Generation of multipotent cell lines from a distinct population of male germ line stem cells

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Abstract

Spermatogonial stem cells (SSCs) maintain spermatogenesis by self-renewal and generation of spermatogonia committed to differentiation. Under certain *in vitro* conditions, SSCs from both neonatal and adult mouse testis can reportedly generate multipotent germ cell (mGC) lines that have characteristics and differentiation potential similar to embryonic stem (ES) cells. However, mGCs generated in different laboratories showed different germ cell characteristics, i.e., some retain their SSC properties and some have lost them completely. This raises an important question: whether mGC lines have been generated from different subpopulations in the mouse testes. To unambiguously identify and track germ line stem cells, we utilized a transgenic mouse model expressing green fluorescence protein under the control of a germ cell-specific *Pou5f1* (*Oct4*) promoter. We found two distinct populations among the germ line stem cells with regard to their expression of transcription factor *Pou5f1* and c-Kit receptor. Only the POU5F1+/c-Kit+ subset of mouse germ line stem cells, when isolated from either neonatal or adult testes and cultured in a complex mixture of growth factors, generates cell lines that express pluripotent ES markers, i.e., *Pou5f1*, *Nanog*, *Sox2*, *Rex1*, *Dppa5*, *SSEA-1*, and alkaline phosphatase, exhibit high telomerase activity, and differentiate into multiple lineages, including beating cardiomyocytes, neural cells, and chondrocytes. These data clearly show the existence of two distinct populations within germ line stem cells: one destined to become SSC and the other with the ability to generate multipotent cell lines with some pluripotent characteristics. These findings raise interesting questions about the relativity of pluripotency and the plasticity of germ line stem cells.

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Introduction

Application of stem cells for therapeutic purposes has been the focus of stem cell science since the successful derivation of human pluripotent embryonic stem (ES) cells from pre-implantation embryos in 1998 (Thomson *et al.* 1998). Later on, numerous studies (Thomson & Odorico 2000, Weissman *et al.* 2001) have explored the potential of different stem cells, including ES, embryonic germ (EG), and adult stem cells for cell replacement therapy. Recently, multipotent germ cell (mGC) lines have been generated from neonatal mouse using culture-induced reprogramming (Kanatsu-Shinohara *et al.* 2004), indicating the presence of cell populations in the mouse testes with the ability to acquire pluripotency in culture. Multipotent cell lines generated in that study possessed identical ES cell characteristics and were completely devoid of SSC functional properties, i.e., they were unable to repopulate recipient testes following testicular transplantation. Recently, the identity of subpopulation in the mouse testes with the ability to culture-induced reprogramming has been demonstrated (Seandel *et al.* 2007). Interestingly, the cell lines

generated from this cell population acquired ES characteristics and yet maintained their SSC functional properties. mGC lines could also be generated from adult mouse testes without reprogramming growth factors (Guan *et al.* 2006), indicating the presence of a subpopulation of cells with pluripotent characteristics in the adult testes. Altogether, these studies suggest the presence of different subpopulations of mouse testicular stem cells with different abilities.

In the present study, to investigate whether the SSCs or different subpopulations of germ line stem cells generate the mGC lines, we took advantage of a transgenic mouse model expressing green fluorescence protein (GFP) driven by a germ line-specific *Pou5f1* (*Oct4*) promoter (Yeom *et al.* 1996). POU5F1 (previously known as octamer-binding transcription factor 3/4, OCT4) was originally identified as an ES cell and a germ line-specific marker (Okamota *et al.* 1990, Scholer *et al.* 1990). The expression of *Pou5f1* is regulated by a proximal promoter, a germ-specific distal enhancer, and a retinoic acid-responsive element (Saiti & Lacham-Kaplan 2007). At gastrulation, *Pou5f1* expression is down-regulated and thereafter is maintained only in primordial germ cells

(Yeom *et al.* 1996). PGCs, of both males and females, continue to express *Pou5f1* as they proliferate and migrate to the genital ridges. In the males, the expression in germ cells persists throughout fetal development and is maintained postnatally in proliferating gonocytes, prospermatogonia, and undifferentiated spermatogonia, including A single (A_s), A paired (A_{pr}), and A aligned (A_{al}) spermatogonia (Pesce *et al.* 1998, Tadokora *et al.* 2002). As in the mouse only the A_s spermatogonia are considered to be spermatogonial stem cells (SSCs), enriched populations of undifferentiated spermatogonia including SSCs can be isolated by sorting the POU5F1-GFP cells from OG2 transgenic mouse model.

Germ line stem cells could further be subdivided based on the expression of c-Kit receptor molecule. c-Kit, a tyrosine kinase receptor, and its ligand stem cell factor (also known as kit ligand or steel factor) are key regulators of PGC growth and survival (De Miguel *et al.* 2002). c-Kit is expressed in PGCs from their initial segregation to their arrival at the genital ridge. In postnatal mouse testes, it has been shown that c-Kit can be used as a marker for the differentiation of undifferentiated and differentiating type A spermatogonia (Schrans-Stassen *et al.* 1999). Therefore, the expression levels of POU5F1 and c-Kit were used in this study to isolate distinct populations of germ line stem cells. We then analyzed the molecular and phenotypic characteristics of these cells extensively before and after culture and compared their ability to generate multipotent cell lines under a defined culture condition with a mixture of growth factors. In addition, the functionality of these subpopulations and their descendant mGC lines to repopulate recipient testes was evaluated using the SSC transplantation technique.

Results

For enrichment of germ line stem cells, both neonatal and adult testicular tissues were cultured on gelatin-coated culture dishes for 2 h for differential adhesion to remove somatic cells but not germ cells (Kanatsu-Shinohara *et al.* 2004). After differential adhesion, cell suspensions containing GFP-positive cells (4–10% in the neonates, 0.01–0.05% in the adults) could be retrieved (Fig. 1a–c). Harvested germ cells were cultured in a stem cell culture medium with a mixture of growth factors as described. Initial GFP signals disappeared after a few days in culture (Fig. 2a and b). Thereafter, the cells underwent distinct morphological changes forming chain-like colonies that continued to grow without GFP signal (Fig. 2c–e). Up-regulation of POU5F1 indicated by the occurrence of GFP-positive cells within colonies appeared after 3–4 weeks of culture (Fig. 2f). For expansion, the cells were co-cultured with mouse embryonic fibroblast (MEF) feeders in the same culture medium supplemented with 15% fetal bovine serum (FBS). For derivation of cell lines from adult mice, GFP-positive cells harvested after

differential adhesion were sorted using fluorescence-assisted cell sorting (FACS) and were directly cultured on MEF. Using OG2 or OG2-LacZ mice, 19 cell lines (10 neonatal and 9 adult) have been generated. All cell lines expressed either GFP (14 lines) or GFP-LacZ (5 lines) (Fig. 2g–i). In addition, a mGC line has been generated from neonatal wild-type CD-1 mice, indicating that the method is not limited to transgenic OG2 mice (data not shown). Selected cell lines have been frozen/thawed and propagated for more than 40 splittings with an estimated cell doubling time of 72 h (using both manual cell count and GFP sort; Fig. 3).

Next, we separated c-Kit-positive GFP-positive cells from the c-Kit-negative GFP-positive cells by FACS. Among the GFP-positive cells, 60% were c-Kit negative, and c-Kit was dimly expressed on the positive fraction. A linear correlation was observed between the expression level of POU5F1 and c-Kit in germ line stem cells, showing that only the POU5F1 high germ cells were c-Kit positive. Interestingly, the POU5F1 high germ line stem cells were larger than POU5F1 low cells based on their forward scatter distribution (Fig. 1). Only c-Kit-positive populations generated mGC colonies and no cell line could be generated from the c-Kit-negative pool. Among the growth factors used in this study, removal of GDNF resulted in smaller colonies indicating the role of GDNF in the self-renewal of the mGCs. By contrast, removal of FGF2 resulted in the differentiation of the colonies indicating possible role of FGF2 in the maintenance of the mGCs in their undifferentiated stage. Removal of leukemia inhibitory factor (LIF) or EG factor (EGF) did not affect the expansion nor the differentiation of the mGCs (Supplementary Fig. 1, which can be viewed online at www.reproduction-online.org/supplemental).

Marker and imprinting profiles

The majority of cells in the mGC colonies expressed POU5F1, NANOG, SSEA-1, and VASA (Fig. 4a–d). They also expressed pluripotent genes *Sox2*, *Dppa5*, *Rex1*, *eRas*, and *Crypto* along with germ line-specific markers, including *Stella*, *Dazl*, *Vasa*, and *cRet* (Fig. 4q). In addition, the expression of POU5F1, NANOG, and SOX2 was confirmed by Western blot analysis (Fig. 4p). Among the three mGSC lines tested in this study, only one line showed *Nanog* expression and this is the line that has been used for Western blot analysis. The mouse cell line at passage 20 showed high telomerase activity (similar to ES cells) and normal karyotype (40, XY) (Fig. 5).

We also analyzed the global gene expression and imprinting patterns of the mGCs before and after culture and compared that with ES cells. Interestingly, our culture condition did not change the imprinting pattern of the mGCs in all the DMR sites tested. In contrast to the mouse ES cells that showed only a partial androgenetic imprinting, the mGCs clearly exhibited a 100% androgenetic imprinting pattern (Fig. 6). Somewhat

Figure 1 Enrichment of GFP⁺ subpopulations from testicular stem cells isolated from the transgenic OG2 mouse using flow cytometry. POU5F1⁺ cells as indicated by GFP expression were found as a distinct cell population in both neonatal (b) and adult (c) OG2 mouse compared with the wild-type (a). Among the POU5F1⁺ cells, two clear subpopulations consisting of c-Kit positive (R5) and c-Kit negative (R2) were found (d and e). Correlation between expression of GFP and c-Kit is shown in (f–h). Note that the c-Kit dim cells (blue) have also the highest level of GFP (dashed outline) expression. (G) GFP expression in a 2D plot showing the expression of c-Kit-negative cells (red) and c-Kit dim cells (blue). The c-Kit dim cells tend to display overall higher levels of GFP. Scatter plot of the GFP/c-Kit show that GFP-positive c-Kit-negative cells are low in both forward (FSC) and side scatter (SSC), indicating that they are small, round, clear cells. GFP-positive c-Kit-positive cells are forward scatter high and side scatter low/medium, indicating that they are larger cells with not much complexity.

surprisingly, our microarray analysis showed that the global gene expression pattern of the mGCs had 87% similarity before and after culture.

Differentiation potential

When mGCs were aggregated to form embryoid bodies (EBs), gastrulation was observed within 9–15 days (Fig. 7a). Cells in the EBs expressed early developmental markers including E-cadherin and laminin-1 (markers of polarized epithelium, Fig. 7b and c); ZIC1, PAX6, and SOX1 (early ectoderm markers, Fig. 7d and f); GATA4 and FOXA2 (early endoderm markers, Fig. 7e and f); and BRACHURY, BMP4, and COL2A1 (early mesoderm markers, Fig. 7f). In culture, mGC colonies spontaneously differentiated into phenotypes expressing markers of cardiomyocytes (Fig. 7g–j), adipocytes (Fig. 7k), and neural cells (Fig. 7l and m). Some of the cells that spontaneously differentiated to cardiomyocytes exhibited rhythmic contractions for up to 3 days (Fig. 7i, the beating sequence of representative cells are shown in the Supplementary Video, which can be viewed online at www.reproduction-online.org/supplemental). Using directed differentiation protocols, mGC lines could be induced to differentiate into neural cells representing neural progenitors (nestin, NEUROD1), neurons (MAP2,

NF-68, GAD67), and glial cells (GFAP, MBP, A2B5, O4, NG2), as shown in Fig. 8a–g and j. They could also be induced to form cardiomyocytes (troponin-1, cardiac myosin, desmin, NKX2.5, GATA4) or chondrocytes (collagen Xa1 and staining by alcian blue) (Fig. 8h–l and k–l).

In a separate differentiation study with mGCs, we counted the number of cells (nuclei) with and without staining of neural markers in seven colonies within a culture, and the average percentage was estimated as 17.6% for GFAP⁺ cells, 2.5% for Tuj-1⁺ cells, and 2.3% for MAP2⁺ cells. In general, the efficiency of induced differentiation by these protocols was much higher in ES cells compared with the mGCs.

Testes regeneration

Four weeks after transplantation, testes of the control animals as well as those that received POU5F1-positive c-Kit-positive cells showed no spermatogenesis in the majority of the seminiferous tubules. On the contrary, 80% of those that received freshly isolated POU5F1-positive c-Kit-negative cells showed some degrees of spermatogenesis throughout the testes, indicating the presence of functional SSCs in the cell suspension.

Figure 2 Morphological changes during the development of multipotent germ cell lines in culture. A few mouse POU5F1-GFP cells were observed in cell preparations before culture. Note the presence of large (arrow) and small (asterisk) GFP-positive cells (a). Shortly after culture, down-regulation of POU5F1 was observed (b). After the attachment of the cells in the second week of culture, obvious morphological changes occurred (c and d). Approximately, 3 weeks after culture, colonies containing small round cells were formed (e). Up-regulation of POU5F1 was observed about 1 month after culture (f). Images of three established mGC lines derived from neonatal OG2, adult OG2, or neonatal OG2-LacZ are presented in (g-i). Scale bars: 50 µm.

Similarly, our short-term transplantation experiment using the cell trace marker carboxyfluorescein diacetate succinimidyl ester (CFSE) showed that only the c-Kit-negative subpopulation of germ line stem cells colonized the recipient testes (Fig. 9). No spermatogenesis was found in the majority of seminiferous tubules of the recipient mice testes transplanted with the mGCs, indicating that these cells do not have SSC properties (Table 1).

Teratomas and chimera formation

For teratoma formation, we injected equal numbers of mouse ES cells (as positive control) or POU5F1-GFP/LacZ mGC cells into the skin, muscle, and testis of different groups of nude mice (one million cells/site). All recipient mice (6/6) receiving ES cells developed teratomas in all three tissue types. By contrast, none of the mice (0/20) receiving mGSCs gave rise to teratomas (Fig. 9a-f; Supplementary Fig. 2, which can be viewed online at www.reproduction-online.org/supplemental). Six weeks after transplantation, POU5F1-GFP/LacZ cells were found in skin, muscle, and testicular tissues (Fig. 9g-l). These data show that mGCs, unlike ES cells, are non-tumorigenic. We tested chimera formation by injecting cultured POU5F1-GFP/LacZ cells into eight-cell embryos and blastocysts of CD-1 mice, an approach considered by many to be the highest standard for proving the pluripotency of cells. As shown in Fig. 10a-d, POU5F1-GFP/LacZ cells were incorporated into the inner cell mass of the mouse

Figure 3 Doubling time of the mGS cells. Multipotent germ cell lines (mGC) were cultured on mouse embryonic fibroblast (MEF) feeders in culture medium supplemented with 15% fetal bovine serum (FBS). At different time points during culture, the number of GFP+ cells was analyzed using fluorescence-activated cell sorting (FACS). The data are representative of three replicates. Note that the size of the GC colonies increased during culture (a-d). The analysis of GFP+ cell count during culture is shown in (e) with a cell doubling time estimated at 3 days (72 h). Scale bars: 60 µm.

blastocysts. The embryos were transferred to the uterus of pseudopregnant mice (a total of 45 fetuses from 119 transferred embryos). At 12.5 dpc, staining of whole embryos for LacZ (β -galactosidase activity) showed distinctive patterns in the eye, brain, and limbs (Fig. 10e). The intensity of LacZ staining was much higher in chimeric embryos receiving mouse ES cells than those injected with mGC lines. The distribution of chimeric cells is also demonstrated in histological sections of the brain, heart, gonadal ridge, and liver (Fig. 10l-o). The intensity and the number of LacZ-positive cells were much higher in chimeric embryos injected with LacZ-ES cells than those injected with LacZ-GS cells (data not shown). Confirmation of POU5F1-GFP/LacZ chimeric tissues was supported by the presence of GFP DNA sequence in the ectodermal (brain), mesodermal (heart), endodermal (liver), and testis of the chimeric pups (Fig. 10p), as well as the presence of LacZ DNA (Fig. 10q) in all four tissues. These combined results clearly demonstrate that cultured mGCs are non-teratogenic stem cells with some pluripotent characteristics.

Figure 4 Phenotypic and molecular characterization of multipotent germ cells. Immunofluorescence of pluripotent markers are shown for (a–d) POU5F1, (e–h) NANOG, and (m–o) SSEA-1. (i–l) The germ cell marker, VASA, in mGC colonies. Scale bars: (a–h) 25 μ m, (i–o) 20 μ m; expression of pluripotent and germ-specific markers determined by RT-PCR is shown in (q). The Western blot analysis of protein contents of POU5F1, NANOG, and SOX2 in mGC cells before and after immunoprecipitation (IP) is presented in (p). Note in the RT-PCR panel (q), among three mGC lines tested only line 3 shows *Nanog* expression.

Discussion

In this study, using a transgenic mouse model, we specifically isolated germ line stem cells from both neonatal and adult testes based on their POU5F1 expression. Germ line stem cells were further separated into two populations according to their expression level of c-Kit. Our FACS analysis showed two distinct populations regarding the expression levels of POU5F1 and c-Kit in germ line stem cells. Interestingly, only the POU5F1/GFP+ cells that possess c-Kit receptor molecule responded to culture and generated mGC lines and only the c-Kit-negative subpopulations repopulated the testis after SSC transplantation. This indicates the existence of two distinct subsets of cells within germ line stem cells, i.e., a c-Kit-positive pool containing larger cells with higher POU5F1 expression with the ability to become multipotent germ line stem cell and a smaller population of germ line stem cells that have lower POU5F1 and no c-Kit expression with SSC properties. Apparently, at least in the mouse, some germ stem cells in the testis are either at

different developmental stages or possess various signaling molecules that allow them to respond to growth factors at different speed. A recent study in the mouse showed that a G-protein (GP-125)-coupled receptor might play an important role as a signaling molecule in this process (Seandel *et al.* 2007). The existence of stem cells at different developmental stages has been proposed in other neonatal and adult tissues (Ratajczak *et al.* 2008).

We have derived multipotent cell lines from postnatal mouse testicular stem cells with some but not all pluripotent characteristics. These cell lines are distinctively different from the mGC lines obtained by the other laboratories (Kanatsu-Shinohara *et al.* 2004, Seandel *et al.* 2007), most notably, with regard to the extent of pluripotentiality and teratoma formation. Based on microarray analysis, our mGC lines express pluripotent genes, i.e., *Nanog* and *Crypto* respectively 1000 and 5000 times less than ES cells. Similarly, our cell lines express oncogenes, i.e., *p53*, *Eras*, *Bak*, *Int2*, and *c-myc*, several folds lower than the ES cells (Supplementary Fig. 3, which can be viewed online at www.reproduction-online.org/

Figure 5 Telomerase activity and karyotype analysis. (a) Telomerase activity in adult adipose stem cells (ADSC), mouse ES cells, freshly isolated germ line stem cells after c-Kit sorting, and multipotent germ line stem cells at passage 10 (neonatal OG2). The telomerase activity in the germ line stem cells is comparable with mouse ES cells and higher than the ADSC cells. (b) Karyotype of the same neonatal OG2 cell line. The picture is representative of 80 metaphase spreads analyzed. Note that after 15 passages the cells exhibit normal karyotype.

supplemental). Comparisons of the present findings with published reports suggest that mGC lines generated by other groups and our cell lines might differ in terms of developmental stages, imprinting profiles, and differentiation potential. Indeed, germ line-derived cells in the present report have properties of ES cells such as marker expression, broad differentiation potential, and limited chimera formation. However, these cells also seem to have retained germ cell-specific imprinting patterns and non-tumorigenic characteristics.

Several lines of evidence support the notion that our cell lines retain their germ cell properties more than they resemble the reported properties of ES cells. First, these

Figure 6 The imprinting analysis of multipotent germ cells before (NGC) and after culture (GC) and mouse ES cells with differentially methylated regions such as *Meg3*, *Peg10*, *Pou5f1*, *Igf2r*, and *Rasgrf1*. Note, except a slight difference in the *Meg3* locus, that the mGC showed strong similarity to NGC in their imprinting profile.

cells doubled their cell numbers in about 72 h (determined by both GFP sorting and manual counting). This cell doubling time, similar to that of germ line stem cells, is three times longer than that of the ES cells. Secondly, they seem to have molecular characteristics different from those in ES cells or other mGC lines. Our results on global gene expression analysis show that our cell lines have 65% similarity to ES cells and 87% to germ line stem cells. Among the genes tested, our cell lines showed significantly higher expression level of germ line-specific genes (*Vasa*, *Plzf*, *Gfra1*, *Dazl*) and lower expression level of pluripotent genes (*Pou5f1*, *Nanog*, *Dppa5*, *Sox2*, *Crypto*). Thirdly, our cell lines are more dependent on GDNF for their self-renewal than LIF or FGF2. GDNF has been shown to be the key regulator of the self-renewal of male germ line stem cells (Kubota *et al.* 2004, Ryu *et al.* 2005, Oatley *et al.* 2006), while LIF and FGF2 play crucial role in the self-renewal of the ES cells (Cartwright *et al.* 2005, Levenstein *et al.* 2005). Fourthly, the expression level of SSEA-1 in our cell lines was lower than that found either in mouse ES cells (Supplementary Fig. 4, which can be viewed online at www.reproduction-online.org/supplemental) or other mGC line as reported

Figure 7 Spontaneous differentiation of multipotent germ cells. Gastrulation of embryoid body (EB); a) and the expression of markers indicative of polarized epithelium (E-cadherin and laminin-1; b, c) and early development of the three germ layers, i.e., ectoderm (ZIC1, PAX6, SOX1), endoderm (GATA4, FOXA2), and mesoderm (BRACHURY, BMP4, and COL2A1) are shown (d–f) during culture reprogramming mGCs also differentiated spontaneously into cardiomyocytes (g–j), adipocytes (k), and neural cells (l and m). A video showing the rhythmic contraction of cells and cell aggregates derived from mouse GCs is presented in the Supplementary Video. Scale bars: (a and i) 50 μ m, (c and e) 30 μ m, (b and d) 25 μ m, (g, h and l) 45 μ m, (k) 12 μ m.

by Kanatsu-Shinohara *et al.* (2004). It has been shown that SSEA-1 may be involved in tumor invasion and metastasis in certain animal model systems (Kajiwaru *et al.* 2005), suggesting that higher expression may reflect higher potential for tumorigenesis. Finally, our multipotent GCs exhibited an androgenic imprinting pattern that is different from mouse ES cells or other mGC lines reported by other laboratories (Kanatsu-Shinohara *et al.* 2004, Seandel *et al.* 2007).

Despite all of the similarities to their germ line ancestors, our cell lines did not regenerate testes following transplantation. One possibility is that mGC

lines might have changed their phenotypic and/or molecular signature during culture condition. Indeed, a slight difference in gene expression profile between the mGCs and the non-cultured germ line stem cells was observed. As some of the altered genes were cell adhesion molecules, we speculate that at least some of these alterations might be due to the *in vitro* condition, i.e., attachment to MEF instead of basement membrane of testicular epithelium and the absence of the nursing somatic Sertoli cells. On the contrary, mouse SSCs cultivated for a long period of time are reported to repopulate recipient testes following SSC

Figure 8 Induced differentiation of multipotent germ cells into lineage-specific phenotypes. Confocal images of the cells that expressed neural markers are shown in (a–g). Expression of the neural gene markers is shown in (j). Confocal image of mGCs differentiated into cardiomyocytes are presented in (i). Expression of the cardiac gene markers is shown in (l). Alcian blue-positive chondrocyte after differentiation of mGCs is shown in (h). Expression of the chondrocyte-specific genes is shown in (k). Scale bars: (a, c and g) 20 μ m, (b and h) 50 μ m, (d–f and i) 10 μ m.

Figure 9 Formation of teratomas after transplantation of mouse ES cells (a–f) but not multipotent LacZ-GFP mGCs (g–l) into the skin, muscle, and testis. The morphology of ESC-derived teratomas was identified by H&E staining on thin paraffin sections, whereas the fate of transplanted multipotent germ cells (mGCs) was identified by LacZ staining shown in blue. Six weeks after transplantation, GFP-LacZ⁺ mGCs were found in the skin, i.e., in the bulging area of hair follicles and adjacent sebaceous glands (arrow head; g and h), in the muscle (arrows; i and j), and in the testis (arrows; k). Testes regeneration following transplantation of germ line stem cells before and after culture is presented in (m–r). Cross section of the normal testis of an immune-deficient mouse is shown in (m). One month after busulfan treatment, the majority of the seminiferous tubules were depleted from endogenous spermatogenesis (n). While 73% of seminiferous tubules of the mice transplanted with POU5F1-positive c-Kit-negative cells showed some degree of spermatogenesis (o), the majority of tubule cross sections of the mice received POU5F1-positive c-Kit-positive cells were empty (p). CSFE-tagged positive colony shortly after transplantation of POU5F1-positive-c-Kit- cells is shown in (r). Transplanted mGC also failed to repopulate recipient testes indicating that they do not have SSC property (q). Scale bars: (a and k) 275 µm, (b, d, and i) 60 µm, (c) 140 µm, (e) 100 µm, (f) 50 µm, (g and j) 125 µm, (h and j) 40 µm, (m–r) 40 µm.

transplantation, indicating that *in vitro* condition should not affect SSC properties (Shinohara *et al.* 2003). On the other hand, some of the growth factors present in our culture medium are reported to reprogram germ line stem cells to embryonic stage. However, the gene profile analysis and imprinting results do not support the reprogramming. Therefore, the most likely explanation would be that our multipotent germ line stem cells might have been generated from a subpopulation of germ line stem cells other than SSCs. Indeed, our transplantation study comparing the POU5F1+/c-Kit+ cells (being the germ line progenitors) versus the POU5F1+/c-Kit- cells (destined to become SSCs) showed that germ line progenitor cells did not repopulate recipient testes. This is also supported by the fact that only the germ line progenitor cells can generate multipotent germ line stem cells.

In summary, the present study demonstrates that a distinct subpopulation of germ line stem cells maintained under defined culture conditions generate multipotent cell lines that has some pluripotent characteristics and do not form teratomas. The findings raise interesting questions about what constitutes pluripotency, as well as an intriguing view into the potentiality of the germ line.

Irrespectively, the development of similar cell lines from human germ cells, such as those isolated from an adult testis, could provide a novel and highly valuable autologous cell source for clinical applications, particularly since germ line cells contain some of the best protected DNA in the adult body.

Materials and Methods

Cell isolation and culture

Mouse testicular stem cells were isolated from neonatal OG2 or OG2-LacZ males (0–3 days old, ~30 pups/trial) or adult OG2 mice (2–5 months old, 1 male/trial). After mincing, the testes were digested in DPBS (Dulbecco's 10 mM phosphate-buffered (pH 7.2), 0.14 M saline) containing collagenase (1 mg/ml), DNase-1 (1 µg/ml), and EDTA (5 mM). Testicular cell culture was performed according to the previously published protocols (Izadyar *et al.* 2003, Shinohara *et al.* 2003, Kanatsu-Shinohara *et al.* 2004). In brief, germ cell enrichment was accomplished by differential adhesion. The cells were dispensed into gelatin-coated (0.1%) culture dishes. The following day the floating cells were collected and passed to a secondary culture plate (1×10^5 cells per 1.2 cm^2) in a culture medium used by Kanatsu-Shinohara *et al.* (2004) with modifications (Supplementary Table, which can be viewed

Table 1 Restoration of spermatogenesis following transplantation of subpopulations of germ line stem cells and multipotent germ cell line in recipient mouse testes.

Transplanted cells	Recipient animals	Tubules with incomplete spermatogenesis ^a (mean ± s.d.)	Tubules with complete spermatogenesis (mean ± s.d.)	Empty tubules (mean ± s.d.)
POU5F1 + c-Kit+	1	17	4	98
	2	11	3	75
	3	12	2	92
	4	15 (14 ± 2.7)*	4 (3 ± 0.9)	97 (90 ± 10.6)
POU5F1 + c-Kit–	1	114	6	33
	2	96	8	41
	3 ^b	85	25	8
	4	108 (104 ± 12) [†]	5 (11 ± 9.4)	34 (29 ± 14.4)
mGC	1	12	11	78
	2	18	13	87
	3	11	8	69
	4	18 (14 ± 3.7)*	9 (10 ± 2.2)	86 (80 ± 8.3)
Sham	1	21	4	112
	2	15	7	95
	3	18	3	107
	4	16 (17 ± 2.6)*	6 (5 ± 1.8)	86 (100 ± 11.7)

*, [†] $P < 0.01$. mGC, multipotent germ cell line.

^aIncomplete spermatogenesis defined as the tubule cross sections containing spermatogenic cells up to the spermatids. ^bMouse with the least number of empty tubules and the highest number of tubules with complete spermatogenesis probably due to ineffective busulfan treatment.

online at www.reproduction-online.org/supplemental). After 2–4 weeks in culture, GFP-positive colonies were mechanically transferred to culture dishes containing mitomycin C-treated murine embryonic fibroblast (MEF) feeder layers (see below). After passage for three to four times, via mechanical transfer, to new MEF cultures, the colonies were established and could be removed from the culture plate enzymatically (collagenase 1 mg/ml, 5–10 min) for further expansion and/or storage in liquid nitrogen. To enhance germ line stem cell derivation, spermatogenesis was arrested in adult OG2 mice (4–6 weeks old, $n=4$), i.e., testes were surgically secured to the abdominal wall to become cryptorchid as described previously (de Rooij *et al.* 1999). mGC lines were also generated from neonatal and non-cryptorchid adult OG2 mice by sorting the GFP-positive cells. To further study which subpopulation of germ line stem cells generate these cell lines, the GFP-positive cells were sorted based on their c-Kit expression and were cultured on MEF feeders as described above. Finally, the effect of the growth factor removal on self-renewal or differentiation of mGC lines was investigated.

Preparation of MEF feeders

MEF feeders were made by the standard procedures using 12.5 dpc CD-1 mouse embryos. The embryos were eviscerated before trypsinization and the dissociated cells plated onto 150 mm plates at a plating density of ~1.5 embryos per plate. After initial plating, MEFs were split 1:5 and then frozen/thawed (passage 1). Thawed MEFs (P1) were passed only once for expansion purposes prior to mitomycin C treatment. MEF feeders were plated at a density of 50×10^3 to 60×10^3 per cm^2 . New MEF feeders were used for pluripotent germ cell culture every 7–10 days. All animal experiments were conducted in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals.

Evaluation of telomerase activity and karyotyping

For the determination of telomerase activity, cell extracts were isolated from germ cell lines (passage 10 and higher), freshly isolated POU5F1 + c-Kit+ sorted, and POU5F1 + c-Kit– sorted cells using CHAPS lysis buffer containing 150 U/ml RNase. Cell lysates were centrifuged for 20 min at 12 000 g , 4 °C, and the supernatants were stored at –80 °C. Protein concentration was measured by Bradford assay using BSA as the standard. Telomerase activity was measured by PCR-based assay using TRAPEZE detection kit (Chemicon, Temecula, CA, USA). Two microliters of the cell extract at 750 $\mu\text{g}/\mu\text{l}$ were added to a total volume of 50 μl PCR mix containing the TRAP reaction buffer, dNTPs, substrate oligonucleotide, telomerase primer, internal standard primer, and *Taq* polymerase. Two microliters of mESC cell extract were added to the reaction mix as positive control, and CHAPS lysis buffer and heat-inactivated telomerase were used as negative control for each experimental sample. Each sample was incubated at 30 °C for 30 min for telomerase extension, followed by PCR amplification. Karyotyping was performed at Coriell Cell Repositories, Cytogenetics Laboratory. For karyotyping, proliferating cells were incubated in culture with 0.1 $\mu\text{g}/\text{ml}$ KaryoMAX Colcemid (Invitrogen) for 3–4 h before they were resuspended in hypotonic solution (0.075 M KCl) and incubated at room temperature for 10 min. The cells were then resuspended in a cold fixative (3:1 methanol:acetic acid) and stored at 4 °C for at least 30 min. After washing with the fixative, the cells were applied to clean glass slides and air dried. Metaphase chromosomes were prepared and karyotypes created using an Applied Spectral Imaging Band View digital imaging system.

In vitro differentiation

For generating EBs, mGSC colonies were dissociated with collagenase and plated at a concentration of $1 \times 10^6/\text{well}$ to $2 \times 10^6/\text{well}$ on 6-well non-adhesive culture plates in the

Figure 10 Chimeras formation after incorporation of multipotent GCs into blastocysts and host embryos. The incorporation of LacZ-GFP⁺ mGC cells during early embryonic development and blastocyst formation is presented in (a–d). The majority of the GFP-LacZ cells injected at eight-cell stage have been incorporated at day 2 of the embryonic development (arrow head) and some cells have not been incorporated yet (arrows). GFP-positive cells were further found at day 3.5 incorporated in inner cell mass (arrow) of the blastocyst. An example of four chimeric embryos showing different degree of chimerism is shown in (e) as whole embryo staining. To visualize the internal organs, sagittal sections of two of the embryos (indicated by asterisks) are also shown (f and g). (h–k) Chimeric pattern in dissected organs and (l–o) chimeric cell population in histological sections of the brain, heart, liver, and gonadal ridge (chimeric LacZ-GFP cells appear in blue). Amplification of the GFP and LacZ DNA in the tissues of the chimeric pups is shown in (p and q). Scale bars: (a and c) 50 μ m, (b and d) 25 μ m, (e–g) 1250 μ m, (h–k) 625 μ m, (l–n) 50 μ m, (o) 10 μ m.

complete medium containing 15% FBS (Hyclone, Logan ,UT, USA). In some experiments, EBs were formed in hanging drops by aggregating 50×10^3 to 100×10^3 cells in 50 μ l medium. For differentiation into cells representing the three germ layers, the EBs were cultured for 15 days, and every 3 days 20–30 EBs

were collected for RT-PCR analysis and 20–30 EBs were used for histological examinations. For induced differentiation, the EBs were cultured in the complete medium for 4 days before they were cultured in the serum-free N1 medium for lineage selection, i.e., DMEM/F12 (Invitrogen) supplemented with ITS

(insulin, 10 mg/l; transferrin, 5.5 mg/l; selenium, 0.67 mg/l) and fibronectin (50 µg/ml). After 5–7 days, N1-treated cell aggregates were transferred to gelatin-coated culture plates (Ying *et al.* 2003) in the N2 medium for expansion of neural progenitor cells, i.e., N1 medium with ITS, without fibronectin, and supplemented with bFGF (10 ng/ml). For differentiation into cardiomyocytes, the EBs were cultured for 2 weeks in the presence of different cardiogenic compounds including DMSO (0.06 M), 5'-aza-2'-deoxycytidine AZA (5 mM), and Cardiogenol C (25–50 µM; Calbiochem, San Diego, CA, USA) (Paquin *et al.* 2002, Choi *et al.* 2004). During the differentiation process, the morphology of cells was analyzed and the samples were taken both for gene expression analysis by RT-PCR and immunohistochemical (IHC) staining (see below). Chondrocyte differentiation (Lee *et al.* 2004) of mGSCs was induced by adding a chondrogenic induction medium (Chondrogenic SingleQuots, Cambrex, Walkersville, MD, USA) supplemented with transforming growth factor- β (10 ng/ml) and 20% FBS.

Immunocytochemical and IHC staining

Cultured cells were fixed in 4% paraformaldehyde for 10–30 min at room temperature and stored in PBS at 4 °C. For fluorescent immunocytochemistry, the cells were permeabilized with 1×Cytoperm (BD Biosciences, San Jose, CA, USA) or 0.2% Triton X-100 (Sigma) for 15 min and subsequently incubated

in 2% (w/v) BSA (Sigma) and 2% (v/v) normal goat serum (GS)/1× Cytoperm–PBS for 30–60 min both at room temperature. Primary antibody was either diluted at the optimal concentration in 2% BSA and 2% GS/1× Cytoperm–PBS and incubated for 3 h at 4 °C or diluted in blocking buffer overnight at 4 °C. After two washes, fluorescent secondary antibody was diluted accordingly (Supplementary Fig. 2) in 2% BSA and 2% goat serum/1× Cytoperm–PBS and incubated for 1 h at 4 °C in the dark. The cells were washed twice with PBS, wrapped in foil, and stored at 4 °C until microscopic analysis. Images were recorded using an Olympus IX71 microscope or Zeiss LSM510 confocal microscope equipped with digital image hardware and software.

For bright-field immunocytochemistry, the cells were washed once with 1× PBS. Endogenous peroxidase activity was blocked with 3% (v/v) H₂O₂ for 15 min followed by permeabilization – blocking with 2% BSA and 2% GS/1× Cytoperm–PBS for 30 min. The primary antibody was diluted accordingly (Supplementary Fig. 2) in 2% BSA and 2% GS/1× Cytoperm–PBS and incubated for 3 h at 4 °C. The remainder of the staining was accomplished using ABC staining kits (Vector Labs, Burlingame, CA, USA), according to the manufacturer's instructions. Visualization was done with enhanced diaminobenzidine substrate (Sigma) tablet dissolved in purified water and incubated for 5–10 min. For negative controls, the primary antibody was omitted. The primary antibodies used in this study were obtained from various

Table 2 Antibodies and reagents used for immunohistochemical staining.

Lineage	Name of antigen	Antibody source	Working dilution
Pluripotent	POU5F1	Santa Cruz	1:50
	NANOG	Bethyl laboratories	1:50 to 1:100
	SOX2	Stem cell technologies	1:1000
	SSEA-1	Chemicon	1:50
	Alkaline phosphatase	Abcam, Cambridge, MA, USA	1:50 to 1:100
	Alkaline phosphatase	Cell Biolabs Inc., San Diego, CA, USA	According to instructions
Germ layer markers	E-cadherin	R&D Systems	1:40
	Laminin-1	Santa Cruz	1:100
	SOX1	Affinity Bio reagents	1:100
	FOXA2	Chemicon	1:100
	Nestin	Chemicon	1:200
Neural	NG2	Chemicon	1:200
	MAP2	Chemicon	1:200
	GFAP	Chemicon	1:1000
	O4	Chemicon	1:100
	A2B5	Chemicon	1:100
	Neurofilament	Chemicon	1:200
	Troponin-1	Chemicon	1:200
Cardio	Myosin heavy chain	Abcam	1:200
Adipocyte	Oil red dye	American MasterTech Inc., Lodi, CA, USA	Solution supplied by kit
Chondro	Alcian blue dye	Fluka, St Gallen, Switzerland	1% solution
Germ cells	VASA	Abcam	1:100 to 1:200
Second antibodies	Alexa 488	Molecular Probes, Eugene, OR, USA	1:200
	Alexa 568	Molecular Probes	1:500
	Alexa 488 IgG1	Molecular Probes (Carlsbad, CA, USA)	1:500
	Alexa 488 IgG2b	Molecular Probes (Carlsbad, CA, USA)	1:500
	Anti-mouse IgG	Jackson Labs, Bar Harbor, ME, USA	1:200
	Anti-mouse IgM	BD Pharmingen, San Diego, CA, USA	1:200
	Anti-rabbit Ig	Jackson Labs	1:200
Nuclear dye	DAPI	Molecular Probes	1:1000
Nuclear dye	TO-PRO-3	Molecular Probes	1:100
Nuclear dye	Hoechst 33342	Molecular Probes	1:1000
LacZ staining kit	β -gal	Sigma	According to instructions

companies. Each antibody was validated and the concentrations optimized in our laboratory. The source and working dilutions of these antibodies are presented in Table 2.

Flow cytometry

Specific antibodies, including SSEA-1 and c-Kit, were optimized in our laboratory for FACS analysis. Cells were sorted on the inFlux Cell Sorter (Cytospeia, Seattle, WA, USA). For c-Kit sorting, freshly isolated testicular cells containing the POU5F1-GFP construct were stained with CD117 APC (BD Pharmingen, San Diego, CA, USA). GFP excitation was attained with 488 nm solid-state Coherent laser (Coherent, Santa Clara, CA, USA) and emission light collected through a 530-40 dichroic mirror. APC excitation was attained with 638 nm solid-state Coherent laser (Coherent) and emission light collected through a 710-40 dichroic mirror. The cells were stained at a final dilution of 1:200 c-Kit APC in the complete culture medium for 45 min on ice in the dark. After washing twice with the fresh cold complete medium, the cells were concentrated at 10×10^6 cells/ml and kept on ice for sorting. To sort for GFP/c-Kit+/- cells, first we gated the whole GFP population with the non-stained cells to establish a baseline for APC channel. A region was placed based on the baseline to discriminate between negative and positive c-Kit-stained cells. Any cells/events falling into this region was considered to be positively stained for c-Kit and was sorted from

the negative c-Kit cells. For some experiments, fresh germ cell colonies were dissociated and the cells were stained with anti-SSEA-1 antibody followed by goat-anti mouse IGM conjugated with PE-Cy7 (BD Biosciences).

Gene expression, imprinting analysis, and GFP amplification

Total RNA was isolated using RNeasy mini kit (Qiagen) and the RNA was used for RT-PCR, quantitative PCR, or microarray analysis. For RT-PCR, cDNA was synthesized with the Sensi-script RT kit (Qiagen), and the PCR was performed with HotStarTaq DNA Polymerase (Qiagen). All PCRs began with an initial incubation at 95 °C for 15 min to activate the enzyme. This was followed by 35 cycles of 95 °C for 15 s, the appropriate annealing temperature for 1 min, and 72 °C for 1 min, which was then followed by 1 cycle of 72 °C for 10 min for final extension. The reactions were carried out using an iCycler Thermal Cycler (Bio-Rad). RT-PCR was carried out using specific primers including *Pou5f1*, *Nanog*, *Rex1*, *Dppa5*, *Dazl*, β -actin (*Actb*), *Nkx2.5*, *nestin*, *Mab2*, and *Gfap* (primer sequences are presented in Table 3). For internal controls, *Gapdh* was used as a housekeeping gene for cellular samples and β -actin or interleukin-2 (*IL2*) was used in mouse embryos.

Imprinting patterns in mGSCs and mESCs were determined by a PCR-based analysis. PCR amplification of each dimethylated

Table 3 Primers for marker determination by RT-PCR and primers used for imprinting study.

Lineage	Name of gene	5'-Sequence	3'-Sequence	DNA size	
(A)					
Pluripotent	<i>Pou5f1</i>	CCCCCTGTCCCCATTCTCTA	CGACCATCTGCCGCTTTGAG	577	
	<i>Nanog</i>	AGAAGGCCTCAGCACCTAC	GGCCTGATTGTTCCAGGATT	205	
	<i>Dppa5</i>	GAAAGATCCAGAGGTGTTT	TGGTTCACTTCATCCAAG	297	
	<i>Rex1</i>	GCGTACGCAAATTAAGTCCAGA	CAGATCCTAAACAGCTCGCAG	306	
	<i>Sox2</i>	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT	448	
Gonadal	<i>Dazl</i>	GGAGCTATGTTGTACCTCC	CCATGTAAGTATAGATAAGCCAG	203	
Neural	<i>Nestin</i>	GCCTGACCACTCCAGTT TA	GGAGTCCTGGATTTCTCTCC	199	
	<i>NeuroD1</i>	AAGCCATGAACGCAGAGGAGGACT	AGCTGTCCATGGTACCGTAA	579	
	<i>Vimentin</i>	CAGGAAGAGCAGAAGAACTGG	GGTAAAGAAGCTGGTTGTGG	750	
	<i>NF-68 (Nefl)</i>	ACCCGACTCAGTTTACCAG	CTCATCCTTGGCTTCCTCAG	216	
	<i>GAD67</i>	CGAGGACTCTGGACAGTAGAGG	GATCTTGAGCCCCAGTTTCT G	182	
	<i>GFAP</i>	ACATCGAGATCGCCACCTAC	ACATCACATCCTTGTGCTCC	219	
	<i>MBP</i>	GGAAACCACGCAGGCAAACGAGAG	AAAAGAGGCGGATCAAGTGGGG	284	
	Cardio	<i>Nkx2.5</i>	CTTCAAGCCAGAGGCCTACG	TCATAGACCTGCTCCTGCG	305
		<i>GATA4</i>	GACGGGTCACTATCTGTGCAAC	AGACATCGCACTGACTGAGAAC	475
		α -Actin	ACTGGGACGACATGGAGAAG	GCAAAGCGTAGCCCTCATAG	282
<i>ANP</i>		GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTAGGAG	406	
Chondro		<i>Collagen IIa1</i>	GGGAGTAATGCAAGGACCAA	ACCATCATCACCAGGCTTTC	178
	<i>Collagen Xa1</i>	GATATGGTGCTCCTGGTCGT	CACCTCTTTTCCCCTCCA	96	
	<i>Aggrecan</i>	ACAGCTGGGGACATTAGTGG	GTGGAATGCAGAGGTGGTTT	189	
	<i>Perlecan</i>	TCCACCTGAGTACCCGAAAC	CTGAAGTGACCAGGCTCCTC	130	
Housekeeping	β -Actin	ATCTGGCACACACCTTCTACAATGAGCTGCG	CGTCATACTCTGCTTGCTGATCCACATCTGC	838	
Housekeeping	<i>Gapdh</i>	ACCCAGAAGACTGTGGATGG	TTCACTCAGGGATGACCTT	125	
Name of gene		5'-sequence	3'-sequence		
(B)					
<i>Meg3 IG</i>		GGTTTGGTATATATGGATGTATTGTAATATAGG	ATAAAACACCAAATCTATACCAAAATATACC		
<i>Rasgrf1</i>		GTGTAGAATATGGGGTTGTTTTATATTG	ATAATACAACAACAACAATAACAATC		
<i>Igfr2</i>		TTAGTGGGGTATTTTTATTTGTATGG	AAATATCCTAAAAATACAAACTACACAA		
<i>Peg10</i>		GTAAGTGATTGGTTTGTATTTTAAAGTG-3	TTAATTACTCTCTACAACCTTCCAAATT		
<i>Pou5f1</i>		GGTTTTTATAGAG GATGGTTGAGTG	TCCAACCCTACTAACCCATCACC		

region (DMR) from bisulfite-treated DNAs was carried out by specific primers as described (Kanatsu-Shinohara *et al.* 2004). For analysis of the imprinted genes, we used the UVP image software to quantify the band intensity. For GFP and LacZ amplification, individual tissues from chimeric embryos were carefully collected by dissection, minced into small pieces, and placed in DNA extraction buffer (DNeasy kit, Qiagen) for DNA isolation and purification, according to the manufacturer's protocol. PCR (1 µl DNA template containing 100 ng DNA) was carried out as described previously (Izadyar *et al.* 2000).

Microarray analysis

Total cellular RNA was isolated using RNeasy mini kit (Qiagen Inc.), according to the manufacturer's recommendations. To eliminate DNA contamination, the samples were treated with 2.0 U DNase I (Amplification grade, Invitrogen) at 37 °C for 15 min, and the enzyme is inactivated by the addition of EDTA (2 mM final) at 65 °C for 10 min. The samples were concentrated by ethanol precipitation and resuspended in RNase-free water. Two micrograms of total RNA of each sample were sent to the UCI DNA Microarray Facility, where the samples were prepared and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips (Affymetrix Inc., Santa Clara, CA, USA) and scanned, according to the manufacturer's protocols.

SSC transplantation

To test the functionality of the subpopulations of germ line stem cells and our mGCs for regeneration of spermatogenesis, we used the SSC transplantation technique. Sixteen 6–8 weeks immune-deficient nude male mice (Harlan, Indianapolis, IN, USA) have been treated with busulfan (40 mg/kg) and used as recipients. One month after busulfan treatment, 2×10^5 cells were transplanted into the seminiferous tubules via rete testis injection as described previously (Ogawa *et al.* 2000). Four mice were transplanted with freshly isolated POU5F1-positive c-Kit-positive sorted cells, four mice injected with freshly isolated POU5F1-positive c-Kit-negative sorted cells, and four mice transplanted with mGCs. As the mGCs are cultured on MEFs, they were sorted for GFP to avoid MEF contamination during transplantation. The remaining four mice served as sham control and not injected. One month after transplantation, the animals were killed and the testes harvested and used for histological evaluations. To evaluate the efficiency of transplantation, 100–150 tubule cross sections of each animal was examined and the number of tubules with different stages of spermatogenesis was counted. Statistical analysis was carried out using ANOVA and $P < 0.05$ was considered significant. To better identify the transplanted cells in the recipient testes, a fluorescent cell trace marker, CFSE, (Invitrogen) was used. CFSE is colorless and non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent product. This fluorescent product is well retained and can be fixed with an aldehyde fixative; however, it diminishes following multiple cell divisions and can be used successfully only in short-term studies. Two mice were transplanted with POU5F1-positive c-Kit-negative cells and two other mice received POU5F1-positive c-Kit-positive cells. Ten days after transplantation, the mice were killed and the number of

CFSE-positive colonies was determined. Considering 72 h for each cell doubling of germ line stem cells (Fig. 2), at this time after transplantation, SSCs could have gone through two to three cell doublings. Therefore, colonies of four to eight cells could have been formed.

Tests for teratoma and chimera formation

To test the ability of the mGCs to form teratomas or chimeras, OG2 mice (Jackson Laboratories, Bar Harbor, ME, USA) were bred with Rosa26 mice (Jackson Laboratories) and a new strain (OG2-R26) was generated. These mice have both GFP and LacZ constructs in their germ cells. Culture was performed as described and the new POU5F1-GFP/LacZ germ cell lines were produced for testing teratoma and chimera formation. Mouse POU5F1-GFP/LacZ mGSCs were examined for their ability to form teratomas *in vivo* by s.c., i.m., or injection into the seminiferous tubules of nude mice (Harlan). As positive controls for teratoma formation, the ES cells were injected into some mice. For s.c., i.m., or testicular injections, $\sim 1 \times 10^6$ cells were injected. Mice were killed 6 weeks later and the tissues harvested for morphological and histological analysis.

The ability of mouse POU5F1-GFP/LacZ GSCs to form chimeric cell populations was determined after injection into host blastocysts, or by their aggregation with morula stage embryos or eight-cell stage embryos (Bradley 1987). Blastocyst injections of 15–20 cells were administered using 3.5-day blastocysts collected from CD-1 mice following the procedure as described (Chatot *et al.* 1990). After injection, the blastocysts were transferred (7–8 blastocysts in each horn of the uterus) to 2.5-day pseudopregnant CD-1 females, previously mated with vasectomized males. Incorporation of LacZ cells was examined in different areas of the chimeric 12.5 dpc embryos by the β-galactosidase staining kit (Sigma). In addition, LacZ and GFP PCRs were performed in DNAs isolated from the brain, heart, liver, and gonadal ridges of the chimeric embryos formed from POU5F1-GFP/LacZ cells.

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