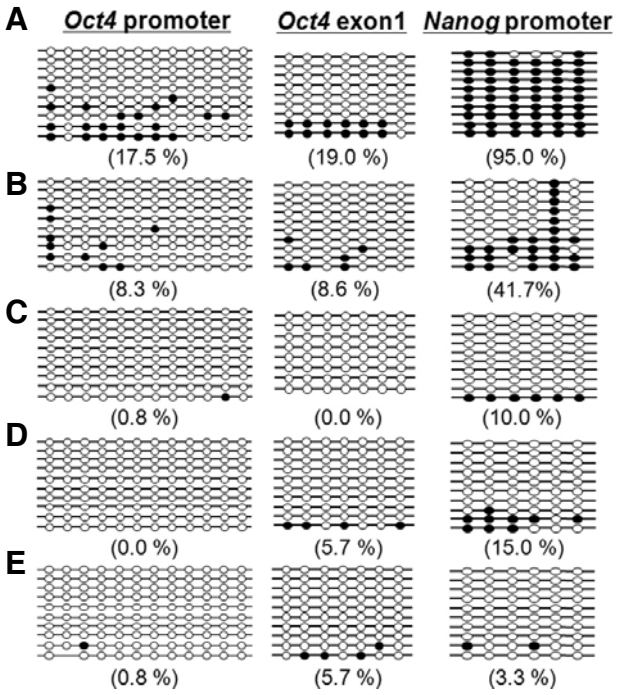

Fig. 5. DNA methylation analysis of SSCs, iSSCs, and mSSCs. DNA methylation of promoter (*Oct4* and *Nanog*) and *Oct4* exon1 regions were analyzed in (A) SSCs, (B) iSSCs and (C and D) CHA-mSSCs-1 to 2 lines by bisulfite genomic sequencing. Black spots indicate methylated cytosine-guanine sites (CpGs), and white spots indicate unmethylated CpGs. (E) mESCs were used as a control.

expression level of these genes, we analyzed each of the three types of cell (SSCs, iSSCs and mSSCs) for DNA methylation patterns in the promoters of the *Oct4 and Nanog* genes, as well as in the region of exon 1 of the *Oct4* gene, using bisulfite genomic sequencing (Fig. 5). The promoter and exon 1 region of *Oct4* were mostly demethylated in SSCs (Fig. 5A) and iSSCs (Fig. 5B) (promoter: 17.5% and 19.0% for SSC and iSSC, respectively; exon 1: 8.3% and 8.6% for SSC and iSSC, respectively); the CHA-mSSC-1 and -2 lines (Figs. 5C and 5D) were even less methylated (promoter: 0.8% and 0% for CHA-mSSC-1 and -2, respectively; exon 1: 0% and 5.7% for CHA-mSSC-1 and -2) and were similar to the mES cells used as an experimental control (Fig. 5E) (0.8% and 5.7% for promoter and exon 1, respectively). However, the DNA methylation patterns at the promoter region of *Nanog* were strikingly different for all groups. The *Nanog* promoter in the SSCs was highly methylated (Fig. 5A) (95%), but CHA-mSSC-1, -2, and mES cells were only 10.0%, 15.0% and 3.3% methylated, respectively (Figs. 5C-5E). Additionally, to confirm the demethylation in iSSCs, we compared the DNA methylation pattern of the *Nanog* promoter among four different iSSC samples (I-IV) (Fig. 6). Surprisingly, all iSSCs showed evidence of demethylation at the *Nanog* promoter, where the DNA methylation level dropped to 53.56% ± 21.66% (mean ± SD; $n = 4$). These results strongly suggest that *Nanog* is released from a transcriptionally inactive state by demethylation during the spontaneous reprogramming of SSCs.

miRNA and stem-loop real-time RT-PCR analysis

Although miRNAs have not been profoundly studied in stem cells to date, they are thought to have an important role in regulating pluripotency during the spontaneous reprogramming of ES cells or the generation of iPS cells through the introduction of pluripotency factors (Lin et al., 2008; Suh et al., 2004). We analyzed the miRNA expression profiles in mSSCs, Sertoli cells, MEFs and mouse ES cells (Supplementary Fig. S3). Among the array data, we focused on two clusters that had distinctive patterns of expression in the three germ-derived cells. Cluster 1, corresponding to the *miR-290* cluster (*miR-290, 291a, 291b, 292, 293, 294, 295*) of miRNAs that is known to be specifically expressed in mES cells (Houbaviy et al., 2003), was not detected in SSCs, Sertoli cells or somatic cells; however, this cluster was highly upregulated in the two mSSC lines as well as in mouse ES cells (Fig. 7A). Cluster 2, corresponding to the *let-7* cluster (*let-7a-7f*), which is known to be expressed in all tissues and adult stem cells including SSCs (Pasquinelli et al., 2000; Wulczyn et al., 2007), was not expressed in mSSCs or mouse ES cells (Fig. 7B). The expression patterns of selected miRNAs from the two clusters were compared and confirmed in the three SSC types by stem-loop real-time RT-PCR. miRNAs *miR-293* and *-295* of cluster 1 were upregulated in iSSCs and the mSSC lines, as shown in Fig. 7A. Another miRNA, *miR-290,* was not differentially expressed between SSCs and iSSCs (Fig. 7C), but it was highly upregulated in mSSCs. miRNA *let-7f* of cluster 2 was only upregulated in SSCs. Interestingly, this miRNA, a member of the *let-7* family of miRNAs, was suppressed not only in mSSCs but was also downregulated in iSSCs (Fig. 7D). These results imply that the conversion of SSCs into mSSCs may be similar to the spontaneous reprogramming described in ES cells and suggest that specific miRNAs may regulate the expression of pluripotency-related genes.

Analysis of *in vitro* and *in vivo* differentiation potency

To determine the *in vitro* differentiation potential, we collected iSSCs and mSSCs for embryoid body (EB) formation. EBs from mSSCs were cultured and analyzed by RT-PCR and immuno-

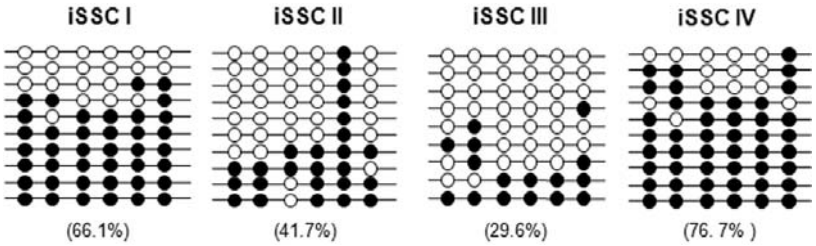

Fig. 6. DNA methylation patterns of the *Nanog* promoter region in different iSSC groups. The *Nanog* promoter region was analyzed in four different groups (I to IV) of iSSCs by bisulfite genomic sequencing. Black spots indicate methylated cytosine-guanine sites (CpGs), and white spots indicate unmethylated CpGs.

cytochemistry (Supplementary Fig. S4). In contrast to the suc cessful EB formation of mSSCs, iSSCs could not be assessed because EBs failed to form or only partially formed (data not shown). To confirm the *in vivo* differentiation potential, we injected iSSCs and mSSCs subcutaneously into the testes of immune-deficient mice and examined their ability to form teratomas *in vivo*. Injected mSSCs gave rise to typical teratomas in recipients (two of two) by five to six weeks after transplantation. The teratomas contained derivatives of the three embryonic germ layers (Supplementary Fig. S5). However, similar to the *in vitro* system, iSSCs transplanted into the mice failed to form tumors (3 of 3) (data not shown). Additionally, we demonstrated the pluripotency of mSSCs through chimera production (Supplementary Fig. S6) and confirmed that the cultured mSSC lines have a normal karyotype through karyotypic analysis (Supplementary Fig. S7).

DISCUSSION

The reprogramming of somatic cells (Cowan et al., 2005; Takahashi and Yamanaka, 2006) or unipotent stem cells into a pluripotent state (Clarke et al., 2000; Gimble et al., 2007; Jiang et al., 2002) has been the most prominent progress in the stem cell research field. However, unanswered questions still remain, and an urgent area that needs to be understood is the molecular and cellular mechanisms of the reprogramming process. To answer this question, we investigated and identified the transitional states between the somatic cell state and the multipotent stem cell state. Previous reports have made reference to ES-like or epiblast-like colonies that arise as an outcome of the dedifferentiation of SSCs (Guan et al., 2006; Seandel et al., 2007; Shinohara et al., 2004); however, to date, no study has clearly shown a transitional state or intermediate state during reprogramming. Furthermore, the timing of when the unipotent state of SSCs begins to change to the pluripotent state and the distinguishing features of dedifferentiating SSCs during reprogramming are not understood. Here, we showed that during a three-step culture of SSCs, clumped and multilayered SSC colonies attached firmly onto a MEF feeder layer and showed distinct colony morphology compared with SSC colonies (Fig. 1B). At this stage, SSCs started to express pluripotent ES cell markers, such as SSEA1 and Nanog, and *Oct4* expression was highly upregulated. Furthermore, the colony shape was much more similar to a mouse ES cell colony, which is flat and forms a monolayer, and we designated this state as intermediate SSCs. To determine the kinds of cellular and molecular differences present in our culture system, we further analyzed the characteristics of SSCs, iSSCs, and mSSCs. AP staining increased from SSCs and iSSCs (Figs. 1a and 1b) to mSSCs (Fig. 1c). The dedifferentiation of SSCs can be observed in the gene expression analyses and the FACS analysis. These results suggest that iSSCs may represent a transitional state of culture-induced reprogramming. iSSCs required continual culture conditions in order to progress toward a fully reprogrammed

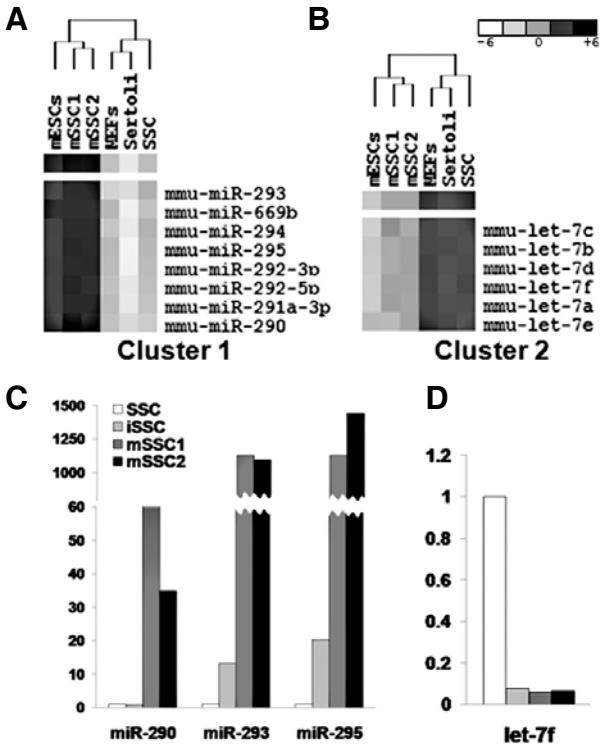

Fig. 7. microRNA and stem-loop real time-PCR analysis. (A) Cluster 1 (*miR-290* family, ES-specific) showed expression in mES and two mSSC lines. (B) Cluster 2 (*let-7* family) was specifically expressed in SSCs and differentiated cells. (C and D) Using real time-PCR, selected miRNAs from each clusters were analyzed and displayed similar results to those obtained with the miRNA array data. ES cells, Sertoli cells, and MEFs were used as control groups; and SSC was used as a relative control in the real time-PCR.

pluripotent state. This observed process is similar to the direct reprogramming of mouse somatic cells into iPS cells via the transfection of four defined factors (Brambrink et al., 2008).

Guan and colleagues reported that their isolated and cultured SSCs could restore spermatogenesis and embryogenesis (Guan et al., 2006). However, they did not evaluate whether the mSSCs already existed in the cultured SSCs or whether a cell type of transitional state existed in the cultured SSCs. Here, we clearly showed that morphologically distinguished colonies are present during the three-step culture. The intermediate state SSCs have characteristics that are very similar to those of mSSCs with respect to the expression of stem cell markers, such as Oct4, Nanog, Sox2, and SSEA1. A significant difference between SSCs and iSSCs is the expression of major markers of pluripotency. During the transition of SSCs into iSSCs, the promoter and exon 1 region of *Oct4* became slightly demethylated.

By contrast, the DNA methylation status of *Nanog* changed markedly, dropping to a methylation percentage in iSSCs that was about half that of SSCs. After conversion to mSSCs, the promoter became fully demethylated (Fig. 5). This demethylation may be a reason for the morphological and phenotypical changes in the SSC colonies, and further study is needed to elucidate whether this is the case.

To characterize the reprogramming process of SSCs, we analyzed the expression pattern of mouse ES cell-specific and somatic cell-specific miRNA clusters (Fig. 7). Our results showed that the miR-290 cluster, called the mouse ES cell-specific cell cycle-regulating miRNA cluster (Kim et al., 2009), was dramatically upregulated after reprogramming. Recently, Marson et al. (2008) revealed that the expression of the miR-290 cluster is regulated by *Oct4*, *Sox2* and *Nanog* and is a key mES cell transcription factor. This study integrated miRNAs into the regulatory circuitry that controls the stemness of mES cells and this explains the upregulation of the miR-290 cluster. In addition, the let-7 family, which is known to be a key regulator of neurons (Wulczyn et al., 2007), male germ cell developmental differentiation (Hayashi et al., 2008), and a cancer tumor suppressor (Takamizawa et al., 2004), was highly expressed in SSCs and somatic cell groups and was dramatically downregulated in mSSCs. This finding implies that SSCs are tightly restricted in their gene-regulation state relative to other cell types. Collectively, these results suggest that mouse ES cell-specific miRNAs may be involved in the reprogramming of SSCs and the maintenance of pluripotency.

In the present study, we induced reprogramming using a three-step culture system without genome modification or cell sorting. We successfully established two mSSC lines from outbred ICR mice. This is the first report that proves the existence of an intermediate state of SSCs during the conversion of unipotent SSCs into multipotent SSCs. The intermediate state of SSCs has similar expression profiles of pluripotent stem cell markers to that of mSSCs, yet the expression of germ cell markers is preserved. Although iSSCs did not support pluripotency, fully reprogrammed cells (mSSCs) from iSSCs displayed powerful developmental potency *in vivo* and *in vitro,* like mES cells (Supplementary Figs. S4-S6). These results clearly suggest that the morphological transformation of SSCs into an intermediate state indicates the start of the expression of specific stemness-regulating genes and specific miRNAs that reprogram SSCs during long-term culture under specific conditions. Additionally, this information suggests that our method could be used to screen candidate cells that could become pluripotent and would provide unique insight into the initiation mechanisms for reprogramming SSCs and other stem cells.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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