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ORIGINAL ARTICLE

Sphere-formation culture of testicular germ cells in the common marmoset, a small New World monkey

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Abstract Spermatogonia are specialized cells responsible for continuous spermatogenesis and the production of offspring. Because of this biological property, in vitro culture of spermatogonia provides a powerful methodology to advance reproductive biology and engineering. However, methods for culturing primate spermatogonia are poorly established. We have designed a novel method for culturing spermatogonia in the common marmoset (*Callithrix jacchus*), a small primate. By using our method with a suite of growth factors, adult marmoset testis-derived germ cells could be cultured in the form of a floating sphere for several weeks. Notably, this method could be applied not only to freshly isolated cells but also to cryopreserved cell stocks. The spheres enriched spermatogonia and early spermatocytes, and could be assembled from a C-KIT^{+} spermatogonial population. Techniques for culturing spermatogonia could facilitate increased understanding of primate reproduction as well as the preservation of valuable biomaterials from nonhuman primates.

Keywords Common marmoset · Testis · Germ cell culture

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Introduction

Spermatogonia are specialized cells responsible for producing sperm throughout a male's life. To guarantee continuous spermatogenesis, spermatogonial stem cells renew themselves while giving rise to a large number of differentiated cells. In the context of advancing our understanding of spermatogonial physiology, in vitro culture systems provide a powerful method of investigating their biological characteristics and the underlying molecular mechanisms. In rodents, this has led to successful long-term culture of spermatogonia, denoted germline stem cells (GSCs; Kanatsu-Shinohara et al. 2003). GSCs proliferate stably in culture and resume spermatogenesis upon transplantation into seminiferous tubules. Notably, GSCs are available as a biological vector to preserve or create animals with a particular genetic mutation (Takehashi et al. 2010). Thus, techniques for culturing spermatogonia could potentially allow the conservation of valuable bioresources and reproductive engineering.

In contrast, the methodology for culturing primate spermatogonia has not been established, due mainly to a lack of accessibility to primate samples, although the importance of primate research has increased in recent years. Among nonhuman primates, the common marmoset (*Callithrix jacchus*), a small New World monkey, has many advantages as an experimental model for life

sciences: it produces many offspring during its lifetime and displays physiological and developmental similarities to humans (Li et al. 2005; Okano et al. 2012). Furthermore, artificial reproductive technologies have been developed to produce offspring in marmosets (Sasaki et al. 2009; Tomioka et al. 2012; Takahashi et al. 2014). A recent study also attempted to culture marmoset spermatogonia (Langenstroth et al. 2014); however, long-term culture has not been achieved.

In this study, we aimed to develop a long-term culture of marmoset testicular germ cells, especially spermatogonia. Using our method, we were able to maintain marmoset testicular germ cells in culture for at least 2 months. Cell culture could be initiated not only from freshly isolated testis cells but also from cryopreserved cells. This technique may therefore represent a conventional method of preserving and culturing testicular germ cells from valuable nonhuman primate species for reproductive biology.

Methods

Animals and tissue collection

The marmosets used in this study were housed in cages measuring 82 × 61 × 160 cm with wire mesh floors, which were maintained at 25–26 °C with 45–55 % humidity and illuminated for 12 h per day at the Central Institute for Experimental Animals (CIEA). Animal care was conducted in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). All animal experiments were approved by the Institutional Animal Care and Use Committee of CIEA (license numbers: 12025, 13071, 14030) and performed in accordance with CIEA and Keio University guidelines concordant with the *Guidelines for Proper Conduct of Animal Experiments* by the Science Council of Japan (2006). For tissue collection, testes were obtained from more than 10 adult marmosets (2, 3, 4, 7, 8, and 10 years of age) that were euthanized because of disease, accidents, or other experiments.

Cell culture

For cell culture, seminiferous tubules were physically excised and digested in Dulbecco's modified Eagle's medium (DMEM) containing 10 mM Hepes, 20 ng/ml collagenase, and 4 mg/ml dispase II at 37 °C for 15 min with shaking. The cell suspension was consecutively passed through 70- and 40-μm cell drainers, and then seeded at a density of 1.5×10^5 cells/well in 6-well low-attachment plates with knockout DMEM supplemented with 20 % knockout serum replacement, 100 μM non-essential amino acids, 1 mM L-glutamine, 1 mM 2-mercaptoethanol, 20 ng/ml human GDNF, 200 ng/ml human GFR-α1, 10 ng/ml human bFGF, 10 ng/ml mouse SCF, 12.5 ng/ml human FGF9, and 10^3 U/ml LIF. The cells were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Half of the medium was exchanged every 2 days. For storage, the cell suspension was cryopreserved with CELLBANKER 2 (Takara) in liquid nitrogen.

Cell sorting

For cell sorting, cells were suspended in ice-cold Hank's balanced salt solution at 1×10^7 cells/ml and incubated with a mouse anti-C-KIT antibody (Mar117-22; Oriental Yeast) for 30 min on ice. After two washes with ice-cold phosphate-buffered saline (PBS), 1×10^6 cells were gently resuspended in ice-cold 70 % ethanol and incubated at 4 °C for 2 h. The fixed cells were washed again with PBS and then treated with 25 μg/ml RNase A. After incubation at 37 °C for 1 h, propidium iodide (PI) was added at a final concentration of 50 μg/ml, and the cell suspension was incubated at 4 °C in the dark for 1 h. Flow-cytometric analysis and cell sorting were performed using a FACS Aria III (BD Biosciences).

Immunofluorescence microscopy

Alkaline phosphatase (AP) staining was performed according to the manufacturer's instructions (Sigma). For whole-mount immunofluorescence microscopy, spheres were adhered to fibronectin-coated coverslips on the day before experiments. The spheres were then fixed with 10 % formaldehyde, permeabilized with 0.5 % Triton-X, blocked with 5 % skim milk, and incubated with primary antibodies. For microtomy, spheres were solidified in iPGell (Genostaff), fixed in 4 % PFA, embedded in paraffin, and sliced at a thickness of 5 μm, as previously described (Lin et al. 2015). The primary antibodies used were rabbit anti-DDX4 antibody (ab13840; Abcam), rabbit anti-DAZL antibody (ab34139; Abcam), mouse anti-MARWI antibody (2D9-1H6 clone), mouse anti-Ki67 antibody (NCL-Ki67 MM1; Leica Biosystems), and rabbit anti-SALL4 antibody (ab29112; Abcam). Nuclei were stained with 10 ng/ml Hoechst 33342.

RT-PCR analysis

RNA extraction and RT-PCR were performed as described previously (Lin et al. 2012). Briefly, total RNA prepared with TRIzol reagent was isolated using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) and an oligo-dT_{18} primer. PCR was semiquantitatively performed

with Ex Taq Hot Start Version (Takara) using three different escalatory cycles, and only representative images are shown in the results.

Results

Surgically isolated adult marmoset testes were dissociated for cell culture. The testicular cells were cultivated in suspension, in which they formed cell clumps around day 3. Small spheres formed by week 1 and grew during 4 weeks of culture (Fig. 1a). The spheres could be formed not only from freshly isolated testicular cells but also from cryopreserved cells (Fig. 1b). Compared with freshly isolated cells, cryopreserved cells formed morphologically good spheres with less debris, possibly due to the elimination of inappropriate somatic cells via the freeze-thawing process (Langenstroth et al. 2014). Sphere formation was induced in a growth-factor-dependent manner; no spheres were formed in the absence of growth factor cocktail (Fig. 1c). These spheres, named testicular spheres (TeS), enclosed AP-positive germ cells (Fig. 1d). TeS could be

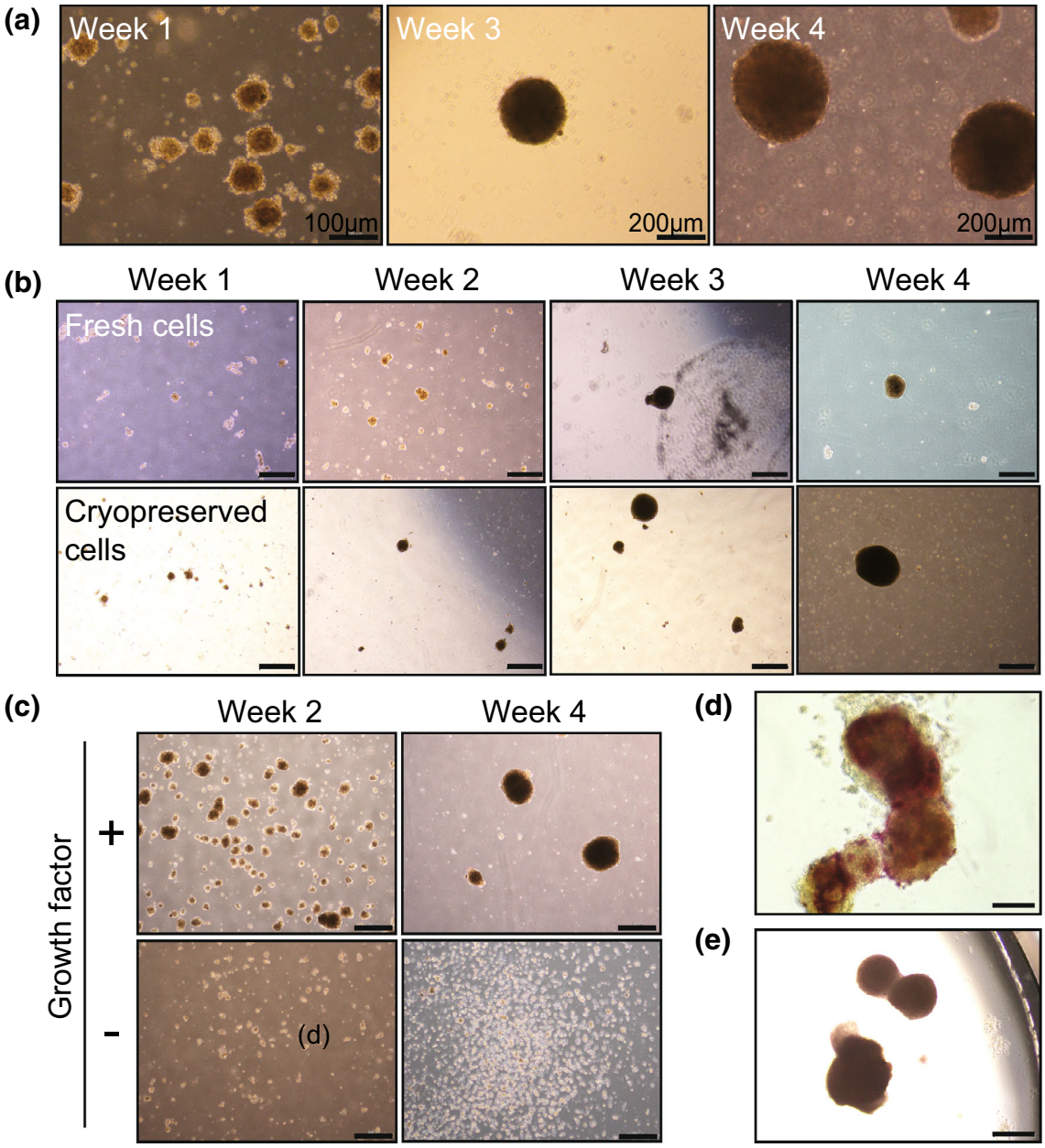

Fig. 1 Sphere formation culture of marmoset testicular cells. **a** Morphology of TeS during 4 weeks of culture. **b** TeS formation culture from freshly isolated or cryopreserved testicular cells. *Scale bar* 500 μm. **c** Growth-factor-dependent formation of TeS. **d** Alkaline phosphatase staining. *Scale bar* 100 μm. **e** Morphology of 60-day TeS

cultured for at least 2 months without passaging while retaining AP activity, although there were fewer spheres in culture that had been performed for over a month (Fig. 1e).

Immunofluorescence microscopy showed that $DAZL^+$ (spermatogonia-spermatocyte) and $VASA^+$ (spermatocyte-spermatid) cells were incorporated in the 1-week TeS (Fig. 2a). In the 2-week TeS, quiescent (SALL4) and active (Ki67) spermatogonia markers as well as a spermatocyte-spermatid marker (MARWI) were detected (Fig. 2b). However, MARWI protein disappeared by week 4, while SALL4 protein was still detectable. Time-course RT-PCR analyses of TeS revealed that genes associated with later stages of spermatogenesis disappeared earlier during culture. The 1-week TeS expressed all spermatogenesis-related genes examined except for *STRA8* (Fig. 3). However, in prolonged culture, the expression of post-meiotic markers (*TEKT1*, *HAPRIN*. etc.) decreased or stopped, in contrast to that of spermatogonia and early spermatocyte

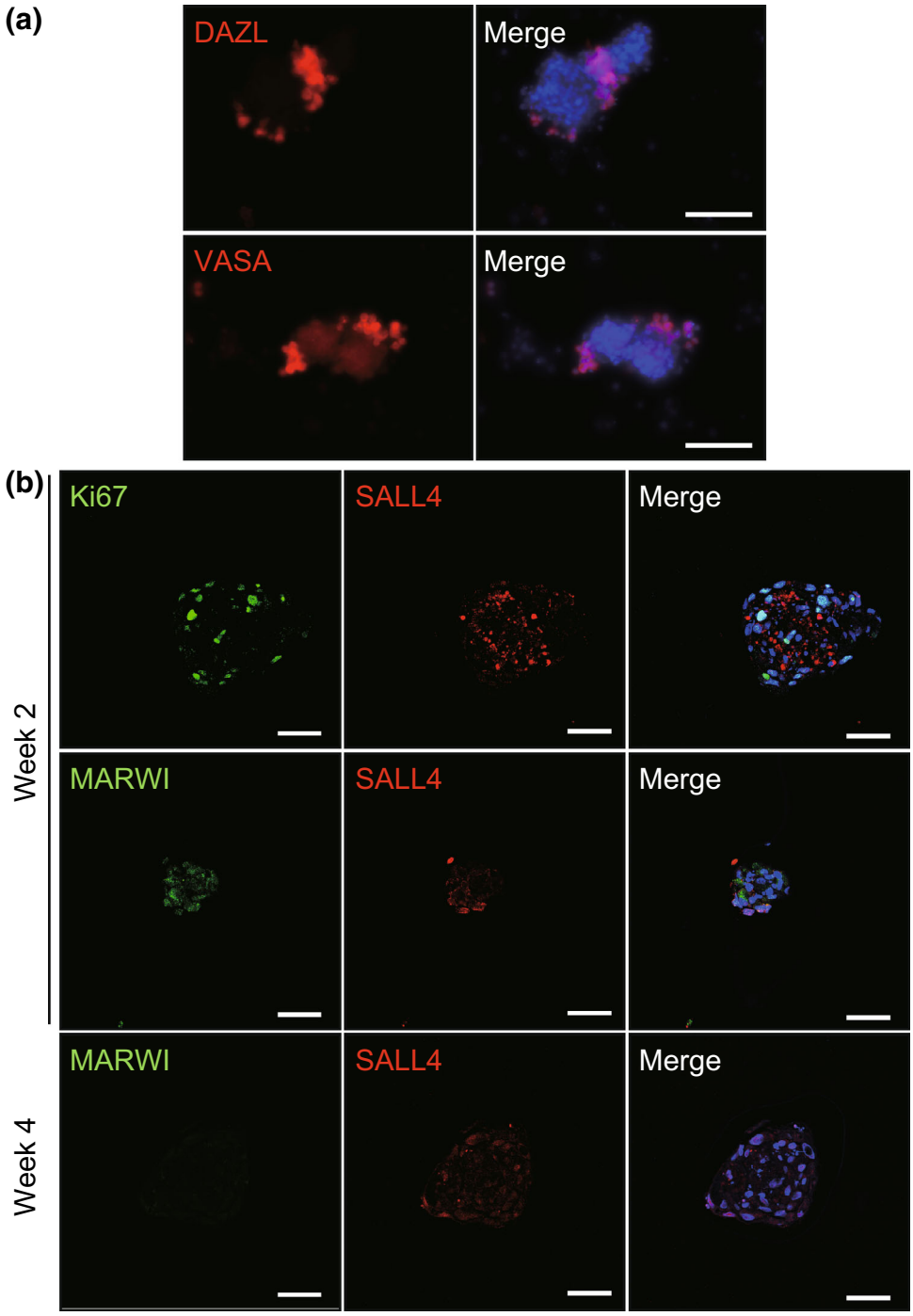

Fig. 2 Immunofluorescence analyses of spermatogonia and spermatocyte markers in TeS. **a** Whole-mount immunofluorescence analyses of DAZL and VASA proteins. Nuclei were counterstained with Hoechst 33342. *Scale bar* 200 μm. **b** Immunofluorescence analyses of Ki67, SALL4, and MARWI in the TeS sections. *Scale bar* 50 μm

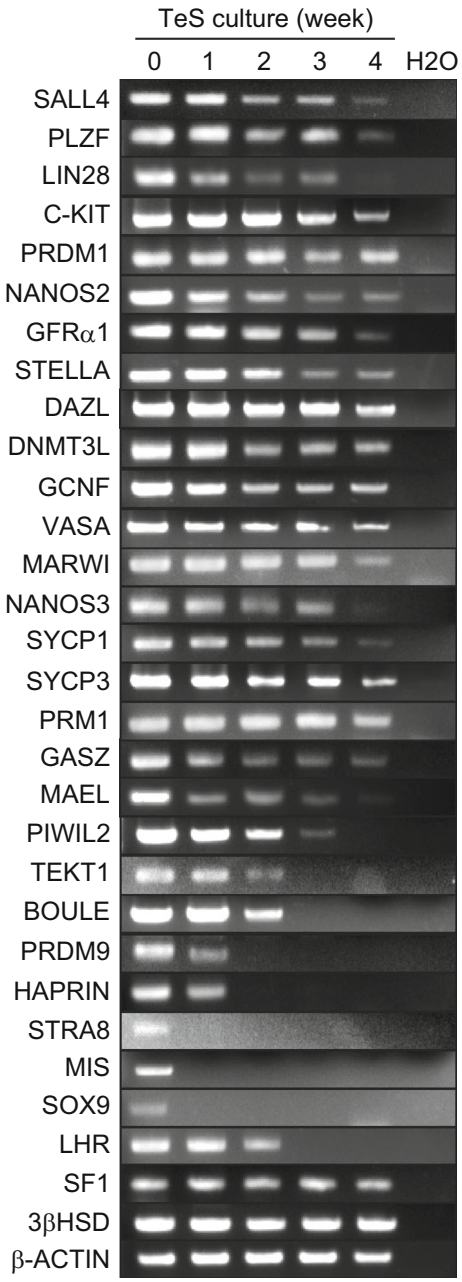

Fig. 3 Enrichment of early spermatogenic cells during TeS culture. RT-PCR analyses of spermatogenesis-related genes during 4 weeks of TeS culture. β-actin was analyzed as an internal control and water was used as a negative control

markers, which remained even later during culture. As for somatic cells, the expression of Sertoli cell markers (*MIS*, *SOX9*) was regressed upon cultivation, while the expression of Leydig cell markers gradually decreased (*LHR*) or remained constant (*SF1*, *3βHSD*).

To confirm whether the genes that were highly enriched in the later TeS reflected the character of early spermatogenic cells, especially spermatogonia, we sorted and characterized C-KIT$^+$ dividing/differentiating spermatogonia (Fig. 4a). Indeed, the transcripts of spermatogonia markers (*SALL4*, *PLZF* etc.) were enriched in C-KIT$^+$ cell populations (Fig. 4b), while meiotic, post-meiotic, and Leydig cell markers were predominant in C-KIT$^-$ cell populations. Furthermore, when both cell populations were supplied for TeS culture, only C-KIT$^+$ cell populations formed spheres (Fig. 4c), suggesting that TeS formation in culture could be attributed to spermatogonial activity.

Discussion

Sophisticated methods have enabled us to culture rodent spermatogonia (Kanatsu-Shinohara et al. 2003), while the situation in primates is quite different; no methods for germ cell culture have been established yet (Imamura et al. 2013). Previous studies have addressed primate testicular cell culture, but they achieved only short-term culture of spermatogonia and/or considerable expansion of somatic cells (Eildermann et al. 2012; Albert et al. 2012; Kossack et al. 2013; Langenstroth et al. 2014; Zheng et al. 2014). Our recent work has revealed some of the characteristics of marmoset spermatogenesis (Lin et al. 2012; Hirano et al. 2014; Lin et al. 2015), so methods of cultivating marmoset spermatogonia are now required. In the present work, we succeeded in cultivating testicular germ cells of adult marmosets in the form of floating spheres named TeS. Gene expression and cell sorting analyses suggested that TeS were preferentially constituted by spermatogonia and early spermatocytes. This is reasonable because, among spermatogenic cells, only spermatogonia undertake mitotic division, and therefore proliferate continuously. Notably, the TeS formation culture was applicable not only to freshly isolated cells but also to cryopreserved cell stocks. Thus, cryopreservation and culture of nonhuman primate spermatogonia, together with other stem cell technologies including induced pluripotent stem cells (Imamura et al. 2012, 2014), appears be a promising method of preserving unique genetic material of endangered or mutant nonhuman primates (Oishi et al. 2014; Suzuki et al. 2010; Onishi et al. 1999) as well as a useful research tool for basic and applied reproductive biology.

Two major concerns remain in relation to further improving marmoset spermatogonia culture. The first is the clonal propagation of marmoset spermatogonia in culture. Marmoset TeS comprise heterogeneous cell populations while rodent GSC can be clonally derived from single spermatogonia (Kanatsu-Shinohara et al. 2005). We and others have tried to establish clonal culture of marmoset spermatogonia but have not yet succeeded (Langenstroth et al. 2014). The second concern is reconstructing the entire process of spermatogenesis in vitro. In prolonged culture, TeS lost the ability to express post-meiotic genes,

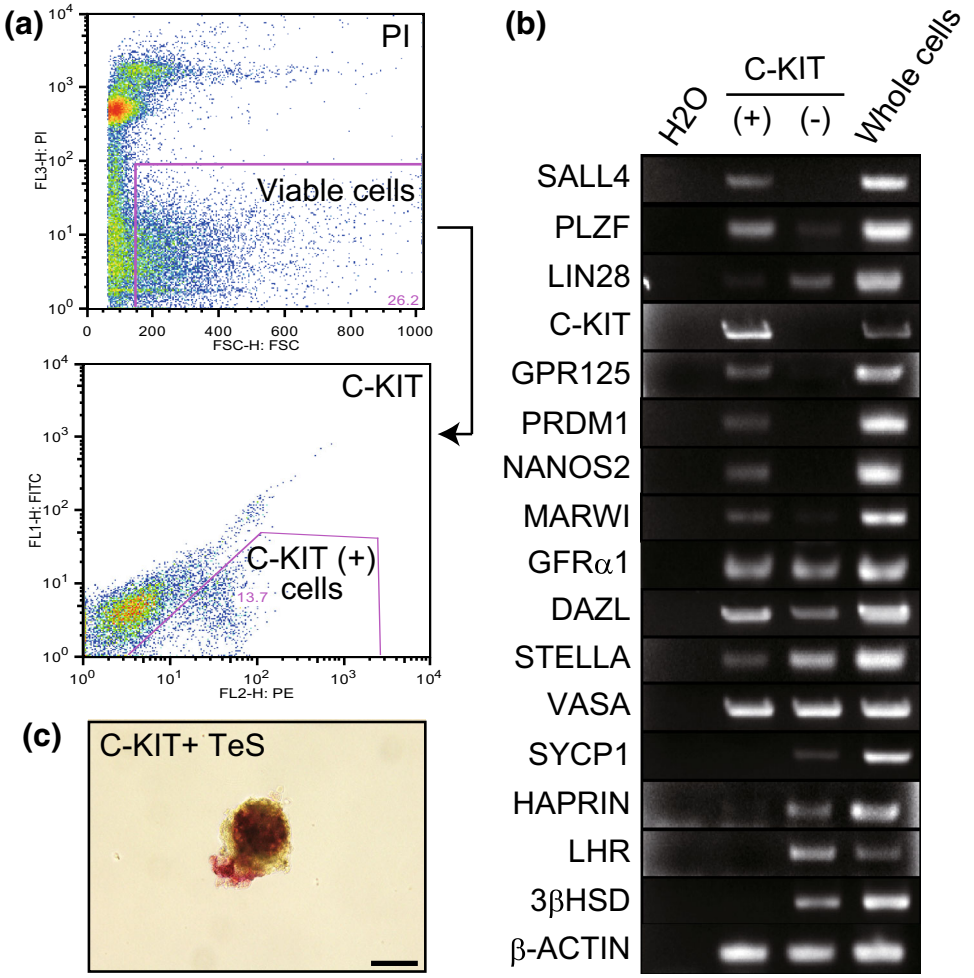

Fig. 4 Isolation and TeS formation culture of C-KIT$^+$ testicular cells. **a** Sorting of C-KIT$^+$ cells from cryopreserved testicular cells. After live cell selection by PI staining, the C-KIT$^+$ cell fraction enclosed in a *box* with *purple borders* were collected. **b** Gene expression of C-KIT$^+$ and C-KIT$^-$ cell populations separated from whole testicular cells. β-actin was analyzed as an internal control and water was used as a negative control. **c** TeS formation from a C-KIT$^+$ cell population. AP staining was performed. *Scale bar* 200 μm

suggesting that (1) meiotic germ cells left over from testis samples gradually become diminished, and (2) de novo spermatogenesis does not occur in TeS culture. To resume spermatogenesis, especially meiosis, in vitro, we stimulated TeS with retinoic acid and/or testosterone; however, the meiotic/post-meiotic genes were not induced (data not shown). Since spermatogenesis can be reconstructed by culturing testis fragments in mice (Sato et al. 2011), similar methods should be developed for primates as well. Advances in these techniques, combined with germ cell transplantation (Langenstroth et al. 2014), will provide the fundamental steps needed to produce cultured spermatogonia-derived sperm.

In conclusion, we have demonstrated a method of cultivating germ cells derived from freshly prepared or cryopreserved marmoset testes. This should make it easy to address primate reproductive biology and should also facilitate the preservation of valuable biomaterials in nonhuman primates.

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Compliance with ethical standards

Conflict of interest H.O. is a Scientific Advisory Board Member of San Bio Co. Ltd. The other authors have no competing interests.

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