Analysis of molecular and cellular mechanisms regulating medaka (*Oryzias latipes*) spermatogenesis, using a newly established cell culture system

（新規に開発した細胞培養系を用いたメダカ（*Oryzias latipes*）精子形成を制御する分子細胞機構の解析）

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CONTENTS

ABBREVIATIONS

AMH, anti-Müllerian hormone
BrdU, bromodeoxyuridine
CMV, cytomegalovirus
DHP, 17α, 20β-dihydroxy-4-pregnen-3-one
DIG, digoxigenin
Egf, epidermal growth factor
ENU, *N*-ethyl-*N*-nitrosourea
ES, embryonic stem
Fgf, fibroblast growth factor
FSH, follicle-stimulating hormone
Gdnf, glial cell line derived neurotrophic factor
Gsdf, gonadal soma derived factor
GST, glutathione-S-transferase
HCG, human chorionic gonadotropin
11βHSD2, 11β-hydroxysteroid dehydrogenase 2
Igf, insulin-like growth factor
Il, interleukin
IMPS, Iwamatsu's medaka physiological saline
ITRs, inverted terminal repeats
JAK, Janus kinase
KO, knockout
11-KT, 11-ketotestosterone
LB, latex beads
LH, luteinizing hormone
Lif, leukemia inhibitory factor
Mif, macrophage (migration) inhibitory factor
ORF, open reading frame
p45011β, cytochrome P450 11β
PBS, phosphate buffered saline
PFA, paraformaldehyde
pFSH, porcine follicle-stimulating hormone
PGCs, primordial germ cells
PI, propidium iodide
PAGE, polyacrylamide gel electrophoresis
PMSG, pregnant mare serum gonadotropin
qRT-PCR, quantitative reverse transcription-polymerase chain reaction
RA, retinoic acid
RT-PCR, reverse transcription-polymerase chain reaction
SDS, sodium dodecyl sulfate
Sox, SRY-related HMG-box
STAT, signal transducers and activators of transcription
WT, wild-type
Zfp91-Cntf, zinc finger protein 91 homolog-ciliary neurotrophic factor transcription unit

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GENERAL INTRODUCTION

Proliferative differentiation of germ cells that produce gametes under various controls ensures the continuity of life in multicellular organisms. Spermatogenesis is a complex process that involves highly regulated cell growth and differentiation, including proliferation of spermatogonia by mitosis, genetic modifications in spermatocytes through meiosis, and differentiation of spermatids to spermatozoa with drastic morphological and functional changes to carry the genetic information to the eggs. Therefore, spermatogenesis provides an attractive model system to analyze the cellular and molecular mechanisms of developmental processes accompanying mitotic and meiotic cell divisions and cellular maturation and differentiation. *In vivo*, many molecules such as cell growth factors, cell differentiation factors and cell adhesion factors are intimately intertwined, making it difficult to understand the precise control mechanisms of spermatogenesis (Chen et al., 2015). It would be expected that this complexity is unraveled by using a simple experimental system, the *in vitro* culture system, which tells us when and how these factors work in the process of spermatogenesis.

Attempts have been made to establish testicular cell culture system in various animal species for more than a century (Komeya et al., 2018). Recently, it has become possible to reproduce an entire spermatogenic process in mice by an organ culture system (Sato

et al., 2011) and in zebrafish by a dispersion cell culture system with feeder cells (Kawasaki et al., 2016). In the case of mice, however, a dispersion cell culture system can reproduce spermatogonial proliferation but not meiosis (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004; Ogawa et al., 2004). The failure of mouse spermatogenic cells to undergo meiosis in the dispersion culture system is largely due to a mammal-specific situation in the interaction between male germ cells and somatic cells (Sertoli cells), in which a single Sertoli cell contacts with male germ cells throughout the process of spermatogenesis from spermatogonial proliferation/differentiation to spermiogenesis via meiosis. This situation means that the somatic cell-mediated regulatory mechanisms of male germ cells in mammalian testis are very complicated; a single Sertoli cell regulates the germ cells in a spatio-temporal manner, in which one Sertoli cell locally produces and secretes plural and different regulatory factors for spermatogenesis depending on the stage of germ cells. In contrast, a fish testis consists of many pouches (called cysts) surrounded by Sertoli cells, in which germ cells at the same developmental stage reside. Therefore, the fish testis seems to have a system simpler than that in mammals from the viewpoint of somatic cell-germ cell interaction; namely, all Sertoli cells constituting a cyst synchronically regulate the germ cells only in a temporal manner depending on the stage of germ cells existing in the cyst, in

striking contrast to the spatio-temporal manner in mammals as described above. Indeed, the difference in testicular structure between fish and mammals should be the reason why the whole spermatogenesis can be reproduced in zebrafish but not in mice by a simple dispersion cell culture system (Kawasaki et al., 2016). It is obvious that molecular and cellular mechanisms regulating spermatogenesis can be easily analyzed by dispersion cell culture rather than organ culture, prompting us to use fish as experimental animals for a better understanding of basic regulatory mechanisms of spermatogenesis in vertebrates.

Medaka and zebrafish are well known as model organisms for vertebrates. These two small fish speciated about 200 million years ago (Inoue et al., 2015) and differ in several important points such as genome size, reproduction and developmental patterns. Thus, the two species are very useful for examining similarities and differences in spermatogenesis, enhancing our understanding of the mechanisms regulating spermatogenesis in general and species-specific aspects. In comparison with zebrafish, I aimed to analyze molecular and cellular mechanisms of medaka spermatogenesis by a system similar to that used in zebrafish (Kawasaki et al., 2016), namely a dispersion cell culture system that recapitulates the entire spermatogenic process from spermatogonia to spermatozoa.

In this study, I established a new *in vitro* medaka male germ cell culture system to enable analyses of gene and protein functions in medaka meiosis and mitosis. So far, *in vitro* spermatogenesis from spermatocytes to spermatozoa by a dispersion cell culture system has been reported for medaka (Saiki et al., 1997; Shimizu et al., 1997; Sasaki et al., 2005); however, a culture system for the whole process of spermatogenesis from spermatogonia to functional fertilizable spermatozoa has not been established yet. In Chapter I, I established a culture system that can produce functional spermatozoa from spermatogonia by improving the culture medium. In addition, I established a system in which spermatozoa can be produced more stably by using a newly established feeder cell line. In Chapter II, I studied growth and differentiation factors involved in spermatogenesis, using the new culture system launched in Chapter I. Based on findings that the number of spermatogonia and the expression level of leukemia inhibitory factor (Lif) are increased in the testes of *p53* (a tumor suppressor gene) –knockout (*p53*-KO) medaka, I examined the function of Lif in medaka spermatogenesis, by adding recombinant Lif proteins to the cell culture medium or co-culture with Lif-overexpressing Mtp1 cells.

Chapter I

In vitro transmeiotic differentiation of medaka spermatogonia into functional spermatozoa

INTRODUCTION

Although the molecular and cellular mechanisms of spermatogenesis have been extensively investigated by using mammals, the use of fish has some experimental advantages compared with mammals and, indeed, results obtained from studies using fish have continuously provided new insights into the regulatory mechanisms of spermatogenesis (Schulz et al., 2010). In particular, it is notable that fish spermatogenesis can be reproduced *in vitro*. This property facilitates analyses of the molecular mechanisms of spermatogenesis because we can directly and simply assess the effects of substances to be tested by addition to the culture medium. *In vitro* spermatogenesis has been reported in some fish species: eel (*Anguilla japonica*) (Miura et al., 1991; Ohta et al., 2007; Miura et al., 2011), medaka (*Oryzias latipes*) (Saiki et al., 1997; Shimizu et al., 1997; Shimizu et al., 2000; Song and Gutzeit, 2003; Hong et al., 2004; Sasaki et al., 2005; Iwasaki et al., 2009), zebrafish (*Danio rerio*) (Sakai, 2002; Kawasaki et al., 2012; Wong and Collodi, 2013; Kawasaki et al., 2016) and tilapia (*Oreochromis niloticus*) (Tokalov and Gutzeit, 2005). Among them, the medaka fish has received much attention as an experimental animal in various fields of biological science, including reproductive and developmental biology (Naruse et al., 1994; Wittbrodt et al., 2002; Shima and Mitani, 2004), and, accordingly, various wide-ranging

experimental tools have become available, including genomic and cDNA information, technical manuals, wild-type, inbred, mutant and transgenic strains (see Medaka Book, https://www.shigen.nig.ac.jp/medaka/medakabook/index.php; National BioResource Project (NBRP) Medaka, https://www.shigen.nig.ac.jp/medaka/ March 17, 2019).

In medaka, an *in vitro* culture system, in which spermatocytes can differentiate into spermatozoa, has already been established (Saiki et al., 1997; Shimizu et al., 1997; Sasaki et al., 2005); however, a culture system that reproduces all processes of spermatogenesis *in vitro* has not been established yet. In previous culture systems, spermatogonia neither differentiate nor produce fertile spermatozoa. Therefore, I developed a new *in vitro* male germ cell culture system that allows transmeiotic differentiation of medaka spermatogonia into functional spermatozoa, for analysis of gene and protein functions in medaka spermatogenesis.

MATERIALS AND METHODS

Fishes

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (permission No. 08-0013 and 13-0099). Sexually mature hi-medaka (an orange-red variety) were obtained from a local fish farm. *olvas*-GFP medaka (Tanaka et al., 2001) was kindly provided by Prof. Minoru Tanaka (Nagoya University). The fish were cultured in fresh water at 27˚C under artificial light conditions (14-hour light and 10-hour dark) to induce and maintain the daily reproductive cycle. The use of these animals for experimental purposes was in accordance with the guidelines of the Hokkaido University.

Cell culture

Culture of medaka spermatogenic cells was performed according to the procedure described previously (Shimizu et al., 1997), with several crucial modifications. The original medium (called S medium in this study) consisted of Leibovitz L-15 (Life Technologies, Tokyo, Japan) supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin (Sigma-Aldrich, St Louis, United States of America), 1 mg/L bovine insulin and 10 mM HEPES (pH 7.4) (Table 1). In the

present study, I added the following substances to the medium (called MS medium in this study): 50 ng/ml retinol, 3% carp serum (*Cyprinus carpio*; obtained from mature carps purchased by a local fish farm: The serum was produced from blood centrifuged and clotted at room temperature for 30 minutes and stored at -80˚C until use.), 10 IU/ml human chorionic gonadotropin (HCG, Teikoku Hormone Medical, Kanagawa, Japan), 10 IU/ml pregnant mare's serum gonadotropin (PMSG, Teikoku Hormone Medical, Kanagawa, Japan) and porcine follicle-stimulating hormone (pFSH, Teikoku Hormone Medical, Kanagawa, Japan). The pH of culture medium was also changed from 7.4 (S medium) to 7.3 (MS medium) (Table 1).

In some experiments, the medium was further supplemented with 10^{-7} M retinoic acid (RA, Sigma-Aldrich, St Louis, United States of America), 50 ng/ml 11-ketotestosterone (11-KT, Sigma-Aldrich, St Louis, United States of America) and 10 ng/ml 17α, 20β-dihydroxy-4-pregnene-3-one (DHP, Sigma-Aldrich, St Louis, United States of America); the latter two steroids are major androgen and progestin in the teleost fish, respectively, and known to play important roles in inducing spermatogenesis (Nagahama, 1994). These supplements were added to MS culture medium from 5 days before fertilization experiments.

Mtp1 feeder cells

Mtp1, a somatic cell line derived from *p53*-KO medaka testes (Kawasaki et al., 2009), was cultured by MS medium. Mtp1 was kindly provided by Dr. Noriyoshi Sakai (Genetic Strains Research Center, National Institute of Genetics).

Cell sorting

Medaka testes were sterilized for 2 minutes in 0.5% bleach in Iwamatsu's medaka physiological saline excluding $CaCl_2$ (IMPS (-); 111 mM NaCl, 5.4 mM KCl, 0.6 mM $MgSO_4$, adjusted to pH 7.3 with 0.1 M $NaHCO_3$). After washing 2 times in IMPS (-), 3 or 4 testes were minced with scissors, put in a tube containing 1 ml of 1.2 mg/ml dispase (Life Technologies) and 2 mg/ml collagenase (Wako, Osaka, Japan) in IMPS (-), and incubated at 28˚C for 2 hours with pipetting at intervals of 20 minutes to dissociate the tissue into single cells. The cell suspension was filtered through 70-μm mesh and centrifuged at 420 *g* for 5 minutes at room temperature. The pellet was suspended in MS medium (Sheath solution, which is commonly used for cell sorting, is toxic to medaka spermatogenic cells.) containing 2 μg/ml of propidium iodide (PI), filtrated through 40-μm mesh, and sorted by a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). To remove dead cells, PI-negative cells (living cells) were

sorted according to the levels of forward scatter and side scatter, which roughly represent the size and internal complexity, respectively, and collected in silicon-coated tubes ($1.5x10^5$ cells/tube).

The obtained cell fractions were examined by a phase-contrast microscope, and the types of cells in each fraction were identified by their morphology (Fig. 1 and 2). To identify the cell types, I also used *olvas-GFP* medaka, which contain GFP-labeled spermatogonia and spermatocytes (GFP expression levels in spermatogonia being higher than those in spermatocytes) (Tanaka et al., 2001). Immunocytochemistry with an antibody against the meiosis marker Sycp3 (Iwai et al., 2006) was also performed to identify primary spermatocytes.

Labeling of germ cells with bromodeoxyuridine (BrdU) in culture

Spermatogonia-rich fractions obtained by cell sorting were labeled with 0.5 μM BrdU (Sigma-Aldrich, St Louis, United States of America). BrdU was added on 3, 5, and 7 days of culture using S medium, MS medium, and MS medium supplemented with Mtp1 feeder cells. After culture for 24 hours, the cells were harvested, suspended in PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4), attached to a cover slip coated with 0.1% poly-L-lysine and fixed with 4%

paraformaldehyde (PFA) diluted in PBS (4% PFA/PBS pH 7.4) for 15 minutes. The fixed cells were washed two times with PBS and treated with 0.1% Triton-X100 for 5 minutes and then 2N HCl for 20 minutes. After washing in PBS three times, the cells were blocked in 10% goat serum in PBS for 30 minutes, treated with anti-BrdU antibody (Chemicon, Temecula, United States of America) at a 1:200 dilution in blocking buffer (Roche Diagnostics, Tokyo, Japan) for 45 minutes at room temperature and then washed two times in PBS. BrdU signals were visualized by Alexa 488-conjugated anti-mouse IgG antibody (Life Technologies, Tokyo, Japan). Following washing two times in PBS, the samples were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, United States of America) and observed under a Zeiss Axioskop microscope. Since somatic cells, secondary spermatocytes, spermatids and spermatozoa can be distinguished by their morphology, only BrdU-positive spermatogonia and primary spermatocytes were counted (spermatogonia and primary spermatocytes are indistinguishable when fixed for immunological detection of BrdU.)

Phagocytosis assay of Mtp1

A 1,000-fold diluted suspension of polystyrene beads (LATEX BEADS, LB-11 Sigma-Aldrich, St Louis, United States of America; average diameter, 1.1 μm) was added to a culture plate of Mtp1 in an 80% confluent state and the plate was incubated at 28℃ for 24 hours. Internalization of the beads into Mtp1 cells was determined by phase-contrast microscopic observation after washing the cell layer at least 3 times by PBS.

Detection of medaka Sertoli and Leydig cell markers by reverse transcription-polymerase chain reaction (RT-PCR)

Following isolation of total RNA samples with ISOGEN (Nippon Gene, Tokyo, Japan), cDNAs were produced with a Super Script III First Strand Synthesis System (Life Technologies, Tokyo, Japan). According to the amino acid sequence data obtained by similarity searches of human, mouse, chicken, *Xenopus* and zebrafish against Ensemble databases, I designed primer sets that amplify cDNA fragments (ca. 500 bp) encoding medaka Sertoli cell markers, Leydig cell markers, housekeeping genes and cytokines. Those include epidermal growth factor (Egf), Fgf2, Fgf16, Fgf20a, Fgf20b, Gdnfa, Gdnfb, insulin-like growth factor 1 (Igf1), interleukin-11a (Il-11a), Il-11b,

macrophage migration inhibitory factor (Mif), and zinc finger protein 91 homolog-ciliary neurotrophic factor (Zfp91-Cntf) (Table 2). The cDNA fragments were sequenced to confirm that the primer sets specifically amplify the target cDNAs.

Isolation of a single colony from Mtp1 cells

Limiting dilution was carried out using a 96-well plate. After dilution, the wells containing only one cell were checked and used for a long-term culture. Conditional medium of Mtp1 was used as a culture medium for isolated Mtp1 cells. Initially, isolated Mtp1 cells were cultured on gelatin-coated 96-well plates, then transferred to a larger plate after treating the cells by 0.05% trypsin-EDTA (Thermo Fisher Scientific K. K., Tokyo, Japan).

Primary cultures of medaka male germ cells

Testes were removed carefully from mature medaka with a pair of fine forceps under a dissecting microscope to prevent contamination with bacteria from the fish body and gut and with other tissues such as fat and blood. Each testis was transferred into sterile IMPS (-) in a 35-mm dish.

Testes were treated with 0.5% bleach in IMPS (-) for 2 minutes. After washing 2 times with IMPS (-) each for 2 minutes, the testes were minced with fine micro scissors, put in a 15-ml tube containing 2 mg/ml of collagenase (Wako, Osaka, Japan) and 1.2 mg/ml of dispase (Life Technologies, Tokyo, Japan) in IMPS (-), and incubated at 28℃ for 2 hours. At intervals of 20 minutes, the tissue was pipetted with a Pasteur pipette to dissociate it into single cells. The cells suspension was filtered through 70-μm mesh and centrifuged at 400 *g* for 5 minutes at room temperature after 7-fold dilution with IMPS (-). The supernatant containing mature spermatozoa was discarded. The resulting cell pellet was suspended in a culture medium and the number of cells was counted. The cells were cultured in a gelatin-coated (0.1% gelatin (Wako, Osaka, Japan) in PBS, pH 7.3) 35-mm plastic dish (Corning, New YorK, United States of America) at the density of $6x10^6$ cells/dish in the presence of 50 μg/ml kanamycin at 28℃ in humidified air. When feeder cells were used, Mtp1 cells in an approximately 80% confluent state were reseeded to a plate at twice the initial concentration on the day before cultivation. The medium was changed on days 1, 4, 7 and 10 to remove dead cells and spermatozoa, both of which were liberated from the bottom of dish to the culture medium. In some experiments, the isolated cells were subjected to cell sorting to obtain a cell fraction enriched by spermatogonia. For artificial fertilization experiments, MS medium was

supplemented with RA, 11-KT and DHP on day 10 (5 days before fertilization experiments) as described above.

Insemination of naturally ovulated eggs with spermatozoa produced *in vitro*

After cultured for 15 days, cells were collected by normal trypsin treatment. To concentrate the spermatozoa, the cell suspension was centrifuged at 420 *g* for 5 minutes and resuspended in IMPS (IMPS (-) containing 1 mM $CaCl_2$). The spermatozoa were washed 3 times with IMPS and stored on ice until use for insemination.

Naturally ovulated unfertilized eggs were released from isolated ovaries by tearing the ovarian cavity with fine forceps, and their attaching filaments were cut off with scissors. The isolated eggs were put into a plastic dish (35 mm in diameter) filled with IMPS. Artificial fertilization was performed in a medium containing 0.5% BSA and 10 mM HEPES (pH 7.9) to prevent spontaneous activation of unfertilized eggs (Sakai et al., 1997). Since spermatozoa were liberated to the culture medium, eggs were inseminated by adding several drops of sperm suspension at room temperature.

RESULTS

Cell sorting of medaka spermatogenic cells

Medaka testicular cells were dissociated as described above and suspended in MS medium at the concentration of $2x10^6$ cells/ml, filtrated through a 40-μm nylon mesh, and sorted by a JSAN desktop cell sorter (Bay Bioscience, Kobe, Japan). Since I found that Sheath solution, which is used commonly for cell sorting, is toxic to medaka spermatogenic cells, MS medium was used in this study. The cells were treated with 5 μg/ml PI to remove dead cells in the course of cell sorting.

The obtained cell fractions were examined by a phase-contrast microscope, and the types of cells in each fraction were identified by their morphology (Fig. 1 and 2): spermatogonia were identified by a large nucleus and prominent nucleolus (Fig. 1A and 1B), primary spermatocytes were recognized by their large size and chromatin structure characteristic of meiosis (Fig. 1C), secondary spermatocytes were identified by their intermediate size (Fig. 1D), spermatid and spermatozoa were recognized by their small size and a highly differentiated morphology equipped with a flagellum (Fig. 1E), and somatic cells were recognized by their flattened and irregular shape (Fig. 1F).

I obtained a cell fraction rich in spermatogonia. Cell sorting based on the levels of forward scatter and side scatter yielded four fractions, G-I to G-IV (Fig. 2A). An

experiment using the *olvas*-GFP medaka that has GFP-labeled spermatogonia and spermatocytes indicated that the percentage of spermatogonia and spermatocytes is about 90% in Fraction G-IV (Fig. 2B). Morphological identification (Fig. 1) of cells in the fractions revealed that Fractions G-I, G-II, G-III and G-IV are rich in spermatids/spermatozoa, secondary spermatocytes/spermatogonia, primary spermatocytes and spermatogonia, respectively (Fig. 2C). According to the morphological criteria, I estimated that Fraction G-IV contains 10% somatic cells, 30% primary spermatocytes and 60% spermatogonia. To assess the cell population in Fraction G-IV more accurately, I specifically detected primary spermatocytes by anti-Sycp3 (a meiosis-specific protein) immunocytochemistry and found that they accounted for about 30% (The percentages of Sycp3-positive cells were 15.7±4.5%, 81.3±4.8% and 27.5±2.3% in Fractions G-II, G-III and G-IV, respectively [mean ± SD, n=3]). In harmony with the conclusion obtained by the morphological identification, these results confirmed that the ratio of spermatogonia in Fraction G-IV is about 60%, a much higher ratio than that in the wild-type medaka testis (about 2%, cf., Fig. 13B). I used the spermatogonia-rich fraction (Fraction G-IV) for studies described below.

Improvement of culture conditions

Previous cell culture systems have failed to recapitulate the differentiation from spermatogonia to mature spermatozoa in medaka (Saiki et al., 1997; Shimizu et al., 1997; Shimizu et al., 2000; Song and Gutzeit, 2003; Hong et al., 2004; Sasaki et al., 2005; Iwasaki et al., 2009). I modified the previous systems to support spermatogonial proliferation and differentiation *in vitro*. A critical modification was supplements to a culture medium. Among several substances examined, I found that retinol, carp serum and gonadotropins (HCG, PMSG, pFSH) are effective (Fig. 3).

To analyze changes in the proliferation rates of spermatogonia under different culture conditions, I performed BrdU labeling experiments (Fig. 3). Following the addition of BrdU to the culture of the spermatogonia-rich fraction (Fraction G-IV) on day 3, 5 or 7, the cells were harvested on day 4, 6 or 8, respectively, and the number of BrdU-labeled cells (including spermatogonia and primary spermatocytes but excluding secondary spermatocytes, spermatid, spermatozoa and somatic cells) was counted (Fig. 4). Spermatogonia and primary spermatocytes were indistinguishable when fixed for immunological detection of BrdU, but it is reasonable to assume that the dynamics of BrdU-labeled cells chiefly reflects spermatogonial proliferation, since the number of spermatogonia is about twice the number of primary spermatocytes in Fraction G-IV as

stated above. In the culture using S medium, the number of BrdU-labeled cells was smaller than that in other cultures and continued to decrease during the culture (Fig. 4), indicating that spermatogonial proliferation is not sustained in this medium as was shown previously (Shimizu et al., 1997; Shimizu et al., 2000). In the cultures using MS medium and Mtp1 feeder cells, obvious improvement of spermatogonial proliferation was found compared with that in the culture using S medium, although the number of BrdU-labeled cells continued to decrease in the course of culture (Fig. 4). In accordance with the improvement of spermatogonial proliferation, the number of spermatozoa produced in the cultures using MS medium and Mtp1 feeder cells was increased (data not shown).

Fertility of spermatozoa produced *in vitro*

I assessed the effect of a new culture medium (MS medium) and new feeder cells (Mtp1) on medaka spermatogenesis by examining the production rate of functional spermatozoa, which was estimated by the fertilization rate of eggs inseminated with the spermatozoa produced *in vitro* (Fig. 5). Medaka testicular cells were cultured for 15 days with MS medium, which was changed on days 1, 4, 7 and 10. MS medium was supplemented with 11-KT, DHP and RA on day 10, since they were expected to

enhance the differentiation of spermatocytes to spermatozoa (Nagahama, 1994). The resulting spermatozoa were collected from the culture supernatant by centrifugation, washed with IMPS, resuspended in 50 μl IMPS and added to naturally ovulated eggs.

In contrast to the original medium (S medium), the fertilization rate was significantly increased when eggs were inseminated by spermatozoa produced in MS medium. Co-culture of testicular cells and Mtp1 cells further increased the fertilization rate (Fig. 5). In addition to the fertilization rate, the rate of normal embryonic development also increased when eggs were fertilized with spermatozoa produced in the presence of Mtp1 cells (Table 3). The resultant adult medaka yielded a next generation, confirming that the spermatozoa produced *in vitro* are entirely normal. These findings indicate that the newly established cell culture system greatly improves medaka spermatogenesis *in vitro*.

Characterization of Mtp1 cells

It has been reported that Sertoli cells are necessary for spermatogonial proliferation and differentiation in zebrafish and eel (Miura et al., 1991; Sakai, 2002). I found that the culture system using Mtp1 as feeder cells greatly improves spermatogonial proliferation (Fig. 4), suggesting that Mtp1 cells contain Sertoli cells. Actually, Mtp1 cells exhibit

various morphology (Fig. 6A), which implies that Mtp1 cells are not a single origin but a mixture of testicular somatic cells including Sertoli cells, Leydig cells, fibroblasts, and so on. I thus tried to characterize Mtp1 cells by phagocytosis assay and gene expression analysis.

One of characteristic features of Sertoli cells is phagocytic activity as demonstrated in mammals (Tokuda et al., 1992; Rassoulzadegan et al., 1993) and zebrafish (Kurita et al., 2004). I examined phagocytic activity of Mtp1 cells (Fig. 6) and found that 32% of the cells showed the activity, indicating that Mtp1 cells include a population of Sertoli cells.

I then analyzed gene expression patterns of Mtp1 cells. RT-PCR analysis showed that Mtp1 cells express both Sertoli cell markers (*Gsdf* and *Sox9b*) and Leydig cell markers (*p45011β* and *11βHSD2*) but not a germ cell marker (*olvas*, Yoon et al., 1997) (Fig. 7). These results reveal that Mtp1 cells are heterogeneous, consisting at least of Sertoli cells and Leydig cells.

Isolation of single cell lines from Mtp1 cells

On the basis of findings described above, it is evident that Mtp1 cells is at least a mixture of Sertoli and Leydig cells. I then intended to isolate cell lines (clones), each of which is originated from a single Mtp1 cell. I isolated 8 clones (named MA1 to 8, Fig. 8

and 9). They are roughly classified into the following categories according to their morphology; large (Fig. 8A, MA4), middle (Fig. 8B, MA1), and small scale-like cells (Fig. 8C, MA2, 3, 6), triangular cells (Fig. 8D, MA5, 7) and fibroblast-like cells (Fig. 8E, MA8). RT-PCR analysis demonstrated that the isolated 8 cell lines express various cytokines specific to each clone (Fig. 9). My preliminary experiments showed that when male germ cells were co-cultured with one of MA1 to 8 cell lines, neither remarkable spermatogonial proliferation nor improvement of fertilization rate was observed, as compared with the original Mtp1 cells.

DISCUSSION

To understand regulatory mechanisms of medaka spermatogenesis and to manipulate medaka meiosis genetically as its application, I established a new culture system that promotes proliferation and differentiation of spermatogonia. In previous culture systems (Shimizu et al., 1997; Shimizu et al., 2000), germ cells were not surrounded by Sertoli cells, which are known to regulate proliferation and differentiation of spermatogonia under the influence of hormonal signals and growth factors (Miura et al., 1991; Sakai, 2002). I improved the culture system by changing the constitution of culture medium and by using feeder cells. In the newly established culture system in this study, spermatogonia differentiated into functional spermatozoa via spermatocytes and spermatids *in vitro* (Table 3 and Fig. 5; see also Fig. 17). The major difference between MS medium and S medium is the presence of hormones (Table 1). It is likely that HCG and PMSG are especially effective on the differentiation of spermatogonia and that carp serum works mainly on somatic cells (Miura et al., 1996).

Proliferation rates of spermatogonia in MS medium with and without Mtp1 cells were higher than those in S medium in the early period of the culture, but the rates rapidly decreased later (Fig. 4). These findings suggest that, unlike *in vivo*, spermatogonial proliferation is not correctly regulated under these conditions. Consistent with this

notion, it has been reported that spermatogenesis is abnormally accelerated in the absence of interaction between germ cells and somatic cells *in vitro* (Iwasaki et al., 2009). The present cell culture system is thought to lack some factors important for regulating spermatogonial proliferation via the interaction between germ cells and somatic cells, which is required to maintain the constant rate of spermatogonial proliferation as *in vivo*. In the next chapter, I deal with this point, focusing on the function of the cytokine Lif in spermatogonial proliferation in the medaka.

In previous fertilization assays, ovulated eggs were simply put in a dish in which germ cells were cultured in various conditions. In this assay, even if the spermatozoa produced *in vitro* can fertilize the eggs, the resulting embryos died in the middle of development. I therefore changed the method of artificial fertilization: The spermatozoa produced in culture were washed with a medaka physiological saline, IMPS, and then used for insemination. This modification increased the rate of normal development of embryos fertilized with *in-vitro*-produced spermatozoa (Table 3). The previous method using culture medium for insemination might be toxic to medaka embryos, since HCG, PMSG, pFSH, 11-KT, DHP and RA are likely to inhibit normal embryonic development.

To improve cell culture of medaka spermatogenesis, I also used somatic cells derived from *p53*-KO medaka testes, Mtp1 cells, as feeder cells, in expectation that they would promote spermatogenesis *in vitro* and useful to investigate the molecular mechanisms of spermatogenesis. I succeeded in cloning 8 cell lines from Mtp1 cells. Each of Mtp1 cell lines (MA1-8) expresses various cytokines (Fig. 8). My preliminary experiments showed that when male germ cells were co-cultured with one of these cell lines, neither remarkable spermatogonial proliferation nor improvement of fertilization rate was observed, as compared with the original Mtp1 cells. However, I noticed that each cell line shows different effects on spermatogonial differentiation, suggesting that each line expresses different growth and/or differentiation factors. Further studies that reveal the details of gene and protein expression in each Mtp1 cell line will make a great contribution to the understanding of molecular mechanisms regulating medaka spermatogenesis, as well as to construct a better culture system by selecting an appropriate combination of cell lines from MA1 to 8 as feeder cells.

A cell culture system from spermatogonia to functional spermatozoa has been established in zebrafish (Sakai et al., 2002). A major difference between my medaka system and the zebrafish system is feeder cells. Feeder cells must be prepared for each species, since it is known that germ cells die rapidly *in vitro* when somatic cells of other

species are used as feeder cells. The zebrafish system can reproduce the entire process of spermatogenesis from spermatogonial stem cells to functional spermatozoa *in vitro* (Kawasaki et al., 2016). It is uncertain whether my culture system can maintain spermatogonial stem cells or not. To clarify this point, I need to carry out further studies, in which medaka spermatogonial stem cells are isolated and characterized by gene expression patterns specific to spermatogonial stem cells in other vertebrates.

Chapter II

Involvement of leukemia inhibitory factor in medaka spermatogonial proliferation

INTRODUCTION

Spermatogenesis is principally regulated by gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) and steroids (androgens, estrogens and progestins), which in turn stimulate testicular somatic cells, such as Sertoli cells and Leydig cells, to produce various molecules necessary for proper proliferation and differentiation of spermatogenic cells (Chen et al., 2015; Shiraishi et al., 2017; Zhou et al., 2019). Among them, cytokines are of particular interest because they are key molecules in local regulation (paracrine regulation) mediated by cell-to-cell communications (Weinbauer and Wessels, 1999; Hedger and Meinhardt, 2003; Oatley and Brinster, 2008).

Leukemia inhibitory factor (Lif), one of the well-known cytokines, exerts pleiotropic actions in various tissues. Lif maintains the undifferentiated state of mouse embryonic stem (ES) cells and allows their proliferation *in vitro*, although it is unnecessary for human and rabbit ES cells (Humphrey et al., 2004; Honda et al., 2009). Lif also promotes the survival or proliferation of mouse primordial germ cells (PGCs) (De Felici and Dolci, 1991; Chuma and Nakatsuji, 2001; Farini et al., 2005). It is thus plausible that a general role of Lif is to maintain the pluripotent ability and proliferation of various stem cells and their descendants, such as spermatogonial stem cells and

spermatogonia in the testis. In fact, Lif has been reported to support the proliferation of rat spermatogonia (Dorval-Coiffec et al., 2005) under culture conditions, consistent with the reports showing that its mRNAs are expressed in spermatogonia and somatic cells (chiefly in peritubular cells) in the rat testis (Jenab and Morris, 1998; Dorval-Coiffec et al., 2005). In zebrafish, feeder cells that express Lif, fibroblast growth factor 2 (Fgf2) and glial cell line-derived neurotrophic factor (Gdnf) have been reported to enhance spermatogonial cell proliferation under culture conditions (Wong and Collodi, 2013). Therefore, it is likely that Lif contributes to the regulation of spermatogenesis in fish, in addition to mammals. However, the function of Lif *in vivo* (in the testis) is not fully understood (Curley et al., 2018), and the generality of its function in mammals and other vertebrates including fish remains to be elucidated.

Here, I report that the proportion of spermatogonia is increased in the testis of *p53*-KO medaka, in which the mRNA expression levels of Lif are up-regulated. By means of a culture system established in Chapter I, I examined the effect of Lif on spermatogonia and found that the addition of recombinant medaka Lif proteins to the culture medium increased the number of spermatogonia. Culture of spermatogonia with Lif-overexpressing Mtp1 cells showed similar effects. *In situ* hybridization and immunohistochemical analyses of the medaka testes showed that Lif proteins, as well as

its mRNAs, are expressed restrictedly in spermatogonia and Sertoli cells that surround the spermatogonia. These findings strongly suggest that Lif plays an important role in the regulation of spermatogonial cell proliferation in the medaka.

MATERIALS AND METHODS

Fishes

The *p53*-KO medaka ($p53^{E241X/E241X}$) was isolated by high-throughput sequencing for point mutations induced by *N*-ethyl-*N*-nitrosourea (ENU) as described previously (Taniguchi et al., 2006) and was provided by NBRP Medaka, Japan. Six-month-old males of the mutant medaka and two wild-type medaka, Kyoto-Cab (a sub strain of Cab, to which ENU-mutagenesis was performed) and hi-medaka (an orange-red variety purchased from a local fish farm), were used in this study. The fish were cultured in fresh water at 27˚C under artificial light conditions (14-hour light and 10-hour dark).

Morphometrics

Testes were fixed in Bouin's solution and prepared for hematoxylin/eosin-stained histological sections. The proportion of each type of spermatogenic cells was estimated by morphometric analysis as follows. Regions of spermatogonia, spermatocytes, spermatids/spermatozoa, and somatic tissues were marked in Photoshop software (Adobe Systems, Tokyo, Japan). The area occupied by each type of cells was calculated using ImageJ software and expressed as a percentage of the total area of the histological section. Three arbitrary sections were analyzed for one testis.

Detection of medaka cytokines by RT-PCR

Medaka cytokines were detected by RT-PCR as described in Chapter I. Following isolation of total RNA samples from the testis, ovary and brain with ISOGEN (Nippon Gene, Tokyo, Japan), cDNAs were produced with a Super Script III First Strand Synthesis System (Life Technologies, Tokyo, Japan). A cDNA fragment encoding medaka Lif was amplified by PCR using a primer set (Table 4), which was designed according to a sequence found by a BLAST search in the medaka genome database (http://asia.ensembl.org/Oryzias_latipes/Info/Index) using the amino acid sequence of goldfish Lif (AAU94362.1) (Hanington and Belosevic, 2005) as a query.

Quantitative RT-PCR (qRT-PCR)

Total RNAs (2 μg) isolated from wild-type and *p53*-KO medaka testes were reverse-transcribed in a volume of 20 μl. Following 5-fold dilution of the reaction mixture with water, mRNAs encoding Lif, Fgf20b, Il-11b, Mif and Zfp91-Cntf were quantified by a real-time PCR system with SYBR green PCR Master Mix (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions, using specific primer sets (Table 2). Reactions were carried out at 50˚C for 2 minutes, 95˚C for 10 minutes, 40 cycles of 15 seconds at 95˚C and then a final extension for 1 minute at 60˚C. The cDNA samples

were evaluated in triplicate for each mRNA, and results were normalized to *β-actin* mRNA. Specific amplification of each PCR product was confirmed by creating a dissociation curve.

Production of baculovirus-expressed medaka Lif

Using a PCR-derived Lif cDNA fragment as a screening probe, I isolated full-length Lif cDNA (DDBJ/EMBL/GenBank accession number AB766229, Fig. 10) from a Lambda Zap II medaka ovary cDNA library. Since the cDNA contains two putative start methionines, methionine 1 (Met^1) and methionine 14 (Met^{14}), the open reading frame (ORF) from Met^1 (Lif-F1) was amplified by PCR using a Topo-Lif-F1/Topo-Lif-R primer set, and the ORF from Met^{14} (Lif-F2) was amplified using a Topo-Lif-F2/Topo-Lif-R primer set (Fig. 10 and Table 4). The resulting ORFs were inserted into the pENTR vector by TOPO cloning (Life Technologies, Tokyo, Japan). After confirming the sequences, the plasmids were recombined with the destination vector pET161-DEST to produce Lif proteins with a polyhistidine tag at the C-terminus (Lif-F1-His and Lif-F2-His).

ORFs encoding Lif-F1-His and Lif-F2-His were amplified from Lif cDNAs in pET161-DEST by PCR using a *Bam*HI-Lif-F1/*Not*I-His primer set and a *Bam*HI-Lif-

F2/*Not*I-His primer set, respectively (Table 4). The resulting ORFs were cloned into the pGEM-T Easy vector (Promega, Tokyo, Japan) by TA cloning, digested with *Bam*HI and *Not*I, ligated into the corresponding site of pFastBac1, and transformed into DH10Bac (Life Technologies, Tokyo, Japan) to obtain recombinant bacmid DNAs. Sf9 cells to which the recombinant bacmid DNA had been transfected with Cellfectin II reagent (Life Technologies, Tokyo, Japan) were cultured for 72 hours at 28˚C, and viruses producing Lif-F1-His or Lif-F2-His proteins were collected (P1 viruses). P1 viruses were further transfected into Sf9 cells for amplification, and after cultivation of the cells for 72 hours, P2 viruses were collected.

Sf9 cells and their culture supernatant were collected by centrifugation 72 hours after P2 virus infection. The cells were washed once in PBS, sonicated in extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol, 3 μg/ml leupeptin, pH 7.5), and centrifuged to clarify the extract before immunoblotting. The presence of Lif-F1-His and Lif-F2-His proteins in the culture supernatant and cell extract was confirmed by immunoblotting as described below. Since purification of Lif-F1-His or Lif-F2-His proteins from the culture supernatant or cell extract was difficult, the culture supernatant from Sf9 cells was used to examine the effect of Lif-F1-His or Lif-F2-His on spermatogonia. As a control, I also produced

glutathione-S-transferase (GST)-expressing Sf9 cells by a method similar to that for medaka Lif. The culture supernatant of Lif- or GST-producing Sf9 cells was added to the spermatogonial cell culture in the ratio of 1:1 to adjust the protein concentration of Lif or GST to 250 μg/ml in the culture medium.

Production of Mtp1 cells overexpressing medaka Lif

Mtp1 cells that overexpress medaka Lif were produced in cooperation with Prof. Hisanori Bando (Research Faculty of Agriculture, Hokkaido University). Exogenous genes can be transfected into mammalian and fish cells, as well as insect cells, by a baculovirus (Leisy et al., 2003; Yan et al., 2009; Yokoo et al., 2013). I noticed that baculovirus-mediated gene transfer is less harmful than electroporation- or retrovirus-mediated methods. To produce cells that overexpress medaka Lif continuously, I therefore used a baculovirus-mediated transgenic system that employs two baculovirus vectors, one encoding the piggyBac transposase (a helper plasmid) and the other consisting of the *piggyBac* inverted terminal repeats (ITRs) flanking a fusion of the cytomegalovirus (CMV) promoter and the medaka Lif-F2-His cDNA (a donor plasmid) (Tamura et al., 2000) (Fig. 11 and 12). The resulting plasmids were introduced into the genome of Mtp1.

Mtp1 cells were cultured in the presence of the helper and donor viruses for transfection, and 48 hours later, hygromycin B (Thermo Fisher Scientific K. K., Tokyo, Japan) was added to the culture medium at the final concentration of 500 μg/ml. Cell culture was continued for more than 2 weeks to select cells to which the transgene was stably introduced. The culture supernatant and cell extract from the drug-selected cells were analyzed by immunoprecipitation followed by immunoblotting to confirm the expression of Lif-F2-His. The Lif-overexpressing Mtp1 cells, as well as the wild-type Mtp1 cells, were used as feeder cells to culture medaka spermatogenic cells. The Lif-overexpressing Mtp1 cells were also used to check the specificity of anti-Lif antibody.

Spermatogenesis of PKH26-labeled spermatogonia *in vitro*

To trace the fate of germ cells under culture conditions, cells in spermatogonia-rich fractions (Fraction G-IV, see Chapter I) were labeled with the fluorescent vital staining dye PKH26 (Sigma-Aldrich, St Louis, United States of America) according to the manufacturer's instructions. PKH26-labeled cells (20 cells/well) were added to PKH26-untreated spermatogonia-rich fractions ($1.5x10^5$ cells/well) to maintain a sufficient amount of spermatogonia per well to promote the proliferation and differentiation of PKH26-labeled spermatogonia in culture. A mixture of PKH26-labeled and -unlabeled

cells was co-cultured with Mtp1 or Lif-overexpressing Mtp1 cells in a gelatin-coated 24-well plate. On days 0, 3, 5 and 7, the cultured cells were harvested by centrifugation following treatment with 0.05% trypsin-EDTA (Life Technologies, Tokyo, Japan) and suspended in PBS in a 35-mm dish. After the types of cells had been identified by the morphological criteria, PKH26-labeled spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids/spermatozoa were counted under a fluorescent microscope using 3 wells at each time point to calculate the means and standard deviations. After counting living PKH26-labeled cells for each cell type, the cells were attached to a cover slip coated with 0.1% poly-L-lysine, fixed with 4% PFA/PBS for 15 minutes, and stained with anti-Sycp3 antibody (Iwai et al., 2006) to confirm the number of anti-Sycp3-positive cells (primary spermatocytes).

Section in situ hybridization

RNA probes were prepared by in vitro transcription with SP6 or T7 RNA polymerase using adigoxigenin (DIG) RNA-labeling Kit (Roche Diagnostics, Tokyo, Japan). Medaka testes were fixed in 4% PFA/PBS overnight at 4 ˚C, dehydrated in ethanol, and embedded in paraffin. Sections (7μm in thickness) were successively treated with 0.3% Triton X-100 in PBS, 0.2N HCl, and 1 μg/ml proteinase K in PBS at 37˚C and re-fixed

with 4% PFA/PBS (each for 5 minutes). After hybridization with DIG-labeled sense or antisense probes, samples were incubated with anti-DIG-HRP antibody at 1:500 dilution (Roche Diagnostics, Tokyo, Japan) for 30 minutes. Following amplification of signals with tyramide-DNP by a TSA Plus Fluorescence kit (PerkinElmer, Waltham, United States of America), the samples were incubated with anti-DNP-Alexa Fluor 488 antibody at 1:500 dilution (Thermo Fisher Scientific K. K., Tokyo, Japan) overnight at 4˚C, stained with 10 μg/ml Hoechst 33258 for 10 minutes to visualize nuclei, mounted with a Prolong Anti Fade Kit (Life Technologies, Tokyo, Japan), and observed under a Zeiss LSM-DUO confocal laser microscope.

Production of anti-medaka Lif antibodies

Anti-medaka Lif antibodies were raised by injecting recombinant medaka Lif proteins into guinea pigs. The recombinant proteins were produced as follows. A cDNA fragment encoding Lif-F3 was amplified by a primer set of Topo-Lif-F3 and Topo-Lif-R (Fig. 10 and Table 4). The amplified cDNA was inserted into the pENTR/D-TOPO Gateway vector with a pENTR Directional TOPO Cloning Kit (Thermo Fisher Scientific K. K., Tokyo, Japan), and the resulting plasmids were recombined with the destination vector pDEST15 using a Gateway cloning system (Life Technologies,

Tokyo, Japan) to produce proteins with a glutathione-S-transferase (GST) tag at the N-terminus (GST-Lif-F3). GST-Lif-F3 was expressed in *E. coli* and purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by electro-elution in Tris-glycine buffer without SDS, according to the method described previously (Hirai et al., 1992). The antigenic proteins were injected into guinea pigs, and the antisera were affinity-purified with GST-Lif-F3 electroblotted onto Immobilon membranes (Millipore, Billerica, United States of America). The anti-GST-Lif-F3 guinea pig antibody works in immunoprecipitation and immunocytochemistry but not in immunoblotting, probably because the antibody can recognize the native epitopes but not those modified by SDS.

Immunoprecipitation, immunoblotting and immunostaining

Immunoprecipitation and immunoblotting were performed according to the procedures described previously (Ota et al., 2011; Yamashita *et al.,* 1991) using anti-GST-Lif-F3 guinea pig antibody (this study) and anti-His G-18 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, United States of America), respectively. For immunostaining, medaka testes were fixed with 4% PFA/PBS overnight at room temperature. Following dehydration with ethanol and benzene, samples were embedded

in paraffin and cut into 7-μm-thick sections. After deparaffinization and rehydration, the slides were immersed in 1 mM EDTA (pH 8.0) and heated in a microwave oven for 10 minutes. Following overnight incubation with anti-GST-Lif-F3 guinea pig antibody at 1:200 dilution, the slides were treated with Alexa 546-conjugated anti-guinea pig IgG antibody (Life Technologies, Tokyo, Japan). The expression of GFP in *olvas-GFP* medaka testes was immunologically detected by anti-GFP mouse antibody (Roche Diagnostics, Tokyo, Japan) and Alexa 488-conjugated anti-mouse IgG antibody. Immunostaining with anti-Sycp3 antibody was performed as described previously (Iwai et al., 2006).

RESULTS

Increase in the proportion of spermatogonia in *p53*-KO medaka

Protein p53 monitors the integrity of DNA and stops cell division when damaged DNA is detected in the cell, thereby acting as a tumor-suppressor gene. Indeed, it has been reported that various tumors develop spontaneously in the *p53*-KO medaka (Taniguchi et al., 2006). With the expectation that abnormalities caused by *p53* mutation would provide insights into the regulatory mechanisms of spermatogenesis *in vivo*, I investigated spermatogenesis in the *p53*-KO medaka.

Since p53 is a key protein that regulates cell growth and death through the control of apoptosis, its dysfunction would cause various biological abnormalities with multiple phenotypes. The formation of oocyte-like cells in the testes (testis-ova) has been reported in the *p53*-KO medaka (Yasuda et al., 2012). I confirmed this abnormality in some but not all individuals. I also noticed that the *p53*-KO medaka testes exhibit abnormal structures with disordered tubules and cysts, with a tendency for older individuals to show more severe defects, and that the testes are vestigial in some individuals. Among various abnormalities found in the *p53*-KO medaka, I focused on the abnormal proportion of spermatogonia in the testes. Spermatogonia are localized to the peripheral region of the testis in wild-type medaka, and the mean area occupied by

spermatogonia is about 2%. In contrast, the area occupied by spermatogonia expanded in the *p53*-KO medaka testis, reaching 4.5% (Fig. 13A and 13B). The expansion was associated with decreases in the areas occupied by spermatocytes and spermatids/spermatozoa (Fig. 13B), although the extent varied from individual to individual. An increase in the proportion of spermatogonia implied that the loss of *p53* causes defects in spermatogonial cell proliferation and differentiation.

Increase in mRNA levels of Lif in the *p53*-KO medaka testis

To identify the molecules involved in the increase in the proportion of spermatogonia in the *p53*-KO medaka testis, I examined the expression levels of mRNAs encoding several cytokines and growth factors by RT-PCR. Among 13 molecules examined in the testis (and in the brain and ovary as controls), mRNAs encoding Lif, Fgf20b, Il-11b, Mif and Zfp91-Cntf were expressed in the wild-type medaka testis (Fig. 14A). I then compared their expression levels in the wild-type and *p53*-KO medaka testes by qRT-PCR analysis and found that mRNAs encoding Lif, Il-11b and Mif were overexpressed in the *p53*-KO medaka testis (Fig. 14B), although the levels varied by individuals. Because an increase in Lif-encoding mRNA levels was generally observed in the *p53*-KO medaka testis, I focused on Lif and examined its function in spermatogonial cell

proliferation and differentiation in the medaka using the *in vitro* culture system described in Chapter I.

Increase in the number of spermatogonia in the presence of recombinant Lif

I produced recombinant medaka Lif proteins by a baculovirus expression system. Since two start methionines were presumed from its cDNA sequence (Fig. 10), two versions of Lif proteins (Lif-F1-His and Lif-F2-His) were produced. Immunoblotting analysis following immunoprecipitation showed that cell extracts from Lif-F1-His-expressing Sf9 cells contained 30-kDa and 25-kDa proteins, whereas those from Lif-F2-His-expressing cells contained 28-kDa and 25-kDa proteins. In contrast, only the 25-kDa protein was found in both of the culture supernatants (Fig. 15A). According to the molecular masses, it is conceivable that the 30-kDa and 28-kDa proteins are full-length Lif-F1-His and Lif-F2-His, respectively. Since Lif is functionally activated (matured) by removal of a signal peptide from the N-terminus (Fig. 10), the 25-kDa protein is probably a mature Lif-His. Thus, it is most likely that a single mature Lif-His protein is produced from both Lif-F1-His and Lif-F2-His and secreted to the culture medium. The finding described below that the culture supernatants of Lif-F1- and Lif-F2-producing

cells showed increases in the number of spermatogonia to similar extents supported this idea.

I used spermatogonia-rich fraction (Fig. 2C, G-IV) to evaluate the effect of Lif on spermatogonia. Spermatogonia-rich fractions obtained by cell sorting were cultured in the presence of Mtp1 feeder cells, and the culture supernatant of Lif-overexpressing Sf9 cells or that of GST-overexpressing Sf9 cells as a control was added to the medium. Results clearly showed that irrespective of the use of Lif-F1 or Lif-F2, the number of spermatogonia significantly increased in the presence of recombinant Lif proteins (Fig. 16A and 16B).

Dynamics of spermatogenic cells in the presence of Lif

The increase in spermatogonia in the presence of Lif strongly suggests that Lif plays an important role in the control of spermatogonial cell proliferation and differentiation. To examine the dynamics of spermatogenic cells *in vitro*, I performed PKH26 label and chase experiments, in which PKH26-labeled spermatogonia were co-cultured with Mtp1 or Lif-overexpressing Mtp1 cells (Fig. 17). Following identification of germ cells according to the morphological criteria, the number of PKH26-labeled cells was counted on days 0, 3, 5 and 7 for each cell type (Fig. 18).

The number of PKH26-labeled spermatogonia cultured with Lif-overexpressing Mtp1 cells increased during the culture, with a higher rate of increase than those cultured with wild-type Mtp1 cells (Fig. 18A). PKH26-labeled primary spermatocytes, the cell type of which was confirmed by anti-Sycp3 antibody, were observed on day 0, because of their contamination in the spermatogonia-rich fraction (Fig. 18B). In striking contrast to spermatogonia, the number of PKH26-labeled primary spermatocytes did not change significantly during the culture period from days 0 to 7, with no significant differences between the cultures with Mtp1 and Lif-overexpressing Mtp1 cells (Fig. 18B). PKH26-labeled secondary spermatocytes were absent on day 0. They increased during the early period of culture (until day 3), but the increasing rate later reached a plateau (Fig. 18C), probably because of an appropriate balance between the increase caused by differentiation of primary spermatocytes into secondary spermatocytes and the decrease by the differentiation of secondary spermatocytes into spermatids/spermatozoa. No significant differences in the number of PKH26-labeled secondary spermatocytes were observed between Mtp1 and Lif-overexpressing Mtp1 cells (Fig. 18C). PKH26-labeled spermatids/spermatozoa, the end product of spermatogenesis, were not found on day 0. They continued to increase during the cultures, with no significant differences between Mtp1 and Lif-overexpressing Mtp1 cells except for day 7 (Fig. 18D).

Expression of Lif in the medaka testis

Since *in vitro* experiments demonstrated that Lif contributes to the regulation of spermatogonial cell proliferation and differentiation, I decided to study the function of Lif *in vivo*. To this end, I examined the mRNA expression of Lif in the medaka testis by section *in situ* hybridization. Positive signals were found in the peripheral regions of the testis, where spermatogonia and surrounding Sertoli cells exist (Fig. 19). Closer observations confirmed that spermatogonia and Sertoli cells surrounding them express the mRNAs, whereas other spermatogenic cells, including primary spermatocytes, and surrounding somatic cells did not.

I then examined Lif protein expression by immunohistochemistry with an antibody against medaka Lif, the specificity of which was confirmed by using Lif-overexpressing Mtp1 cells (Fig. 15). In contrast to the absence of signals by a control antibody that was pre-absorbed by antigenic proteins (Fig. 20A), Lif signals were found in spermatogonia and surrounding Sertoli cells (Fig. 20B). To identify the Lif-expressing cells more accurately, I examined the testes of *olvas*-GFP medaka, in which the intensity of GFP signals of spermatogenic cells is higher in the following order: type A (undifferentiated) spermatogonia, type B (differentiated) spermatogonia, primary spermatocytes, and secondary spermatocytes (no signals in spermatids, spermatozoa and somatic cells)

(Tanaka et al., 2001). Triple staining with anti-Lif antibody, anti-GFP antibody and Hoechst 33258 showed that the signal intensity of Lif corresponded well to that of GFP (Fig. 20C), indicating that the protein levels of Lif in type A spermatogonia are higher than those in type B spermatogonia. These results are consistent with results obtained by *in situ* hybridization analyses (Fig. 19). Similar observations of the testes of *p53*-KO medaka showed that the expression levels of Lif proteins in spermatogonia and surrounding Sertoli cells were clearly higher in the *p53*-KO medaka than in the *olvas*-GFP medaka, which retains the intact *p53* gene (Fig. 20D), in accordance with the results of qRT-PCR analysis (Fig. 14B).

DISCUSSION

On the basis of findings that the proportion of spermatogonia (Fig. 13) and the expression level of mRNA encoding Lif (Fig. 14) are increased in the testis of *p53*-KO medaka, I examined the function of Lif in spermatogonial cell proliferation. I found that addition of Lif to the culture medium or co-culture with Lif-overexpressing testicular somatic cells increases the number of spermatogonia (Figs. 16 and 18). Lif proteins (Fig. 20), as well as its mRNAs (Fig. 19), are expressed in spermatogonia and surrounding Sertoli cells, with higher expression levels in type A spermatogonia than in type B. These results strongly suggest that Lif plays a critical role for the paracrine and/or autocrine regulation of spermatogonial cell proliferation in the medaka.

The relationship between increase in mRNA expression of Lif and loss of p53 function in the medaka testis remains a mystery. It has been reported that p53 in female, but not male, mice up-regulates *Lif* mRNA levels via the p53-consensus binding element in the *Lif* gene (Hu et al., 2007). If this were the case in the medaka, loss of p53 should result in a decrease in mRNA levels of Lif, contradictory to the up-regulation in *p53*-KO medaka. Although I cannot exclude the possibility that p53 directly regulates the expression of medaka Lif through its transcriptional activity, I propose that the increased mRNA expression in the medaka testis is not directly caused by the loss of

p53 function. It must be one of various events that occur concomitantly with abnormal regulation of the cell cycle, cell growth/differentiation and apoptosis by the defect in p53-mediated tumor-suppression mechanisms. A possible scenario leading to the increase in Lif levels in *p53*-KO medaka testes is as follows: 1) The loss of p53-mediated apoptosis increases the number of spermatogonia, as reported in mice (Beumer et al., 1998; Chen et al., 2012), 2) the proliferated spermatogonia produce and secrete higher levels of Lif, and 3) the secreted Lif proteins induce neighboring spermatogonia and Sertoli cells to produce large amounts of Lif by a positive feedback mechanism.

Taking advantage of a cell culture system that recapitulates the process of medaka spermatogenesis from spermatogonia to spermatozoa *in vitro*, I examined the behavior of spermatogenic cells co-cultured with Mtp1 or Lif-overexpressing Mtp1 cells. Results of PKH26 label and chase experiments revealed that the increase in the number of spermatogonia is enhanced by Lif (Fig. 18A). This finding strongly suggests that Lif regulates the proliferation of spermatogonia. How does Lif regulate spermatogonia? The number of spermatogonia can be increased by the following events if I do not take cell death or cell survival into account: 1) increase in the production rate and/or self-multiplication rate of spermatogonia and 2) inhibition of the differentiation of type B

spermatogonia into primary spermatocytes (inhibition of entry into meiosis). The numbers of PKH26-labeled primary spermatocytes were not significantly different in Mtp1 and Lif-overexpressing Mtp1 cells (Fig. 18B), and the number of their descendants (secondary spermatocytes and spermatids/spermatozoa) increased irrespective of Lif (Fig. 18C and 18D). These findings indicate that spermatogenesis continuously proceeds even in the presence of Lif, thereby contradicting the possibility that the differentiation of type B spermatogonia into primary spermatocytes is inhibited by Lif.

The number of PKH26-labeled spermatogonia co-cultured with Lif-overexpressing Mtp1 cells increased at a higher rate than those co-cultured with wild-type Mtp1 cells (Fig. 18A). In contrast, the number of PKH26-labeled primary spermatocytes neither increased significantly during the cultures nor showed differences between the two conditions (Fig. 18B). In addition, the number of PKH26-labeled spermatids/spermatozoa, the end product of spermatogenesis, did not show apparent differences between Mtp1 and Lif-overexpressing Mtp1 cells (Fig. 18D). These findings suggest that although Lif promotes the proliferation of spermatogonia, the resulting "extra" spermatogonia do not enter meiosis on schedule (after undergoing 9-10 mitotic divisions *in vivo*) (Shibata and Hamaguchi, 1988). It is uncertain at present whether the

"extra" spermatogonia enter meiosis behind schedule (after mitotic divisions more than 10 times) or whether they disappear due to apoptosis. Culture of PKH26-labeled cells for a longer time might provide an answer to this question, although it is technically difficult at present and I need to improve the culture conditions. In the case of delayed entry into meiosis, the number of PKH26-labeled spermatids/spermatozoa will become larger in the presence of Lif according to a longer culture period. Regarding this question, it is important to examine whether Lif is involved in the survival of spermatogonia, because my present data do not exclude the possibility that the main function of Lif is to improve germ cell survival rather than to promote their proliferation. To verify this possibility, I need to analyze the apoptosis of germ cells cultured for a longer time in the presence and absence of Lif.

On the basis of the results obtained from tissue or cell culture experiments and gene knockout experiments in mammals (De Miguel et al., 1996; Jenab and Morris, 1998; Piquet-Pellorce et al., 2000; Dorval-Coiffec et al., 2005; Kanatsu-Shinohara et al., 2007; Mirzapour et al., 2012; Curley et al., 2018), Lif is thought to be required for Sertoli cells to maintain the normal spermatogenesis through regulation of spermatogonial proliferation and/or survival in mammals. Since it has been reported that zebrafish feeder cells expressing Lif, Fgf2 and Gdnf enhance spermatogonial cell proliferation in

cultures (Wong and Collodi, 2013), Lif is likely to function not only in mammalian spermatogenesis but also in teleost spermatogenesis. However, the expression and localization of Lif mRNAs and proteins in the testes have not been fully examined in any vertebrate. To my knowledge, this is the first report showing the expression patterns of Lif in vertebrate testes that were determined by *in situ* hybridization analysis and immunohistochemistry. The finding that Lif is expressed in spermatogonia and Sertoli cells surrounding them in the medaka testes (Fig. 19 and Fig. 20) strongly suggests that Lif-mediated paracrine and autocrine signals function among these cells *in vivo*. Further studies including studies to characterize Lif receptors and downstream signal transduction pathways consisting of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (Haan et al., 2006) will provide deeper insights into the mechanisms underlying Lif-mediated spermatogonial cell proliferation. In addition, I need to elucidate the functional relationships between Lif and its upstream players, such as pituitary hormones (LH and FSH), steroid hormones (androgens, estrogens and progestins), proteinaceous hormones (activin, anti-Müllerian hormone (AMH)) and a virilization factor (gonadal soma-derived factor (Gsdf)) (Schulz et al., 2010; Tang et al., 2018), for a comprehensive understanding of the mechanism for regulating the switch from proliferation to differentiation of spermatogonia.

GENERAL DISCUSSION

Spermatogenesis is strictly regulated by the endocrine systems comprising gonadotropins and androgens. In response to these molecules, the testicular somatic cells such as Sertoli cells produce various molecules that control the proper proliferation and differentiation of spermatogenic cells through their paracrine and autocrine actions. However, the entity of the molecules that act downstream of hormonal stimulation and their functions are poorly understood.

In Chapter I, I described the establishment of an experimental system to produce functional spermatozoa from spermatogonia in medaka fish. In vertebrates, production of functional and fertilizable spermatozoa from spermatogonia under dispersed cell conditions but not under organ culture conditions is the second success following zebrafish (Sakai et al., 2002). Both zebrafish and medaka are small fish, but they have diverged about 200 million years ago (Inoue et al., 2015). Since there are large interspecific differences, zebrafish and medaka are extremely useful for examining the similarities and differences in spermatogenesis, leading to a better understanding of the mechanisms regulating spermatogenesis in both species, as well as the mechanisms responsible for reproductive isolation. The culture system established in this study is a powerful tool that allows us to analyze each stage of spermatogenesis in detail and to examine how spermatogonia proliferate and differentiate with the aid of various factors.

In addition, several Sertoli cell lines (MA1 to 8) expressing various cytokines should be a useful tool for researchers who challenge unrevealed mechanisms of spermatogenesis.

In Chapter II, I noticed that the number of spermatogonia and the expression level of mRNA encoding Lif increased in the testis of *p53*-knockout medaka. Lif is known to maintain the pluripotency of stem cells including embryonic stem cells and primordial germ cells, at least *in vitro*, but its actual roles *in vivo* remain to be elucidated. To clarify Lif functions in the medaka testes, I examined the proliferation of spermatogonia in the presence of baculovirus-produced recombinant medaka Lif in a newly established culture system described in Chapter I. I found that the addition of Lif to culture medium or the co-culture with Lif-overexpressing Mtp1 cells promotes spermatogonial proliferation (Fig. 16 and 18). *In situ* hybridization and immunocytochemical analyses of the medaka testes showed that mRNA and protein of Lif are expressed spermatogonia and the surrounding Sertoli cells, with higher expression levels in undifferentiated spermatogonia than differentiated spermatogonia (Fig. 19 and 20). These results suggest that Lif plays an important role in the regulation of spermatogonial proliferation in the medaka.

The new medaka spermatogonial cell culture system described in Chapter I allows detailed analysis of the factors involved in spermatogenesis, as demonstrated in

Chapter II. In vertebrates, dispersion cell culture systems that recapitulate spermatogonial proliferation and differentiation into spermatocytes have been established only in zebrafish and medaka. These experimental systems should contribute to the advancement of comparative biology of spermatogenesis, since it is difficult to study in other organisms.

SUMMARY

This thesis consists of two chapters, Chapter I "*In vitro* transmeiotic differentiation of medaka spermatogonia into functional spermatozoa" and Chapter II "Involvement of leukemia inhibitory factor in medaka spermatogonial proliferation", with general introduction and discussion.

In response to gonadotropins and androgens, testicular cells produce various molecules that control proper proliferation and differentiation of spermatogenic cells through their paracrine and autocrine actions. However, molecules functioning downstream of the hormonal stimulation are poorly understood. In Chapter I, I established a new cell culture system by improving a culture medium and using a cell line (named Mtp1) derived from medaka testicular somatic cells as feeder cells, to clarify the mechanisms regulating medaka spermatogenesis. This culture system recapitulates the whole process of spermatogenesis from spermatogonia to spermatozoa *in vitro*, and the resulting spermatozoa are functional, as revealed by normal development of embryos inseminated with spermatozoa produced *in vitro*.

In Chapter II, I noticed that the number of spermatogonia and the expression level of mRNA encoding Lif were increased in the testis of *p53* (a tumor-suppressor gene) -knockout medaka. To clarify the function of Lif in medaka testes, I examined

spermatogonial proliferation in the presence of baculovirus-produced recombinant medaka Lif in the culture system established in Chapter I. I found that addition of Lif to the culture medium or co-culture with Lif-overexpressing Mtp1 cells promotes spermatogonial proliferation. I also found that mRNAs and proteins of Lif are expressed in spermatogonia and surrounding Sertoli cells. These results suggest that Lif regulates spermatogonial proliferation in the medaka.

The new medaka spermatogonial cell culture system established in this study allows detailed analysis of regulatory mechanisms of spermatogenesis. This experimental system should contribute to the advancement in the field of reproductive and developmental biology.

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Table 1. Components of culture media used in this study

Components	S medium	MS medium
Leibovitz L-15 (pH 7.3)	+	+
Hepes (10 mM)	pH 7.4	pH 7.3
L-prolin (1.7 mM)	+	+
L-aspartic acid (0.1 mM)	+	+
L-glutamic acid (0.1 mM)	+	+
Bovine serum albumin (0.5%)	+	+
Bovine insulin (1 μg/ml)	+	+
Penicillin (50 U/ml)	+	+
Streptomycin (50 U/ml)	+	+
Kanamycin (50 U/ml)	+	+
Fetal bovine serum (10%)	+	+
Retinol (50 ng/ml)		+
Carp serum (3%)		+
Human chorionic gonadotropin (10 IU/ml)		+
Pregnant mare serum gonadotropin (10 IU/ml)		+
Porcine follicle-stimulating hormone (10 IU/ml)		+

Table 2. Primer sets for RT-PCR

Target	Forward primer	Reverse primer
Sox9b	CTCCAGGAGAACATTCAGGT	CAAAGAATGCTGCTGTTTGG
Amh	GATCTGGCAGAGCAGGAAAC	CTCCTCAGCCCACAGATGTT
p45011β	CAGGTTATACCCAGTAGGAATC	GTCTTAGGTTGCAGGATCAG
11βHSD2	CCACTATCCTGCCATCTTCCTATAD	GCTGATGCTGAAGGGGAAGTA
β-actin	CTCGTTATTGACAATGGATCTGG	AGCCTTCATAGATGGGTACTGTG
Lif	AAAGCTTCTCAAGGAGAAGG	GAACAGTGCATTTGTCTTCATC
Egf	GTGAACGTGTGTTCCAGTCC	TCACCAGCGTCTGATCCTCA
Fgf2	GGAGAAATCACAACACTCCC	TGGCAGACATAGGCAGAAAG
Fgf16	CGCTCGACTTGGATTTACAAAG	TACGCCTGTGGAATCCTTTC
Fgf20a	GCGTCCCACTTCTTCCTGAC	GTACAGCTCTGGCACGCGAT
Fgf20b	CGGTTGGCTCGCATTTCGTT	TCTGGAACTCGGTCTGGATC
Gdnfa	GGCCACGTGTTTGTTGCTGC	CAGCCCCAAATCCGTCACATTG
Gdnfb	GACCACTTGTTTGATTCTGCTG	GATTTTGTCGTAGTTGGTGTAGG
Igf1	CGCTCATTTCTCTCAATGGCAT	GTTGCCTCGACTGGAGTTTT
Il-11a	TGCTCGACTCCTCCTCGTCT	GTGTTGGAGAAGTTCATGGGTGG
Il-11b	TCGTCCCACCCAGAACTTCT	CAGCCAGATCTTCTTCTGATCAAC
Mif	AGTCGATCAGTGTGAGGATTG	GACAGTGAAATCAGTGCAATGC
Zfp91-Cntf	TCAGGGACGACCTGAACGAT	CTTGAAGGCTCGAGCACAGAA

Table 3. Number of eggs fertilized with spermatozoa produced *in vitro*

Medium	Number of eggs exposed to spermatozoa	Number of fertilized eggs	Number of normally developed eggs
S	123	1 (0.8%)	0 (0%)
MS	131	12 (9.2%)	12 (9.2%)
MS with Mtp1	142	29 (20.4%)	29 (20.4%)

Table 4. List of primers for DNA construction

Name	Sequence
*Bam*HI-His	GGATCCCGGATCAAACTCAATGA
*Bam*HI-Lif-F1	GGATCCACCATGATAGGTCTTCAATTCGCC
*Bam*HI-Lif-F2	GGATCCACCATGAATGGTCATGCAAAGAAT
*Nhe*I-Lif-F2	GCTAGCGCCACCATGAATGGTCA
*Not*I-His	GCGGCCGCCGGATCAAACTCAATGATGATG
Topo-Lif-F1	CACCATGATAGGTCTTCAATTCGCCT
Topo-Lif-F2	CACCATGAATGGTCATGCAAAGAATATG
Topo-Lif-F3	CACCATGAAATCAATAGCAACGTTACTC
Topo-Lif-R	GAACAGTGCATTTGTCTTCATC

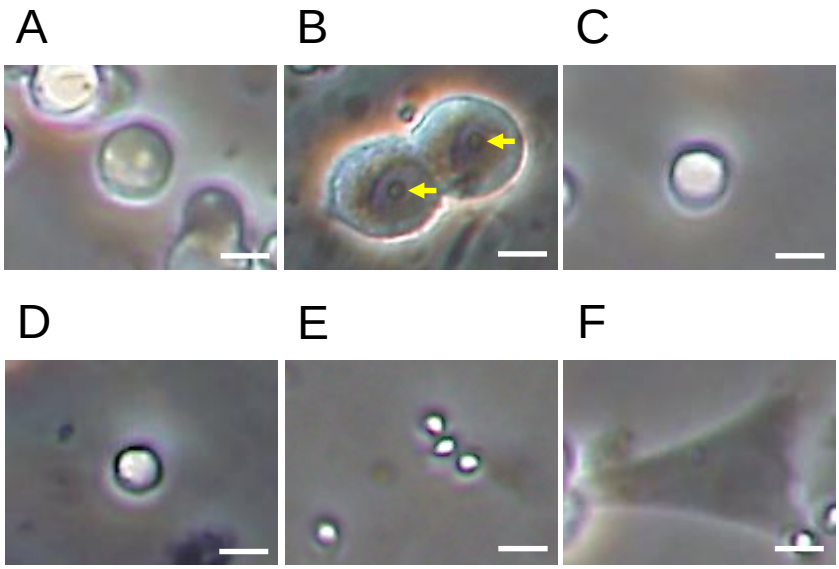

Figure 1. Morphology of medaka testicular cells.
Living cells isolated from the testis were examined under a phase-contrast microscope. (A) spermatogonium; (B) Flattened spermatogonia immediately after mitosis. Note a prominent nucleolus (arrows) in the nucleus, one of their diagnostic characteristics; (C) primary spermatocyte; (D) secondary spermatocyte; (E) spermatids/spermatozoa; (F) somatic cell. Scale bar, 10 μm.

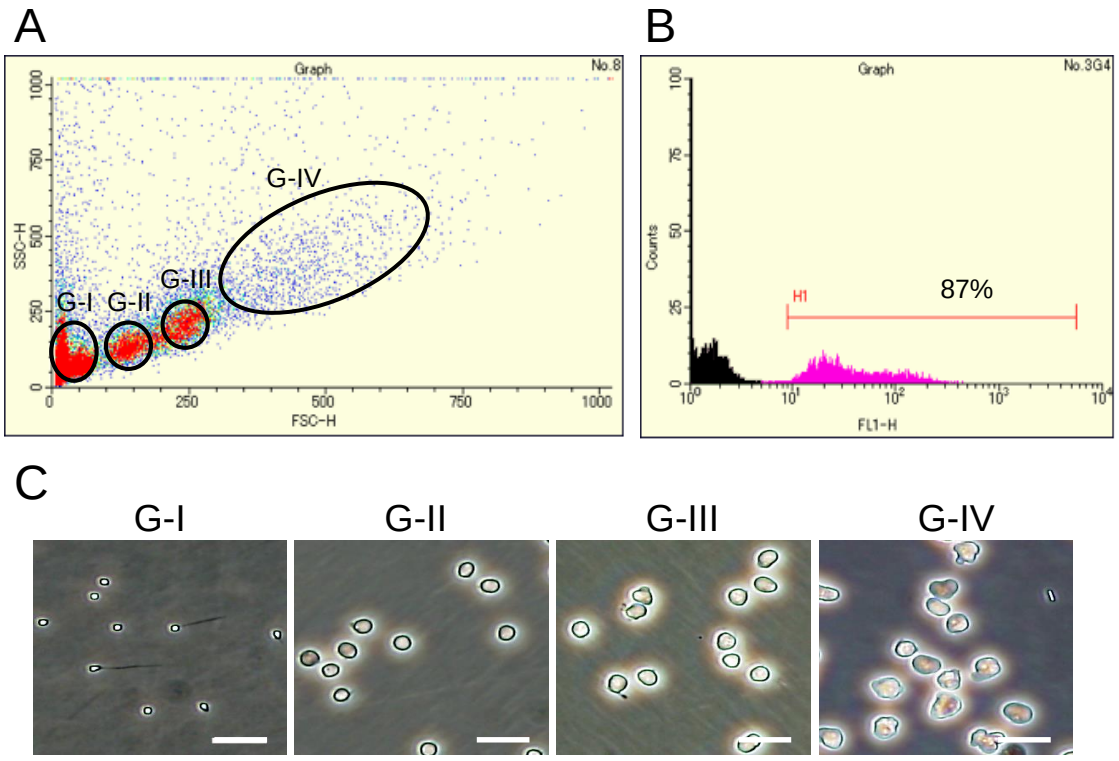

Figure 2. Cell sorting of medaka testicular cells.
(A) Sorting of medaka testicular cells according to the levels of forward scatter (FSC-H) and side scatter (SSC-H). Four fractions (Fractions G-I to G-IV) were obtained.
(B) The number (Counts) and level of GFP fluorescence (FL1-H) of cells in Fraction G-IV obtained from wild-type (black) and *olvas*-GFP (magenta) medaka. Spermatogonia and spermatocytes are labeled with GFP in the *olvas*-GFP medaka. In Fraction G-IV, about 90% of the cells expressed GFP when the *olvas*-GFP medaka was used for cell sorting, indicating that spermatogonia and spermatocytes account for 90% of cells in this fraction.
(C) Phase-contrast microscopic observation of cells present in each fraction. Scale bar, 20 μm. According to the cell morphology, Fraction G-I is rich in spermatozoa and spermatids (The haploid DNA content of cells in Fraction G-I was confirmed by DNA fluorescence.), Fraction G-II is rich in secondary spermatocytes and spermatogonia (probably at the late stage), Fraction G-III is rich in primary spermatocytes, and Fraction G-IV is rich in spermatogonia (probably at the early stage). Scale bar, 10 μm.

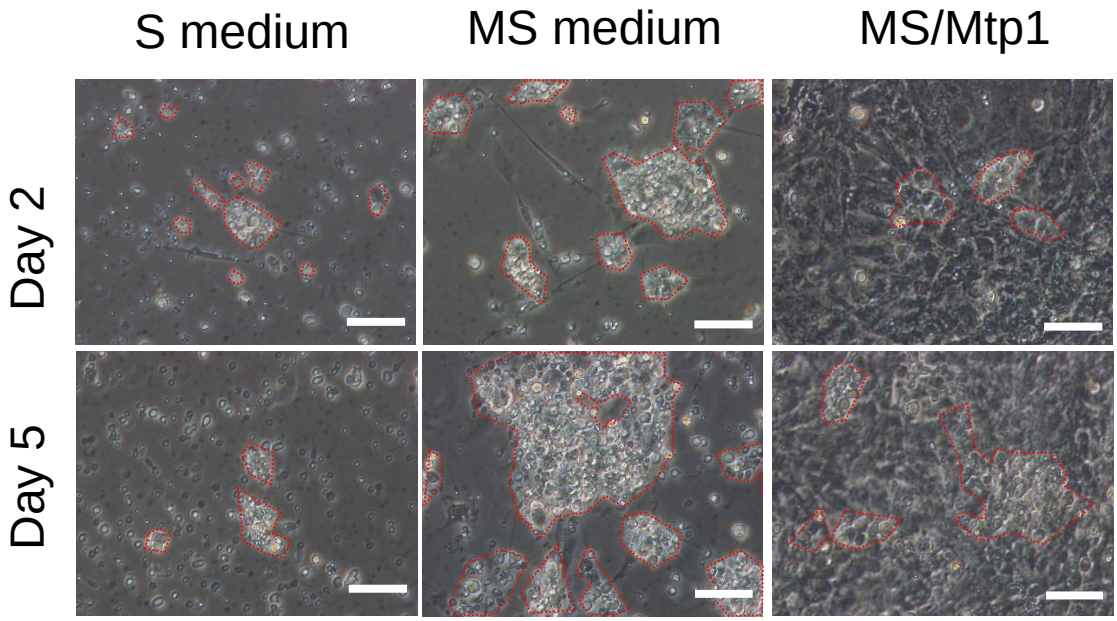

Figure 3. Recapitulation of medaka spermatogenesis *in vitro*.
Spermatogonia cultured in the original medium (S medium, S), modified medium (MS medium, MS) and MS medium with Mtp1 cells (MS/Mtp1) for 2 days (day 2) and 5 days (day 5). Spermatogonia-rich clusters are marked by red dotted lines. These cells were labeled with BrdU. Scale bar, 100 μm.

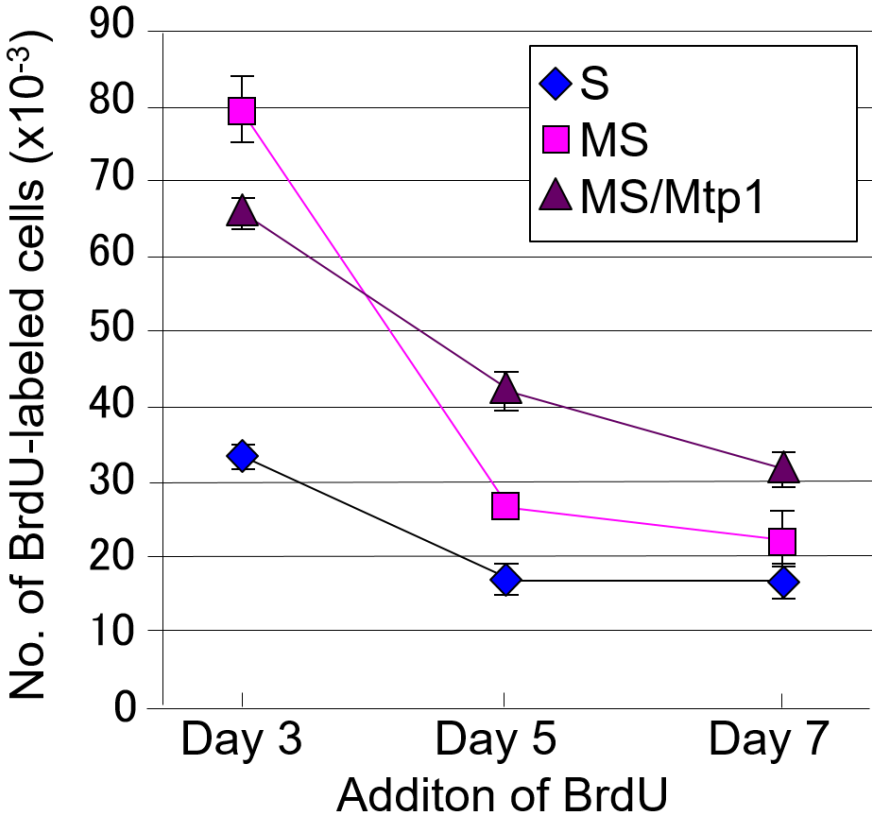

Figure 4. Changes in the number of BrdU-labeled cells.
Spermatogonia-rich fractions were cultured with S medium (S), MS medium in the absence (MS) or presence (MS/Mtp1) of Mtp1 cells. BrdU was added on day 3, 5 or 7 and the cells were harvested 1 day later.

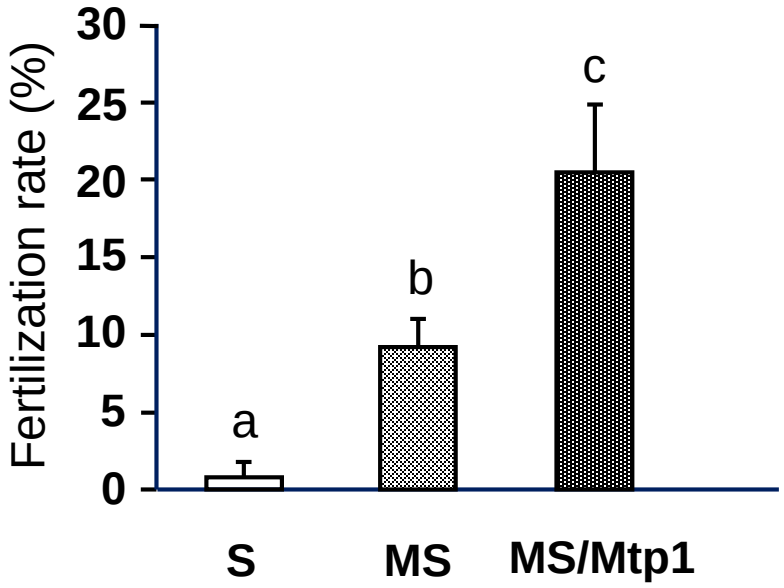

Figure 5. Fertilization rate of eggs inseminated with spermatozoa produced *in vitro*.
Eggs were inseminated with spermatozoa produced under 3 different culture conditions; S medium (S), MS medium in the absence (MS) or presence (MS/Mtp1) of Mtp1 cells. Values with different letters are significantly different (mean ± SD; n=3; $P<0.01$, Tukey-Kramer test).

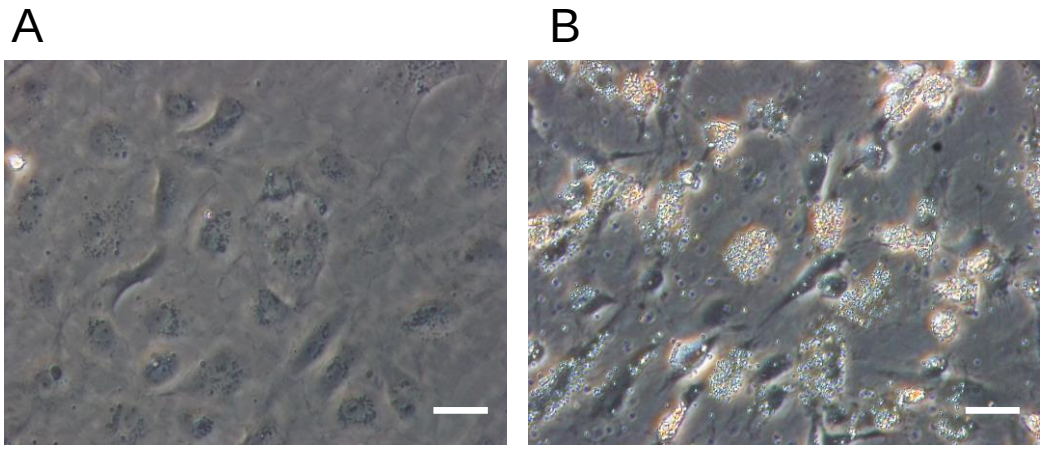

Figure 6. Phagocytosis activity of Mtp1 cells.
After 24 hours of culture in the absence (A) or presence (B) of a 1000-fold diluted suspension of polystyrene beads (Latex beads, LB-11; Sigma-Aldrich, Saint Louis, MO), cells were washed 3 times with PBS and observed under a phase-contrast microscope. Scale bar, 50 μm.

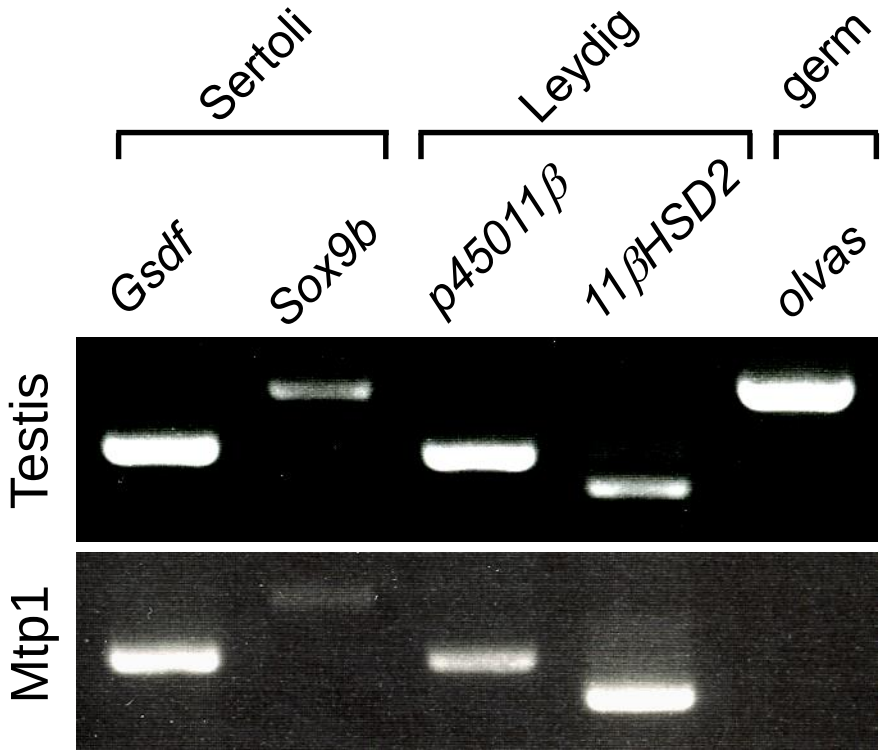

Figure 7. Expression of marker genes in Mtp1 cells.
Total RNA samples from the testis and Mtp1 cells were analyzed by RT-PCR for Sertoli cell markers (*Gsdf*, *Sox9b*), Leydig cell markers (*p45011β*, *11βHSD2*) and a germ cell marker (*olvas*).

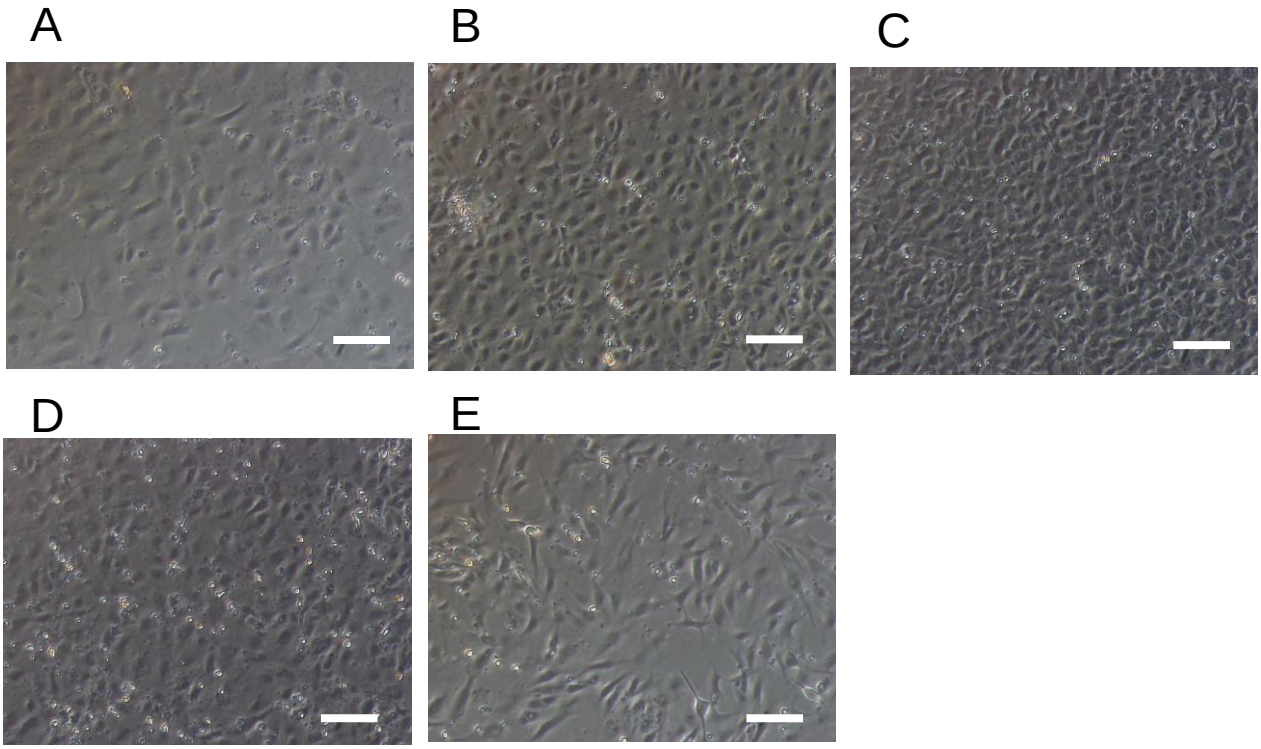

Figure 8. Morphology of cloned Mtp1 cells.
(A) large scale-like cells, (B) middle scale-like cells, (C) small scale-like cells, (D) triangular cells, (E) fibroblast-like cells. Scale bar, 50 μm.

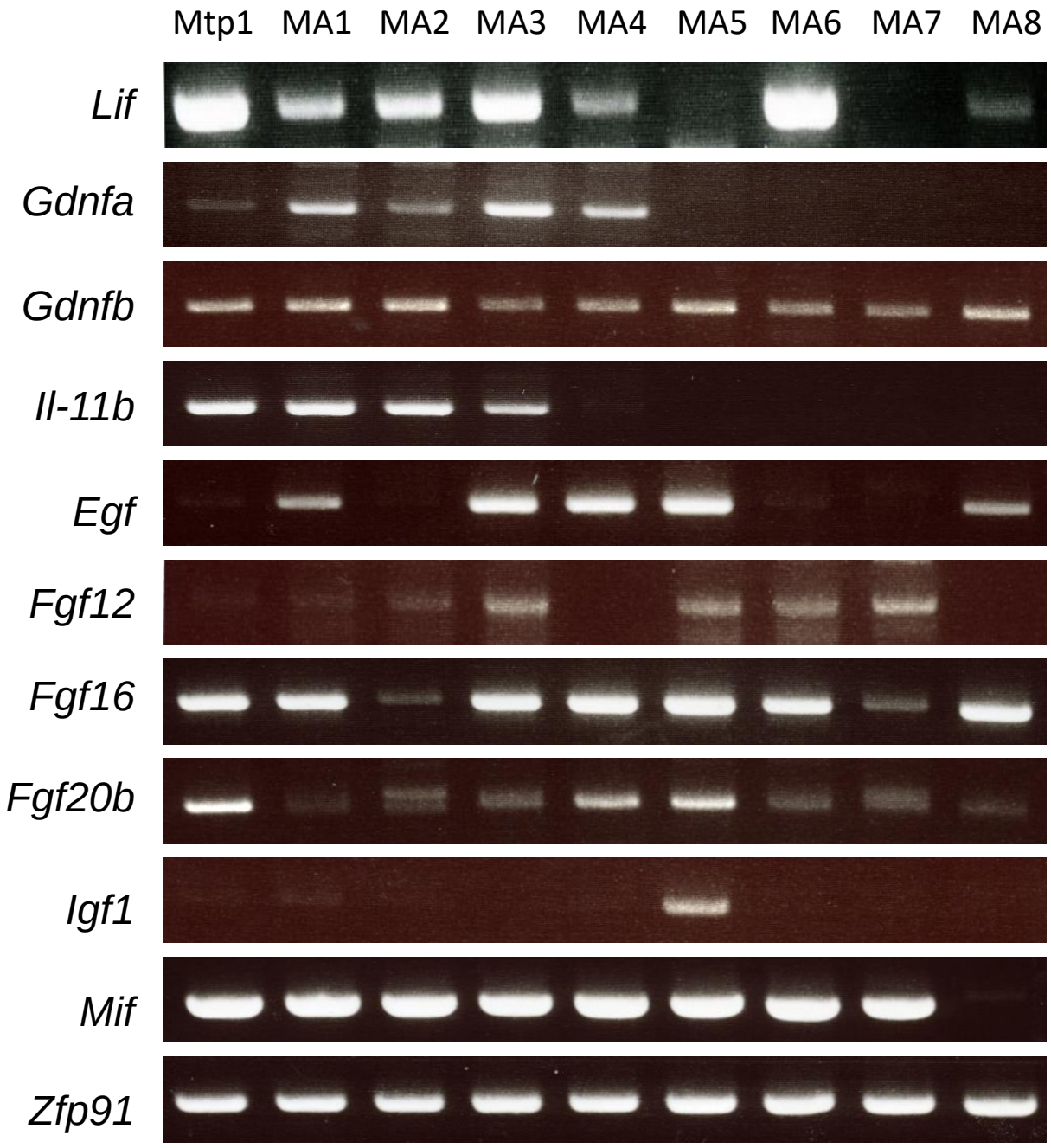

Figure 9. Expression of cytokines in Mtp1 cells and cloned Mtp1 cells. Total RNA samples from the original Mtp1 cells and cloned Mtp1 cells were analyzed by RT-PCR for various cytokines. 8 cell lines (MA1 to MA8) with different expression patterns of cytokines were obtained.

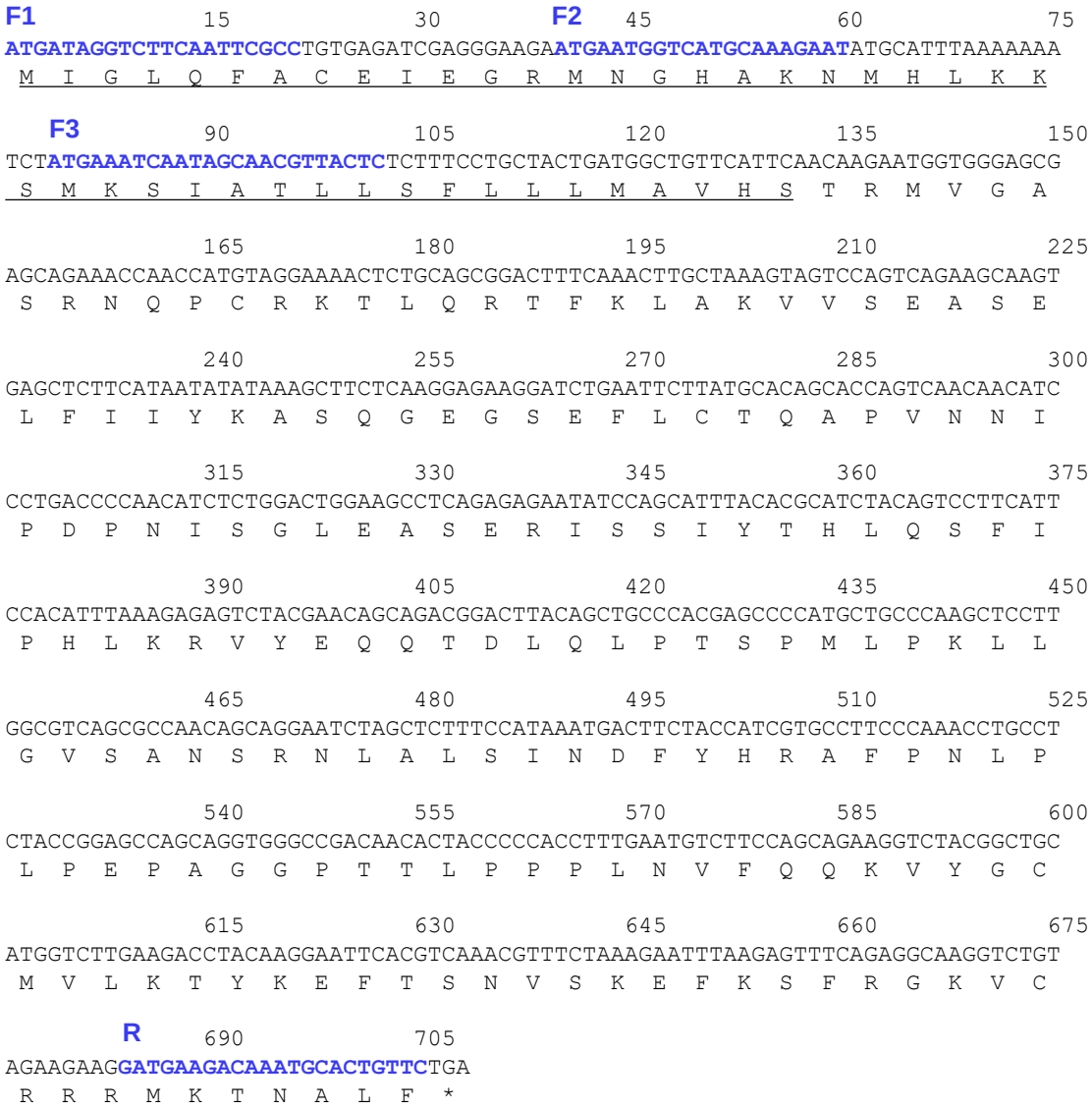

Figure 10. Nucleotide and amino acid sequences of the coding region of medaka Lif cDNA.

The N-terminal sequence indicated by an underline is a signal peptide that will be removed when Lif protein is secreted as a mature form. F1, F2, F3 and R show the positions of primers used for various versions of Lif cDNA (see MATERIALS AND METHODS).

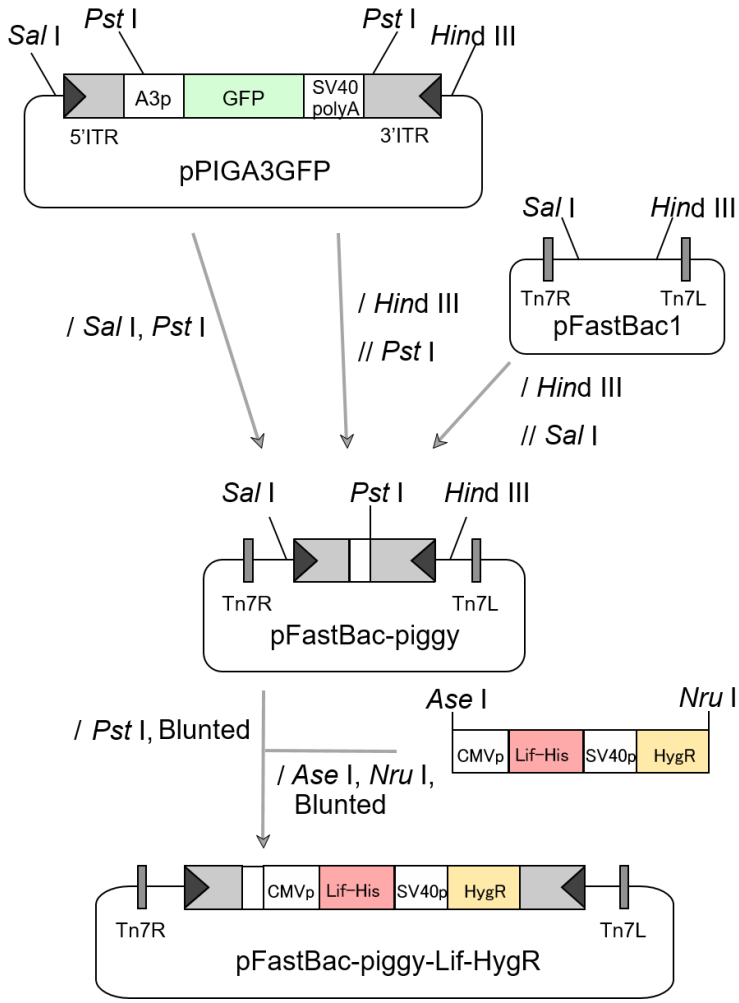

Figure 11. Construction of the donor plasmid to produce Lif-overexpressing Mtp1 cells.

The 5' and 3' piggyBac inverted terminal repeats (5' ITR, 3' ITR) were isolated from pPIGA3GFP by digestion with S*al*I/*Pst*I and *Hind*III/*Pst*I, respectively, and the resulting fragments were ligated into *Sal*I/*Hind*III-cut pFastBac1 (Life Technologies) that lacks a polyhedrin promoter (pFastBac-dphp) to yield pFastBac-piggy. Using medaka Lif in pET161-DEST as a template, cDNA encoding Lif-F2-His was amplified with a *Nhe*I-Lif-F2/*Bam*HI-His primer set (Table 4). The resulting cDNA was digested with *Nhe*I/B*am*HI and ligated into *Nhe*I/*Bam*HI-cut pAcGFP-Hyg-C1 (Clontech Laboratories, Mountain View, CA) to yield Lif-F2-His/pAcGFP-Hyg-C1, from which a cDNA fragment including medaka Lif-F2-His (Lif-His) and hygromycin resistant gene (HygR) was obtained by digestion with *Ase*I/*Nru*I. The cDNA fragment was blunted with a DNA Blunting Kit (Takara Bio, Shiga, Japan) and ligated into *Pst*I-digested, blunted pFastBac-piggy to produce pFastBac-piggy-Lif-HygR that contains CMV-driven Lif and SV40-driven HygR.

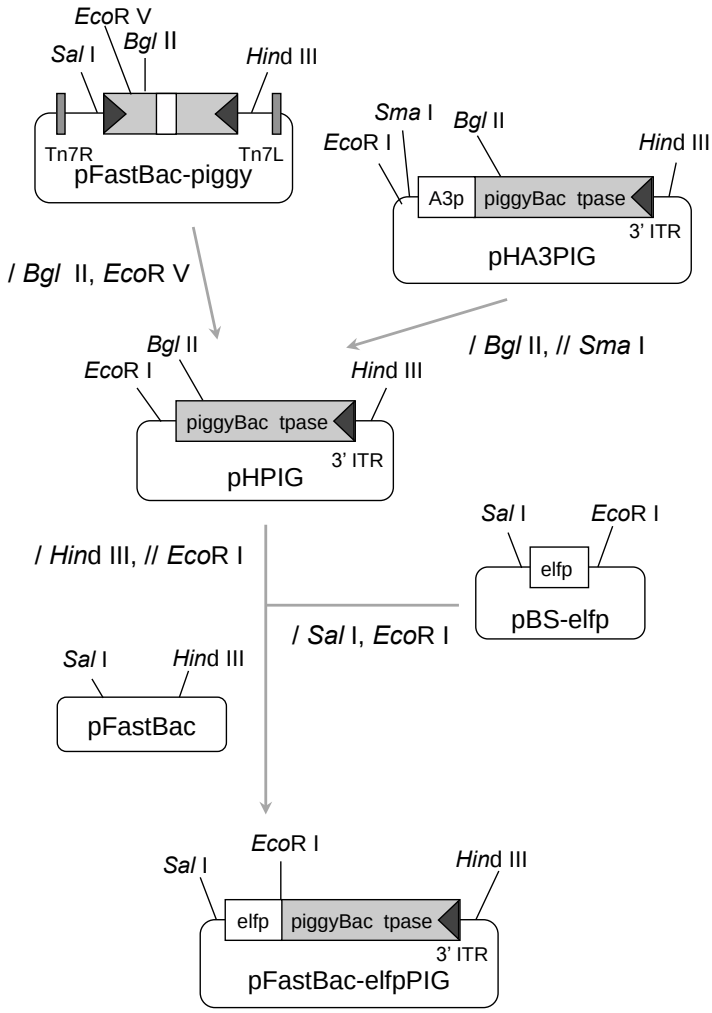

Figure 12. Construction of the helper plasmid to produce Lif-overexpressing Mtp1 cells.

The helper plasmid carrying piggyBac transposase (tpase), the expression of which is under the control of human elongation factor 1α promoter (elfp), was produced as follows. A 5' region of piggyBac transposase was isolated from pFastBac-piggy by *Eco*RV/*Bgl*II and ligated into the *Sma*I/*Bgl*II site of pHA3PIG to produce pHPIG. A DNA sequence including a full-length transposase and 3' ITR was isolated from pHPIG by digestion with *Eco*RI/*Hind*III. pEF-BOS was digested with *Hind*III/*Eco*RI and the resulting elfp-containing DNA was ligated into *Hind*III/*Eco*RI-treated pBluescript SK (-) (Stratagene) to produce pBS-elfp. Finally, two DNA fragments, elfp isolated from pBS-elfp by *Sal*I/*Eco*RI digestion and piggyBac transposase with its 3' ITR isolated from pHPIG by *Hind*III/*Eco*RI digestion, were ligated into the *Sal*I/*Hind*III site of pFastBac-dphp to produce pFastBac-elfpPIG, which contains elfp-driven piggyBac transposase. pFastBac-elfpPIG and pFastBac-piggy-Lif-HygR (Fig. 11) were transfected into Sf9 cells to produce helper and donor viruses, respectively, and after amplification of P1 viruses, the resulting P2 viruses were used to transfect Mtp1 cells.

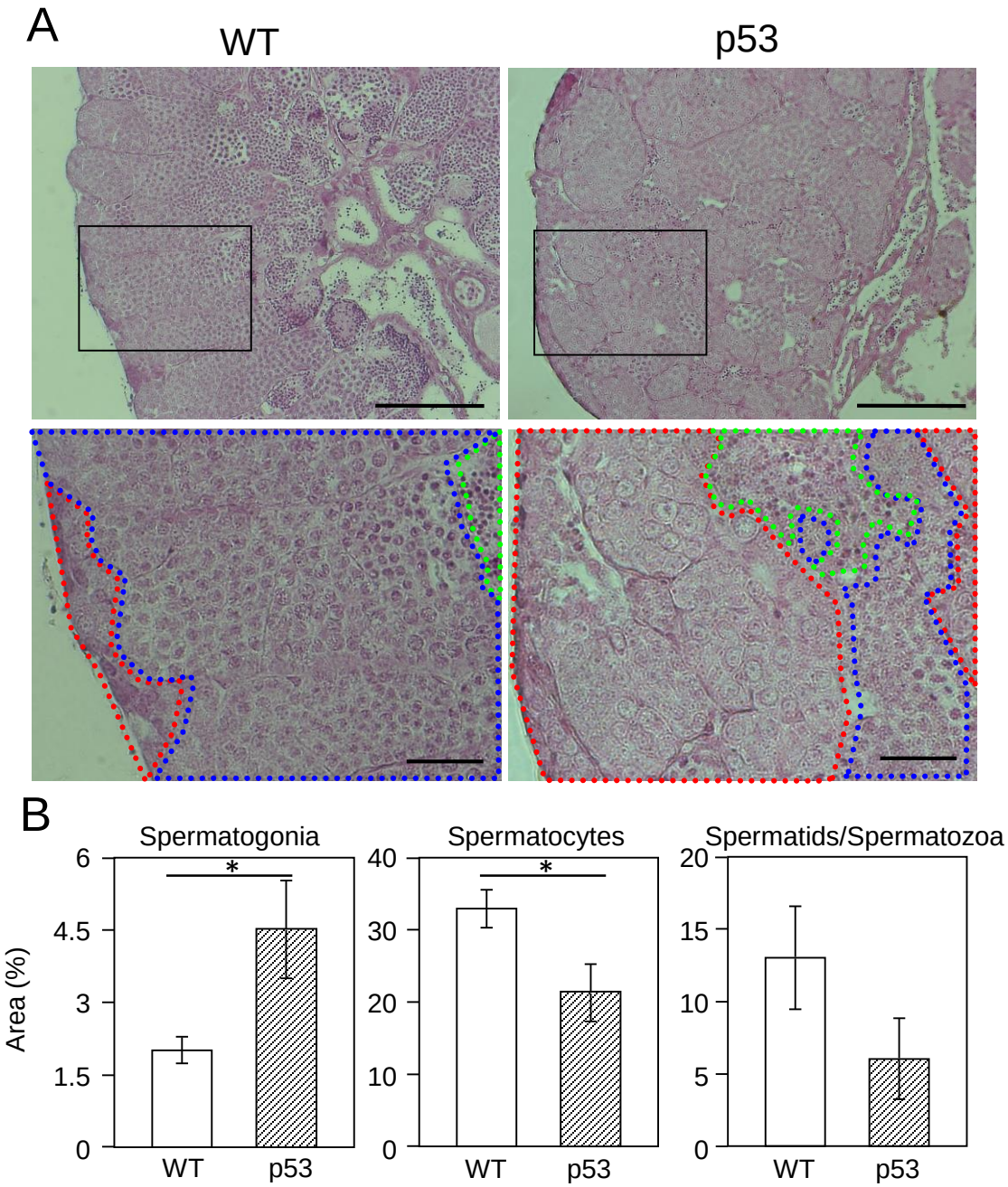

Figure 13. The area occupied by spermatogonia in the *p53*-KO medaka. (A) Histological sections of wild-type (WT) and *p53*-KO (p53) medaka testes. The regions indicated in the upper figures are magnified in the lower figures to show each type of spermatogenic cell. Areas of spermatogonia, spermatocytes and spermatids/sperm are surrounded by red, blue and green dots, respectively. Scale bar; 100 μm (upper figures), 20 μm (lower figures). (B) Morphometric analyses of wild-type (WT) and *p53*-KO (p53) medaka testes. The area occupied by spermatogonia in the *p53*-KO medaka testis is significantly larger than that in the wild-type medaka testis and, conversely, the area occupied by spermatocytes is significantly smaller in the *p53*-KO medaka (mean ± SD; n=3; *, $P<0.05$, Student's *t*-test).

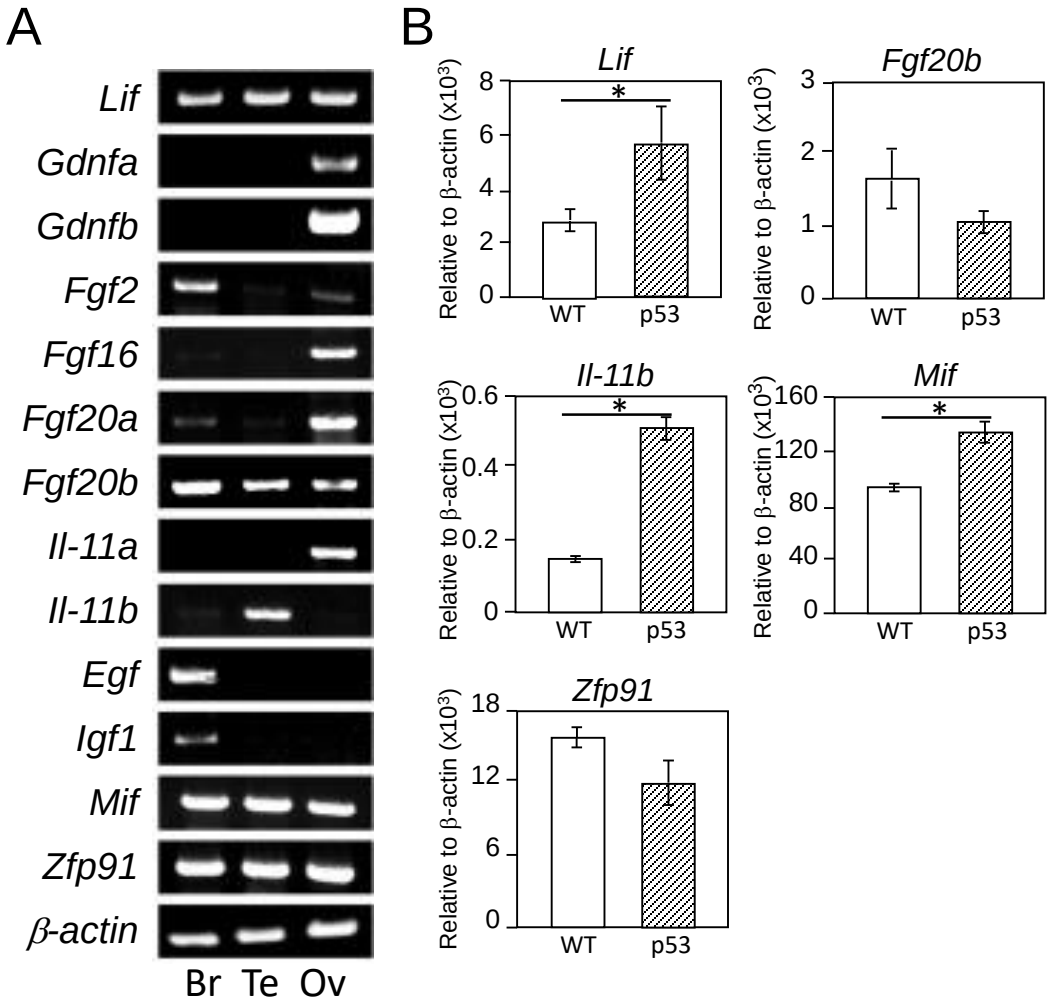

Figure 14. Expression of cytokine mRNAs in medaka tissues.
(A) RT-PCR analyses of mRNAs encoding several cytokines in the brain (Br), testis (Te) and ovary (Ov) of wild-type medaka. *β-actin* mRNA is a control. (B) Quatitative RT-PCR analyses of cytokines in wild-type (WT) and *p53*-KO (p53) medaka testes. The expression levels of mRNAs encoding *Lif*, *Il-11b* and *Mif* are significantly higher in the *p53*-KO medaka testis than the wild-type testis (mean ± SD; n=3; *, $P<0.01$, Student's *t*-test).

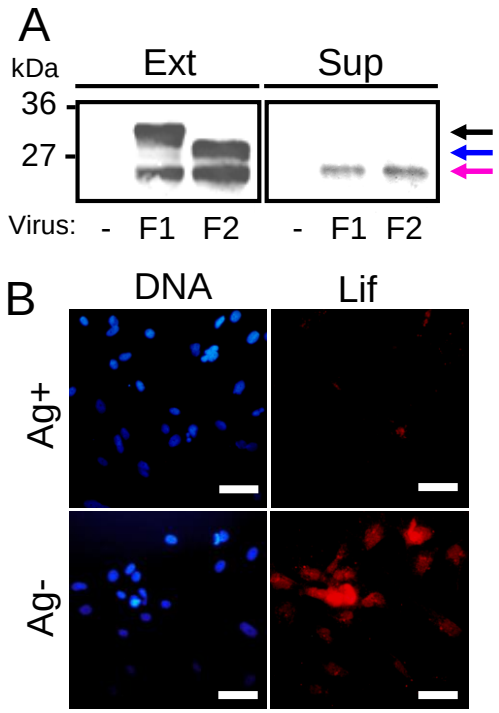

Figure 15. Characterization of baculovirus-produced medaka Lif proteins and anti-Lif antibody.

(A) Detection of baculovirus-produced medaka Lif proteins (Lif-F1-His, Lif-F2-His). Protein extracts (Ext) from Sf9 cells not infected with (-) or infected with viruses producing Lif-F1-His (F1) or Lif-F2-His (F2) and culture supernatants of the cells (Sup) were immunoprecipitated with anti-Lif antibody, and the resulting precipitates were immunoblotting with anti-His antibody. A truncated form of Lif (a mature Lif indicated by a magenta arrow) and full-length Lif-F1-His (a black arrow) and Lif-F2-His (a blue arrow) were observed in the extracts, whereas only the mature protein was present in the culture supernatants.

(B) Anti-Lif immunocytochemistry of Lif-overexpressing Mtp1 cells with an antibody pre-absorbed with (Ag+) or not pre-absorbed with (Ag-) antigenic proteins. Lif-stained sections (Lif) were also stained with Hoechst 33258 (DNA) to visualize the cell nuclei. Positive Lif signals (red) were found by the intact antibody (Ag-) but not by the antigen-absorbed control antibody (Ag+). Scale bar, 50 μm.

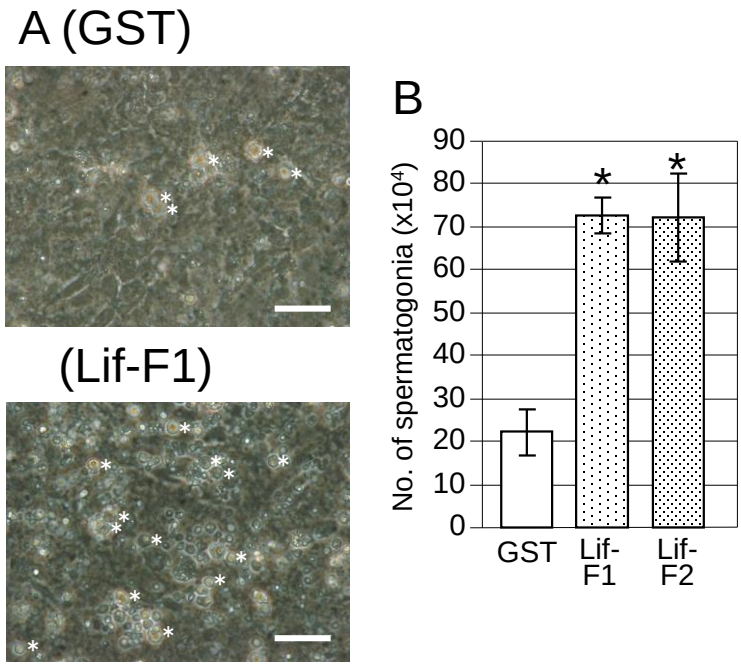

Figure 16. Effects of Lif on spermatogonial proliferation under culture conditions.

(A) Culture of spermatogonia-rich fractions in the presence of baculovirus-produced GST as a control or baculovirus-produced medaka Lif protein (Lif-F1) for 4 days. Spermatogonia identified by their morphology (see Fig. 1) were counted. The number of spermatogonia (indicated by asterisks) was increased at a higher rate in the presence of Lif-F1 than in the presence of GST. Lif-F2 had a similar effect (data not shown). Scale bar, 10 μm.

(B) Number of spermatogonia in the culture on day 7 in the presence of GST or Lif (added to the culture on day 3). The number of cells at the beginning of culture (on day 0) was 15×10^4. Recombinant Lif proteins (Lif-F1, Lif-F2) increased the number of spermatogonia significantly compared to the control (GST) (mean ± SD; n=3; *, $P<0.01$; Student's *t*-test).

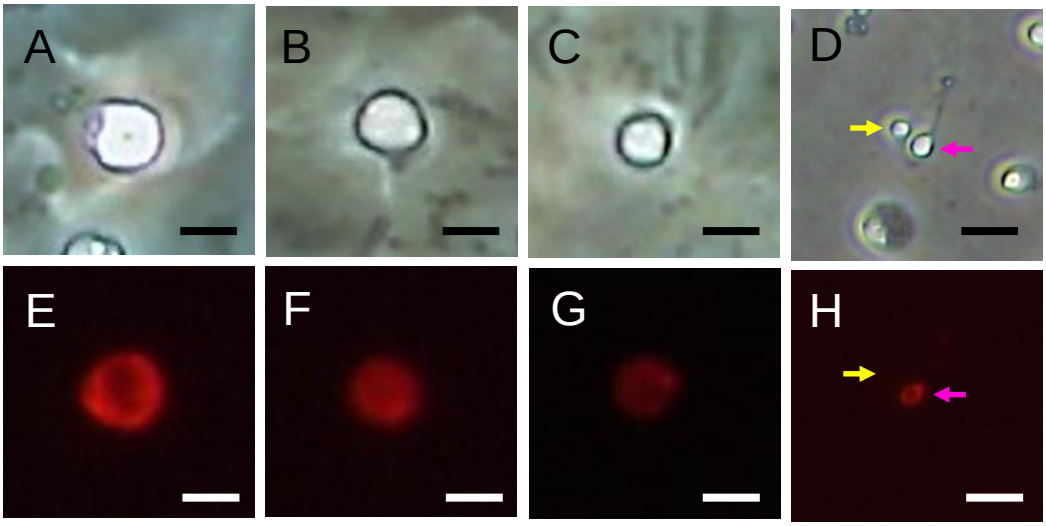

Figure 17. *In vitro* spermatogenesis of PKH26-labeled spermatogonia. Differentiation of a PKH26-labeled spermatogonium (A, E) into a primary spermatocyte (B, F), secondary spermatocyte (C, G) and spermatid/spermatozoon (D, H) is shown. PKH26-labeled spermatogonia were co-cultured with PKH26-unlabeled spermatogonia in the presence of Lif-overexpressing Mtp1 cells, and on day 7, PKH26-labeled cells were observed under a phase-contrast microscope (A-D) and a fluorescent microscope (E-H). The spermatid/spermatozoon indicated by a magenta arrow has been labeled with PKH26, but the cell indicated by a yellow arrow has not (D, H), demonstrating that the former is derived from the PKH26-labeled spermatogonium and the latter is from the unlabeled spermatogonium. Scale bar, 10 μm.

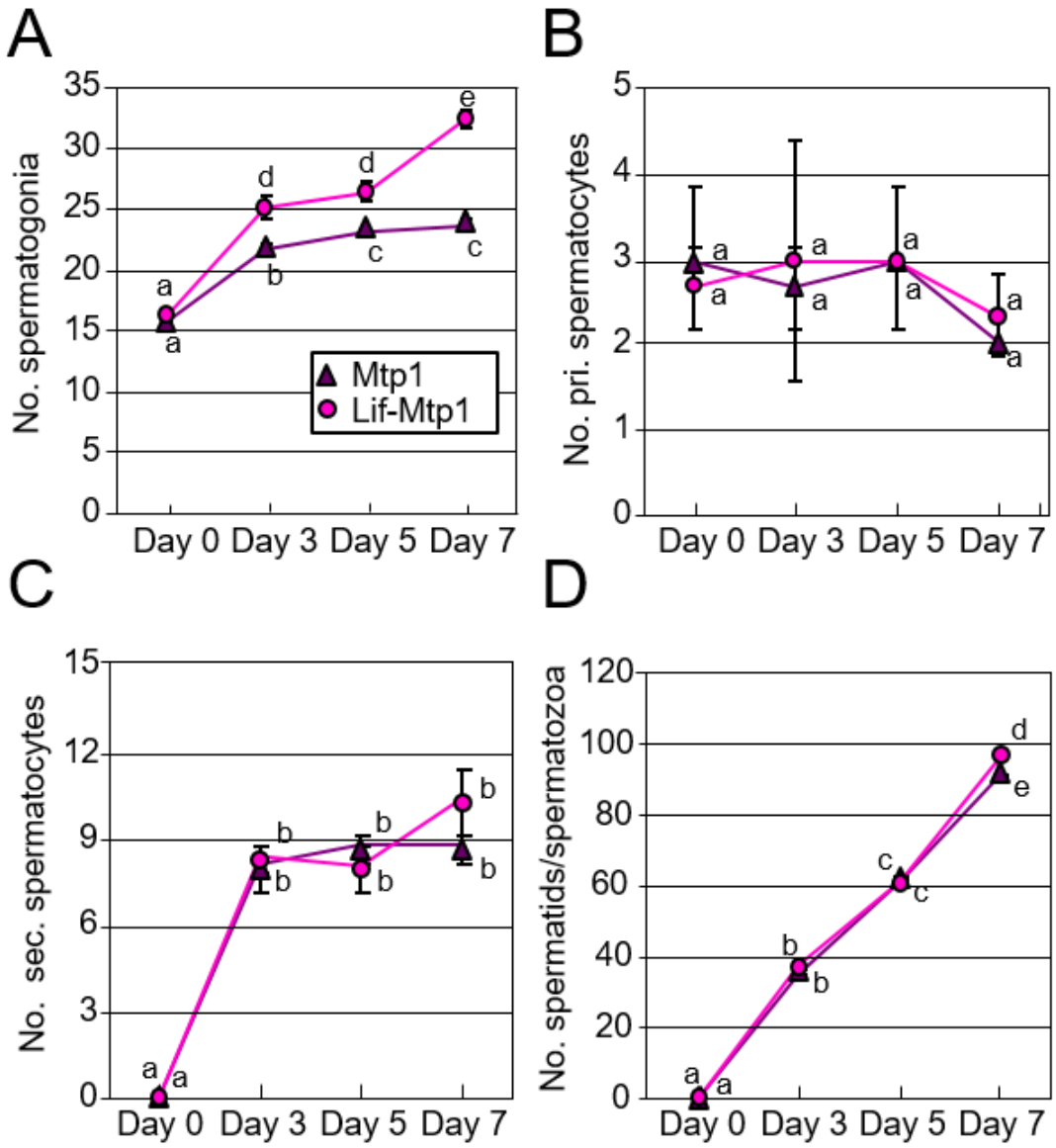

Figure 18. Dynamics of PKH26-labeled cells co-cultured with Mtp1 or Lif-overexpressing Mtp1 (Lif-Mtp1) cells.
Cells were harvested on days 0, 3, 5 and 7 and PKH26-labeled cells were counted after identification of cell types by their morphology (A, spermatogonia; B, primary spermatocytes; C, secondary spermatocytes; D, spermatids/spermatozoa; see also Fig. 1 and 17). The data were obtained by three independent experiments. Values with different letters are significantly different (mean ± SD; n=3; $P<0.01$, Tukey-Kramer test).

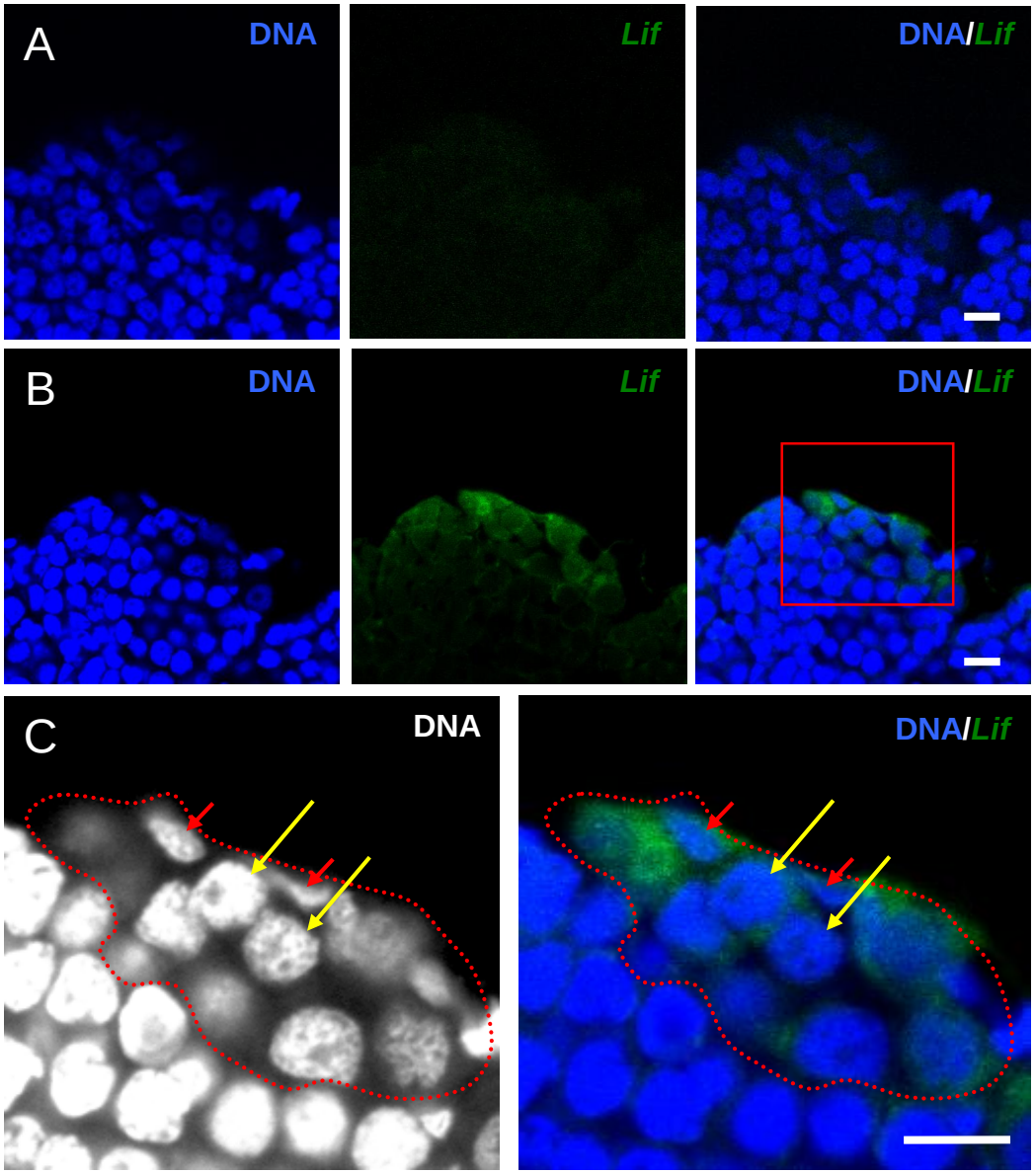

Figure 19. mRNA expression of Lif in medaka testes.
Histological sections of wild-type medaka testes were subjected to *in situ* hybridization analysis using DIG-labeled sense (A) and anti-sense (B) probes. The localization of probes was visualized with anti-DIG antibody (*Lif*, green) and the cell nuclei were stained with the DNA dye Hoechst 33258 (DNA, blue) to identify the cell type. Positive signals were detected in the peripheral region. (C) Enlargement of the Lif-positive area indicated in B (red rectangle). The DNA image (DNA) and DNA and Lif merged image (DNA/*Lif*) are shown. The area occupied by spermatogonia and associated Sertoli cells is marked by dots, and the nuclei of spermatogonia and Sertoli cells are indicated by yellow arrows and red arrows, respectively. The expression of mRNAs encoding Lif was detected in spermatogonia and Sertoli cells surrounding them but not in primary spermatocytes existing outside the marked area. Scale bar, 10 μm.

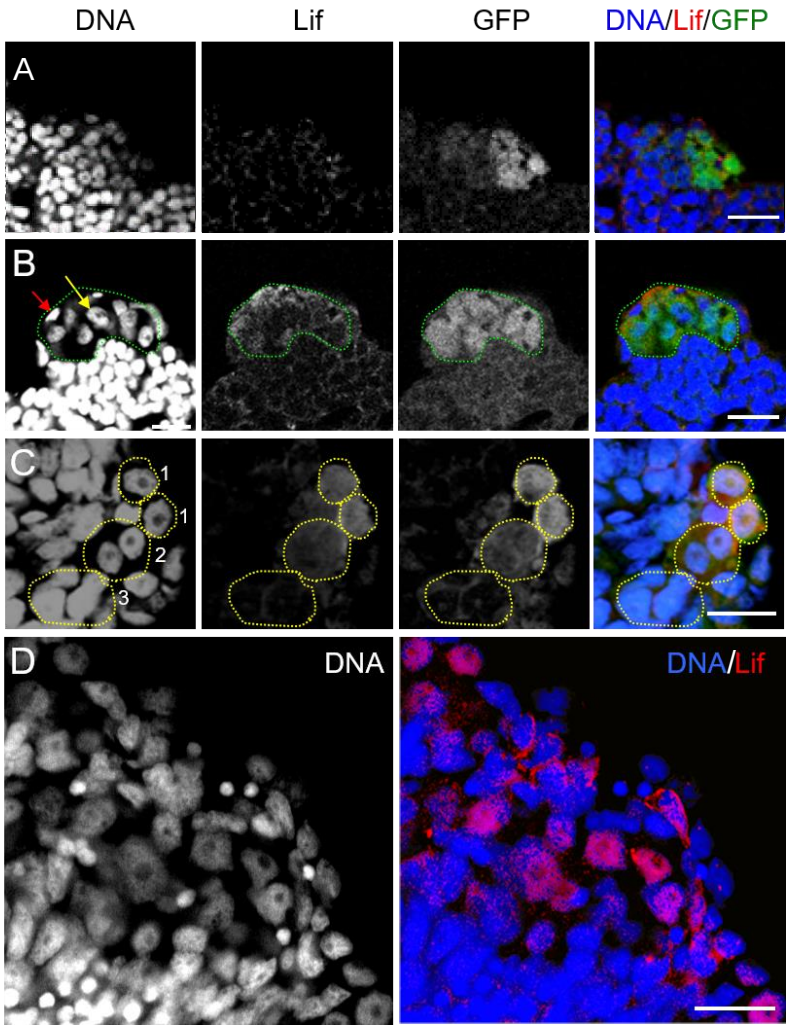

Figure 20. Lif protein expression in medaka testes.
Histological sections of the *olvas*-GFP medaka testis (A-C) triple-stained with the DNA dye Hoechst 33258 (DNA, blue), anti-Lif antibody (Lif, red) and anti-GFP antibody (GFP, green) and of the *p53*-KO medaka testis (D) double-stained with Hoechst 33258 (DNA, blue) and anti-Lif antibody (Lif, red). Scale bar, 10 μm. (A) A control experiment using an antigen-absorbed anti-Lif antibody. Clear Lif signals are not found. (B) Triple staining (DNA/Lif/GFP) of the *olvas*-GFP medaka testis. The area occupied by Lif-expressing spermatogonia (type A spermatogonia judged by their high GFP expression levels) is encircled by green dots. A yellow arrow shows the nucleus of a type A spermatogonium and a red arrow shows the nucleus of a Sertoli cell. (C) Enlargement of the *olvas*-GFP medaka testis. Spermatogonia are characterized by a prominent nucleolus that is not stained with Hoechst 33258. GFP expression levels are higher in the following order: type A spermatogonia, type B spermatogonia and primary spermatocytes. According to their nuclear morphology and GFP expression levels, the cells enclosed by dots can be identified as type A spermatogonia (1), type B spermatogonia (2) and primary spermatocytes (3). Lif protein expression levels in type A spermatogonia are higher than those in type B spermatogonia and no expression is seen in primary spermatocytes. (D) Double staining (DNA/Lif) of the *p53*-KO medaka testis. Lif protein signals (red) in spermatogonia and surrounding Sertoli cells are stronger in the *p53*-KO medaka than in the *olvas*-GFP medaka, in which the *p53* gene is intact.