Regulation of pluripotency in male germline stem cells by Dmrt1

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Spermatogonial stem cells (SSCs) present the potential to acquire pluripotency under specific culture conditions. However, the frequency of pluripotent cell derivation is low, and the mechanism of SSC reprogramming remains unknown. In this study, we report that induction of global DNA hypomethylation in germline stem (GS) cells (cultured SSCs) induces pluripotent cell derivation. When DNA demethylation was triggered by Dnmt1 depletion, GS cells underwent apoptosis. However, GS cells were converted into embryonic stem (ES)-like cells by double knockdown of Dmrt1 and p53. This treatment down-regulated Dmrt1, a gene involved in sexual differentiation, meiosis, and pluripotency. Dmrt1 depletion caused apoptosis of GS cells, but a combination of Dmrt1 and p53 depletion also induced pluripotency. Functional screening of putative Dmrt1 target genes revealed that Dmrt1 depletion up-regulates Sox2. Sox2 transfection up-regulated Oct4 and produced pluripotent cells. This conversion was enhanced by Oct1 depletion, suggesting that the balance of Oct proteins maintains SSC identity. These results suggest that spontaneous SSC reprogramming is caused by unstable DNA methylation and that a Dmrt1–Sox2 cascade is critical for regulating pluripotency in SSCs.

[Keywords: DNA methylation; pluripotency; reprogramming; spermatogonia; teratoma]

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Germ cells are thought to have pluripotency potential because they form teratomas. Teratomas are spontaneously formed in mice on a 129 background, and this strain was used to derive embryonic stem (ES) cells [Evans and Kaufman 1981; Stevens 1984]. Although the frequency of spontaneous teratoma development was found to be limited to ~2%, studies have shown that primordial germ cells (PGCs) in the fetus can be induced to form teratomas by transplanting the genital ridges of midgestational embryos into ectopic locations in other animals [Stevens 1984]. In the most successful cases, teratomas were found in ~80% of the PGC transplants. PGCs have pluripotency potential up until 12.5 d post-coitum (dpc). Although these classic experiments provided the foundation for teratoma studies, the mechanism of teratoma development has remained unknown. However, in 1992, two groups [Matsui et al. 1992, Resnick et al. 1992] demonstrated that PGCs can become ES-like pluripotent embryonic germ (EG) cells in vitro when they are cultured with Kit ligand [KitL], fibroblast growth factor2 [FGF2], and leukemia inhibitory factor [LIF]. EG cells are similar to ES cells, except in their DNA methylation patterns, and formed chimeras when the cells were transferred into blastocysts [Matsui et al. 1992, Labosky et al. 1994]. The frequency of EG cell formation decreases gradually during development, and such potential pluripotency is no longer found in the germline later than 12.5 dpc [Labosky et al. 1994].

In 2003, a long-term culture system for spermatogonial stem cells (SSCs), in which SSCs proliferated in vitro in the presence of FGF2 and glial cell line-derived neurotrophic factor (GDNF), was developed [Kanatsu-Shinohara et al. 2003]. Cultured SSCs, designated as germline stem (GS) cells, form grape-like clusters of spermatogonia in vitro but reinitiate spermatogenesis when transplanted into seminiferous tubules of infertile testes. Although GS cells are unipotent and produce sperm, they were subsequently found to transform into ES-like cells [Kanatsu-Shinohara et al. 2004]. These ES-like cells, called multipotent GS (mGS) cells, often appear as sheets of epiblast-like cells, which transformed into ES-like compact colonies upon passaging. Despite their spermatogonial origin, they proliferate without GDNF and produce teratomas.
in seminiferous tubules but are able to contribute to the blastocyst, thereby producing germline chimeras. Additional studies revealed that GS cells directly transform into mGS cells [Kanatsu-Shinohara et al. 2008], and similar cells were also derived from other species, including humans, although some of these results are being questioned [Ko et al. 2010b; Tapia et al. 2011].

While these results showed the pluripotency potential of SSCs, several critical issues remain to be answered. One is their low derivation frequency: mGS cells develop in only one out of every ~30 tests during GS cell derivation [Kanatsu-Shinohara et al. 2004]. Although several groups also reported the derivation of pluripotent cells from postnatal male germ cells, discrepancies exist among these studies in terms of the nature and efficiency of reprogramming [Geijsen and Hochedlinger 2009]. For example, one study showed the usefulness of LIF and obtained four lines from 21 mice [Guan et al. 2006]. However, multipotent adult GS [maGS] cells produced in this study were unique in that they contribute to not only spermatogenesis but also embryogenesis, but the result of this study is being questioned [Geijsen and Hochedlinger 2009]. Although beneficial effects of coculturing on testicular CD34+ stromal cells in pluripotency induction were reported in another study [Seandel et al. 2007], even better results were obtained by low-cell-density culture on mouse embryonic fibroblasts [MEFs], and six ES-like germline-derived pluripotent stem (gPS) cells were derived from eight testes within 4 wk in the most successful case [Ko et al. 2012]. However, the quality of MEFs is still a critical factor, and an additional 3–4 wk are required when ES-like cells do not appear by low-cell-density cultures [Ko et al. 2010a]. In addition to the variation in induction procedure, the difference in DNA methylation patterns in the differentially methylated region [DMR] of H19 is also pointed out. While gPS cells show androgenetic DNA methylation patterns, maGS cells exhibit somatic cell DNA methylation patterns [Guan et al. 2006; Ko et al. 2009]. It also should be noted here that many of these studies claimed derivation of ES-like cells from 129 or C57BL/6 (B6) mice, whose SSCs never proliferate without augmenting GDNF signal by GFRA1 supplementation [Kubota and Brinster 2008].

Such low or inconsistent derivation efficiency has made it difficult to study the molecular mechanism underlying pluripotency induction. We initially noticed that mGS cells often develop during initiation of GS cell cultures and that p53 deficiency improves their derivation [Kanatsu-Shinohara et al. 2004]. We also found that mGS cells occasionally appear after freezing–thawing or electroporation [Kanatsu-Shinohara et al. 2005, 2008]. Unexpectedly, GS cells were resistant to transfection of Yamanaka vectors. Real-time PCR confirmed down-regulation of Dmrt1 expression after reprogramming into mGS cells, we directly examined the role of Dnmt1, which maintains DNA methylation. To induce DNA demethylation, we used a Dnmt1 knockdown vector. When we induced demethylation by Dnmt1 knockdown, the majority of cells underwent p53-dependent apoptosis [Fig. 1B], but no mGS cells were found. However, because Dnmt1 is a maintenance methylase and passive demethylation due to Dnmt1 depletion requires multiple cell divisions [Jackson-Grusby et al. 2001], poor proliferation of GS cells in these cultures probably prevented efficient global DNA demethylation at 7 d. Therefore, GS cells from p53 knockout mice were transduced with a Dnmt1 knockdown vector and kept for ~1 mo. Although extensive apoptosis occurred after knockdown, some cells divided slowly and formed GS cell colonies. Using this protocol, mGS cells were obtained within 4 wk after Dnmt1 knockdown [Fig. 1C, Table 1, Supplemental Table S1]. The morphology and the proliferation pattern of the Dnmt1 knockdown-induced mGS [Dnmt1-mGS] cells were indistinguishable from those of cells that developed spontaneously. Real-time PCR confirmed down-regulation of Dnmt1 expression after knockdown [Supplemental Fig. S1A]. Examination of global DNA methylation showed 3.7% ± 0.6% reduction in total methylcytosine levels by Dnmt1 knockdown 2 wk after transfection [n = 3; P < 0.05 by t-test]. Combined bisulfite restriction analysis [COBRA] showed progressive demethylation of H19 [Fig. 1D].

To examine whether the wild-type GS cells also transform into mGS cells, we established GS cells from transgenic pups carrying an enhanced green fluorescent protein [EGFP] gene [green mouse]. Logarithmically growing GS cells were transduced with Dnmt1 and p53 knockdown vectors. Real-time PCR confirmed down-regulation of both p53 and Dnmt1 [Supplemental Fig. S1B,C]. Wild-

Results

Reprogramming of GS cells by induction of DNA demethylation

Global methylation of genomic DNA in GS cells is significantly higher than those in mGS and ES cells [Fig. 1A]. Because DNA demethylation is often found in DMRs of H19-imprinted genes after reprogramming into mGS cells, we directly examined the role of Dnmt1, which maintains DNA methylation. To induce DNA demethylation, we used a Dnmt1 knockdown vector. When we induced demethylation by Dnmt1 knockdown, the majority of cells underwent p53-dependent apoptosis [Fig. 1B], but no mGS cells were found. However, because Dnmt1 is a maintenance methylase and passive demethylation due to Dnmt1 depletion requires multiple cell divisions [Jackson-Grusby et al. 2001], poor proliferation of GS cells in these cultures probably prevented efficient global DNA demethylation at 7 d. Therefore, GS cells from p53 knockout mice were transduced with a Dnmt1 knockdown vector and kept for ~1 mo. Although extensive apoptosis occurred after knockdown, some cells divided slowly and formed GS cell colonies. Using this protocol, mGS cells were obtained within 4 wk after Dnmt1 knockdown [Fig. 1C, Table 1, Supplemental Table S1]. The morphology and the proliferation pattern of the Dnmt1 knockdown-induced mGS [Dnmt1-mGS] cells were indistinguishable from those of cells that developed spontaneously. Real-time PCR confirmed down-regulation of Dnmt1 expression after knockdown [Supplemental Fig. S1A]. Examination of global DNA methylation showed 3.7% ± 0.6% reduction in total methylcytosine levels by Dnmt1 knockdown 2 wk after transfection [n = 3; P < 0.05 by t-test]. Combined bisulfite restriction analysis [COBRA] showed progressive demethylation of H19 [Fig. 1D].

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type GS cells produced mGS cells within 4 wk [Fig. 1E; Supplemental Table S1]. RT–PCR confirmed Nanog expression in Dnmt1-mGS cells, which was accompanied by loss of Nanos3 expression, suggesting that GS cells lost their spermatogonial identity and became ES-like cells [Fig. 1F]. We did not find mGS cells using 5-azacytidine treatment using both wild-type and p53 knockout GS cells.

Dmrt1 knockdown induces mGS cells

Because Dnmt1 knockdown causes tumors in somatic cells without p53 [Gaudet et al. 2003], we hypothesized that DNA demethylation might have changed the expression of genes responsible for germ cell tumor (GCT) development. We therefore examined the impact of 14 GCT candidate genes by deregulating their expression in a p53 knockout GS cell line. Dnmt1 knockdown down-regulated the expression of several genes, including Dnd1 and Dmrt1, both of which are implicated in the formation of teratomas from PGCs [Fig. 2A; Supplemental Fig. S2A,B; Gilbert et al. 2011]. When we carried out knockdown experiments, knockdown of Dnd1 or Dmrt1 yielded mGS cell colonies within 4 wk [Supplemental Table S1]. However, none of the other genes showed evidence of conversion.

To confirm the effects of Dnd1 and Dmrt1 knockdown on wild-type GS cells, we cotransfected each knockdown vector with a p53 knockdown vector. Although we obtained no mGS cells with Dnd1 knockdown, Dmrt1 knockdown successfully yielded mGS cell colonies within 4 wk [Supplemental Table S1]. Compared with Dnmt1 knockdown, Dmrt1 knockdown induced mGS cells at a higher frequency with a shorter incubation time, and colony development was observed as early as 12 d.

Table 1. Summary of mGS induction efficiency

<table>
<thead>
<tr>
<th>Gene transduction</th>
<th>Multiplicity of infection</th>
<th>Number of wells cultured</th>
<th>Number of wells with mGS cells</th>
<th>mGS colonies per 10⁶ cells</th>
<th>Day colonies examined</th>
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<tr>
<td>p53 knockdown</td>
<td>10</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Dnmt1 knockdown, p53 knockdown</td>
<td>10</td>
<td>12</td>
<td>9</td>
<td>13.7 ± 4.1</td>
<td>28</td>
</tr>
<tr>
<td>Dmrt1 knockdown, p53 knockdown</td>
<td>2,10</td>
<td>21</td>
<td>12</td>
<td>47.1 ± 17.1</td>
<td>28</td>
</tr>
<tr>
<td>Sox2 overexpression, p53 knockdown</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2.3 ± 1.0</td>
<td>21</td>
</tr>
<tr>
<td>Oct4 overexpression, p53 knockdown</td>
<td>10</td>
<td>31</td>
<td>12</td>
<td>2.0 ± 0.7</td>
<td>21</td>
</tr>
<tr>
<td>Oct1 knockdown, p53 knockdown</td>
<td>10</td>
<td>12</td>
<td>3</td>
<td>1.0 ± 0.6</td>
<td>21</td>
</tr>
<tr>
<td>Oct4 overexpression, Oct1 knockdown, p53 knockdown</td>
<td>10</td>
<td>15</td>
<td>8</td>
<td>6.3 ± 1.9</td>
<td>21</td>
</tr>
<tr>
<td>Oct4 overexpression, Oct1 knockdown, p53 knockdown</td>
<td>10</td>
<td>8</td>
<td>3</td>
<td>2.5 ± 1.3</td>
<td>21</td>
</tr>
<tr>
<td>Oct6 knockdown, p53 knockdown</td>
<td>10</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
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Values are mean ± SEM. Cells (5 × 10⁵) were cultured in six-well plates. All experiments were carried out with wild-type GS cells.
Concurrent overexpression of Dmrt1 cDNA with a Dnmt1 knockdown vector significantly reduced mGS cell development (Fig. 2B), which suggests that Dmrt1 down-regulation is responsible for Dnmt1 knockdown-induced pluripotency. As in Dnmt1-mGS cells, simultaneous p53 knockdown was necessary because cells underwent apoptosis due to Dmrt1 knockdown (Fig. 2C). Although Bax knockdown was able to rescue cells from Dmrt1 knockdown-induced apoptosis, we were unable to observe mGS cells (Fig. 2C), suggesting that p53 knockdown has an additional function to promote pluripotency.

Because of the relatively high efficiency of mGS cell formation, we used this system to characterize the reprogramming process. As previously noted for spontaneously developed mGS cells, Dmrt1-mGS cell development is often accompanied by sudden generation of an epiblast-like sheet, which has Nanog expression in the center (Supplemental Fig. S3A). All Dmrt1-mGS cell colonies expressed Nanog on the day when colonies were discovered. This protocol was useful in deriving mGS cells from all seven tested wild-type GS cell lines, and mGS cells were obtained from not only pups but also adults (Supplemental Fig. S3B). These results suggest that Dmrt1 regulates pluripotency in GS cells.

Characterization of Dnmt1- and Dmrt1-mGS cells

RT–PCR and flow cytometry indicated that both Dnmt1- and Dmrt1-mGS cells have typical features of ES cells [Supplemental Fig. S4A,B]. They expressed high levels of Oct4 and Sox2 and were positive for Nanog in immunohistochemical analyses [Supplemental Fig. S4C]. However, several lines of Dnmnt1- or Dmrt1-mGS cells expressed Neurog3 or Sohlh1, which suggested that the cells retain a spermatogonial phenotype [Supplemental Fig. S4A]. We carried out bisulfite sequencing analysis to check the DNA methylation patterns in imprinted genes. Consistent with previous studies [Kanatsu-Shinohara et al. 2004], GS cells showed typical androgenetic DNA methylation patterns: hypermethylation in H19 and hypomethylation in Igf2r [Supplemental Fig. S4D]. In contrast, Dnmt1- and Dmrt1-mGS cells showed demethylation in H19. Although we occasionally observed Igf2r methylation in mGS cells, no apparent difference in Igf2r was found, regardless of the induction method.

Functional characterization of Dnmt1- and Dmrt1-mGS cells

To determine whether Dnmt1- and Dmrt1-mGS cells are functionally similar to ES cells, we examined their differentiation potential. In these experiments, Dnmt1-mGS cells were produced by shRNA transfection, and the shRNA was removed by cre treatment (Supplemental Fig. S4E,F). We first induced neuroectodermal differentiation in adherent monocultures (Ying and Smith 2003). The cultured cells successfully differentiated into cells expressing βIII-tubulin, a neuronal marker. We then generated embryoid bodies (EBs) in vitro and examined whether the cells could differentiate into other lineages (Fig. 3A). After 5 d of culture on gelatin-coated dishes, the EBs were
stained with several antibodies. Some cells stained positively for α-smooth muscle actin, a mesodermal marker, while others expressed cells with α-fetoprotein expression, suggesting that they belong to the endodermal lineage. We found no obvious difference between Dnmt1- and Dmrt1-mGS cells in terms of differentiation patterns and efficiencies. We also transplanted both cell types under the subcutaneous tissues of nude mice to examine their teratoma-forming potential. All clones produced tumors within 4 wk after transplantation. Histological analyses showed that the transplanted cells produced teratomas with the ectoderm (neural tube), mesoderm (chondrocytes), and endoderm (gut epithelium) (Supplemental Fig. S4G).

To test whether these mGS cells contribute to embryonic development, we microinjected them into blastocysts [Supplemental Table S2]. In this experiment, GS cells from ROSA26 mice were used to derive Dnmt1-mGS cells, which were treated with cre to remove shRNA for Dnmt1 and p53 before the chimera experiment. On the other hand, we used green GS cells and transfected siRNAs for Dmrt1 and p53 to avoid vector integration for Dmrt1-mGS cell production. Both Dnmt1- and Dmrt1-mGS cells could produce chimeric offspring, albeit at low efficiency [Fig. 3B–D, Supplemental Table S2]. Low efficiency of chimera production could be due to residual expression of Neurog3 in these cells [Supplemental Fig. S4H]. Nevertheless, both offspring were apparently normal, and Dmrt1-mGS-derived F1 offspring were born by natural mating. No abnormalities in H19 were found by COBRA in these offspring [Supplemental Fig. S4I].

Identification of Dmrt1 target genes

In the next set of experiments, we sought to identify target genes regulated by Dmrt1. A previous study showed that Dmrt1 regulates >1400 genes in spermatogonia [Murphy et al. 2010]. Among the diverse types of genes, we focused on those associated with cell cycle regulation (p18 and p19) and pluripotency (Nr5a1, Utf1, Sox2, and Zic3) because we reasoned that deregulation of these genes can potentially stimulate GCT formation. We analyzed the expression levels of these candidate genes in p53 knockout GS cells by real-time PCR after Dmrt1 knockdown [Fig. 4A]. The analyses revealed that while Sox2 and Utf1 were up-regulated by Dmrt1 knockdown, Nr5a1 and Zic3 were down-regulated, which suggests that Nr5a1 and Zic3 are dispensable for reprogramming. Both p18 and p19 cyclin-dependent kinase inhibitors (CDKIs) were down-regulated, which may facilitate teratoma formation. Although similar results were obtained using wild-type GS cells with p53 knockdown, we failed to find statistically significant down-regulation of p19 [Supplemental Fig. S5].

Based on this observation, we screened for genes that trigger pluripotency in GS cells. We overexpressed pluripotency-related genes or depleted the expression of CDKIs in wild-type GS cells. All transfection experiments were carried out by cotransfection with a p53 knockdown vector. Although Utf1 transfection did not result in mGS cell development, transfection of Sox2 yielded mGS cells using three different wild-type GS cell lines [Table 1; Supplemental Table S1; Supplemental Fig. S2C]. Many cells underwent p53-dependent apoptosis after Sox2 overexpression [Supplemental Fig. S6A], but the remaining cells transformed into mGS cells, a pattern similar to that for Dmrt1 knockdown. Sox2 knockdown inhibited Dmrt1-mGS cell development [Fig. 4B, Supplemental Fig. S2A]. Neither p18 nor p19 knockdown resulted in mGS cell production. Because concurrent Dmrt1 overexpression suppressed Sox2 expression induced by Dnmt1 depletion, the Dmrt1–Sox2 axis also appears to operate in Dnmt1-mGS cell development [Fig. 4C, Supplemental Fig. S2C].

Sox2 is necessary to regulate transcription factors that affect Oct4 expression in ES cells [Masui et al. 2007]. Although GS cells express Oct4, its level is significantly lower than that found in ES cells [Inamura et al. 2006]. However, we noted that Oct4 mRNA and protein expression were up-regulated in Sox2 transfected GS cells [Fig. 4D, Supplemental Fig. S7]. Moreover, Oct4 knockdown prevented mGS cell development after Sox2 overexpression [Fig. 4E]. Although apparent changes in Oct4 expression were not observed after Dnmt1 knockdown [Fig. 4C], we reasoned that this could be due to the relatively variable timing and small number of pluripotent colonies.

Figure 4. Development of mGS cells after Sox2 or Oct4 overexpression (OE). [A] Real-time PCR analysis of Dmrt1 target genes in p53 knockout (KO) GS cells after Dmrt1 knockdown (KD) [n = 3]. pLKO.1 EGFP was used as a control. [B] Suppression of mGS cell development by Sox2 knockdown 28 d after Dmrt1 knockdown (n = 13). pLKO.1 EGFP was used as a control. [C] Real-time PCR analysis of Dmrt1, Dnmt1, Sox2, and Oct1/4 expression in p53 knockout GS cells 14 d after Dmrt1 knockdown (n = 4). pSicoR was used as a control. For concurrent Dmrt1 overexpression, CSII-EF1α-IRES2-Venus was a control. [D] Real-time PCR analysis of Oct4 expression in p53 knockout GS cells 14 d after Sox2 overexpression [n = 3]. CSII-EF1α-IRES2-hKO1 was used as a control. [E] Reduced Sox2-mGS cell development 28 d after Oct4 knockdown (n = 9).
by Dnmt1 knockdown. Because Sox2 maintains the requisite level of Oct4 in ES cells and Oct4 overexpression can sustain Sox2-null ES cells (Masui et al. 2007), we tested the possibility that Oct4 overexpression may abolish the need for Sox2 transfection. Transfection of Oct4 induced mGS cell formation using three different wild-type GS cell lines (Table 1; Supplemental Table S1). Unlike Sox2 overexpression, Oct4 overexpression did not induce GS cell apoptosis (Supplemental Fig. S6B) but did require p53 knockdown for mGS cell formation. These results suggest that Sox2 is responsible for Dmrt1-mGS cell generation and that increased Sox2 expression up-regulates Oct4, thereby inducing the formation of pluripotent cells.

**Increased mGS cell development following Oct1 knockdown**

Although these results show the importance of Oct4 regulation, the role of this protein in SSCs has been controversial. One study reported that Oct4 down-regulation induces GS cell apoptosis (Dann et al. 2008), while another study showed that Oct6, but not Oct4, is indispensable for SSCs (Wu et al. 2010). However, we noted that GS cells express Oct1 (Fig. 5A); Oct1 mRNA was down-regulated upon FGF2 or GDNF treatment, and Western blot analysis also confirmed this result (Fig. 5B,C). Although we did not observe apparent changes in Oct4 and Oct6 expression at mRNA levels, Western blotting showed that both Oct4 and Oct6 protein levels were slightly up-regulated by cytokine treatment. These results suggested that regulation of Oct1 and Oct4/6 expression is different. Because Oct1 often has the same targets as Oct4 (Kang et al. 2009), we reasoned that the relative balance of Oct proteins is important for regulating pluripotency in SSCs.

To test this hypothesis, we examined the impact of Oct knockdown in GS cells. Knockdown of Oct1 or Oct4 induced GS cell apoptosis in a p53-dependent manner (Supplemental Fig. S6C). Oct6 knockdown also caused apoptosis, which was not rescued by p53 deficiency. We transfected wild-type GS cells with Oct1 or Oct6 knockdown vectors together with a p53 knockdown vector. Knockdown of Oct1, but not Oct6, yielded mGS cells using two different wild-type GS cell lines (Table 1). The efficiencies of Oct4 overexpression and Oct1 knockdown were comparable, but combining Oct4 overexpression and Oct1 knockdown synergistically improved mGS cell derivation efficiency ($P < 0.05$ by ANOVA), and mGS cells were obtained (Table 1). While Oct4 overexpression increased Sox2 expression (Fig. 5D), Oct1 knockdown increased not only Sox2 expression but also Oct4 expression (Fig. 5E). On the other hand, Oct4 knockdown decreased Sox2 expression, suggesting that Oct4 is positively regulating Sox2 (Fig. 5F). Although Oct1 overexpression decreased Oct4 and Sox2 expression (Fig. 5G), it was not possible to suppress Oct4-mGS cell development, suggesting that Oct1 is necessary but not sufficient for suppressing pluripotency. Because neither Oct4 nor Sox2 overexpression influenced Oct1 expression (Fig. 5H), it is likely that Oct1 is regulated independently of Oct4 and Sox2.

When we examined whether changes in Oct1/4 expression in GS cells can influence genes that are regulated by Oct4 in ES cells (van den Berg et al. 2010), several genes changed expression by Oct1 overexpression or knockdown (Supplemental Fig. S8). In particular, Utf1, one of the Dmrt1 target genes, was down-regulated by Oct1 overexpression, while it was up-regulated by Oct1 knockdown. These results suggest that Oct1 suppresses pluripotency in GS cells by competing with Oct4 for several pluripotency-related target genes.

**Characterization of Sox2- and Oct4-mGS cells**

Both Sox2- and Oct4-mGS cells were phenotypically similar to Dnmt1- and Dmrt1-mGS cells and were able to differentiate into cells of the three germ layers in vitro (Supplemental Fig. S9A–E). However, their global gene expression profiles were slightly different from those of ES cells (Fig. 6A, Supplemental Fig. S10). To confirm their pluripotency, we introduced Sox2 or Oct4 cDNA into a floxed vector that was transfected with a p53 knockdown vector. After transformation into mGS cells, the transgenes were removed by cre-mediated deletion (Supplemental Fig. S9F,G). The ability of the resulting cells to form chimeras by blastocyst injection was then tested (Supplemental Table S2). Chimeric offspring were obtained.
Germline reprogramming

Figure 6. Development of normal offspring from Sox2- or Oct4-mGS cells. [A] Hierarchical clustering of gene expression by microarray. [B,C] A live chimera formed by microinjection of Sox2-mGS cells (B) and Oct4-mGS cells (C) into B6 and ICR blastocysts, respectively. (Inset) Offspring produced after germline transmission. While Sox2-mGS cell origin was identified by the cinnamon coat color (B), Oct4-mGS cell origin was confirmed by the cinnamon coat color [C]. (D) A model to explain the mechanism of pluripotency regulation in SSCs.

from both Sox2- and Oct4-mGS cells [Fig. 6B,C]. Both types of mGS cells showed germline transmission by natural mating. These results suggest that Sox2- and Oct4 mGS cells are pluripotent.

Discussion

The molecular mechanism of culture-induced pluripotency is not well understood. While DNA methylation patterns in ES cells often change within several months, GS cells maintain a stable karyotype and DNA methylation patterns in imprinted genes for >2 yr [Kanatsu-Shinohara et al. 2005]. Because demethylation of H19 DMRs is almost always found in spontaneously developed mGS cells, we reasoned that DNA demethylation might have contributed to the reprogramming of GS cells. To test our hypothesis directly, we used Dmrt1 knockdown to induce hypomethylation in GS cells. Unlike ES cells, which can proliferate without Dnmt genes [Tsumura et al. 2006], GS cell survival depends critically on Dmrt1 expression [Takashi et al. 2009]. Although Dmrt1 knockdown resulted in apoptosis of GS cells, simultaneous p53 knockdown successfully induced mGS cell formation, suggesting that DNA demethylation and suppression of p53 function are necessary for dedifferentiation of GS cells into pluripotent cells.

In our previous study, we did not observe mGS cells by Dmrt1 knockdown, probably because of the higher virus titer and shorter incubation time [Takashi et al. 2009], and H19 DMR demethylation was not confirmed. The higher virus titer induced extensive apoptosis of GS cells, and the shorter incubation time (~3 wk) was probably insufficient to induce passive demethylation by cell replication because the current study shows that most of the mGS cell colonies developed after 3 wk. Because deficiency of Dmrt1 causes somatic cell tumors [Gaudet et al. 2003], acquisition of pluripotency in GS cells is suggested to share similarities with tumorigenesis. Consistent with this notion, H19 is often demethylated in human GCTs [Looijenga et al. 1998]. In particular, seminomas and nonseminomas generally show biallelic expression of the H19 gene. Moreover, we also recently showed H19 demethylation in ES-like colonies produced in vitro transformation of mouse SSCs with activated Ras, c-myc, and p53 dominant-negative constructs [Morimoto et al. 2012]. Although the roles of imprinted genes in GCTs are unknown, similarities between GCT formation and GS cell reprogramming led us to examine whether GCT candidate genes are deregulated as a result of global DNA hypomethylation.

Dmrt1 was one of the several candidate genes that were influenced by Dnmt1 knockdown, and Dmrt1 knockdown successfully induced mGS cell formation. Dmrt1 is one of a group of conserved transcriptional regulators of sexual differentiation that share a Doublesex/Mab-3 (DM) domain DNA-binding motif and is required for testicular development in vertebrates [Raymond et al. 2000]. In mice, this gene is expressed only in the gonad and is essential for differentiation of germ cells and Sertoli cells. Strikingly, tests without Dmrt1 show ovarian differentiation even at the adult stage [Matson et al. 2011]. Humans lacking one copy of Dmrt1 exhibit testicular dysgenesis and in some cases are feminized [Krentz et al. 2009]. In germ cells, this gene is responsible for the formation of teratomas from PGCs, but it is limited to the 129 background [Krentz et al. 2009]. In the postnatal testis, Dmrt1 has been considered as a transcriptional gatekeeper that controls mitosis versus meiosis in germ cells [Matson et al. 2010]. Undifferentiated spermatogonia without Dmrt1 showed precocious entry into meiosis and reached meiotic prophase by skipping amplifying divisions of the differentiating spermatogonia population, but no tumor formation was reported in postnatal animals without Dmrt1. In contrast, Dmrt1 overexpression is thought to cause spermatocytic seminomas in human adults by increasing Ret expression [Krentz et al. 2009]. Although regulation of pluripotency by Dmrt1 in spermatogonia has not been reported, we speculate that the discrepancy between our results and previous studies is due to two factors. One is the suppression of apoptosis by p53 knockdown. Dmrt1 is involved in germ cell survival, and while Dmrt1 deficiency in PGCs caused teratomas on a 129 background, germ cell loss was observed in non-129 strains [Krentz et al. 2009]. Apoptosis of germ cells is probably caused by reduced GDNF signaling because analyses of mutant testes showed that both GFRa1 and Ret are down-regulated, which suggests that Dmrt1 influences the responsiveness to GDNF. In the present study, we also observed that simple Dmrt1 knockdown induced apoptosis of GS cells, but simultaneous p53 knockdown rescued them from apoptosis, resulting in mGS cell formation. The other factor is the differentiation status of the target cells. In the previous study, analysis of Dmrt1 function in spermatogonia was carried out using cre
driven by a *Neurog3* promoter [Matson et al. 2010]. However, *Neurog3* is thought to be expressed in a subpopulation of SSCs in a reversible manner [Yoshida 2010]. Therefore, some SSCs may not have undergone *Neurog3*-cre-mediated *Dmr1* deletion, and the function of *Dmr1* in the remaining SSC population is not known. The differentiation level of the target cell population is important for germ cell transformation because cells enriched for SSCs showed a higher frequency of development into transformed cells than committed spermatagonia after transfection of oncogenes or Yamanaka factors [Morimoto et al. 2012].

Knockdown of two GCT candidate genes, *Dnd1* and *Dmr1*, induced pluripotency in p53 knockout GS cells. *Dnd1* is an RNA-binding protein that regulates PGC viability and suppresses teratoma formation [Zhu et al. 2007]. It is similar to *Dmr1* in that it only causes teratomas on a 129 background. However, the mechanism of pluripotency regulation seems to differ between these two genes. Although we were able to obtain mGS cells by *Dnd1* or *Dmr1* knockdown using p53 knockout GS cells, only *Dmr1* was useful for deriving mGS cells from wild-type cells. Indeed, studies on PGCs also suggest that they have independent functions [Krentz et al. 2009]. For example, *Dnd1* mutants undergo a severe loss of germ cells before 11.5 dpc, whereas those in *Dmr1* mutants survive until birth. Although *Pten* deficiency induces teratomas in PGCs on non-129 backgrounds, *Dmr1* deficiency did not change levels of *Pten* or *Akt* phosphorylation [Kimura et al. 2003; Krentz et al. 2009]. These results suggested that *Dmr1* acts either independently of *Pten* or downstream from the *Pten* pathway in PGCs. Although p53 deficiency also causes teratomas on non-129 backgrounds, it is distinct from *Pten* in that *Pten*-deficient gonocytes and spermatagonia do not show pluripotency [Goertz et al. 2011]. Therefore, p53 and *Dmr1* seem to be guardians of pluripotency at both embryonic and postnatal stages.

Of the *Dmr1* target genes, our functional screening suggested that Sox2 acts downstream from *Dmr1* to induce reprogramming. Sox2 mRNA is expressed from the early stages of PGC development, but Sox2 protein decreases from 13.5 dpc to 17.5 dpc in fetal gonocytes and is absent in spermatogonia [Imamura et al. 2006; Campolo et al. 2013]. *Dmr1* protein expression is absent by 15.5 dpc but is re-expressed in spermatogonia [Lei et al. 2007]. This suggests that *Dmr1* protein expression is discrepant with Sox2 protein expression, which apparently involves additional molecules. On the other hand, although the Oct4 protein is expressed in GS cells, its level is only 10% of that found in ES cells; Oct4/Sox2 sites in GS cells are not occupied by Oct4 and Sox2 despite their hypomethylated status, possibly due to the smaller amount of Oct4 and the absence of Sox2 [Imamura et al. 2006]. Nevertheless, our results showed that Sox2 and Oct4 expression levels are closely correlated in GS cells. Although the role of Oct4 in SSCs is still under debate [Wu et al. 2010], high expression of Sox2 appears to overcome Sox2 repression at the translational level and tip the balance toward pluripotency by up-regulating Oct4. Although changes in Oct4 levels were modest, this may have a large impact on pluripotency regulation, considering that the threshold for inducing differentiation in ES cells is set at 50% above or below the normal Oct4 expression [Niwa et al. 2000]. Because Oct1 knockdown also induced up-regulation of Oct4, Sox2, and Ut1, relative reduction in Oct1 may perhaps induce pluripotency genes by increasing the access of Oct4 to target genes to regulate several pluripotency-related genes [Fig. 6C]. However, given our production of mGS cells by *Dnd1* knockdown in p53 knockout GS cells and the fact that Oct1 overexpression could not suppress Oct4-mGS cell development, suppression of pluripotency probably involves additional molecules. Identifying other genes that modulate the response of GS cells to Sox2 overexpression is warranted.

Because mGS cells can be derived without genetic manipulation, SSCs represent a unique resource for deriving pluripotent cells. However, conflicting reports on the induction method and nature of SSC-derived PS cells need to be reconciled. In particular, it still remains unknown why mGS cell development is often accompanied by epiblast-like colony formation, while similar colonies were not reported in other studies [Seandel et al. 2007; Geijsen and Hochedlinger 2009]. Such discrepancies could be due to the differences in the cell of origin and/or mode of reprogramming. This may also explain why mGS cell formation occurred at relatively lower frequency compared with gPS cells (0.0047% vs. 0.01%), which gradually form ES-like colonies over several weeks. We also do not know how p53 is involved in pluripotency regulation. Because mGS cell development often occurred after freezing-thawing or electroporation, we think that such changes in culture conditions induce cellular stress, which may influence p53 levels. However, because Bax knockdown could not induce mGS cells, the role of p53 knockdown is not simply rescuing apoptosis but has an additional role in pluripotency regulation. Because we can reproducibly obtain mGS cells with our new protocol, analysis of reprogramming mechanisms is now possible, which will improve our understanding of how pluripotency is suppressed in germ cells despite their similarity to ES cells.

**Materials and methods**

**Cell culture**

GS cells were derived from the transgenic mouse lines C57BL6/Tg14(act-EGFP-OsbY01) and B6-Tg[Rosa26]26Sor, as previously described [Kanatsu-Shinohara et al. 2003, 2004, 2011]. GS cells were also established from the transgenic mouse line Tg[Nanog-GFP, Puro]1Yam (Okita et al. 2007). GS cells from p53 knockout mice were previously described [Kanatsu-Shinohara et al. 2004]. Culture medium was based on StemPro-34 SFM [Invitrogen] as previously described [Kanatsu-Shinohara et al. 2003]. We also used mGS cells on a DBA/2 background that spontaneously developed during green GS cell derivation (Kanatsu-Shinohara et al. 2003). Growth factors used were 10 ng/mL human FGF2 and 15 ng/mL recombinant rat GDNF (both from Peprotech). ES cells [R1] were a generous gift from Dr. M. Ikawa (Osaka University, Suita, Japan). ES cells (BRC1 and B6-6) were provided by RIKEN BRC.
All pluripotent cell lines were maintained on MEFs in Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 15% fetal bovine serum [FBS], 1000 U/mL LIF [ESGRO; Merck Millipore], nonessential amino acid mixture [Invitrogen], and 0.1 mM 2-mercaptoethanol [2-ME]. We also used 2 μM PD0325901 [Selleck Chemicals], 3 μM CHIR99021 [Biovision, Inc.], and 1000 U/mL LIF in N2B27 medium to maintain these cells for chimera production [Ying and Smith 2003].

For differentiation into neuronal lineages, cells were cultured on gelatin-coated plates for 8 d in N2B27 medium. Neural cell differentiation was induced by replating on a LAB-TEK chamber slide [Thermo Fisher Scientific] coated with 0.2 mg/mL fibronectin [Invitrogen] and maintenance in N2B27 medium supplemented with 20% FBS, 10 μM 2-ME, and nonessential amino acid mixture and plated on a low-cell-binding plate at a density of 7.5 × 10⁵ cells per 9.6 cm². Two days after culture, the serum concentration was reduced to 15% FBS, and the cells were cultured for 8 d. EBs were then transferred to a LAB-TEK chamber slide coated with 0.1% gelatin and cultured for 5 d.

Accession number
Raw data sets have been submitted to the Gene Expression Omnibus database [http://www.ncbi.nlm.nih.gov/geo] and are available under the accession number GSE43850.

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