Title: Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture

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Summary statement

The entire spermatogenesis process, from stem cell propagation to differentiation of functional sperm, has been successfully achieved solely in culture, using zebrafish testicular hyperplasias that accumulate early stage spermatogonia.

Abstract

Molecular dissection and chemical screening on a complex process such as spermatogenesis could be facilitated by cell culture approaches that allow easy access for experimental manipulation and live imaging of specific molecules; however, technical limitations have thus far prevented the complete reconstruction of spermatogenic events in cell culture. Here, we describe production of functional sperm from self-renewing spermatogonial stem cells (SSCs) in cell culture conditions, using zebrafish testicular hyperplasia cells that accumulate early stage spermatogonia. By serially transplanting hyperplasias into immuno-deficient rag1 mutant zebrafish, we succeeded in long-term maintenance and efficient production of starting material for SSC culture. Through improvements of culture conditions, we achieved efficient propagation of SSCs derived from the hyperplasia. When SSCs that underwent the SSC-propagating step for 1 month were transferred onto Sertoli feeder cells, they differentiated to functional sperm that gave rise to offspring. Oxygen at the concentration of air proved to be detrimental for sperm differentiation from SSCs, but not for propagation of SSCs. These results indicate that the whole spermatogenic process can be represented in cell culture in zebrafish, facilitating analyses of molecular mechanisms of spermatogenesis in vertebrates.
Introduction

Spermatogenesis is characterized by sequential transitions of multiple processes: self-renewal of spermatogonial stem cells (SSCs), differentiation of SSCs into differentiating spermatogonia, and meiotic events leading to the production of functional sperm. Understanding spermatogenic processes is facilitated by cell culture experimental approaches, which are easily accessible for experimental manipulation and live imaging of specific molecules within germ cells. Thus far, only organ culture systems in eel and mice have represented the whole process of spermatogenesis (Miura et al., 1991; Sato et al., 2011). While the maintenance of SSCs in culture has been performed in various species in mammals, such as mice (Kanatsu-Shinohara et al., 2003), rats (Hamra et al., 2005), and hamsters (Kanatsu-Shinohara et al., 2008), differentiation of these SSCs to fertile sperm or spermatids usually requires testis organ environment through transplantation into the testis (Kanatsu-Shinohara et al., 2003; Hamra et al., 2005; Kanatsu-Shinohara et al., 2008) or testicular organ culture (Sato et al., 2013); cell culture approach has so far failed to reproduce either the conversion of SSCs to differentiating spermatogonia (Sato et al., 2013) or the subsequent meiotic processes (La Salle et al., 2009). For example, premeiotic germ cells derived from mice and cultured on a Sertoli-like cell line undergo the meiotic and postmeiotic differentiation into haploid spermatids (Rassoulzadegan et al., 1993). Mouse germ cells that were immortalized either with the simian virus 40 large tumor antigen and a temperature-sensitive p53 mutation or by over-expression of telomerase catalytic component, TERT, differentiate into haploid spermatids in cell culture (Hofmann et al., 1994; Feng et al., 2002). A spermatogonial cell line of medakafish without immortalization also differentiates to haploid sperm-like cells in culture (Hong et al., 2004). However, the next
generation has not been produced from these cultured spermatids and sperm-like cells. Instead, the next generation has been obtained only by nuclear injection using haploid spermatids that are differentiated from primary spermatocytes in culture (Marh et al., 2003).

In contrast, differentiating spermatogonia of zebrafish can undergo meiosis and differentiate to functional sperm when cultured on Sertoli cell-derived cell lines (Sakai, 2002), making it a promising system to develop culture conditions that represent the whole spectrum of spermatogenesis from SSCs to sperm. To achieve this goal two protocols must be developed in order to efficiently generate functional sperm that can produce the next generation offspring. One is the conversion of SSCs to differentiating spermatogonia under defined culture conditions. The other is the maintenance and propagation of SSCs and/or a stable source of SSCs to serve as the starting material for SSC culture, because so far zebrafish SSCs cannot be propagated efficiently in culture (Kawasaki et al., 2012; Wong and Collodi, 2013). To develop a new experimental source of SSCs, we focused our attention to testicular hyperplasias which are found at a low frequency in laboratory strains of zebrafish. Unlike benign seminomas which are tumors of predominantly one cell type, testicular hyperplasias are enlarged testes containing all stages of spermatogenesis (Neumann et al., 2011), suggesting that they include SSCs that are capable of undergoing normal differentiation process including differentiation to spermatogonia and subsequent meiosis.

Despite potential usefulness of testicular hyperplasia as the source of SSC culture, such hyperplasias are found only sporadically in laboratory strains of zebrafish and cannot serve as a stable source of SSC culture. We thus first established a system for long-term maintenance of hyperplasia by serial transplantation using immune-deficient adult zebrafish as a host. Then, we
improved culture conditions for propagation and differentiation of SSCs using cells of the hyperplasia, allowing efficient propagation of the SSC and differentiation to functional sperm that can give rise to the next generation. In addition, we examined effects of oxygen as an important condition for spermatogenesis by using the cell culture system established.
Results

Transplantation of a testicular hyperplasia into \textit{rag1} mutant zebrafish

Before we examined a system for long-term maintenance of testicular hyperplasias, we examined whether SSCs of the hyperplasia can differentiate into sperm. Dissociated hyperplasia cells from fish harboring a \textit{vas::egfp} transgene (Krøvel and Olsen, 2002) were cultured for 4 weeks as previously described (Kasasaki et al., 2012). GFP positive cells were then mixed with testicular aggregates derived from the inbred IM line (Shinya and Sakai, 2011) and transplanted subcutaneously into IM recipients, as described previously (Kawasaki et al., 2010). After 3 months, we performed artificial insemination using sperm from the transplanted aggregates and obtained fertilized eggs. The aggregates did not grow to hyperplasias, similar to subcutaneous transplants of aggregates from normal testis tissue mixed with SSCs of normal \textit{vas::egfp} testis (supplementary material Fig. S1), suggesting that hyperplasia-derived SSCs self-renewed and differentiated the same as SSCs of normal testes.

A general problem in tissue transplantation is immunological rejection, which is caused by self-nonself recognition by T cells (Ingulli, 2010). Because the generation of mature B and T lymphocytes requires the activities of the RAG (Recombination-activating genes) proteins, one way to alleviate rejection is to use mutations in the \textit{rag} genes (Belizario, 2009; Tang et al., 2014; Tenente et al., 2014). As the host of transplantation we chose \textit{rag1}^{26683} mutant zebrafish (hereafter \textit{rag1} mutant), which harbors a nonsense mutation in the catalytic domain of the encoded RAG1 protein resulting in loss of mature functional T and B cells (Wienholds et al., 2002; Petrie-Hanson et al., 2009). To examine if the \textit{rag1} endures surgery and accepts allografts, we grafted wild-type testis fragments into wild type and \textit{rag1} mutant fish as described
previously (Kawasaki et al., 2010). Grafts of testis fragments to wild type host were immediately rejected and fragments were not found in hosts after 4 weeks; however, when testis fragments were transplanted under abdominal skin of \textit{rag1} mutants, the graft was found in 8 out of 10 transplants (supplementary material Table S1). Spermatogenic cells of grafts recovered from \textit{rag1} mutant hosts incorporated BrdU (supplementary material Fig. S2), indicating that spermatogenesis of the grafted testis proceeded normally. These results indicate that \textit{rag1} mutant could be an efficient vehicle for long-term maintenance of spontaneous testicular hyperplasias.

To test the validity of this transplantation method to maintain testicular hyperplasia, we collected four testicular hyperplasias from various laboratory strains: two (named A, B) from wild-type strain (kindly provided by Dr. H. Hirata), and two (named C, D) from transgenic fish carrying a \textit{sox17} promoter::\textit{egfp} reporter gene (Mizoguchi et al., 2008). By histology, we confirmed that the testis contained all stages of spermatogenesis, and found a large number of single spermatogonia surrounded by Sertoli cells, which population includes SSCs (Fig. 1A, B). Testicular hyperplasias were cut down to transplantable small size including the outer layer, and the fragments were transplanted under the abdominal skin of \textit{rag1} mutant and wild-type fish (Fig. 1C, E, F). While fragments grafted into the wild-type host disappeared completely by four weeks after transplantation (supplementary material Table S2), fragments grafted into \textit{rag1} mutants grew remarkably, achieving more than 20-fold increase in volume at 3 months after transplantation (Fig. 1D, G). Grafts that grew in hosts were histologically indistinguishable from the original hyperplasia (Fig. 2), suggesting that this transplantation protocol can be used to maintain testicular hyperplasia without causing any drastic changes in its character.
Maintenance and amplification of testicular hyperplasia by serial transplantation

We next examined if testicular hyperplasias can be amplified by serial transplantation. The four transplanted hyperplasia A through D that grew in the host animal were cut down again to transplantable size, re-transplanted into several *rag1* mutant zebrafish and maintained for 1 to 16 months. Grafts were then recovered and serially transplanted to new host animals. This protocol enabled maintenance of the hyperplasia for more than 3 years (Fig. 2; supplementary material Fig. S3). Most grafted testes contained many single spermatogonia and sperm, indicating that SSCs in hyperplasia continued to proliferate and differentiate in the adult host. In some cases, however, the graft appeared to change its character. In hyperplasia B, recipients receiving graft fragments from a particular third serial transplant often failed to survive, implying occurrence of malignant transformation (B4-1 in supplementary material Fig. S3A). Testis-ova were observed in some of the grafts conducted after two serial passages (A3-8 in Fig. 2B; B3-1 and B4-1 in supplementary material Fig. S3B). Because such events were infrequent, we conclude that long-term maintenance and amplification of testicular hyperplasia is possible by transplantation into multiple host animals in each generation and histological checking. Thus, this technique can be used to propagate an unlimited resource of testicular hyperplasia material from which relatively large quantities of SSCs can be derived.
Propagation of SSCs in culture

Since testicular hyperplasia contains all stages of spermatogenesis, we wished to propagate and enrich SSCs by culturing in conditions that favor stem cell maintenance. To this end we improved culture conditions for SSCs previously described (Kawasaki et al., 2012). To monitor differentiation of spermatogonia we used sox17::egfp transgenics which express green fluorescent protein (GFP) in early stages of spermatogonia in small clusters (Kawasaki et al., 2012). Dissociated cells of the sox17::egfp hyperplasia testis were cultured in modified conditions, and the number of GFP-positive spermatogonia was counted. Changes of the basal culture medium and gas phase from Leibovitz’s L-15 in air to DMEM in 5% CO$_2$ increased the number of GFP-positive spermatogonia to twofold after 21 days of culture (Fig. 3A). We could not observe any significant differences in the number of spermatogonia between different O$_2$ concentration at 10% and 20% (Fig. 3A).

We also examined the effect of heparin, because other members of the glycosaminoglycan family are known as essential regulators of germline stem cell niches in Drosophila (Hayashi et al., 2009). In addition, glial cell line-derived neurotrophic factor (GDNF), which is supplemented in our medium, was originally purified by heparin affinity chromatography (Lin et al., 1993) and has later been shown to interact with heparan sulfates (HSs; Rickard et al., 2003). HSs are required for GDNF signaling through the GFR-α1–RET complex (Barnett et al., 2002; Parkash et al., 2008). The addition of heparin at a final concentration of 25-50 U/ml resulted in more than a twofold improvement in the yield of GFP-positive spermatogonia after 21 days of culture (Fig. 3B).
Combining these modifications with an addition of a BMP inhibitor dorsomorphin, which is known to promote proliferation of zebrafish spermatogonia (Wong and Collodi, 2013), GFP-positive spermatogonia could be maintained for more than 48 days (Fig. 3C), a period surpassing the time required for zebrafish spermatogonia to produce functional sperm (Haffter et al., 1996). Most GFP-positive cells had the morphology of the early stage of spermatogonia with large nuclei and one or a few nucleoli (Fig. 3C). We regard these GFP-positive spermatogonia as SSCs because the spermatogonia proliferated continuously and had characteristics of early stage spermatogonia. Therefore, this demonstrates that this culture condition can efficiently maintain and propagate SSCs in culture.

Stemness of cultured GFP-positive spermatogonia was further confirmed in vivo by the functional transplantation assay carried out in aggregates with dissociated testicular cells of spermatogenesis-defective minamoto (moto) mutants. The moto mutant testis lack germ cells after late stage spermatogonia due to a germ cell-autonomous defect (K. R. S. et al., unpublished). After transplantation of co-aggregates of cultured GFP-positive spermatogonia and moto testicular cells into the rag1 mutant, the spermatogonia self-renewed, and re-initiated spermatogenesis and produced sperm (supplementary material Fig. S4), suggesting that the spermatogonia indeed include SSCs that are capable of generating sperm.
Differentiation of cultured SSCs to functional sperm in culture

Having enriched for SSCs we tested whether these SSCs can undergo a complete differentiation step to produce functional sperm in culture. To induce differentiation, we utilized the culture system using a Sertoli cell line ZtA6-12, which supports all steps of differentiating spermatogonia to sperm (Sakai, 2002; Kurita and Sakai, 2004), with slight modification in which the basal culture medium and gas phase were changed to DMEM in 5% CO₂. SSCs that underwent the SSC-propagating step described above for 1 month were removed and transferred onto mitomycin-treated ZtA6-12 cells. One day after the transfer, most clumps flattened and SSCs attached tightly to the Sertoli feeders (Fig. 4A). Spermatocytes appeared around after 9-10 days (Fig. 4A). Most cells expressed Synaptonemal complex protein 3 (Sycp3), a marker of meiosis; Sycp3 protein was localized as a small particle in one side of the nucleus and extended in the nucleus, as observed in intact zebrafish testis (Ozaki et al., 2011; Saito et al., 2011; Fig. 4B; compare with SSC clumps under SSCs propagating condition shown in Fig. 4C). Sperm-like cells appeared after 17 days of transfer (Fig. 4A). Functionality of these sperm-like cells was demonstrated by artificial insemination; we obtained embryos that expressed GFP (Mizoguchi et al., 2008) and grew up to normal fertile adult fish without testicular hyperplasia formation (Table 1; supplementary material Fig. S5). Differentiating spermatogonia differentiate to sperm within 15 days on Sertoli feeders as described previously (Sakai, 2002), but fertile embryos were obtained after 20 days in the present study. These results indicate that our culture condition using ZtA6-12 cells induces differentiation of SSCs and supports the entire differentiation process from SSCs to functional sperm. This condition, however, appeared not to support SSC maintenance; unlike the propagation culture, the GFP signal representing early
spermatogonia decreased during culture and was nearly gone after 10 days (Fig. 4A). This suggests that our two culture conditions preferentially support either SSC propagation or differentiation of testicular hyperplasia.

Since we improved culture conditions with testicular hyperplasia cells, we also examined the effectiveness of these culture conditions on dissociated normal testicular cells of the sox17::egfp adult fish. The improved culture conditions resulted in more than a twofold increase in the yield of GFP-positive spermatogonia after 21 days of culture, and GFP positive cells continuously proliferated in the propagation culture of SSCs (supplementary material Fig. S6A, B). However, a small number of differentiating spermatogonia were still observed after 31 days. The number of Sycp3 positive cells in cultures of normal testicular cells was larger than those derived from hyperplastic testis after 40 days (supplementary material Fig. S6C, D). These results indicate that the improved culture method is effective on SSCs of normal testis, but some of the SSCs were induced to differentiate even in the propagation culture. Since this culture contains somatic cells that are from the initial dissociated testis, it is likely that somatic cells in the culture supported some spermatogonia differentiation.
Effect of oxygen on the differentiation of SSCs to sperm

Recent evidence suggests that oxygen has two distinct effects on spermatogenesis; while oxidative stress is generally thought to cause male infertility, reactive oxygen species (ROS) have been shown to be required for the self-renewal of SSCs and the fertility of sperm (Agarwal et al., 2008; Morimoto et al., 2013). To begin dissecting the roles of oxygen concentration in spermatogenetic processes, we compared the efficiency of SSC differentiation under normal and low oxygen concentrations. Cultured SSCs were transferred to Sertoli feeders and cultured in either 10% or 20% O$_2$ conditions, and the effect on spermatogenesis was assayed by measuring the efficiency of sperm production through artificial insemination. While cultures at 20% O$_2$ produced functional sperm only rarely, cultures at 10% O$_2$ resulted in more than four-fold increase in the production of fertilized egg and the growth to adult fish (Table 2); under low oxygen concentration fertilized embryos were stably obtained from cultured sperm (Table 3). On the other hand, no significant effect of low oxygen concentration was observed for the SSC propagation step (Fig. 3A). These results suggest that, in zebrafish, oxygen at the concentration of air causes some damage in differentiation steps from SSCs to sperm.
Discussion

The present study highlights significant technical advances in two fronts: the use of immuno-deficient \textit{rag1} mutants to maintain allogeneic hyperplasias, and the establishment of culture conditions promoting SSCs to self-renew and differentiate to functional sperm that can give rise to the next generation by simple artificial insemination. Although zebrafish SSCs are not able to be maintained as long as those of mammalian SSCs in culture (Kanatsu-Shinohara et al., 2003; Hamra et al., 2005; Kanatsu-Shinohara et al., 2008), relatively large quantities of SSCs can be derived from an unlimited resource of testicular hyperplasia material, propagated through the transplantation technique. The utilization of hyperplasias makes it possible to select desired cell types under the culture condition for SSC propagation.

In the present study, we used SSCs of testicular hyperplasias because they differentiated into sperm in the aggregate of normal testis after transplantation and did not induce hyper-growth of the aggregate. Although we do not know the underlying cause of the hyperplasia used in our study, differentiated sperm from hyperplasia-derived SSCs gave rise to normal fertile adult fish without later testicular hyperplasia formation. Therefore, SSCs from hyperplasias seem to have proper stem cells function, and somatic cells rather than SSCs are likely the cause of the hyperplasia. In support of this notion, loss of anti-mullerian hormone receptor in the somatic cells of testis causes testicular hyperplasia in the medaka fish (Morinaga et al., 2007).

When adult SSCs cells from normal testes were cultured under the conditions for SSCs propagation, we observed a small number of differentiating spermatogonia after 31 days of culture, probably due to somatic cells in the culture supporting differentiation of spermatogonia.
This indicates that there is still room for improvement for culturing SSCs of normal testes. The present culture system is favorable for the analysis of factors to induce differentiation of SSCs because Sertoli feeders can induce their differentiation in vitro. Identification of factors that induce differentiation of SSCs and improvement of culture conditions to prevent such factors will facilitate development of efficient culture conditions for SSCs of normal testes.

Subcutaneous grafting to rag1 mutants demonstrated not only spermatogenesis of testes but also growth and amplification of zebrafish testicular hyperplasias by serial transplantation. Since the hyperplasia accumulates SSCs, this amplification protocol would facilitate analyzing the properties of SSCs in zebrafish. The rag1 mutant fish lack all mature T and B cells (Wienholds et al., 2002; Petrie-Hanson et al., 2009) but have not been widely used for transplantation approaches because of reduced viability of adult fish and failure to thrive following injury (Tang et al., 2014). However, the present study shows that the mutant can thrive by the treatment to prevent from infection after surgery and allows us to transplant a fragment of organs and hyperplasia, which regenerated in the host. While this study concentrated on spermatogenesis in testis, rag mutants are likely to accept other allogeneic tissues and hyperplasia since immunocompromised rag2^{E450fs} mutant accepts various cell types (Tang et al., 2014; Tenente et al., 2014). Long-term maintenance and amplification that can be achieved in rag1 mutants will enhance our understanding of stem cell function and regeneration in various allogeneic organs and cancers in zebrafish.

An advantage of the cell culture system is the ease in manipulating conditions that may affect various developmental processes. One important condition for spermatogenesis that we examined was the effect of oxygen concentration. In contrast to SSCs of mice which show more
efficient propagation of SSCs in an atmosphere of 10% O$_2$ than 21% O$_2$ (Kubota et al., 2009), we could not observe differences between at 20% and 10% O$_2$ on the propagation of zebrafish SSCs. However, larger number of fertilized sperm were produced from SSCs in the present conditions at 10% O$_2$ than that at 20%. It has been indicated that germ cells at advanced stages such as spermatocyte, spermatids and sperm undergo intense apoptosis and oxidative DNA damage by hypoxanthine-induced ROS in testicular organ cultures of eel (Celino et al., 2011). Furthermore, proliferating spermatogonia are also susceptible to ROS compared with nonproliferating primary spermatogonia (Celino et al., 2012). The complete reconstruction of spermatogenesis achieved under our culture conditions should help pinpoint the process that is susceptible to oxygen within germ cells, and may unveil new effects of oxygen beside DNA damage and reducing sperm motility (Agarwal, 2008). Similarly, chemicals that disrupt germ cell development, including endocrine disruptors, by inhibition or induction of certain signaling pathways could be studied with this culture system, thus pinpointing the process that is susceptible to the chemical.

There are many subcellular processes inherent to germ cells for which this cell culture system can facilitate the study of. For example, it is well known that germ cells have special RNA-rich cytoplasmic bodies, so called germ granules, which developmentally regulates post-transcriptional gene expression (Voronina et al., 2011). Additionally, during meiosis, chromosomes behave dynamically to achieve meiotic recombination (Roeder, 1997). The cell culture method developed here would also allow genetic manipulation to dissect individual spermatogenic processes and their transitions by specific molecules. Combined with the ease of manipulating condition in culture, the ease of imaging specific molecules and subcellular
structures in germ cells should further advance our understanding of molecular and cellular mechanisms behind processes of spermatogenesis such as SSC differentiation and meiotic events, which are likely controlled by common sets of genes in vertebrates (Howe et al., 2013).
Materials and Methods

Zebrafish

sox17::egfp transgenic fish (Mizoguchi et al., 2008) and rag1 mutants (rag1^{2668}; Wienholds et al., 2002; Petrie-Hanson et al., 2009) were provided by Dr. Yutaka Kikuchi and Dr. Lora Petrie-Hanson, respectively. moto mutant fish were isolated by N-ethyl-N-nitrosourea mutagenesis screening (Saito et al., 2011). As wild-type fish, we used India or AB lines. The use of these animals for experimental purposes was conducted in accordance with the guidelines of the National Institute of Genetics.

Subcutaneous transplantation

To transplant fragments of normal testes and testicular hyperplasias, we applied the testis transplantation method (Kawasaki et al., 2010) with slight modifications. Briefly, one day prior to transplantation, male recipients were maintained without feeding. The recipient fish were anesthetized with 0.01% ethyl p-aminobenzoate (Wako) and an incision of about 10 mm was made into their abdominal skin with a razor blade. The tip of a forceps was then inserted between the muscle and skin through this wound to enable transplantation of the fragment. The graft was inserted subcutaneously through the wound. Immediately after transplantation, the recipient fish were then reared at dark condition in 0.4x phosphate buffered saline (PBS) containing 10 µg/ml gentamicin (Life Technologies) without sewing of the wound for four days to facilitate wound healing. The fish were then reared normally and after an appropriate period were anesthetized to enable removal of the graft. The removed graft was fixed with 4% paraformaldehyde (PFA) in PBS or Bouin’s fixative overnight at room temperature and
sectioned in paraffin at 5-10 µm thick for histological or immunohistochemical analysis.

The functional transplantation assay of SSCs was performed with aggregates of dissociated testicular cells previously described (Kawasaki et al., 2012), using testes of the sterile moto mutant. The moto mutant testis lacks late stage germ cells due to a germ cell-autonomous defect and therefore does not contribute to spermatogenic germ cells in transplants (K. R. S. et al., unpublished). Cultured SSCs derived from a sox17:egfp testicular hyperplasia were co-aggregated with testicular cells from moto mutant testis. These aggregates were transplanted to rag1 mutant as described above to allow spermatogenesis to occur in situ. Aggregates were removed from the host after 1 month and examined for the presence of sperm and EGFP-positive spermatogonia.

**BrdU staining**

The grafted fragment of a testis was removed from recipients after 1 month, and treated with 0.1% BrdU labeling reagent (GE Healthcare) in 80% L-15 for three hours. Grafts were then fixed with Bouin’s fixative and section and processed in paraffin at 5 µm. Incorporated BrdU was detected immunohistochemically using a Cell Proliferation Kit (GE Healthcare) in accordance with the manufacturer’s instructions.

**Cell culture**

To maintain and propagate SSCs in culture (propagation culture), testicular hyperplasias were dissociated in 500 U/ml of collagenase (Sigma) in Leibovitz’s L-15 for three hours as described previously (Sakai, 2002). Dissociated testicular cells containing germ cell populations were then
collected and suspended in culture medium described previously (Kawasaki et al., 2012) or modified as following; basal medium was changed from L-15 to DMEM, and 25-50 U/ml of heparin (Sigma) was supplied. Dorsomorphin dehydrochloride (Wako) at 2 µM was also added for long-term culture of SSCs (Wong and Collodi, 2013). The cells were then plated on gelatin-coated dishes and incubated at 28˚C in air (for L-15), or 5% CO_2 and 10% or 20% O_2 (for DMEM). The cells were replated using Accutase (Life Technologies) before the somatic cells reached confluence.

For differentiation of SSCs (differentiation culture), cells that underwent propagation culture were removed by using Accutase, and then transferred on mitomycin-treated ZtA6-12 cells and cultured as described (Sakai, 2002; Kurita and Sakai, 2004) with a change of the basal medium from L-15 (in air) to DMEM (in 5% CO_2 and 10% or 20% O_2). The medium was changed every 4 days.

**Immunostaining**

To detect germ cells derived from the cultured *sox17::egfp* spermatogonia in the testicular cell aggregates, EGFP immunohistochemistry was performed as described previously (Kawasaki et al., 2004). Briefly, paraffin sections (5 µm) of the testicular cell aggregates were immersed in citrate buffer (10 mM tri-sodium citrate; pH 6.0) and autoclaved (120˚C, 5 minutes) to enable antigen retrieval. The sections were then incubated with 5 µg/ml solution of a GFP mouse monoclonal antibody (Clontech Laboratories) for 1 h. After washing, the sections were further incubated with 2 µg/ml alkaline phosphatase-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) for 1 h. Immunoreactivity was detected using NBT/BCIP (Roche).
For the immunocytochemical analysis of Symp3, the samples were fixed with 4% paraformaldehyde for 10 min. After pre-incubation with a blocking buffer (10% fetal bovine serum and 0.5% Triton X-100 in phosphate buffered saline), the samples were incubated with an anti-zebrafish Symp3 rabbit polyclonal antibody (Ozaki et al., 2011), at a 1:300 dilution in blocking buffer for 1 h. After washing, the primary antibody was detected with anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Life Technologies). Samples were counterstained with DAPI (Life Technologies).

**Artificial insemination**

A single grafted testis was minced and ground with a microtube pestle in Hank’s saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4, 4.2 mM NaHCO3). Cultured sperm were collected by centrifugation of culture medium at 190 x g for 10 minutes. The majority of the supernatant was discarded. The pellet was then resuspended in the remaining medium (<100 μl), and stored on ice. Unfertilized eggs were collected onto a dish from a single wild-type female by pressing gently on the belly after anesthetization, according to Westerfield (1995). The sperm suspension was added and the dish was shaken gently for 2 minutes to mix well. PBS (100 μl) was added gradually with shaking. After an additional 2 minutes, 5 ml of fish water was gradually added. Fertilization success was assessed at 3-5 hour past fertilization. The fertility depends on the concentration of functional sperm in the suspension (Hagedorn and Carter, 2011).
Statistical analysis

Data are analyzed using paired t-test. A minimum of four replicates was performed for each experiment.

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Competing interests

The authors declare no competing or financial interests.

Author Contributions

T. K. and N. S. conceived and designed the work. T. K. performed all transplantation experiments, histological analysis and immunostaining. T. K. and N. S. performed SSC culture experiments, artificial insemination and data analyses. K. R. S. isolated the \textit{moto} mutant. All authors wrote the manuscript.

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Fig. 1. Maintenance and growth of testicular hyperplasias following subcutaneous transplantation. (A, B) Histological observation of testicular hyperplasia (A) and normal testis (B). Arrows indicate a single spermatogonium surrounded by Sertoli cells. DG: differentiating spermatogonia in large clusters surrounded by Sertoli cells. PC: primary spermatocytes, SP: sperm. Note that the hyperplasia contains a large number of single spermatogonia. Scale bar, 50
μm. (C-G) Views of the rag1^{26683} mutant just after transplantation beneath the abdominal skin (C), and after 3 months (D). A testicular hyperplasia (E) was cut down to transplantable size (F). After three months of transplantation, the grafted fragment was removed (G). Note that the grafted region (arrows) swelled out (C, D), and that the transplanted fragment grew to almost original size (E, G). Scale bar, 1 cm (C, D), and 3.3 mm (E-G).
Fig. 2. Serial transplantation of the testicular hyperplasia A (A series). (A) Each box shows the number of the grafted fragments, the number of recipients survived for more than 1 month, and the number of the grown graft in each transplantation steps. The number in parenthesis indicates day/month/year of the transplantation. (B) Histological observation of a grafted hyperplasia. Sections of each testis correspond to the grafts shown in the panel A. Many single
spermatogonia (arrows) were observed in grafts, and sperm (arrowhead) was present. Testis-ova (the presence of oocytes in testis) were observed in A3-8 (fish #8 of the third serial transplantation of hyperplasia A; double arrowheads). Scale bar, 50 μm. Serial transplantation of other testicular hyperplasia lines are shown in Figure S2.
Fig. 3. Propagation of SSCs in culture. (A, B) Effects of basal medium (A) and heparin at 25-50 U/ml (B) on proliferation of early spermatogonia. Grafts of the sox17::egfp testicular hyperplasias recovered from rag1<sup>126683</sup> mutant host were dissociated and cultured under various conditions. The gas condition was adjusted to the basal culture medium; in air for L-15 and in 5% CO<sub>2</sub> for DMEM. The culture medium in B was DMEM with 5% CO<sub>2</sub> and 20% O<sub>2</sub>. Bars indicate the average (n=4). (C) Dissociated sox17::egfp testicular hyperplasias were cultured for 1, 31, and 48 days under the SSC propagation condition. Inset shows the morphology of the early stage of spermatogonia. Upper panels: phase contrast; bottom panels: fluorescent. Arrows indicate GFP-negative differentiated spermatogonia. Scale bars, 50 µm.
Fig. 4. Differentiation of SSCs to sperm in culture. (A) SSCs that underwent propagation culture for 31 days were transferred onto Sertoli ZtA6-12 feeders and cultured under the differentiation condition. After 1 day of transfer, SSCs attached tightly on Sertoli feeders. Spermatocytes (inset) and sperm (inset) appeared around 10 and 17 days after the transfer, respectively. Upper panels: phase contrast; bottom panels: fluorescent. (B) Sycep3 expression in
cells under differentiation culture. SSCs that underwent propagation culture for 31 days were transferred onto ZtA6-12 feeders and cultured for 9 days. Sycep3 signals are observed as a small particle in one side, and extended in the nucleus (inset), suggesting normal meiotic initiation.

(C) Sycep3 immunostaining of SSC clumps cultured under the SSC propagation condition for 40 days. Cells expressing Sycep3 was observed only rarely, at the periphery of SSC clumps (arrows). Scale bars, 50 µm.
### Tables

**Table 1. Production of functional sperm by two successive cultures of SSCs.**

| Exp. # | propagation culture§ | differentiation culture† | oocytes used | fertilized | growth to adult |
|--------|-----------------------|--------------------------|--------------|------------|-----------------|
|        | culture length | culture length            |              |            |                 |
| Exp. 1 | 31 days          | 24 days                  | 209          | 1          | 1               |
|        | 31 days          | 397                      | 1            | 1          |                 |
| Exp. 2 | 31 days          | 24 days                  | 373          | 1          | 1               |
|        | 31 days          | 309                      | 0            | -          |                 |
|        | 43 days          | 199                      | 1            | 1          |                 |

§ Cells from testis hyperplasia amplified by serial transplantation in *rag*<sup>l<sup>26683</sup> mutant host were cultured in the optimum condition for maintenance and propagation of SSCs: DMEM with 50 U/ml heparin and 2 μM dorsomorphin in 5% CO<sub>2</sub>, 20% O<sub>2</sub>.

† To promote spermatogonial differentiation, SSCs that underwent propagation culture were transferred onto ZtA6-12 feeders and cultured for the period indicated in 5% CO<sub>2</sub>, 20% O<sub>2</sub>. The production of functional sperm was then determined by artificial insemination.
Table 2. Effect of \( \text{O}_2 \) concentration on the efficiency of sperm production in culture

| Exp. # | Propagation culture§ | Differentiation culture† | Oocytes used | Fertilized | Growth to adult |
|--------|----------------------|--------------------------|--------------|------------|----------------|
|        | Culture length | \( \text{O}_2 \) conc. | Culture length | \( \text{O}_2 \) conc. |              |               |
| Exp. 1 | 34 days          | 10%                      | 21-36 days*   | 10%        | 1167          | 7             | 6             |
|        |                  |                          |              | 20%        | 1217          | 1             | 1             |
| Exp. 2 | 31 days          | 10%                      | 20-35 days*   | 10%        | 1174          | 12            | 10            |
|        |                  |                          |              | 20%        | 934           | 0             | 0             |
| Exp. 3 | 31 days          | 20%                      | 20-36 days*   | 10%        | 1342          | 43            | 39            |
|        |                  |                          |              | 20%        | 1396          | 11            | 10            |
| Exp. 4 | 31 days          | 20%                      | 21-36 days*   | 10%        | 885           | 9             | 7             |
|        |                  |                          |              | 20%        | 811           | 0             | 0             |

§ Cells from testis hyperplasia amplified by serial transplantation in \( \text{rag}^{L26683} \) mutant host were cultured in conditions that support maintenance and propagation of SSCs in DMEM with 50 U/ml heparin and 2 \( \mu \)M dorsomorphin. Oxygen concentrations were set as indicated.

† To promote spermatogonial differentiation SSCs that underwent propagation culture were transferred to two dishes with ZtA6-12 feeders and cultured under two different oxygen concentrations. The efficiency of production of functional sperm was then determined by artificial insemination.

*Fertilization experiments were performed every 7-8 days. The total sum of three fertilization experiments is indicated.
Table 3. Production of functional sperm in culture from SSCs under low oxygen.

| Exp. # | Propagation culture§ | Differentiation culture† | Oocytes Used | Fertilized | Growth to Adult |
|--------|----------------------|--------------------------|--------------|------------|----------------|
| Exp. 1 | 31 days              | 24 days                  | 265          | 14         | 13             |
|        |                      | 31 days                  | 421          | 1          | 1              |
|        |                      | 43 days                  | 212          | 21         | 20             |
|        |                      | 51 days                  | 399          | 32         | 27             |
|        |                      | 59 days                  | 430          | 3          | 3              |
| Exp. 2 | 31 days              | 19 days                  | 282          | 1          | 1              |
|        |                      | 27 days                  | 288          | 23         | 22             |
|        |                      | 34 days                  | 498          | 32         | 29             |
| Exp. 3 | 31 days              | 19 days                  | 495          | 5          | 4              |
|        |                      | 27 days                  | 486          | 45         | 40             |
|        |                      | 34 days                  | 511          | 18         | 16             |

§ Testis hyperplasia amplified by serial transplantation in rag1t26683 mutant host were dissociated and cultured in DMEM with 50 U/ml heparin and 2 μM dorsomorphin in 5% CO₂, 20% O₂.

† SSCs from the propagation culture were cultured on ZtA6-12 feeders in 5% CO₂, 10% O₂ and processed for the sperm functional assay after the culture period indicated.