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Induction of Pluripotency in Adult Unipotent Germline Stem Cells

Kinarm Ko,[1] Natalia Tapia,[1] Guangming Wu,[1] Jeong Beom Kim,[1] Marcos J. Araúzo Bravo,[1] Philipp Sasse,[2] Tamara Glaser,[3] David Ruau,[4,5] Dong Wook Han,[1] Boris Greber,[1] Kirsten Hausdörfer,[3] Vittorio Sebastiano,[1] Martin Stehling,[1] Bernd K. Fleischmann,[2] Oliver Brüstle,[3] Martin Zenke,[4,5] and Hans R. Schöler[1,*]
[1]Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Münster 48149, Germany
[2]Institute of Physiology I, Life & Brain Center, University of Bonn, 53105 Bonn, Germany
[3]Institute of Reconstructive Neurobiology, Life & Brain Center, University of Bonn and Hertie Foundation, 53105 Bonn, Germany
[4]Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University Medical School, Aachen 52074, Germany
[5]Helmholtz Institute for Biomedical Engineering, RWTH Aachen University, Aachen 52074, Germany
*Correspondence: office@mpi-muenster.mpg.de
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SUMMARY

Mouse and human stem cells with features similar to those of embryonic stem cells have been derived from testicular cells. Although pluripotent stem cells have been obtained from defined germline stem cells (GSCs) of mouse neonatal testis, only multipotent stem cells have been obtained so far from defined cells of mouse adult testis. In this study we describe a robust and reproducible protocol for obtaining germline-derived pluripotent stem (gPS) cells from adult unipotent GSCs. Pluripotency of gPS cells was confirmed by in vitro and in vivo differentiation, including germ cell contribution and transmission. As determined by clonal analyses, gPS cells indeed originate from unipotent GSCs. We propose that the conversion process requires a GSC culture microenvironment that depends on the initial number of plated GSCs and the length of culture time.

INTRODUCTION

Germline stem cells (GSCs) are unipotent precursor cells for sperm generation in the testis. Although GSCs represent an extremely low proportion (0.02%–0.03%) of the cells of the testis (Tegelenbosch and de Rooij, 1993), they can be isolated and propagated in vitro (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). Survival of GSCs and maintenance of their stemness property requires expression of Oct4, which is a pluripotency- and germ-cell-specific marker (Kehler et al., 2004). The role of Oct4 in germ cell development was elucidated by the demonstration that knockout of the Oct4 gene led to apoptosis (Kehler et al., 2004). Apart from expression in the inner cell mass and the epiblast in pre- and early postimplantation embryos, Oct4 expression is otherwise restricted to the germ cell lineage, including primordial germ cells (PGCs), GSCs, and oocytes. To date, GSCs are the only adult stem cells shown to exhibit significant Oct4 expression. Disruption of Oct4 activity in GSCs cultured in vitro caused the loss of self-renewal and spermatogenetic activity (Dann et al., 2008). It has been suggested that Oct4 contributes to abnormal conversion of germ cells into tumorigenic cells in the testis (Looijenga et al., 2003, 2007). Recent evidence has shown that Oct4 is essential for reprogramming somatic cells into induced pluripotent stem cells (iPSCs) (Kim et al., 2008, 2009; Takahashi and Yamanaka, 2006).

In 1992, it was first reported that mouse unipotent PGCs, which are fetal germ cells, can be converted into embryonic stem cell (ESC)-like cells, so-called embryonic germ (EG) cells (Matsui et al., 1992; Resnick et al., 1992). Subsequent research also demonstrated that EG cells can be established from human PGCs (Shamblott et al., 1998). Mouse EG cells share the feature of pluripotency with ESCs, as evidenced by in vitro differentiation and in vivo developmental potential, including germline contribution and transmission. The derivation of ESC-like cells is not limited to fetal germ cells in both mouse and human models (Conrad et al., 2008; Guan et al., 2006; Kanatsu-Shinohara et al., 2004, 2008; Kossack et al., 2009; Seandel et al., 2007). In 2004, it was demonstrated that ESC-like pluripotent cells could be obtained from testicular cells during derivation of GSCs (Kanatsu-Shinohara et al., 2004). However, generation of ESC-like pluripotent cells was only possible from postnatal day (PND) 0–2 testicular cells, but not from adult testis (Kanatsu-Shinohara et al., 2004). A subsequent report showed that retinoic acid 8 (Stra8)-GFP-positive cells from adult testis could become multipotent GSCs (mGSCs) (Guan et al., 2006), but their cellular source and their germline competency were not evaluated. Three independent groups reported that mGSCs could be obtained from established GSC lines derived from testes samples taken from mice of different ages (Izadyar et al., 2008; Kanatsu-Shinohara et al., 2008; Seandel et al., 2007). However, these cells were not fully pluripotent, as evidenced by their inability to form teratomas, contribute to the germline, or demonstrate germline transmission. Two recent studies demonstrated that cell clusters formed from either isolated human spermatogonial cells (Conrad et al., 2008) or testicular cells (Kossack et al., 2009) have certain ESC properties. All these cited studies support the notion that, in contrast to somatic cells, germ cells have the distinct potential to be converted into ESC-like stages without the introduction of exogenous reprogramming factors.

In this study, we introduce a robust protocol for the generation of pluripotent cells from adult unipotent GSCs. Unlike previous reports that demonstrated—using a mouse model—that the

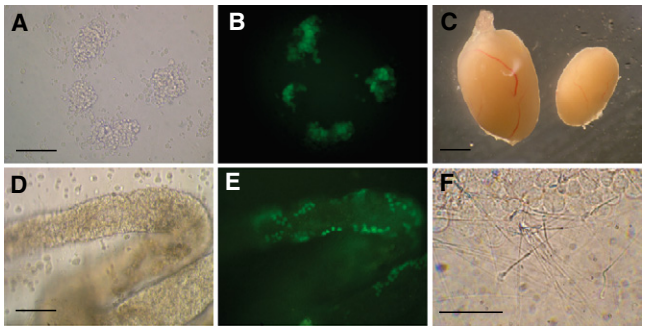

Figure 1. Establishment of a GSC Line from the Testis of PND 35 Oct4-GFP Mice

(A) Typical GSC colonies formed in culture.

(B) GSCs express the GFP gene under control of the Oct4 promoter (details as in A).

(C) Three months after transplantation of GSCs into the W/W recipient mice, the testes were larger than those of controls.

(D and E) Photomicrographs of the testicular tubules of a recipient mouse. Note that transplanted Oct4-GFP-positive GSCs (E) colonized the seminiferous tubules.

(F) The testes of recipient mice produced spermatozoa. Spermatogenesis in the testes of W/W mice was restored by the transplantation of GSCs. Scale bars, 100 μm (A and D), 2 mm (C), 25 μm (F).

corresponding ESC-like cells were multipotent, we demonstrate that adult GSCs can be converted into pluripotent cells, designated as germline-derived pluripotent stem (gPS) cells. Pluripotency of gPS cells was confirmed by in vitro and in vivo differentiation, germline contribution in chimeras, and germline transmission to the next generation. The DNA methylation status of the imprinted genes *H19* and *Igf2r* was the same in gPS cells as in GSCs, even after 20 passages, suggesting that conversion of GSCs into gPS cells does not alter the imprinting status. Thus, imprinting marks can be used to provide evidence of the origin of gPS cells: adult GSCs. Furthermore, we described the critical time frame in which subpopulations of GSCs were converted into gPS cells. Our results indicate that the conversion process requires a specific microenvironment of GSC culture that depends on the initial number of plated GSCs. The mouse model developed in this study can be used to enhance our understanding of the mechanisms underlying the reprogramming of unipotent cells into pluripotent cells as well as germline-related tumor formation.

RESULTS

Establishment and Characterization of Adult GSCs

GSC lines were established from the testes of PND 35 Oct4-GFP transgenic mice. Expression of a GFP transgene under the control of the Oct4 promoter is indicative of Oct4 expression in GSCs, as Oct4 is a GSC-specific marker gene (Kehler et al., 2004). For derivation of GSCs, the testes were enzymatically dissociated and plated onto gelatin-coated plates in our GSC culture medium. Colonies of GSCs formed within 7 days of culture. GSCs were collected by pipetting and were replated onto mouse embryonic fibroblasts (MEFs). For expansion of GSCs, cells were split every 4 to 7 days on MEFs using a 1:2 to 1:3 dilution. Established GSCs have a typical grape-like morphology and express Oct4-GFP (Figures 1A and 1B). Germ-cell-specific genes, including Oct4, were detected by reverse transcriptase polymerase chain reaction (RT-PCR) (see Figure S1A available online). The presence of GSC-specific proteins in the established cell line was confirmed by immunocytochemistry (Figure S1B). To further determine the functionality of the cultured GSCs, we transplanted GSCs into the seminiferous tubules of germ-cell-depleted W/W mutant mice (Figure S1C) or busulfan-treated mice. Three months after injection, we observed colonization of the transplanted GSCs and restoration of spermatogenesis in the host mice without teratoma formation (Figures 1C–1F), confirming the functionality and unipotency of the established GSCs. Furthermore, after 37 passages in culture, GSCs were still capable of restoring spermatogenesis in the germ-cell-depleted males. Although restoration of spermatogenesis was observed in busulfan-treated males after GSC transplantation, the number of offspring generated was smaller than in normal males. Overall, the established adult GSC cell lines have retained the same properties of previously described GSCs (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). We were able to derive GSC lines from mice testis of different ages (10 days up to 7 months, the oldest in our study) with different genetic backgrounds (129Sv and FVB) using our GSC establishment protocol.

Conversion of GSCs into ESC-like Cells

To obtain ESC-like cells, approximately 1000 GSCs were plated per well in 24-well plates containing MEFs and maintained in GSC culture medium without splitting. Within 3 to 4 weeks, colonies with high GFP intensity and a morphology distinct from typical GSC colonies appeared (Figures 2A and 2B); the GFP-positive colonies were of round shape, while GSC colonies had an irregular appearance. To expand the ESC-like cells, the colonies were isolated mechanically, dissociated by trypsinization, and plated onto MEFs in ESC medium (Figures 2C and 2D). The oldest male used for derivation of GSCs and further ESC-like cell conversion was 7 months old.

It is unlikely that the established GSCs contained a pluripotent subpopulation capable of forming ESC-like cell colonies. Even after 16 passages under GSC expansion culture conditions (splitting every 4–7 days), we could not detect ESC-like cell colonies. Furthermore, if pluripotent cells had existed within the GSC population, ESC-like cell colonies would have formed within 2–3 days in culture, as established ESC-like cells can be maintained in GSC culture medium (Figure S2). ESC-like cell colonies were only observed when GSCs were cultured under conversion culture conditions (for 3–4 weeks without splitting). A schematic diagram of the conversion protocol is shown in Figure S3. Further experiments that excluded the existence of a pluripotent subpopulation are described below.

We called these ESC-like cells "germline-derived pluripotent stem (gPS) cells." The morphology of gPS cell colonies was comparable to that of ESCs (Figure S4A). gPS cells expressed high levels of Oct4-GFP (Figure 2D) and stained positive for alkaline phosphatase (Figure 2E) and SSEA-1 (Figure 2F). We examined the expression of genes specific to ESCs by RT-PCR (Figure S4B). The gene expression pattern of gPS cells derived from GSCs was similar to that of ESCs. Hierarchical cluster analysis revealed that global gene expression of gPS cells is more similar to that of ESCs than to other reprogrammed iPSCs

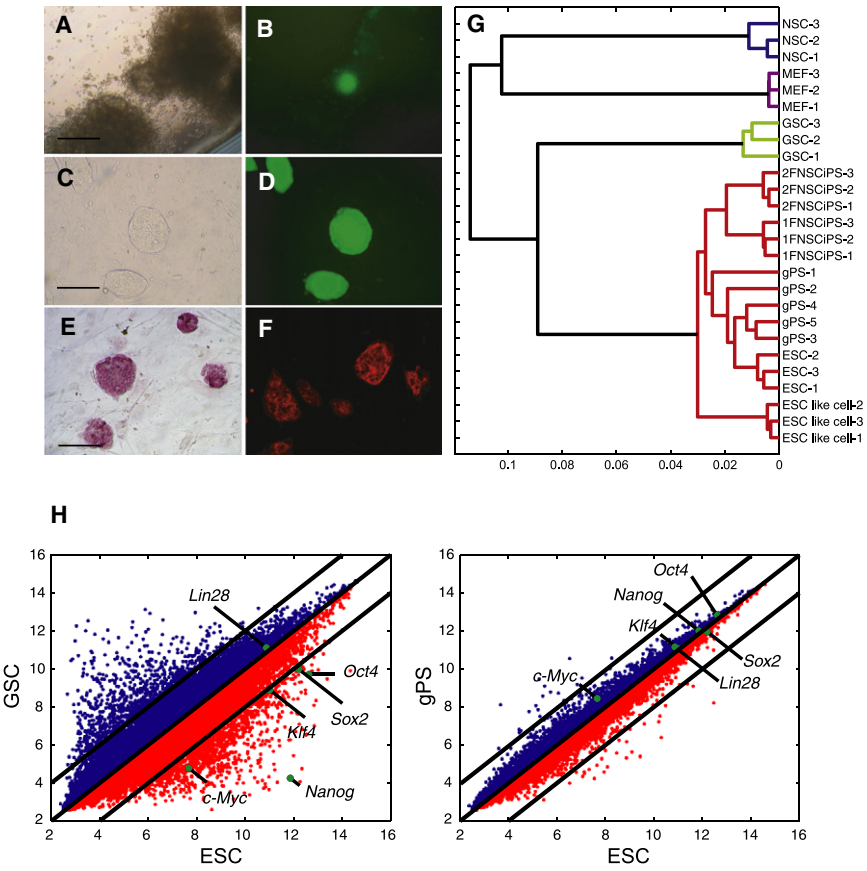

Figure 2. Establishment of gPS Cells and Cellular and Molecular Characterization of gPS Cells

(A and B) Conversion of GSCs into ESC-like cells (gPS cells) after 4 weeks in culture. Distinct Oct4-GFP-expressing colonies were observed in the induction culture.

(C and D) Established ESC-like cells from Oct4-GFP-expressing colonies. The colonies displayed morphology similar to that of ESC colonies and were positive for Oct4-GFP.

(E) ESC-like cells (gPS cells) stained positive for alkaline phosphatase.

(F) Immunofluorescence staining revealed SSEA1 expression in the ESC-like cells (gPS cells).

(G) Hierarchical analysis of different cell types: ESCs, GSCs, neural stem cells (NSCs), MEFs, gPS cells, 2FNSC-iPSCs (Kim et al., 2008), 1FNSC-iPSCs (Kim et al., 2009), and ESC-like cells from PND 0-2 testis (Kanatsu-Shinohara et al., 2004).

(H) Comparison of global gene expression between ESCs and GSCs (left), and between ESCs and gPS cells (right). Scale bars, 200 μm (A), 100 μm (C and E).

(Figure 2G). Scatter plots of microarray analyses highlight the differences between GSCs and gPS cells and demonstrate the similarity between gPS cells and ESCs (Figures 2H and S10). DNA methylation analysis showed that the Oct4 and Nanog promoter regions were completely unmethylated in gPS cells, as in ESCs (Figure S4C). Taken together, these results demonstrate that the cellular and molecular characteristics of the gPS cells are very similar to those of ESCs.

DNA Methylation Patterns in DMR Regions of *H19* and *Igf2r* Are Not Altered in gPS Cells

To determine the DNA methylation pattern of the imprinted genes *H19* and *Igf2r* in three different cell types (GSCs, gPS cells, and ESCs), we performed bisulfite sequencing analyses (Figure 3). Our data show that differentially methylated regions (DMRs) of *H19* in ESCs displayed a somatic imprinting pattern, while those in GSCs showed an androgenetic pattern. The androgenetic imprinting pattern of the DMRs of *H19* in gPS cells is a particularly interesting result, as it suggests that the DNA methylation pattern of *H19* is maintained (to the level of GSCs) even after conversion of GSCs into gPS cells. We also investigated the imprinting control region (ICR) of the maternally imprinted gene *Igf2r*. gPS cells were completely unmethylated, like GSCs, whereas ESCs exhibited a somatic methylation pattern. The imprinted pattern in gPS cells is maintained even after 20 passages. Therefore, the DNA methylation status in DMRs and ICRs of major imprinted genes in gPS cells provides evidence that gPS cells did originate from adult GSCs.

Analysis of In Vitro and In Vivo Pluripotency of gPS Cells

To determine the ability of gPS cells to undergo in vitro differentiation into derivatives of all three embryonic germ layers, embryoid bodies (EBs) were generated from gPS cells and plated onto gelatin-coated 24-well plates. The EBs attached and differentiated into a variety of cell types. We observed cells that stained positive for Flk1, a mesodermal cell lineage marker, and Tuj1, a neuronal marker. We used anti-α1-fetoprotein to detect endodermal derivatives. Taken together, our data suggest that gPS cells can differentiate in vitro into cells of the three germ layers (Figures S5A–S5F).

To assess the differentiation capability of gPS cells in vivo, a teratoma assay was performed injecting subcutaneously gPS cells into athymic mice. Within 4 weeks of transplantation, teratomas had formed in all recipients (3/3). Histological assessment of the teratomas revealed the presence of derivatives of the three embryonic germ layers: mesoderm (muscle and blood cells), endoderm (pancreas and respiratory epithelium), and ectoderm (sebaceous gland, skin, and brain) (Figures S5G–S5L). Teratomas were not observed after transplantation of GSCs, confirming their lack of pluripotency. Of note, testicular transplantation of ESCs and gPS cells into W/W mice led to teratoma formation in the transplanted testis, while GSCs cells restored spermatogenesis in the absence of teratoma formation—proof of their unipotency (data not shown).

To further confirm the pluripotency of gPS cells, a chimera assay was performed to investigate their capability to contribute to all three germ layers and to germ cells using an aggregation protocol. Aggregation was performed with 8-cell-stage C57BL6/C3H/CD1 or CD1 embryos and a clump of gPS cells (Figure 4A). We detected the GFP gene in all three germ layers of embryonic day (E)14.5 embryos by genotyping (Table S1), and we observed

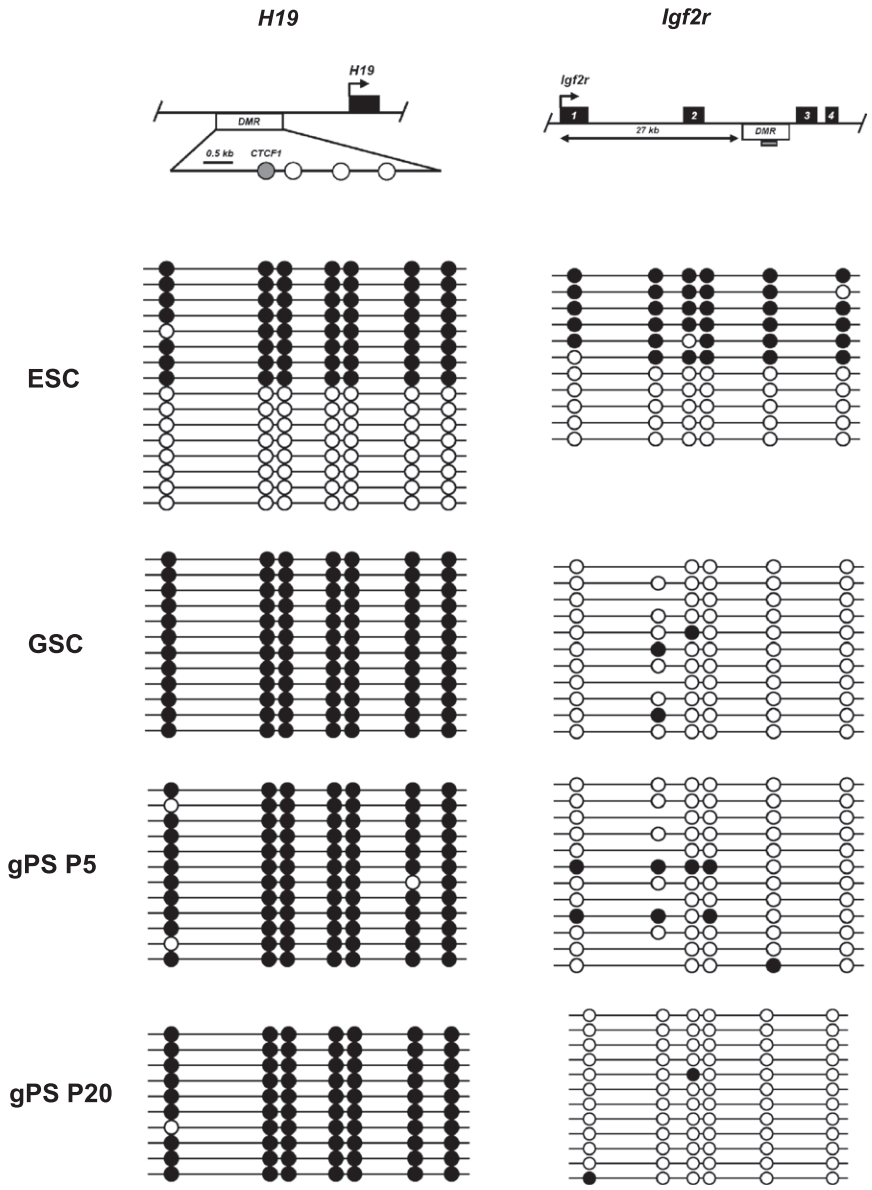

Figure 3. Methylation Status of Differentially Methylated Region of *H19* and Imprinting Control Region of *Igf2r* in ESCs, GSCs, Passage 5 gPS Cells, and p20 gPS Cells

DNA methylation was analyzed by bisulfite genomic sequencing. Open and filled circles indicate unmethylated and methylated CpGs, respectively. DMR, differentially methylated region; P, passage.

Oct4-GFP expression in fetal gonads (Figures 4B and 4C). Furthermore, we found skin chimerism in adult male mice (C57BL6/C3H/CD1) (1/7) (Figure 4D) and in CD1 background chimeric male (1/18) (Figure S5M). Germline transmission was confirmed by genotyping of the GFP gene in F1 pups from the C57BL6/C3H/CD1 chimeric male mouse (3/73) (Figure 4E), and the identity of PCR bands was confirmed by sequencing, while the CD1 chimeric male has not shown F1 germline transmission so far. Therefore, gPS cells, which originated from an established unipotent adult GSC line, are fully pluripotent and show germline transmission, though at a lower level than ESCs.

Functional Analysis of In Vitro Differentiated Cells from gPS Cells

We examined the functionality of gPS cell-derived cardiomyocytes and neural cells. gPS cells were differentiated into α-actinin-positive cross-striated cardiomyocytes (Figure 5A), which contracted spontaneously (Movie S1) with 1.45 ± 0.13 Hz (n = 8). The cardiomyocytes displayed action potentials, as revealed by intracellular recordings (n = 5, Figure 5B). Ca^{2+} transients of different cardiomyocytes in a beating cluster were synchronized, indicating electrical coupling (Figure 5C); this was supported by positive connexin 43 staining (Figure 5D). The chronotropy of gPS cell-derived cardiomyocytes was modulated by hormones of the autonomic nervous system: The muscarinergic agonist CCh reduced the frequency to 22% ± 6% (n = 4), while the adrenoceptor agonist ISO increased the frequency to 155% ± 8% (n = 7, Figure 5E).

gPS cells were also capable of differentiating into neural and glial cells expressing O4 (oligodendrocytes: 33% ± 6%) and GFAP (astrocytes: 55% ± 10%), respectively (Figures 5F–5H). To assess whether gPS cell-derived glial precursors are capable of forming myelin in vivo, we transplanted these cells into the brains of 2- to 3-day-old myelin-deficient (md) rats. Md rats develop severe central nervous system hypomyelination due to a point mutation in the X-linked proteolipid protein (PLP) gene and serve as an animal model for the study of Pelizaeus-Merzbacher disease (Boison and Stoffel, 1989; Koeppen et al., 1988). Due to the lack of endogenous myelin formation and the absence of PLP expression in md rats, donor-derived internodes can be easily detected by PLP immunolabeling (Duncan et al., 1997). Following injection into the cerebral hemispheres, gPS cell-derived donor cells were found in several fiber tracts, including corpus callosum, fimbria, and axon bundles in the septum, where they generated parallel PLP-positive profiles characteristic of myelinating oligodendrocytes (Figures 5I and 5K). Regions with PLP-positive cells also contained donor-derived astrocytes, which were identified using the mouse-specific M2 antibody (Figure 5I and 5K). Overall, these results prove that gPS cells are capable of giving rise to functional somatic cells in vitro.

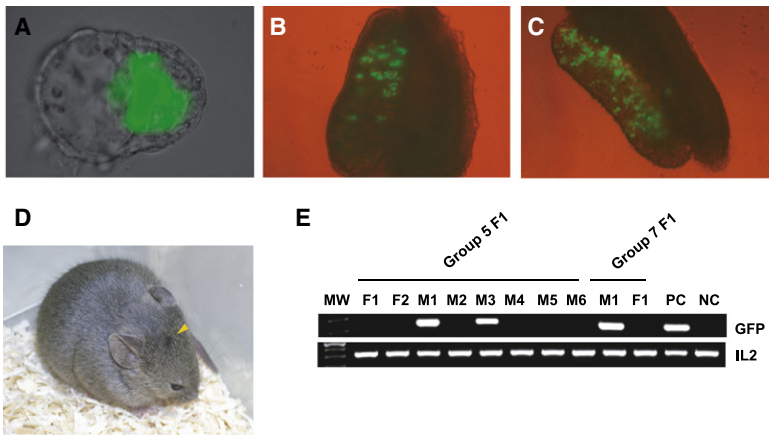

Figure 4. Analysis of Chimera Formation

(A) Blastocyst stage of an embryo created by the aggregation of an 8-cell-stage embryo with gPS cells. Note that Oct4-GFP-positive gPS cells contributed to the development of the inner cell mass.

(B and C) Male (B) and female (C) fetal gonads from E14.5 embryos. Oct4-GFP-positive germ cells were detected in both male and female gonads.

(D) Arrowhead indicates black coat color chimerism by donor gPS cells (C57BL6 background).

(E) Genotyping of GFP gene in F1 offspring using IL2 gene as endogenous control. M, male; F, female; PC, positive control; NC, negative control; MW, molecular weight marker.

The Origin of gPS Cells Is Unipotent GSCs

To exclude the possibility that any pre-existing pluripotent cells or GSCs were likely to develop into gPS cells within the established GSCs, we clonally established GSC lines from single GSCs and further converted the clonal GSCs into gPS cells (Figure S6). Single GSCs were plated and expanded on 96-well plates containing MEFs. After 7 days of single-cell culture, GSC clusters had formed in 15 of 192 wells. The GSC colonies were expanded to obtain approximately 0.5×10^6 cells (Figure 6A). Using two clonal GSC lines (Plate#1Clone#1:P1C1 and P2C3), we analyzed the expression of GSC-specific genes by RT-PCR and FACS to confirm their GSC property (Figures S7A and S7B). The established GSCs were mostly c-kit negative, which is consistent with the previous report (Kanatsu-Shinohara et al., 2003). Oct4-GFP expression in the GSCs varies from 50% to 90% depending on the density of GSCs, the size of colonies, and the length of culture during expansion culture (data not shown). However, we found that 95% of Oct4-GFP-positive cells are c-kit negative, suggesting that Oct4-GFP-positive cells are mostly undifferentiated cells in the established GSCs. We further performed a GSC cluster-forming assay (Yeh et al., 2007), which is an alternative method to assess the functionality of the cloned GSCs (Figure 6B). We did not observe any teratoma formation after transplantation of the GSCs into athymic mice. When P1C1 and P2C3 GSCs were applied to the gPS cell conversion culture, we obtained gPS cells (Figures 6C and 6D). RT-PCR analysis confirmed pluripotent gene expression in the gPS cells (Figure S7D). Microarray analyses were performed on the gPS cells converted from the GSCs (Figure S7E). The chimera assay showed that gPS cells contributed to germ cells in E14.5 fetal gonads (2/19) (Figures 6E and 6F). Differentiation ability of the gPS cells was also proven by in vitro differentiation assay (Figures S7F–S7I and Movie S2) and teratoma assay (Figures S7J and S7K). Overall, these results suggest that the pluripotent cells did indeed originate from unipotent GSCs.

Since our GSC lines were established after culturing the whole testis, we needed to exclude the possibility that they originated from circulating somatic stem cells in the adult testis, rather than from GSCs. We sorted GSCs directly from the adult testis on the basis of Oct4-GFP and c-kit expression (Figure S8A). A previous study has shown that c-kit is expressed in differentiated germ cells but not in undifferentiated GSCs (Shinohara et al., 2000). Oct4-GFP$^+$ and c-kit$^-$ cells (0.023% of total testicular cells) that represent the GSC population in the testis were plated as single cells into 96-well plates containing MEFs to establish clonal GSC lines (Figures S8A and S8B). We found that GSC colonies formed from Oct4-GFP$^+$/c-kit$^-$ cells (12 out of 192 wells), whereas no GSC colonies were observed to originate from c-kit$^+$ cells, even if Oct4-GFP was expressed (0/192). When Oct4-GFP$^+$/c-kit$^-$ cells isolated from the testis or clonally established GSCs from Oct4-GFP$^+$/c-kit$^-$ cells were directly cultured under ESC culture conditions, we could not observe the formation of either mGSCs or gPS cells. However, we could obtain gPS cells upon applying the gPS cell conversion protocol to the Oct-GFP$^+$/c-kit$^-$ established GSCs (Figure S8C). Microarray analyses show that the global gene expression of gPS cells is similar to ESCs (Figure S8D). The gPS cells can be differentiated into three germ layers in vitro and in vivo (Figures S8E–S8J and Movie S3). These data exclude the possibility that gPS cells originate from circulating adult stem cells and provide additional proof that the origin of gPS cells is the unipotent GSCs.

Microniche for the Generation of gPS Cells Based on the Initial Number of Plated GSCs and Length of Culture

In the initial attempt to generate gPS cells, we found distinct gPS cell colonies after about 3 weeks of culture (Figure 2B). It was first unclear whether the gPS colonies had developed from GSC-only colonies or whether they had originated from a subpopulation of cells residing within a GSC colony. To address this question, we began to closely examine the growth of each culture. After about 2 weeks of culture, a subpopulation of cells with high Oct4-GFP expression was detected inside a GSC colony (Figure 7A), which had just started to form a gPS cell colony (Figure 7A). The cells grew so rapidly that a gPS cell colony had formed within 5 days. Positive immunostaining with Nanog confirmed that the colony actually consisted of gPS cells (Figure 7A). We also found that converted cells kept appearing continuously even after 2 weeks of culture (Figure 7B).

We hypothesized that the initial number of plated GSCs was critical for gPS cell conversion. To test this, GSCs were plated at a range of 1,000 to 20,000 cells per well in 24-well plates and cultured for 4 weeks to observe gPS cell conversion. We could not detect gPS cell colonies in the wells plated with more than 10,000 cells, while we found colonies in the wells

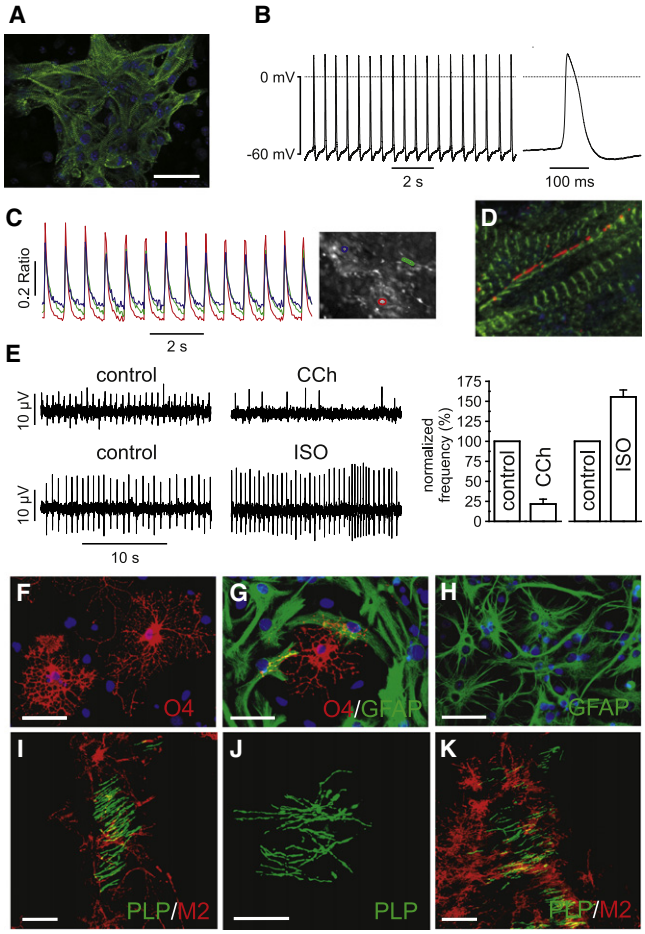

Figure 5. Differentiation and Function of gPS Cell-Derived Cardiomyocytes and Neural Cells

(A and D) α-actinin-positive (green) cross-striated cardiomyocytes. (B) Action potentials recorded with a sharp electrode from a beating area (d5 + 7 EB); right panel: enlarged single action potential. (C) Spontaneous and synchronous Ca^{2+} transients (left panel) recorded from three different (cells are marked in the right panel, ~200–300 μm apart) Fura-2-loaded cardiomyocytes. (D) Connexin 43 staining in adjacent cardiomyocytes (red). (E) Extracellular field potentials recorded with multielectrode arrays from beating areas of differentiated (d4 + 14) EBs. Negative (top traces, charbachol [CCh]) and positive (lower traces, isoprenaline [ISO]) chronotropic modulation is observed. Hormonal modulation statistics of normalized beating frequencies (right panel; $p < 0.05$; CCh: n = 4, ISO: n = 7, error bars: SEM). (F–H) Following in vitro growth factor withdrawal, gPS cell-derived-glial precursors (GP) produced 33% ± 6% O4-positive oligodendrocytes and 55% ± 10% GFAP-positive astrocytes. (I–K) Upon transplantation of gPS cell-derived-GP cells into the brain of myelin-deficient (md) rats, myelination of host axons in the corpus callosum was observed. Newly formed internodes are identified using an antibody specific to PLP, which is absent in the PLP-deficient md rats. Astrocytes are labeled with the mouse-specific M2 antibody. (F–H) Conventional fluorescence photomicrograph. (I–K) Confocal image. Scale bars: 50 μm (A), 230 μm (C), 8 μm (D), 50 μm (F–K).

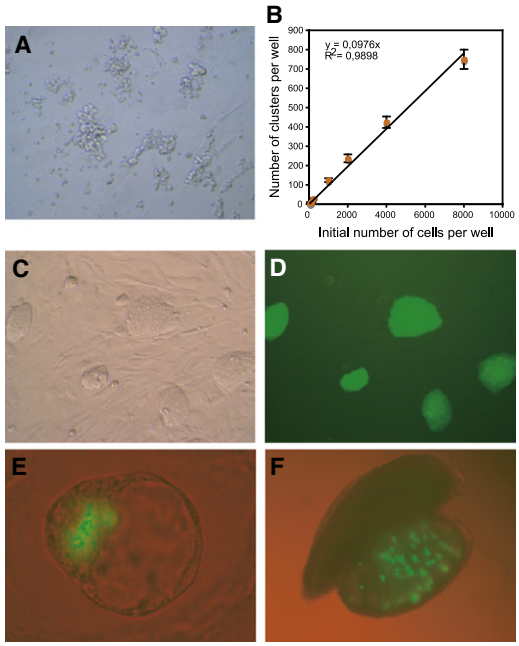

Figure 6. Characterization of GSCs from Single GSCs and gPS Cells from the Clonal GSCs

(A) Phase contrast photomicrograph of P1C1 GSCs.

(B) Clonally established formation, which is a functional characteristic. Error bars indicate standard deviations.

(C and D) Phase contrast photomicrograph (C) and GFP fluorescence (D) of P1C1 gPS cells.

(E) Blastocyst stage of an embryo created by the aggregation of an 8-cell-stage embryo with P1C1 gPS cells.

(F) Oct4-GFP-positive germ cells were detected in male fetal gonads from E14.5 embryos.

plated with fewer than 8,000 cells (Figure 7C). We estimated that about 0.01% of the plated GSCs (4,000 per well) had been converted into gPS cells within 4 weeks of culture. For GSC expansion, the density of GSCs is approximately 5- to 20-fold higher than that for the gPS cell conversion culture. Thus, in the ordinary GSC expansion culture, the seeding density might be too high and the culture time too short for the development of a microniche that would support gPS cell conversion. Further experiments are required to elucidate the molecular mechanisms underlying the effect of this microniche environment on gPS cell conversion.

DISCUSSION

Our study demonstrated that unipotent GSCs established from adult testis can be converted into pluripotent cells. Recently, several research groups claimed to have obtained multi- or pluripotent stem cells from adult or newborn mice (Guan et al., 2006; Izadyar et al., 2008; Kanatsu-Shinohara et al., 2004, 2008; Seandel et al., 2007). Yet these studies were all limited in scope, inasmuch as samples were either obtained from neonatal mice or lacked the full pluripotency potential (i.e., germ cell contribution and chimera germline transmission) and that a detailed description of the potency and origin of the initial GSCs was not provided. A comparison of previous studies is shown in Table S2.

Using clonally established GSCs, we proved that gPS cells originated from unipotent adult GSCs. We observed that pluripotent cells, such as gPS cells and ESCs, formed teratomas in the transplanted testis in the absence of spermatogenesis,

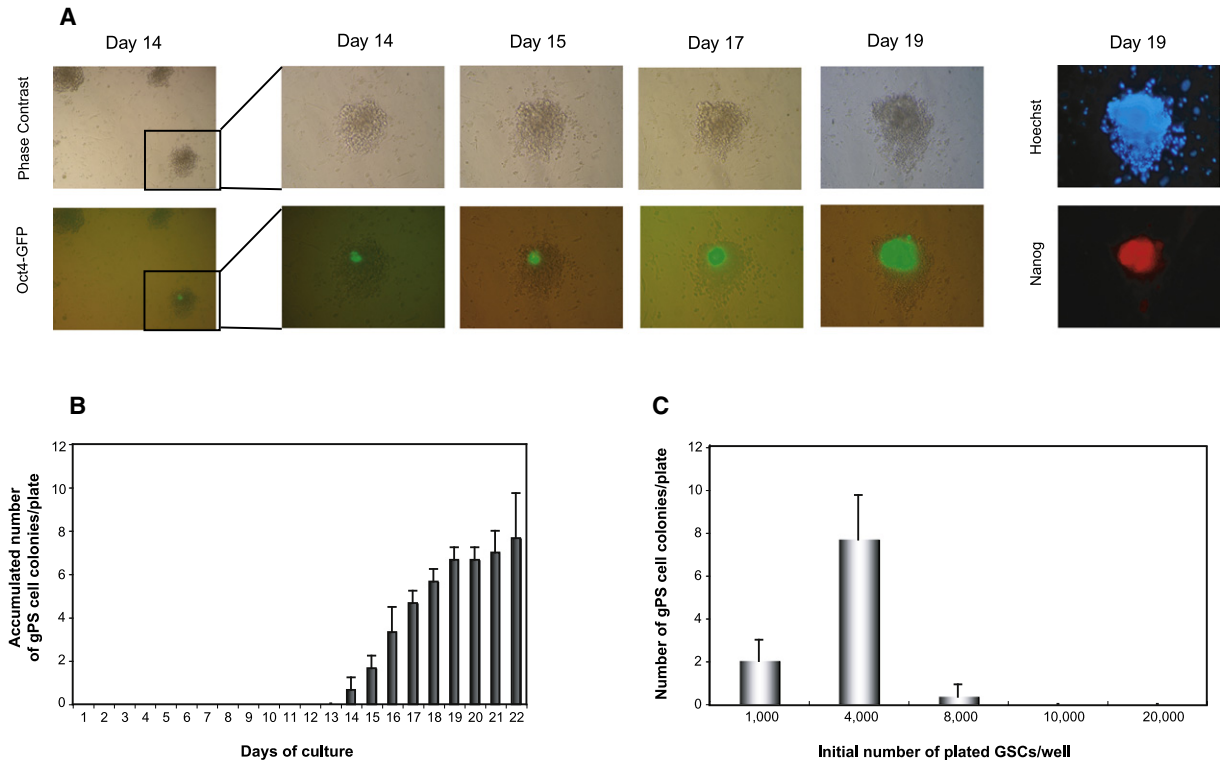

Figure 7. Critical Time Frame and Cell-Number-Dependent Microniche for the Conversion of GSCs into gPS Cells

(A) A subpopulation of GSCs was converted into gPS cells at about 14 days of culture. Time course observation of the growth of gPS cells. Nanog expression was confirmed by immunofluorescence staining.

(B) The number of gPS cell colonies was counted from day 0 to day 21 of culture. Error bars indicate standard deviations.

(C) The number of converted gPS cell colonies depends on the initial number of GSCs plated per well in 24-well plates. Error bars indicate standard deviations.

while unipotent GSCs restored spermatogenesis but did not form teratomas or chimeras. This observation is consistent with those of previous reports (Kanatsu-Shinohara et al., 2004, 2008; Kubota et al., 2004; Seandel et al., 2007). In contrast to previous studies and our current data, Guan et al. reported that GSCs isolated from testis using a Stra8-GFP marker display both unipotency and pluripotency. The authors argued that these cells show double characteristics (Guan et al., 2006), as Stra8-GFP-positive cells both restored spermatogenesis without forming teratomas when transplanted into testis and contributed to the germline in chimeras after injection into blastocysts. Stra8-GFP-positive cells (unipotent/pluripotent) could be converted (dedifferentiate/differentiated) into mGSC under ESC culture conditions. Although Guan et al. showed that the initial Stra8-GFP-positive GSCs were pluripotent, but also unipotent, the generated mGSCs were not tested for chimera contribution and germline competency—the hallmark of true pluripotency. The inconsistencies between the study of Guan et al. and those cited above can perhaps be attributed to different cell sources. Only a clonal experiment can shed light on the origin of Guan et al. cells. Despite the fact that a recent study has suggested that Stra8 plays a role in initiation of meiotic differentiation (Anderson et al., 2008), we analyzed whether Stra8-positive germ cells could have better competency for ESC-like cell conversion, as Guan et al. claimed. We found that increasing Stra8 expression in GSCs by treatment of retinoic acid did not improve the gPS conversion (data not shown).

gPS cells exhibited characteristics of ESCs with respect to gene expression, in vitro and in vivo differentiation potential, and germline chimera contribution—all characteristics of true pluripotency. gPS cells did not result in born pups after tetraploid complementation (0/82), which is likely due to the imprinting status of gPS cells, whose DMRs of *H19* and ICRs of *Igf2r* are maintained as androgenetic patterns. This result is also supported by a previous report showing that DNA methylation of imprinted genes is critical for fetal development (Feil et al., 1994). It is important to note that paternal imprinting patterns of *H19* and *Igf2r* in gPS cells are not altered, even after 20 passages. Unlike pluripotent stem cells from newborn testis (Kanatsu-Shinohara et al., 2004), gPS cells from adult GSCs still maintain an androgenetic pattern in DMRs of *H19*, while the unmethylated status of ICRs of *Igf2r* remained unchanged for our gPS cells and also for pluripotent stem cells established from newborn mice (Kanatsu-Shinohara et al., 2004) (Figure S9). This divergence is possibly related to the fact that gPS cells are originated from adult GSCs, in which de novo paternal DNA methylation of imprinted genes has been completed, whereas pluripotent cells from newborn testis possibly originated from early GSCs, which had not yet acquired a full paternal imprinting status. Interestingly, mGSCs from the study by Guan et al. showed somatic patterns in both *H19* and *Igf2r*, similar to ESCs but clearly not to GSCs

(Figure S9). These results led us to propose two possibilities: (1) in the derivation protocol of Guan et al., the paternal imprinting patterns could have changed to somatic patterns even though mGSCs may have truly originated from adult GSCs; or (2) the cells possibly originated from circulating somatic stem cells in the adult testis. In human, it has been reported that adult spermatogonial cells acquire a paternal imprinting pattern in *H19* (Kerjean et al., 2000). Interestingly, isolated spermatogonial cells and human pluripotent GSCs in the studies by Conrad et al. and Kossack et al. did not show clear paternal imprinting patterns in *H19* and *Igf2r* (Conrad et al., 2008; Kossack et al., 2009), which is also inconsistent with our notion that DNA imprinting methylation patterns in GSCs are not altered during the gPS cell conversion. The androgenic imprinted pattern of our gPS cells also explains the observed low rate of germline transmission (3/73, 4% for the C57BL6/C3H/CD1 chimeric male mouse and 0% for the CD1 chimeric male mouse), since Surani et al. have reported that androgenetic ESCs had a germline transmission rate between 0% and 4% (Narasimha et al., 1997).

Two reports cited above (Conrad et al., 2008; Kossack et al., 2009) claimed the generation of ESC-like cells from human pluripotent GSCs of adult human testis, but these ESC-like cells had not fully acquired pluripotency, as determined by pluripotent gene expression and/or teratoma formation. This may also explain the high methylation level of the Oct4 and Nanog promoters in these ESC-like cells. These two key pluripotent promoter genes were fully demethylated in our gPS cells (Figure S4C). Oct4 promoter methylation is a very relevant criterion of complete pluripotency. The first iPSCs described by Yamanaka et al. in 2006 showed a partially methylated Oct4 promoter, and although they were capable of forming teratomas, they could not contribute to the germline (Takahashi and Yamanaka, 2006). In the following reports (Okita et al., 2007), fully reprogrammed iPSCs were generated with completely unmethylated Oct4 promoters. These cells were capable of forming teratomas and showed germline transmission. Since teratoma formation and microarray analysis can be used to assess the pluripotency of human cells, we also compared the microarray data published by Conrad et al. with our scatter plot data (Figure S10). Whereas our gPS cells were highly similar to mouse ESCs, the cells derived from human testis were not similar at all to human ESCs. Thus, we conclude that the cells published by Conrad et al. (2008) are not pluripotent. However, it is possible that these cells are multipotent, which still would be quite adequate for therapeutic purposes.

Our findings suggest that the rate for conversion of GSCs into gPS cells is about 0.01% of the initially plated GSCs. This phenomenon consistently occurs within 2 to 4 weeks of GSC culture under our culture conditions. Modification of the composition of the medium used in our study from that used in previous studies describing the generation of mGSCs from adult testis may account for the reported differences in pluripotency. However, we do not have clear evidence that this modification affects the conversion status (multipotency/pluripotency) of ESC-like cells. Another possible explanation is that our culture system created a microenvironment, distinct from those of previous studies, thus supporting a different outcome. Our data suggest that plating fewer than 8000 GSCs per well in 24-well plates supports the generation of gPS cells; this number of GSCs is approximately 5- to 20-fold lower than that in regular expansion culture. Overall, the observed differences between our study and previous others may be due to a combination of factors, including medium composition, criteria used for identification of ESC-like cells, and a different microenvironment created within our culture system.

Conversion of unipotent PGCs into pluripotent EG cells was first demonstrated in mouse and human models (Matsui et al., 1992; Resnick et al., 1992; Shamblott et al., 1998). This is consistent with our finding that unipotent GSCs can be converted into pluripotent cells under specific in vitro culture conditions. Notably, abnormal conversion of germ cells into pluripotent cells can be found in vivo. It has been suggested that germ-cell-related tumorigenesis and pluripotency of germ cell tumors (GCTs) are associated with the expression of Oct4 (Looijenga et al., 2003). Although the pathogenic mechanism underlying the formation of GCTs remains unknown, it is assumed that accumulation of genetic and molecular changes mediates the transformation of Oct4-expressing germ cells to GCTs (Looijenga et al., 2007).

Here we report that GSCs established from adult testis can be fully reprogrammed into pluripotent stem cells under relatively simple culture conditions. Just as somatic cells can only be converted into pluripotent cells upon introduction of a cocktail of exogenous transcription factors, so can the generation of gPS cells result from a microenvironment of GSC colonies created within 2 weeks of culture depending on the initial number of plated GSCs. Our study provides a robust and reproducible in vitro model to study the mechanisms underlying the conversion of unipotent germ cells into pluripotent stem cells. Furthermore, our mouse model could help to uncover mechanisms involved in germ-cell-related testicular teratoma formation and thus further our understanding of human testicular cancer.

EXPERIMENTAL PROCEDURES

Establishment of GSCs from Adult Testis

GSCs were established from Oct4-GFP transgenic mice as previously described (Kanatsu-Shinohara et al., 2003) with modifications. Testicular cells in GSC culture medium were plated onto gelatin-coated culture dishes (2×10^5 cells/3.8 cm^2). GSC colonies were observed under the microscope within 7 days in culture. GSC colonies were collected by gentle pipetting. Collected GSCs were reconstituted with 500 μl of GSC medium and replated on one well of a 24-well plate containing mitomycin C-inactivated or irradiated MEFs for expansion. Detailed procedures are provided in the Supplemental Experimental Procedures.

Conversion of gPS Cells from GSCs

GSCs cultured under expansion conditions were dissociated into single cells by trypsinization. Approximately 1,000 cells were plated per well in 24-well plates containing fresh MEF feeder cells in GSC culture medium. For analysis of gPS cell generation efficiency, about 1,000 to 20,000 GSCs were plated per well in 24-well plates. The medium was changed every 2 to 3 days, but the culture was maintained without splitting. gPS cell colonies with high Oct4-GFP expression appeared within 2 to 4 weeks of culture. To expand the gPS cells, the colonies were enzymatically dissociated into single cells and cultured under ESC culture conditions at 37°C in an atmosphere of 5% CO_2 in air. Figure S3 shows a schematic representation of the conversion protocol.

RT-PCR and Bisulfite Sequencing Analysis

RT-PCR and bisulfite sequencing analyses were performed as previously described (Kim et al., 2008). The primer sequences are listed in Table S3.

Immunocytochemistry and Flow Cytometric Analysis

Immunocytochemistry analysis was performed according to the protocol previously described (Kim et al., 2008). Flow cytometric analysis was conducted as described elsewhere (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). Antibodies used in this study are listed in the Supplemental Experimental Procedures.

Testicular Transplantation

The transplantation experiments were performed as previously described (Ogawa et al., 1997). Two- to four-week-old germ-cell-depleted male mice were used as the recipient mice. Approximately 3×10^5 cells were injected with a micropipette (80 μm diameter tips) into the seminiferous tubules of the testes of recipient mice through the efferent duct. For further details, see the Supplemental Experimental Procedures.

In Vitro Differentiation of gPS cells

For the differentiation of gPS cells into cardiomyocytes and neural cells, embryoid bodies (EBs) from gPS cells were applied to the protocols described in previous studies (Brustle et al., 1999; Igelmund et al., 1999). For further details, see the Supplemental Experimental Procedures.

Chimera Formation

Chimera assay was performed using the protocol previously described (Wood et al., 1993) with modifications. Briefly, 8-cell-stage embryos were flushed from mice at 2.5 days post coitum (dpc) and placed in M2 medium. Clumps of ESCs (10 to 20 cells) from short trypsin-treated day 2 cultures were aggregated with a single embryo. The aggregates were cultured overnight at 37°C in an atmosphere of 5% CO_2 in air. After 24 hr of culture, the majority of the aggregates had formed blastocysts. Approximately 11 to 14 aggregated embryos were transferred into the uterine horn of each 2.5 dpc pseudopregnant mouse. For further details, see the Supplemental Experimental Procedures.

GSC Cluster Analysis

GSC cluster analysis was conducted using the previously described methods with minor modifications (Yeh et al., 2007). For quantification of cluster formation, GSCs were plated at a range of 100 to 8000 cells per well in 24-well plates containing MEFs and GSC culture medium. The number of clusters was counted after 7 days of culture. The experiment was performed in three different plates.

Microarray Analysis

The microarray study was carried out using either Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) or Illumina MouseRef-8 v2.0 Expression BeadChips. For further details, see the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The microarray data are available from the Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE11274 and GSE16178.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, three tables, ten figures, and three movies and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00283-5.

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REFERENCES

Anderson, E.L., Baltus, A.E., Roepers-Gajadien, H.L., Hassold, T.J., de Rooij, D.G., van Pelt, A.M., and Page, D.C. (2008). Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc. Natl. Acad. Sci. USA *105*, 14976–14980.

Boison, D., and Stoffel, W. (1989). Myelin-deficient rat: a point mutation in exon III (A→C, Thr75→Pro) of the myelin proteolipid protein causes dysmyelination and oligodendrocyte death. EMBO J. *8*, 3295–3302.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science *285*, 754–756.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Buhring, H.J., Mattheus, U., Mack, A., et al. (2008). Generation of pluripotent stem cells from adult human testis. Nature *456*, 344–349.

Dann, C.T., Alvarado, A.L., Molyneux, L.A., Denard, B.S., Garbers, D.L., and Porteus, M.H. (2008). Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. Stem Cells *26*, 2928–2937.

Duncan, I.D., Grever, W.E., and Zhang, S.C. (1997). Repair of myelin disease: strategies and progress in animal models. Mol. Med. Today *3*, 554–561.

Feil, R., Walter, J., Allen, N.D., and Reik, W. (1994). Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. Development *120*, 2933–2943.

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Lee, J.H., Nolte, J., Wolf, F., Li, M., Engel, W., et al. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature *440*, 1199–1203.

Igelmund, P., Fleischmann, B.K., Fischer, I.R., Soest, J., Gryshchenko, O., Bohm-Pinger, M.M., Sauer, H., Liu, Q., and Hescheler, J. (1999). Action potential propagation failures in long-term recordings from embryonic stem cell-derived cardiomyocytes in tissue culture. Pflugers Arch. *437*, 669–679.

Izadyar, F., Pau, F., Marh, J., Slepko, N., Wang, T., Gonzalez, R., Ramos, T., Howerton, K., Sayre, C., and Silva, F. (2008). Generation of multipotent cell lines from a distinct population of male germ line stem cells. Reproduction *135*, 771–784.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. *69*, 612–616.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. Cell *119*, 1001–1012.

Kanatsu-Shinohara, M., Lee, J., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Ikawa, M., Nakamura, T., Ogura, A., and Shinohara, T. (2008). Pluripotency of a single spermatogonial stem cell in mice. Biol. Reprod. *78*, 681–687.

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomeli, H., Nagy, A., McLaughlin, K.J., Scholer, H.R., et al. (2004). Oct4 is required for primordial germ cell survival. EMBO Rep. *5*, 1078–1083.

Kerjean, A., Dupont, J.M., Vasseur, C., Le Tessier, D., Cuisset, L., Paldi, A., Jouannet, P., and Jeanpierre, M. (2000). Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. Hum. Mol. Genet. *9*, 2183–2187.

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Araúzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., et al. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature *454*, 646–650.

Kim, J.B., Sebastiano, V., Wu, G., Araúzo-Bravo, M.J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M., van den Boom, D., et al. (2009). Oct4-induced pluripotency in adult neural stem cells. Cell *136*, 411–419.

Koeppen, A.H., Barron, K.D., Csiza, C.K., and Greenfield, E.A. (1988). Comparative immunocytochemistry of Pelizaeus-Merzbacher disease, the jimpy mouse, and the myelin-deficient rat. J. Neurol. Sci. *84*, 315–327.

Kossack, N., Meneses, J., Shefi, S., Nguyen, H.N., Chavez, S., Nicholas, C., Gromoll, J., Turek, P.J., and Reijo-Pera, R.A. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells *27*, 138–149.

Kubota, H., Avarbock, M.R., and Brinster, R.L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. USA *101*, 16489–16494.

Looijenga, L.H., Stoop, H., de Leeuw, H.P., de Gouveia Brazao, C.A., Gillis, A.J., van Roozendaal, K.E., van Zoelen, E.J., Weber, R.F., Wolffenbuttel, K.P., van Dekken, H., et al. (2003). POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res. *63*, 2244–2250.

Looijenga, L.H., Gillis, A.J., Stoop, H.J., Hersmus, R., and Oosterhuis, J.W. (2007). Chromosomes and expression in human testicular germ-cell tumors: insight into their cell of origin and pathogenesis. Ann. N Y Acad. Sci. *1120*, 187–214.

Matsui, Y., Zsebo, K., and Hogan, B.L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841–847.

Narasimha, M., Barton, S.C., and Surani, M.A. (1997). The role of the paternal genome in the development of the mouse germ line. Curr. Biol. *7*, 881–884.

Ogawa, T., Arechaga, J.M., Avarbock, M.R., and Brinster, R.L. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. Int. J. Dev. Biol. *41*, 111–122.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313–317.

Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550–551.

Seandel, M., James, D., Shmelkov, S.V., Falciatori, I., Kim, J., Chavala, S., Scherr, D.S., Zhang, F., Torres, R., Gale, N.W., et al. (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature *449*, 346–350.

Shamblott, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R., and Gearhart, J.D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc. Natl. Acad. Sci. USA *95*, 13726–13731.

Shinohara, T., Orwig, K.E., Avarbock, M.R., and Brinster, R.L. (2000). Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc. Natl. Acad. Sci. USA *97*, 8346–8351.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Tegelenbosch, R.A., and de Rooij, D.G. (1993). A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat. Res. *290*, 193–200.

Wood, S.A., Allen, N.D., Rossant, J., Auerbach, A., and Nagy, A. (1993). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. Nature *365*, 87–89.

Yeh, J.R., Zhang, X., and Nagano, M.C. (2007). Establishment of a short-term in vitro assay for mouse spermatogonial stem cells. Biol. Reprod. *77*, 897–904.