Establishment of a normal medakafish spermatogonial cell line capable of sperm production *in vitro*

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Spermatogonia are the male germ stem cells that continuously produce sperm for the next generation. Spermatogenesis is a complicated process that proceeds through mitotic phase of stem cell renewal and differentiation, meiotic phase, and postmeiotic phase of spermiogenesis. Full recapitulation of spermatogenesis *in vitro* has been impossible, as generation of normal spermatogonial stem cell lines without immortalization and production of motile sperm from these cells after long-term culture have not been achieved. Here we report the derivation of a normal spermatogonial cell line from a mature medakafish testis without immortalization. After 140 passages during 2 years of culture, this cell line retains stable but growth factor-dependent proliferation, a diploid karyotype, and the phenotype and gene expression pattern of spermatogonial stem cells. Furthermore, we show that this cell line can undergo meiosis and spermiogenesis to generate motile sperm. Therefore, the ability of continuous proliferation and sperm production in culture is an intrinsic property of medaka spermatogonial stem cells, and immortalization apparently is not necessary to derive male germ cell cultures. Our findings and cell line will offer a unique opportunity to study and recapitulate spermatogenesis *in vitro* and to develop approaches for germ-line transmission.

germ stem cells | meiosis | *Oryzias latipes* | spermiogenesis | testis

Throughout adult life of most animals, male germ cells in the testis produce sperm that transmit genetic information between generations. Male germ cells originate from primordial germ cells, which are segregated from somatic cells early in development and migrate to the embryonic gonad, where they become gonocytes (1, 2). At the onset of spermatogenesis, testicular gonocytes or prospermatogonia resume proliferation and become undifferentiated type-A spermatogonia, the male germ stem cells that self-renew themselves to maintain the stem cell pool or differentiate through meiosis into fertile sperm (3, 4). Because spermatogenesis provides a unique opportunity to study and control animal reproduction and, more importantly, offers an excellent system for genetic manipulations of the germ line in vertebrates, efforts are increasingly put into the recapitulation *in vitro* of this process. Recapitulation has faced two major challenges: long-term cultivation of normal spermatogonial stem cells into stable cell lines and full range of spermatogenesis from spermatogonial stem cells to sperm in culture. Attempts have steadily been made toward the establishment of spermatogenic cell lines in vertebrate species. In mice, the number of spermatogenic cells decreases 50% and 90% after 2 and 7 days of culture, respectively (4, 5). Only by immortalization have male germ cell lines been established. One cell line was derived from 6-week-old mice and was able to generate meiotic cells at 6 months of culture (6), but lost this ability subsequently (7). Similarly, a spermatogonial cell line has been obtained by telomerase immortalization from 6-day-old mice (8). In the cattle, type-A spermatogonia from 5-month-old testes have been cocultured with Sertoli-like cells for 100 days (9). Recently, mouse testicular cells after culture for up to 5 months in the presence of growth factors have been shown to maintain their spermatogonial stem cell potential (10, 11), and germ cells capable of meiotic differentiation *in vitro* have been derived from mouse embryonic stem (ES) cells (12–14). In lower vertebrates such as fish, cultivation of spermatogenic cells has been limited to the primary culture system (15–17).

Efforts have been attempted to obtain meiotic and postmeiotic progression in culture. The two immortalized mouse spermatogenic cell lines were able to produce round spermatids (6, 8), and mouse spermatocyte precursors in primary coculture with Sertoli cells generated haploid postmeiotic cells (18). After 3 months of culture, mouse testicular cells were able to produce functional sperm upon transplantation into infertile testes (10, 11). Bull type-A spermatogonia in coculture generated meiotic products, and after 1 month in culture, colonized recipient testes (9). However, *in vitro* spermatogenesis in mammals has so far ended up with the spermatid stage (6, 8).

In contrast to mammals, spermatogenesis in lower vertebrates can proceed fully *in vitro*. Specifically, sperm production *in vitro* has been reported in three fish species using primary cultures. In the eel, all stages of spermatogenesis were established in organ culture of immature testes (15). In the medaka, fertile sperm were obtained during 10 days of primary cultures of spermatocytes at the meiotic prophase (16). In the zebrafish, dissociated testicular cells during 15 days of coculture on a feeder layer of Sertoli-like cells gave rise to fertile sperm (17). However, stable cultivation of germ cells has not been described in fish.

Here we report a success in the derivation of a normal spermatogonial cell line from the adult testis of medaka (*Oryzias latipes*) and in the recapitulation of spermatogenesis from this line to produce motile sperm *in vitro*. Medaka represents one of the unique vertebrate species in which fertile sperm can be obtained *in vitro* in the complete absence of any somatic cells (16). This fish is an excellent model for analyzing vertebrate development (19), and allows for derivation of ES cell lines (20–22).

Materials and Methods

Plasmids. pEGFP-N1 and pDsRed-N1, which express the enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) from the human cytomegalovirus early gene enhancer/promoter, respectively, were from Clontech. pVegfp contains EGFP between the 5.1-kb promoter and the 0.6-kb 3′ untranslated region of medaka vasa gene (23). To construct pOct4gfp, a 2.4-kb fragment of the mouse Oct4 distal enhancer

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Abbreviations: AP, alkaline phosphatase; ES, embryonic stem; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein.

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plus the promoter linked to EGFP was PCR-amplified from pGOF18dPEgfp (24) by using primers ODE (GTACGCGTGAATTCAGACAGGACTGCTGGGC) and SVA (AGCATCACAAATTTCACAAATAAAGAATTCACGGCTTT) and cloned into pBluescript KS (Stratagene). Plasmid DNA was prepared by using the Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany).

Fish and Cell Culture. Medaka fish was maintained at 26°C. Fertile male fish of the albino strain i^3 were selected from a spawning population. They were incubated in ice-cold water for 2 min, sterilized with 70% ethanol, and decapitated. Testes were dissected and minced with a fine scissors in PBS. Testicular fragments from single fish were incubated in 1 ml of cold trypsin–EDTA for 4 h on ice followed by 30 min at 28°C. The fragments sunk down to the bottom. Trypsin-EDTA was aspirated, leaving 200 μl without disturbing the fragments. A total of 200 μl of the germ cell culture medium (GCM; see *Supporting Text*, which is published as supporting information on the PNAS web site) was added to the tube, and cells were released by pipetting. Dissociated cells were seeded in 1 ml of GCM into a well of a gelatin-coated 12-well plate. Cells were cultured at 28°C in air. Sperm and spermatids were removed by medium change daily during the first week. Cell subculture, clonal expansion, and chromosome examination were performed as described (20, 21). To observe cell divisions *in situ*, 10^3 SG3 cells were seeded in gelatin-coated 6-cm tissue culture plates. After 5 days of culture, the cells were incubated with colchicine (10 ng/ml) for 2 h, hypotonically treated with 40 mM KCl, fixed in methanol-acetic acid (3:1), air-dried, and stained with Giemsa solution.

Cell Transfection. SG3 cells at 70% confluence in gelatin-coated 12-well plates were transfected with 1–2 μg of plasmid DNA by using the GeneJuice reagent (Novagen) as described (25).

Induced Differentiation and Flow Cytometry. Cells were grown at confluence without subculture. Cell detachment occurred. Detached cells were maintained for 1–7 days or transferred into 6-cm bacteriological dishes for suspension culture. For flow cytometry, detached cells were collected at intervals of 12 h; attached cells were harvested by trypsinization, and testicular cells dissociated from mature testes were used as reference. Cells were fixed in 70% ethanol, incubated at 37°C for 15 min in PBS containing RNase A (200 μg/ml) and propidium iodide (20 μg/ml) for DNA staining, and analyzed with a Coulter Elite ESP flow cytometer equipped with a WINMDIV2.8 software (Beckman Coulter).

Coculture. Stromal cells of the embryonic rainbow trout gonad cell line RTG were maintained at 21°C in EMEM containing 10% FBS and antibiotics (EMEM/10% FBS). RFP-positive SG3 cells were obtained at passage 70 by transfection with pDs-Red-N1 and clonally expanded (21) for 2 months. After trypsinization, 10^6 SG3 single cells were combined with 3×10^5 RTG cells in EMEM/10% FBS and seeded into a 6-cm bacteriological Petri dish for suspension culture at 21°C.

Expression Analysis. Total RNA was isolated by using the Trizol Reagent (GIBCO/BRL). Reverse transcription was done with 1 μg of total RNA by using the cDNA synthesis kit (Clontech). PCR was run for 30 (β-actin) and 35 cycles (the remainder) at 95°C for 20 s, 60°C for 20 s, and 72°C for 1 min. cDNA from 10 ng of total RNA was used for PCR with cDNA-specific primers (see supporting information).

Results

Derivation of Testicular Cell Cultures. To initiate medaka testicular cell cultures, we adopted the feeder-free conditions we previously developed for derivation of chimera-competent medaka ES cell lines (20–22). Dissociated testicular cells were seeded in gelatin-coated tissue culture plates in the germ cell culture medium. Cell attachment initiated within 24 h. At day 5, the majority (94%; 392 of 419 cells) of attached cells displayed strong staining for alkaline phosphatase (AP) (Fig. 1*A*) and appeared to comprise presumptive germ cells. The remainder of attached cells was negative for AP staining and heterogeneous in morphology. Sperm and spermatids displayed no attachment and

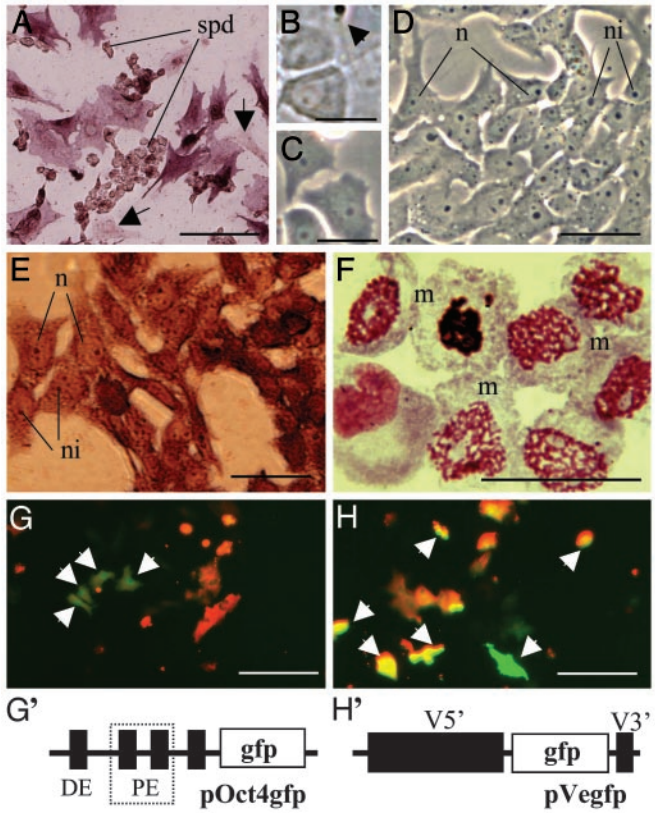

Fig. 1. Derivation and characterization of SG3 cells. (*A*) Testicular cells after 5 days in culture. The majority of attached cells are AP-positive; sperm and spermatids, as well as few attached cells (arrows), are AP-negative. (*B*) Morphology of spermatogonia at attachment after one day in primary culture. A sperm is visible (arrow). (*C*) After 436 days of culture, attaching SG3 cells at passage 72 retained the typical morphology of spermatogonia, such as sparse cytoplasm, and large and spherical nuclei with prominent nucleoli. (*D–F*) Micrographs of SG3 cells at passage 70. (*D*) SG3 cells under normal culture. (*E*) SG3 cells showing positive AP-staining. (*F*) Colony of dividing SG3 cells. Cells at passage 44 were maintained for undifferentiated proliferation at moderate density (3×10^5 cells per well of 12-well plate) for 5 days and fixed for Giemsa staining *in situ*. m, metaphase; n, nuclei; ni, nucleoli; spd, spermatid; spm, sperm. (*G–H′*) Analysis of spermatogonial gene expression by reporter assay. (*G*) Merged green and red fluorescent micrograph showing GFP expression from pOct4gfp. (*G′*) Schematic structure of pOct4gfp. The four conserved sequences (black boxes) in the 5′ regulatory region of the mouse Oct4 are shown with the deleted part boxed. DE, germ cell-specific distal enhancer; PE, epiblast-specific proximal enhancer. (*H*) Merged green and red fluorescent micrograph showing GFP expression from pVegfp. (*H′*) Schematic structure of pVegfp. The EGFP is flanked by a 5.1-kb 5′ regulatory sequence (V5′) and a 0.6-kb untranslated 3′ region (V3′) of the medaka vasa gene. SG3 cells at passage 70 were cotransfected with pDsRed-N1 plus pOct4gfp (*G*) or pVegfp (*H*) and photographed 3 days after transfection. Red cells are caused by ubiquitous expression of RFP from pDsRed-N1 that serves as an internal control; green cells result from GFP expression from pOct4gfp (*G*) or pVegfp (*H*); yellow cells result from the merge of both RFP and GFP. GFP-expressing cells are highlighted by arrows. (Bars = 20 μm in *A*, *D*, and *F*; 10 μm in *B* and *C*; and 50 μm in *G* and *H*.)

little AP staining. AP is a marker for the mouse primordial germ cells (1, 2), dividing spermatogenic cells (26) and ES cells (27) as well as medaka ES cells (20, 21).

Establishment of a Spermatogonial Cell Line. AP-negative cells were abundant during the initial 10 days and disappeared around day 60. Concurrently, AP-positive cells stably proliferated and eventually became the single cell type in culture. From two adult fish, we obtained one cell line that has so far been grown for >140 passages during 2 years in culture without any senescence. It is termed SG3 for spermatogonial cell line from i^3 medaka, because it closely resembles the freshly isolated spermatogonia (Fig. 1 *B* and *C*). SG3 cells are polygonal at low densities, and become round or oval and 10–15 μm in diameter at high densities. They display a smooth contour, sparse cytoplasm, and relatively large nuclei with prominent nucleoli (Fig. 1 *B–E*). They retain high AP activity (Fig. 1*E*). Under normal culture conditions, SG3 cells actively undergo self-renewing divisions (Fig. 1*F*).

Characterization of the Spermatogonial Property. To determine the identity of SG3 cells by reporter assay, we used two constructs expressing EGFP from the mouse Oct4 promoter (pOct4gfp; Fig. 1*G′*) or from the medaka vasa promoter (pVegfp; Fig. 1*H′*). Oct4 is a transcription factor that is expressed specifically in mouse primordial germ cells, type-A spermatogonia, and undifferentiated ES cells (24). pOct4gfp is similar to gcOct4-GFP that has been demonstrated for specific expression in mouse ES cells and ES-derived germ cells (12). Vasa is an RNA helicase of the DEAD-box protein family, and its mRNA expression is limited to germ cells in medaka (28) and mouse (29). Germ cell-specific expression from pVegfp has been demonstrated in transgenic medaka (23). SG3 cells after transfection with either construct gave rise to GFP-positive cells (Fig. 1 *G* and *H*). This coincides with the germ cell origin.

We further analyzed the germ cell identity of SG3 by using the transcripts of germ cell markers vasa, dazl, piwi, and c-kit. Vasa is expressed in spermatogonia and spermatocytes of medaka (28), mouse (29), and in mouse ES-derived germ cells (12–14). Dazl encodes an RNA-binding protein of the deleted-in-azoospermia gene family and is expressed in spermatogonia and spermatocytes in mice (13, 30, 31) and medaka (dada not shown). Piwi encodes a cytoplasmic protein expressed in mouse spermatogenic cells (32), and its expression in the adult testis is colocalized with vasa in zebrafish (33) and medaka (data not shown). C-kit is a tyrosine kinase receptor that, in the adult mouse testis, is highly expressed by spermatogonia and spermatocytes (34). SG3 transcribes all four of these genes (Fig. 2*A*), and thus appears to comprise male germ cells including spermatogonia.

We ruled out possible contamination with somatic cells, in particular, the Sertoli cell that is abundant in adult testes. Several Sertoli cell markers are known, including WT1, the Wilms' tumor suppressor (35), and DMRT1Y, a doublesex/mab3-related transcription factor necessary for male sex determination in medaka (36). SG3 transcribes neither WT1 nor DMRT1Y (Fig. 2*B*), and is thus virtually devoid of Sertoli cells.

We eliminated the possible transformation of SG3 line. SG3 cells underwent growth arrest after 2 days of culture in a medium from which growth factors were depleted. We monitored the chromosomes of SG3 cells at different passages to exclude gross chromosomal changes indicative of transformation and malignancy. Approximately 90% of SG3 cells have a diploid karyotype consisting of 48 chromosomes (Table 1 and Fig. 3*A*), characteristic of this organism (21). A diploid constitution was also indicated by flow cytometry analysis (see below). Interestingly, a low frequency (1%) of haploid metaphases was consistently detected (Fig. 3*B*). Apparently, SG3 is not transformed and shows genetic stability during long-term culture.

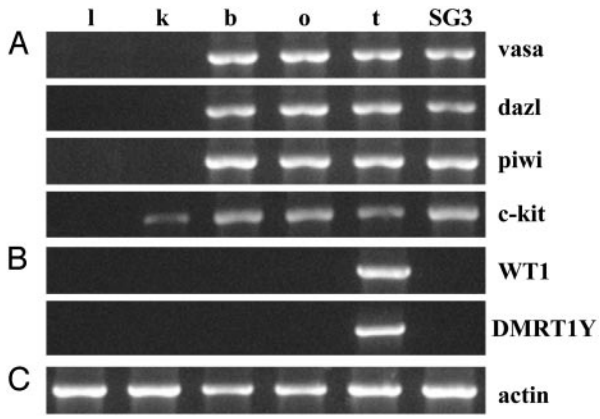

Fig. 2. Gene expression pattern of SG3 cells. (*A*) Expression of germ cell markers by RT-PCR of total RNA from blastula embryos and adult tissues with primers for vasa, dazl, piwi, and c-kit. (*B*) Absence of Sertoli cell markers by RT-PCR with primers for WT1 and DMRT1Y. (*C*) Actin expression was determined for calibration. b, blastula embryos; k, head kidney; l, liver; o, ovary; t, testis; SG3, SG3 cells under normal culture conditions for undifferentiated proliferation; SG*, SG3 cells maintained at confluence without subculture for induced meiotic differentiation. The liver and kidney were used as representative somatic tissues. A moderate level of the c-kit transcript is seen in the head kidney, in accordance with the fact that the head kidney is the fish hematopoietic organ equivalent to the adult mammalian bone marrow, where c-kit is expressed in hematopoietic stem cells.

Meiotic Differentiation. In the adult testis, type-A spermatogonia undergo a series of differentiating divisions to produce type-B spermatogonia, which become primary spermatocytes with a tetraploid DNA content and go through meiosis I to generate secondary spermatocytes with a haploid metaphase but a diploid DNA content; the second spermatocytes in turn go through meiosis II to give rise to spermatids with a haploid DNA content. Usually, the four round spermatids from a single primary spermatocyte are connected together by cytoplasmic bridges to form a tetrad (3). Telomerase-immortalized mouse type-A spermatogonia are capable of differentiation into spermatids (8) in response to stem cell factor (SCF), the ligand of c-Kit. Addition of recombinant human SCF to the culture medium did not promote meiotic differentiation of SG3 into spermatids (data not shown). Activation of c-Kit by SCF is highly species-specific (34). The inaccessibility to a fish SCF provoked alternative approaches. Because medaka ES cells can be induced for differentiation by enhanced cell–cell interactions (21), we maintained SG3 cells at full confluence without subculture for >2 weeks. Cell detachment occurred under this condition. Newly detached cells usually formed clusters of four round cells like tetrads (Fig. 3*C*). In the medaka, single tetrads are present in freshly isolated cells from mature testes (data not shown) and also in primary cultures of spermatocytes (16). Detached single cells took a round shape and often displayed a cytoplasmic extension, resembling elongating spermatids (Fig. 3*D*). After 1 day of suspension culture in bacteriological dishes, some of the detached cells generated sperm with a forming residual body (Fig. 3*E*). Thus, SG3 cells appear to be capable of meiosis.

We determined the meiotic gene expression of SG3 cells by using the mRNAs for DMC1 (disrupted meiotic cDNA) and protamine as markers of meiosis and its progression. DMC1 is a homolog of the bacterial RecA and transcribed exclusively in mouse primary spermatocytes at the meiotic transition into the leptotene stage of meiosis prophase I (37) and specifically in the meiotic spermatocytes of the adult medaka testis (data not

Table 1. Chromosome numbers of SG3 cells after different passages and days of culture

Passages/days of culture	Total counts, *n*	Counts of different chromosome numbers, *n*				
		24	44–47	48	49–52	96*
10/100	100	1	6	81	2	10
30/220	100	0	4	83	3	10
52/360	200	1	11	166	5	17
50/360†	200	6	10	163	4	17
84/466	200	3	22	159	3	13

*Metaphases with ≈96 chromosomes.
†Cells seeded at high density and maintained at full confluence without subculture for 2 weeks and attached cells were used for chromosome preparation.

shown). Protamine is an essential protein that replaces histones for tight package of chromosomes into the sperm head. In species ranging from man to fish, protamine mRNA is restricted to postmeiotic cells of the testis (3, 38). A low level of mRNAs of both genes was present in SG3 cells under normal culture conditions, in accordance with spontaneous meiotic differentiation as revealed by a low frequency of haploid metaphases. Noticeably, an elevated expression level of both genes was found in SG3 cells that were maintained at confluence without subculture (Fig. 3*F*). Thus, SG3 cells can show a gene expression pattern of meiosis.

Cell detachment at full confluence without subculture is reminiscent of nonattachment of spermatids and sperm in primary culture. Therefore, the attached and detached cells were collected separately for cytogenetic analyses by chromosome examination and flow cytometry. As a reference, adult testicular cells had three peaks of haploid (60% of total cells), diploid, and tetraploid DNA contents (Fig. 4*A*). SG3 cells under normal culture conditions exhibited, as expected for a diploid cell line of active proliferation, two peaks of diploid and tetraploid DNA contents (Fig. 4*B*). The detached cells displayed a significant haploid peak (19% of total cells) plus a diploid peak, with a tetraploid peak being missing (Fig. 4*C*). The attached cells generated a slightly increased frequency (3%) of haploid metaphases (Table 1) and exhibited just two peaks of diploid and tetraploid DNA contents (Fig. 4*D*). The DNA contents of the three peaks of SG3 cells are identical to those of testicular cells. Therefore, SG3 cells appear to be able to enter and complete meiosis to generate haploid products.

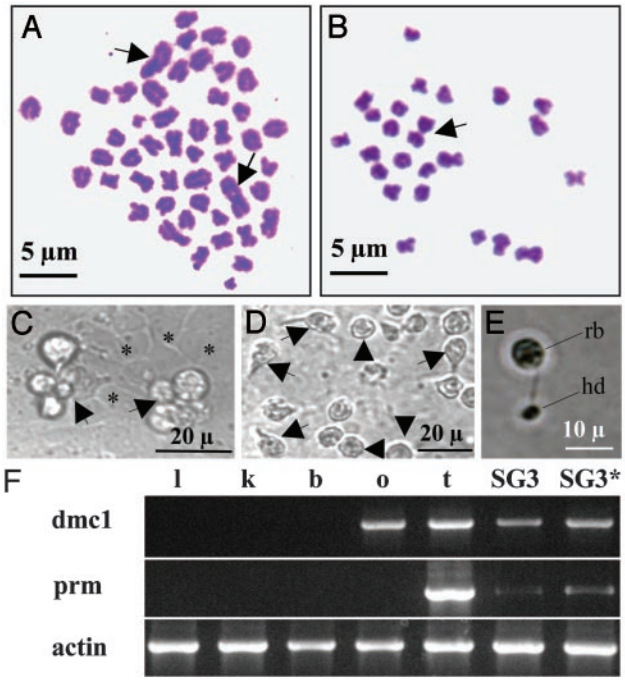

Fig. 3. Meiosis of SG3 cells *in vitro*. (*A* and *B*) Chromosomes of SG3 cells at day 360 of culture (passage 52). (*A*) Diploid metaphase of 48 chromosomes as seen in the majority of SG3 cells. (*B*) Haploid metaphase of 24 chromosomes occasionally seen in SG3 cells. Arrows indicate the largest chromosome. (*C–E*) Meiotic products formed at confluent growth without subculture. (*C*) Tetrad-like clusters (arrows) consisting of four round cells released from attached SG3 cells (stars). (*D*) Detached single cells showing similarities to round spermatids (arrowheads) and elongating spermatids (arrows). (*E*) Sperm associated with the residual body from detached SG3 derivatives after one day in suspension culture. hd, sperm head; rb, residual body. (*F*) Expression of meiotic genes by RT-PCR with primers for dmc1 and protamine. Expression of the dmc1 and protamine transcripts is low in undifferentiated SG3 cells. Elevated expression of both genes is seen in SG3 cells maintained at confluence without subculture. For further explanations, see the legend to Fig. 2.

Spermiogenesis. During spermiogenesis, the meiotic products round spermatids are converted into sperm through drastic morphological changes including the package of chromosomes into a condensed sperm head, formation of a flagellar tail, and extrusion of most cytoplasm as the residual body (3). Observa-

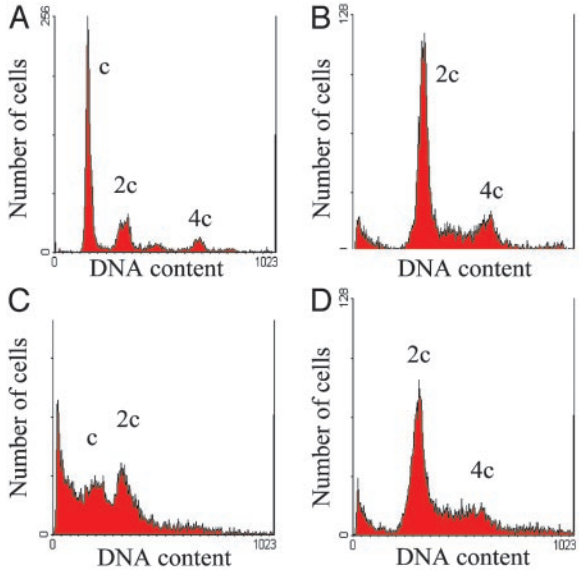

Fig. 4. Flow cytometry analysis. (*A*) Adult testicular cells showing a prominent haploid peak in addition to diploid and tetraploid peaks. (*B*) SG3 cells at passage 64 maintained under normal culture conditions for undifferentiated proliferation, showing diploid and tetraploid peaks. (*C* and *D*) SG3 cells at passage 68 maintained at confluence without subculture for 2 weeks, showing a haploid peak in detached cells (*C*) but not in attached cells (*D*). c, 2c, and 4c, haploid, diploid, and tetraploid DNA contents, respectively.

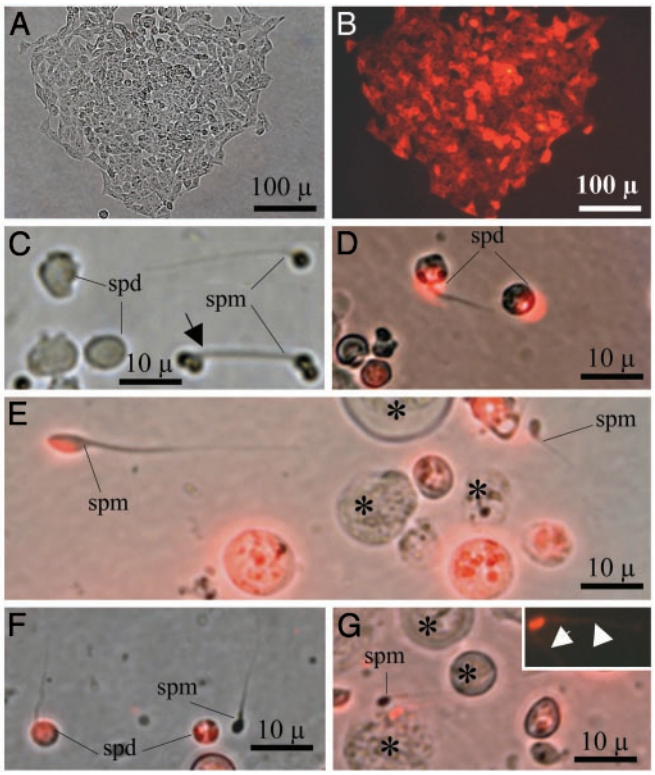

Fig. 5. Spermiogenic progression and sperm production *in vitro*. (*A* and *B*) Single colony of SG3 cells labeled with RFP by transfection with pDsRed-N1 at passage 70. (*C*) Sperm from adult medaka. A cluster of four sperm from a tetrad is indicated by an arrow. (*D–G*) Sperm production from clonally expanded, RFP-labeled SG3 cells in suspension coculture with rainbow trout gonadal RTG stromal cells. (*D*) Round spermatids and early elongating spermatids. (*E*) Maturing sperm showing a compacted head and a long tail of up to 50 μm. (*F*) RFP-negative, matured sperm with a fully (by darkness) condensed head of 3.5 μm in length and a thin and long tail. (*G*) RFP-positive, matured sperm. The motility of this sperm is reflected by different positions on the merged micrograph and the appearance of two fluorescent tails (arrows in *Inset*) during fluorescent image capturing. Note that SG3-derived sperm resemble natural sperm in morphology (*C*). (*A* and *C*) Differential interference contrast (DIC) micrographs. (*B* and *G Inset*) Fluorescent micrographs. (*D–G*) DIC-fluorescent merges. spd, spermatid; spm, sperm; asterisk, RTG cells.

tion of the residual body-associated sperm in detached SG3 derivatives after 1 day of suspension culture (Fig. 3*E*) indicated that SG cells had the potential to generate sperm. We confirmed this by using a coculture system. SG3 cells were mixed in suspension with stromal cells of the cell line RTG that was derived from the embryonic rainbow trout gonad. Chimeric aggregates formed between SG3 and RTG cells within 2 h after cell seeding. At day 2–3 of coculture, we sequentially obtained round spermatids, elongating spermatids, and motile sperm that were morphologically indistinguishable from their counterparts from the testis (data not shown). To unambiguously identify the SG3-derived spermatids and sperm during spermiogenesis, we labeled SG3 cells at passage 70 with RFP by transfection with pDsRed-N1. The RFP-expressing SG3 cells were clonally expanded for 2 months (Fig. 5 *A* and *B*), and then used for coculture in suspension again with RTG cells. After 2–3 days of coculture, the RFP-expressing SG3 cells gave rise to postmeiotic products comparable to their counterparts in freshly isolated testicular cells (Fig. 5*C*). These SG3-derived spermatids were RFG-positive; elongating spermatids possessed developing flagella of varying lengths (Fig. 5 *D* and *F*). Progressively maturing sperm displayed a condensing head of 5–8 μm in length and a tail of up to 60 μm (Fig. 5*E*). Matured sperm were characterized by a dense head of 3.5 μm in length, a long and thin flagellum, and little cytoplasm evidenced by the absence of cytoplasmic RFP signal (Fig. 5*F*), in accordance with the extrusion of bulk cytoplasm into the residual body. Fortunately, we observed a small but reproducible proportion (1%) of maturing and matured sperm that were partly or entirely RFG-labeled, allowing for unambiguous distinction by fluorescent microscopy. Most importantly, we obtained motile sperm that, like freshly isolated testicular sperm, measured 50 μm in total length and moved around (Fig. 5 *E–G*). Like their counterpart in primary culture from the adult testis, these SG3-derived mature sperm moved fast during a period of 5–10 min and slowed down the speed and distance of movement. Taken together, by morphology, gene expression pattern, cytogenetics, morphogenesis, and physiology, the SG3 cell line retains the potential to undergo meiosis and postmeiotic progression to form normal sperm in culture.

Discussion

This study reports the development of a normal spermatogonial cell line from an adult medaka testis and the production of motile sperm from this cell line in culture as a first success in the recapitulation *in vitro* of spermatogenesis in a vertebrate. The spermatogonial identity of the cell line SG3 is characterized by several lines of evidence. These include the testicular origin, spermatogonial phenotype, expression of germ cell markers, and more convincingly, the ability of meiosis and sperm production. In the adult testis, only the type-A spermatogonia have the ability of self-renewal and differentiation, the defining property of stem cells. The SG3 cell line shows this spermatogonial property by its long-term self-renewing cell divisions and differentiation potential during 2 years in culture, and thus appears to comprise type-A spermatogonia. The cell line also appears to consist of normal cells that have not become transformed during long-term cultivation, because it retains a normal karyotype and growth factor-dependent growth. The ability to obtain a normal spermatogonial cell line from an adult medaka testis demonstrates that the capability of continuous proliferation in culture is an intrinsic property of spermatogonial stem cells and that immortalization apparently is not necessary to derive male germ cell cultures.

We attribute the success to the use of a suitable culture system. The feeder-free culture condition similar to those used for SG3 derivation in the present study has previously enabled the derivation of medaka ES cell lines (20–22). We reasoned that this system could also support stable proliferation of adult germ stem cells in culture. Indeed, this feeder-free culture system has allowed for reproducible derivation of testicular cell cultures from different strains of medaka (T.L. and Y.H., unpublished data). Intriguingly, the established SG3 cells undergo rapid growth arrest upon depletion of growth factors from the culture medium. This finding underscores that, although spermatogonia have the potential of continuous proliferation, their long-term cultivation depends strongly on the proper culture conditions. However, at the present, it is not known whether this culture system can also be adopted for other species. In the mouse, glial cell-derived neurotrophic factor is important for spermatogonial proliferation (10), and in the eel, platelet-derived endothelial cell growth factor is a spermatogonial stem cell renewal factor (39). It will be interesting to determine what a factor(s) in the germ cell culture medium is essential for the cultivation of medaka testicular germ cells, as one important medium component is a crude embryo extract whose composition is unknown because of its heterogeneity.

Spermatogenesis proceeds through three major stages: mitotic phase of proliferation and differentiation, meiosis, and postmeiotic spermiogenesis. Meiosis results in round spermatids, whereas spermiogenesis leads to sperm. Much progress has

recently been reported for male germ cell culture and their meiotic differentiation. In mammals, male germ cells can be cultured for several months, and their meiotic differentiation can proceed up to but not beyond the spermatid stage *in vitro* (6, 8–11, 13, 14). It is unknown whether the inability to obtain sperm *in vitro* from spermatogenic cells is intrinsic to mammals or acquired by immortalization or culture conditions. In fish, fertile sperm can be obtained from spermatocytes in primary culture (16, 17). We have demonstrated that the SG3 cell line retains the ability of long-term self-renewal, meiosis, and spermiogenesis to generate motile sperm. Therefore, at least in the medaka, sperm production can be obtained from established germ cell lines *in vitro*. Our work corroborates and extends these reports by demonstrating the establishment of a stable cell line of normal spermatogonial stem cells and the successful recapitulation *in vitro* of all three spermatogenic stages, including postmeiotic progression into motile sperm.

Spermatogenesis is an excellent model system for stem cell biology. The ability to recapitulate spermatogenesis *in vitro* makes the SG3 cell line a unique paradigm for studying the mechanisms underlying mitotic proliferation and differentiation of stem cells, meiosis, and their regulation in a vertebrate. The SG line will also offer a powerful system to study the molecular basis of male infertility caused by genetic factors and gonotoxins.

Transgenic mice have been obtained by retroviral transduction of primary spermatogonial cultures (4, 5). The efficiency remains to be improved because of the difficulty in transfecting and drug-selecting primary cultures for desired genotypes. Use of spermatogonial cell lines may resolve this difficulty. Specifically, it would be interesting to examine whether the male germ stem cells can be used as an alternate to ES cells for germ-line transmission. In mammals, germ-line transmission of male germ cell cultures has been achieved by cell transplantation into the testis (9–11) and by nuclear transfer of haploid cells into the oocytes (13, 14, 18). A third approach exists in fish. This approach is *in vitro* spermatogenesis followed by artificial insemination, as has been shown with the primary culture system where sperm production from piscine spermatocytes proceeds in a programmed manner (16, 17). In the present study, we have shown the motility of the SG3-derived sperm, an essential property of functional sperm. However, the fertility of these SG3-derived sperm remains to be determined by artificial insemination: under the present culture conditions, a short life span of the SG-derived sperm in combination with their continuous but nonprogrammed production did not provide a number of motile sperm sufficient for artificial insemination. Sperm nuclear transfer has been established in zebrafish (40). Whether the SG3-derived sperm can be used for this approach remains to be addressed. Therefore, the establishment of SG3 as a first normal spermatogonial cell line capable of sperm production *in vitro* makes the medaka a unique vertebrate model for developing approaches to reproductive engineering. It is of particular interest to test whether the SG3 cell line can be used for gene targeting and for germ-line transmission by cell transplantation, nuclear transfer, and/or *in vitro* sperm production followed by artificial insemination.

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