Characterization of type A spermatogonia and development of the culture system in zebrafish

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2007

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1. Abstract

Continuation of spermatogenic process throughout life relies on a proper regulation of self-renewal and differentiation of spermatogonial stem cells which is present in a special cellular organization called a niche. In spite of biological significance of the spermatogonial stem cells, little is known about their behavior and properties because of a lack of model system to approach the stem cell maintenance and differentiation in several organisms.

In this study, I show the presence of subpopulations in zebrafish type A spermatogonia which is classically considered homogeneous population, varying in size, location pattern and cell cycle phase. Morphological analysis showed two types of type A spermatogonia that are localized distant from basement membranes (termed type A_{dt}) and close to basement membranes (A_{cl}). The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) incorporation experiments showed that duration of cell cycle of the type A_{cl} spermatogonia was longer than that of the type A_{dt} spermatogonia. Tracing label-retaining cells (LRCs), after 4 weeks of chase, I observed occasionally BrdU-positive type A_{cl} spermatogonia. Immunohistochemical analysis using antibody against zebrafish homologue of Synthetic lethal mutant of dpb11-1 (zSld5) which is able to distinguish between the quiescent cells and the proliferation cells showed that type A_{cl} spermatogonia could be divided into two subpopulations. One population is zSld5-positive type A_{cl} spermatogonia (A_{cl}^{+}) and the other is zSld5-negative type A_{cl} spermatogonia (A_{cl}^{-}). In addition, a lot of LRCs are contained in

a latter population. All the results suggest that type A_{cl}^{-} spermatogonia are most undifferentiated type A spermatogonia.

It is generally known that spermatogonial stem cells have ability to restore spermatogenesis when a testis was damaged by a drug or irradiation. To elucidate the characterization of type A spermatogonia, I examined the effect of cytotoxic agent busulfan on male germ cells. As a result, only type A_{cl} spermatogonia survived after treatment of busulfan, and then recovered spermatogenesis. The morphological observation suggests that type A_{cl} spermatogonia are the most resistant cell population against busulfan in the zebrafish testis. Detailed analysis revealed that the number of type A_{cl}^{+} spermatogonia increased transiently after busulfan treatment, while the number of type A_{cl}^{-} spermatogonia relatively stabilized. These results suggest that both type A_{cl}^{-} and A_{cl}^{+} spermatogonia would posses a feature of spermatogonial stem cells, like an actual stem cell and a potential stem cell, respectively.

Furthermore, I developed the *in vitro* culture condition for supporting the maintenance and proliferation of type A spermatogonia by the use of Sertoli feeder ZtA6-6 cells for 1 month or so.

This study represents the first step towards further understanding the character of spermatogonial stem cells and molecular mechanisms to maintain the undifferentaition status in teleosts.

2. Introduction

Spermatogenesis typifies the development of a stem cell derived tissue. Continuous production of highly differentiated, short-lived sperm is maintained throughout reproductive life by a small, dedicated population of spermatogonial stem cells. As precursors of the spermatogonial lineage, spermatogonial stem cells must maintain a balance between the production of mature sperm and the self-renewal of stem cell potential throughout adult reproductive life, as defined by their function as persistent, clonogenic founders of differentiating germ cells. It is known that spermatogonial stem cells can be identified by multiple criteria, including anatomical position in the testis and distinct behavioral phenotypes. They are a rare, relatively quiescent population that lies in a protected region in the testis among support cells, which may regulate their behavior. Furthermore, spermatogonial stem cells exhibit many similarities to other stem cell systems. Like all stem cells, spermatogonial stem cells are the most resistant cells to irradiation of chemical damage. As with hematopoietic stem cells, spermatogonial stem cells in mammals are transplantable (Brinster and Zimmermann, 1996), with an ability to both expand the stem cell pool and to regenerate an entire depleted spermatogenic linage (Kiger and Fuller, 2001).

Generally, a stem cell is present in a special cellular organization called a niche, which provides a microenvironment designed to maintain the proliferation or survival of stem cells (Schofield, 1987). In a niche, the stem cell has a high probability of self-renewal and divides rarely. When a stem cell divides, it has been proposed that only

one daughter can remain in the niche, and the other cell will be committed to differentiate unless another niche is available. Therefore, by definition, if space with the necessary microenvironment were limited, the number of stem cells also would be limited (Schofield, 1987). Although the niche theory was proposed originally to explain the behavior of transplanted hematopoietic stem cells (Schofield, 1987), this theory now has been extended to stem cells in several organs (Xie and Spradling, 2000; Tran et al., 2000; Kiger et al., 2001; Williams, 1992), and the niche is thought to be involved in the transdifferentiation of stem cells (Bjornson et al., 1999). The niche must provide factors that maintain stem cells and exclude factors that induce differentiation (Schofield, 1987; Xie and Spradling, 2000; Tran et al., 2000; Kiger et al., 2001).

In *Drosophila*, approroximately nine spermatogonial stem cells lie at the apical tip of the testis, forming a ring that closely surrounds a cluster of post-mitotic somatic cells called the hub (Hardy et al., 1979). When the spermatogonial stem cell divides, it normally give rise to one cell that retains stem cell identity and one cell, called a gonialblast, that is displaced away from the hub and initiates differentiation. Therefore, the hub functions as a stem cell niche. Hub cells express the signaling ligand Unpaired (Upd), which activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway within spermatogonial stem cells to maintain stem cell identity (Kiger et al., 2001; Rawlings et al., 2004; Tulina and Matunis, 2001). Recent studies suggest that BMP homologue, *decapentaplegic* (Dpp), which is expressed in both hub cells and somatic stem cells also plays a role in spermatogonial

stem cells maintenance or survival (Kawase et al., 2004; Schulz et al., 2004; Shivdasani and Ingham, 2003). Spermatogonial stem cells are anchored to the hub cell with E-cadherin, and spindle orientation ensures a reliably asymmetric outcome of spermatogonial stem cell divisions, consistently placing one daughter within the reach of short-range signals from the hub and positioning the other away from the niche (Yamashita et al., 2003).

Unlike in *Drosophila*, in the seminiferous tubules in the mammalian testis, obvious anatomical niche has not been identified. The Sertoli cell divides the seminiferous tubule into a basal compartment comprising mainly spermatogonia and an adluminal compartment where more advanced germ cells are sequestered behind a blood-testis barrier formed by tight junctions between adjacent Sertoli cells. Although spermatogonial stem cells are in direct contact with Sertoli cells, that nourish developing spermatogonia, and the basal lamina that surrounds each tubule, no functional subcompartment has been identified among the vast area delimited by these components. Transplanted germ cells colonize sites on the wall of the tubule, each of them corresponding morphologically to the basal compartment. There is the only place in which spermatogonial stem cells can reside to maintain themselves and must therefore be regarded as the niche for spermatogonial stem cells (Nanano et al., 1999). The successful restoration of spermatogenesis in infertile *Steel/Steel^{dickie}* mice by wild-type Sertoli cell transplantation (Shinohara et al., 2003) has confirmed that a Sertoli cell is one of the major contributors to stem cell niche.

However, it is likely that other players are involved in spermatogonial stem cells niche formation. These players include the basement membrane of the seminiferous tubules, which is shared with the surrounding peritubular cells, and, possibly, some signals external to the seminiferous tubules. Recently, Yoshida et al. (2007) revealed that branching of blood vessels in the testes may define the location of niches of spermatogonial stem cells. The limited number of active niches suggests that all Sertoli cells cannot be equal, and only Sertoli cells near interstitial that harbor a branched vasculature can support spermatogonial stem cells.

A key molecule to contribute to the mammalian spermatogonial stem cells niche is glial cell line-derived neurotrophic factor, GDNF, a protein member of the transforming growth factor (TGF)-β superfamily, which is produced and secreted by Sertoli cells from birth through adulthood (Meng et al., 2000; Tadokoro et al., 2002). Spermatogonial stem cells express GDNF receptors, i.e., GFRα1 and c-RET tyrosine kinase receptor (Meng et al., 2003; Yomogida et al., 2003) which acts downstream by activating phosphatidyl-inositol3' (PI-3')- kinase and/or the Src family of tyrosine kinases (Sariola and Saarma, 2003). The GDNF has been shown to be critical for the proliferation of spermatogonial stem cells in culture (Kanatsu-Shinohara et al., 2003a; Kubota et al., 2004). Another Sertoli cell product, ERM, a transcription factor which is exclusively expressed in mature Sertoli cells, has been shown to be required for spermatogonial stem cells self-renewal and maintenance of spermatogenesis in adult mice (Chen et al., 2005). It has been hypothesized that spermatogonial stem cell niche

regulation changes with age; GDNF could control spermatogonial stem cell self-renewal during the perinatal period of development, while ERM could during the pubertal period (Hess et al., 2006).

Although there are reports in the *Drosophila* or the mouse in regarding the molecules and pathways that underlie stem cell maintenance and differentiation, little is known about the common conserved mechanisms between both organisms. This is due to the lack of a model system which is able to approach the stem cell maintenance and differentiation in several organisms.

In a teleost testis, spermatogenesis occurs in cysts within the seminal tubules isolated from interstitial cells by basement membranes. A cyst unit is formed when Sertoli cells enclose a single primary spermatogonium during the male gonad developmental period. An isolated single germ cell in each cyst is defined as a type A spermatogonium (Billard, 1986). During spermatogenesis, the type A spermatogonia divide and produce isogenic differentiating germ cells (termed type B spermatogonium) that are committed to enter meiosis within the cyst after five or six divisions in zebrafish (Ewing, 1972). The characteristics of Sertoli cells in each cyst would correspond to the stage of the germ cells within the cysts, having advantages in the analysis for the functions of Sertoli cells in the niche for spermatogonial stem cells.

In addition, the co-culture system of Sertoli cells and germ cells has been established in zebrafish, in which the differentiation from spermatogonia to functional sperm can occur *in vitro* (Sakai, 2002). The large-scale saturated mutagenesis project

has generated literally thousands developmental mutants, including a lot of defects in spermatogenesis (Bauer and Goetz, 2001). The combination of the mutants in spermatogonial stem cell development together with *in vitro* culture could lead to better understanding of the niche for spermatogonial stem cells in vertebrate. Despite these advantages, unequivocal characterization and identification of the spermatogonial stem cells in zebrafish has not been achieved.

In the present study, I performed characterization of zebrafish type A spermatogonia and development of the culture system *in vitro*. Initially, I ascertained whether all type A spermatogonia have a feature of spermatogonial stem cells, and identified subpopulations of type A spermatogonia. This examination is based on their location, morphometric features, and on cell cycle phases with 5-bromo-2'-deoxyuridine (BrdU) and zSld5 expression which is a member of DNA pre-replication complex. Second, I examined the kinetics of type A spermatogonia against cytotoxic agent, busulfan. Finally, I developed the culture condition that enable to maintain and proliferate type A spermatogonia with a Sertoli cell line as feeder cells. This study represents the first step towards understanding mechanisms of not only spermatogenesis in teleost but also stem cell systems in multicellular organism.

3. Materials and methods

Fish stock

Zebrafish Danio rerio wild-type were obtained from Dr. N. Hopkins (Center for Cancer Research, Massachusetts Institute of Technology). Transgenic *vas::EGFP* zebrafish were obtained from Dr. L. Olsen (Sars International Centre for Marine Molecular Biology, Bergen High Technology Centre). Zebrafish were maintained as described (Westerfield, 1995).

Sample preparation for histological analysis

Testes were collected from adult zebrafish at 3-10 months of age, fixed in Bouin's solution at 4°C for over night, dehydrated through an ethanol series, embedded in paraffin, and sectioned at 5 μm thickness using rotary microtome 2065 Supercut (Leica microsystems Co. Ltd., Tokyo, Japan).

Hematoxylin and Eosin (HE) staining

Paraffin was removed from the sections by rinse in 100% xylene for 5 minutes at three times, and the sections were hydrated progressively in 100%, 90%, 80%, 70% ethanol series for 5 minutes at each step. The sections were stained with hematoxylin (Wako, Japan) for 5 minutes. After washing with running water for more than 30 minutes, the sections were stained with eosin (Muto Pure Chemicals Co. Ltd, Japan) for 1 minute. Then the sections were dehydrated progressively in 70%, 80%, 90%, 100% ethanol

series for 5 minutes at each steps, and dehydrated in 100% xylene for 5 minutes at three times. These procedures were done at room temperature. Samples were observed using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

BrdU labeling

Two protocols were established for labeling the type A spermatogonia with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) *in vivo*. For short term labeling, zebrafish were allowed to swim for 24, 48 h at 28°C in fish water containing BrdU (1 mg/ml; Sigma Chemical Co., St. Louis, MO) and were then put into fresh fish water. For long term labeling to detect slow-cycling cells, zebrafish were allowed to swim for 3 days in fish water containing 1 mg/ml BrdU, then were put into fresh fish water for 1 day, and this labeling was repeated three times. After the labeling, zebrafish were sacrificed to determine the initial BrdU labeling at day 0. After the several chase periods, testis were collected and analyzed for BrdU-labeled nuclei by immunohistochemistry and immunofluorescence.

For labeling of ZE cells, BrdU labeling reagent (Amersham) was added to the culture medium during the last 24 hour of the culture period. Cells were fixed with acid-alcohol (90% ethanol: 5% acetic acid: 5% water) at room temperature for 30 minutes, washed with PBS containing 0.05% Tween 20, and detected BrdU-labeled cells as described bellow.

For labeling of zebrafish type A spermatogonia in culture, BrdU labeling reagent

(Amersham) was added to the culture medium during the last 2 days of the culture period. Cells were fixed with acid-alcohol at room temperature for 30 minutes. The remaining steps of the detection of BrdU were performed same conditions in case of ZE cells.

Detection of BrdU

The detection of BrdU both sections and culture cells was performed with a Cell Proliferation Kit (Amersham) according to the manufacturer's manual. In case of immunofluorescence, the sections were stained with hematoxylin solution and then incubated for 1 h at RT with Alexa Fluor 488-conjugated anti-mouse IgG (1:800) (Molecular Probes) as the secondary antibodies. For the quantification of BrdU-labeled cells, randomly selected three sections from each sample were counted at least 200 cells.

For detection of BrdU-labeled cells in ZE cells, unlabeled cells were visualized by propidium iodide (5 μg/ml) staining. Cells were counted using a Nikon ECLIPSE TE2000-S fluorescent microscope (Nikon, Tokyo, Japan). From each sample, at least 1000 cells were counted, and mean values from three independent experiments.

Measurement of type A spermatogonia

Type A spermatogonia were measured to determine the length, area of the nucleus, area of the cytoplasm and the ratio of the area of the nucleus to the cell size. Images of the

cells were obtained from an Olympus BX51 microscope. All the images were converted to binary images using the NIH Image J software. The cell edge was traced manually using Adobe PhotoshopCS3 (Adobe System Inc., USA). The length and area of cells in the image were then automatically measured.

Cloning of zSld5

To determine whether zebrafish possesses a gene orthologous to mammalian Synthetic lethal mutant of dpp11-1, Sld5, I searched the zebrafish genome database by tBLSTn comparison with the mouse Sld5 (NM_024240). The best zebrafish hit (BC078242) resulting from this search, was then compared with GenBank to infer its nature, resulting as best hit mammalian Sld5, followed by *Xenopus laevis* Sld5. This finding provided the first evidence that zebrafish possesses a Sld5 ortholog.

Culture of ZE cells

ZE (zebrafish embryonic) cell line was established from 5 day-old embryo and cultured in L-15 (Leibovitz's L-15 medium, Gibco) supplemented with 2mM L-glutamine (Gibco), 50U/ml penicillin–50 μg/ml streptomycin (Gibco), 100 μg/ml Kanamycin sulfate (Gibco), 800 μM CaCl2 (Sigma), 10% fetal bovine serum (FBS, Gibco) 20 embryos per ml of embryo extract (Westerfield, 1995). For serum starvation, cells at 60-80% confluence (in exponential growth phase) were rinsed with PBS, and then cultured in L-15 containing 0.5% FBS for 7 days. For serum stimulation, cells after

serum starvation were passaged, and then cultured for 4 days.

RT-PCR

Samples from testis and ZE cells were homogenized in Isogen (Nippon gene, Tokyo, Japan), and total RNA was extracted following the manufacturer's instructions. Total RNA was treated with RNase-free DNase I (Roche Diagnostics, Tokyo, Japan). The reverse transcripts and the PCR products of total RNA were obtained using a TaKaRa RNA PCR Kit with *zsld5* and *α-tubulin* primers for 30 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec. The *zsld5* primers (forward, 5'-CTGGTACAGTTTGCGCTGAA-3'; reverse, 5'-CTGATTCCTTCATGGCGTCT-3') and the *α-tubulin* primers (forward, 5'-AACTCCATCCTGACCACCCACACC-3'; reverse, 5'- AGCCAGATCACCACCTGGAACCAC-3') amplified a 813-bp fragment, and a 552-bp fragment, respectively.

Production of polyclonal antibody against zSld5

To use as antigen, recombinant zSld5 was prepared by bacterial expression. cDNA fragments encoding a full-length of zSld5 was amplified by RT-PCR using the primers with *Sph* I site, 5′-TATGCATGCATGACCCCGGCAGAGCTCAT (forward primer) -3′ and *Hind* III site, 5′-TCGAAGCTTTTAAATGAGCTGCACTGCTC (reverse primer) -3′. After digestion with *Sph* I and *Hind* III, the digested fragments were subcloned into a pQE-30 expression vector (QIAGEN, Hilden, Germany) with the sequence encoding 6

× histidine. After subcloning into the pQE vector, recombinant zSld5 was expressed in *E. coli* following induction by isopropyl β-d-thiogalactopyranoside (IPTG) and purified using the QIAexpressionist (QIAGEN). Briefly, the fusion proteins were purified from the bacterial lysate by affinity chromatography using the metal chelate adsorbent nickel–nitrilotriacetic acid agarose (QIAGEN). The fusion protein was eluted with elution buffer (8 M urea, 1 M NaCl, 0.1 M sodium phosphate, 10 mM Tris, pH 4.0), and dialyzed for 3 days against 10 mM phosphate buffered saline (PBS), pH 7.5. Approximately 1.5 mg of purified protein was immunized four times into a rabbit (Keari Co. Ltd., Osaka, Japan).

Serum was collected after the fourth injection. The antiserum against recombinant zSld5 was mixed with bacterial lysate, which was transformed pQE30 vector only, to absorb the antibodies against the bacterial lysate that might have been generated by contamination during the purification of the recombinant zSld5. The mixed solution was centrifuged at 14,000×g for 1 h and the supernatants were collected. Moreover, in order to purify rabbit IgG against recombinant zSld5 (anti-zSld5), the supernatant was mixed with saturated ammonium sulfate in 10 mM phosphate buffer (PB) (pH 7.0) and were adjusted to 40% saturated solution. After stirring overnight at 4°C, the precipitate was sedimented by centrifugation for 15 min at 14,000×g. The pellet was resuspended in 10 mM PBS (pH 7.5) and dialyzed overnight against 17.5 mM PB (pH 6.3). Then, the IgG against zSld5 (anti- zSld5) was purified by anion-exchange chromatography on a DEAE-cellulose (DE52, Whatman, Kent, UK) column equilibrated with 17.5 mM PB

(pH 6.3).

Electrophoresis and Western blot analysis

The homogenate of the testes or ZE cell was mixed with an equal volume of sample buffer (0.125 M Tris–HCl, 4% (W/V) sodium dodecyl sulfate (SDS), 20% (V/V) glycerol, 0.05% (W/V) bromophenol blue) with reduction by 10% 2-β-mercaptoethanol (2ME). After mixing with sample buffer, all samples were boiled for 10 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Tris-glycine buffer system (Laemmli, 1970). For Western blot analysis, the proteins separated on the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated and shaken for 1 h in Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 0.5 M NaCl) containing 5% skim milk to block non-specific binding site, and then immersed overnight at 4°C in a solution containing anti-zSld5 at dilution to 1:500 by TBS containing 5% skim milk. After washing with TBS containing 0.05% Tween 20 and then with TBS, the membrane was incubated with bovine anti-rabbit IgG-HRP (Santa Cruz Biotechnology) diluted to 1:5000 in TBS for 1 h. After washing, HRP activity was detected using Immobilon Western (Millipore, Bedford, MA, USA), and analyzed using a Lumi-Imager F1 (Boehringer Mannheim).

Immunohistochemistry of zSld5

Testis samples were fixed with Bouin's and embedded in paraffin. Paraffin was removed from section, and the sections were hydrated with same conditions as described above. Paraffin sections were microwaved in 5 mM EDTA buffer (pH 8.0) for 5 minutes to unmask the antigen. After washing in water, the sections were blocked in 5% skim milk and 10% normal goat serum in PBS for 1 hour at room temperature and then incubate overnight at 4°C in a solution containing anti-zSld5 at dilution to 1:200 by PBS containing 5% skim milk and 10% normal goat serum. Sections were washed with PBS, incubate with biotinylated anti-rabbit IgG for 1 hour and treated with avidin-biotin alkaline-phosphatase complex for 30 min prior to staining with NBT/BCIP as substrate for 10-20 min. In addition to immunohistochemical staining of zSld5, some sections were incubated with anti-BrdU antibody as described above after stained with zSld5.

For detection of zSld5 in ZE cells, the cultured cells were fixed in ice-cold acetone-methanol (50% acetone: 50% methanol) for 10 min, washed with PBS containing 0.05% Tween 20 and then incubate overnight at 4°C in a solution containing anti-zSld5 at dilution to 1:500 by PBS containing 5% skim milk and 10% normal goat serum. The remaining steps of the immunohistochemical staining were performed as described above.

Busulfan treatment

In the first set of experiment with effect of busulfan on male germ cells, busulfan (Sigma Chemical Co., St. Louis, MO) was first dissolved in dimethyl sulfoxide (Sigma),

and then busulfan was added to fish water a final concentration of 6.6, 13.2, 33 and 66 μg/ml. Adult zebrafish were allowed to swim for 24 h at 28°C in busulfan containing fish water, and then put into fresh fish water. After 1 week, testes were collected and fixed (see above).

In the second set of experiments with busulfan-induced regeneration, busulfan (66 μg/ml) was used to eliminate differentiating germ cells. After a particular incubation period in fresh fish water, testes were collected and fixed.

Culture of type A spermatogonia

To develop the culture system of type A spermatogonia, co-culture system of zebrafish male germ cells and Sertoli cell line (Kurita and Sakai, 2004) was used in this study. Busulfan-treated testes were dissociated with 500 units/ml of collagenase (ICN Biomedicals) in L-15 at 28°C for 2 h by pipetting every 20 min. The cell suspension was diluted seven times with L-15 containing BSA and Hepes (pH 7.9) at the final concentrations of 1% and 20 mM, respectively. After removal of undissociated fragments, the suspension was centrifuged at 35g for 10 min. The centrifugation pellet was resuspended in testicular cell culture medium (TCCM) supplemented with 3% FBS (Sakai, 2002).

Feeder cells for the male germ cell culture were prepared from ZtA6 cell-derived ZtA6-2 and ZtA6-6 cell line (Kurita and Sakai, 2004), which exhibited features characteristic of Sertoli cells, and treated with mitomycin C (10 μg/ml in L-15, Sigma)

for 3 h. Typically, dissociated testicular cells of four males were plated on the feeder cells of a 35-mm culture dish. The next day, half of the medium was replaced with fresh TCCM supplemented with 7% crucian carp (Carassius carassius) serum, 50 ng/ml 11-ketotestosterone (Fukube chem, Japan) and 1 μg/ml forskolin (Sigma). The medium was changed every 3 days.

In the latter experiments, a transgenic zebrafish strain in which spermatogonia and premeiotic spermatocytes are labeled by expression of enhanced green fluorescence protein (EGFP) under the control of *vas*-gene regulatory regions, known as *vas::EGFP* (Krøvel and Olsen, 2002) was used for culture.

In situ hybridization

To detect the expression of *vas*, *in situ* hybridization was performed in cultured cells. Cells were fixed by 4% paraformaldehyde for 30 min at 4°C and were hybridized with digoxigenin-labeled riboprobes transcribed *in vitro* according to the manufacturer's manual (Roche, Tokyo, Japan).

Statistical analysis

All data represent the average of the indicated number of samples ± the standard deviation (SD). Statistical significance was analyzed with Aspin-Welch's t-test or Student's t-test, following F test.

4. Results

4.1 Characterization of type A spermatogonia

4.1.1 Type A spermatogonia can be classified into two types by their location and morphology

Cysts of germ cells are always separated from the testicular interstitial cells by a basement membrane (Fig. 1B, black arrowheads). The basement membrane of the germinal epithelium is usually well-stained by eosin stain. To examine the location of type A spermatogonia within the tubule, serial sections of the testes were stained with hematoxylin and eosin. The results showed that type A spermatogonia not only lie immediately close to basement membranes (termed type A_{cl}) (Fig. 1A,B, white arrowhead) but also locate distant from basement membranes (termed type A_{dt}) (Fig. 1B, white arrow).

To examine the morphometric features between these cells, the length of nucleus, areas of the nucleus and cytoplasm were measured, and the ratio of the nucleus to whole cell size was calculated (Fig. 2). The average of the longest length in type A_{cl} spermatogonia was 7.6 ± 1.1 μm (n = 65), and that in the type A_{dt} spermatogonia was 8.8 ± 1.0 μm (n = 33) ($P < 0.001$). The cell length of the type A_{dt} spermatogonia was thus ≈ 1.2 μm (≈ 13.6%) longer than that of the type A_{cl} spermatogonia (Fig. 2A). In addition, the area of the nuclei of the type A_{cl} spermatogonia was 34.3 ± 8.5 μm^2 (n = 65), while that of the type A_{dt} spermatogonia was 46.5 ± 8.1 μm^2 (n = 33) ($P < 0.001$). The area of the nuclei of the type A_{cl} spermatogonia was larger than that of the type A_{dt}

spermatogonia by about ≈ 12.2 μm^2 (≈ 26.2%) (Fig. 2B). Although the nucleus/cell size (N/C) ratio of the type A_{cl} spermatogonia was a slight decrease as compared with that the type A_{dt} spermatogonia, the difference was not significant (Fig. 2C). The results indicate that the type A_{cl} spermatogonia and type A_{dt} spermatogonia showed distinct morphometric features.

4.1.2 The cell cycle of type A_{cl} spermatogonia was longer than that of the other spermatogonia

It was reported that undifferentiated type A spermatogonia, which contained spermatogonial stem cells, are known to divide randomly and their cell cycle is much longer than that of the other spermatogonia in mouse (Monsei, 1962; Huckins, 1971). To compare the length of the cell cycle of type A_{cl} spermatogonia, type A_{dt} spermatogonia and type B spermatogonia, I performed DNA labeling experiments with administration of BrdU to zebrafish. Administration of BrdU had no effect on subsequent growth. After 24 or 48 hr administration, a labeling index of BrdU in type A spermatogonia and type B spermatogonia was determined (Fig. 3). Within 24 hr, 18.8% of type A_{cl} spermatogonia were labeled with BrdU, and 46.8% of type A_{dt} spermatogonia were labeled with BrdU. 77.3% of type B spermatogonia were labeled with BrdU. Within 48 hr, 79.5% of type A_{dt} spermatogonia were labeled with BrdU and almost all of type B spermatogonia had taken up BrdU, while only 42.5% of type A_{cl} spermatogonia were labeled with BrdU. This result indicates that duration of cell cycle

of the type A_{cl} spermatogonia was much longer than that of the type A_{dt} spermatogonia and type B spermatogonia.

4.1.3 Fate of labeled type A spermatogonia

To analyze the fates of the labeled type A spermatogonia, zebrafish were labeled by BrdU administration for 48 hr. As shown in Figure 4B, both type A_{cl} spermatogonia and type A_{dt} spermatogonia were decreased immediately after pulse-label. At day 4 of chase, I observed that, in most cases, BrdU-positive cells had a low reactivity against BrdU antibody compared with initial control (Fig. 4C,D). The label of BrdU was diluted through mitotic divisions, as unlabeled nucleotides are incorporated into newly synthesized DNA. The result showed that the initial labeled cells undergo mitotic divisions after administration of BrdU. During the following 4 days, type B spermatogonia could divide and differentiate into more differentiated type B spermatogonia or spermatocytes, since the length of the cell cycle of type B spermatogonia was shorter than type A spermatogonia. In spite of this feature of type B spermatogonia, I observed that small clones of type B spermatogonia which are considerable low reactivity against BrdU antibody compared with initial control (Fig. 4C,E). The weakly labeled type B spermatogonia could be differentiated cells that initial labeled type A_{dt} spermatogonia through mitotic divisions. Hence, the result indicates that initial labeled type A_{dt} spermatogonia were proliferate and differentiate into type B spermatogonia.

4.1.4 Detection of label-retaining cells

The results of preceding experiment suggest that type A_{cl} spermatogonia contain slow-cycling cells that do not incorporate BrdU during 48 hr administration. Slow-cycling cells are widely accepted as a common feature of adult stem/progenitor cells; this has been demonstrated in the studies of various epithelial (Bickenbach, 1981; Cotsarelis et al., 1990; Lehrer et al., 1998; Morris and Potten, 1999; Taylor et al., 2000), hematopoietic (Bradford et al., 1997; Cheshier et al., 1999) and testicular stem cells (Ehmcke et al., 2005). Thus, a method of tracing label-retaining cells (LRCs) is able to directly identify stem/progenitor cells without the specific markers.

On day 0, after long term BrdU labeling of zebrafish was completed, the majority of type A_{cl} spermatogonia (88.9% ± 5.1%, n = 3) and almost of all other germ cells, which contained type B spermatogonia, were stained by BrdU (Fig. 5B,C). During a week, the percentage of type A_{cl} spermatogonia declined from approximately 89% to 43% (Fig. 5J). After 1 weeks of chase, the majority of BrdU-labeled cells in the initial labeling differentiate into mature sperm. By 2 weeks of chase, BrdU staining was detected small clones of type B spermatogonia and a portion of spermatocytes. However the majority of BrdU staining was diffuse and showed diminished intensity in many type of cells, while obvious signals was detected in type A_{cl} spermatogonia (22.3% ± 6.8%) (Fig. 5D,E,J). After 4 weeks of chase, I observed occasionally BrdU-positive type A_{cl} spermatogonia (0.8% ± 0.4%) (Fig. 5F,G,J) and very rarely small clones of

BrdU-positive type B spermatogonia (Fig. 5H,I). Since a slow decline of labeled-cells was observed compared with the chase of 48 hr labeling experiment (Fig. 5J), the labeled cells contained slow-cycling cells. These results indicate that type A_{cl} spermatogonia have characteristics of LRCs.

4.1.5 cDNA cloning of *zsld5*

The results of long term BrdU labeling experiment suggest that type A_{cl} spermatogonia contain the quiescent cells in cell cycle which exhibit the stem cell characteristic. To characterize the cell cycle phase of type A_{cl} spermatogonia, I focused on the protein which regulates chromosomal DNA replication. Because most of the genes encoding pre-replication complex factors are not transcribed in G0 (Ohtani et al., 1996; Leone et al., 1998), I examined zebrafish Sld5 expression in type A_{cl} spermatogonia. Sld5 is required for chromosomal DNA replication and a component of GINS (Takayama et al., 2003). GINS consists of Psf1, Psf2, Psf3, Sld5, all of which are well conserved in eukaryotic cells, and the abundance of GINS was constantly maintained during the cell cycle. It was reported that PSF1 and Sld5 cooperate in the proliferation of immature cell populations in mice (Kong et al., 2005; Ueno et al., 2005).

I isolated zebrafish homologue of Sld5 based on their sequence similarity to mouse and yeast Sld5. The predicted zSld5 protein consisted of 203 amino acids. The deduced amino acid sequences of zSld5, mouse SLD5 (NM_024240) and yeast SLD5 (NP_010777) were compared. Based on the primary amino acid sequence, zSld5

contained a coiled-coil domain and a basic region. When the amino acid sequences were compared, two regions of significant homology were found (Fig. 6A); (black box) aa 61 to aa 83 of zebrafish Sld5 and aa 81 to aa 103 of mouse SLD5 (73.9%), and aa 132 to aa 154 of yeast Sld5 (52.1%) and (gray box) aa 176 to aa 203 of zebrafish Sld5 and aa 196 to aa 223 of mouse SLD5 (82.1%), and aa 267 to aa 294 of yeast Sld5 (52.1%). I detected *zsld5* expression in the testis and zebrafish embryonic cell line derived from 5 days post fertilization larvae cells, ZE cells, by RT-PCR (Fig. 6B). To analyze localization of zSld5 proteins, I generated polyclonal antibody against zSld5 by immunizing rabbit with recombinant zSld5. Western blot analysis for zSld5 protein was performed using testis and ZE cell protein. A single band immunoreacted with anti-zSld5 of approximately 24 kDa was detected (Fig. 6C).

4.1.6 Expression analysis of zSld5 in ZE cells

First, I examined relationship between the expression of zSld5 protein and cell cycle by the use of cultured cell line ZE cells. The G0/G1 phase in cultured cells can be obtained by serum starvation. This is a commonly used method to arrest the donor cell state at the G0/G1 phase of the cell cycle prior to nuclear transfer such as sheep (Campbell et al., 1996; Wilmut et al., 1997), pig (Boquest et al., 1999) and zebrafish (Lee et al., 2002). DNA synthesis was measured via BrdU incorporation in standard medium containing 10% FBS and serum-starved medium containing 0.5% FBS (Lee et al., 2002). When DNA synthesis was monitored by BrdU incorporation, 90.1% of BrdU-positive cells

were observed (Fig. 7B). When cells were starved for 7 days, no more than approximately 4.0% BrdU incorporation was detected (Fig. 7B). Stimulation of starved cells by serum addition triggered cell cycle progression (Fig. 7B). Thus, serum starvation was sufficient to inhibit DNA synthesis and entering the G0/G1 arrest phase of the cell cycle.

Second, to examine the effects of cell cycle on the expression of zSld5 protein, I carried out immunohistochemical analysis with anti-zSld5 antibody in normal, serum starved and serum stimulated culture conditions in ZE cells. In normal culture condition, it was expressed in almost all of cells regardless of the cell cycle phases (Fig. 7F). In contrast, I observed almost no expression of zSld5 protein in serum starved culture condition (Fig. 7G). After stimulation by serum addition, its expression was recovered (Fig. 7H). Thus, it appeared that zSld5 is down-regulated at the protein level when ZE cells exit the cell cycle into G0, and its expression is induced as cells re-enter the cell cycle. These results indicate that zSld5 antibody is able to distinguish the proliferating cells from the G0/G1 arrest phase of the cell in ZE cells.

4.1.7 Expression analysis of zSld5 in the zebrafish testis

Immunohistochemical examinations of zSld5 in the testis showed that it was localized in nucleus of a portion of type A_{cl} spermatogonia, type B spermatogonia and spermatocytes, but not in spermatids and sperm (Fig. 8A,B). In addition, 51.2% of type A_{cl} spermatogonia were zSld5-positive (Fig. 8A,C), on the other hand, 92.3% of type

A_{dt} spermatogonia were zSld5-positive (Fig. 8B,C).

To examine relationship between the expression of zSld5 proteins and proliferation activity, I carried out immunohistochemistry with anti-BrdU and anti-zSld5 antibodies after the BrdU-labeled for 24 hr (Fig. 9). Double staining showed that BrdU-positive and zSld5-positive (18.5% ± 2.3%), BrdU-positive and zSld5-negative (0.7% ± 0.2%), BrdU-negative and zSld5-positive (33.1% ± 4.6%), BrdU-negative and zSld5-negative (48.1% ± 2.9%) type A_{cl} spermatogonia were observed (Fig. 8). The result showed that almost all of BrdU-positive type A_{cl} spermatogonia were zSld5-positive and about half of type A_{cl} spermatogonia were both negative. Agreement with the results of ZE cells, these findings suggested that type A_{cl} spermatogonia entering proliferative phase express zSld5, whereas type A_{cl} spermatogonia entering G0/G1 arrest phase do not.

4.1.8 Comparison of morphometric features of zSld5-negative and zSld5-positive type A_{cl} spermatogonia

To examine the morphometric features between zSld5-negative and zSld5-positive type A_{cl} spermatogonia, the length of nucleus, areas of the nucleus and cytoplasm, and the ratio of the nucleus to whole cell size were measured. The difference between these cells, however, was not significant (Fig. 10), and I couldn't found any morphometric features.

4.1.9 Expression of zSld5 in label-retaining cells

The results of expression analysis of zSld5 in type A_{cl} spermatogonia and ZE cells suggest that zSld5 is down-regulated during G0/G1 arrest phase. To characterize the expression of zSld5 in LRCs, I performed immunohistochemistry with anti-BrdU and anti-zSld5 antibodies of testis after 4 weeks following long term BrdU labeling. The result showed that the majority of LRCs (82.3% ± 8.4%, n = 71) were zSld5-negative (Fig. 11A-C), indicating that the cells entering G0/G1 arrest phase do not possess zSld5 proteins.

4.1.10 Localization of zSld5-positive type A_{cl} spermatogonia

Examination of immunohistochemistry of zSld5 in the zebrafish testis, I observed several type of zSld5 expression pattern in a small subset of type A_{cl} spermatogonia. In normal adult zebrafish testis, as shown by Figure 8A, adjacent to two types of type A_{cl} spermatogonia that is zSld5-positive and zSld5-negative are located along the basement membranes, while both positive type A_{cl} spermatogonia are also located along the basement membranes (Fig.12A). In contrast, type A_{cl} spermatogonia and type A_{dt} spermatogonia that located vertically against the basement membranes was observed (Fig.12B,C). An opposite case against pattern detected in Figure 12C was not observed. It suggested that divisions of type A_{cl} spermatogonia are regulated near the basement membranes.

4.2 Assessment of type A spermatogonia following cytotoxic damage

4.2.1 Effect of busulfan at different concentrations on male germ cells

It is known that spermatogonial stem cells have an ability to restore spermatogenesis when a testis was damaged by a drug or irradiation (Bucci and Meistrich, 1987). In the first set of experiments, I examined the effect of busulfan on differentiating germ cells. I initially determined the optimal dose to eliminate differentiating germ cells such as type B spermatogonia, spermatocytes and spermatids. Adult zebrafish were soaked in busulfan solution at four different doses (6.6, 13.2, 33 and 66 μg/ml) for 24 hr, and the zebrafish were killed after 7 days of the busulfan treatment to determine the extent of differentiating germ cells. No significant reduction in differentiating germ cells was observed in fish treated with busulfan at doses of 6.6 μg/ml (Fig. 13B). In contrast, decline of differentiating germ cells was observed in animals given busulfan at doses of 13.2, 33 and 66 μg/ml, respectively (Fig. 13C-E). Particularly in fish treated with busulfan at 66 μg/ml, virtually differentiating germ cells were eliminated, and only type A spermatogonia close to basement membranes and spermatozoa in the sperm duct were observed (Fig. 13E). These observations indicated that the effects of busulfan were dose-dependent. Thus, I selected a dose of 66 μg/ml for further analysis.

4.2.2 Regeneration of spermatogenesis after busulfan treatment

In the second set of experiments, I counted the number of germ cell cysts that contain different types of a germ cell during busulfan-induced regeneration. Adult zebrafish

were soaked in busulfan solution at 66 μg/ml for 24 hr, and the zebrafish were killed from 1 to 30 days after busulfan treatment to determine the extent of ongoing spermatogenesis. As shown in Figure 14, the number of cysts that contain differentiating germ cells declined at 3 days after busulfan treatment, while cysts of a type A spermatogonium gradually increased with time. After 11 days, type B spermatogonia and spermatocytes appeared, and the cysts increased, while the cysts of a type A spermatogonium decreased. On day 30 after treatment, virtually all tubules of testis showed apparently normal spermatogenesis and produced functional sperm. The results suggest that surviving type A_{cl} spermatogonia can regenerate to show spermatogenesis following treatment of 66 μg/ml of busulfan.

4.2.3 The expansion of zSld5-positive type A_{cl} spermatogonia

It was reported that the number of spermatogonial stem cells undergoes dynamic expansion during the course of regeneration after busulfan treatment in mice (Kanatsu-Shinohara, 2003b). To elucidate the characterization of type A_{cl} spermatogonia responsible for regeneration after busulfan treatment, I performed immunohistochemical analysis using anti-zSld5 antibody. As shown in Figure 15A, the observation at 4 or 8 days after the treatment revealed that transient expansion of zSld5-positive type A_{cl} spermatogonia was observed. Although the number of zSld5-negative type A_{cl} spermatogonia showed a slight increase as compared with control, the difference was not significant. The number of type zSld5-positive A_{cl} spermatogonia was reduced at 12

or 15 days. The result showed that zSld5-positive type A_{cl} spermatogonia increase their number transiently after busulfan treatment. These results suggest that zSld5-positive type A_{cl} spermatogonia have a function of stem cells, because the behavior of these cells agrees with the previous study (Kanatsu-Shinohara, 2003b).

4.3 *In vitro* culture of zebrafish type A spermatogonia

4.3.1 Development of the culture system to proliferate type A spermatogonia

To establish the culture system capable of supporting the maintenance and proliferation of type A spermatogonia *in vitro*, I examined two Sertoli cell lines as feeder cells. Adult zebrafish testes on day 7 after busulfan treatment were enzymatically dissociated. Testicular cells were plated on mitomycin C-inactivated ZtA6-2 or ZtA6-6 feeder cell lines that were isolated from ZtA6 cells derived from a spontaneous tumor-like hypertrophied testis (Sakai, 2002; Kurita and Sakai, 2004). The suspended germ cells tended to form clumps and attached to feeder cells after overnight incubation. ZtA6-2 cells were previously shown to stimulate the proliferation of spermatogonia (Kurita and Sakai, 2004). ZtA6-2 and ZtA6-6 cells, showed similar features of Sertoli cells, such as expression of Sertoli cell marker genes and phagocytic activity (Kurita and Sakai, 2004). However, I observed that ZtA6-2 cells tend to detach from the culture dish when forskolin were added in the culture medium. Therefore, I used ZtA6-6 cells (Fig. 16A), which served as feeder cells for further analysis.

Morphology of the cultured cells from busulfan-treated adult testis resembles to

type A spermatogonia of paraffin section and to that of freshly isolated type A spermatogonia from the busulfan-treated testis. They had a flattened and somewhat elongated shape, round/oval nuclei with relatively large nucleoli (Fig. 16B). These features were retained during the culture period (Fig 16C-F). As shown by *in situ* hybridization, the cells expressed *vas* which is a marker of pre-meiotic germ cells in the zebrafish testis (Fig. 17).

To detect the proliferative activity of cultured germ cells under this culture condition, I performed BrdU incorporation experiments. BrdU was added to the culture medium during the last 48 hr of the culture period. Immunolocalization of BrdU showed that during the 5-16 days of culture, some BrdU-positive germ cells with large nuclei were detected in the colony. Furthermore, asynchronous proliferations were observed (Fig. 18). Since differentiating germ cells, such as type B spermatogonia and spermatocytes, divide synchronously in a cyst, these asynchronous proliferated cells were not differentiated germ cells. These results indicated that the cultured cells from busulfan-treated zebrafish maintained both proper characteristics of type A spermatogonia and proliferating ability *in vitro*.

4.3.2 Culture of type A spermatogonia isolated from normal adult testis

The results of preceding experiment suggest that a co-culture using ZtA6-6 cell line and germ cells isolated from busulfan-treated testis maintained the survival and mitotic activity of type A spermatogonia. Therefore, I attempted to culture type A

spermatogonia isolated from normal testis of adult *vas::EGFP* transgenic zebrafish. Many germ cells attached to ZtA6-6 cells after overnight incubation. Within 2 wk, almost all clumps of the differentiating germ cells had disappeared because of the differentiating into mature sperm (Fig. 19A,B). In contrast, I observed that the cells with morphological characteristics of type A spermatogonia and GFP expression were retained even after 1 month of culture (Fig. 19C,D). However, growth of these cells did not seem to be enough to recover the amount of lost cells at re-plating, and GFP expression was decreased gradually. The result showed that type A spermatogonia isolated from normal adult testes were able to maintain under this condition at least 1 month.

5. Discussion

In this study, I demonstrated the presence of subpopulations in zebrafish type A spermatogonia varying in size, location pattern and duration of cell cycle. Label retaining cells were found among small size of type A spermatogonia that were located close to basement membranes, which fulfilled the characteristics of spermatogonial stem cells. The observation of a busulfan-treated testis showed the transit increase of zSld5-positive type A spermatogonia close to basement membranes, and suggest that these cells can reconstitute spermatogenesis, indicating that the spermatogonia possess a feature of stem cells. Furthermore, I developed the *in vitro* culture condition for supporting the maintenance and proliferation of type A spermatogonia for 1 month or so. This study represents the first step towards further understanding the character of spermatogonial stem cells and molecular mechanisms to maintain the undifferentaition status in teleosts.

5.1 Heterogeneous population of type A spermatogonia in zebrafish

In mammalian testes, spermatogonia are classified as type A, intermediate (In), or type B according to their amount of nuclear heterochromatin. Type A spermatogonia are the most primitive spermatogonia and have little heterochromatin. They are subdivided into seven categories according to their topographical arrangement on the basement membrane (Huckins, 1971a; Oakberg, 1971). The most primitive spermatogonium is the A_{single} (A_s). A_s gives rise to A_{paired} (A_{pr}) and then divides to make chains of 4, 8 and 16

cells known as $A_{aligned}$ (A_{al}). A_s, A_{pr}, and A_{al} spermatogonia together have been called undifferentiated type A spermatogonia. Most A_{al} and some A_{pr} spermatogonia differentiate into A1 spermatogonia at a certain stage and go through a series of six divisions and via A2, A3, A4, In, and B spermatogonia, become primary spermatocytes. Many lines of investigations suggest that the spermatogonial stem cell activity resides in undifferentiated type A spermatogonia (Aponte et al., 2005; de Rooij, 1998; McLean, 2005). The undifferentiated type A spermatogonia lie at the tubule periphery next to the basement membrane (Hadley and Dym, 1987). Although the undifferentiated type A spermatogonia were all morphologically similar to one another with the exception of topographical arrangement on the basement membrane, A_{al} spermatogonia became progressively enlarged during stages V to VII accompanying the changes to A1 spermatogonia (Huckins, 1971a).

In teleost testis, considering the capability of producing repetitive waves of spermatogenesis in seasonally reproducing species, undifferentiated type A spermatogonia would have stem cell capacities. Recently, a technique for spermatogonial transplantation has been established in rainbow trout and demonstrated the existence of functional spermatogonial stem cells among type A spermatogonia in teleosts for the first time using transplantation technique (Okutsu et al., 2006). Classically type A spermatogonia are considered to equal spermatogonial stem cells based on morphological criteria (large size and isolated single germ cell in each cyst) and are homogeneous population (Billard, 1986). However, there is no formal proof yet

in fish that the type A spermatogonia are homogeneous population and all of them have stem cell capacities. Therefore, at first I performed the observation of type A spermatogonia in criteria such as the size and the location.

It was reported that type A spermatogonia locate along the entire length of the tubules, generally immediately beneath the tubular basement membrane in the unrestricted spermatogonial type of a testis in Salmoniformes, Perciformes, and Cypriniformes (Grier, 1980). The present study, however, showed two types of type A spermatogonia that close to basement membranes (termed type A_{cl}) and distant from basement membranes (type A_{dt}) from the morphological analysis in Cypriniformes zebrafish testis (Fig. 1). Furthermore, I found that type A_{dt} spermatogonia were large in size compared with type A_{cl} spermatogonia (Fig. 2A,B).

These results agree in part with the previous morphological study in a seasonal breeding teleost, the gilthead seabream, which described two subpopulation of type A spermatogonia based on morphological criteria (Chaves-Pozo et al., 2005). They claimed that primitive type A spermatogonia close to basement membranes that are the residual type observed post-spawning or resting stages are much lager (approximately 2.1 μm nuclear diameter) than that of type A spermatogonia observed spermatogenesis stage throughout tubule. Although the locations of two subpopulations agree with this study, the differences of cell size between two subpopulations to be conflict with this study. A possible explanation for this discrepancy is that the difference of the species. Another possible reason for the discrepancy is that the difference of the reproductive

cycle, because in a seasonal breeding teleost, spermatogonia divide actively in spermatogenesis stage after resting stage.

Considering the differentiating state of mammalian type A spermatogonia, basement membranes may play an important role in maintaining spermatogonial stem cells in the zebrafish testis. Therefore, it is suggested that zebrafish type A_{dt} spermatogonia are differentiated cells compared with that of close to basement membranes. In addition, these results suggest that the cyst of type A spermatogonium migrates throughout the tubule in the zebrafish testis, although it is considered that cysts do not move during the maturation process (Grier, 1980).

5.2 Differences in cell cycle duration of type A spermatogonia

The cell cycle properties of the type A spermatogonia have been studied in mammals. Undifferentiated type A spermatogonia are known to divide randomly and their cell cycle is much longer than that of the other spermatogonia (Huckins, 1971b; Monesi, 1962). The results in the present study showed that 18.8% of type A_{cl} spermatogonia incorporate BrdU during 24 hours administration of BrdU. Therefore, it is estimated that the length of the cell cycle of the type A_{cl} spermatogonia is 128 hours (5.3 days). Under the same condition, 46.8% of type A_{dt} spermatogonia incorporate BrdU for 24 hours administration of BrdU. The length of the cell cycle of them is 51.3 hours (2.1 days). Similarly, the length of the cell cycle of type B spermatogonia is estimated as 31 hours (1.3 days) because 77.3% of them incorporate BrdU. This shows that the length of cell

cycle of type A_{cl} spermatogonia was much longer than the type A_{dt}.

Using the LRC technique, I identified the LRCs, which exhibit a stem cell-like property (quiescence), in the zebrafish testis by chasing the BrdU-labeled cells after long term administration of BrdU for 9 days. The LRCs were found among type A spermatogonia after 4 weeks of chase, and they were situated along the basement membranes. Nevertheless the long term administration of BrdU, 11.2% of type Acl spermatogonia did not incorporate in BrdU (Fig. 4I). The BrdU-labeled type A spermatogonia decreased from 88.8% to 43.2% (the reduction rate is 51.4%) during one week. After 2 weeks of chase, the BrdU-labeled type A spermatogonia decreased to 22.3% (the reduction rate is 75.6%). In contrast, the 48 hours BrdU administration showed that the BrdU-labeled type A_{cl} spermatogonia decreased from 42.5% to 4.7% (the reduction rate is 88.9%) after one week (Figure 4B). After 2 weeks, the BrdU-labeled type A spermatogonia almost diminished (it decreased to 0.08%, and the reduction rate is 99.8%). These results suggest that the length of cell cycle of LRC is longer than the other type A spermatogonia that can be labeled by administration for 48 hours. These results agree with the results of Huckins (1971c) in mice, which suggested that there is a long-cycling subpopulation in A_s spermatogonia which have a cell cycle length longer than 13 days. Therefore, I propose the heterogeneity among type A_{cl} spermatogonia.

5.3 zSld5 is useful to identify proliferating cells

Staining with the antibody against zSld5, I found two subpopulations of type A_{cl} spermatogonia. The result revealed that the zSld5 antibody is able to distinguish between the quiescent cells and the proliferating cells. From the immunohistochemistry against zSld5 in the zebrafish testis, it is revealed that zSld5-potitive type A_{cl} spermatogonia (termed type A_{cl}^{+}) and zSld5-negative (termed type A_{cl}^{-}) are 51.2% and 48.8%, respectively. The result means that about half of the type A_{cl} spermatogonia are proliferating cells because of the expression zSld5, and that about half of them are quiescent cells because of the non-expression of zSld5 like serum starved culture ZE cells. In addition, it is also revealed that zSld5-potitive and zSld5-negative type A_{dt} spermatogonia are 92.3% and 7.7%, respectively. These results consisted with the experiment of BrdU incorporation, which showed that type A_{dt} spermatogonia were more proliferating than the type A_{cl}. In addition, 82.3% of LRCs were type A_{cl}^{-} spermatogonia after 4 weeks of chase. Because it is considered that LRCs are quiescent cells, this result support the character of zSld5 antibody, which is able to distinguish proliferating cells from quiescent cells. To detect proliferating cells, generally, anti-proliferating cell nuclear antigen (PCNA) antibody is used (Matsumoto et al., 1987; Miyachi and Tan, 1979; Prelich et al., 1987). Since PCNA protein level increase two to three times between early G1 and early S phases, become maximal during S phase and declines during G2 and M phase, and anti-PCNA antibody mainly detect S phase of cells (Landberg and Roos, 1991), zSld5 antibody would be easier to distinguish proliferating cells from quiescent cells than anti-PCNA antibody.

The result from double immunohistochemistry using zSld5 and BrdU antibodies for 24 hours BrdU administration showed that the BrdU-labeled cells account for 35.5% of the total type A_{cl}^{+} spermatogonia (the ratio of BrdU-labeled cells, 18.1% divided by the ratio of zSld5-positive cells, 51.2%). Therefore, the length of the cell cycle of type A_{cl}^{+} spermatogonia is estimated as 67.9 hours (2.8 days). On the other hand, the length of the cell cycle of type A_{cl}^{-} spermatogonia is estimated as follows. In the experiment of identification of LRCs, 88.8% of type A_{cl} spermatogonia were labeled during 9 days BrdU administration. I can assume that all type A_{cl}^{+} spermatogonia incorporate BrdU, which account for 51.2% of type A_{cl} spermatogonia. Thus, 37.6% of type A_{cl}^{-} spermatogonia incorporate BrdU. Since type A_{cl}^{-} spermatogonia account for 48.8% of total type A_{cl} spermatogonia, it means that 77.1% (37.6/48.8) of type A_{cl}^{-} spermatogonia incorporate BrdU during 9 days. Therefore, the length of the cell cycle of type A_{cl}^{-} spermatogonia should be 11.7 days.

The estimation of the length of cell cycle from this study generally agrees with previous studies of the length of cell cycle of rat spermatogonia. It is reported that the length of the cell cycle of type A2 through type B spermatogonia has been estimated to be 42 hours (Monesi, 1962; Hilscher, 1969), and that of A_s, A_{pr}, and A_{al} spermatogonia to be 56 hours (Huckins, 1971b). Furthermore, it is suggested that there is a long-cycling subpopulation in A_s spermatogonia which have a cell cycle length longer than 13 days (Huckins, 1971c). The length is similar to the estimation of the cell cycle length of type A_{cl}^{-} spermatogonia of zebrafish, suggesting that these spermatogonia are

the most undifferentiated cells in the zebrafish testis. Therefore, I define type A_{cl}^{-} spermatogonia as most undifferentiated type A spermatogonia. In this view, the next longer length of the cell cycle of type A_{cl}^{+} spermatogonia would be equivalent to the mammalian A_s, A_{pr}, and A_{al} spermatogonia. The shortest length of the cell cycle of type B spermatogonia would be correspond to the mammalian type A2 through type B spermatogonia. In the present study, I could not determine whether type A_{dt} spermatogonia that have intermediate length of the cell cycle were differentiate state or undifferetiate state. Further studies will be required such as transplantation system of spermatogonia or discovery of a new cell marker.

5.4 Polarity of type A spermatogonia near the basement membrane

The result reveals that a small subset of type A spermatogonia locate along the basement membrane or toward the lumen (Fig. 12). In *Drosophila* testes, orientation of stem cells toward the niche appears to play a critical role in the mechanism that ensures a reliably asymmetric outcome of spermatogonial stem cell divisions, consistently placing one daughter within the reach of short-range signals from the hub and positioning the other away from the niche (Yamashita et al., 2003). To examine spindle orientation and centrosome position in type A spermatogonia in the zebrafish testis, it will likely reveal further information about regulation of the process of stem cell division in vertebrates.

5.5 Assessment of type A spermatogonia following busulfan treatment

It has been shown that undifferentiated type A spermatogonia were resistant to cytotoxic damage or irradiation, whereas other spermatogonia were sensitive. Busulfan is an alkylating agent and does not have any effect on DNA synthesis; however, it causes inhibition of the next mitosis when it intoxicates the cells in the G1 phase (Kramer and de Rooij, 1970). The results in present study revealed that differentiated germ cells such as type B spermatogonia and spermatocytes were eliminated while only type A_{cl} spermatogonia were survived after busulfan treatment at a dose of 66 μg/ml. Then, survived type A_{cl} spermatogonia can proliferate and regenerate to recover spermatogenesis. The results suggest that type A_{cl} spermatogonia are the most resistant cell population against busulfan in the zebrafish testis.

It has been shown that stem cells begin to increase their number immediately after cytotoxic damage by morphological studies (van Beek et al., 1986; van Beek et al., 1990) and transplantation assays (Kanatsu-Shinohara et al., 2003b) in mouse. In addition, there were some studies to characterize surviving type A spermatogonia using molecular markers after busulfan treatment in mouse. Choi et al. (2004) reported that expression of the c-kit was reduced in busulfan-treated testis. c-kit is a marker for differentiated spermatogonia containing differentiating type A, In, B type spermatogonia, but not express in undifferentiated type A spermatogonia (Schrans-Stassen et al., 1999). Another study showed that busulfan-treated testis enrich type A spermatogonia which express α6-integrin, β1-integrin and GFRα-1 (Choi et al., 2006), markers for undifferentiated type A spermatogonia (Meng et al., 2000; Shinohara et al., 1999;

Widenfalk et al., 2000). These studies support that the busulfan-resistant type A spermatogonia correspond to undifferentiated type A spermatogonia in mice.

In present study, detail analysis after busulfan treatment showed that the number of type A_{cl}^{+} spermatogonia increased and reached the highest value at 8 days, and then decreased. In contrast, the number of type A_{cl}^{-} spermatogonia relatively unaltered. The results show that type A_{cl}^{+} spermatogonia respond to regeneration of spermatogenesis after busulfan treatment, suggesting that type A_{cl}^{+} spermatogonia possess a feature of spermatogonial stem cells.

Recently, a model for the composition of the mouse spermatogonial stem cells was proposed with the combination with transplantation and regeneration assays after pulse-labeling (Nakagawa et al., 2007). Interestingly, these analyses with transplantation and regeneration assays gave different results, suggesting that the stem cell population is not homogeneous. Instead, their data suggest that the A_s, A_{pr}, and A_{al} cells exhibit differing degrees of stem cell potential, with the highest potential perhaps in the A_s cells. Spermatogonial stem cell potential is revealed by the degree of niche occupancy. In normal testes, niches are generally occupied by stem cells with the highest potential for self-renewal. These stem cells can be referred to as the "actual stem cells". But in recipient testes depleted of resident spermatogonial stem cells, transplanted cells can fill unoccupied niches, even if the cells have a low potential for self-renewal. These transplantable cells called the "potential stem cells". In response to actual stem loss or emptied stem cell niche, the potential stem cells also would shift their mode from transit

amplification to self-renewal and give rise to new actual stem cells. In mouse, the beginning of spermatogonial stem cell niche formation occurs during early postnatal development, while Sertoli cells are maturing. Although the number of available spermatogonial stem cell niches dramatically increase from birth to sexual maturity (Shinohara et al., 2001), and then niche become stable or decrease with age. In addition, there is no report that increases of the niche after cytotoxic damage or irradiation. Therefore, previous study that have shown the increase of spermatogonial stem cells after busulfan treatment (Kanatsu-Shinohara et al., 2003b) appear to responsible for the potential stem cells, because they used transplantation assays. Considering this new aspect and a series of classic morphological investigations, type A_{cl}^{+} spermatogonia in zebrafish, which increase transiently after busulfan treatment, appeared to be the potential stem cells. Therefore, type A_{cl}^{+} spermatogonia may have ability to colonize in the niche if these cells transplant into recipient testes depleted of resident spermatogonial stem cells. In addition, it may be reasonably postulated that type A_{cl}^{-} spermatogonia are actual stem cells in the zebrafish testis because their number did not change after the busulfan treatment correlate with the theory.

Surrounding support cells secrete growth factors that activate signaling within adjacent stem cells to specify stem cell self-renewal and block differentiation. In addition, cell-cell adhesion between stem cells and surrounding support cells is important for holding stem cells close to self-renewal signals. These components of the stem cell niche are largely unknown in vertebrates. In the zebrafish testis, it appears that

the components of niche are present on the basement membrane since type A_{cl}^{-} spermatogonia locate around there. However, what type of somatic cells is responsible for niche structures is not clear in zebrafish yet. In mouse, it is thought that a Sertoli cell is one of the major contributors to stem cell niche regulation (Chen et al., 2005; Hess et al., 2006; Ogawa et al., 2005; Shinohara et al., 1999; Shinohara et al., 2001). Sertoli cells are in direct contact with germ cells and secreted GDNF which affects spermatogonial stem cell behavior (Meng et al., 2000; Tadokoro et al., 2002). But whether this factor is made by all or only subset of Sertoli cells is not known. Unlike the structure of mammalian that Sertoli cells attach all developmental stages of germ cells, each developmental stages of germ cell are isolated from other stages of germ cells by Sertoli cells in teleosts. Therefore, it is possible that a specific type of Sertoli cell which encloses only type A_{cl}^{-} spermatogonia may present and provide the niche.

Recently, it is reported that a branching of blood vessels in the testes may define the location of niches of spermatogonial stem cells in mouse (Yoshida et al., 2007). It means that the position of spermatogonial stem cells near the interstitial cells is the site of the existence of secreted factors that must act over a distance to influence the niche. If so in zebrafish, Sertoli cells near the interstitial cells may regulate the behavior of type A_{cl}^{-} spermatogonia on the basement membrane. A successful identification of type A_{cl}^{-} spermatogonia which behave the actual stem cells has significance in understanding the mechanisms of the niche in the zebrafish. Since the structure of teleost testis has advantage to analyze functions of Sertoli cells in the niche, it is worth examining a

future approach, such as an establishment of the culture and transplantation technique.

5.6 Development of the culture system to proliferate type A spermatogonia

The present study performed to culture the type A spermatogonia of a busulfan-treated testis on the ZtA6-6 Sertoli cell line. The results showed the asynchronous proliferation of the type A spermatogonia determined by BrdU incorporation experiments, and the maintenance of the type A spermatogonia for > 1month. Because differentiating type B spermatogonia proliferate synchronously in zebrafish, these results suggest that type A spermatogonia can proliferate without differentiation in this culture system. The Sertoli cell line is easily accessible for experimental genetic manipulation, and the type A spermatogonia would be so. This represents an establishment of culture system which can analyze the interaction between type A spermatogonia and Sertoli cells. Because of the complexity of these interactions between spermatogonial stem cells and surrounding somatic cells *in vivo*, it would be useful to have a culture system that provides the opportunity for direct analysis of the interactions occurring during these processes.

Several cell culture systems of spermatogonial stem cells were established in mouse (Kanatsu-Shinohara., 2003a) and the *Drosophila* (Niki et al., 2006), and new aspects that signaling pathway involved in the self-renewal division of spermatogonial stem cells were obtained (Hofmann et al., 2005; Braydich-Stolle et al., 2007; Lee et al., 2007). In contrast, type A spermatogonia could be cultured for a limited period under the condition in the present study. Our culture condition might lack a certain special

growth factor. Recently, it has been reported that gonadal soma-derived growth factor (GSDF), which is a novel member of transforming growth factor TGF-β superfamily, expressed in Sertoli cells enhances spermatogonial proliferation in rainbow trout (Sawatari et al., 2007). In addition, it also has been reported that GDNF is a crucial factor for the self-renewal of rodent spermatogonial stem cells, and that epidermal growth factor and leukemia inhibitory factor could also support the growth of spermatogonial stem cells *in vitro* (Kanatsu-Shinohara et al., 2003a, 2005). *Drosophila* stem cell culture requires a growth factor Dpp (BMP4 homologue) (Niki et al., 2006). Further study of the effective factor to proliferate the type A spermatogonia will be required toward a longer culture duration in zebrafish.

Recently, several spermatogenesis defect mutants were found in zebrafish, including some defects on spermatogonial stem cells or type A spermatogonia. Analysis of these mutants with the criteria in the present study would lead to new evidence on the spermatogonial stem cells in zebrafish. Culture of type A spermatogonia with the Sertoli cells derived from the mutant testis or *vice versa* would also lead to understanding the character of spermatogonial stem cells and the mechanism to proliferate and differentiation. Combining development of the culture condition and some other experimental approaches such as the transplantation system of spermatogonia, the criteria of type A spermatogonia shown in present study open the door to further understanding spermatogonial stem cells in fish.

6. Acknowledgments

I wish to express my sincere gratitude to my supervisor, Dr. Noriyoshi Sakai, for his continuous advice, stimulating discussion and encouragement during all the stages of this work. I am grateful to Dr. Minori Shinya for valuable advice and discussion. I also thank Dr. Mika Tokumoto, Yuich Ozaki and Toshihiro Kawasaki for helping and supporting for this work. I greatly appreciate Ms. Aki Masuda and Megumi Hashiguchi for valuable suggestion and supporting for this work. I also thank Mrs. Michiyo Kojima, Rie Maeda and Namie Suzuki for taking care of the fish used in this study.

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Figure 1

Localization of type A spermatogonia in the testis of zebrafish. Serial testis cross sections (5 μm) were performed to identify a single type A spermatogonia (A-C). Cysts of germ cells are always separated from the testicular interstitial cells by a basement membrane (black arrowheads) which is usually well-stained by eosin stain. Type A spermatogonia not only lie immediately near the tubule basement membrane (white arrowhead) but also locate distant from basement membranes (white arrow). Bar = 50 μm.

Figure 1

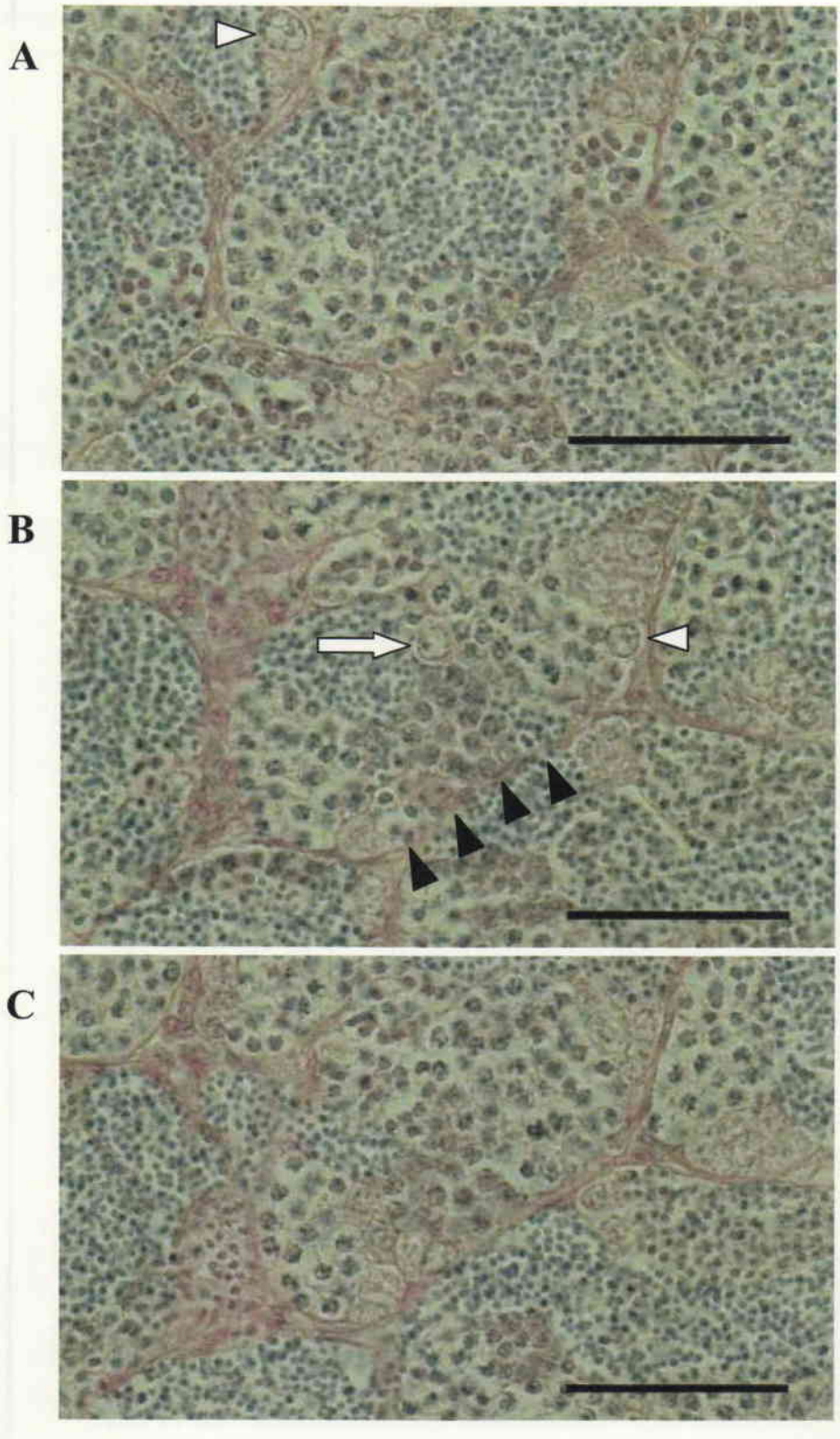


Figure 2

Comparison of morphometric features between type A_{cl} type spermatogonia and type A_{dt} spermatogonia (A–C). Average values for type A_{cl} spermatogonia (n = 65) are shown by black bars, and average values for type A_{dt} spermatogonia (n = 33) by white bars. (A) Comparison of longest diameter (μm) and shortest diameter (μm) of cells. The cell size of type A_{dt} spermatogonia was larger than that type A_{cl} spermatogonia, as measured by the average longest diameter and shortest diameter of the cells. (B) Comparison of areas of nucleus and cytoplasm in type A_{dt} spermatogonia versus type A_{cl} spermatogonia. The areas of the nucleus and cytoplasm of type A_{dt} spermatogonia were larger than type A_{cl} spermatogonia. (C) Comparison of the ratio of nucleus to cell size (N/C). The N/C ratio was not significant between these cell types. (D) The percentage of each type of type A spermatogonia (n = 240). Gray bar shows the percentages of unclassified cells. Values are mean (bars) and SD (A, B, C and D). *$P < 0.001$. **$P < 0.05$.

Figure 2

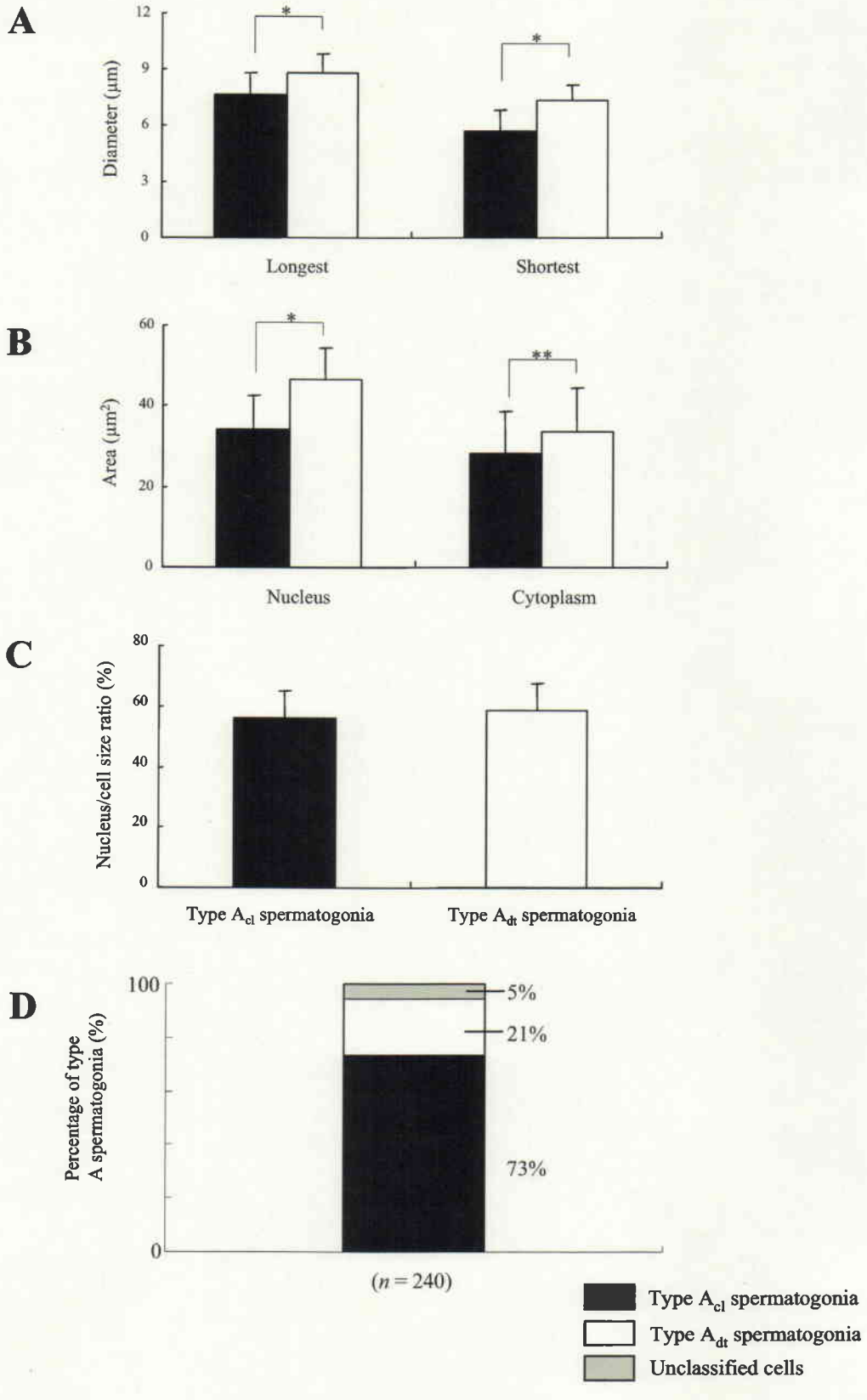

A
Diameter (μm)
12
9
6
3
0
*
*
Longest
Shortest
B
Area (μm²)
60
40
20
0
*
**
Nucleus
Cytoplasm
C
Nucleus/cell size ratio (%)
80
60
40
20
0
Type A_cl spermatogonia
Type A_dt spermatogonia
D
Percentage of type A spermatogonia (%)
100
0
5%
21%
73%
(n = 240)
Type A_cl spermatogonia
Type A_dt spermatogonia
Unclassified cells

Figure 3

Percentage of BrdU-labeled cells in testis of zebrafish after 24 and 48 hr administration of BrdU. In both labeling periods, percentage of BrdU-labeled type A_{cl} spermatogonia was lower than that type A_{dt} spermatogonia. In addition, percentage of BrdU-labeled type B spermatogonia was highest among these spermatogonia. Data are shown as the mean ± SD ($n = 3$).

Figure 3

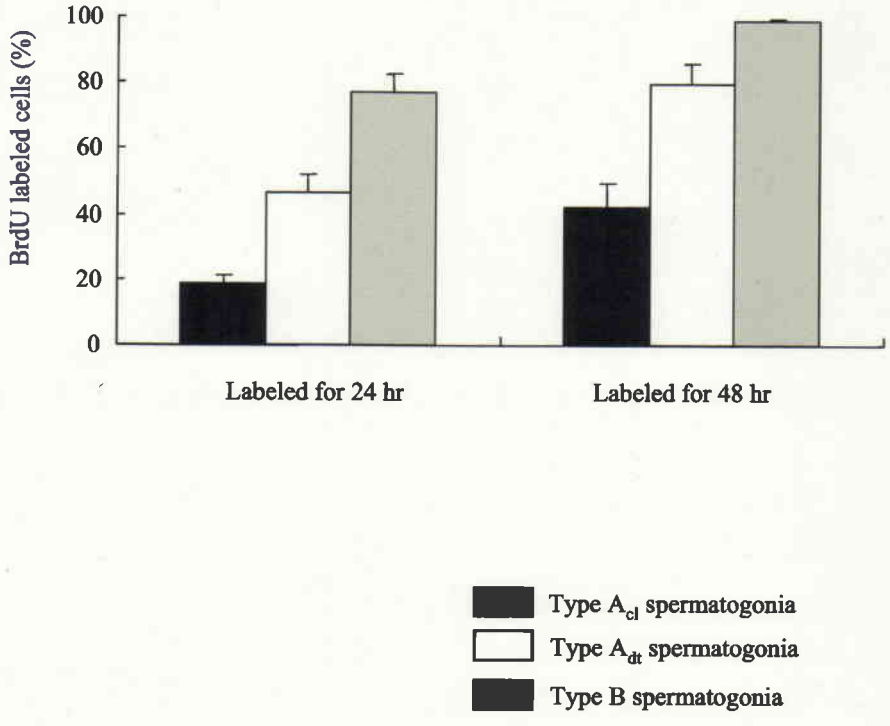
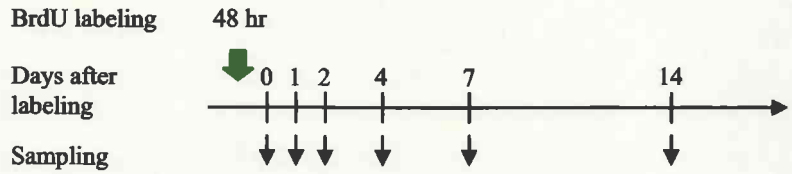

BrdU labeled cells (%)
100
80
60
40
20
0
Labeled for 24 hr
Labeled for 48 hr
Type A_{cl} spermatogonia
Type A_{dt} spermatogonia
Type B spermatogonia

Figure 4

BrdU-labeled type A spermatogonia in testis of zebrafish at various chase periods. (A) Schematic outline of the experimental procedures. The arrows below the timeline indicate the dates of sampling and those above the timeline indicate the dates of BrdU labeling. The cells were labeled for 48 hr administration of BrdU. (B) Quantitation of BrdU-labeled type A spermatogonia in testis at 0, 1, 2, 4, 7 and 14 days of chase. Data are shown as the mean ± SD (n = 3). (C) BrdU immunohistochemistry of the labeled cells at 0 day of chase. Note the heavily immunostained BrdU nuclei was detected. (D,E) BrdU immunohistochemistry of labeled at 4 day of chase. Note the progressive loss of BrdU signal was observed. Type A_{cl} spermatogonia were indicated as black arrowhead, type A_{dt} spermatogonia as black arrow, and type B spermatogonia as white arrowhead. Bar = 50 μm.

Figure 4 (1/2)

A



B

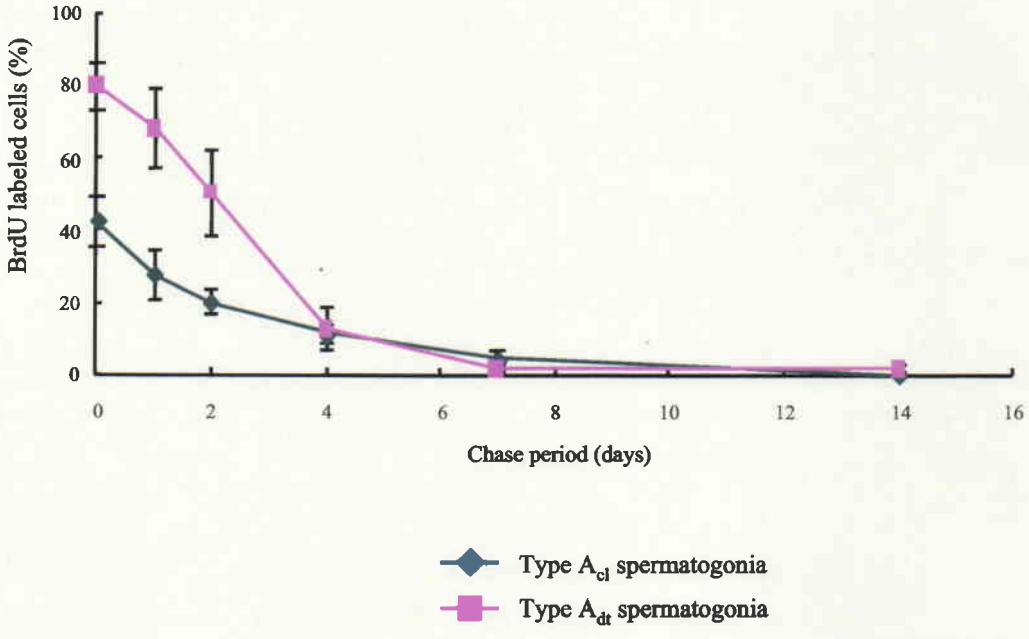


Figure 4 (2/2)

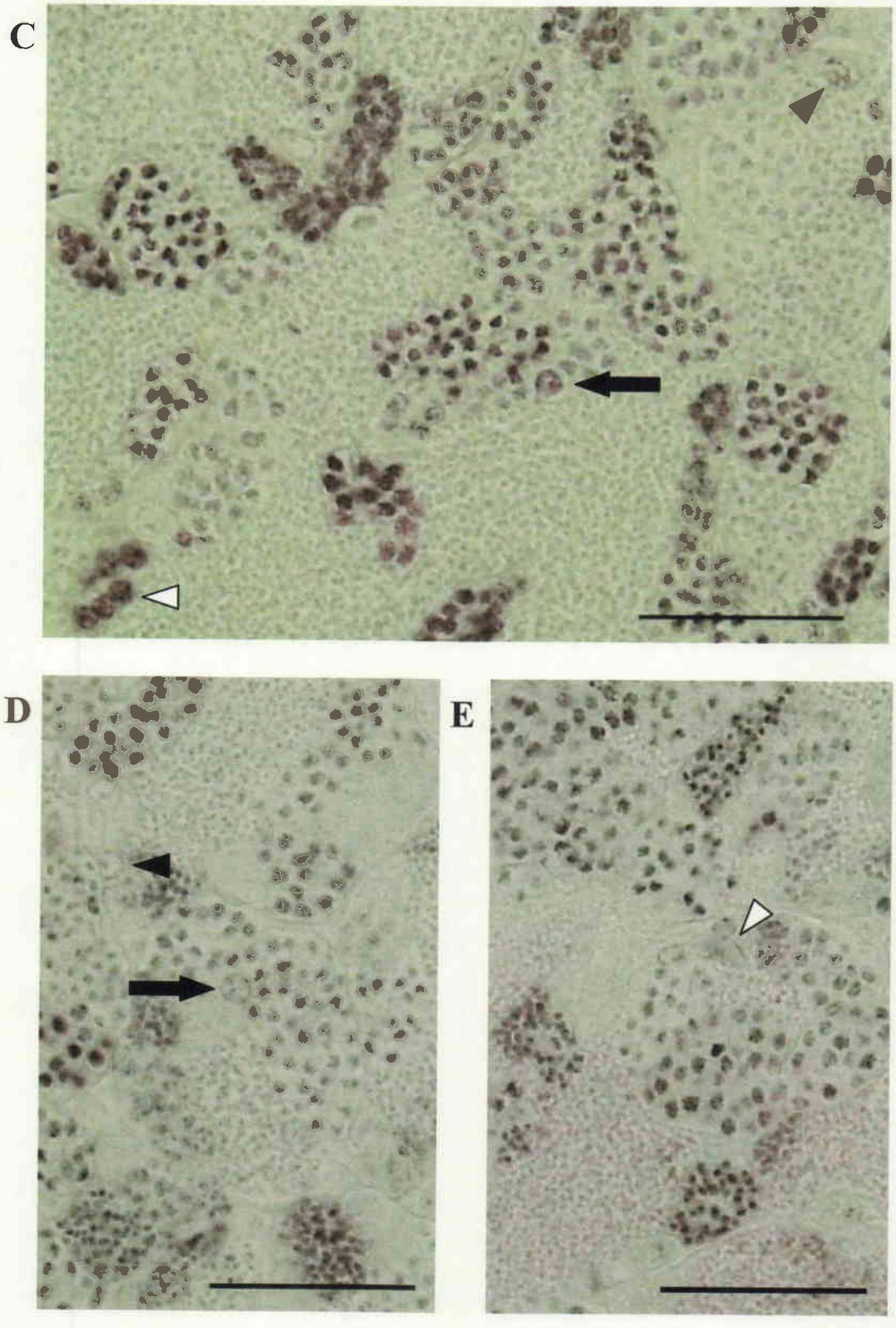
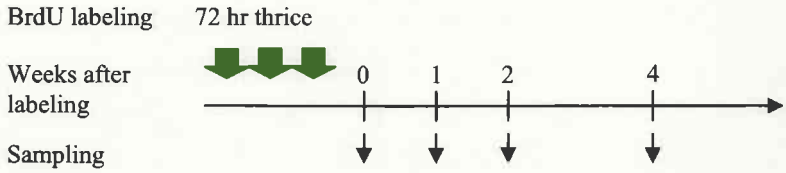


Figure 5

Identification of label-retaining cells (LRCs) in testis of zebrafish. (A) Schematic outline of the experimental procedures. The arrows below the timeline indicate the dates of sampling and those above the timeline indicate the dates of BrdU labeling. Testis section of stained both with immunohistochemically for the detection of BrdU (B,D,F,H, green label) and hematoxylin (C,E,G,I). (B,C) At initial labeling (day 0), note that the majority of type A_{cl} spermatogonia (arrowhead) and almost of all other germ cells stained for BrdU. (D,E) 2 weeks of chase, note that the progressive loss of BrdU-positive cells except for sperm compared with A. (F-I) 4 weeks of chase, note that the majority of cells was BrdU-negative, while some type A_{cl} spermatogonia (F,G, arrowhead) and very rarely small cysts containing type B spermatogonia were stained for BrdU (H,I, arrow). Bar = 50 μm. (J) Quantitation of BrdU-labeled type A_{cl} spermatogonia in testis at 0, 1, 2 and 4 weeks of chase. Data are shown as the mean ± SD ($n = 3$).

Figure 5 (1/2)

A


BrdU labeling
72 hr thrice
Weeks after labeling
0
1
2
4
Sampling

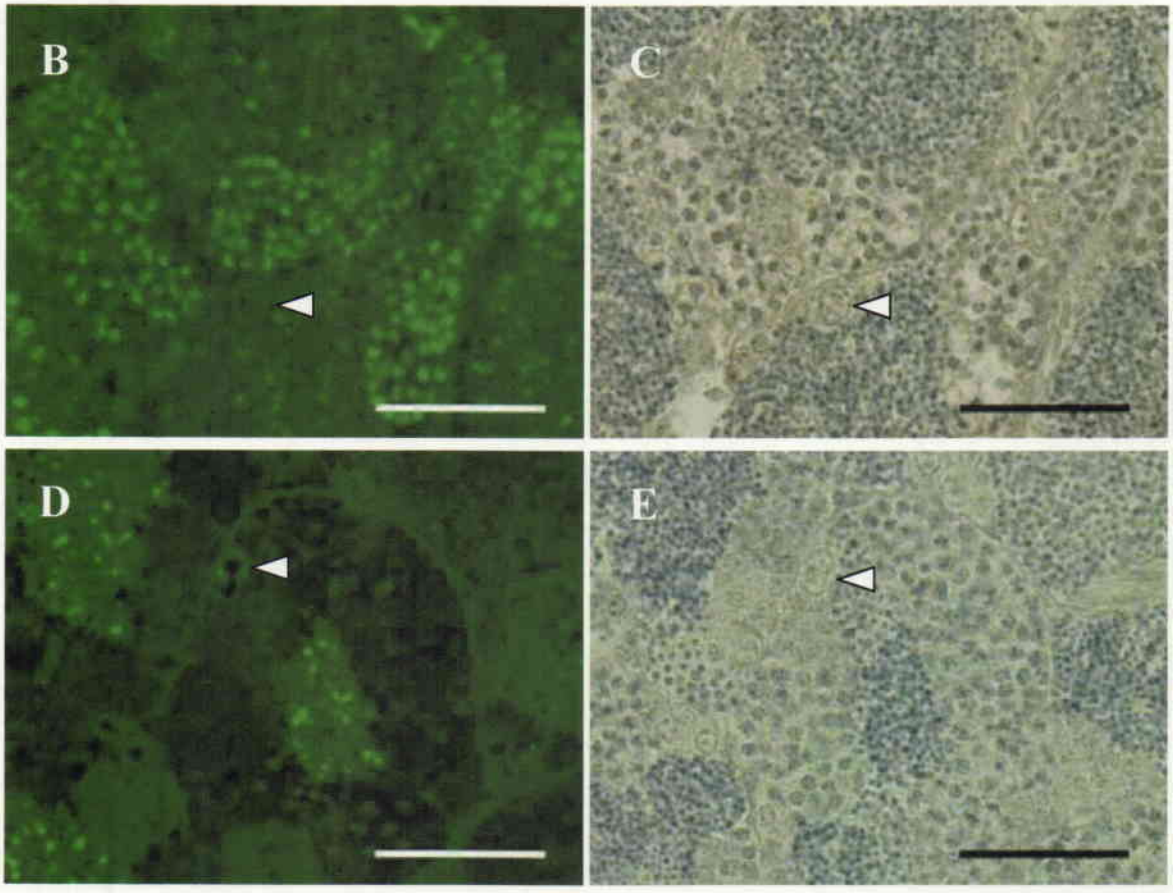
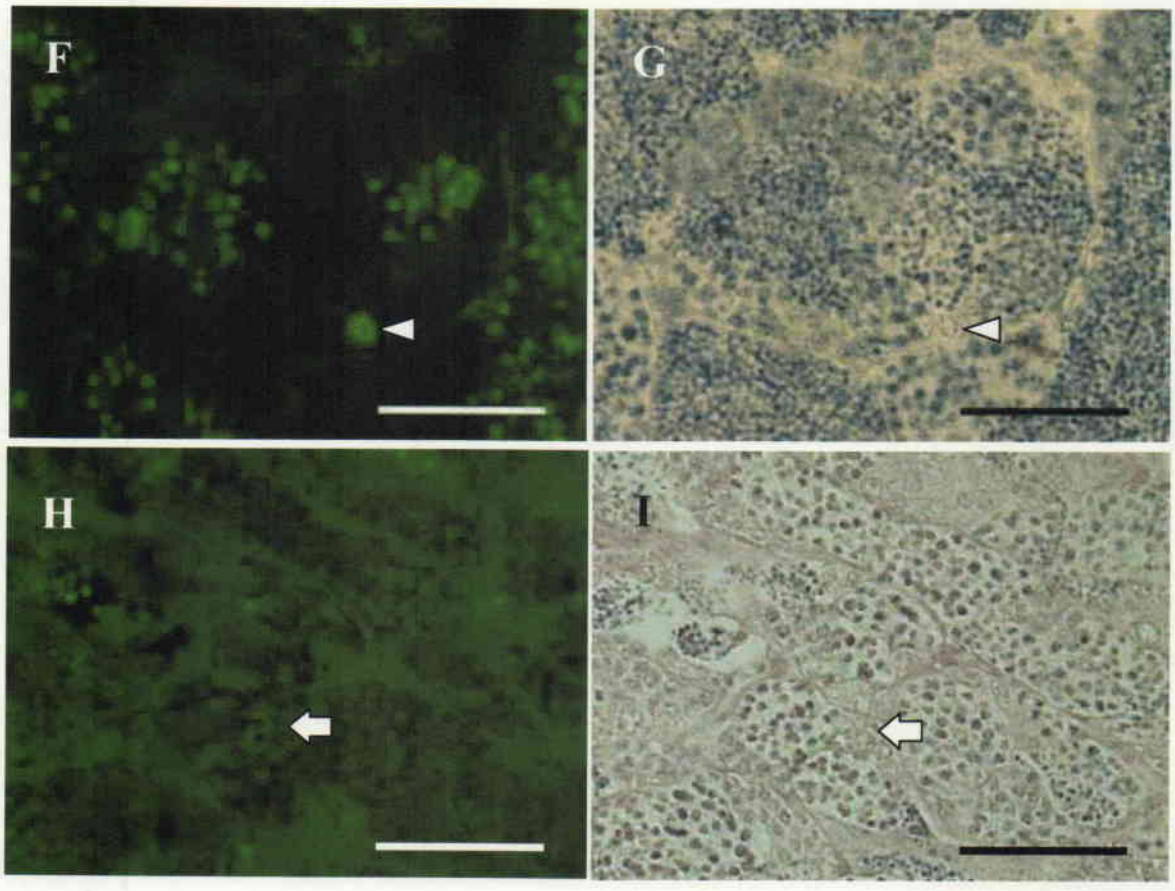

B
C
D
E

Figure 5 (2/2)


F
G
H
I

J

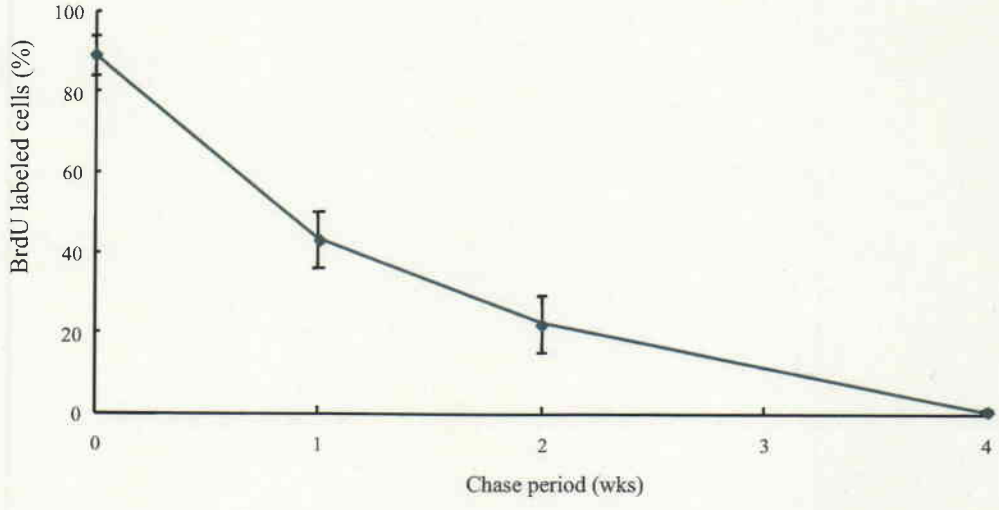
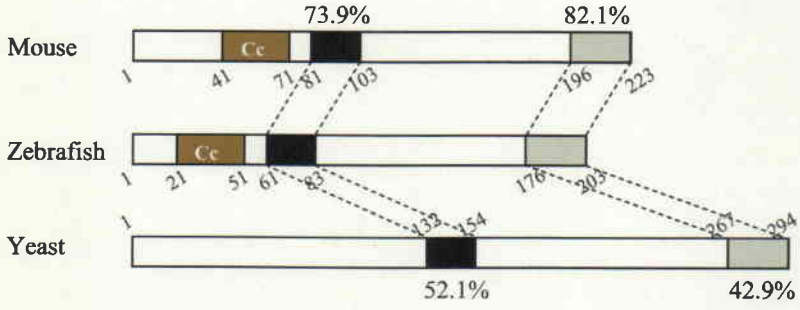

BrdU labeled cells (%)
100
80
60
40
20
0
0
1
2
3
4
Chase period (wks)

Figure 6

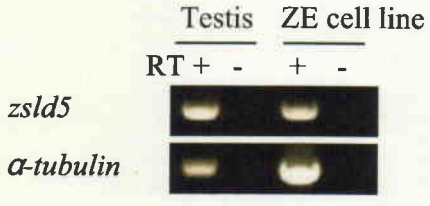
Cloning and expression analysis of zebrafish Sld5. (A) Schematic representation of mouse, zebrafish and yeast Sld5. The percentage homology between two species is indicated. Cc, Coiled-coil. (B) Expression of *zsld5* in the testis and in the ZE cell line was detected by RT-PCR. Total RNAs were extracted from each and used for synthesis of *zsld5* and *α-tubulin* cDNA. (C) Western blot analysis of the testis and the ZE cell line using specific antibody against zSld5.

Figure 6

A


73.9%
82.1%
Mouse
Cc
1
41
71
81
103
196
223
Zebrafish
Cc
1
21
51
61
83
176
203
1
133
154
267
294
Yeast
52.1%
42.9%

B


Testis
ZE cell line
RT + - + -
zsld5
α-tubulin

C

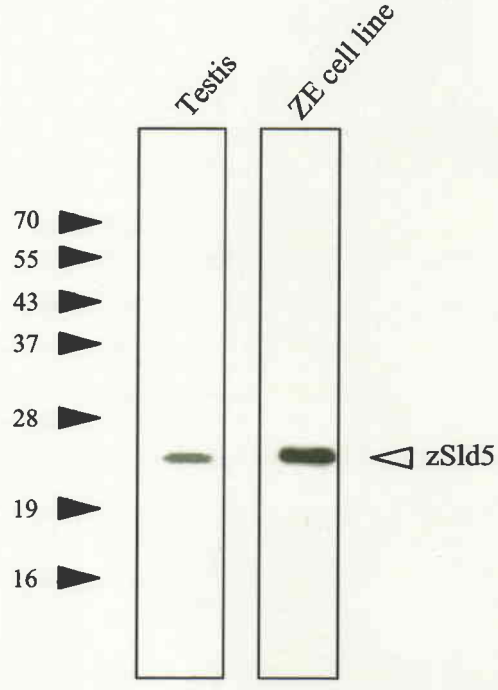
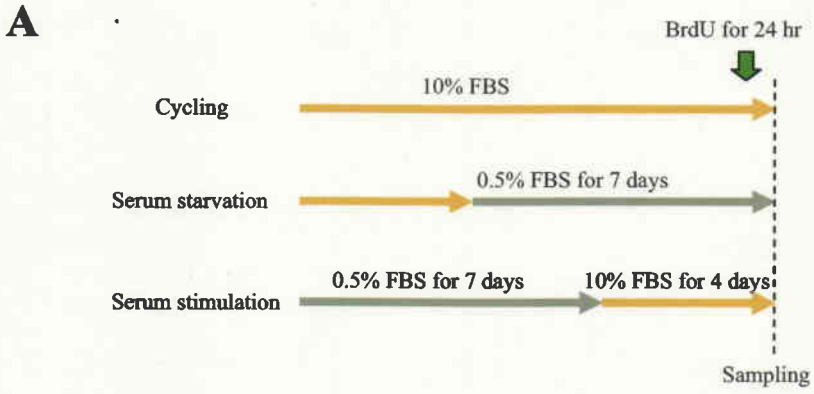

Testis
ZE cell line
70
55
43
37
28
19
16
zSld5

Figure 7

Analysis of zSld5 protein levels in ZE cell line. (A) Schematic outline of the experimental procedures. The orange lines indicate the cultured period in the medium containing 10% FBS. The gray lines indicate the culture period in the serum-starved medium containing 0.5% FBS. (B) Effect of serum starvation on cell proliferation. Cells were maintained in the medium containing 10% FBS or in the serum-starved medium containing 0.5% FBS. Over 1000 cells were measured for each point and percent of BrdU-positive cells was scored. Data are shown as the mean ± SD (n = 3). (C-H) Morphology and immunohistochemical staining of zSld5 were shown in control (C,F), serum starvation (D,G) and serum stimulation condition (E,H). Note that in serum staved condition, zSld5 protein level progressively decreased. Bar = 50 μm.

Figure 7 (1/2)


A
BrdU for 24 hr
10% FBS
Cycling
0.5% FBS for 7 days
Serum starvation
0.5% FBS for 7 days
10% FBS for 4 days
Serum stimulation
Sampling

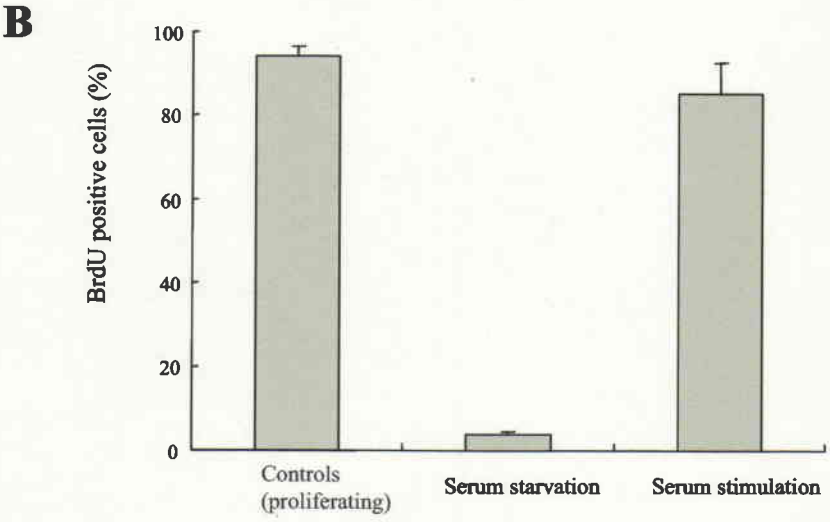

B
BrdU positive cells (%)
100
80
60
40
20
0
Controls (proliferating)
Serum starvation
Serum stimulation

Figure 7 (2/2)

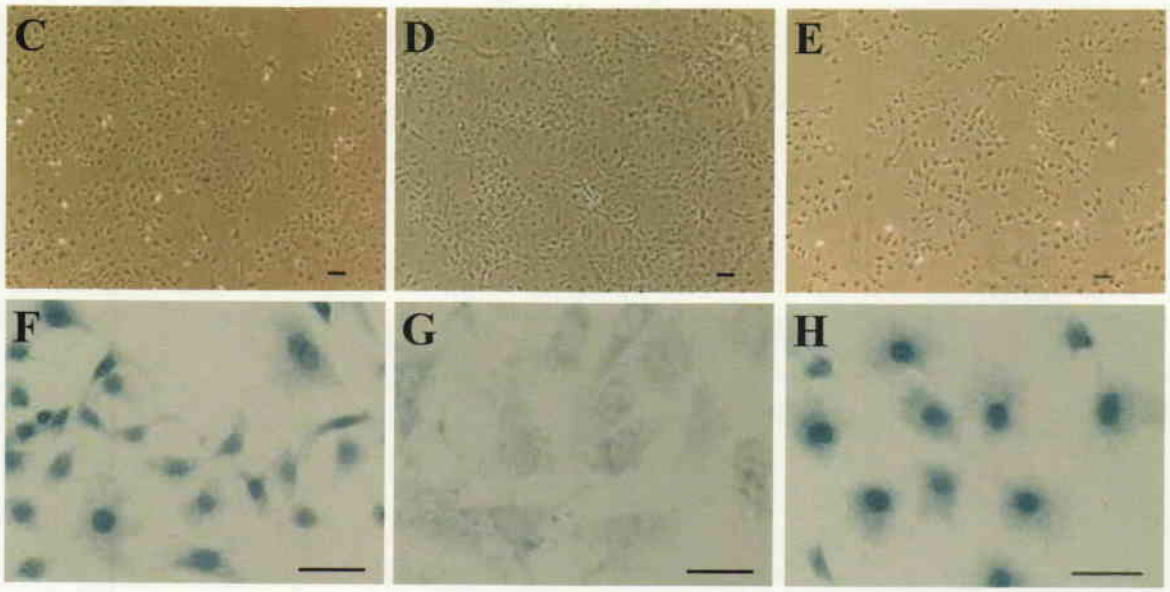


Figure 8

Expression analysis of zSld5 in the zebrafish testis. (A,B) Immunohistochemistry of zSld5 in the testis. (A) Note that both zSld5-positive (white arrowhead) and zSld5-negative (black arrowhead) type A_{cl} spermatogonia were found close to basement membrane. zSld5 signals were also observed in type B spermatogonia (Sgb) and a portion of spermatocytes (Sc), but not in spermatids and sperm. Inset in A shows higher magnification of the dot boxed region. (B) Note that zSld5-positive type A spermatogonia (white arrowhead) were found distant from basement membrane. (C) Analysis of zSld5 expression between type A_{cl} spermatogonia and type A_{dt} spermatogonia in the zebrafish testis. Percent of zSld5-positive and zSld5-negative type A spermatogonia were scored for each sample. Data are shown as the mean ± SD (n = 3). Bar = 50 μm.

Figure 8 (1/2)

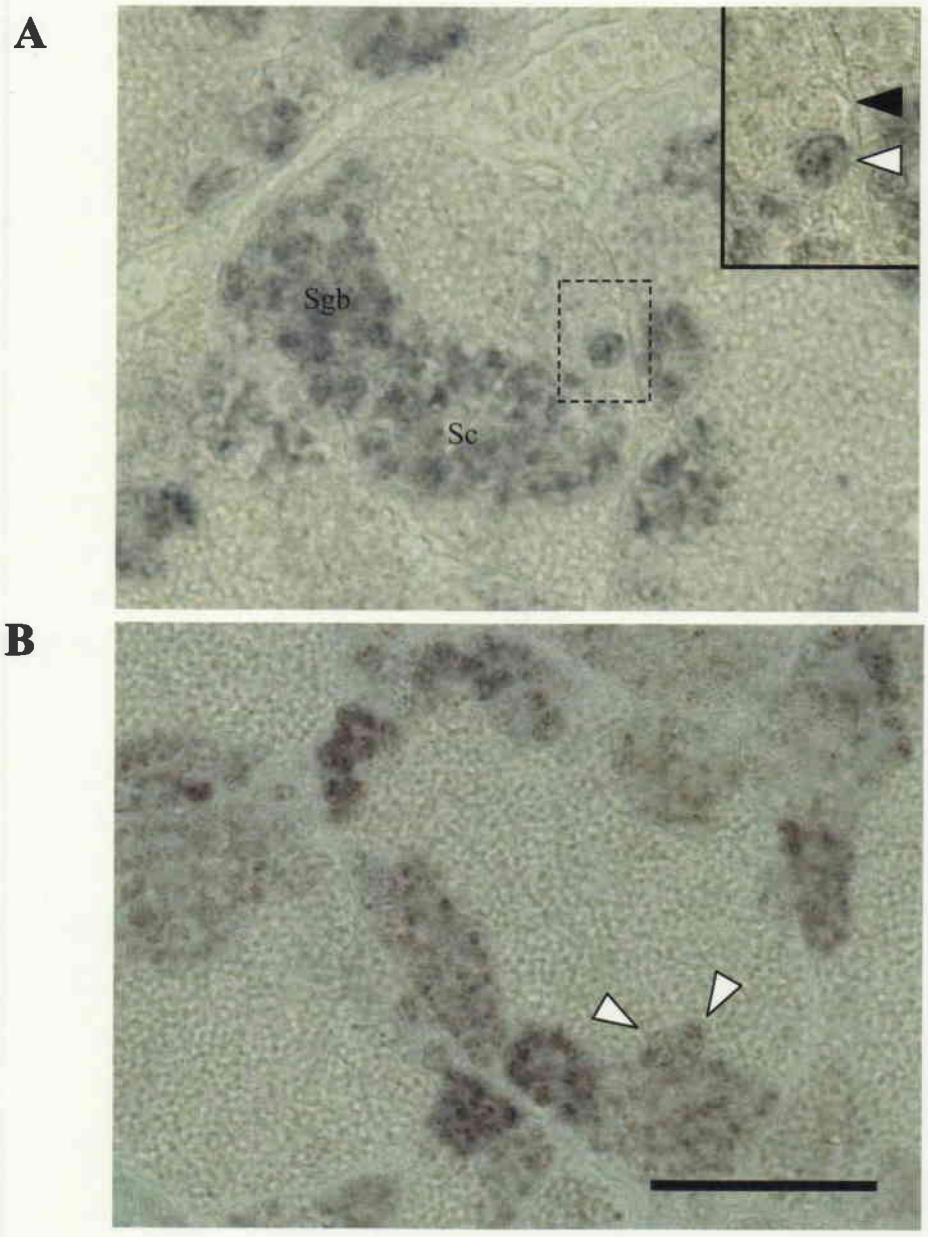


Figure 8 (2/2)

C

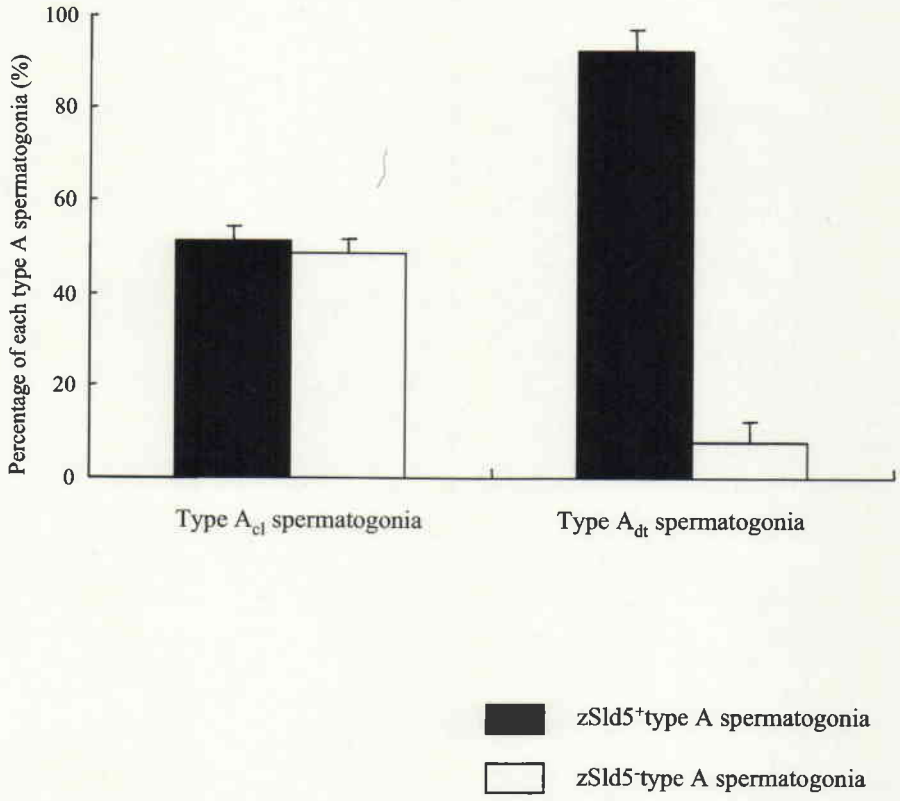

Percentage of each type A spermatogonia (%)
100
80
60
40
20
0
Type A_{cl} spermatogonia
Type A_{dt} spermatogonia
zSld5^{+}type A spermatogonia
zSld5^{-}type A spermatogonia

Figure 9

Analysis of zSld5 expression and incorporation of BrdU after 24 hr administration in the zebrafish testis. Over 600 type A_{cl} spermatogonia were analyzed and percent of BrdU-positive cells was scored for each sample. Data are shown as the mean ± SD (n = 3).

Figure 9

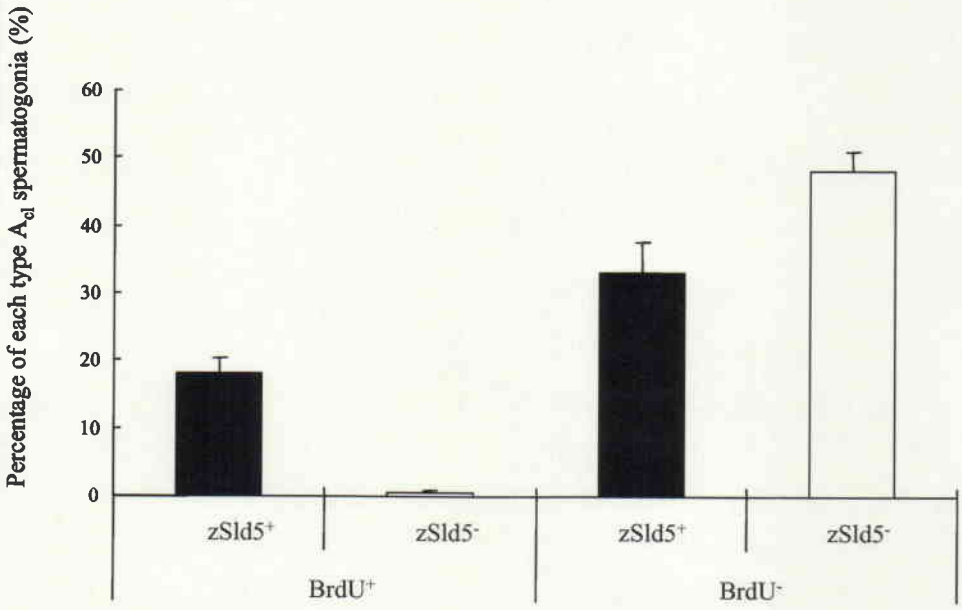

Percentage of each type A_{al} spermatogonia (%)
60
50
40
30
20
10
0
zSld5+
zSld5-
zSld5+
zSld5-
BrdU+
BrdU-

Figure 10

Comparison of morphometric features between zSld5-positive and zSld5-negative type A_{cl} spermatogonia. (A–C) Average values for zSld5-negative type A_{cl} spermatogonia are shown by white bars, and average values for zSld5-positive type A_{cl} spermatogonia by black bars. (A) Comparison of longest diameter (μm) and shortest diameter (μm) of cells. (B) Areas of nucleus and cytoplasm. (C) The ratio of nucleus to cell size. All features were not significant between zSld5-positive and negative cells. Number of cells analyzed: zSld5-negative type A_{cl} spermatogonia, n = 32; zSld5-positive type A_{cl} spermatogonia, n = 33. Values are mean (bars) and SD.

Figure 10

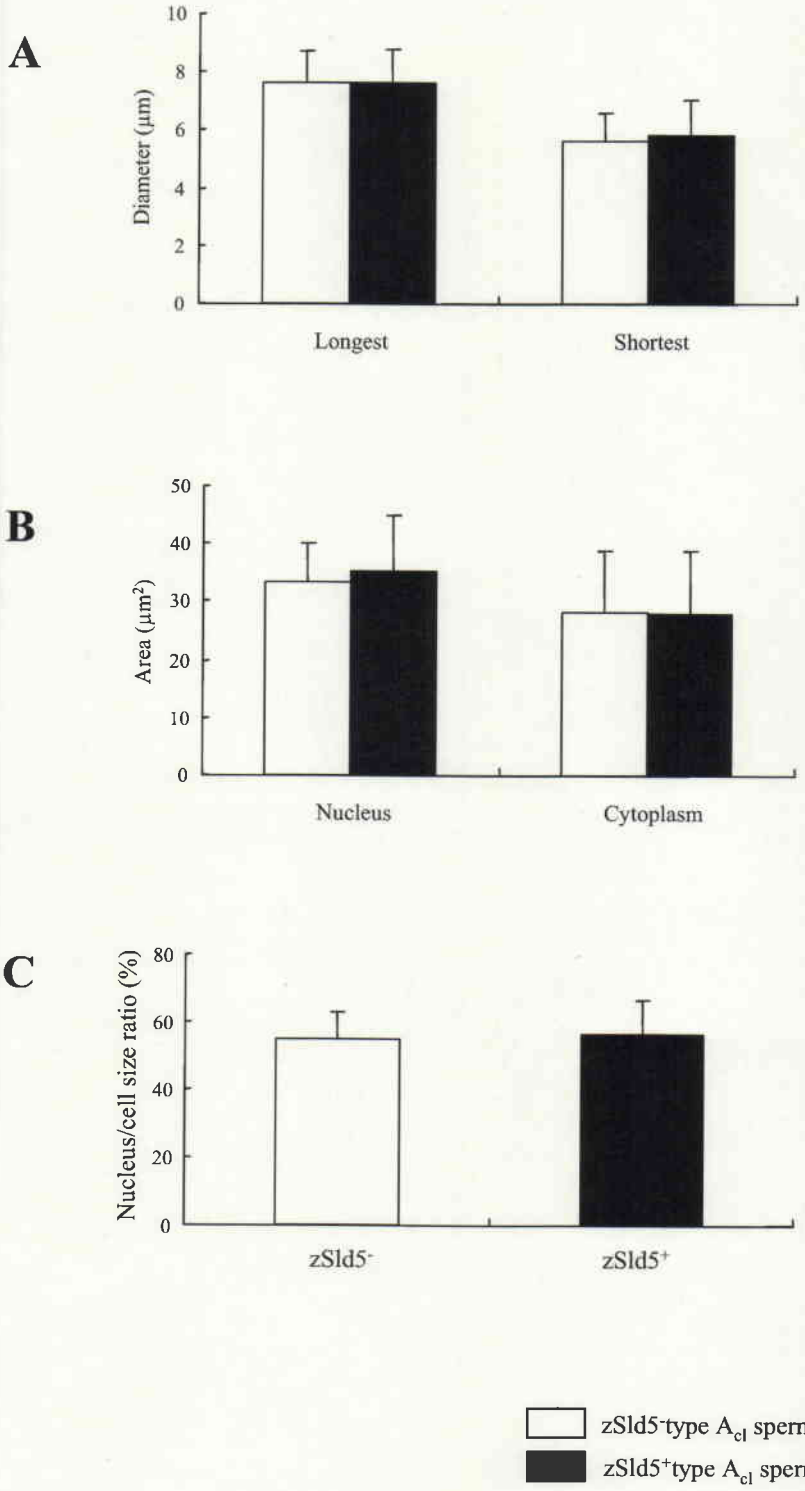

A
Diameter (μm)
10
8
6
4
2
0
Longest
Shortest
B
Area (μm²)
50
40
30
20
10
0
Nucleus
Cytoplasm
C
Nucleus/cell size ratio (%)
80
60
40
20
0
zSld5⁻
zSld5⁺
zSld5⁻type A_cl spermatogonia
zSld5⁺type A_cl spermatogonia

Figure 11

Expression of zSld5 on label-retaining cells (LRCs) in the zebrafish testis. (A) Fluorescence micrograph of immunostained for BrdU after 4 weeks of chase, showing BrdU-retaining type A_{cl} spermatogonia (arrowhead). (B) Bright-field micrograph of immunostained for zSld5 of the same section as presented in (A). (C) Digital overlay of (A) and (B). Note that LRCs do not localize zSld5. Bar = 50 μm.

Figure 11

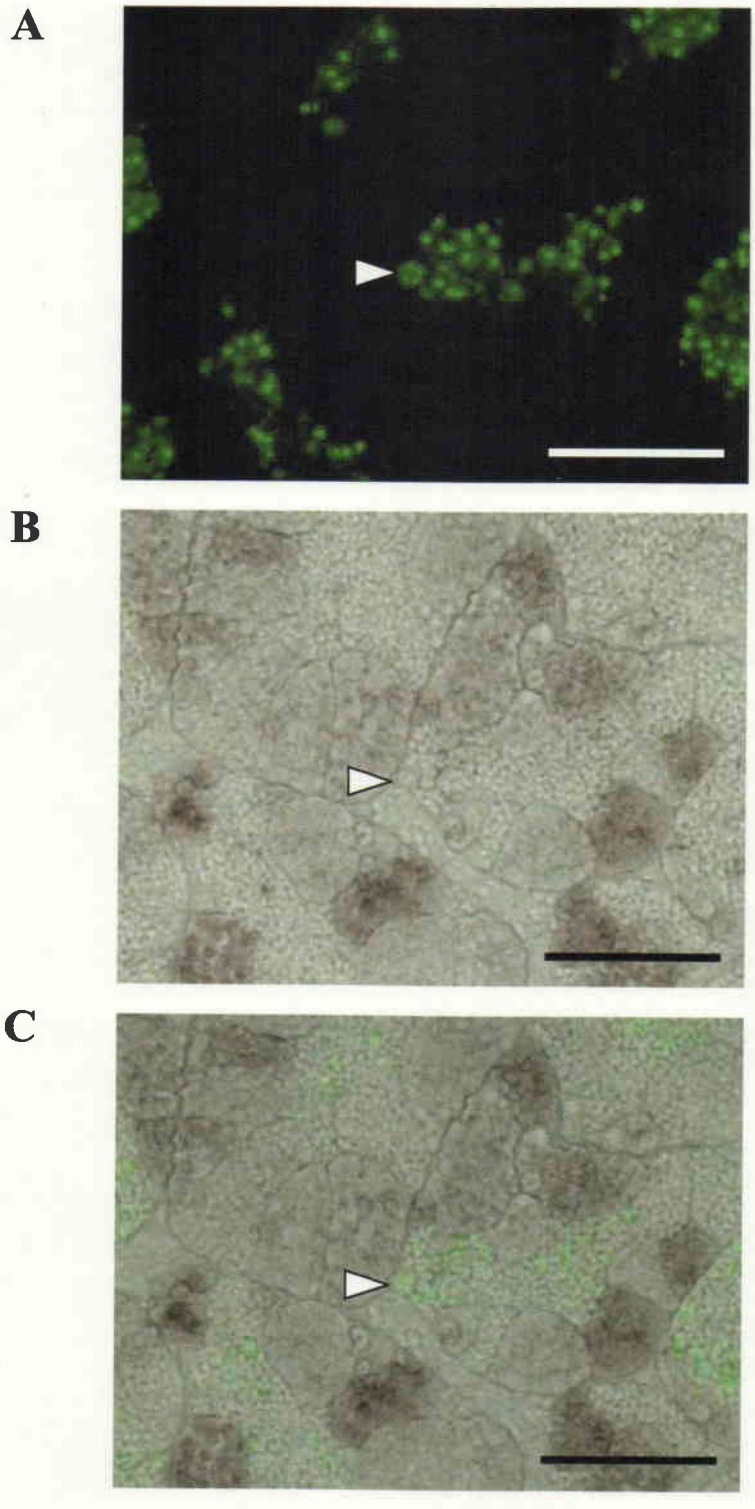


Figure 12

Localization of zSld5-positive type A_{cl} spermatogonia. (A) Two zSld5-positive type A_{cl} spermatogonia (white arrowhead) are located along basement membranes. (B) Two zSld5-positive two type A spermatogonia (white arrowhead) are vertically located against basement membranes. (C) zSld5-positive type A spermatogonia (white arrowhead) and zSld5-negative type A spermatogonia (black arrowhead) are vertically located against basement membranes. An opposite case in C was not observed. White dots represent basement membranes. Bar = 20 μm.

Figure 12

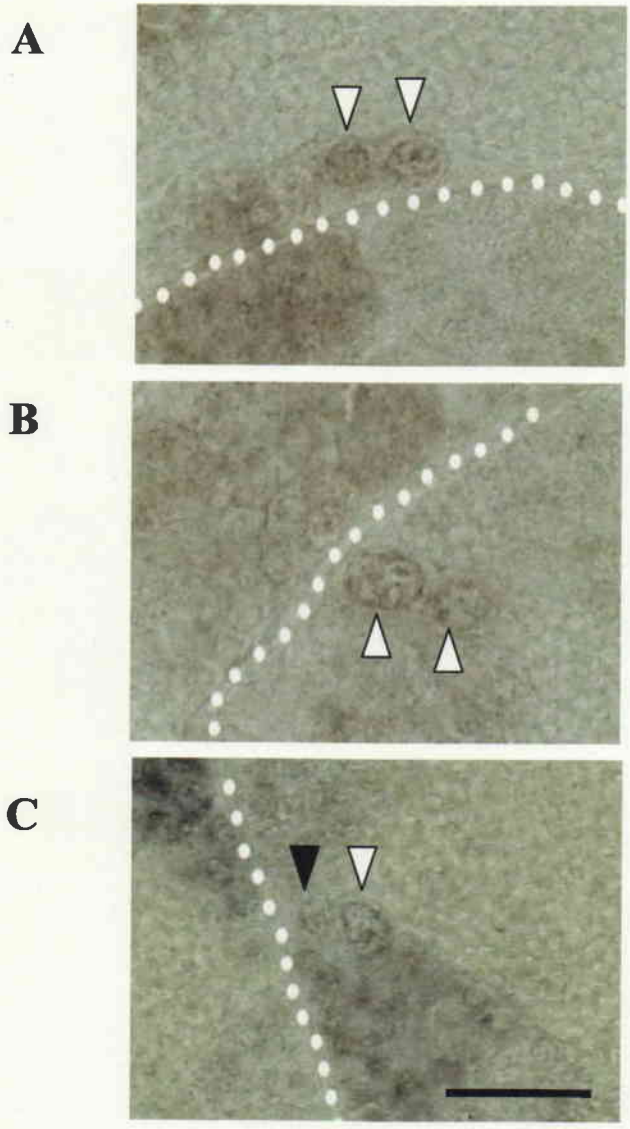


Figure 13

Effect of busulfan at different concentrations on male germ cells. Histological changes of testes were observed after 7 days of busulfan treatment at various doses. (A) control; (B) 6.6 μg/ml; (C) 13.2 μg/ml; (D) 33 μg/ml; (E) 66 μg/ml.

Figure 13

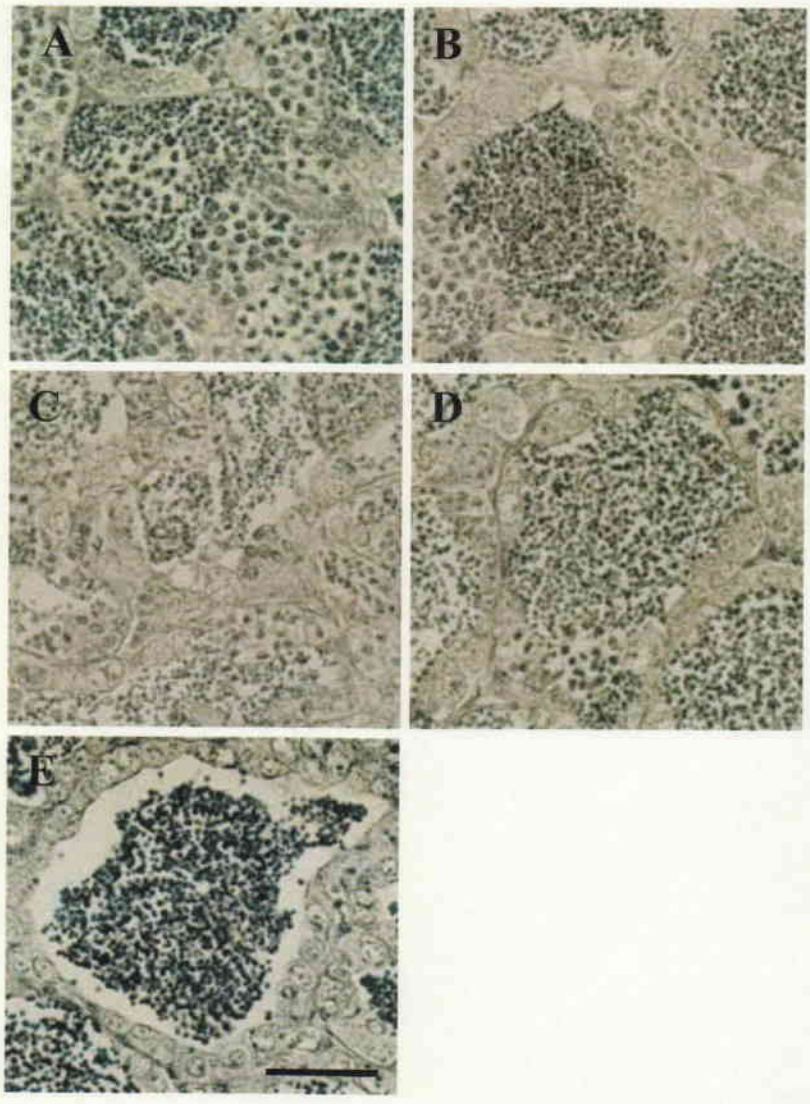


Figure 14

Recovery process of spermatogenesis after busulfan treatment. Changes of germ cells in each cyst were observed and the number of the cysts was counted after busulfan treatment at a dose of 66 μg/ml.

Figure 14

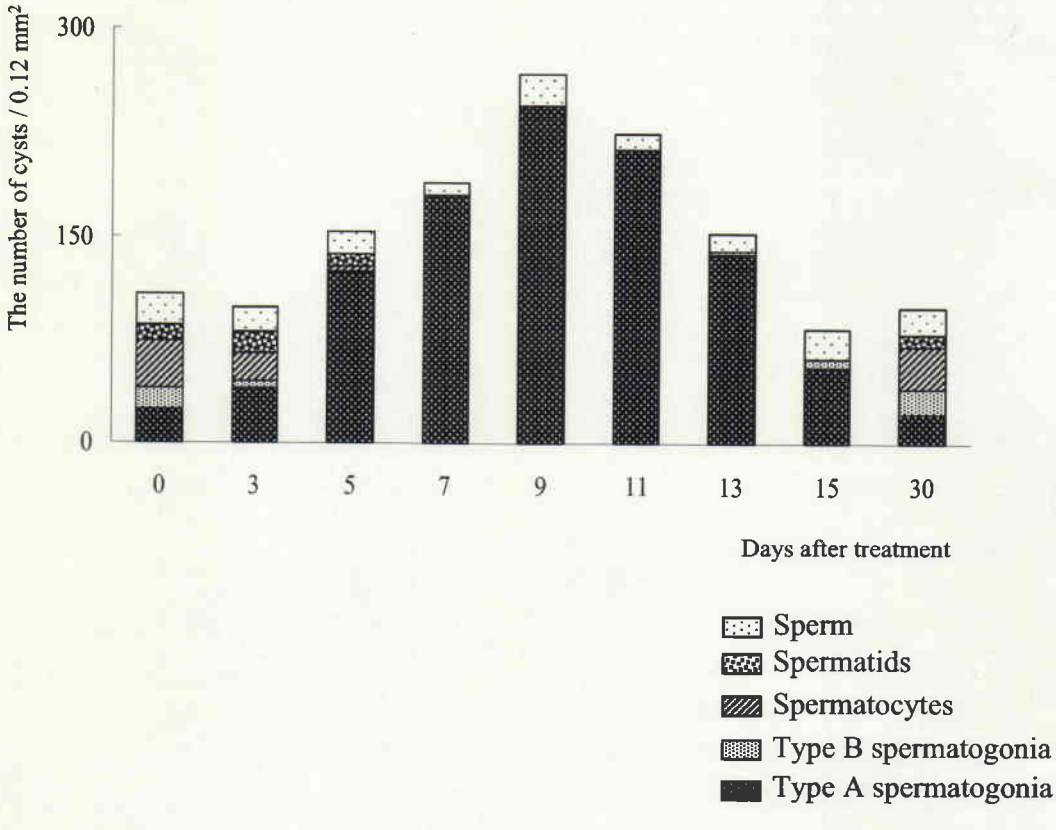
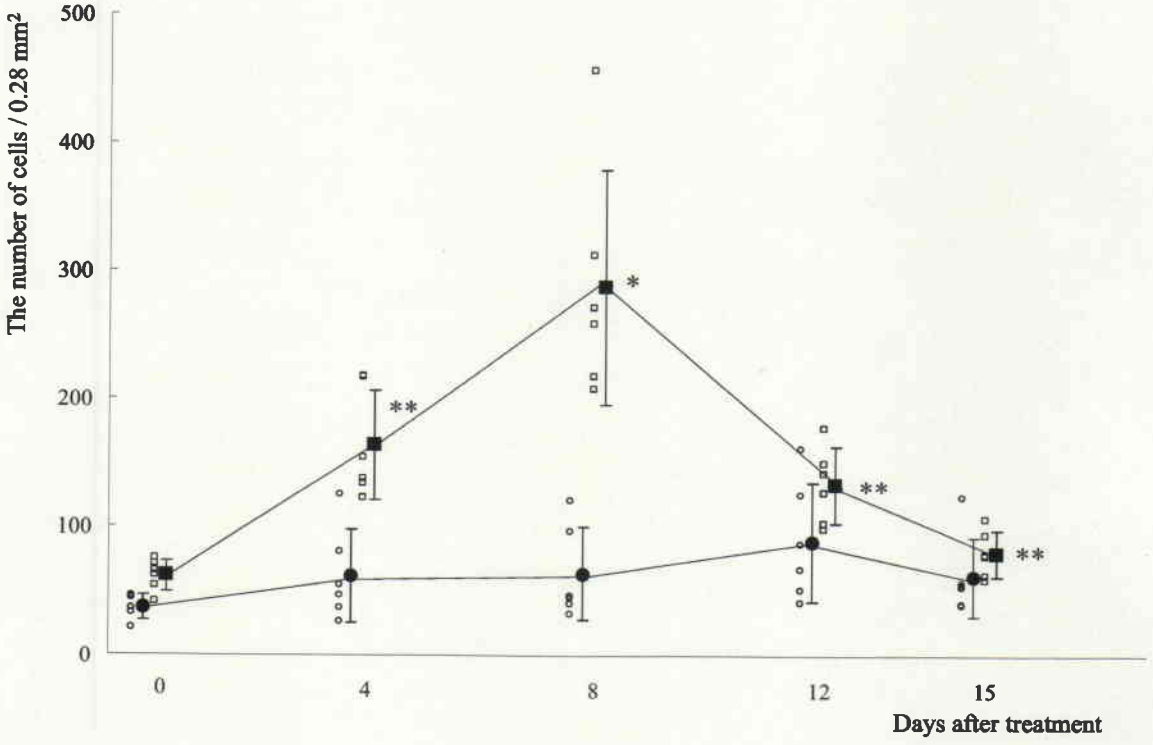

The number of cysts / 0.12 mm²
300
150
0
0
3
5
7
9
11
13
15
30
Days after treatment
Sperm
Spermatids
Spermatocytes
Type B spermatogonia
Type A spermatogonia

Figure 15

Recovery process of zSld5-positive type A_{cl} spermatogonia after busulfan treatment. (A) Changes in the number of zSld5-positive and zSld5-negative type A spermatogonia after busulfan treatment. Open squares and open circles represent each data of the number of zSld5-positive and zSld5-negative type A_{cl} spermatogonia from individual testes, respectively. Black squares and black circles indicate the averages of the number of zSld5-positive and zSld5-negative type A_{cl} spermatogonia, respectively. Values represent the means ± SD (n = 6) of each type A_{cl} spermatogonia. Statistically significant differences in the number of cells to the previous time point are denoted as follows: * $P < 0.05$, ** $P < 0.01$ (Aspin-Welch's t-test). (B) Testis sections of normal zebrafish and (C) busulfan-treated zebrafish at day 8 stained with anti-zSld5 antibody. zSld5-negative type A_{cl} spermatogonia were indicated as white arrowheads. Bar = 50 μm.

Figure 15 (1/2)

A



The number of zSld5⁺type A_{cl} spermatogonia of individual testis

The number of zSld5⁻type A_{cl} spermatogonia of individual testis

Average number of zSld5⁺type A_{cl} spermatogonia

Average number of zSld5⁻type A_{cl} spermatogonia

Figure 15 (2/2)

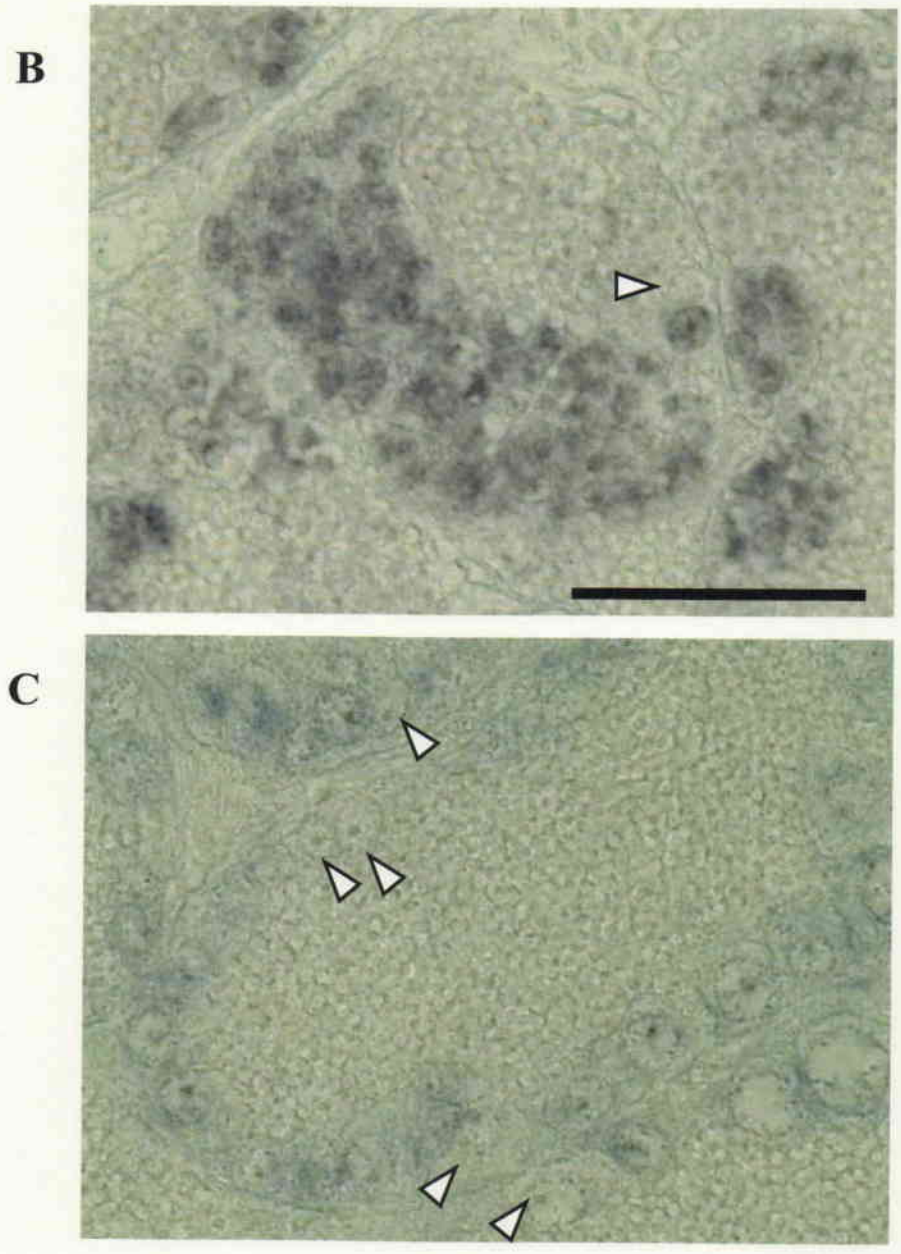


Figure 16

Culture of zebrafish type A spermatogonia isolated from busulfan-treated testes. (A) A Sertoli cell line, ZtA6-6. (B) Type A spermatogonia immediately after isolation. Note that some cells have one or some nucleoli in their nucleus. Morphology of type A spermatogonia at day 2 (C), day 5 (D), day 8 (E) and day 15 (F) in culture are shown. These cells resemble to type A spermatogonia of paraffin section, and the feature did not change during the culture. Bar = 250 μm (A), Bar = 50 μm (B-F).

Figure 16

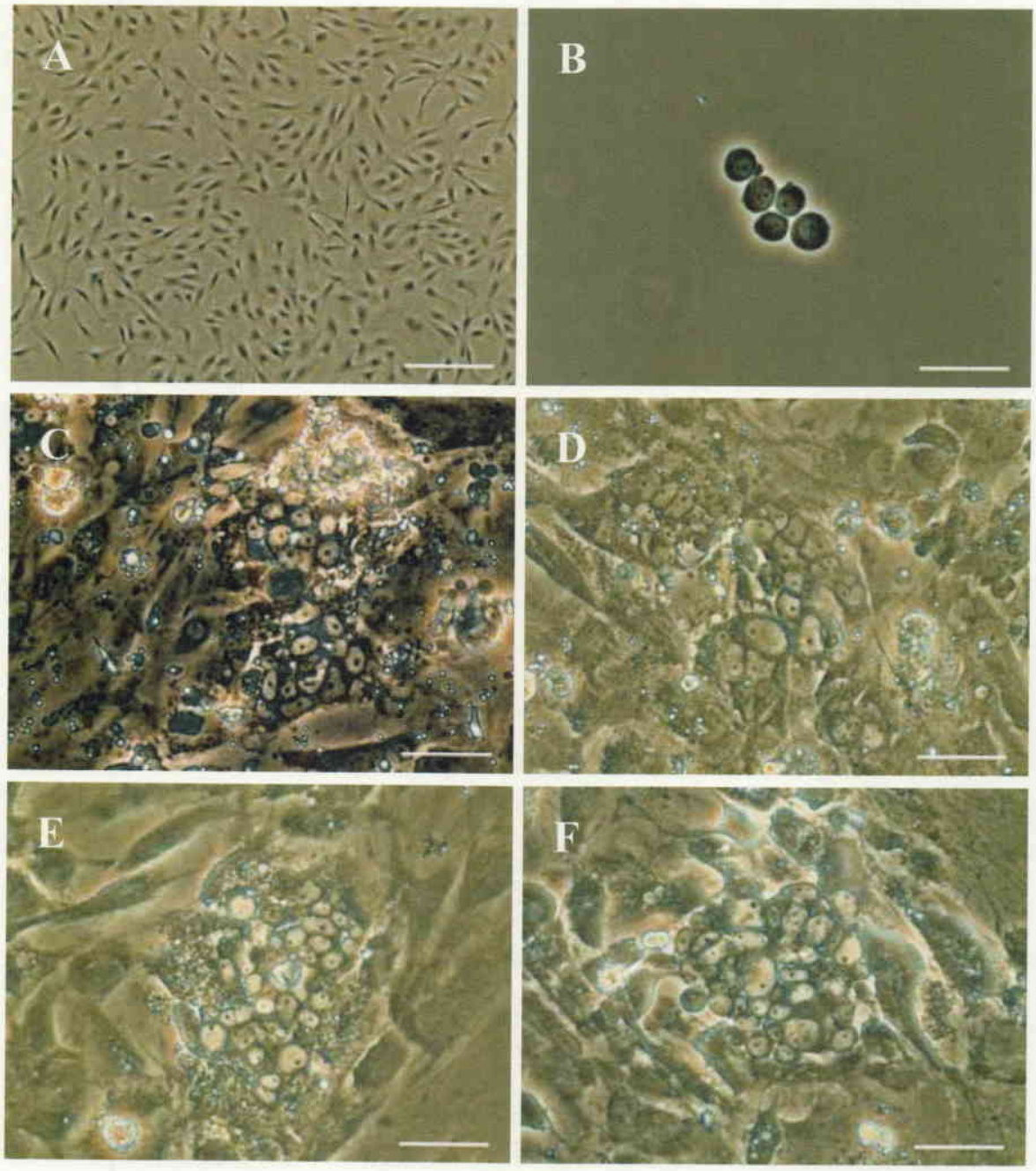


Figure 17

Expression analysis of *vas* in the cultured type A spermatogonia on ZtA6-6 cells. (A,B,D,E) The cells after day 6 (A and D) and day 14 (B and E) of culture were hybridized with *vas* antisense probe. (C,F) The control, the cells after day 6 were hybridized with sense probe. Phase contrast micrographs (A-C) and bright field images (D-F) are shown. Bar = 50 μm.

Figure 17

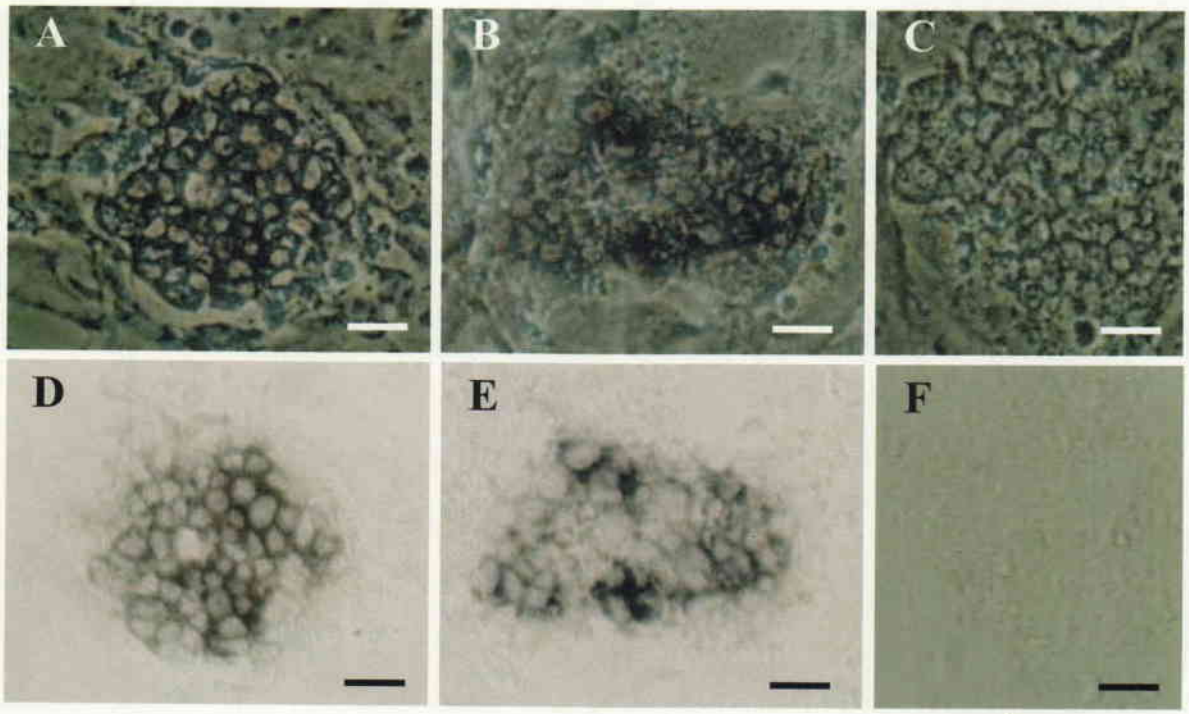


Figure 18

BrdU incorporation in the cultured type A spermatogonia on ZtA6-6 cells. The cells after day 7 (A and E), day 10 (B and F), day 13 (C and G) and day 16 (D and H) of culture were stained with anti-BrdU antibody. Phase contrast micrograph (A-D) and bright field image (E-H) are shown. Note the presence of some BrdU-positive cells in the colony during the culture. Bar = 50 μm.

Figure 18

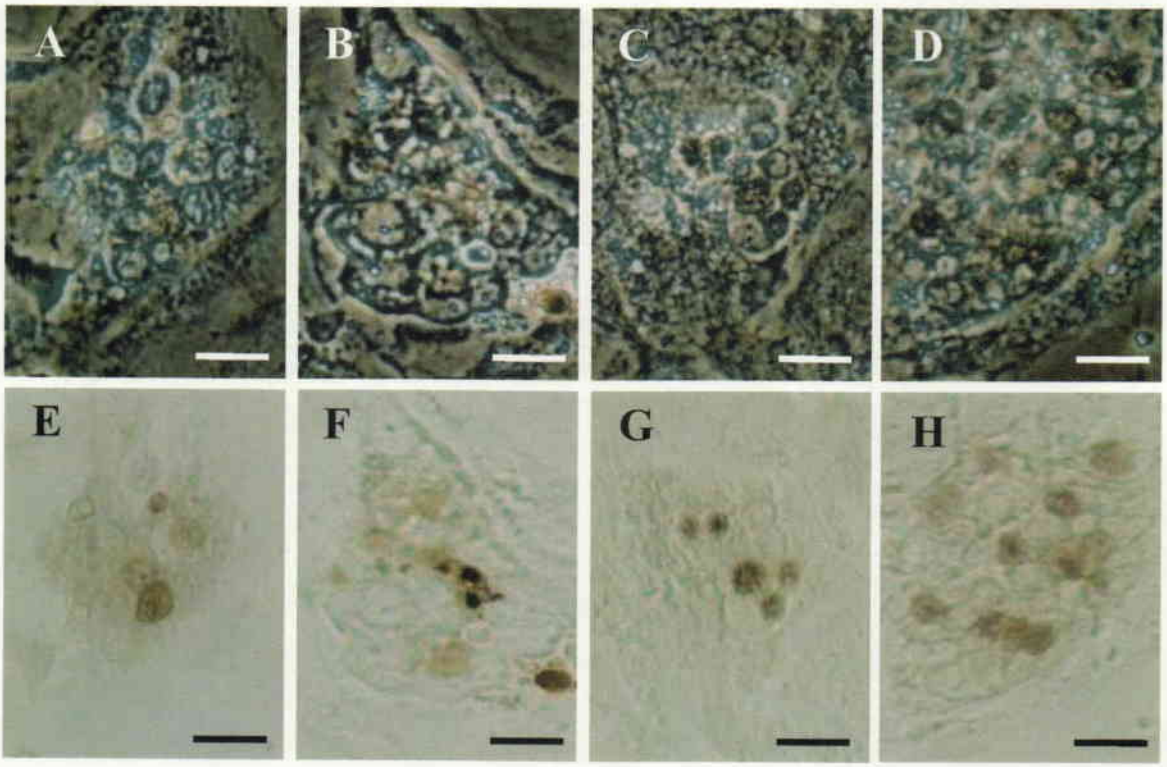


Figure 19

GFP expression of type A spermatogonia in culture isolated from normal testes of *vas::EGFP* transgenic zebrafish. Morphology of the cells after day 20 (A), day 30 (C) and day 60 (E) of culture are shown in phase contrast. *vas::EGFP* expression of the cells after day 20 (B) and 30 (D) of culture are shown in fluorescence micrographs. At day 60 of culture, expression of *vas::EGFP* did not observed in the colony. Bar = 50 μm.

Figure 19