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Pluripotency of spermatogonial stem cells from adult mouse testis

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Embryonic germ cells as well as germline stem cells from neonatal mouse testis are pluripotent and have differentiation potential similar to embryonic stem cells[1,2], suggesting that the germline lineage may retain the ability to generate pluripotent cells. However, until now there has been no evidence for the pluripotency and plasticity of adult spermatogonial stem cells (SSCs), which are responsible for maintaining spermatogenesis throughout life in the male[3]. Here we show the isolation of SSCs from adult mouse testis using genetic selection, with a success rate of 27%. These isolated SSCs respond to culture conditions and acquire embryonic stem cell properties. We name these cells multipotent adult germline stem cells (maGSCs). They are able to spontaneously differentiate into derivatives of the three embryonic germ layers *in vitro* and generate teratomas in immunodeficient mice. When injected into an early blastocyst, SSCs contribute to the development of various organs and show germline transmission. Thus, the capacity to form multipotent cells persists in adult mouse testis. Establishment of human maGSCs from testicular biopsies may allow individual cell-based therapy without the ethical and immunological problems associated with human embryonic stem cells. Furthermore, these cells may provide new opportunities to study genetic diseases in various cell lineages.

For the isolation of spermatogonial stem cells, we used the spermatogonia-specific marker *Stra8* (ref. 4). The activity of its regulatory sequences enables the enrichment of germline stem cells from transgenic mice[5,6]. Testicular cells (2–3 × 10^7 per mouse) were isolated from Stra8–enhanced green fluorescent protein (EGFP)/Rosa26 adult mice ($n = 15$; Supplementary Fig. 1) and cultured in medium containing glial-derived neurotrophic factor (GDNF) for 4–7 days. Thereafter, GFP^+ cells (1–2 × 10^6 cells) were isolated using fluorescence-activated cell sorting (FACS). To prove the stem-cell activity of sorted GFP^+ cells, we transplanted these cells into seminiferous tubuli of germ-cell-depleted mice. After 5–6 months, regeneration of normal spermatogenesis was observed (data not shown). Testicular teratomas (tumours derived from pluripotent cells) are rare in most inbred mouse strains, and occur spontaneously at a rate of 1–5% in 129/Sv inbred strains[7]. In a mixed C57BL6/129Sv genetic background, fewer mice deficient for the tumour-suppressor gene *Trp53* develop testicular tumours than in a pure 129/Sv background[8]. We observed no testicular tumours in 15 Stra8–EGFP/Rosa26 males with a mixed FVB/C57BL6/129Sv genetic background.

The same Stra8–$EGFP^+$ cells that had been tested for their contribution to spermatogenesis were used to establish the conditions required to convert these cells into pluripotent stem cells. The GFP^+ SSCs were propagated in basic medium (condition I) for two weeks and then cultured under four different conditions (Supplementary Fig. 1). In condition I, most of the colonies formed were similar to cultured epiblast cells (Fig. 1a). However, when the cells were cultivated in medium containing leukaemia inhibitory factor (LIF; condition II) or grown on mitomycin-C-treated mouse embryonic fibroblasts (MEFs; condition III), tightly packed embryonic stem cell (ESC)-like colonies appeared (Fig. 1b). These ESC-like colonies can be expanded under standard ESC culture conditions (both MEFs and LIF applied; condition IV). We successfully established four cell lines (SSC5, SSC6, SSC10 and SSC15) from fifteen mice (27% success rate). Two cell lines, SSC5 and SSC6, have now been passaged for more than 30 passages (Fig. 1c).

The phenotype of our cultured GFP^+ SSCs depends on the culture conditions. Cultured GFP^+ SSCs expressed the cell-surface marker SSEA-1 (stage-specific embryonic antigen-1; ref. 9) and the germline-specific transcription factor Oct3/4 (ref. 10) (Fig. 1d–f and Supplementary Fig. 2) that characterize undifferentiated mouse ESCs. In common with ESCs, the SSCs in conditions III and IV did not express SSEA-3 (ref. 11), but some cells in conditions I or II stained positively for SSEA-3 (Fig. 1g and Supplementary Fig. 2b). c-Kit is expressed in differentiating spermatogonia but not in SSCs[12], and more cells stained positively for c-kit in conditions I and II than in conditions III and IV (Supplementary Fig. 2b). Thy1 is a surface marker of SSCs[13], and can also be detected on ESCs[14]. GFP^+ cells cultured in the presence of LIF (conditions II and IV) are negative for Thy1. Like ESCs, they show only low positive staining for the lymphocyte surface marker Sca1, and are negative for Ter119 and CD34. Alkaline phosphatase is highly expressed in ESCs but not in SSCs[2]. In our study, alkaline phosphatase was strongly expressed in the GFP^+ cells in condition IV (Fig. 1h), but a 'mixed colony' phenotype was revealed in conditions I–III (Fig. 1i).

These results suggest that SSCs respond to culture conditions[15,16], and acquire ESC properties under standard ESC culture conditions. We therefore name these cultured ES-like cells multipotent adult germline stem cells (maGSCs), to distinguish them from SSCs. Cytogenetic analysis showed that maGSCs have a normal karyotype (40 chromosomes, XY) in 80–90% of metaphase spreads. Additionally, and similar to ESCs, maGSCs express higher levels of *Ras1* (also known as *Rasd1*) and *Gro1* (the chemokine *Cxcl1*), but lower levels of *Mcm4* compared to the teratocarcinoma cell line F9 (derived from teratocarcinoma of testis from the 129 mouse strain) and embryonic carcinoma cell (ECC) line P19 (derived from teratocarcinoma of a C3H/He mouse embryo) (Supplementary Fig. 3a), consistent with previous studies[17].

Polymerase chain reaction with reverse transcription (RT–PCR) analysis showed that under all four culture conditions, the cultured cells expressed the transcription factors *Oct3/4* (also known as *Pou5f1*), *Nanog* (ref. 18), *Utf1* (ref. 19), *Esg1* (also known as *Dppa5*; ref. 20) and *Rex1* (also known as *Zfp42*; ref. 21) (Fig. 1j), similar to

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ESCs. Notably, cells cultured with LIF (conditions II and IV) expressed higher levels of *Stra8* than those cultured without LIF (conditions I and III).

On the basis of our experience with isolating SSCs from the GFP$^+$ transgenic mice, we were able to identify the cells morphologically, which allowed us to rule out the possibility that the SSC isolation was restricted to the transgenic mice. We have successfully derived ES-like cell lines from mice with three different genotypes (C57BL/6, 129/Ola and FVB) without genetic selection. ES-like cells were isolated from two out of eleven C57BL/6 mice (18%), one out of seven FVB mice (14%) and one out of three 129/Ola (33%) mice. These cultures, similar to the GFP$^+$ cells, express *Stra8*, *Oct3/4*, *Nanog*, *Utf1*, *Esg1* and *Rex1*, and are susceptible to LIF (Supplementary Fig. 3b).

Analysis of the DNA microsatellite profile of the established cultures rules out the possibility of contamination with ESCs, ECCs or MEFs cultured in the same facility (Supplementary Tables 1–4). Furthermore, we show that the two lines from the C57BL/6 inbred strain differ in the four DNA microsatellite markers, indicating they are two separate lines (Supplementary Table 1).

To induce *in vitro* differentiation of maGSCs, we applied the 'hanging drop' method used for ESC differentiation[22]. We observed an overall decrease in GFP$^+$ cell populations upon embryoid body differentiation (Supplementary Fig. 4). In parallel, the expression of *Oct3/4*, *Nanog*, *Utf1*, *Esg1* and *Rex1* gradually decreased during differentiation (Fig. 1k). To determine whether maGSCs can spontaneously differentiate into derivatives of the three primary germ layers, we examined the expression of a panel of cell-specific genes and proteins during embryoid body differentiation (Figs 2–4).

Differentiation of mesodermal lineages (for example, cardiac, skeletal muscle and vascular cells) was confirmed by expression of the early mesoderm marker *brachyury* (*T*)[23] as well as lineage-specific genes and proteins. The expression of *brachyury* is maximal at early differentiation stages (Fig. 2). The cardiac transcription factors *islet-1* (ref. 24), *Gata4*, *Nkx2.5* and *Mef2c* are already expressed at high levels at day 5, whereas cardiac-specific genes such as α-myosin heavy chain (α-MHC, known as *Myh6*), ventricular isoform 2 of myosin light chain (*Mlc2v*, also known as *Myl2*) and atrial natriuretic factor (*ANF*, also known as *Nppa*) are expressed at high levels two days later

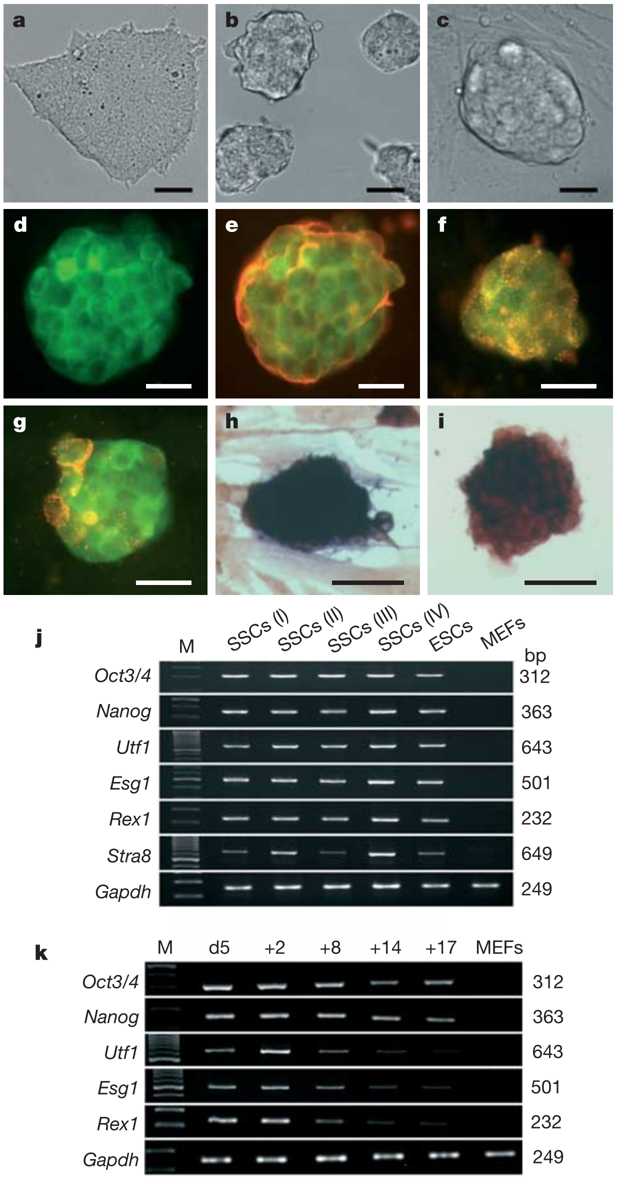

Figure 1 | Cellular and molecular characterization of cultured SSCs and maGSCs. **a**, Epiblast-like colony formed under culture condition I. **b**, ESC-like colonies appeared under culture condition II. **c**, A typical colony of established culture under condition IV at passage 30. **d**–**g**, Double immunostaining of maGSCs in culture condition IV (**d**–**f**) or condition II (**g**) with antibodies against GFP (green, **d**–**g**) and SSEA-1 (red, **e**), Oct4 (red, **f**) or SSEA-3 (red, **g**). **h**, **i**, Alkaline phosphatase staining. SSCs cultured under condition IV (maGSCs, **h**) are strongly positive for alkaline phosphatase, whereas SSCs under condition II (**i**) show a mixed colony phenotype. **j**, **k**, RT–PCR analyses of transcription factors essential for undifferentiated cells in SSCs cultured under conditions I, II, III and IV (**j**) and during differentiation of embryoid bodies after plating at day 5 (d5; **k**). M, 100-bp DNA markers; numbers to the right indicate the sizes of the resolved DNA fragments (in bp). Scale bars, 50 μm (**a**–**c**, **h**, **i**), 25 μm (**d**–**g**).

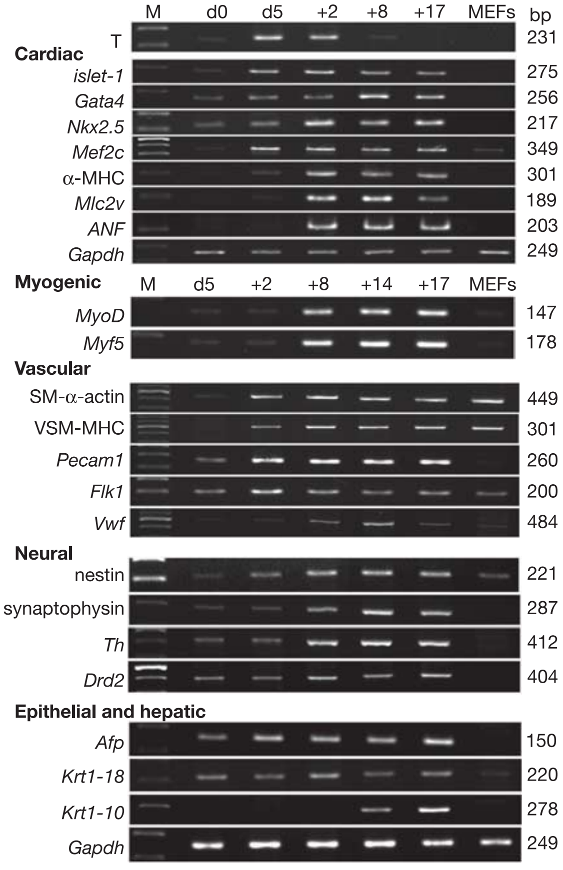

Figure 2 | RT–PCR analysis of lineage-specific transcription factors and genes. Analyses were performed at different stages during the differentiation of embryoid bodies after plating at day 5 (d5). M, 100-bp DNA markers. d0, maGSCs before embryoid body formation.

(Fig. 2). Spontaneously contracting cells appeared as clusters (Supplementary Video 1) and were identified in approximately 90% of the embryoid bodies ($n = 144$; three independent experiments) at day 5 + 5 (at day 5 after plating of 5-day-old embryoid bodies), comparable to the cardiac differentiation of ESCs (86%). The cardiomyocyte phenotype of these contracting areas can be shown by immunostaining for proteins relevant for myocyte contraction. Single cardiomyocytes isolated from beating areas show sarcomeric striations when stained for α-sarcomeric actinin (Fig. 3a), sarcomeric MHC and cardiac troponin T, organized in bundles (Fig. 3b, c). Expression of the gap-junction protein connexin 43 at cell-to-cell contacts in cardiac clusters (Fig. 3d–f) indicates cell-to-cell communication. In addition, single cardiomyocytes show spontaneous action potentials, as analysed using patch-clamp electrophysiology (Fig. 3g).

The differential expression of cardiac- and skeletal-muscle-specific transcription factors makes it possible to distinguish maGSC-derived cardiomyocytes from skeletal muscle cells. The myogenic regulatory factors *MyoD* and *Myf5* are strongly expressed from day 5 + 8 (Fig. 2), whereas cardiac-specific transcription factors are strongly expressed by day 5. Moreover, the first myoblasts positive for nebulin[25] appear at day 5 + 7, whereas cardiac cells are never positive for nebulin. The formation of multinucleated contracting myotubes positive for nebulin was only observed at late stages (Fig. 3h). These data show that the cardiac and skeletal muscle lineages are different from early on in the differentiation process.

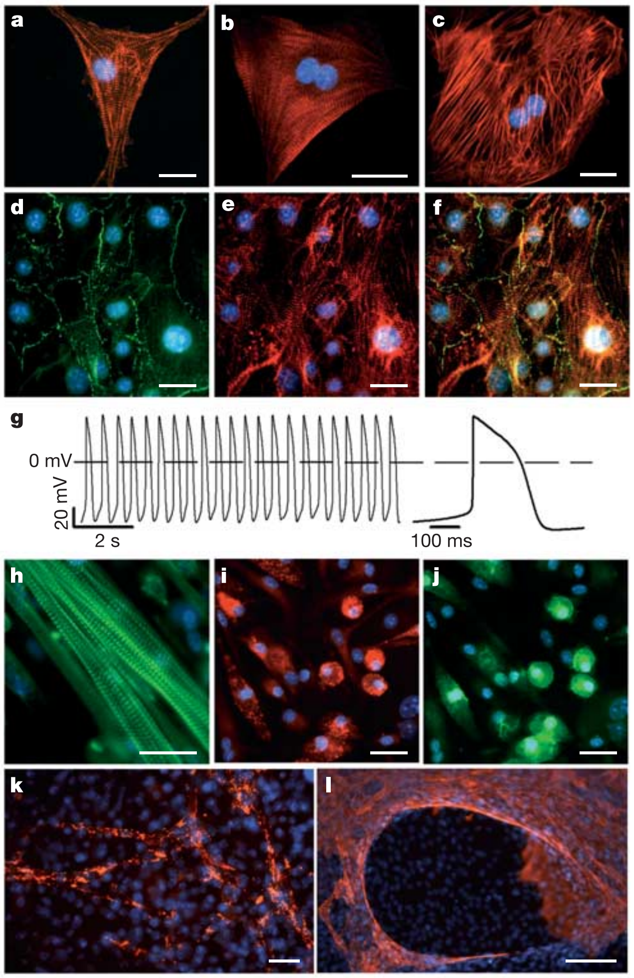

Figure 3 | Mesoderm differentiation of maGSCs. **a**–**c**, Organization of the sarcomeric proteins α-actinin (**a**), sarcomeric MHC (**b**) and cardiac troponin T (**c**) in isolated cardiomyocytes at day 5 + 7. **d**–**f**, Connexin 43 staining (**d**, green) in a cluster of uninucleate cardiac cells stained for sarcomeric α-actinin (**e**, red), with an overlay of **d** and **e** shown in **f**. Nuclei are stained with DAPI. **g**, Original traces of ventricle-like action potentials in a cardiomyocyte derived from maGSCs. **h**, Nebulin-positive myotubes at differentiation day 5 + 23. **i**, **j**, DiI-acLDL uptake (**i**, red) and lectin binding (**j**, green) of endothelial cells in embryoid body outgrowths at day 5 + 14. **k**, Vwf-positive endothelial cells (red) at day 5 + 17. **l**, Smooth muscle α-actin-positive cells (red) of tube-like structure in embryoid body outgrowths at day 5 + 14. Scale bars, 25 μm (**a**–**f**, **h**–**k**), 100 μm (**l**).

The differentiation of vascular endothelial and smooth muscle cells was confirmed by the expression of genes encoding smooth muscle α-actin (SM-α-actin, known as *Acta2*), vascular smooth muscle MHC (VSM-MHC, known as *Myh11*), platelet/endothelial-cell adhesion molecule 1 (*Pecam1*), *Flk1* (also known as *Kdr*) and von Willebrand factor (*Vwf*) (Fig. 2). In embryoid body outgrowths we found cells positive for both the uptake of DiI-conjugated acetylated low-density lipoproteins (ac-LDL-DiI; (Fig. 3i) and lectin binding (Fig. 3j), which are characteristic of endothelial cells[26]. We also observed the formation of cellular networks and tube-like structures in embryoid body outgrowths at later stages using antibodies against Vwf (Fig. 3k) and smooth muscle α-actin (Fig. 3l), indicating that these net-like and tubular structures consist of vascular endothelial and smooth muscle cells.

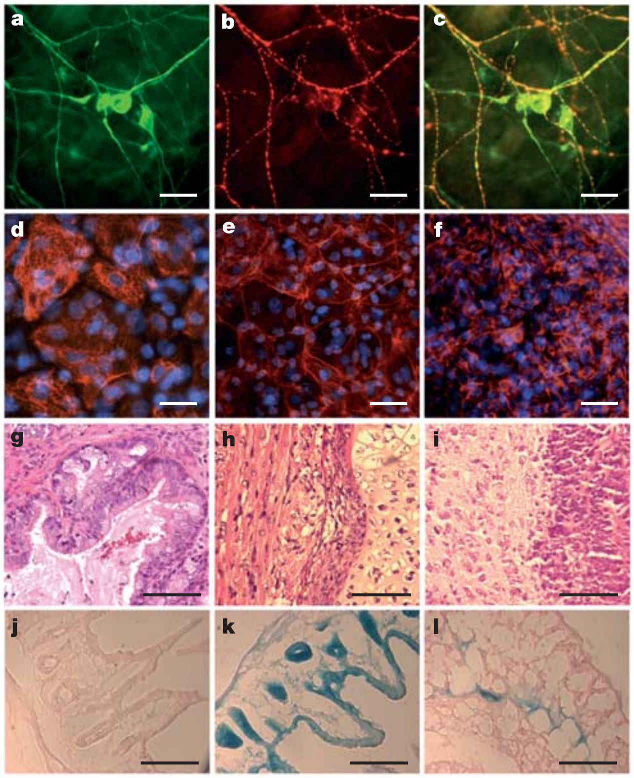

Figure 4 | Differentiation of maGSCs *in vitro* and *in vivo*. **a**–**c**, Neuronal differentiation of maGSCs. TH-positive dopaminergic neurons (**b**) appeared among neurofilament protein M (NFM)-positive neurons (**a**) on day 12 after plating of embryoid bodies at day 5 (day 5 + 12). **c**, Overlay of **a** and **b**. **d**–**f**, Epithelial cell/hepatocyte differentiation. **d**, Pan-cytokeratin-positive epithelial cells on day 5 + 14. **e**, CK18-positive large epithelioid cells on day 5 + 17. **f**, CK7-positive bile duct cells on day 5 + 14. Nuclear staining with DAPI. **g**–**i**, Teratomas from maGSCs. The tumours contained abundant differentiation of advanced derivatives of all three embryonic germ layers. Shown are epithelium with intestinal differentiation (**g**), striated muscle, cartilage (**h**) and neural tissue (**i**). **j**–**l**, Representative images of LacZ-stained intestine tissue from a wild-type mouse (**j**), a mouse with high grade of chimaerism (**k**) and a mouse with low grade of chimaerisms (**l**). Scale bars, 25 μm (**a**–**f**), 50 μm (**g**–**i**), 100 μm (**j**–**l**).

Neuroectoderm differentiation was confirmed by the expression of nestin, a marker for neuroepithelial precursors, and of synaptophysin, tyrosine hydroxylase (*Th*) and dopamine receptor 2 (*Drd2*), markers for differentiated neurons (Fig. 2). We found high numbers of nestin-positive neuroprogenitors at day 5 + 5 (data not shown). Although the spontaneous differentiation of maGSCs into neuronal cells (Fig. 4a–c) was low (only 25% of embryoid bodies contained neuronal cells at day 5 + 11), maGSCs could differentiate into dopaminergic neurons under standard embryoid body differentiation conditions (Fig. 4a–c).

We characterized epithelial-like cells in embryoid bodies using an anti-pan cytokeratin antibody, which reacts with a wide variety of epithelial tissues (Fig. 4d). Cytokeratin 10 (*Krt1-10*), which is present in keratinizing stratified epithelia, was expressed at late differentiation stages (Fig. 2). We also found cells with phenotypic characteristics of hepatocytes in embryoid bodies. Expression of early (alpha fetoprotein, *Afp*) and late (*Krt1-18*) markers of hepatocyte differentiation was observed during embryoid body differentiation (Fig. 2). At day 5 + 8, cells expressing AFP have one of two different morphologies: round or spindle shapes. At day 5 + 17, many large epithelioid cells stained positive for cytokeratin 18 (CK18) (Fig. 4e). In addition, we found many cells positive for CK7, a maturation marker in bile duct cells (Fig. 4f).

To confirm the pluripotency of maGSCs *in vivo*, we subcutaneously injected the cells into SCID/beige mice. The transplanted cells formed mature teratomas in all recipients (10/10) by 6 weeks after inoculation. The teratomas contained derivatives of three embryonic germ layers, including epithelium with intestinal differentiation (endoderm), striated muscle, smooth muscle, fat, bone and cartilage (mesoderm), and neural tissue (ectoderm) (Fig. 4g–i).

To determine the developmental potential of SSCs, we injected 10–15 GFP^+ SSCs into 3.5-day-old blastocysts. Sixty-five injected blastocysts were transferred into the uterus of pseudopregnant mice. The number of litters born (42 animals) and number of animals per litter (6–7) were consistent with the birth rate seen with ESC implantation[27]. Animals born from microinjected blastocysts were of similar size as normal animals and did not have overt abnormalities. Chimaerism could be detected in 39 of 42 of mice (~93%) by *LacZ* PCR of genomic DNA isolated from tail biopsies (Supplementary Fig. 5a). We identified a contribution of $LacZ^+$ SSCs to many tissues, including heart, brain, intestine, lung, skeletal muscle, liver, kidney, spleen and testis, by both PCR analysis (Supplementary Fig. 5b) and LacZ staining (Fig. 4j–l, Supplementary Fig. 6). In addition, six male chimaeric mice were each mated with two female chimaeric mice for 2–3 weeks. Two litters (from two different males) were born. DNA analysis from one litter (6 animals) showed three animals positive for LacZ and two animals positive for GFP (segregation of the locus in which *Gfp* and *LacZ* integrated; Supplementary Fig. 5c), indicating germline transmission.

The most striking observation from our experiments is the pluripotency of adult SSCs and SSC-derived maGSCs, similar to ESCs. A previous study failed to establish ES-like cells from adult mouse testis[2]. Here we used a genetic selection[5] to enrich SSCs, which is more efficient than CD9 antibody selection[2,28]. Unlike the previous study, we did not apply basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and LIF for the initial culture of testicular cells, growth factors that may result in the overgrowth of other cells, including fibroblasts, endothelial and Sertoli cells. Although GDNF is essential for the self-renewal of SSCs *in vivo*[29], we found that the proliferation of mouse SSCs *in vitro* is not dependent on GDNF but instead on LIF. Generation of ESC-like maGSCs *in vitro* may result from genetic reprogramming of SSCs in culture, similar to that which occurs during the 'cloning process'[30]. Alternatively, SSCs themselves may be multipotent. In this case, under *in vivo* physiological conditions, the interaction with Sertoli cells in the testis may lead SSCs to spermatogenesis and inhibit multilineage differentiation. However, when SSCs are expanded in the absence of Sertoli cells, as in our *in vitro* culture and in blastocysts, some SSCs might be released from this inhibition and converted into pluripotent stem cells. This is similar to the conversion of embryonic germ cells from primordial germ cells[1]. Cell fusion has been suggested as an explanation for stem-cell plasticity. Our *in vitro* studies show the multi-lineage differentiation potential of maGSCs in the absence of cell fusion, as no co-culture system is applied.

Human SSCs may have great potential for cell-based organ regeneration therapy and for studying genetic diseases in various cell lineages. Therefore, development of a culture system for establishing human maGSCs from testicular biopsies is of paramount importance.

METHODS

Isolation and culture of SSCs. SSCs were isolated from testis tissues of adult transgenic (Stra8–EGFP/Rosa26) and inbred (C57BL/6, FVB and 129/Ola) mice (4–6 weeks old). Details are available in the Supplementary Information.

GFP^+ cells from Stra8–EGFP/Rosa26 mice were separated by FACS. To maintain the GFP^+ cells in an undifferentiated state, four different culture conditions were tested. For condition I, cells were cultured on gelatine-coated culture dishes in basic medium (DMEM medium supplemented with 15% FCS, 2 mM L-glutamine (Gibco), 50 μM β-mercaptoethanol (Promega), 1× nonessential amino acides (NEAA, Gibco)). For condition II, cells were cultured on gelatine-coated culture dishes in basic medium containing 10^3 units ml^{-1} LIF (ESGRO, Chemicon). For condition III, cells were cultured on MEFs in basic medium. For condition IV, cells were cultured on MEFs with basic medium containing LIF (ESC culture condition).

Details of SSC transplantation into seminiferous tubules of testes, cytogenetic analysis, *in vitro* differentiation using the 'hanging drop' method, teratoma formation, chimaeras and germline transmission, isolation of single cardiomyocytes, immunocytochemistry, alkaline phosphatase staining, RT–PCR, action potential recordings and LacZ staining are presented in the Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature. A summary figure is also included.

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