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In Vitro Murine Spermatogenesis in an Organ Culture System[1]

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ABSTRACT

Achieving mammalian spermatogenesis in vitro has a long history of research but remains elusive. The organ culture method has advantages over the cell culture method, because germ cells are in situ albeit the tissue as a whole is in vitro. The method was used in the 1960s and 1970s but encountered difficulties in inducing complete meiosis, i.e., in getting meiosis to proceed beyond the pachytene stage. In the present study, we reevaluated the organ culture method using two lines of transgenic mice, *Acr-GFP* and *Gsg2* (haspin)-*GFP* mice, whose germ cells express green fluorescent protein (GFP) at the mid and end stages of meiosis onward, respectively. Immature testicular tissues from these mice, ranging from 4.5 to 14.5 days postpartum, were cultured on the surface of the medium, providing a liquid-gas interface. Culturing testicular tissues of all ages tested resulted in the expression of both *Acr*- and *Gsg2-GFP*. Round spermatids were identified by a combination of *Gsg2-GFP* expression, cell size, and the presence of a single nucleus with a dot stained by Hoechst. In addition, the chromosome number of one of such presumptive spermatids was found to be 20 by the premature chromosome condensation method. As our semiquantitative assay system using GFP expression grading was useful for monitoring the effects of different environmental factors, including temperature, oxygen concentration, and antiretinoic molecules, further improvement of the culture conditions should be possible in the future.

haploid, in vitro, meiosis, organ culture, spermatid, spermatogenesis, testis

INTRODUCTION

Research on spermatogenesis in vitro has been ongoing for nearly a century [1, 2]. To the best of our knowledge, however, for mammals, it is not yet possible to make spermatogonia differentiate into haploid cells in vitro [3–5]. After a long nascent period, the research progressed rapidly in the 1960s, mostly owing to extensive studies performed by A. Steinberger and E. Steinberger using an organ culture system [6–8]. They succeeded in inducing gonocytes to differentiate up to the pachytene stage of meiosis using immature rat testes [6]. One of the important aspects of their culture method was the placement of fragments of testis tissue on the surface of the medium so as to allow the easy exchange of oxygen while also allowing them full access to the nutrients in the medium. Trowell [9], who developed this so-called gas-liquid interphase method, applied it to a variety of tissues including the testes, but it is not clear whether it successfully promoted spermatogenesis. In addition to the gas-liquid interphase method, the Steinbergers adopted a new culture medium developed by Eagle [10]. The combination of these innovations contributed to the in vitro differentiation of gonocytes/spermatogonia into meiosis, which was confirmed histologically [6]. However, the above researchers did not observe the completion of meiotic division or the production of spermatids in their experiments. Even with more developed testis fragments from 14-day-old rats, in which meiosis should have started, the progression of meiotic differentiation beyond the pachytene stage did not take place [8]. These results therefore unequivocally demonstrated a limitation of the organ culture method and presented a new challenge to the research community, i.e., to produce haploid spermatids in vitro. In fact, thereafter, several research groups tackled this subject and reportedly succeeded in producing haploid cells in vitro [11–14]. These studies abandoned the organ culture method and adopted the cell culture method instead. However, most of these studies used samples consisting of spermatocytes, and the rest included spermatocytes in their samples, as starting materials of the culture; i.e., meiosis had already commenced at the initiation of culturing. In addition, no protocol for in vitro meiosis has been repeated and verified by other independent research groups. Therefore, the in vitro replication of the whole meiotic process of mammals has not yet been achieved.

After the Steinbergers' study, the organ culture method for spermatogenesis has been used only very limitedly. It has mostly been used as a biological assay of soluble factors to assess their effects on cells of testis tissue, including germ cells and Sertoli cells [15–18]. Regarding the meiotic progression of germ cells in vitro, the organ culture system has not even been reevaluated, leaving the results of the Steinbergers' study untested and unimproved for nearly 40 yr. We thought therefore that it would be worthwhile to verify their results using modern equipment. We especially thought that it would be useful to use transgenic mice that express green fluorescent protein (GFP) specifically at meiosis during spermatogenesis. We also thought that it would

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be useful to use the new culture media now available for germline stem cells. Using such advanced technologies, we reevaluated the achievements of the Steinbergers.

MATERIALS AND METHODS

Animals

Two lines of transgenic mice, *Acr-GFP* (C57BL/JC3HF1), official symbol *Tg(Acr-EGFP)1Osb*, and *Gsg2-GFP* transgenic mice (C57BL6/JICRF1), were used. The *Acr-GFP* transgenic mice were produced by Okabe [19, 20], and the *Gsg2-GFP* transgenic mice were produced by Tanaka [21, 22]. Both transgenic mouse lines were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Female mice of the ICR (Japan SLC), C57BL/6 (Japan SLC), or ICRxC57BL/6F1 were mated with a sire male of the transgenic mice to produce pups. The pups were used for the culture experiments at 4.5 to 14.5 days postpartum (dpp). All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

Culture Media and Reagents

The culture media used were α-minimum essential medium (α-MEM; 12561–056; Invitrogen), Dulbecco modified Eagle medium (DMEM; 12100–46; Invitrogen), and StemPro-34 SFM (10639–011; Invitrogen). Each medium then had 10% (v/v) of fetal bovine serum (FBS; SH30396.03; Hyclone) added to it. StemPro-34 SFM was supplemented with StemPro supplement (Invitrogen) and 10% FBS. LE540, a retinoic acid receptor (RAR) pan-antagonist [23], dissolved in dimethyl sulfoxide to a concentration of 1 mM was used to block the action of retinoic acid (RA) by being added to the medium, giving a final concentration of 1 μM.

Culture Method

The testes of the pup mice were removed and, after being weighed, decapsulated. The testis tissues were gently separated by forceps into two to eight pieces of 2–3 mm in diameter. These testis tissue fragments were then placed on stands made of agarose gel placed in culture plate wells. To make the agarose gel stand, agarose-1 (Dojindo Molecular Technologies) was heated to dissolve it in distilled water (1.5% [w/v]) and then poured in to a 10-cm dish. After cooling down, the gels were cut into hexahedrons of about $10 \times 10 \times 5$ mm in size. They were then soaked in the culture medium for more than 24 h to replace the water in them with the medium. Three to four pieces of the agarose gels were placed in the wells of a six-well plate (MS-80060; Sumitomo Bakelite Co.). Each gel was loaded with one to three testis tissue fragments. The medium in each well came up to just below the upper surface of the agarose gel stand and was changed once a week. The culture incubator was supplied with 5% carbon dioxide in air and maintained at 34°C unless otherwise noted.

Observations

The cultured tissues were observed at least once a week under a stereomicroscope equipped with an excitation light for GFP (SZX12; Olympus) to identify GFP-positive cells in the seminiferous tubules. For Hoechst staining of nuclei, GFP-expressing tissues were treated with collagenase type 4 (2 mg/ml) and DNase (10 μg/ml) at 37°C for 5 min. After they were rinsed with PBS, dissociated cells were stained with Hoechst 33342 solution (Dojindo Molecular Technologies), which was diluted to give a final concentration of 25 μg/ml, for 20 min at 4°C. They were then rinsed again with PBS and observed under a microscope for GFP and nuclear Hoechst staining. For histological examination, the specimens were fixed with Bouin fixative and embedded in paraffin. Thin sections were then stained with hematoxylin and eosin. For periodic acid-Schiff (PAS)-hematoxylin staining and immunofluorescence staining, tissues fixed with 4% paraformaldehyde in PBS were cryo-embedded in OCT compound (Sakura) and cut into 7-μm-thick sections. *Gsg2-GFP* was detected using anti-GFP Alexa Fluor 488 conjugate (1:50; Invitrogen). Nuclei were counterstained with Hoechst 33342 dye.

Chromosome Analysis

Oocytes collected from the oviducts of mature B6D2F1 females that were induced to superovulate using 5 IU equine chorionic gonadotropin and 5 IU human chorionic gonadotropin were injected with the nuclei of haspin-GFP-positive round spermatids using a piezo-micromanipulator. The nuclei of round spermatids do not activate mouse oocytes, but rather they undergo premature chromosome condensation during the following incubation period; therefore, the chromosome number becomes countable. After 2–3 h of incubation, the oocytes were briefly rinsed in 1% hypotonic sodium citrate solution supplemented with 0.5% human serum albumin. After performing light fixation by adding a small volume of fixative (methanol:acetic acid = 3:1), each oocyte was placed individually on a glass slide before final fixation with the same fixative. After drying, the samples were stained with Hoechst and observed under a fluorescent microscope [24].

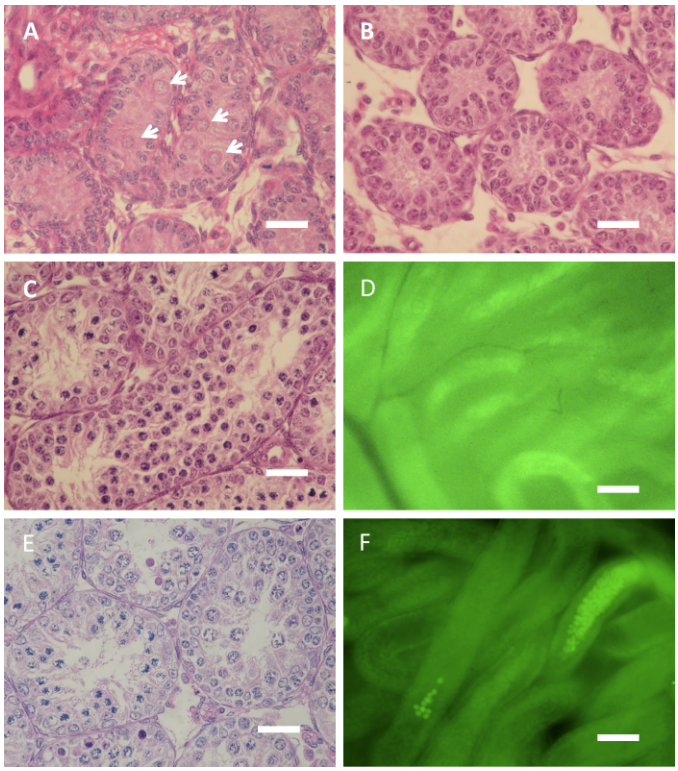

FIG. 1. Histological (**A**–**C**, **E**) and stereomicroscopic (**D**, **F**) views of two lines of transgenic mouse testes, *Acr-GFP* (**C**, **D**) and *Gsg2-GFP* (**A**, **B**, **E**, **F**), from the neonatal to the adolescent period are shown. **A**) At 3.5 dpp, germ cells observed were only gonocytes (arrows). **B**) At 7.5 dpp, the gonocytes had disappeared after migrating to the basement membrane to become spermatogonia. **C**) At 14.5 dpp, meiotic figures of spermatocytes were seen. **D**) At 14.5 dpp, *Acr-GFP* was first expressed. **E**) At 19.5 dpp, many spermatocytes had accumulated in the tubules. **F**) After 19.5 dpp, *Gsg2-GFP* expression began in several parts of the seminiferous tubules. Bar = 30 μm (**A**–**C**, **E**), 200 μm (**D**, **F**).

Statistical Analysis

The chi-square method was used for statistical analyses. Differences for which $P < 0.05$ were considered statistically significant.

RESULTS

In order to confirm the timing of the expression of *Acr-GFP* and *Gsg2-GFP* in the testes of pup mice, the testes of the transgenic mice were examined throughout the course of their development. At 3.5 dpp testes contained only gonocytes as germ cells in the center of their seminiferous tubules (Fig. 1A). At 7.5 dpp, the gonocytes had moved to the basement membrane of the tubule to become spermatogonia. Although it was when meiosis was about to commence, no apparent spermatocytes were recognized (Fig. 1B). At 14.5 dpp, the seminiferous tubules had grown in size, spermatocytes had accumulated in them, and meiotic progression was observed (Fig. 1C). *Acr-GFP* was first expressed around this day (Fig.

1D). As the expression of the *Acr-GFP* starts in the cytoplasm of midpachytene spermatocytes (stage IV), the expression timing at 14.5 dpp corresponds well with the assumption that preleptotene spermatocytes start meiosis at age around 7.5 dpp because it takes about 7 days from type B spermatogonia to midpachytene spermatocytes [25]. At 19.5 dpp, the germ cells increased in number, and each seminiferous tubule showed spermatogenic wave difference (Fig. 1E). This is when *Gsg2-GFP* began to be sporadically expressed throughout the testis (Fig. 1F). This 5-day interval between *Acr-* and *Gsg2-GFP* may not be enough to complete the rest of meiosis after midpachytene, which indicates that *Gsg2* expression is not haploid cell-specific but probably starts during meiotic cell divisions.

For organ culture, we adopted the gas-liquid interphase method originally developed by Trowell [9]. For stands to place the testis tissue on, we tested several materials, including paper filters, nitrocellulose membranes, agar gel, and agarose gel [26]. A preliminary study showed that the testis tissues showed the strongest acrosin- and haspin-GFP expression when they were placed on agar or agarose gel (data not shown). So, we adopted agarose gel of 1.5% (w/v) thereafter in all experiments. The amount of medium was adjusted so that its surface level was just below the upper surface of the agarose gel (Fig. 2A). When medium covered the agarose gel entirely, thereby submerging the tissues, the tissues were compacted and became necrotic and no *Acr-* or *Gsg2-GFP* was ever expressed (data not shown).

Every sample expressed no GFP at the initiation of the culture. Both *Acr-GFP* and *Gsg2-GFP* showed their expression in vitro mostly at the same time as they did in vivo. The 11.5 dpp *Gsg2-GFP* testis (Fig. 2B), for example, expressed GFP at 8–10 days in culture, which corresponds to 19.5–21.5 dpp, its original expression time in vivo, and the expression continued for about 15–20 days (Fig. 2, C and D). Generally, *Acr-GFP* was first expressed around the age corresponding to 14–18 dpp (Fig. 2, E and F), while *Gsg2-GFP* was first expressed around the age corresponding to 19–25 dpp.

Based on these findings on *Acr-GFP* and *Gsg2-GFP*, we tested the effects of several culture condition parameters. Among them, the temperature had quite an effect on testis organ culture. We compared 32, 34, and 37°C for the expression of *Gsg2-GFP*. As was reported in the literature, 37°C was detrimental to the tissue, and no GFP expression was observed. The temperature of 34°C was better than 32°C; the difference was significant when testes of younger mice, ~7.5–10.5 dpp, were used (Table 1).

We also used and evaluated three different culture media, α-MEM, DMEM, and StemPro-34 SFM. Beforehand, we tested the effects of adding FBS to these media and found that it had a profound effect. Without FBS, the tissue architecture was poorly maintained, and GFP expression was rarely observed (data not shown). Although chemically defined medium is ideal for analyzing the effects of ingredients of culture media, we added 10% of FBS to every medium tested thereafter.

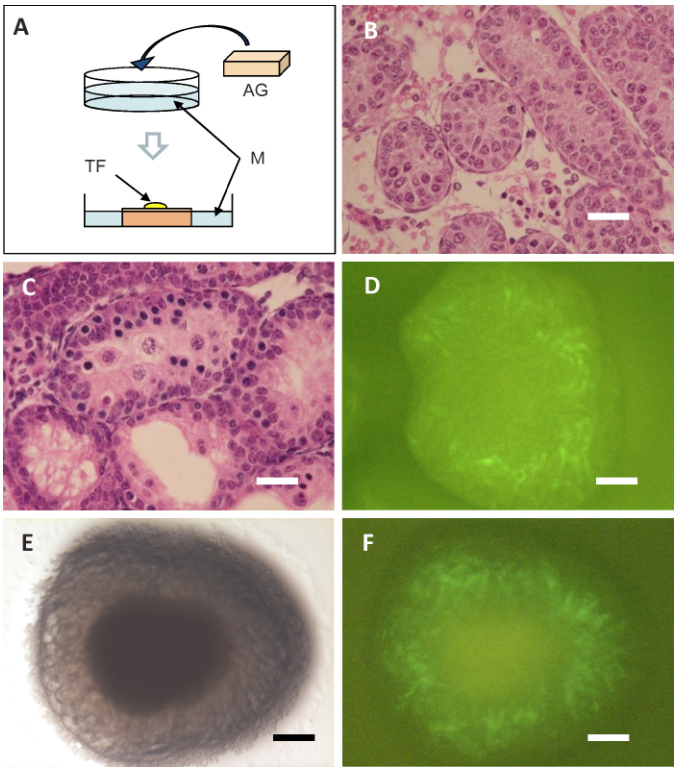

FIG. 2. Organ culture of testis tissues of *Gsg2-GFP* and *Acr-GFP* transgenic mice. **A**) Schematic presentation of the culture method is shown. AG, agarose gel; TF, testis tissue fragment; M, medium. **B**, **C**, **D**) *Gsg2-GFP* mouse testis tissue, 11.5 dpp, histology of starting material (**B**), sample taken on Day 19 (**C**), and stereomicroscopic view showing GFP emission on Day 19 (**D**). **E**, **F**) 8.5 dpp *Acr-GFP* testes tissue fragments cultured for 21 days. Bright field picture (**E**) and GFP emission picture (**F**). Bar = 30 μm (**B**, **C**), 500 μm (**D**–**F**).

Among the above three media, α-MEM showed the best results, although no statistically significant differences were recognized (Table 2).

In order to make this system more quantitative as an assay system, the extent of expression was graded according to the number of tubules expressing GFP in a piece of tissue. They were divided into four grades: grade 0 was no GFP expression, and grades 1, 2, and 3 represented 1–5, 6–15, and over 15 tubules showing GFP expression, respectively (Fig. 3A). Based on this grading, the effects of lower oxygen and anti-RA molecule were tested to confirm the validity of semiquantitative analysis in this organ culture system. For low-oxygen experiments, N_2 gas was infused into the incubator to lower the oxygen concentration to 10%. Under the lower-oxygen condition, *Gsg2-GFP* was expressed, but its grade was lower than that in the control, which was performed under regular air of 20% oxygen (Fig. 3B). This result confirmed that a lower oxygen concentration was disadvantageous for spermatogenesis. To see the effect of RA on spermatogenesis in this organ

TABLE 1. Haspin-GFP expression experiment, temperature comparison.

	32°C			34°C			37°C		
Age of mice (dpp)	No. of experiments	No. of experiments with GFP expression	Success rate (%)	No. of experiments	No. of experiments with GFP expression	Success rate (%)	No. of experiments	No. of experiments with GFP expression	Success rate (%)
7.5–10.5	22	9	40.9[a]	23	19	82.6[a,b]	3	0	0[b]
11.5–14.5	6	5	83.3	10	10	100.0	1	0	0

[a,b] Values with different superscripts are significantly different ($P < 0.05$).

TABLE 2. Haspin-GFP expression experiment, medium comparison.

	α-MEM			DMEM			StemPro-34 SFM[a]		
Age of mice (dpp)	No. of experiments	No. of experiments with GFP expression	Success rate (%)	No. of experiments	No. of experiments with GFP expression	Success rate (%)	No. of experiments	No. of experiments with GFP expression	Success rate (%)
5.5–6.5	4	2	50.0	3	0	0.0	4	0	0.0
7.5–8.5	20	9	45.0	15	3	20.0	14	5	35.7
9.5–10.5	5	4	80.0	6	5	83.3	ND	ND	ND
11.5–13.5	5	3	60.0	8	7	87.5	5	2	40.0

[a] ND, not determined.

culture system, an RAR antagonist, LE540, was added to the medium. Even though LE540 did not completely inhibit meiosis, *Acr-GFP* expression was greatly reduced (Fig. 3C). These results demonstrated that our culture system is able to measure the effects of different environmental conditions on spermatogenesis.

The results of 86 experiments using *Acr-GFP* and *Gsg2-GFP* mice testis tissues at 34°C in α-MEM supplemented with 10% FBS are summarized in Table 3. In the *Acr-GFP* mice testis tissues, GFP expression was observed in every case except one, regardless of the age of the mice used. Namely, the progression of spermatogenesis at least up to the midpachytene stage was demonstrated. Also with *Gsg2-GFP* mice testis, GFP was observed in most cases, but its success rate declined when younger mice were used. Nonetheless, even with mice as young as 4.5–5.5 dpp, the testis tissues showed *Gsg2-GFP* expression with a frequency of more than 85%. Among *Gsg2-GFP* expressing samples, the most evidently GFP-expressing 14 samples from independent experiments were examined histologically. One of them was serially sectioned all through the entire tissue. The remaining 13 were sectioned to show their maximum cut surface for observation. It was, however, difficult to morphologically confirm the presence of spermatids in them. The seminiferous tubules showed different extents of growth and contained meiotic cells (Fig. 2C), but the tubular lumen did not fully enlarge, and the tubular diameter did not compare with that in vivo. This finding suggested that meiotic cell division was disrupted by these special constraints. On the other hand, it seemed that thin-sectioned histological examination was not sufficiently sensitive to detect spermatids.

Then, we adopted another method to detect spermatids among the *Gsg2-GFP*-positive cells, Hoechst nuclear staining. Hoechst staining of the testis cells of *Gsg2-GFP* transgenic mice 23, 27, and 29 days old demonstrated that round spermatids can be distinguished by a combination of GFP expression, cell size (around 11 μm in diameter), and the presence of a single dot stain in the center of the nucleus on Hoechst staining (Fig. 4A). Histological examination of a fully matured *Gsg2-GFP* transgenic mouse testis by serial cryosections showed that round spermatids having acrosomal caps revealed by PAS staining on a section also expressed strong *Gsg2-GFP*, which was demonstrated on the adjacent section. Those round spermatids were recognized also with a single dot

TABLE 3. Summary of organ culture system, 34°C in α-MEM + 10% FBS.

Age of mice (dpp)	No. of experiments	No. of experiments with GFP expression	Success rate (%)
Acrosin-GFP expression experiment			
4.5–5.5	7	7	100.0
6.5–8.5	11	10	90.9
9.5–11.5	6	6	100.0
12.5–14.5	2	2	100.0
Haspin-GFP expression experiment			
4.5–5.5	15	13	86.7
6.5–8.5	24	21	87.5
9.5–11.5	13	12	92.3
12.5–14.5	2	2	100.0

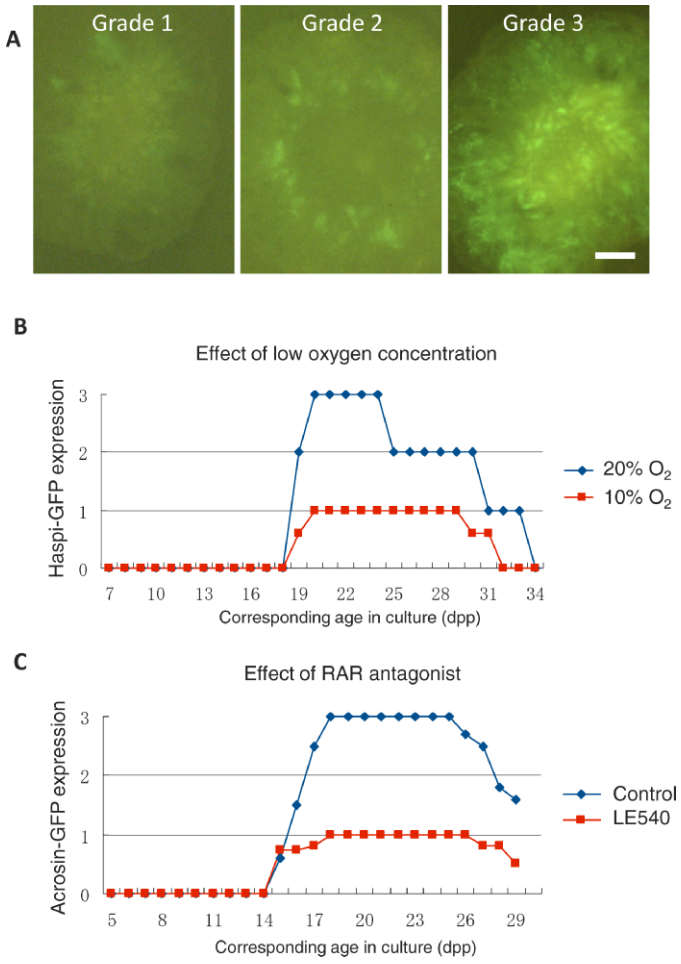

FIG. 3. Semiquantitative evaluation of the extent of GFP expression in seminiferous tubules. **A**) Testis tissues showing no GFP were scored 0. When the GFP expression extended along the tubule for more than 1 mm, it was counted as GFP positive. The extent of GFP positivity was divided into three grades. Grades 1, 2, and 3 represent 1–5, 6–15, and over 15 GFP-positive tubules, respectively. Bar = 500 μm. **B**) Effect of low oxygen concentration on *Gsg2-GFP* expression. The period of *Gsg2-GFP* expression was almost identical in both groups, but the extent of the expression was higher under normal oxygen concentration. **C**) Effect of antiretinoic molecule on *Acr-GFP* expression. LE540, an RAR pan-antagonist, depressed the expression of *Acr-GFP* throughout the culture period.

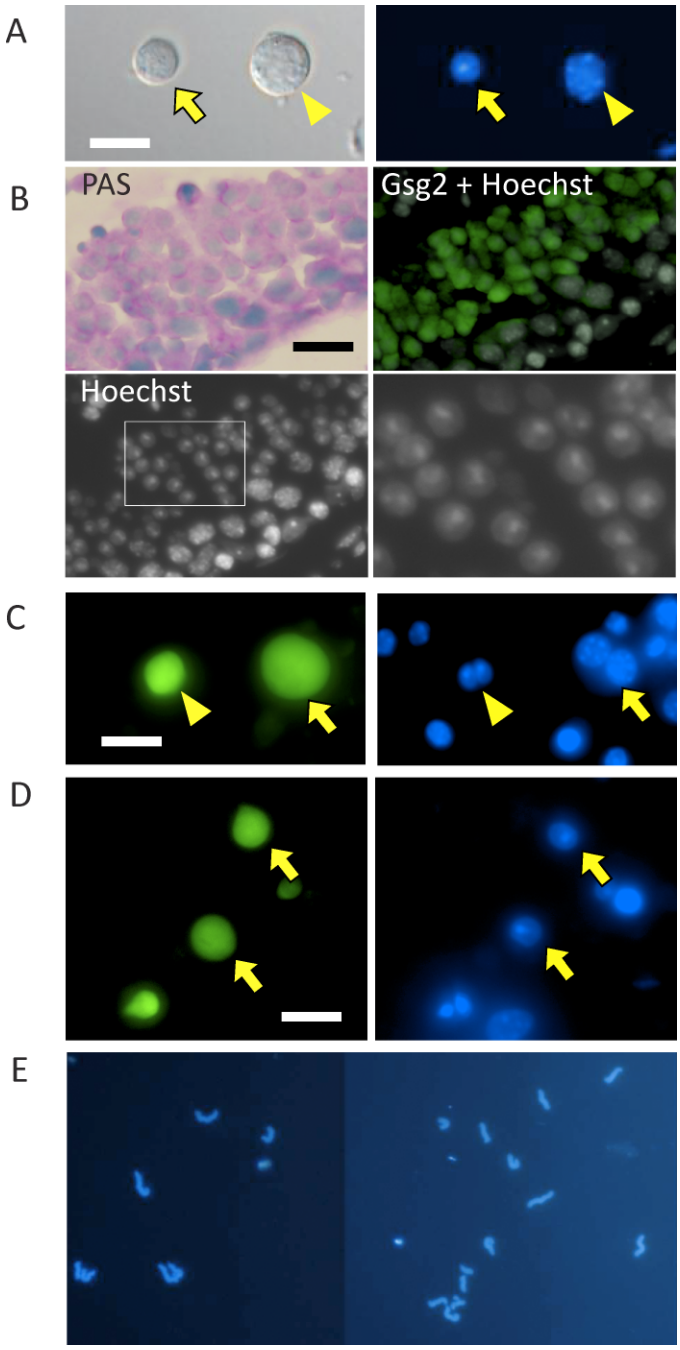

FIG. 4. Hoechst staining of *Gsg2-GFP*-positive cells. Among *Gsg2-GFP*-positive cells, some had a single nucleus with a prominent heterochromatin dot, a sign of typical round spermatids. Others had multiple nuclei each with multiple heterochromatin dots. **A**) Testis cells of adult *Gsg2-GFP* transgenic mouse, 4 wk old, were dissociated and stained with Hoechst. Spermatids (arrow) were recognized as smaller than spermatocytes (arrowhead), around 11 mm in diameter, and showed a prominent single nuclear dot on Hoechst staining (blue). **B**) Cryosection of adult *Gsg2-GFP* transgenic mouse testis stained with PAS-hematoxylin showed round spermatids of which nuclei were covered with acrosomal caps stained by PAS. On the adjacent section, *Gsg2-GFP* expression (green) was confirmed to correspond mostly with those round spermatids. Hoechst stain (gray) demonstrated the nuclear single dot in those round spermatids. White rectangular area is magnified in the left picture. **C**) 11.5 dpp *Gsg2-GFP* transgenic mouse testis tissue cultured for 21 days. Some GFP-positive cells were found to be multinucleated (arrow) and some others showed two dots in a single nucleus (arrowhead). **D**) 6.5 dpp *Gsg2-GFP* transgenic mouse testis tissues cultured for 29 days expressing GFP were considered to be spermatids as they showed a single nucleus with a single dot on Hoechst staining (arrow). **E**) Nuclei of presumptive spermatids were injected into mouse oocytes to induce premature chromosome condensation to allowing the counting chromosome number. Bar = 15 μm (**A**, **C**, **D**), 30 μm (**B**).

TABLE 4. Summary of Hoechst nuclear staining of haspin-GFP positive cells.[a]

Age of mice (dpp)	No. of experiments	No. of spermatids/no. of haspin-GFP positive cells	Proportion of spermatids among haspin-GFP positive cells (%)
6.5	2	3/28	10.7
7.5	2	1/29	3.4
8.5	1	1/20	5.0
11.5	3	17/57	29.8

[a] The testis tissues had been cultured for 17–33 days at the time of analysis.

in the nucleus stained by Hoechst (Fig. 4B). This finding supported the validity of our method for identification of round spermatids by the combination of *Gsg2-GFP*, Hoechst nuclear staining, and cell size. We then applied Hoechst stain to the cultured testis tissue that expressed *Gsg2-GFP*. The *Gsg2-GFP*-positive cells differed in their size (some were larger than 11 μm in diameter) and some were multinucleated, which indicated spermatocytes that had finished nuclear division but not yet cytoplasmic division (Fig. 4C). Among such spermatocytes, spermatids were occasionally observed (Fig. 4D). The frequency of spermatids was up to 30% (17/57) among all GFP-positive cells (Table 4). Finally, we injected the nuclei of these round spermatids into mouse oocytes to induce premature chromosome condensation and counted the number of chromosomes (24). In one case out of 19, we found 20 chromosomes, indicating a haploid genome, in samples cultured from 10.5 dpp mouse testes (Fig. 4E). Taken together, these data supported the assumption that the haploid cells had been produced in our organ culture system.

DISCUSSION

For decades, it has been a challenge to induce mammalian spermatogenic meiosis in vitro. Although a number of improvements and advances have been reported, the completion of the whole process, from spermatogonia to spermatids, has not succeeded [3–5]. With organ culture method in particular, it has been recognized that spermatogenic meiosis would arrest at the pachytene stage [2, 6]. In the present study, we reevaluated the organ culture method and found that spermatids were produced in that culture method, which was the first such achievement, to our best knowledge.

The basics of the present study depended on the methods developed by the Steinbergers in the 1960s. However, the materials and equipment used were different. We tried to make the culture system as simple as possible. One of our novel methods was that we used agarose gel, instead of stainless steel grids covered with a Millipore filter [27], to place testis tissue fragments on. The culture media used were commercially available, and no modifications were made to them. The exact contents of the media used in this study and their quality and those in the Steinbergers' might have differed. The quality of serum might also have been different. These possible differences, or improvements, may have been a reason for the progression of meiosis up to the production of spermatids. More importantly, however, a monitoring system using *Acr-* and *Gsg2-GFP* contributed superbly to our work. Their

expression levels were strong enough to allow them to be detected easily under a stereomicroscope without uncovering the lid of the culture plate, which enabled the experiments to continue. *Gsg2-GFP* was especially useful for detecting the completion of meiosis [28]. On the other hand, histological examination alone seemed insufficient to evaluate the progress of meiosis accurately, and we were not able to find spermatids using histology. Accordingly, it might be possible that the Steinbergers' experiment also produced a few spermatids, which were not possible to identify at the time.

In the present study, we have confirmed several results of previous studies by the Steinbergers. They showed that the progress of spermatogenesis in organ culture samples relates to the developmental stage of those samples. As age increases, the spermatogenesis of their explants is more likely to proceed in culture [7]. However, on the other hand, even using more developed testes, such as of 14-day-old rats, spermatogenesis did not proceed beyond the pachytene stage of meiosis [7]. Our present study used mouse pup testes from 4.5 to 14.5 dpp and showed high rates of *Acr*- and *Gsg2-GFP* expression regardless of the age of the pups. Nonetheless, the rate decreased when younger pups were used, and no differentiation beyond round spermatids, namely spermiogenesis, was observed even when older pups were used. It was reported previously that spermatids had disappeared within several days when adult testis tissues were cultured, while spermatocytes remained for 3–4 wk [6]. Therefore, it seems extremely difficult to induce spermiogenesis in organ culture systems.

The Steinbergers used Eagle synthetic medium [10] supplemented with sodium pyruvate and several amino acids as a basic medium. They found that the addition of vitamin A, E, and C or glutamine fostered spermatogenetic activity [6]. Glutamine was especially useful for producing more spermatocytes in their organ culture system [6]. αMEM, which was the best medium in the present study, includes 2 mM glutamine and 0.28 mM vitamin C. DMEM also contains 4 mM glutamine. As all media used in this study included 10% FBS, it might not be worthwhile to compare the detailed contents of the media and the supplements added to them with those of the Steinbergers' study. In future studies, we would like to omit FBS and replace it with chemically defined supplements in order to enhance our understanding of the environmental factors influencing the spermatogenesis.

It is known that spermatogenesis does not proceed well at 37°C [29]. The Steinbergers tested different temperatures ranging from 31°C to 37°C in their organ culture experiment and found that 33°C or even lower was better for rat spermatogenesis [6, 8]. In the present experiment, the difference between 37°C and the other two temperatures was drastic, which became obvious due to very poor *Gsg2-GFP* expression at 37°C. The difference between 32°C and 34°C was rather subtle, but 34°C appeared to promote spermatogenesis more. The mechanism that induces such temperature sensitivity is not fully understood, but several responsible genes have been postulated [30, 31]. Izu et al. [31], for instance, reported that heat shock transcription factor 1 (HSF1) was activated in male germ cells by heat stress and that HSF1 induced apoptosis of spermatocytes at the pachytene stage. They reasoned that HSF1 contributed to the quality control of spermatogenesis. As high testicular temperature is caused by cryptorchidism and varicocele, which are directly involved in male infertility, the mechanical relationship between high temperature and spermatogenic impairment needs to be explored in more detail. Our organ culture method could be useful for such molecular-level studies because it supported spermatogenesis beyond the pachytene stage.

The liquid-gas interphase method allows each tissue fragment easy access to oxygen [32]. Accordingly, Steinberger et al., seeking the beneficial effects of higher O_2 concentrations, tested 10% CO_2 + 90% O_2 in their organ culture experiments and found that, although the penetration of O_2 into tissues was improved as shown by the smaller area of central necrosis in each tissue, the seminiferous tubules were more distorted and the numbers of spermatogonia and spermatocytes had decreased dramatically [8]. As too much oxygen can damage tissues and cells via the production of reactive oxygen species, the optimal oxygen concentration might be lower than 20%. In fact, in the testis, blood vessels are located exclusively between the tubules, and oxygen reaches the lumen of the tubules only by diffusion. It was reported therefore that luminal oxygen partial pressures were extremely low due to the diffusion distance and the high oxygen consumption by the proliferating germ cells [33–36]. Based on such considerations, we used a lower oxygen concentration of 10% on our organ culture experiments and found that it was disadvantageous for spermatogenesis compared to 20%. Further investigation is necessary to find a more suitable oxygen concentration.

The semiquantitative evaluation of spermatogenic progression in our organ culture method demonstrated that disruption of RA action prevented spermatogenesis as it does in vivo. The effect of RA on spermatogenesis has been well known for decades, mainly due to the fact that a vitamin A-deprived diet prevents spermatogenesis in rodents, leaving only primitive spermatogonia in the seminiferous tubules [37]. Recent studies have revealed the mechanism of this phenomenon and found that RA is a pivotal trigger of meiosis [38]. The present method combined with the use of specific inhibitors to particular molecules in our organ culture system might be useful in future studies for elucidating the roles of such molecules in spermatogenesis.

Recently, several independent groups reported that embryonic stem cells can be induced to differentiate into germ cells in vitro, which then differentiate into spermatogenic cells [39–41]. However, the spermatogenesis seen in these studies did not rely on testicular somatic cells of Sertoli or Leydig cells. Therefore, the role of somatic cells in spermatogenesis was not addressed properly. Authentic spermatogenesis depends on Sertoli cells and other somatic cells outside the seminiferous tubules. Sertoli cells above all are indispensible in supporting various aspects of spermatogenesis. Studies of spermatogenesis from ES cells are certainly important and rewarding, but at the same time it is desirable to have an authentic in vitro spermatogenesis system, which is able to dissect the mutual interplay between germ cells and somatic cells.

In conclusion, we have shown that the induction of spermatogenesis up to haploid spermatids in an organ culture is possible. But it is still a rare event, and further improvements of the culture technique are needed in the future.

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REFERENCES

1. Champy C. Quelques résultats de la méthode de culture des tissues. Arch Zool Exp Gen 1920; 60:461–500.
2. Martinovich PN. Development in vitro of the mammalian gonad. Nature 1937; 139:413.
3. Staub C. A century of research on mammalian male germ cell meiotic differentiation in vitro. J Androl 2001; 22:911–926.
4. Sofikitis N, Pappas E, Kawatani A, Baltogiannis D, Loutradis D, Kanakas

N, Giannakis D, Dimitriadis F, Tsoukanelis K, Georgiou I, Makrydimas G, Mio Y, et al. Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment. Hum Reprod Update 2005; 11:229–259.
5. La Salle S, Sun F, Handel MA. Isolation and short-term culture of mouse spermatocytes for analysis of meiosis. Methods Mol Biol 2009; 558:279–297.
6. Steinberger A, Steinberger E. In vitro growth and development of mammalian testes. In: Johnson AD, Gomes WR, Vandemark NL (eds.), The Testis. New York: Academic Press; 1970:363–391.
7. Steinberger A, Steinberger E. Factors affecting spermatogenesis in organ cultures of mammalian testes. J Reprod Fert 1967; 2(suppl):117–124.
8. Steinberger A, Steinberger E, Perloff WH. Mammalian testes in organ culture. Exp Cell Res 1964; 36:19–27.
9. Trowell OA. The culture of mature organs in a synthetic medium. Exp Cell Res 1959; 16:118–147.
10. Eagle H. Nutritional needs of mammalian cells in tissue culture. Science 1955; 122:501–504.
11. Parvinen M, Wright WW, Phillips DM, Mather JP, Musto NA, Bardin CW. Spermatogenesis in vitro: completion of meiosis and early spermiogenesis. Endocrinology 1983; 112:1150–1152.
12. Rassoulzadegan M, Paquis-Flucklinger V, Bertino B, Sage J, Jasin M, Miyagawa K, van Heyningen V, Besmer P, Cuzin F. Transmeiotic differentiation of male germ cells in culture. Cell 1993; 75:997–1006.
13. Staub C, Hue D, Nicolle JC, Perrard-Sapori MH, Segretain D, Durand P. The whole meiotic process can occur in vitro in untransformed rat spermatogenic cells. Exp Cell Res 2000; 260:85–95.
14. Marh J, Tres LL, Yamazaki Y, Yanagimachi R, Kierszenbaum AL. Mouse round spermatids developed in vitro from preexisting spermatocytes can produce normal offspring by nuclear injection into in vivo-developed mature oocytes. Biol Reprod 2003; 69:169–176.
15. Tajima Y, Watanabe D, Koshimizu U, Matsuzawa T, Nishimune Y. Insulin-like growth factor-I and transforming growth factor-alpha stimulate differentiation of type A spermatogonia in organ culture of adult mouse cryptorchid testes. Int J Androl 1995; 18:8–12.
16. Zhou B, Hutson JM. Human chorionic gonadotropin (hCG) fails to stimulate gonocyte differentiation in newborn mouse testes in organ culture. J Urol 1995; 153:501–505.
17. Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, Loveland KL. Regulation of germ cell and Sertoli cell development by activin, follistatin and FSH. Dev Biol 2000; 220:225–237.
18. Puglisi R, Montanari M, Chiarella P, Stefanini M, Boitani C. Regulatory role of BMP2 and BMP7 in spermatogonia and Sertoli cell proliferation in the immature mouse. Eur J Endocrinol 2004; 151:511–520.
19. Nakanishi T, Ikawa M, Yamada S, Parvinen M, Baba T, Nishimune Y, Okabe M. Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. FEBS Lett 1999; 449:277–283.
20. Ventelä S, Mulari M, Okabe M, Tanaka H, Nishimune Y, Toppari J, Parvinen M. Regulation of acrosome formation in mice expressing green fluorescent protein as a marker. Tissue Cell 2000; 32:501–507.
21. Tanaka H, Yoshimura Y, Nozaki M, Yomogida K, Tsuchida J, Tosaka Y, Habu T, Nakanishi T, Okada M, Nojima H, Nishimune Y. Identification and characterization of a haploid germ cell-specific nuclear protein kinase (Haspin) in spermatid nuclei and its effects on somatic cells. J Biol Chem 1999; 274:17049–17057.
22. Tokuhiro K, Miyagawa Y, Yamada S, Hirose M, Ohta H, Nishimune Y, Tanaka H. The 193-base pair Gsg2 (haspin) promoter region regulates germ cell-specific expression bidirectionally and synchronously. Biol Reprod 2007; 76:407–414.
23. Umemiya H, Fukasawa H, Ebisawa M, Eyrolles L, Kawachi E, Eisenmann G, Gronemeyer H, Hashimoto Y, Shudo K, Kagechika H. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. J Med Chem 1997; 40:4222–4234.
24. Araki Y, Yoshizawa M, Araki Y. Visualization of human sperm chromosomes by using mouse oocytes cryothawed after enucleation. Fertil Steril 2006; 86:348–351.
25. Clermont Y, Trott M. Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of ^{3}H-thymidine and radioautography. Fertil Steril 1969; 20:805–817.
26. Miura T, Yamauchi K, Takahashi H, Nagahama Y. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci U S A 1991; 88:5774–5778.
27. Steinberger A. In vitro techniques for the study of spermatogenesis. Methods Enzymol 1975; 39:283–296.
28. Kita K, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, Nagashima Y, Aoki I, Taniguchi H, Noce T, Inoue K, Miki H, Ogonuki N, et al. Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod 2007; 76:211–217.
29. Setchell BP. The Parkes Lecture. Heat and the testis. J Reprod Fertil 1998; 114:179–194.
30. Nishiyama H, Danno S, Kaneko Y, Itoh K, Yokoi H, Fukumoto M, Okuno H, Millán JL, Matsuda T, Yoshida O, Fujita J. Decreased expression of cold-inducible RNA-binding protein (CIRP) in male germ cells at elevated temperature. Am J Pathol 1998; 152:289–296.
31. Izu H, Inouye S, Fujimoto M, Shiraishi K, Naito K, Nakai A. Heat shock transcription factor 1 is involved in quality-control mechanisms in male germ cells. Biol Reprod 2004; 70:18–24.
32. Freshney RI. Organotypic culture. In: Culture of Animal Cells: A Manual of Basic Technique, 4th ed. NewYork: Wiley-Liss; 2000:395–406.
33. Wenger RH, Katschinski DM. The hypoxic testis and post-meiotic expression of PAS domain proteins. Semin Cell Dev Biol 2005; 16:547–553.
34. Max B. This and that: hair pigments, the hypoxic basis of life and the Virgilian journey of the spermatozoon. Trends Pharmacol Sci 1992; 13: 272–276.
35. Cross BA, Silver IA. Neurovascular control of oxygen tension in the testis and epididymis. J Reprod Fertil 1962; 3:377–395.
36. Free J, Schluntz GA, Jaffe RA. Respiratory gas tensions in tissues and fluids of the male rat reproductive tract. Biol Reprod 1976; 14:481–488.
37. Van Pelt AM, De Rooij DG. The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol Reprod 1990; 42:677–682.
38. Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci U S A 2006; 103:2474–2479.
39. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 2004; 427:148–154.
40. Yu Z, Ji P, Cao J, Zhu S, Li Y, Zheng L, Chen X, Feng L. Dazl promotes germ cell differentiation from embryonic stem cells. J Mol Cell Biol 2009; 1:93–103.
41. Kee K, Angeles VT, Flores M, Nguyen HN, Reijo Pera RA. Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature 2009; 462:222–225.