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STEM CELLS AND REGENERATION

TECHNIQUES AND RESOURCES ARTICLE

Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture

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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 the 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 immunodeficient *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 into 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 the molecular mechanisms of spermatogenesis in vertebrates.

KEY WORDS: Allogeneic graft, Rag1 mutant zebrafish, Spermatogenesis, Cell culture, Oxygen

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. Investigation of 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). Although 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, 2008; Hamra et al., 2005) or testicular organ culture (Sato et al., 2013); cell culture approaches have 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 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 overexpression of the 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, and has been obtained only by nuclear injection using haploid spermatids that are differentiated from primary spermatocytes in culture (Marh et al., 2003).

By 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 a normal differentiation process, including differentiation to spermatogonia and subsequent meiosis.

Despite the potential usefulness of testicular hyperplasias as a source of SSCs, such hyperplasias are found only sporadically in laboratory strains of zebrafish and cannot serve as a stable source of SSCs. Thus, we first established a system for long-term maintenance of hyperplasias by serial transplantation using immunodeficient 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 SSCs and differentiation to functional sperm that can give rise to the next generation. In addition, we examined effects of oxygen as an

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important condition for spermatogenesis by using the cell culture system established.

RESULTS

Transplantation of a testicular hyperplasia into *rag1* mutant zebrafish

Before we examined a system for long-term maintenance of testicular hyperplasias, we examined whether SSCs of the hyperplasia could differentiate into sperm. Dissociated hyperplasia cells from fish harboring a *vas::egfp* transgene (Krøvel and Olsen, 2002) were cultured for 4 weeks as previously described (Kawasaki 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 *vas::egfp* testis (Fig. S1), suggesting that hyperplasia-derived SSCs self-renewed and differentiated in the same manner 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 generation of mature B and T cells requires the activities of the RAG (recombination-activating gene) proteins, one way to alleviate rejection is to use mutations in the *rag* genes (Belizario, 2009; Tang et al., 2014; Tenente et al., 2014). As the host of transplantation we chose *rag1*^{t26683} mutant zebrafish (hereafter *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 whether the *rag1* mutant endures surgery and accepts allografts, we grafted wild-type testis fragments into wild-type and *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 the abdominal skin of *rag1* mutants, the graft was found in 8 out of 10 transplants (Table S1). Spermatogenic cells of grafts recovered from *rag1* mutant hosts incorporated BrdU (Fig. S2), indicating that spermatogenesis of the grafted testis proceeded normally. These results indicate that *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 and B) from the wild-type strain and two (C and D) from transgenic fish carrying a *sox17 promoter::egfp* reporter gene (Mizoguchi et al., 2008). By histology, we confirmed that the testis contained cells at all stages of spermatogenesis and found a large number of single spermatogonia surrounded by Sertoli cells, which included SSCs (Fig. 1A,B). Testicular hyperplasias were cut down to a small, transplantable size including the outer layer and the fragments were transplanted under the abdominal skin of *rag1* mutant and wild-type fish (Fig. 1C,E,F). Whereas fragments grafted into the wild-type host disappeared completely 4 weeks after transplantation (Table S2), fragments grafted into *rag1* mutants grew remarkably, achieving a >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

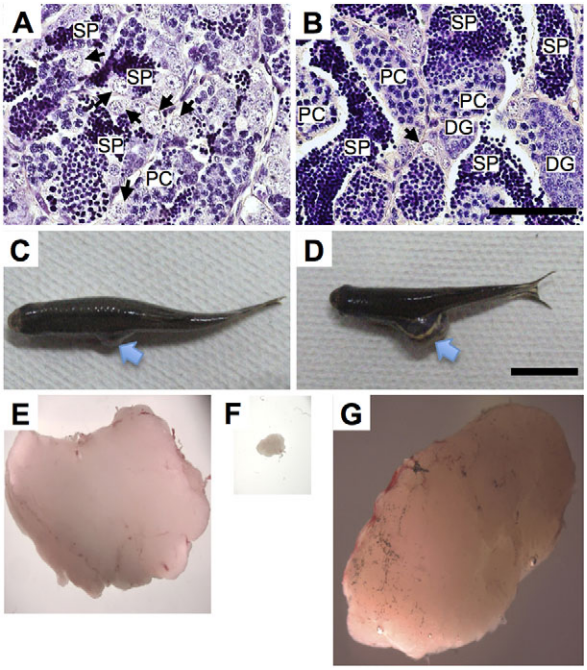

Fig. 1. Maintenance and growth of testicular hyperplasias following subcutaneous transplantation. (A,B) Histological observation of a testicular hyperplasia (A) and normal testis (B). Arrows indicate a single spermatogonium surrounded by Sertoli cells. DG, differentiating spermatogonia in large clusters 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*^{t26683} 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 3 months of transplantation, the grafted fragment was removed (G). Note that the grafted region swelled (arrows in C,D) and the transplanted fragment grew to almost original size (E,G). Scale bar: 1 cm in C,D and 3.3 mm in E-G.

(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 whether testicular hyperplasias could be amplified by serial transplantation. The four transplanted hyperplasia (A-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; 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 the occurrence of malignant transformation (B4-1 in 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 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

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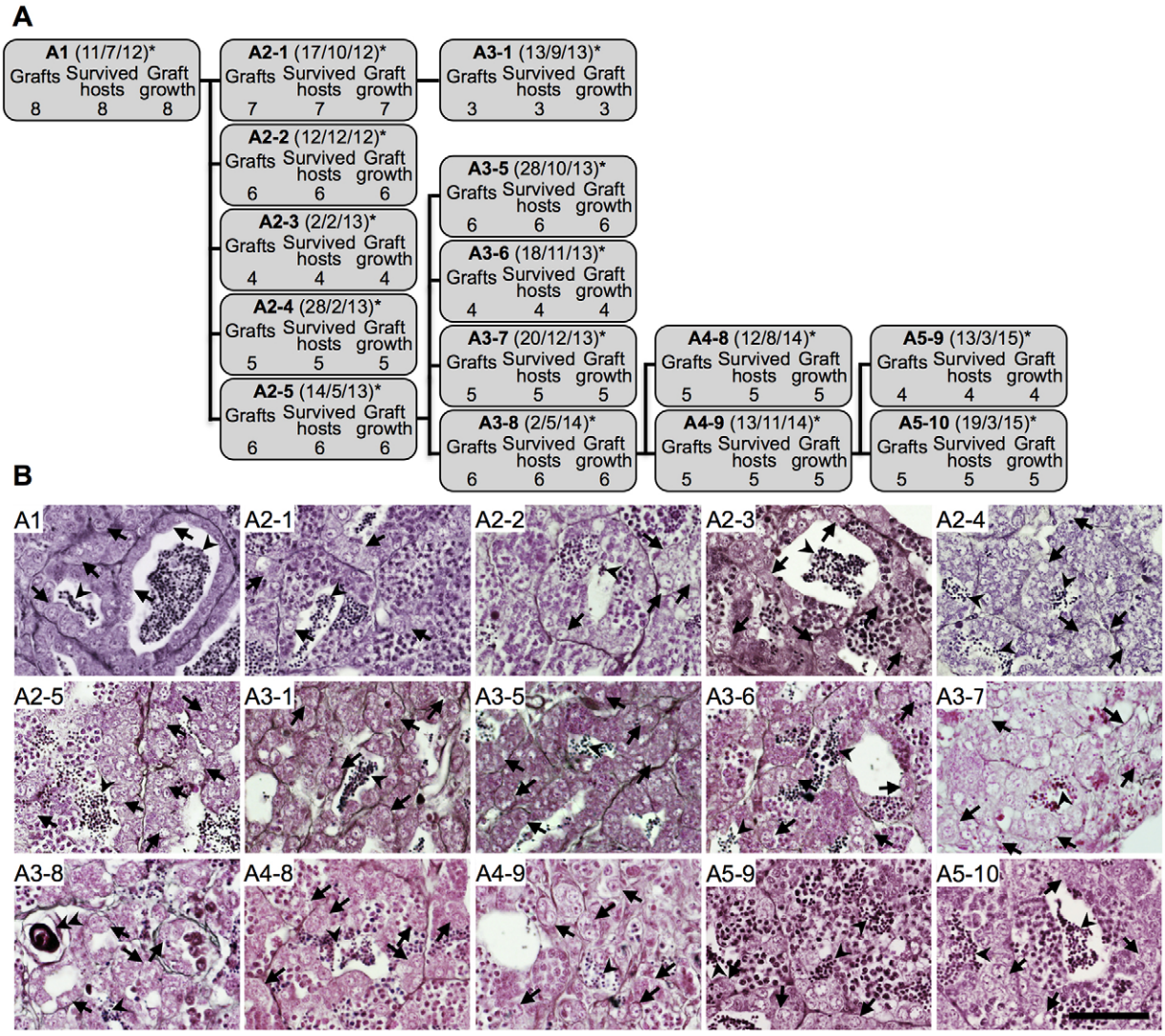

Fig. 2. Serial transplantation of the testicular hyperplasia A. (A) Each box shows the number of the grafted fragments, the number of recipients surviving for more than 1 month, and the number of the grown graft in each transplantation steps. The number in parentheses indicates day/month/year of the transplantation. (B) Histological observation of a grafted hyperplasia. Sections of each testis correspond to the grafts described in A. Many single spermatogonia (arrows) were observed in grafts and sperm (arrowhead) were present. Testis-ova (the presence of oocytes in testis) were observed in A3-8 (fish no. 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 Fig. S2.

an unlimited resource of testicular hyperplasia material from which relatively large quantities of SSCs can be derived.

Propagation of SSCs in culture

As testicular hyperplasias contain cells at 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 the previously described culture conditions for SSCs (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% carbon dioxide increased the number of GFP-positive spermatogonia twofold after 21 days of culture (Fig. 3A). We did not observe any significant differences in the number of spermatogonia between different oxygen concentrations of 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 GFRα1-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 a more than twofold improvement in the yield of GFP-positive spermatogonia after 21 days of culture (Fig. 3B).

Combining these modifications with addition of the BMP inhibitor dorsomorphin, which is known to promote proliferation of zebrafish spermatogonia (Wong and Collodi, 2013), GFP-

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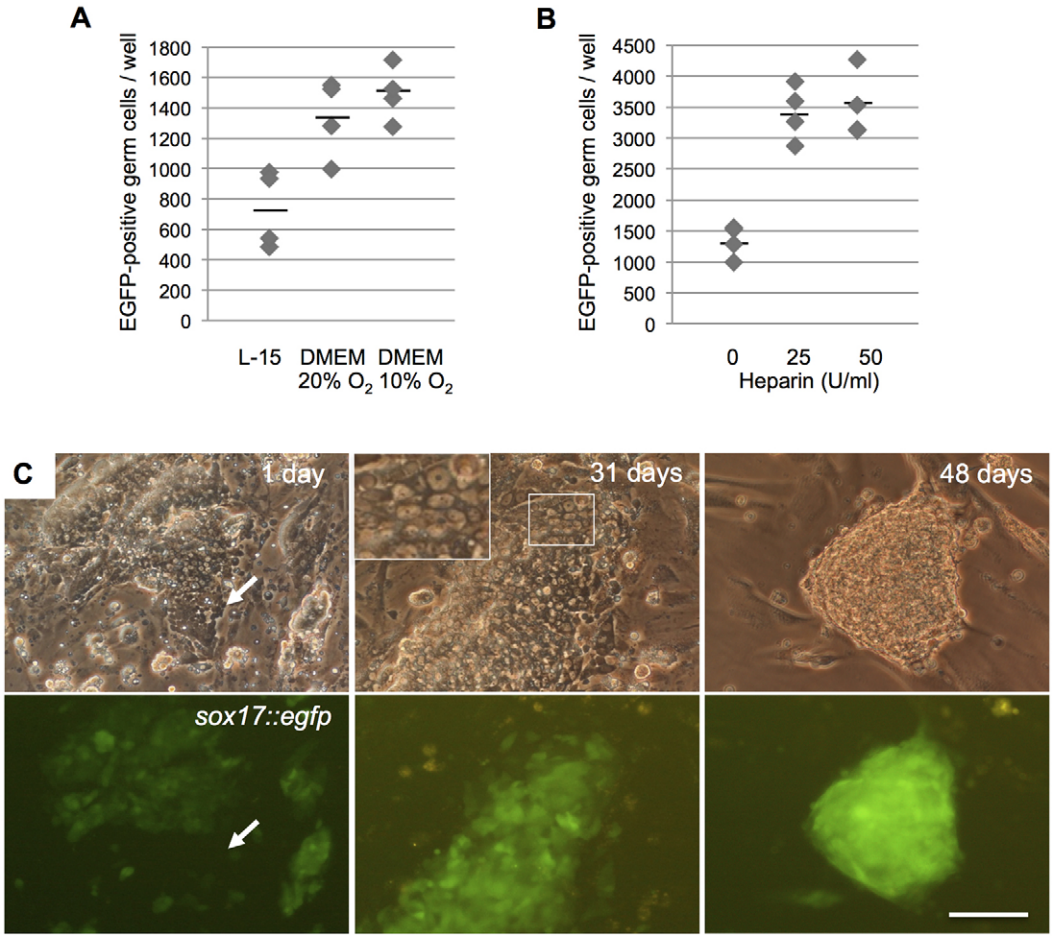

Fig. 3. Propagation of SSCs in culture. (A,B) Effects of basal medium (A) and heparin at 25-50 U/ml (B) on proliferation of early spermatogonia. Grafts of the *sox17::egfp* testicular hyperplasias recovered from *rag1*^{t26683} mutant host were dissociated and cultured under various conditions. The gas condition was adjusted to the basal culture medium; in air for L-15 and in 5% CO_2 for DMEM. The culture medium in B was DMEM with 5% CO_2 and 20% O_2. Bars indicate the mean (n=4). (C) Dissociated *sox17::egfp* testicular hyperplasias were cultured for 1, 31 and 48 days under SSC propagation conditions. Inset shows the morphology of the early stage of spermatogonia. Top panels: phase contrast; bottom panels: fluorescence. Arrows indicate GFP-negative differentiated spermatogonia. Scale bar: 50 µm.

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 early stage 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 our culture conditions efficiently maintain and propagate SSCs in culture.

Stemness of cultured GFP-positive spermatogonia was confirmed *in vivo* by functional transplantation assays 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 because of a germ cell-autonomous defect (T.K., C. Ramos, E. Taylor, T. Nishimura, M. Shinya, C. Sakai, K. Saito, M. Tanaka, N.S. and K.R.S., unpublished results). 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 (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 could 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% carbon dioxide. 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 (Fig. 4B, compare with SSC clumps under SSCs propagating condition shown in Fig. 4C), as observed in intact zebrafish testis (Ozaki et al., 2011; Saito et al., 2011). 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

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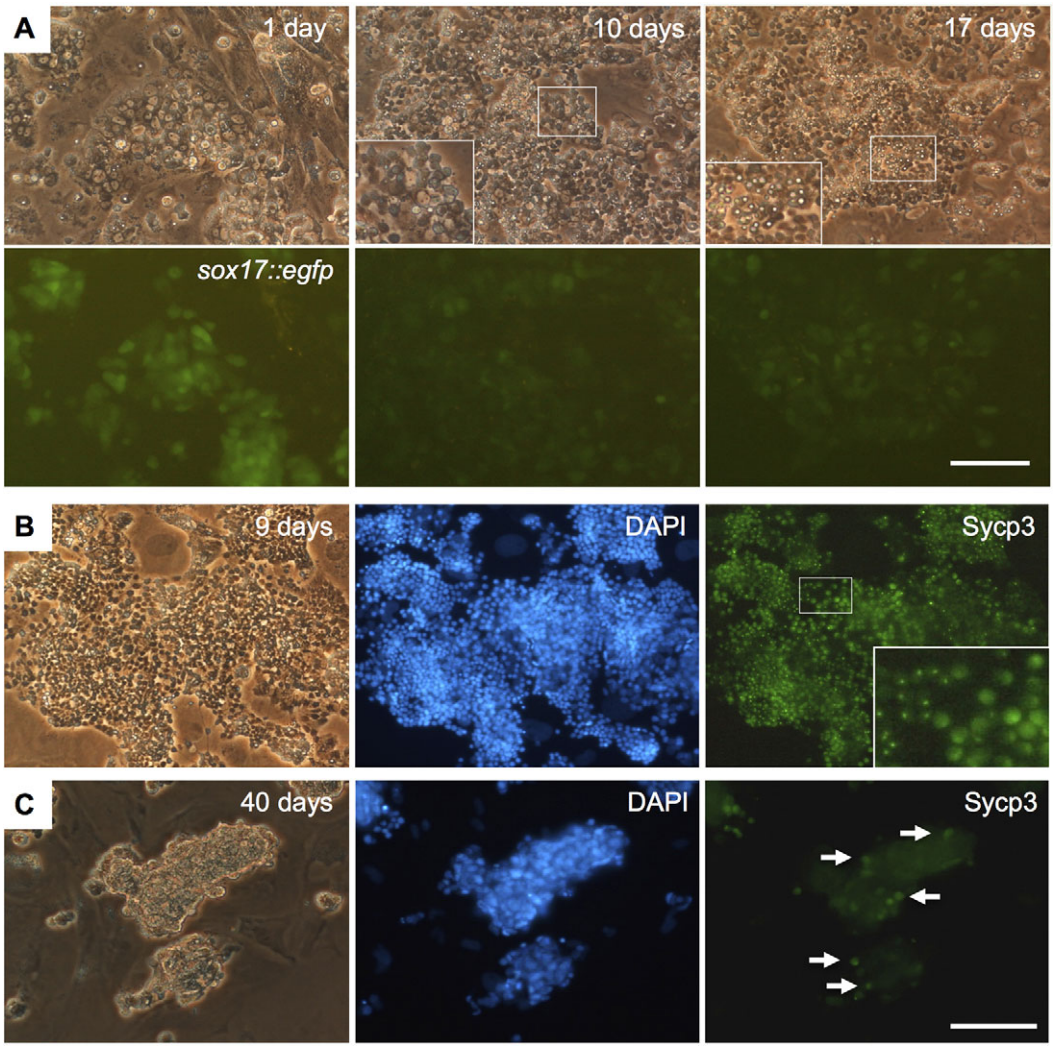

Fig. 4. Differentiation of SSCs to sperm in culture. (A) SSCs that underwent propagation culture for 31 days were transferred onto Sertoli ZtA6-12 feeders and cultured under the differentiation condition. After 1 day of transfer, SSCs attached tightly on Sertoli feeders. Spermatocytes (inset) and sperm (inset) appeared around 10 and 17 days after the transfer, respectively. Top panels: phase contrast; bottom panels: fluorescence. (B) Sycp3 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. Sycp3 signal is observed as a small particle on one side of the nucleus and extends into the nucleus (inset), suggesting normal meiotic initiation. (C) Sycp3 immunostaining of SSC clumps cultured under SSC propagation conditions for 40 days. Cells expressing Sycp3 were observed only rarely, at the periphery of SSC clumps (arrows). Scale bars: 50 µm.

expressed GFP (Mizoguchi et al., 2008) and grew up into normal fertile adult fish without testicular hyperplasia formation (Table 1; 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 the 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

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

Experiment	Propagation culture time*	Differentiation culture time‡	No. of oocytes used	No. fertilized	No. growing to adult
1	31 days	24 days	209	1	1
		31 days	397	1	1
2	31 days	24 days	373	1	1
		31 days	309	0	0
		43 days	199	1	1

*Cells from testis hyperplasia amplified by serial transplantation in *rag1*^{t26683} mutant host were cultured in the optimum conditions for maintenance and propagation of SSCs: DMEM with 50 U/ml heparin and 2 µM dorsomorphin in 5% CO_2, 20% O_2.
‡To promote spermatogonial differentiation, SSCs that underwent propagation culture were transferred onto ZtA6-12 feeders and cultured for the period indicated in 5% CO_2, 20% O_2. The production of functional sperm was then determined by artificial insemination.

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preferentially support either SSC propagation or differentiation of testicular hyperplasia.

As 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 a more than 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 (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 (Fig. S6C,D). These results indicate that the improved culture method is effective for SSCs of normal testis, but some of the SSCs were induced to differentiate even in the propagation culture. This culture contains somatic cells that are from the initial dissociated testis and it is therefore likely that somatic cells in the culture support some spermatogonia differentiation.

Effect of oxygen on the differentiation of SSCs to sperm

Recent evidence suggests that oxygen has two distinct effects on spermatogenesis; whereas 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% oxygen conditions, and the effect on spermatogenesis was assayed by measuring the efficiency of sperm production through artificial insemination. Whereas cultures at 20% oxygen produced functional sperm only rarely, cultures at 10% oxygen resulted in a more than fourfold increase in the number of fertilized eggs that grew into adult fish (Table 2); under low oxygen concentration, fertilized embryos were stably obtained from cultured sperm (Table 3). However, 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 same concentration as in air causes some damage in the differentiation steps from SSCs to sperm.

DISCUSSION

The present study highlights significant technical advances in two fronts: the use of immunodeficient *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 cannot be maintained as long as those of mammalian SSCs in culture (Kanatsu-Shinohara et al., 2003, 2008; Hamra et al., 2005), relatively large quantities of SSCs can be derived from an unlimited resource of testicular hyperplasia material, propagated through the transplantation technique. The utilization of hyperplasia makes it possible to select the desired cell types under the culture conditions optimized for SSC propagation.

In the present study, we used SSCs of testicular hyperplasia 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 hyperplasias 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 cell function, and somatic cells rather than SSCs are likely the cause of the hyperplasias. In support of this notion, loss of anti-mullerian hormone receptor in the somatic cells of testis causes testicular hyperplasias in the medaka fish (Morinaga et al., 2007).

When adult SSCs from normal testes were cultured under the conditions for SSC 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 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 the 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. As the hyperplasias accumulate SSCs, this amplification protocol would facilitate analysis of 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 the reduced viability of adult fish and a failure to thrive following injury (Tang et al., 2014). However, the present study shows that the mutant can thrive by

Table 2. Effect of oxygen concentration on the efficiency of sperm production in culture

	Propagation culture*		Differentiation culture‡				
Experiment	Time	O_2 concentration	Time	O_2 concentration	No. of oocytes used	No. fertilized§	No. growing to adult
1	34 days	10%	21-36 days	10%	1167	7	6
				20%	1217	1	1
2	31 days	10%	20-35 days	10%	1174	12	10
				20%	934	0	0
3	31 days	20%	20-36 days	10%	1342	43	39
				20%	1396	11	10
4	31 days	20%	21-36 days	10%	885	9	7
				20%	811	0	0

*Cells from testis hyperplasia amplified by serial transplantation in *rag1*^{t26683} mutant host were cultured in conditions that support maintenance and propagation of SSCs in DMEM with 50 U/ml heparin and 2 µM 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 of three fertilization experiments is indicated.

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Table 3. Production of functional sperm in culture from SSCs under low oxygen

Experiment	Propagation culture time*	Differentiation culture time ‡	No. of oocytes used	No. fertilized	No. growing to adult
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
2	31 days	19 days	282	1	1
		27 days	288	23	22
		34 days	498	32	29
3	31 days	19 days	495	5	4
		27 days	486	45	40
		34 days	511	18	16

*Testis hyperplasias amplified by serial transplantation in *rag1*^{t26683} mutant host were dissociated and cultured in DMEM with 50 U/ml heparin and 2 µM dorsomorphin in 5% CO_2, 20% O_2.
‡SSCs from the propagation culture were cultured on ZtA6-12 feeders in 5% CO_2, 10% O_2 and processed for the sperm functional assay after the culture period indicated.

treating fish to prevent infection after surgery, allowing us to transplant fragments of organs and hyperplasias, which regenerate in the host. Although this study concentrated on spermatogenesis in the testis, rag mutants are likely to accept other allogeneic tissues and hyperplasias because the immunocompromised *rag2*^{E450fs} mutant accepts various cell types (Tang et al., 2014; Tenente et al., 2014). The 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 our cell culture system is the ease in manipulating the conditions that might affect various developmental processes. One important condition for spermatogenesis that we examined was the effect of oxygen concentration. In contrast to mouse SSCs, which show more efficient propagation of SSCs in an atmosphere of 10% oxygen compared with 21% oxygen (Kubota et al., 2009), we could not observe differences between at 20% and 10% oxygen on the propagation of zebrafish SSCs. However, a larger number of fertilized sperm were produced from SSCs in the present conditions at 10% oxygen compared with 20%. It has been suggested that germ cells at advanced stages such as spermatocytes, 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 levels within germ cells, and might unveil new effects of oxygen in addition to DNA damage and reducing sperm motility (Agarwal et al., 2008). Similarly, chemicals such as endocrine disruptors that disrupt germ cell development 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.

Our cell culture system can facilitate the study of many subcelluar processes inherent to germ cells. For example, it is well known that germ cells have special RNA-rich cytoplasmic bodies, so-called germ granules, which developmentally regulate 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 conditions 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 to be 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*^{t26683}; Wienholds et al., 2002; Petrie-Hanson et al., 2009) were provided by Dr Y. Kikuchi (Hiroshima University, Japan) and Dr L. Petrie-Hanson (Mississippi State University, MS, USA), respectively. *moto* mutant fish were isolated by *N*-ethyl-*N*-nitrosourea mutagenesis screening (Saito et al., 2011). For wild-type fish, we used India lines (provided by Dr W. Driever, University of Freiburg, Germany) or AB lines (provided by Dr U. Strähle, Karlsruher Institute of Technology, Germany). Zebrafish with testicular hyperplasias A and B were provided by Dr H. Hirata (National Institute of Genetics, Japan). The use of these animals for experimental purposes was 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, 1 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 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 in the dark in 0.4× phosphate buffered saline (PBS) containing 10 µg/ml gentamicin (Life Technologies) without sewing of the wound for 4 days to facilitate wound healing. The fish were then reared under normal conditions without PBS and antibiotics, 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 for histological or immunohistochemical analysis.

The functional transplantation assay of SSCs was performed with aggregates of dissociated testicular cells as previously described (Kawasaki et al., 2012), using testes of the sterile *moto* mutant. The *moto* mutant testis lacks late-stage germ cells because of a germ cell-autonomous defect and therefore does not contribute to spermatogenic germ cells in transplants (K.R.S. et al., unpublished results). Cultured SSCs derived from a *sox17::egfp* testicular hyperplasia were co-aggregated with testicular cells from

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moto mutant testis. These aggregates were transplanted to *rag1* mutants 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 3 h. Grafts were then fixed with Bouin's fixative, embedded in paraffin and sectioned at 5 µm using standard protocols. 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 collagenase (Sigma) in Leibovitz's L-15 for 3 h, 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 follows: basal medium was changed from L-15 to DMEM and 25-50 U/ml heparin (Sigma) was added. 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% carbon dioxide and 10% or 20% oxygen (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 onto 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., 2006). 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 min) 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 Nitro Blue tetrazolium chloride in conjunction with 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche).

For immunocytochemical analysis of Sycp3, 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 a rabbit anti-zebrafish Sycp3 polyclonal antibody (Ozaki et al., 2011), at 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 Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Cultured sperm were collected by centrifugation of culture medium at 190 ***g*** for 10 min. 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 in 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 min to mix well. PBS (100 µl) was added gradually with shaking. After an additional 2 min, 5 ml fish water was gradually added. Fertilization success was assessed at 3-5 h after fertilization. The fertility depends on the concentration of functional sperm in the suspension (Hagedorn and Carter, 2011).

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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 *moto* mutant. All authors wrote the manuscript.

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Supplementary information

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