Function of leukaemia inhibitory factor in spermatogenesis of a teleost fish, the medaka *Oryzias latipes*

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Running headline: Lif function in medaka spermatogenesis

Summary

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. Leukaemia inhibitory factor (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 the function of Lif in teleost (medaka) testes, we examined the effects of Lif on spermatogenesis in a newly established cell culture system using a cell line (named Mtp1) derived from medaka testicular somatic cells as feeder cells. We found that addition of baculovirus-produced recombinant medaka Lif to the culture medium or co-culture with Lif-overexpressing Mtp1 cells increased the number of spermatogonia. *In situ* hybridization and immunohistochemical analyses of the medaka testes showed that mRNAs and proteins of Lif are expressed in spermatogonia and surrounding Sertoli cells, with higher expression levels in type A (undifferentiated) spermatogonia than in type B (differentiated) spermatogonia. Our findings suggest that Lif regulates spermatogonial cell proliferation in the medaka.

Keywords: Cell culture; Lif expression; Mitosis; Spermatogenesis; Teleost testis

Introduction

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. 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. Among them, cytokines are of particular interest because they are key molecules in local regulation (paracrine regulation) mediated by cell-to-cell communications (Hedger & Meinhardt, 2003; Loveland *et al.*, 2017; Oatley & Brinster, 2008; Weinbauer & Wessels, 1999).

Leukaemia 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 (Honda *et al.*, 2009; Humphrey *et al.*, 2004). Lif also promotes the survival or proliferation of mouse primordial germ cells (PGCs) (Chuma & Nakatsuji, 2001; De Felici & Dolci, 1991; 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 under culture conditions (Dorval-Coiffec *et al.*, 2005), consistent with reports showing that its mRNAs are expressed in spermatogonia and somatic cells (chiefly in peritubular cells) in the rat testis (Dorval-Coiffec *et al.*, 2005; Jenab and Morris, 1998). 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 & 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 especially in non-mammalian vertebrates, and the generality of its function in mammals and other vertebrates including fish remains to be elucidated.

Although the molecular and cellular mechanisms of spermatogenesis have been 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 different fish species: eel (Miura *et al.*, 2011; Miura *et al.*, 1991; Ohta *et al.*, 2007), medaka (Hong *et al.*, 2004; Iwasaki *et al.*, 2009; Saiki *et al.*, 1997; Sasaki *et al.*, 2005; Shimizu *et al.*, 2000; Shimizu *et al.*, 1997; Song & Gutzeit, 2003), zebrafish (Kawasaki *et al.*, 2012; Kawasaki *et al.*, 2016; Sakai, 2002; Wong & Collodi, 2013), tilapia (Tokalov & Gutzeit, 2005) and catfish (Nayak *et al.*, 2016). Among them, the medaka fish (*Oryzias latipes*) has received much attention as an experimental animal in various fields of biological science, including reproductive and developmental biology (Shima & Mitani, 2004; Wittbrodt *et al.*, 2002), and, accordingly, various wide-ranging experimental tools have become available, including genomic and cDNA information, technical manuals, wild-type, inbred, mutant and transgenic strains, and related species belonging to the genus *Oryzias* (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/).

Here, we report a possible role of Lif in teleost spermatogenesis. A newly established cell culture system revealed that the addition of recombinant medaka Lif proteins to the culture medium increased the number of spermatogonia. Culture of spermatogonia with Lif-

overexpressing somatic cells showed similar effects. *In situ* hybridization and immunohistochemical analyses of the medaka testes showed that Lif proteins, as well as *lif* 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

Six-month-old males of hi-medaka (an orange-red variety purchased from a local fish farm) were used in this study. To visualize spermatogonia and spermatocytes, *olvas*-GFP medaka was also used (Tanaka *et al.*, 2001). The fish were cultured in fresh water at 27˚C under artificial light conditions (14-hr light and 10-hr dark).

Production of baculovirus-expressed medaka Lif

Using a polymerase chain reaction (PCR)-derived Lif cDNA fragment as a screening probe, we isolated full-length Lif cDNA (DDBJ/EMBL/GenBank accession number AB766229, Supplementary Fig. S1) 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 (Supplementary Fig. S1 and Table S1). The resulting ORFs were inserted into the pENTR vector by TOPO cloning (Life Technologies; Thermo Fisher Scientific, 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 (Supplementary Table S1). 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) to obtain recombinant bacmid DNAs. Sf9 cells to which the recombinant bacmid DNA had been transfected with Cellfectin II reagent (Life Technologies) 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 phosphate buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4 $12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4), 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, we 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.

Characterization of Mtp1 cells

Mtp1 is a testicular somatic cell line derived from *p53* (a tumor-suppressor gene) -knockout medaka (Kawasaki *et al.*, 2009; Taniguchi *et al.*, 2006). Its phagocytosis activity was examined as described previously (Sakai, 2002). We also examined expression of marker genes for Sertoli cells (*gsdf, sox9b*), Leydig cells (*p45011β, 11βHSD2*) and germ cells (*olvas*) in Mtp1 cells. Following isolation of total RNA samples from the testis and Mtp1 cells with ISOGEN (Nippon Gene, Tokyo, Japan), cDNAs were produced with a Super Script III First Strand Synthesis System (Life Technologies) and analyzed by reverse transcription PCR (RT-PCR) as described previously (Takahashi *et al.*, 2014). The primer set for *gsdf* was 5'-CTCAGGTTGGTCAAATGAGG-3' and 5'- CCATCATCTGAAGGTTACTTGG-3'. The primer sets for *sox9b*, *p45011β*, *11βHSD2* and *olvas* were described previously (Kurokawa *et al.*, 2007).

Production of Mtp1 cells overexpressing medaka Lif

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). We noticed that baculovirus-mediated gene transfer to medaka cells is less harmful than electroporation- or retrovirus-mediated methods. To produce cells that overexpress medaka Lif continuously, we therefore used a baculovirus-mediated transgenic system that employs two baculovirus vectors, one encoding the piggyBac transpose (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) (Abe *et al.*, 2005; Mizushima & Nagata, 1990; Tamura *et al.*, 2000) (Supplementary Figs. S2 and S3). 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 hour later, hygromycin B 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-F1-His and Lif-F2-His (Supplementary Fig. S4A). The Lif-overexpressing Mtp1 cells, as well as the wild-type Mtp1 cells, were used as feeder cells to culture medaka spermatogenic cells (see ***Cell culture***). The Lif-overexpressing Mtp1 cells were also used to check the specificity of anti-Lif antibody (Supplementary Fig. S4B).

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 a culture medium (see ***Cell culture***) 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). Sheath solution, which is commonly used for cell sorting, is toxic to medaka spermatogenic cells. 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 (Supplementary Fig. S5): spermatogonia were identified by a large nucleus and prominent nucleolus, primary spermatocytes were recognized by their large size and chromatin structure characteristic of meiosis, secondary spermatocytes were identified by their intermediate size, spermatid and spermatozoa were recognized by their small size and a highly differentiated morphology equipped with a flagellum, and somatic cells were recognized by their flattened and irregular shape. To identify the cell types, we also used *olvas-GFP* medaka, which contain GFP-labeled spermatogonia and spermatocytes (GFP expression levels in spermatogonia being larger 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.

Cell culture

Spermatogonia-rich fractions obtained by cell sorting were co-cultured with Mtp1 or Lif-overexpressing Mtp1 cells for 7 or 8 days in a gelatin-coated plastic dish ($1.5x10^5$ cells per well of a 24-well plate) at 28°C. The culture medium consisted of Leibovitz L-15 (Life Technologies) supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin, 1 μg/ml bovine insulin, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml kanamycin, 10% fetal bovine serum, 50 ng/ml retinol, 3% carp serum (obtained from carps purchased at a local fish farm), 10 IU/ml each of human chorionic gonadotropin, pregnant mare serum gonadotropin and porcine follicle-stimulating hormone, and 10 mM HEPES (pH 7.3). The medium was changed on days 1 and 4 to remove dead cells and spermatozoa, both of which were liberated from the bottom of the dish to the culture medium.

Spermatogenesis of PKH26-labeled spermatogonia in cell culture

To trace the fate of germ cells under culture conditions, cells in spermatogonia-rich fractions obtained by cell sorting were labeled with the fluorescent vital staining dye PKH26 (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. PKH26-labeled cells (20 cells/well) were added to PKH26-unlabeled 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 cell culture. A mixture of PKH26-labeled and -unlabeled cells was 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) and suspended in PBS in a 35-mm dish. After the types of cells had been identified by the morphological criteria (Supplementary Fig. S7), 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 a digoxigenin (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 (7 μm in thickness) were successively treated with 0.3% Triton X-100 in PBS, 0.2 N HCl, and 1 μg/ml proteinase K in PBS at 37˚C and re-fixed with 4% PFA in 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) for 30 min. Following amplification of signals with tyramide-DNP by a TSA Plus Fluorescence kit (PerkinElmer Japan, Yokohama, Japan), the samples were incubated with anti-DNP-Alexa Fluor 488 antibody at 1:500 dilution (Molecular Probes; Thermo Fisher Scientific, 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), 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 (Supplementary Fig. S1 and Table S1). The amplified cDNA was inserted into the pENTR/D-TOPO Gateway vector with a pENTR Directional TOPO Cloning Kit (Invitrogen; Thermo Fisher Scientific, Tokyo, Japan), and the resulting plasmids were recombined with the destination vector pDEST15 using a Gateway cloning system (Life Technologies) to produce proteins with a GST tag at the N-terminus (GST-Lif-F3). GST-Lif-F3 was expressed in *Escherichia coli* and purified by SDS-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 as described previously (Takahashi *et al.*, 2014). 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, CA), 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). The expression of GFP in *olvas-GFP* medaka testes was immunologically detected by anti-GFP mouse antibody (Roche Diagnostics) and Alexa 488-conjugated anti-mouse IgG antibody. Immunostaining with anti-Sycp3 antibody was performed as described previously (Iwai *et al.*, 2006).

Results

Characterization of baculovirus-produced medaka Lif proteins

We produced recombinant medaka Lif proteins by a baculovirus expression system. Since two start methionines were presumed from its cDNA sequence (Supplementary Fig. S1), 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 (Supplementary Fig. S4A). 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 (Supplementary Fig. S1), 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.

Spermatogonia-rich fractions used for cell culture

To examine the effects of recombinant medaka Lif proteins on spermatogenesis in cell culture, we 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. 1A). Morphological identification 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. 1B). We estimated that Fraction G-IV contains 10% somatic cells, 15% primary spermatocytes and 75% spermatogonia. To assess the cell population in Fraction G-IV more accurately, we specifically detected primary spermatocytes by anti-Sycp3 (a meiosis-specific protein) immunocytochemistry and found that they accounted for about 15%. 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. 1C). These results confirmed that the ratio of spermatogonia in Fraction G-IV is about 75%. We used this fraction to evaluate the effect of Lif on spermatogenesis.

Cell culture system using testicular somatic cells

Our previous cell culture system without the use of feeder cells recapitulates only the later

process of medaka spermatogenesis from primary spermatocytes to spermatozoa (Shimizu *et al.*, 2000; Shimizu *et al.*, 1997). Since testicular somatic cells, such as Sertoli cells and Leydig cells, are crucial for proper proliferation and differentiation of spermatogenic cells, we established a new cell culture system using a cell line, Mtp1 (Kawasaki *et al.*, 2009), derived from medaka testicular somatic cells as feeder cells in expectation that this system would recapitulate the whole process of medaka spermatogenesis from spermatogonia to spermatozoa.

We characterized Mtp1 cells by their phagocytosis activity and gene expression patterns. When Mtp1 cells were cultured in the presence of polystyrene beads, about 30% of them engulfed the beads, indicating that some of the Mtp1 cells have phagocytosis activity (Supplementary Fig. 6A), a characteristic of Sertoli cells. In addition, RT-PCR analysis showed that Mtp1 cells express Sertoli cell (*gsdf* and *sox9b*) and Leydig cell (*p45011β* and *11β HSD2*) marker genes but not a germ cell marker gene (*olvas*) (Supplementary Fig. 6B). These findings indicate that Mtp1 cells are heterogeneous, consisting at least of Sertoli cells and Leydig cells. The newly established cell culture system using Mtp1as feeder cells recapitulates the whole process of medaka spermatogenesis: Spermatogonia proliferate and differentiate into spermatocytes, which in turn differentiate into spermatids and spermatozoa *in vitro* (Supplementary Fig. S7). The resulting spermatozoa allow the eggs to undergo normal embryonic development after insemination.

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

Spermatogonia-enriched 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. 2A and B).

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. To examine the dynamics of spermatogenic cells *in vitro*, we performed PKH26 label and chase experiments, in which PKH26-labeled spermatogonia were co-cultured with Mtp1 or Lif-overexpressing Mtp1 cells. The number of PKH26-labeled cells (spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids/spermatozoa) was examined on days 0, 3, 5 and 7 for each cell type (Fig. 3).

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. 3A). PKH26-labeled primary spermatocytes, the cell type of which was confirmed by anti-Sycp3 antibody, were observed on day 0 (Fig. 3B), because of their contamination in the spermatogonia-rich fraction. 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. 3B). 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. 3C), 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. 3C). 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. 3D).

mRNA and protein expression of Lif in the medaka testis

Since *in vitro* experiments demonstrated that Lif contributes to the regulation of spermatogonial cell proliferation, we decided to study the function of Lif *in vivo*. To this end, we first examined the expression of *lif* mRNA 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. 4A and B). Closer observations confirmed that spermatogonia and Sertoli cells surrounding them express *lif* mRNAs (Fig. 4C).

We then examined Lif protein expression by immunohistochemistry with a newly produced antibody raised against medaka Lif, the specificity of which was confirmed by using Lif-overexpressing Mtp1 cells (Supplementary Fig. S4B). Specific signals were found in spermatogonia and surrounding Sertoli cells (Fig. 5A and B). To identify the Lif-expressing cells more accurately, we 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. 5C), 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. 4).

Discussion

We investigated the function of Lif in medaka spermatogenesis. We found that addition of baculovirus-produced Lif to the culture medium or co-culture with Lif-overexpressing testicular somatic cells, Mtp1 cells, increases the number of spermatogonia (Figs. 2 and 3). Lif proteins (Fig. 5), as well as its mRNAs (Fig. 4), are expressed in spermatogonia and surrounding Sertoli cells, with higher expression levels in type A (undifferentiated) spermatogonia than in type B (differentiated) spermatogonia. These results strongly suggest that Lif plays a critical role for the paracrine and/or autocrine regulation of spermatogonial cell proliferation in the medaka.

Taking advantage of a cell culture system that recapitulates the process of medaka spermatogenesis from spermatogonia to functional spermatozoa *in vitro*, we 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. 3A). 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 we 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. 3B), and the number of their descendants (secondary spermatocytes and spermatids/spermatozoa) increased irrespective of Lif (Fig. 3C and D). These findings indicate that spermatogenesis continuously proceeds even in the presence 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. 3A). In contrast, the number of PKH26-labeled primary spermatocytes neither increased significantly during the cultures nor showed differences between the two conditions (Fig. 3B). 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. 3D). 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 & 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 we 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 our 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, we 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 (Curley *et al.*, 2018; De Miguel *et al.*, 1996; Dorval-Coiffec *et al.*, 2005; Jenab and Morris, 1998; Kanatsu-Shinohara *et al.*, 2007; Mirzapour *et al.*, 2012; Piquet-Pellorce *et al.*, 2000; Stewart *et al.*, 1992), 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 &

Collodi, 2013), Lif is likely to function not only in mammalian spermatogenesis but also in teleost spermatogenesis. However, the expression and localization of Lif in the testes have not been fully examined in any vertebrate. To our 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 (Figs. 4 and 5) 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 JAKs and STATs (Haan *et al.*, 2006; Loveland *et al.*, 2017) will provide deeper insights into the mechanisms underlying Lif-mediated spermatogonial cell proliferation in the medaka testes. In addition, we 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), and growth factors (activin, anti-Müllerian hormone (AMH)) and 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 in the medaka testes.

In conclusion, a newly established cell culture system has demonstrated that the addition of baculovirus-produced recombinant medaka Lif proteins to the culture medium or co-culture with Lif-overexpressing medaka testicular somatic cells promotes spermatogonial proliferation. *In situ* hybridization and immunocytochemical analyses of the medaka testes have shown that mRNAs and proteins of Lif are expressed restrictedly in spermatogonia and Sertoli cells that surround the spermatogonia, with higher levels in undifferentiated spermatogonia than in differentiated spermatogonia. On the basis of these findings, we propose that Lif plays an important role in the regulation of spermatogonial proliferation in the medaka.

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Conflicts of interest. The authors declare that they have no conflict of interest.

Ethical standards. All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (permission No. 08-0013 and 13-0099). All experiments were performed in accordance with relevant guidelines and regulations.

References

Abe T, Miyake N, Nishijima Y, Fujita R, Sahara K, Asano S and Bando H (2005) Enhancement of cauliflower mosaic virus 35S promoter in insect cells infected with baculovirus. *Virus Res* **112**, 38-41.

Chuma S and Nakatsuji N (2001) Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. *Dev Biol* **229**, 468-79.

Curley M, Milne L, Smith S, Atanassova N, Rebourcet D, Darbey A, Hadoke P W F, Wells S and Smith L B (2018) Leukemia inhibitory factor-receptor is dispensable for prenatal testis development but is required in Sertoli cells for normal spermatogenesis in mice. *Sci Rep* **8**, 11532.

De Felici M and Dolci S (1991) Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM4 feeder layers. *Dev Biol* **147**, 281-4.

De Miguel M P, De Boer-Brouwer M, Paniagua R, van den Hurk R, De Rooij D G and Van Dissel-Emiliani F M (1996) Leukemia inhibitory factor and ciliary neurotropic factor promote the survival of Sertoli cells and gonocytes in coculture system. *Endocrinology* **137**, 1885-93.

Dorval-Coiffec I, Delcros J G, Hakovirta H, Toppari J, Jegou B and Piquet-Pellorce C (2005) Identification of the leukemia inhibitory factor cell targets within the rat testis. *Biol Reprod* **72**, 602-11.

Farini D, Scaldaferri M L, Iona S, La Sala G and De Felici M (2005) Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells. *Dev Biol* **285**, 49-56.

Haan C, Kreis S, Margue C and Behrmann I (2006) Jaks and cytokine receptors—An intimate relationship. *Biochem Pharmacol* **72**, 1538-46.

Hedger M P and Meinhardt A (2003) Cytokines and the immune-testicular axis. *J Reprod*

Immunol **58**, 1-26.

Hirai T, Yamashita M, Yoshikuni M, Lou Y H and Nagahama Y (1992) Cyclin B in fish oocytes: its cDNA and amino acid sequences, appearance during maturation, and induction of $p34^{cdc2}$ activation. *Mol Reprod Dev* **33**, 131-40.

Honda A, Hirose M and Ogura A (2009) Basic FGF and Activin/Nodal but not LIF signaling sustain undifferentiated status of rabbit embryonic stem cells. *Exp Cell Res* **315**, 2033-42.

Hong Y, Liu T, Zhao H, Xu H, Wang W, Liu R, Chen T, Deng J and Gui J (2004) Establishment of a normal medakafish spermatogonial cell line capable of sperm production in vitro. *Proc Natl Acad Sci U S A* **101**, 8011-6.

Humphrey R K, Beattie G M, Lopez A D, Bucay N, King C C, Firpo M T, Rose-John S and Hayek A (2004) Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* **22**, 522-30.

Iwai T, Yoshii A, Yokota T, Sakai C, Hori H, Kanamori A and Yamashita M (2006) Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes*. *Exp Cell Res* **312**, 2528-37.

Iwasaki Y, Ohkawa K, Sadakata H, Kashiwadate A, Takayama-Watanabe E, Onitake K and Watanabe A (2009) Two states of active spermatogenesis switch between reproductive and non-reproductive seasons in the testes of the medaka, *Oryzias latipes*. *Dev Growth Differ* **51**, 521-32.

Jenab S and Morris PL (1998) Testicular leukemia inhibitory factor (LIF) and LIF receptor mediate phosphorylation of signal transducers and activators of transcription (STAT)-3 and STAT-1 and induce c-fos transcription and activator protein-1 activation in Rat Sertoli but not germ cells. *Endocrinology* **139**, 1883-90.

Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Yoshida S, Toyokuni S, Lee J,

Ogura A and Shinohara T (2007) Leukemia inhibitory factor enhances formation of germ cell colonies in neonatal mouse testis culture. *Biol Reprod* **76**, 55-62.

Kawasaki T, Saito K, Mitsui K, Ikawa M, Yamashita M, Taniguchi Y, Takeda S, Mitani K and Sakai N (2009) Introduction of a foreign gene into zebrafish and medaka cells using adenoviral vectors. *Zebrafish* **6**, 253-8.

Kawasaki T, Saito K, Sakai C, Shinya M and Sakai N (2012) Production of zebrafish offspring from cultured spermatogonial stem cells. *Genes Cells* **17**, 316-25.

Kawasaki T, Siegfried K R and Sakai N (2016) Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture. *Development* **143**, 566-74.

Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi K and Tanaka M (2007) Germ cells are essential for sexual dimorphism in the medaka gonad. *Proc Natl Acad Sci U S A* **104**, 16958-63.

Leisy D J, Lewis T D, Leong J A and Rohrmann G F (2003) Transduction of cultured fish cells with recombinant baculoviruses. *J Gen Virol* **84**, 1173-8.

Loveland K L, Klein B, Pueschl D, Indumathy S, Bergmann M, Loveland B E, Hedger M P and Schuppe H C (2017) Cytokines in male fertility and reproductive pathologies: Immunoregulation and beyond. *Front Endocrinol (Lausanne)* **8**, 307.

Mirzapour T, Movahedin M, Tengku Ibrahim T A, Koruji M, Haron A W, Nowroozi M R and Rafieian S H (2012) Effects of basic fibroblast growth factor and leukaemia inhibitory factor on proliferation and short-term culture of human spermatogonial stem cells. *Andrologia* **44 Suppl 1**, 41-55.

Miura C, Shimizu Y, Uehara M, Ozaki Y, Young G and Miura T (2011) Gh is produced by the testis of Japanese eel and stimulates proliferation of spermatogonia. *Reproduction* **142**, 869-77.

Miura T, Yamauchi K, Takahashi H and Nagahama Y (1991) Hormonal induction of all

stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *Proc Natl Acad Sci USA* **88**, 5774-8.

Mizushima S and Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* **18**, 5322.

Nayak S, Ferosekhan S, Sahoo S K, Sundaray J K, Jayasankar P and Barman H K (2016) Production of fertile sperm from *in vitro* propagating enriched spermatogonial stem cells of farmed catfish, *Clarias batrachus*. *Zygote* **24**, 814-24.

Oatley J M and Brinster R L (2008) Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol* **24**, 263-86.

Ohta T, Miyake H, Miura C, Kamei H, Aida K and Miura T (2007) Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. *Biol Reprod* **77**, 970-7.

Ota R, Kotani T and Yamashita M (2011) Biochemical characterization of Pumilio1 and Pumilio2 in *Xenopus* oocytes. *J Biol Chem* **286**, 2853-63.

Piquet-Pellorce C, Dorval-Coiffec I, Pham M D and Jegou B (2000) Leukemia inhibitory factor expression and regulation within the testis. *Endocrinology* **141**, 1136-41.

Saiki A, Tamura M, Matsumoto M, Katowgi J, Watanabe A and Onitake K (1997) Establishment of *in vitro* spermatogenesis from spermatocytes in the medaka, *Oryzias latipes*. *Dev Growth Differ* **39**, 337-44.

Sakai N (2002) Transmeiotic differentiation of zebrafish germ cells into functional sperm in culture. *Development* **129**, 3359-65.

Sasaki T, Watanabe A, Takayama-Watanabe E, Suzuki M, Abe H and Onitake K (2005) Ordered progress of spermiogenesis to the fertilizable sperm of the medaka fish, *Oryzias latipes*, in cell culture. *Dev Growth Differ* **47**, 87-97.

Schulz R W, de Franca L R, Lareyre J J, Le Gac F, Chiarini-Garcia H, Nobrega R H and

Miura T (2010) Spermatogenesis in fish. *Gen Comp Endocrinol* **165**, 390-411.

Shibata N and Hamaguchi S (1988) Evidence for the sexual bipotentiality of spermatogonia in the fish, *Oryzias latipes*. *J Exp Zool* **245**, 71-7.

Shima A and Mitani H (2004) Medaka as a research organism: past, present and future. *Mech Dev* **121**, 599-604.

Shimizu Y, Mita K, Tamura M, Onitake K and Yamashita M (2000) Requirement of protamine for maintaining nuclear condensation of medaka (*Oryzias latipes*) spermatozoa shed into water but not for promoting nuclear condensation during spermatogenesis. *Int J Dev Biol* **44**, 195-9.

Shimizu Y, Shibata N and Yamashita M (1997) Spermiogenesis without preceding meiosis in the hybrid medaka between *Oryzias latipes* and *O. curvinotus*. *J Exp Zool* **279**, 102-12.

Song M and Gutzeit H O (2003) Primary culture of medaka (*Oryzias latipes*) testis: a test system for the analysis of cell proliferation and differentiation. *Cell Tissue Res* **313**, 107-15.

Stewart C L, Kaspar P, Brunet L J, Bhatt H, Gadi I, Köntgen F and Abbondanzo S J (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-9.

Takahashi K, Kotani T, Katsu Y and Yamashita M (2014) Possible involvement of insulin-like growth factor 2 mRNA-binding protein 3 in zebrafish oocyte maturation as a novel *cyclin B1* mRNA-binding protein that represses the translation in immature oocytes. *Biochem Biophys Res Commun* **448**, 22-7.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas J L, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme J C and Couble P (2000) Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nat Biotechnol* **18**, 81-4.

Tanaka M, Kinoshita M, Kobayashi D and Nagahama Y (2001) Establishment of medaka

(*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: A useful model to monitor germ cells in a live vertebrate. *Proc Natl Acad Sci USA* **98**, 2544-9.

Tang H, Chen Y, Wang L, Yin Y, Li G, Guo Y, Liu Y, Lin H, Cheng C H K and Liu X (2018) Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish lacking androgen receptor. *Biol Reprod* **98**, 227-38.

Taniguchi Y, Takeda S, Furutani-Seiki M, Kamei Y, Todo T, Sasado T, Deguchi T, Kondoh H, Mudde J, Yamazoe M, Hidaka M, Mitani H, Toyoda A, Sakaki Y, Plasterk R H and Cuppen E (2006) Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol* **7**, R116.

Tokalov S V and Gutzeit H O (2005) Spermatogenesis in testis primary cell cultures of the tilapia (*Oreochromis niloticus*). *Dev Dyn* **233**, 1238-47.

Weinbauer G F and Wessels J (1999) 'Paracrine' control of spermatogenesis. *Andrologia* **31**, 249-62.

Wittbrodt J, Shima A and Schartl M (2002) Medaka — a model organism from the far East. *Nat Rev Genet* **3**, 53-64.

Wong T T and Collodi P (2013) Dorsomorphin promotes survival and germline competence of zebrafish spermatogonial stem cells in culture. *PLoS One* **8**, e71332.

Yamashita M, Yoshikuni M, Hirai T, Fukada S and Nagahama Y (1991) A monoclonal antibody against the PSTAIR sequence of $p34^{cdc2}$, catalytic subunit of maturation-promoting factor and key regulator of the cell cycle. *Dev Growth Differ* **33**, 617-24.

Yan Y, Du J, Chen T S, Yi M S, Li M Y, Wang S, Li C M and Hong Y H (2009) Establishment of medakafish as a model for stem cell-based gene therapy: Efficient gene delivery and potential chromosomal integration by baculoviral vectors. *Exp Cell Res* **315**, 2322-31.

Yokoo M, Fujita R, Nakajima Y, Yoshimizu M, Kasai H, Asano S and Bando H (2013) Mos1 transposon-based transformation of fish cell lines using baculoviral vectors. *Biochem Biophys Res Commun* **439**, 18-22.

Figure legends

Figure 1. 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) 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). (C) 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 (H1), indicating that spermatogonia and spermatocytes account for 90% of cells in this fraction.

Figure 2. Effects of Lif on spermatogonia under culture conditions. (A) Culture of spermatogonia-rich fractions in the presence of baculovirus-produced GST as a control or baculovirus-produced medaka Lif proteins, Lif-F1 and Lif-F2, for 4 days. Spermatogonia identified by their morphology (see Supplementary Fig. S5) 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 $15x10^4$. 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).

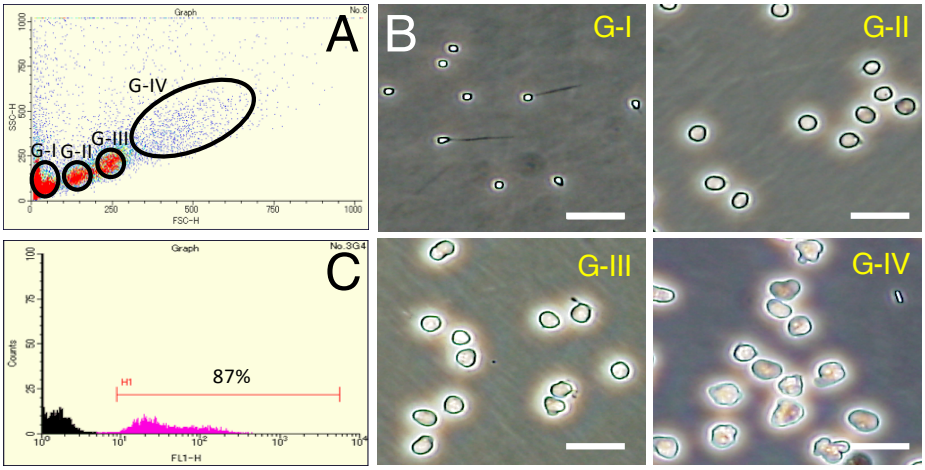
Figure 3. Dynamics of PKH26-labeled cells co-cultured with Mtp1 or Lif-overexpressing Mtp1 (Lif-Mtp1) cells. Cells were harvested on days 0, 3, 5 and 7 and PKH26-labeled cells were counted after identification of cell types by their morphology (A, spermatogonia; B, primary spermatocytes; C, secondary spermatocytes; D, spermatids/spermatozoa; see also Supplementary Figs. S5 and S7). The data were obtained by three independent experiments. [a-e]Values with different letters are significantly different (mean ± SD; n=3; $P<0.01$, Student's *t*-test).

Figure 4. *lif* mRNA expression 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 the TSA Plus DNP system using anti-DIG HRP antibody and anti-DNP-Alexa Fluor 488 antibody (Lif, green). 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 positive area indicated in B. A DNA image (DNA) and a DNA and *lif* mRNA 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 magenta arrows, respectively. The expression of *lif* mRNAs was detected in spermatogonia and Sertoli cells surrounding them but not in primary spermatocytes existing outside the marked area. Scale bar, 10 μm.

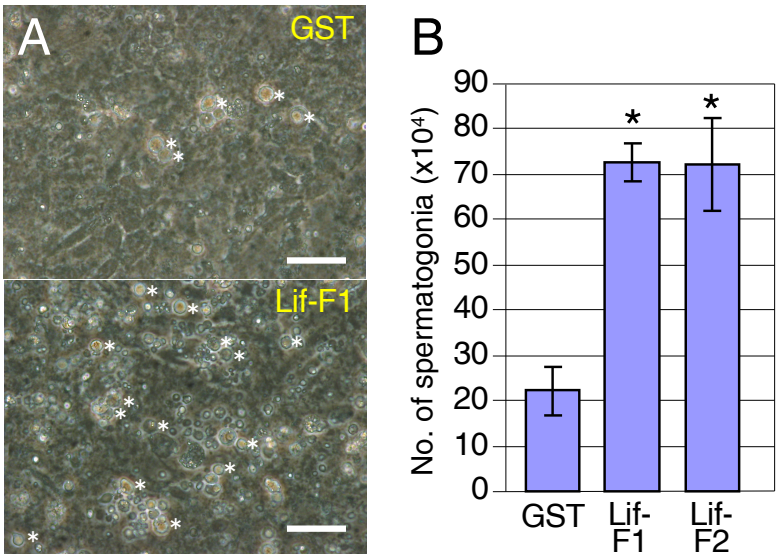
Figure 5. Lif protein expression in medaka testes. Histological sections of the *olvas*-GFP medaka testis triple-stained with the DNA dye Hoechst 33258 (DNA, blue), anti-Lif antibody (Lif, red) and anti-GFP antibody (GFP, green). Scale bar, 10 μm. (A) A control experiment

using an antigen-absorbed anti-Lif antibody. Punctuated signals scattered all over the testis are non-specific signals due to Alexa-conjugated anti-guinea pig IgG secondary antibody. (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 magenta arrow shows the nucleus of a Sertoli cell. The expression of Lif proteins was detected in spermatogonia and surrounding Sertoli cells. (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.

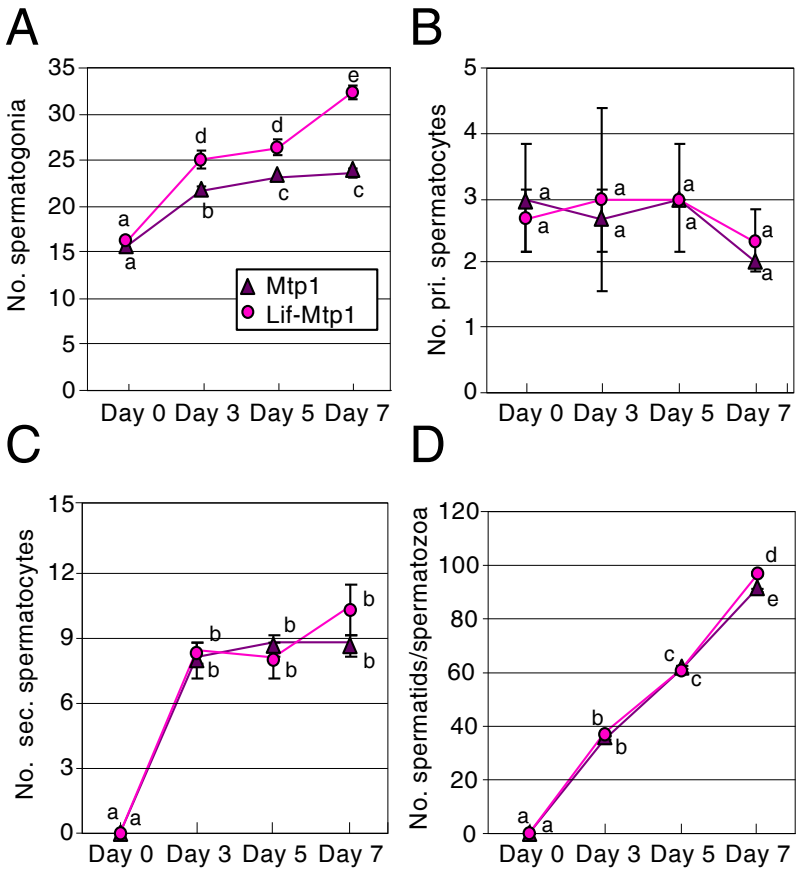
Satoh et al., Figure 1



Satoh et al., Figure 2



Satoh et al., Figure 3



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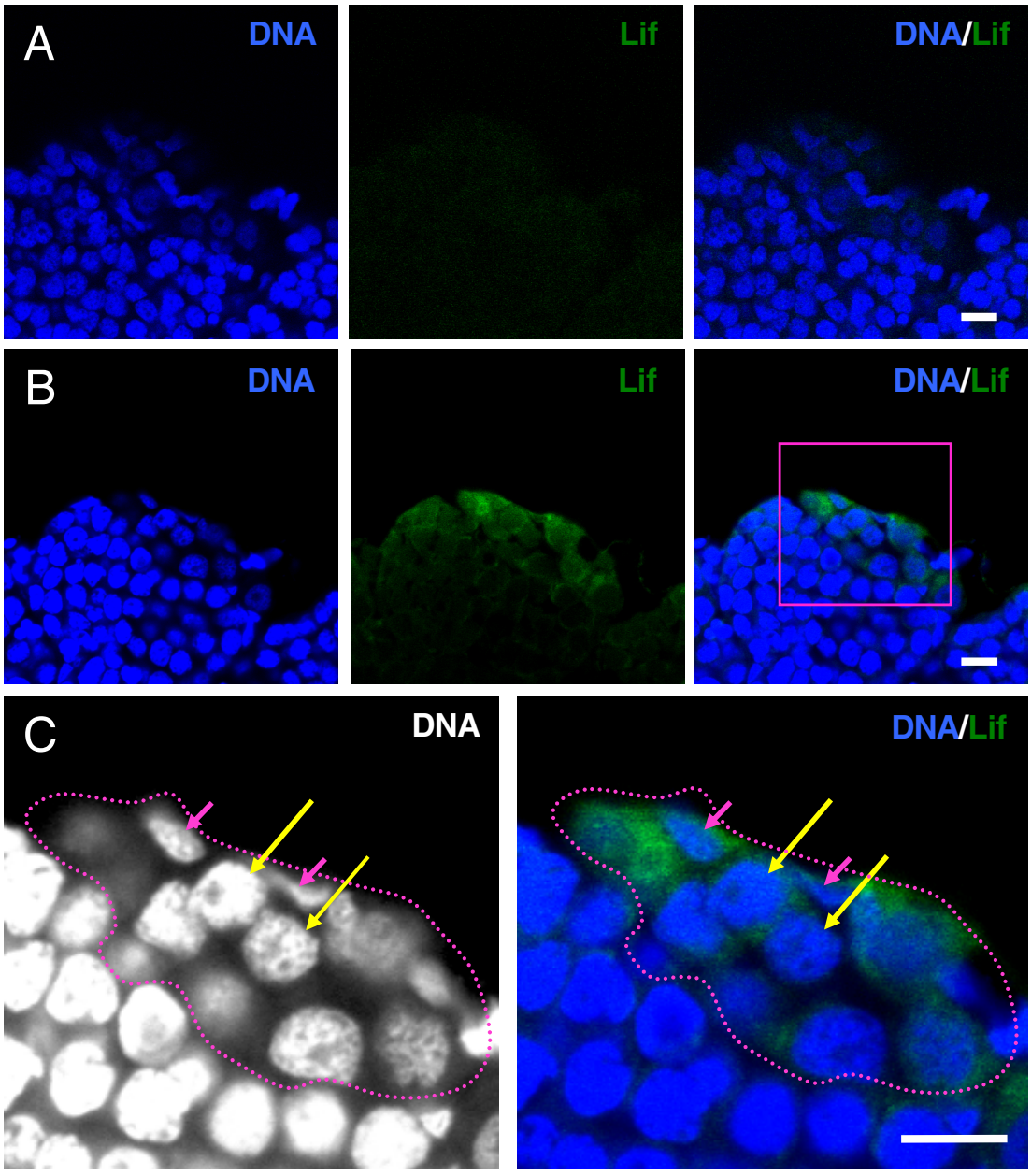

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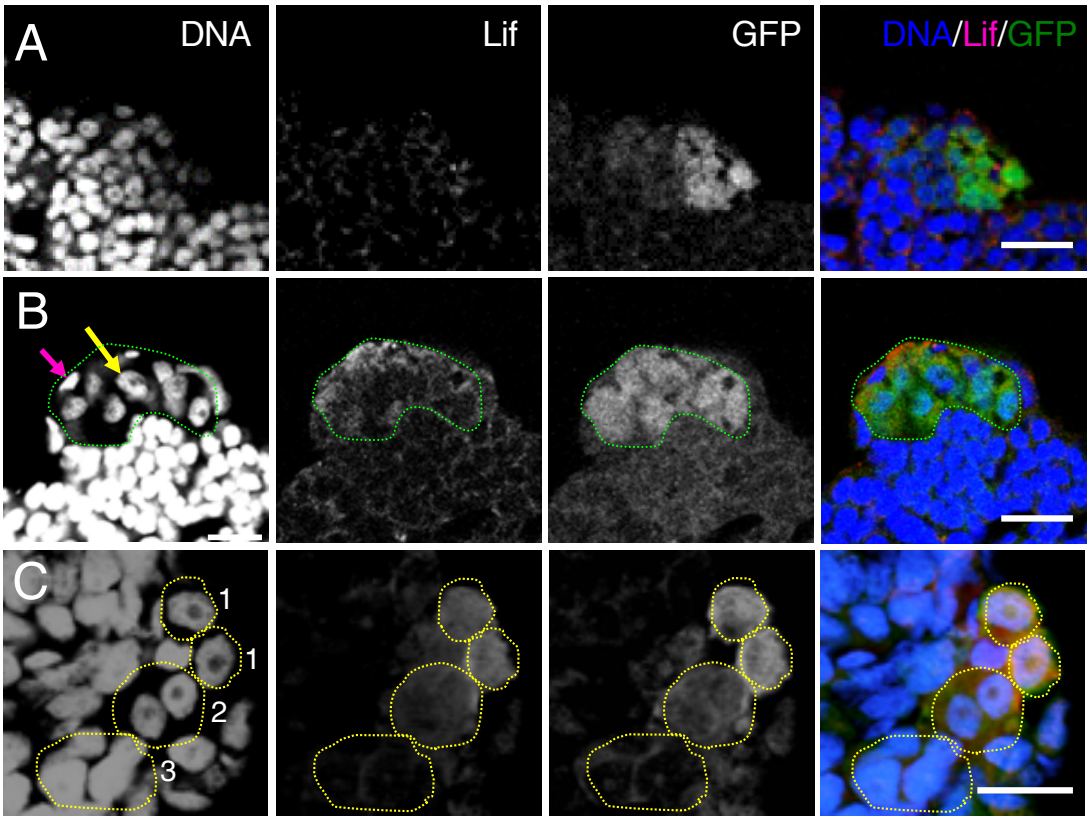
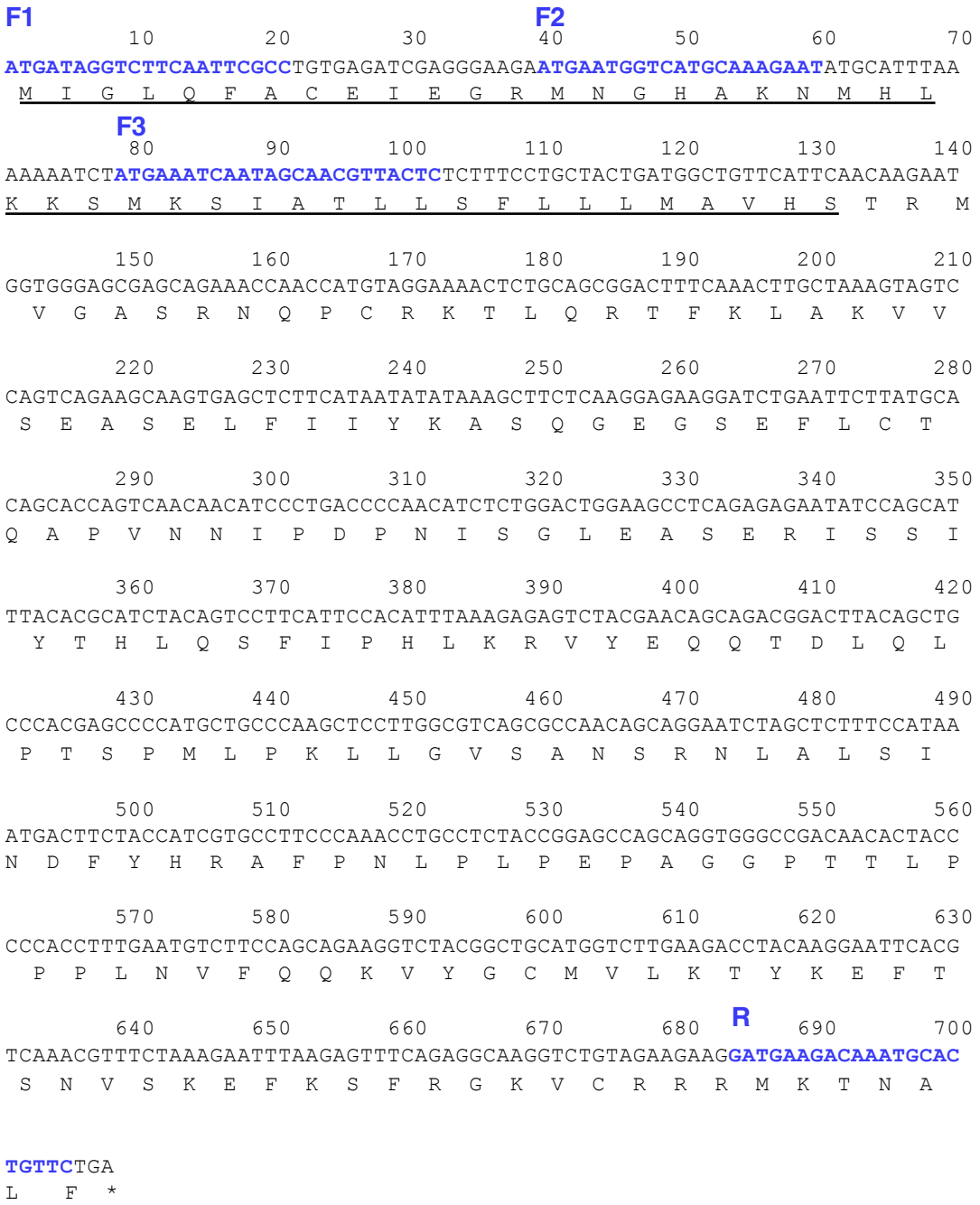
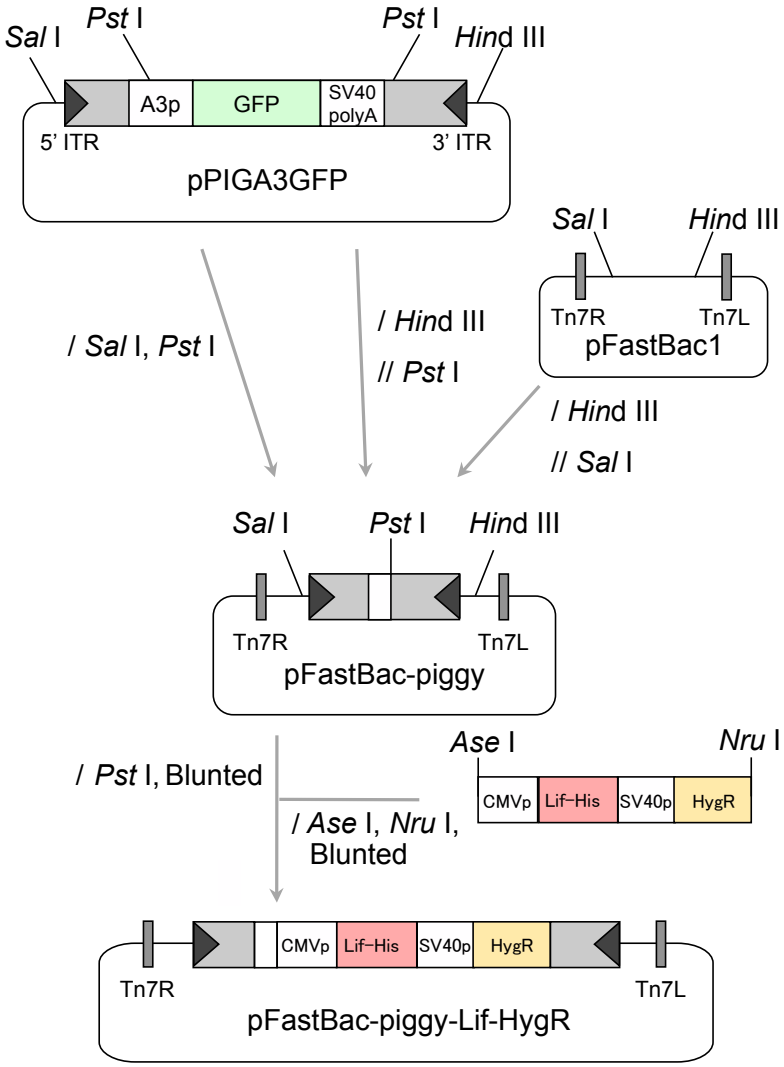


Supplementary Table S1. List of primers for DNA construction.

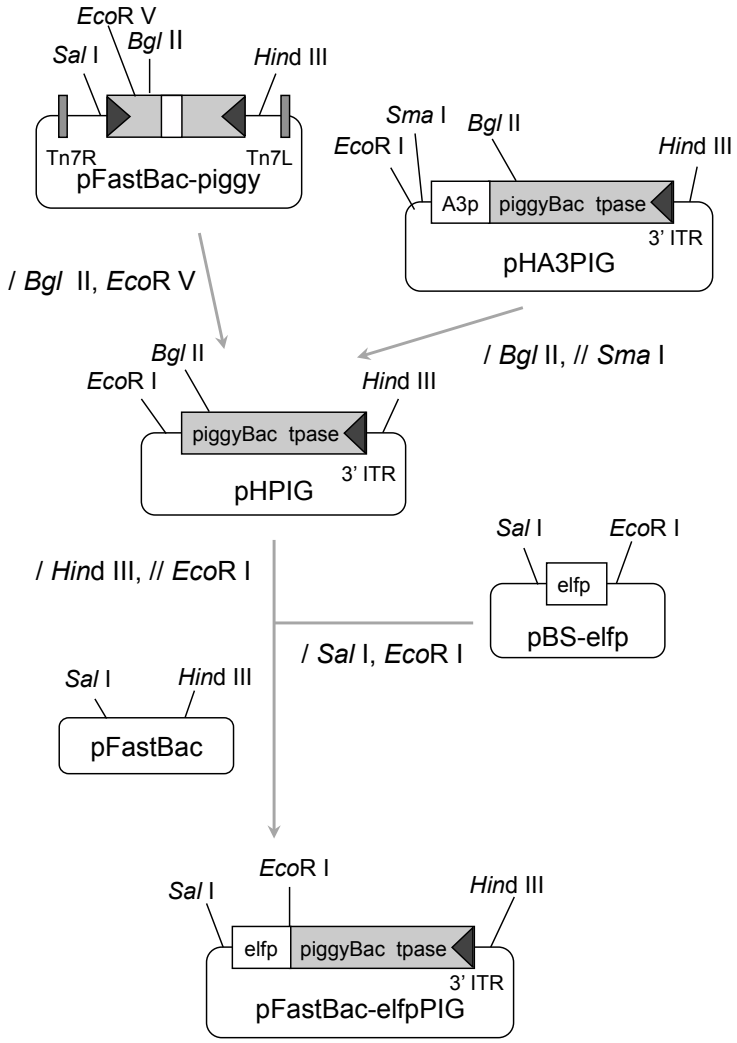
Name	Sequence
Bam HI-His	GGATCCCGGATCAAACTCAATGA
Bam HI-Lif-F1	GGATCCACCATGATAGGTCTTCAATTCGCC
Bam HI-Lif-F2	GGATCCACCATGAATGGTCATGCAAAGAAT
Nhe I-Lif-F2	GCTAGCGCCACCATGAATGGTCA
Not I-His	GCGGCCG*C* CGGATCAAACTCAATGATGATG
Topo-Lif-F1	CACCATGATAGGTCTTCAATTCGCCT
Topo-Lif-F2	CACCATGAATGGTCATGCAAAGAATATG
Topo-Lif-F3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



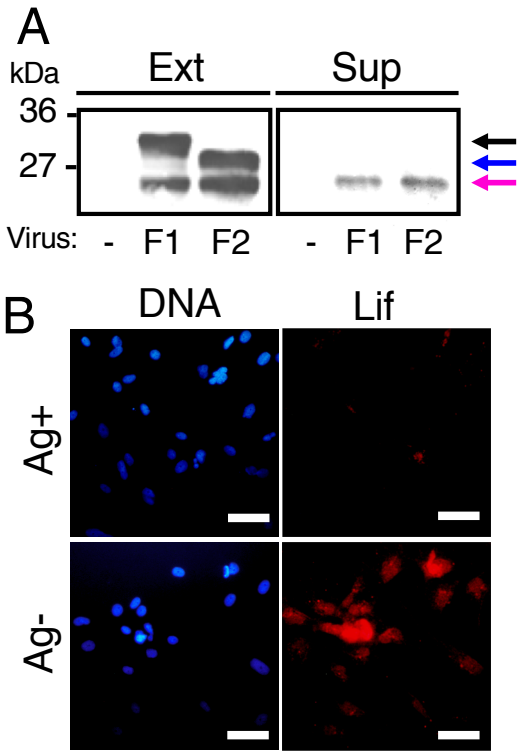
Supplementary Figure S1. Nucleotide and amino acid sequences of the coding region of medaka Lif cDNA. The N-terminal sequence indicated by an underline is a signal peptide that will be removed when Lif protein is secreted as a mature form. F1, F2, F3 and R show the positions of primers used for various versions of Lif cDNA (see **Materials and methods**).



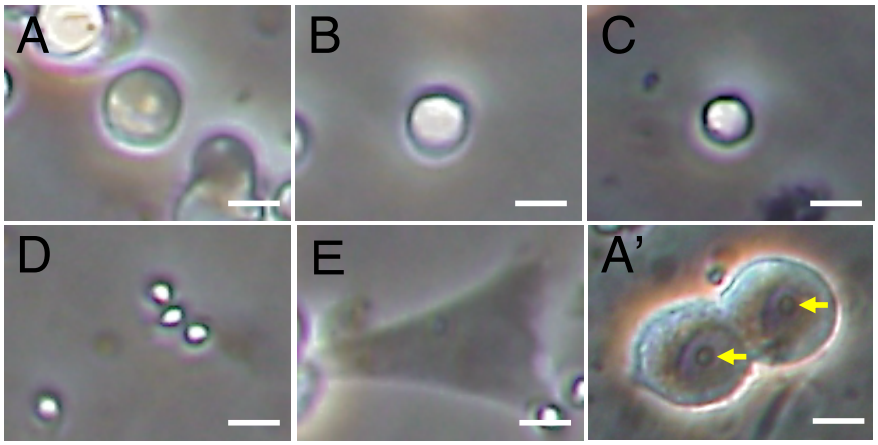
Supplementary Figure S2. Construction of the donor plasmid to produce Lif-overexpressing Mtp1 cells. The 5' and 3' *piggyBac* inverted terminal repeats (5' ITR, 3' ITR) were isolated from pPIGA3GFP (Tamura *et al*., 2000) by digestion with *Sal*I/*Pst*I and *Hin*d III /*Pst*I, respectively, and the resulting fragments were ligated into *Sal* I/*Hin*d III-cut pFastBac1 (Life Technologies; Thermo Fisher Scientific, Tokyo, Japan) that lacks a polyhedrin promoter (pFastBac-dphp) (Abe *et al*., 2005) 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 (Supplementary Table S1). The resulting cDNA was digested with *Nhe*I/*Bam*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.



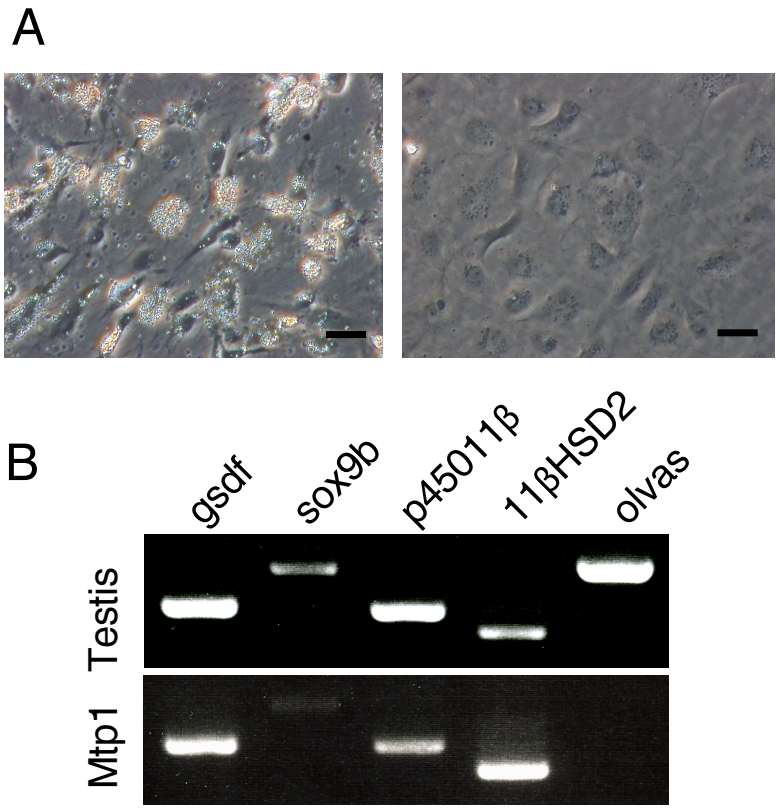
Supplementary Figure S3. Construction of the helper plasmid to produce Lif-overexpressing Mtp1 cells. The helper plasmid carrying piggyBac transposase (tpase), the expression of which is under the control of human elongation factor 1a promoter (elfp), was produced as follows. A 5' region of piggyBac transposase was isolated from pFastBac-piggy by *Eco*RV/*Bgl*II and ligated into the *Sma*I/*Bgl*II site of pHA3PIG (Tamura *et al*., 2000) to produce pHPIG. A DNA sequence including a full-length transposase and 3' ITR was isolated from pHPIG by digestion with *Eco*RI/*Hin*dIII. pEF-BOS (Mizushima & Nagata, 1990) was digested with *Hin*dIII/*Eco*RI and the resulting elfp-containing DNA was ligated into *Hin*dIII/*Eco*RI-treated pBluescript SK (-) (Stratagene; Agilent Technology, Santa Clara, CA) 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/*Hin*d III site of pFastBac-dphp (Abe *et al*., 2005) to produce pFastBac-elfpPIG, which contains elfp-driven piggyBac transposase. pFastBac-elfpPIG and pFastBac-piggy-Lif-HygR (Supplementary Fig. S2) 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.



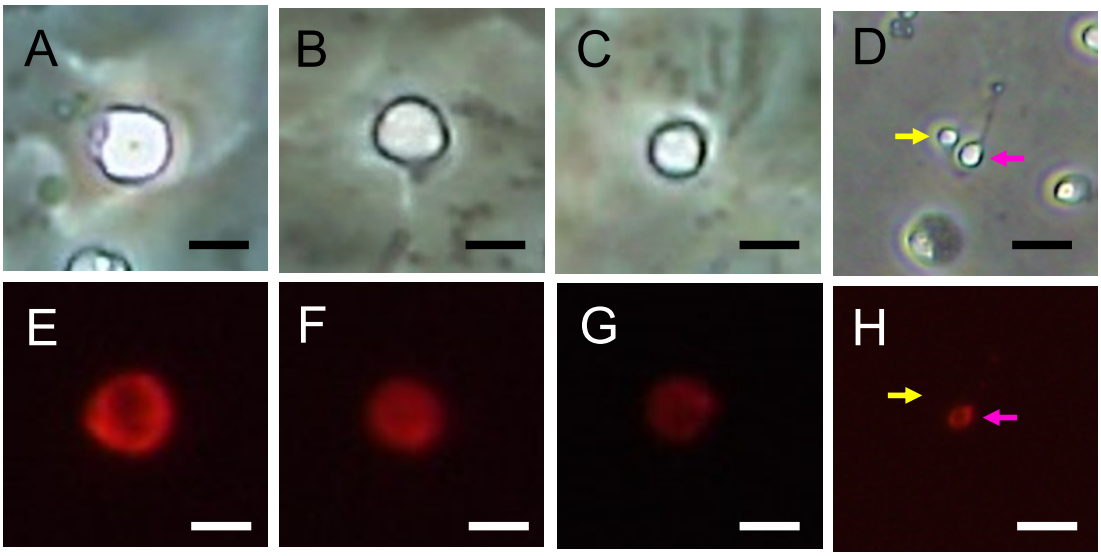
Supplementary Figure S4. Characterization of baculovirus-produced medaka Lif proteins and anti-Lif antibody. (A) Detection of baculovirus-produced medaka Lif proteins (Lif-F1-His, Lif-F2-His). Protein extracts (Ext) from Sf9 cells not infected with (-) or infected with viruses producing Lif-F1-His (F1) 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. Anti-Lif-stained samples (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.



Supplementary Figure S5. Morphology of medaka testicular cells. Living cells isolated from the testis were examined under a phase-contrast microscope. A, spermatogonium; B, primary spermatocyte; C, secondary spermatocyte; D, spermatids/spermatozoa; E, somatic cell. A', Flattened spermatogonia immediately after mitosis. Note a prominent nucleolus (arrows) in the nucleus, one of their diagnostic characteristics. Scale bar, 10 μm.



Supplementary Figure S6. Characterization of Mtp1 cells. (A) Phagocytosis activity of Mtp1 cells. After 24 hours of culture in the presence (left figure) or absence (right figure) 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. (B) Expression of marker genes for Sertoli cells (*gsdf, sox9b*), Leydig cells (*p45011β, 11βHSD2*) and germ cells (*olvas*) in Mtp1 cells. Total RNA samples from the testis and Mtp1 cells were analyzed by RT-PCR.



Supplementary Figure S7. *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.