```
F1                                      F2
        10        20        30        40        50        60        70
ATGATAGGTCTTCAATTCGCCTGTGAGATCGAGGGAAGAATGAATGGTCATGCAAAGAATATGCATTTAA
 M  I  G  L  Q  F  A  C  E  I  E  G  R  M  N  G  H  A  K  N  M  H  L

        F3
        80        90       100       110       120       130       140
AAAAATCTATGAAATCAATAGCAACGTTACTCTCTTTCCTGCTACTGATGGCTGTTCATTCAACAAGAAT
K  K  S  M  K  S  I  A  T  L  L  S  F  L  L  L  M  A  V  H  S  T  R  M

       150       160       170       180       190       200       210
GGTGGGAGCGAGCAGAAACCAACCATGTAGGAAAACTCTGCAGCGGACTTTCAAACTTGCTAAAGTAGTC
  V  G  A  S  R  N  Q  P  C  R  K  T  L  Q  R  T  F  K  L  A  K  V  V

       220       230       240       250       260       270       280
CAGTCAGAAGCAAGTGAGCTCTTCATAATATATAAAGCTTCTCAAGGAGAAGGATCTGAATTCTTATGCA
 S  E  A  S  E  L  F  I  I  Y  K  A  S  Q  G  E  G  S  E  F  L  C  T

       290       300       310       320       330       340       350
CAGCACCAGTCAACAACATCCCTGACCCCAACATCTCTGGACTGGAAGCCTCAGAGAGAATATCCAGCAT
Q  A  P  V  N  N  I  P  D  P  N  I  S  G  L  E  A  S  E  R  I  S  S  I

       360       370       380       390       400       410       420
TTACACGCATCTACAGTCCTTCATTCCACATTTAAAGAGAGTCTACGAACAGCAGACGGACTTACAGCTG
  Y  T  H  L  Q  S  F  I  P  H  L  K  R  V  Y  E  Q  Q  T  D  L  Q  L

       430       440       450       460       470       480       490
CCCACGAGCCCCATGCTGCCCAAGCTCCTTGGCGTCAGCGCCAACAGCAGGAATCTAGCTCTTTCCATAA
 P  T  S  P  M  L  P  K  L  L  G  V  S  A  N  S  R  N  L  A  L  S  I

       500       510       520       530       540       550       560
ATGACTTCTACCATCGTGCCTTCCCAAACCTGCCTCTACCGGAGCCAGCAGGTGGGCCGACAACACTACC
N  D  F  Y  H  R  A  F  P  N  L  P  L  P  E  P  A  G  G  P  T  T  L  P

       570       580       590       600       610       620       630
CCCACCTTTGAATGTCTTCCAGCAGAAGGTCTACGGCTGCATGGTCTTGAAGACCTACAAGGAATTCACG
  P  P  L  N  V  F  Q  Q  K  V  Y  G  C  M  V  L  K  T  Y  K  E  F  T

                                                  R
       640       650       660       670       680       690       700
TCAAACGTTTCTAAAGAATTTAAGAGTTTCAGAGGCAAGGTCTGTAGAAGAAGGATGAAGACAAATGCAC
 S  N  V  S  K  E  F  K  S  F  R  G  K  V  C  R  R  R  M  K  T  N  A

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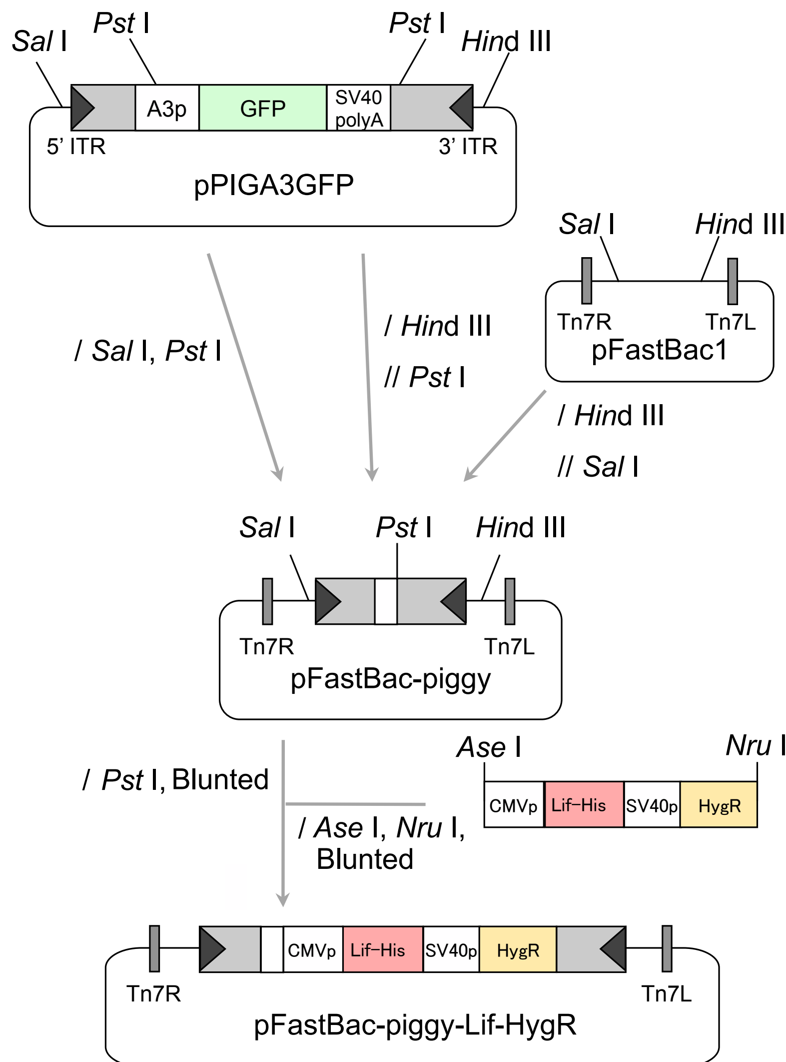
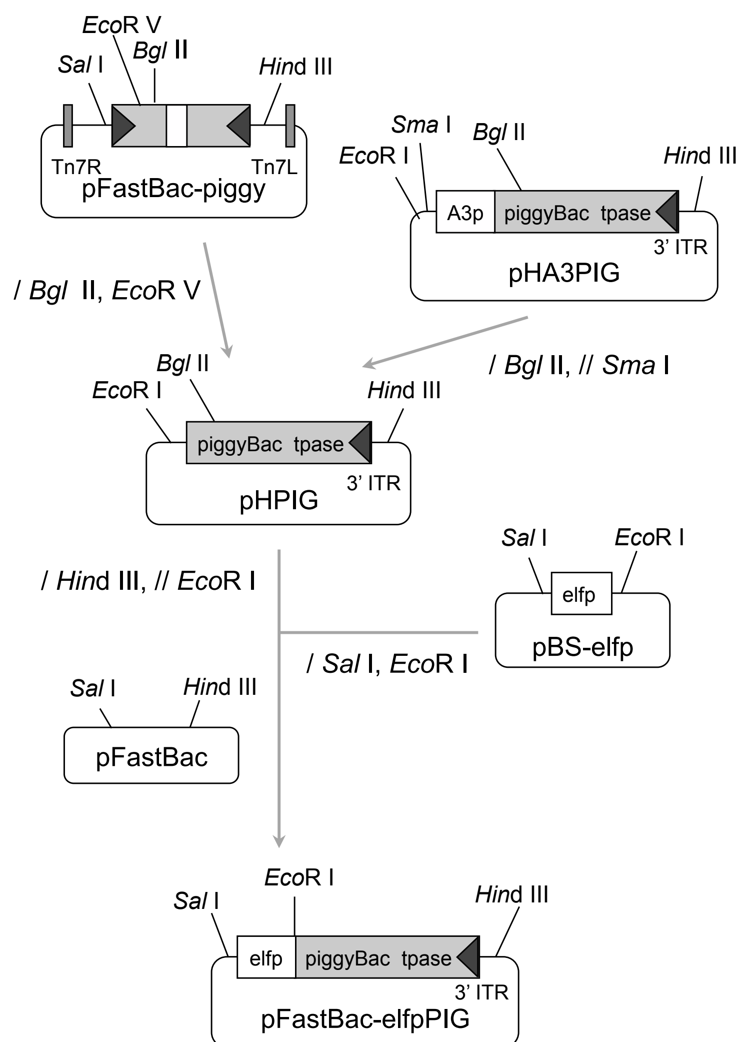
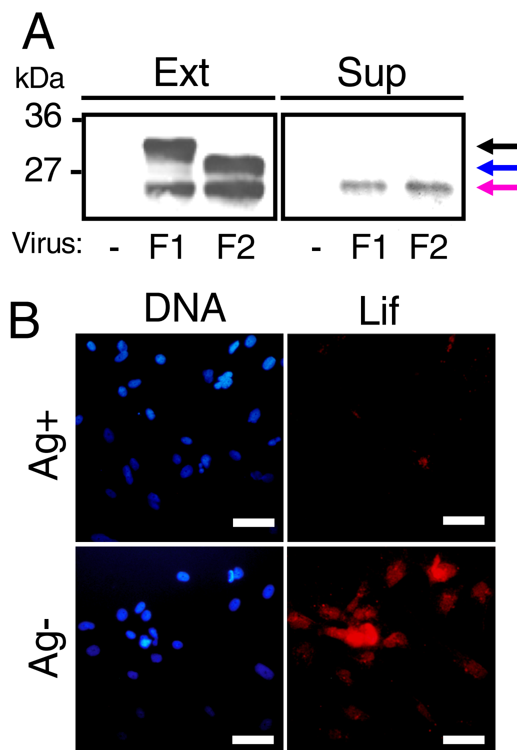
Supplementary Figure S1. Nucleotide and amino acid sequences of the coding region of medaka Lif cDNA. The N-terminal sequence indicated by an underline is a signal peptide that will be removed when Lif protein is secreted as a mature form. F1, F2, F3 and R show the positions of primers used for various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



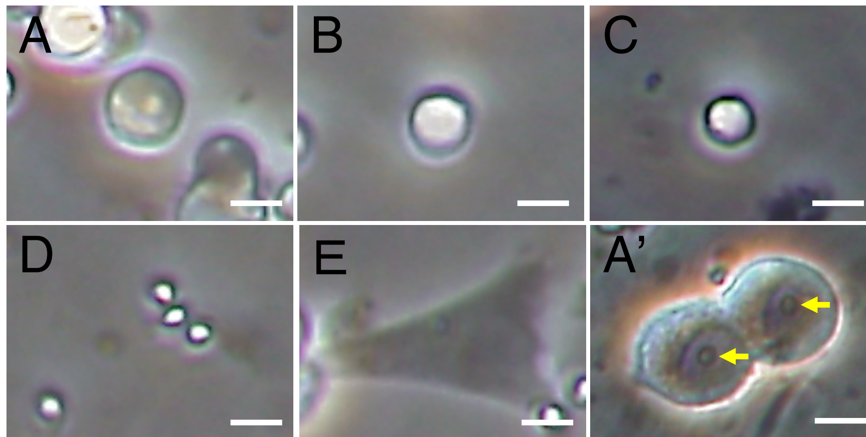
Supplementary Figure S2. Construction of the donor plasmid to produce Lif-overexpressing Mtp1 cells. The 5' and 3' *piggyBac* inverted terminal repeats (5' ITR, 3' ITR) were isolated from pPIGA3GFP (Tamura *et al*., 2000) by digestion with *Sal*I/*Pst*I and *Hin*d III /*Pst*I, respectively, and the resulting 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 *Ps*tI-digested, blunted pFastBac-piggy to produce pFastBac-piggy-Lif-HygR that contains CMV-driven Lif and SV40-driven HygR.



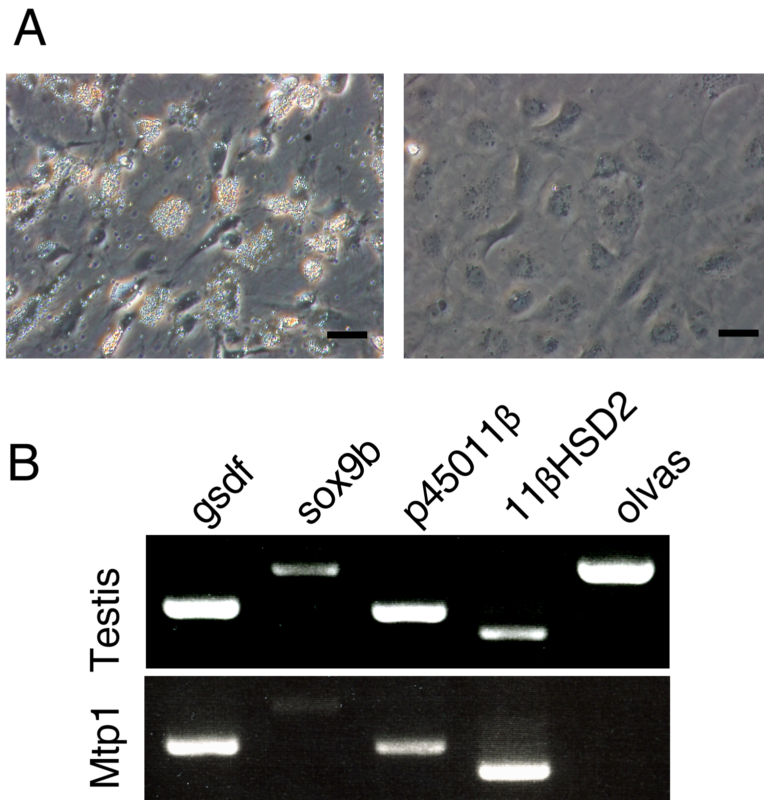
Supplementary Figure S3. Construction of the helper plasmid to produce Lif-overexpressing Mtp1 cells. The helper plasmid carrying piggyBac transposase (tpase), the expression of which is under the control of human elongation factor 1a promoter (elfp), was produced as follows. A 5' region of piggyBac transposase was isolated from pFastBac-piggy by *Eco*RV/*Bgl*II and ligated into the *SmaI/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.



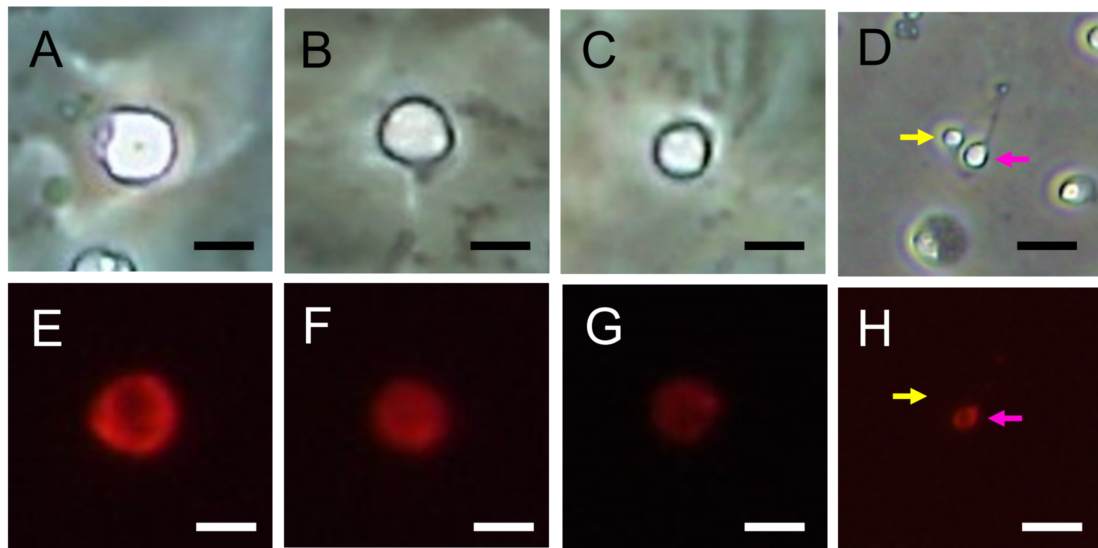
Supplementary Figure S4. Characterization of baculovirus-produced medaka Lif proteins and anti-Lif antibody. (A) Detection of baculovirus-produced medaka Lif proteins (Lif-F1-His, Lif-F2-His). Protein extracts (Ext) from Sf9 cells not infected with (-) or infected with viruses producing Lif-F1-His (F1)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