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Proliferation of Mouse Spermatogonial Stem Cells in Microdrop Culture[1]

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ABSTRACT

It is now possible to make mouse spermatogonial stem cells (SSCs) proliferate in vitro. However, these cultured cells, called germ-line stem (GS) cells, consist of not only SSCs but also a greater number of progenitor spermatogonia. Moreover, isolated GS cells barely proliferate. To elucidate the nature of SSCs and progenitor spermatogonia, we adapted a microdrop culture system to GS cells. Using a micromanipulator, individual microdrops were seeded with clusters or dissociated known numbers of GS cells. The number of surviving colonies was determined after 30 days. The proliferation rate of GS cells in microdrops increased as the number of GS cells seeded increased. It was observed that as few as three GS cells seeded in a microdrop can proliferate and expand the colony size. Those GS cells of expanded colonies were able to proliferate following subculture and underwent spermatogenesis in the host testis after transplantation into the seminiferous tubules of recipient mice. These data revealed that SSCs can multiply in a microdrop culture system. Microdrop culture offers a novel tool to elucidate the nature of SSCs in regard to their self-renewing capacity and can serve as a monitoring system of culture conditions for the self-renewal of SSCs.

culture, microdrop culture, spermatogenesis, spermatogonia, spermatogonial stem cells

INTRODUCTION

The lifelong production of sperm is made possible by spermatogonial stem cells (SSCs), which are defined as possessing the ability to self-renew and the potential to differentiate into spermatozoa. The daughter cells of SSCs can be either SSCs or progenitor spermatogonia. The spermatogonia, defined as mitotic germ cells in the mature testis, represent every premeiotic male germ cell residing on the basement membrane of the seminiferous tubules. SSCs are therefore a subpopulation of spermatogonia, while progenitor spermatogonia work as so-called progenitor cells or transit amplifying cells to expand their population by sequential cell division. This expansion of spermatogonia is the basis for the production of enormous numbers of sperm.

SSCs have long been proposed and generally considered to be singly isolated type A spermatogonia (A_{single}, or A_s spermatogonia), while progenitor spermatogonia are regularly connected to each other by cytoplasmic bridges with siblings forming chains of cells [1, 2]. Recent studies have shown that such a hierarchical stem-progenitor system basically holds true, but it is more flexible and adaptable, meaning that progenitor cells retain some stem cell activity as well [3]. Some molecular markers for undifferentiated spermatogonia, which include SSCs, were also identified, including *Zbtb16* (also known as PLZF), *Gfra1*, *Thy1*, and so on [4]. In addition, it was reported that glial cell line-derived neurotrophic factor (GDNF) was a master factor for the expansion, survival, and/or self-renewal of SSCs [5]. Using GDNF as a soluble factor in culture medium, it became possible to expand and maintain long-term cultures of mouse SSCs in vitro, called germ-line stem (GS) cells [6]. Now, rat and hamster SSCs can also survive and multiply for long periods in culture [7, 8]. The development of these culture methods made the study of SSCs at the molecular level possible [4].

SSCs in vitro are not exclusively self-renewing; instead, there seems to be a balance between self-renewal and differentiation in culture, as there is in vivo. Therefore, in SSC cultures, there are many progenitor spermatogonia at various stages of differentiation [9]. In other words, GS cells consist of both self-renewing SSCs and differentiating progenitor spermatogonia. Self-renewing SSCs make up an estimated 1% or less of the cells in a GS culture based on transplantation assays [10]. One shortcoming of GS cell culturing is that it is not yet possible to readily isolate SSCs from the culture population. Moreover, GS cells are resistant to cloning, in part because of the low frequency of SSCs among GS cells and also because the cultured cells show poor survival and proliferate slowly when plated at low densities. Single GS cells require companion cells for clonal expansion in culture [11], suggesting that direct intercellular interactions are necessary for their proliferation and/or that certain soluble factors are missing from currently used culture medium. In addition, with current GS cell culture methods, it is difficult to trace individual cells over time unless equipped with time-lapsed monitoring systems. We therefore reasoned that microdrop culture may facilitate the study of GS cells by allowing the observation of individual GS cells or of GS microcolonies for long periods.

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In the present study, we applied the microdrop technique to the culture of mouse GS cells. The microdrop culture is very useful for monitoring the proliferation of GS cells and promises to aid in elucidating the nature of SSCs.

MATERIALS AND METHODS

Mice

Male transgenic mice (C57BL/6 genetic background) carrying the pCXN-eGFP transgene (official allele symbol *Tg(CAG-EGFP)50Osb* [12] were mated with ICR females to produce a green fluorescent protein (GFP)-positive GS cell line from F1 (ICRB6F1/GFP) pups. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Yokohama City University, Yokohama, Japan).

Cell Preparation and Maintenance

GS cells were obtained from the testes of male 7- to 10-day-old ICRB6F1/GFP pups and cultured according to methods described elsewhere [6, 9]. The culture medium for GS cells consisted of StemPro-34 SFM (Invitrogen Corp., Grand Island, CA) supplemented with StemPro supplement (Invitrogen), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma Chemical Co., St. Louis, MO), 5 mg/ml bovine albumin (ICN Biomedicals Inc., Irvine, CA), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, minimal essential medium (MEM) vitamin solution (Invitrogen), MEM nonessential amino acid solution (Invitrogen), 10^{-4} M ascorbic acid, 10 μg/ml d-biotin (Sigma), 20 ng/ml mouse epidermal growth factor (BD Biosciences, Bedford, MA), 10 ng/ml human basic fibroblast growth factor (BD Biosciences), 10 ng/ml recombinant human GDNF (Pepro Tech Ltd., Rocky Hill, NJ), and 1% fetal bovine serum (FBS) (Invitrogen). The cells were maintained at 37°C in an atmosphere of 5% CO_2 in air. The GS cells were cultured on a feeder layer of mouse embryonic fibroblasts (MEF), which was cultured in DMEM (Invitrogen) +10% FBS and inactivated by mitomycin C (Wako Pure Chemical Industries, Ltd., Osaka, Japan) treatment. The medium was changed every 3–4 days. Cell passage was performed every 10–15 days with a seeding concentration of $10–30 \times 10^4$ cells/ml. For all experiments, GS cells that were once cryopreserved were used. For cryopreservation, GS cells were suspended in CELLBANKER, a cryopreservative medium, at about 10^6 cells/ml. After thawing, growing populations were cultured for several passages under regular maintenance culture conditions until being used for microdrop experiments.

Preparation of Microdrops

Two sizes of culture dishes, 35 mm (Falcon 3001; Becton Dickinson, Franklin Lakes, NJ) and 60 mm (Falcon 3002; Becton Dickinson), were used. Suspensions of MEF ($1–2 \times 10^6$ cells/ml), inactivated by mitomycin C treatment, of 2.5, 5, and 10 μl, were spotted in a 35-mm dish to make drops for experiment 1. For experiments 2 and 3, 2.5 μl of MEF suspensions were placed to make about 15 microdrops in each 60-mm dish. Those drops were covered by mineral oil (Fuso Pharmaceutical Industries, Ltd., Osaka, Japan). After 4 h or overnight incubation, the MEF medium was removed, and 5-, 10-, or 20-μl drops of GS culture medium were spotted in place of the 2.5-, 5-, and 10-μl MEF drops, respectively. GS cells were transferred to the drops of GS culture medium.

Microdrop Culture of GS Cells

GS cells used for microdrop culture experiments were taken from maintenance cultures at passage.

Experiment 1. GS cell colonies composed of 40–300 cells were picked up with a handheld glass pipette with an inner diameter of 150–200 μm. Each colony was transferred into a GS microdrop with MEF feeders. Pictures were taken of GS cell colonies in each microdrop every several days. The projected area of each colony was traced manually, the encircled area was measured using computer software, ImageJ (NIH), and the doubling times of the area were calculated from these data.

Experiment 2. GS cell colonies in the maintenance culture were dissected with a 26-gauge needle to isolate smaller cell clumps. Clumps consisting of 2–12 cells were picked up in a glass micropipette with an inner diameter of 12–15 μm using a micromanipulator. The cell clumps were transferred into 5.0-μl microdrops with MEF feeders.

Experiment 3. GS cells were disaggregated employing 0.25% trypsin-EDTA (Invitrogen) treatment and then suspended in GS culture medium. The dissociated GS cells were picked up with a micromanipulator in a glass micropipette, and 3, 5, 10, 20, and 40 cells were transferred into 5.0-μl microdrops with MEF feeders, respectively.

In all experiments, cells were cultured at 37°C under 5% CO_2 in air, and the medium was changed every 3–4 days. About half to three-quarters of the medium in each drop was changed using a handheld thin-tipped glass pipette connected to a mouthpiece. Each microdrop was maintained for up to 1 mo without passage.

Transplantation into the Seminiferous Tubules

Colonies, each originating from a separate microdrop in experiment 3, were treated with 0.25% trypsin + EDTA after rinsing with PBS. The dissociated cells were sequentially transferred to a well of a 48-well plate, a well of a 24-well plate, and three wells of a 24-well plate at intervals of about a week. GS cells in the final two wells of the 24-well plate were collected and transplanted into the seminiferous tubules of WBB6F1-W/W^v (W) mice (Japan SLC, Inc., Shizuoka, Japan) [13]. After 2–3 months, the recipient testes were harvested for analysis. GFP-expressing spermatogenic colonies in the seminiferous tubules were observed under a stereomicroscope. The testes were then fixed with Bouin solution and microsectioned and stained with hematoxylin and eosin for histological evaluation.

RESULTS

In the first set of experiments testing the microdrop method, relatively large GS cell colonies (estimated 40–300 cells per colony) were chosen from the maintenance culture dishes. These GS cell colonies were picked up and transferred into microdrops of three different sizes: 5, 10, or 20 μl. Every GS cell colony (N = 27 microdrops) grew and steadily increased in size during the culture period (Fig. 1A). The colony size increased exponentially regardless of the drop size (Fig. 2A). Therefore, the volume of the drop did not affect the proliferation rate of GS cells in this experiment. The proliferation speed of GS cells in microdrops was approximately calculated from the size of the colonies. The colony size doubling time was about 7 days in the first 3 wk. Thereafter, it slowed in all three groups, indicating that the rate of proliferation decreased, probably because of space or nutrient limitations and the accumulation of waste products in the limited culture volume (Fig. 2B).

In the second experiment, we tested the microdrop culture method for smaller colonies of GS cells. The GS cell colonies cultured in regular dishes were fragmented with a 26-gauge needle to produce small cell clusters of 2–12 cells. Each fragment was picked up and seeded into a 5-μl microdrop. In some drops, the GS cell colonies grew and enlarged by day 7. Most of these colonies continued to proliferate (Fig. 1B). In contrast, GS cell clumps that did not show any growth by day 7 generally disappeared at 2 wk. Of the 45 total drops, eight drops showed the sustained expansion of GS cell colonies until the end of the study period (30 days) (Table 1). The expansion efficiency per drop was eight out of 45 drops (17.8%). The 45 drops contained a total of 297 GS cells at the start of the culture period; therefore, the development of eight colonies indicates a proliferation efficiency of each GS cell of 2.7% (8/297), assuming that each colony originated from a single GS cell. This proliferation efficiency may reflect the percentage of SSCs in the GS population, as only SSCs can form colonies.

We then investigated whether GS cells can proliferate in the microdrop after being dissociated into single cells. GS cells were enzymatically disaggregated and picked up individually using a glass micropipette. Exactly 3, 5, 10, 20, or 40 cells were used to seed each microdrop. The cells in the microdrop reaggregated to form cell clusters by the next day. As seen in experiment 2, several colonies grew in size. However, some colonies disappeared in a week, and others remained at nearly the same size for up to 2 wk. Interestingly, there were some that

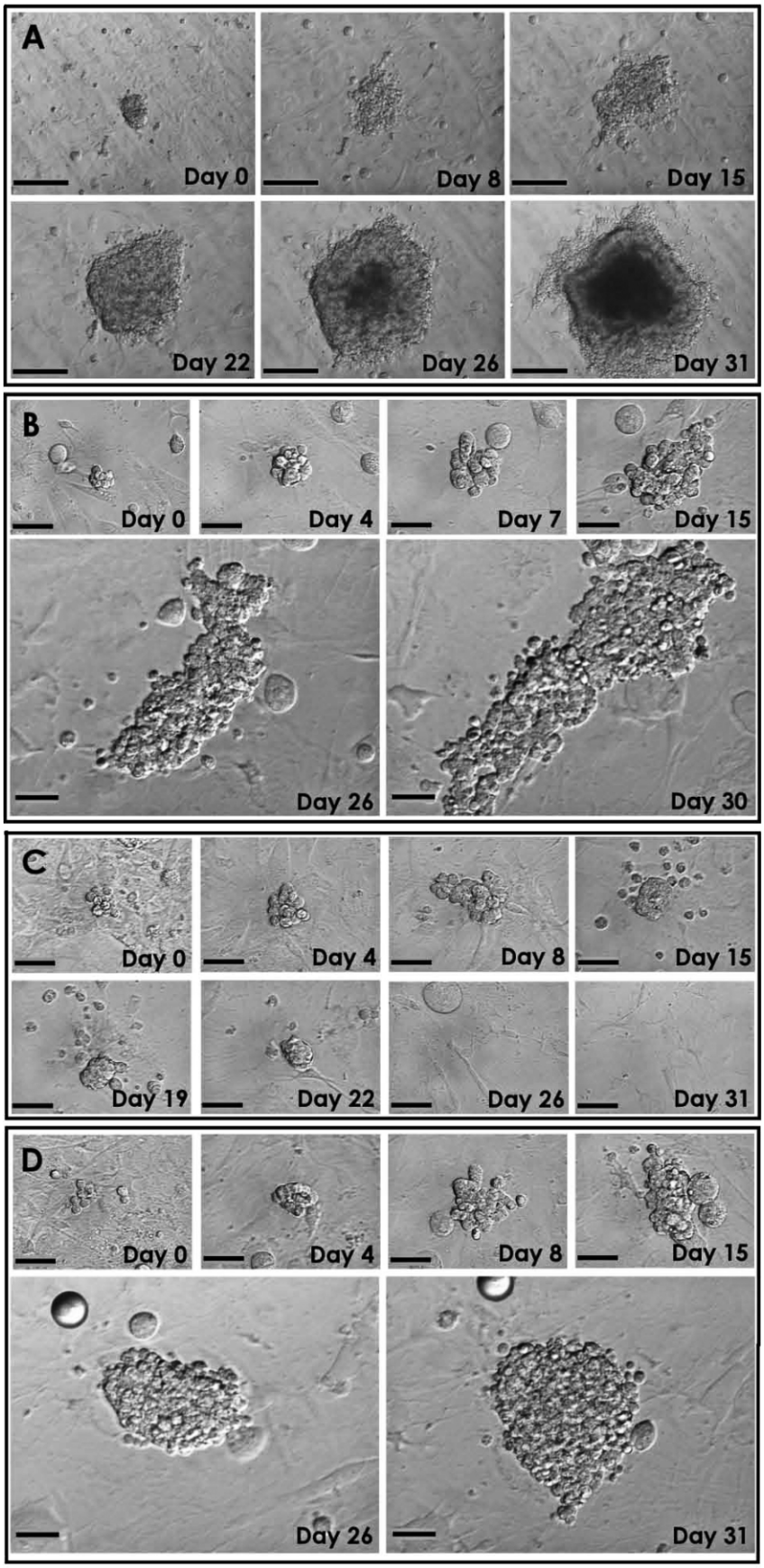

FIG. 1. Proliferation of GS cells in microdrops. **A**) A colony, originally of about 80 GS cells, underwent a 25-fold size increase in a 10-μl drop over a 30-day culture period. **B**) A nine-cell cluster of GS cells in a 5-μl drop enlarged during a 30-day culture period. **C**, **D**) Ten dispersed GS cells introduced into 5-μl microdrops. In some cases, the cells appeared to have proliferated during the initial week. However, they decreased in number and disappeared at 26 days in **C**. In **D**, the proliferation of GS cells was steady and a large colony was formed over the 30-day culture period (**D**). Bars = 200 μm (**A**) and 50 μm (**B**–**D**).

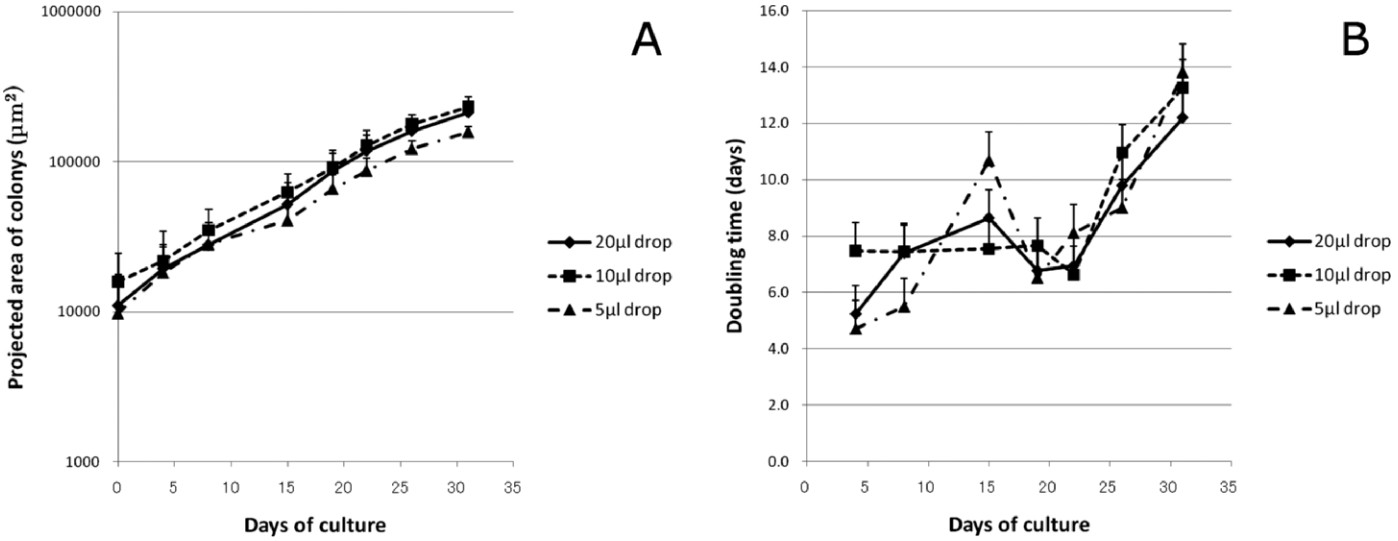

FIG. 2. Growth kinetics of GS cells measured based on the two-dimensional size of the colonies. **A**) The size of every GS cell colony steadily increased over the entire 30-day culture period. **B**) The colony size doubling times was about 7 days during the first 3 wk. Thereafter, the doubling time slowed to approximately 12 days. Error bars indicate SD.

showed proliferation in the first 1–2 wk but stopped proliferating and then disappeared (Fig. 1C). Most clusters that showed expansion at 2 wk grew to become large colonies (Fig. 1D). Twelve out of 64 microdrops, which were seeded with three GS cells, showed the proliferation of GS cells within them. As the number of GS cells seeded increased, the rate of microdrops showing GS cell proliferation increased (Table 2). When the culture started with 40 GS cells, it always ended in proliferation.

Finally, we examined if the expansion of GS cells in the microdrop in vitro culture reflects the expansion of SSCs in vivo by transplanting the daughter cells into recipient mouse testes. The GS colonies from experiment 3 were subcultured for further expansion. Cells from those lines were transplanted into the seminiferous tubules of the testes of W mice. Testes of 11 mice, with each receiving independent GS cells, showed the extensive colonization of donor GS cells and complete spermatogenesis histologically (Fig. 3, A–D). Testes of four mice showed the colonization of GS cells but not complete spermatogenesis (Fig. 3, E and F). The remaining three exhibited no colonization (Table 3).

DISCUSSION

The microdrop culture method was developed by Ralph L. Brinster [14]. In his efforts to establish culture techniques for ova and embryos, he showed that methods using small droplets of media under liquid paraffin oil were effective and especially useful for the observation of ova and embryos and for collecting quantitative data [15]. The microdrop method has since been employed by many researchers and is now a standard practice in many IVF programs [16–18]. The benefits of a microdrop-under-oil culture result from both the oil overlay and reduced incubation volume. The oil provides protection from contamination, reduces evaporation, and stabilizes the temperature and pH. It has also been shown that an oil overlay can act as a sink for toxic substances [19]. However, the use of the microdrop method has been restricted mostly to embryo cultures.

Therefore, the advantages of the microdrop culture method have not been garnered for the culture of cells from other sources. In the present study, we adapted this microdrop culture technique to GS cells and found that it can support the proliferation of GS cells and induce the expansion of colonies

TABLE 1. Proliferation of small clusters of GS cells in microdrops.

Parameter	No. of GS cells seeded in a microdrop											Total no. of drops	Total no. of cells
	2	3	4	5	6	7	8	9	10	11	12		
No. of microdrops studied	3	5	1	6	8	7	4	4	3	1	3	45	297
No. of microdrops showing colony proliferation	0	1	0	0	1	0	2	2	1	1	0	8	8*
Proliferation rate												17.80% (/drops)	2.70% (/cells)

* Assuming that colonies originated from a single SSC.

TABLE 2. Proliferation of dissociated GS cells in microdrops.

Parameter	No. of GS cells seeded in a microdrop				
	3	5	10	20	40
No. of microdrops studied	64	43	202	109	14
No. of microdrops containing proliferated GS cells (proliferation rate, %)	12 (18.8)	17 (39.5)	117 (57.9)	76 (69.7)	14 (100)

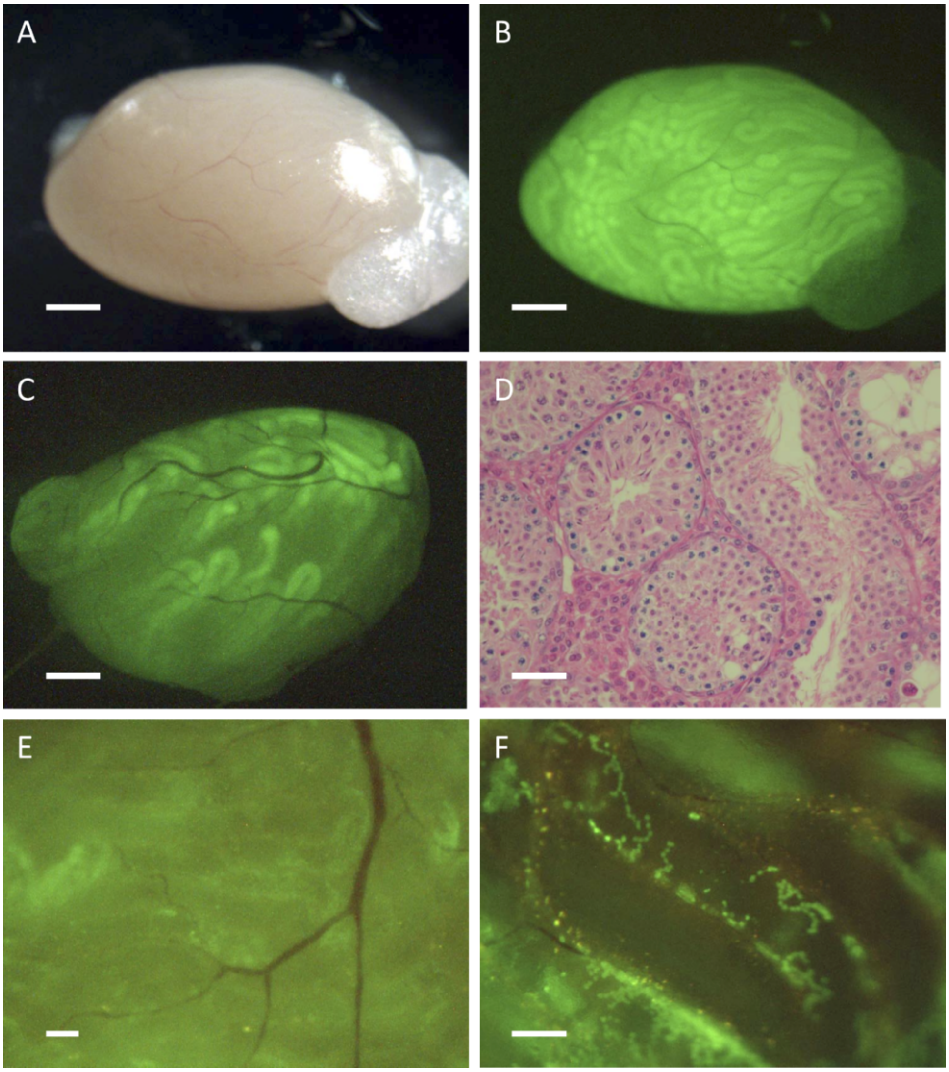

FIG. 3. GS cell transplantation into host testes. **A**, **B**) The testis of a recipient W mouse, 2 mo after transplantation, showed the extensive colonization of GS cells originating from a microdrop culture (**A**: bright picture, **B**: fluorescent picture). **C**) Another host testis showed GFP-positive and -negative tubules. **D**) The histology of such GFP-positive testis showed complete spermatogenesis. **E**) There were host testes that showed faint GFP. **F**) The close examination of such testes revealed GFP-positive GS cells spread inside the seminiferous tubules on the basement membrane and showed aligned cell connections, a typical feature of spermatogonia. Bars = 1 mm (**A**–**C**), 50 μm (**D**), and 100 μm (**E**, **F**).

initiated from a small number of cells. Our estimation of the doubling time of GS cells in the microdrop, about 7 days, might be a bit longer than that in the regular culture condition, which was about 5 days in our experience. In addition, doubling time calculated at each time point fluctuated from time to time and increased after 3 wk. It is therefore possible that the proliferation of GS cells in microdrops tends to be affected more easily by a subtle fluctuation of the culture conditions. This characteristic of microdrop culture might be rather advantageous when trying to study the various effects of culture conditions on GS cells.

In experiment 2, smaller clumps of GS cells comprising 2–12 cells were transferred into microdrops. From a total of 45 microdrops, GS cell colonies expanded in eight of these drops. GS cell colonies require SSCs for expansion. As progenitor spermatogonia can divide only a few to several times, their proliferation is limited. Even SSCs, if their self-renewing probability is less than 0.5, cannot sustain colony expansion. In fact, we observed the expansion of several colonies for a week or two and then their subsequent shrinkage and disappearance. Such limited expansion may represent colonies of progenitor spermatogonia alone or colonies with SSCs that failed to increase their number because of the low probability of self-renewal. We suggest that the colonies that successfully expanded in microdrops contained at least one or more SSCs on the initiation of culturing.

Since the development of the method of spermatogonial transplantation into the seminiferous tubules of the mouse testis, it has been possible to detect SSCs in a given sample of cells [20]. Spermatogonial transplantation has become the

TABLE 3. Results of the transplantation of GS cells in the testes of W mice.

	Starting no. of GS cells (no. of GS cells seeded in a microdrop)					
Parameter	3	5	10	20	40	Total
No. of recipient mice	2	2	10	3	1	18
No. of testes showing colonization by GS cells	2	2	8	2	1	15
No. of testes showing complete spermatogenesis	2	1	5	2	1	11

standard functional assay of SSC identification. However, this method has several limitations and inconveniences. Host mice must be mutants lacking spermatogenesis or pretreated, when using wild-type mice, to deplete germ cells in the testis. The transplantation procedure is difficult and requires practice to master. The time from transplantation to evaluation normally is 2 mo. Above all, the data, which are usually represented as the number of spermatogenic colonies in host testes per number of injected cells, may not be as accurate as those obtained by in vitro experiments. Many factors other than the number of SSCs, including the condition of the host mice and variations among individual transplantation procedures, could affect the results. Yeh et al. [21] devised another method for the estimation of SSC numbers by counting the clusters in culture dishes. Although this method is easy and quick (just 6 days to retrieve data), it may not be accurate either because the early cell clusters might consist of progenitor spermatogonia alone without any SSCs. The microdrop method, on the other hand, can confirm the expansion of SSCs by observing the continuous expansion of GS cells for 30 days. Moreover, the established colonies were confirmed to expand during the following passages to subsequent microdrops. Therefore, we propose microdrop culture as a new method of assaying the SSC activity of GS cells.

We performed transplantation experiments and confirmed that GS cells cultured in microdrops can colonize and cause spermatogenesis in seminiferous tubules of recipient mice. These data finally proved that GS cells proliferated in microdrops reflected the proliferation of SSCs. On the other hand, there were four out of 15 lines of GS cells that did not show spermatogenesis even though they colonized the recipient testes. The reason for this failure in spermatogenesis is not clear, but it may be due to either host or donor factors. We speculate here that the most plausible reason is genetic or epigenetic change(s) in a small population of GS cells. GS cells are reportedly stable for long periods (over a year or two) in terms of their genetic and epigenetic state, karyotype, and the potential for normal spermatogenesis [22]. However, the GS cells were shown to transform into pluripotent stem cells, termed multipotent GS (mGS) cells [23, our unpublished results]. The frequency of appearance of mGS cells was reported to be very low, 1 in 1.5×10^7 cells (equivalent to 35 newborn testes) [23]. Some GS cells may have lost the ability to complete spermatogenesis, which could be a step toward transformation into mGS cells.

The microdrop method could be applicable for the derivation of GS cell lines from a limited amount of testis tissues. When establishing GS cell lines, it is very important to enrich spermatogonial cells and exclude as many fibroblasts and other somatic cells as possible because these other cell types are highly proliferative and can outcompete SSCs [24]. As the population of SSCs is minor among testis cells, estimated to be one in every 3000 [25], or 0.01% of total testis cells [26], the enrichment of mouse and rat SSCs was performed using antibodies to cell surface markers in combination with fluorescence-activated or magnetic-activated cell sorting [27–29]. This enrichment is helpful; however, when only a small amount of testis tissue is available for culturing, such as human testis samples in the case of biopsy, these enrichment strategies might not be appropriate because a substantial portion of the sample could be lost during the procedure. We suggest that the microdrop method can be used as an alternative approach to circumvent this problem. In fact, even without the prior enrichment of SSCs, small colonies of GS cells often appeared in the primary culture of testis cells. The micromanipulation used in the present study could be employed to collect these small colonies of GS cells. This approach may also facilitate the selective transfer of GS cells into drops with the exclusion of somatic cells. In addition, as shown in the present study, the microdrop can support GS cell growth with an efficiency that increases according to the number of GS cells introduced into the drop.

The microdrop method has additional practical advantages over the regular culture method. First, it was easy to identify cells of interest in the microdrop and keep track of them during the culture period. In addition, the manipulation of cells with micromanipulators and glass pipettes is suitable for microdrop culture. With such micromanipulation, it becomes possible to handle a defined number of cells in each experiment. Second, extremely high concentrations of factors that are precious, expensive, or rare can be tested in a microdrop because of the small volume of medium. As these advantages are not specific to GS cells, the microdrop culture method may be widely applicable to the study of other stem cells from different organs and tissues.

In summary, we have shown for the first time that mouse SSCs can expand in microdrop cultures, suggesting that such microdrop cultures could be applicable for future studies on not only SSCs but also other stem cell populations.

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