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Müllerian Inhibiting Substance Is Required for Germ Cell Proliferation during Early Gonadal Differentiation in Medaka (*Oryzias latipes*)

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Müllerian inhibiting substance (MIS) is a glycoprotein belonging to the TGF-β superfamily. In mammals, MIS is responsible for the regression of Müllerian ducts in the male fetus. However, the role of MIS in gonadal sex differentiation of teleost fish, which have no Müllerian ducts, has yet to be clarified. In the present study, we examined the expression pattern of *mis* and *mis* type 2 receptor (*misr2*) mRNAs and the function of MIS signaling in early gonadal differentiation in medaka (teleost, *Oryzias latipes*). *In situ* hybridization showed that both *mis* and *misr2* mRNAs were expressed in the somatic cells surrounding the germ cells of both sexes during early sex differentiation. Loss-of-function of either MIS or MIS type II receptor (MISRII) in medaka resulted in suppression of germ cell proliferation during sex differentiation. These results were supported by cell proliferation assay using 5-bromo-2′-deoxyuridine labeling analysis. Treatment of tissue fragments containing germ cells with recombinant eel MIS significantly induced germ cell proliferation in both sexes compared with the untreated control. On the other hand, culture of tissue fragments from the MIS- or MISRII-defective embryos inhibited proliferation of germ cells in both sexes. Moreover, treatment with recombinant eel MIS in the MIS-defective embryos dose-dependently increased germ cell number in both sexes, whereas in the MISRII-defective embryos, it did not permit proliferation of germ cells. These results suggest that in medaka, MIS indirectly stimulates germ cell proliferation through MISRII, expressed in the somatic cells immediately after they reach the gonadal primordium. (*Endocrinology* 149: 1813–1819, 2008)

MÜLLERIAN INHIBITING SUBSTANCE (MIS), also known as anti-Müllerian hormone, is a glycoprotein belonging to the TGF-β superfamily that is involved in the regulation of growth and differentiation (1). In mammals, MIS induces the regression of Müllerian ducts in the male fetus (2). In mice, MIS is expressed in males during sex differentiation and is first detected in Sertoli cells of the testis shortly after the initial expression of the testis-determining gene *Sry* (3), persisting after regression of the Müllerian ducts (4). On the other hand, ovarian *Mis* mRNA expression is first detected in granulosa cells at d 6 after birth and remains low throughout the reproductive life of the mouse. Thus, MIS is involved in the regression of Müllerian ducts, but also in other physiological functions during the reproduction period.

TGF-β superfamily members signal through a heteromeric receptor complex of type I and II transmembrane serine/threonine kinase receptors (5). Type II receptors probably bind to ligands independently of type I receptors, but the transduction requires the presence of both receptors. The cDNA of *MIS* type II receptor (*MISRII*) has been isolated from mammalian gonads, and its mRNA is detected in the mesenchymal cells adjacent to the Müllerian duct during embryogenesis, and in Sertoli and granulosa cells (6–8). In mice, MISRII-deficient males develop as internal pseudohermaphrodites, possessing a complete male reproductive tract, a uterus, and oviducts (9), similar to the phenotype of MIS mutant males (10). These genetic analyses suggest that the MIS signaling pathway is simple, in contrast to the complex signaling pathways of other TGF-β superfamilies (11). On the other hand, unique type I receptor for MIS has not yet been identified, although the candidates are three activin-like kinases (ALKs) (ALK2, ALK3, and ALK6) known as bone morphogenetic protein type I receptors (12).

In teleost fish, which do not form Müllerian ducts (13), MIS homologs have been isolated in Japanese eel (14), Japanese flounder (15), zebrafish (16), and medaka (17). Eel spermatogenesis-related substances 21 (eSRS21), which is likely to be a MIS homolog in eel, is expressed in Sertoli cells of immature testes and prevents initiation of spermatogenesis (14). In Japanese flounder, *mis* mRNA is initially expressed in the undifferentiated gonads of both sexes; however, from the period of sex differentiation to the adult stage, the mRNA is expressed only in Sertoli cells of gonads in phenotypical

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Abbreviations: ALK, Activin-like kinase; BrdU, 5-bromo-2′-deoxyuridine; DIG, digoxigenin; dpf, d post fertilization; E, embryonic d; EGFP, enhanced green fluorescent protein; eSRS21, eel spermatogenesis-related substances 21; MIS, Müllerian inhibiting substance; MISRII, MIS type II receptor; *Mmis-AS*, mismatched *mis-AS*; ORF, open reading frame; PGC, primordial germ cell; RACE, rapid amplification of cDNA ends; r-eSRS21, recombinant eel spermatogenesis-related substances 21.

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males, but not in the gonads of the phenotypical females (15). On the other hand, in medaka, *mis* mRNA shows no sexually dimorphic expression pattern during the gonadal development, and is expressed in Sertoli cells in the adult testes and in granulosa cells surrounding the oocytes in the adult ovaries, similar to that of *mis* type 2 receptor (*misr2*) (17). These results suggest that MIS signaling plays important roles in gonadal development in teleost fish. However, the role of MIS in early gonadal differentiation of teleost fish remains unclear.

Medaka (*Oryzias latipes*) is a small laboratory fish with several advantages such as a short generation time, small genome size, and the availability of several useful strains (18). A detailed linkage map has been established (19), transgenic techniques are available (20), and mutations affecting embryonic development have been screened (21). Recently, *DMY/dmrt1bY*, the medaka sex-determining gene that is located on the Y-chromosome, has been identified (22–24). Thus, medaka is an excellent vertebrate model for the molecular genetic analysis of various biological phenomena, including embryonic development and sex differentiation. The first appearance of morphological sex differentiation in medaka is the significant difference in the number of germ cells between the sexes at stage 38 before hatching; genetic females (XX) have more germ cells than genetic males (XY), and, subsequently, the germ cells enter mitotic arrest in XY gonads while they initiate meiosis in XX gonads (25–27).

To elucidate the molecular mechanisms underlying germ cell proliferation and differentiation during sex differentiation in medaka, we investigated the role of MIS signaling in sex differentiation in medaka. Here, we show that MIS signaling is required for germ cell proliferation during early gonadal differentiation in medaka. Furthermore, we suggest that MIS functions to stimulate germ cell proliferation through MISRII immediately after they reach the gonadal primordium.

Materials and Methods

Animals

Three medaka stocks were used: orange-red variety, FLFII strain (28), and *olvas-GFP*$^{STII-YI}$ transgenic strain (29). FLFII and *olvas-GFP*$^{STII-YI}$ transgenic strains allow identification of genotypical sex by the appearance of leucophores at 2 d post fertilization (dpf), *i.e.* before the onset of sex differentiation. In the *olvas-GFP*$^{STII-YI}$ transgenic strain, whose genome contains the enhanced green fluorescent protein (pEGFP) vector (Clontech, Palo Alto, CA) fused to the regulatory region of the medaka *vasa* (*olvas*) gene, the germ cells can be visualized by GFP fluorescence in living individuals (29, 30). Fish embryos were maintained in ERM [17 mM NaCl, 0.4 mM KCl, 0.27 mM $CaCl_2 2H_2O$, and 0.66 mM $MgSO_4$ (pH 7)] at 26 C under a 14-h light, 10-h dark cycle. Developmental stages of the embryos were determined according to the descriptions by Iwamatsu (31).

Isolation and sequence analysis of medaka mis and misr2 cDNAs

Total RNA was extracted from the adult testis of an orange-red variety of medaka using ISOGEN (Nippongene, Tokyo, Japan), and the first strand cDNA was synthesized from the total RNA by oligo(dT) priming with RNA PCR Kit (Applied Biosystems, Foster City, CA). PCR was performed with degenerate primers (5′-CTG YTS AAG GCS CTG CAG ACG-3′ and 5′-GTA GCG SAS GGG CAC GCA GCA-3′), using testis cDNA as a template according to the methods described previously (15). The reaction mixture was electrophoresed on a 2% agarose gel, and a 288-bp *mis* cDNA fragment was purified, inserted into the pT7Blue T-vector (Novagen, EMD Chemicals Inc., an affiliate of Merck KgaA, Darmstadt, Germany), and sequenced by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). To isolate medaka *mis* cDNA containing an open reading frame (ORF), a medaka testicular cDNA library was screened with a digoxigenin (DIG)-labeled *mis* cDNA as a probe (32). Both strands of the positive clone were sequenced according to the methods described previously.

To isolate medaka *misr2* cDNA, we first searched the sequence of the *misr2* gene from the medaka genome database (http://dolphin.lab.nig.ac.jp/medaka/) by the BLAST program. Next, to obtain the 5′- and 3′-ends of *misr2* cDNA, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed with primers (first PCR for 5′-RACE; 5′-CAGATGAGTGTCCTCCGTTG-3′, nested PCR for 5′-RACE; 5′-CTTGAAAAACGCACGTTCTC-3′, first PCR for 3′-RACE; 5′-CAGGTTGTGGGACAAGGAC-3′, nested PCR for 3′-RACE; 5′-GAGACTCATCCTCTATGTGTG-3′), designed on the based of the *misr2* sequence information, using the SMART PCR cDNA Library Construction Kit (Clontech). Both strands of the cDNA clones were sequenced according to the methods described previously.

In situ hybridization

FLFII medaka embryos were fixed in 4% paraformaldehyde in 10 mM PBS (pH 7.4) at 4 C overnight, embedded in paraffin, and sectioned serially at 5-μm thickness. *mis* and *misr2* antisense RNA probes were *in vitro* transcribed from the pT7Blue T-vector, into which *mis* or *misr2* cDNA is inserted. *In situ* hybridization was performed using a DIG-labeled *mis* or *misr2* antisense RNA probe as previously described (15).

Construction of RNA expression vectors and RNA synthesis

mis-GFP and *misr2-GFP* vectors were constructed by inserting the antisense gripNA target sequences of *mis* and *misr2*, respectively, into pEGFP vector (*mis-GFP*, 5′-GAAGCATGTTGCTTGTGCCC-EGFP-3′; *misr2-GFP*, 5′-AGATGGGGAAAATGCTGCCC-EGFP-3′). RNA was transcribed *in vitro* from linearized RNA expression vectors using T7 CAP-Scribe (Roche, Nutley, NJ).

Gene knockdown experiments

mis-AS (5′-GCACAAGCAACATGCTTC-3′) and *misr2-AS* (5′-GCAGCATTTTCCCCATCT-3′), antisense gripNAs targeted against *mis* and *misr2*, respectively, were obtained from Active Motif (33). As a control, mismatched *mis-AS* (*Mmis-AS*) (5′-GCAGAACCAAGAAGCATC-3′; mutant sequences are *underlined*), an antisense gripNA in which five bases of the *mis-AS* were changed, was used. Microinjection was performed into medaka embryos at one- or two-cell stage using a Nanoject II (Drummond Scientific Co., Broomall, PA). For control experiments, RNA (100 pg) and gripNA (0.1 or 1.0 mM) were injected into one- and two-cell stage embryos of the orange-red variety, respectively. For the knockdown experiments, gripNA was injected into one-cell stage embryos of the *olvas-GFP*$^{STII-YI}$ transgenic strain. After injection the embryos were maintained in ERM at 26 C using Incubator MIR-153 (SANYO Electric Co., Ltd., Hokkaido, Japan). The germ cells exhibiting GFP fluorescence were counted for each embryo at 4 dpf [before primordial germ cells (PGCs) reach the gonadal region] and 9 dpf (after proliferation of germ cells) using a fluorescent stereomicroscope MZFL III (Leica Microsystems GmbH, Wetzlar, Germany).

Histological analysis

Nine-dpf embryos in the knockdown experiments were fixed in Bouin's solution at 4 C overnight, embedded in paraffin, sectioned serially at 5-μm thickness, and then stained with hematoxylin and eosin. The germ cells were counted in five embryos of both sexes for each group.

Cell proliferation assay

For 5-bromo-2′-deoxyuridine (BrdU) labeling, 6-dpf embryos of *olvas-GFP*$^{STII-YI}$ transgenic strain were dechorionated and cultured in 10 mM

BrdU solution (GE Healthcare, General Electric Co., Fairfield, CT) at 26 C for 3 d. The embryos were fixed as described previously, and the incorporated BrdU was detected immunohistochemically using the Amersham Cell Proliferation Kit (GE Healthcare) according to the manufacturer's protocol. The number of BrdU-positive cells was counted in four embryos for each group and expressed as the percentage of total number of the positive cells.

Culture experiments

Tissue fragments, including germ cells, dissected from 6-dpf embryos of *olvas-GFP*$^{STII-YI}$ transgenic medaka under the fluorescent microscope were cultured at 26 C for 3 d in 1.5 ml of the basal culture medium with 7.5 μl MEM (Life Technologies, Inc., Gaithersburg, MD), or with 1.5 or 7.5 μl MEM containing recombinant recombinant eel spermatogenesis-related substances 21 (r-eSRS21) (14). The basal culture medium consisted of Leibovitz L-15 medium (Life Technologies, Inc.) supplemented with 0.5% BSA and Insulin-Transferrin-Selenium-G Supplement (Life Technologies, Inc.). The germ cells exhibiting GFP fluorescence in the fragments were counted according to the methods described previously.

Statistics

Experimental results were tested using Levene's test for homogeneity of variance. Data were analyzed by one-way ANOVA and then tested with the Dunnett's multiple comparison test.

Results

Isolation and characterization of medaka mis and misr2 cDNAs

To isolate medaka *mis* cDNA containing an ORF, 1.2×10^6 phage clones in a medaka testicular cDNA library were screened, and one positive clone was obtained. The clone had a 1864-bp insert (DNA Data Bank of Japan accession no. AB166790), containing an ORF encoding a potential protein of 496 amino acid residues. The overall identity of the nucleotide sequences of the clone with the already identified *mis* (17) was 99.7%, indicating that the clone is a medaka *mis*.

To isolate medaka *misr2* cDNA, 5′- and 3′-RACE were performed with primers designed according to the *misr2* sequence from the medaka genome database, and the resultant product was 1687-bp in length (DNA Data Bank of Japan accession no. AB258535), containing an ORF encoding a potential protein of 495 amino acid residues. The overall identity of the nucleotide sequences of the product with the already identified *misr2* (17) was 99.9%, indicating that the product is a medaka *misr2*.

mis and misr2 mRNAs are expressed in gonads of both sexes before proliferation of their germ cells

To investigate the expression of *mis* and *misr2* mRNAs during sex differentiation, *in situ* hybridization was performed with a DIG-labeled *mis* or *misr2* antisense RNA probe using FLFII medaka. *mis* mRNA was expressed only in the somatic cells surrounding the germ cells of both sexes on 4 dpf (stage 32), 6 dpf (stage 36), and 9 dpf (stage 39), whereas *misr2* mRNA was expressed predominantly in the somatic cells of both sexes on 6 and 9 dpf, but not in 4 dpf, embryos (Fig. 1). There was no signal in the gonads using a *mis* or *misr2* sense RNA probe (data not shown). These results indicate that *mis* and *misr2* mRNAs are expressed in gonads of both sexes before proliferation of germ cells.

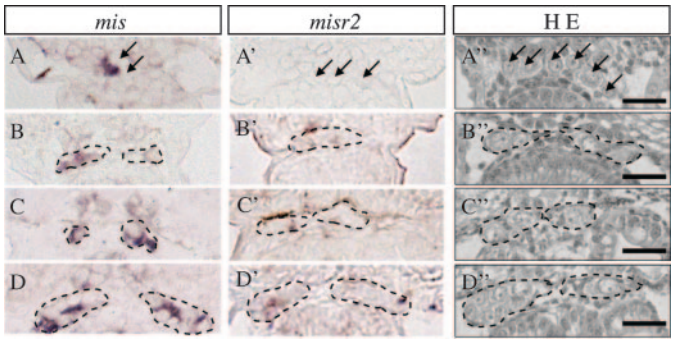


FIG. 1. Cellular localization of *mis* and *misr2* mRNAs during early gonadal differentiation in medaka. *In situ* hybridization of *mis* (A–D) and *misr2* transcripts (A'–D') in the individuals at 4 dpf (A, A', and A"), 6 dpf (B, B', and B"), and 9 dpf (XY: C, C', and C"; XX: D, D', and D"). A"–D", Hematoxylin/eosin-stained (HE) sections. *Arrows* and *dotted lines* indicate the PGCs and the outline of the gonadal regions, respectively. *Scale bar* represents 20 μm.

Loss-of-function of either MIS or MISRII results in suppression of germ cell proliferation during sex differentiation

To elucidate the role of MIS signaling during early sex differentiation in medaka, we performed loss-of-function experiments of MIS and MISRII using the antisense knockdown system (33).

For control experiments, *GFP* RNA containing the antisense gripNA-target sequence and the gripNA were injected into one- and two-cell stage embryos of the orange-red variety, respectively. When *mis-GFP* RNA was injected into one-cell stage embryos, 1-dpf embryos expressed GFP fluorescence (Fig. 2A), but the fluorescence was completely blocked by injection of *mis-AS* (Fig. 2B), whereas five base-*Mmis-AS* did not affect GFP expression (Fig. 2C). When *misr2-GFP* RNA was injected into one-cell stage embryos, the

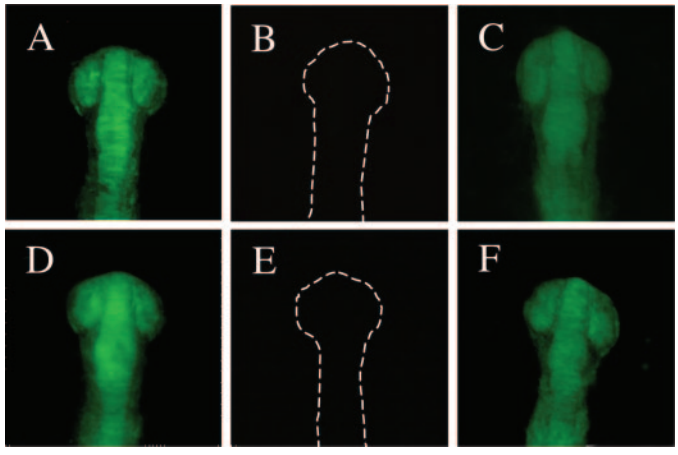


FIG. 2. Specific interference with gene translation using *mis-antisense* gripNA (*mis-AS*), five base-*Mmis-AS*, and *misr2-AS*. *Photographs* show dorsal views of 1-dpf embryos under excitation light. A, An embryo injected with RNA containing the respective *mis-AS* target sequence fused to the *GFP* gene (*mis-GFP* RNA). B, An embryo injected with *mis-GFP* RNA and *mis-AS*. C, An embryo injected with *mis-GFP* RNA and *Mmis-AS*. D, An embryo injected with *misr2-GFP* RNA. E, An embryo injected with *misr2-GFP* RNA and *misr2-AS*. F, An embryo injected with *misr2-GFP* RNA and *mis-AS*. *Dotted lines* indicate the outlines of the embryos.

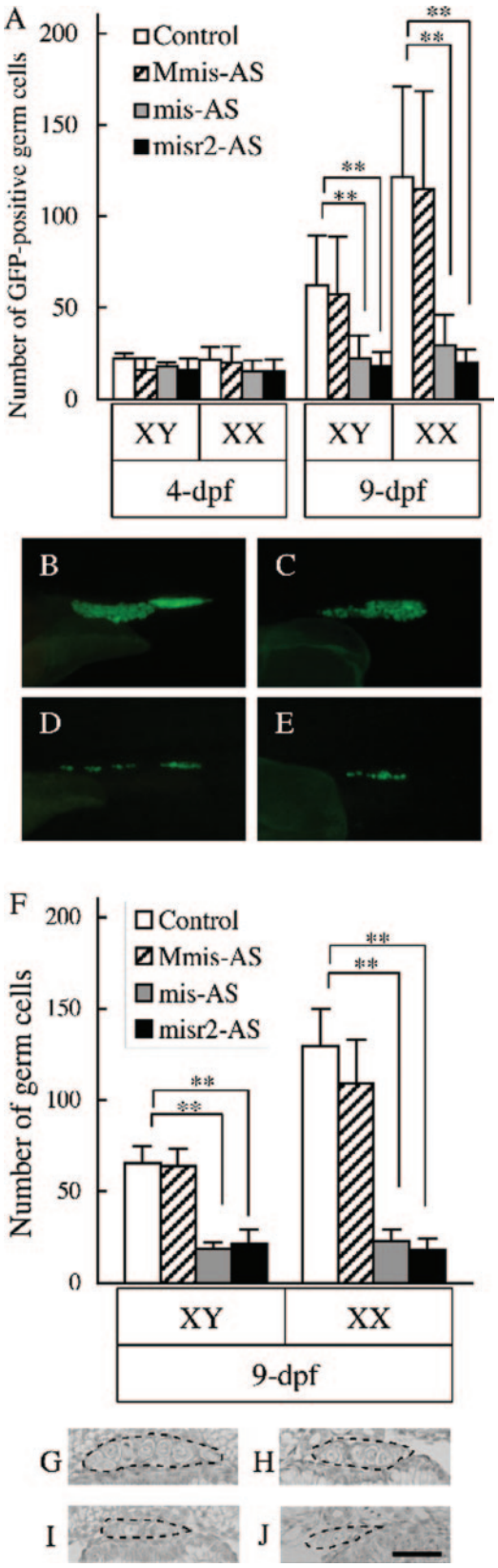


FIG. 3. Number of germ cells in MIS- and MISRII-defective embryos in medaka. A, Number of GFP-positive germ cells in the individuals uninjected (control), or injected with *mis-AS*, *Mmis-AS*, or *misr2-AS*. Results are expressed as mean ± SEM of GFP-positive germ cell number ($n \geq 5$; **, $P < 0.01$). *Photographs* show GFP fluorescent germ cells in 9-dpf embryos (XX individuals) uninjected (B), or injected with *Mmis-AS* (C), *mis-AS* (D), or *misr2-AS* (E). F, Number of germ cells in the individuals uninjected (control), or injected with *mis-AS*, *Mmis-AS*, or *misr2-AS*. Results are expressed as mean ± SEM of germ cell number ($n = 5$; **, $P < 0.01$). *Photographs* show germ cells in 9-dpf embryos (XX individuals) uninjected (G), or injected with *Mmis-AS* (H), *mis-AS* (I), or *misr2-AS* (J). *Dotted lines* indicate the outline of the gonadal regions. *Scale bar* represents 20 μm.

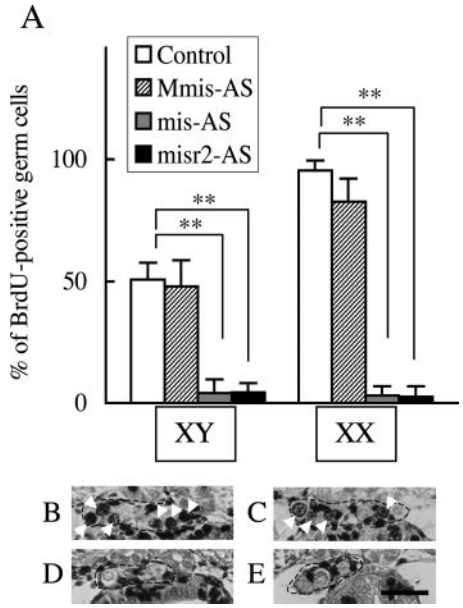


FIG. 4. Proliferation activity of germ cells in MIS- and MISRII-defective embryos in medaka. A, Percentage of BrdU-positive germ cell number in the individuals uninjected (control), or injected with *mis-AS*, *Mmis-AS*, or *misr2-AS*. Results are expressed as mean ± SEM of the percentage of BrdU-positive germ cell number ($n = 4$; **, $P < 0.01$). *Photographs* show BrdU-positive germ cells in 9-dpf embryos (XX individuals) uninjected (B), or injected with *Mmis-AS* (C), *mis-AS* (D), or *misr2-AS* (E). *Arrows* and *dotted lines* indicate the BrdU-positive germ cells and the outline of the gonadal regions, respectively. *Scale bar* represents 20 μm.

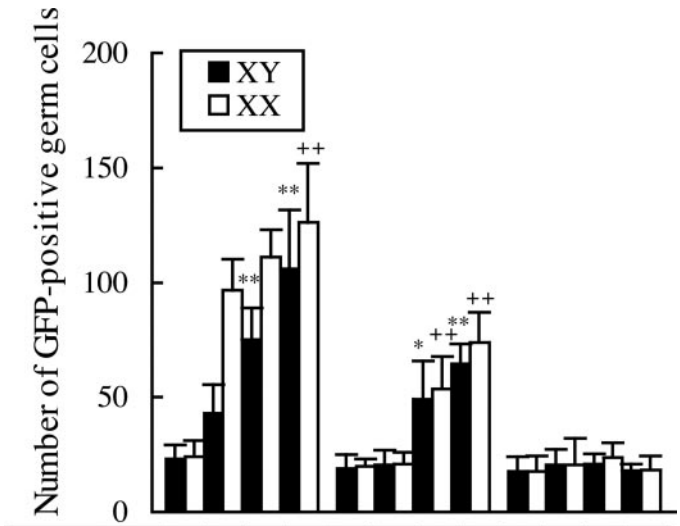
1-dpf embryos expressed GFP fluorescence (Fig. 2D), but the fluorescence was completely blocked by injection of *misr2-AS* (Fig. 2E), whereas *mis-AS* did not affect GFP expression (Fig. 2F). Blockade of the fluorescence maintained in the embryos until at least 9 dpf (data not shown). No obvious difference was observed between the gripNA-injected and uninjected embryos in externally embryonic development (Fig. 2). These experiments showed that both *mis-AS* and *misr2-AS* specifically blocked translation by interfering with their targets and allowed us to conduct loss-of-function experiments *in vivo*.

We then performed loss-of-function experiments of MIS and MISRII by injecting *mis-AS*, *Mmis-AS*, or *misr2-AS* into fertilized eggs of *olvas-GFP*$^{\text{STII-YI}}$ transgenic strain, allowing us to monitor germ cells by GFP fluorescence (29, 30). Indeed, we made sure that all germ cells on paraffin sections of their embryos were positive for anti-GFP antibody (data not shown). On 4 dpf before PGCs reached the gonadal region, there were no significant differences in the number of GFP-positive PGCs in both sexes of *mis-AS*-, *Mmis-AS*-, or *misr2-AS*-injected embryos compared with the uninjected control

embryos (Fig. 3A). After the proliferation of germ cells (9 dpf), there were fewer GFP-positive germ cells in both sexes of *mis-AS-* (Fig. 3, A and D) or *misr2-AS*-injected embryos (Fig. 3, A and E) compared with the control (Fig. 3, A and B) and *Mmis-AS*-injected embryos (Fig. 3, A and C). However, the recovery of germ cell number was observed in the MIS- and MISRII-defective fries at 5 d after hatching, when this loss-of-function system is invalid (data not shown). Next, to investigate the proliferation of germ cells histologically, we counted the number of all germ cells in *mis-AS-*, *Mmis-AS-*, or *misr2-AS*-injected embryos. Similar to counts by GFP fluorescence, there were fewer germ cells in both sexes of *mis-AS-* or *misr2-AS*-injected embryos compared with the control and *Mmis-AS*-injected embryos (Fig. 3, F–J). To confirm the proliferation activity of germ cells, we performed BrdU labeling analysis on paraffin sections of 9-dpf embryos. Germ cells having nuclei positive for BrdU were observed numerously in both sexes of the control and *Mmis-AS*-injected embryos (Fig. 4, A–C). In contrast, BrdU-positive germ cells were hardly detected in both sexes of *mis-AS-* or *misr2-AS*-injected embryos (Fig. 4, A, D, and E). On the other hand, BrdU-positive somatic cells were found not only in the gonads of the control and *Mmis-AS*-injected embryos, but also in *mis-AS-* or *misr2-AS*-injected embryos (Fig. 4, B–E). These results demonstrate that loss-of-function of either MIS or MISRII results in suppression of germ cell proliferation specifically in both sexes during the sex differentiation.

Effect of recombinant MIS on germ cell proliferation in vitro

To clarify further the role of MIS signaling on germ cell proliferation, we examined the effect of MIS protein in the tissue culture system. Tissue fragments containing germ cells were dissected from 6-dpf embryos of *olvas-GFP*$^{\text{STII-YI}}$ transgenic medaka and cultured in 1.5 ml of the basal medium with 7.5 μl MEM (control), or with 1.5 or 7.5 μl MEM containing recombinant eel MIS (r-eSRS21) for 3 d. Subsequently, the germ cells exhibiting GFP fluorescence in the fragments were counted. Treatment of tissue fragments in the uninjected embryos with r-eSRS21 resulted in a significant increase in the number of germ cells in both sexes compared with the control without r-eSRS21 (Fig. 5). Culture of tissue fragments from *mis-AS*-injected embryos inhibited proliferation of germ cells in both sexes compared with the uninjected embryos ($P < 0.05$ and $P < 0.01$ in XY and XX individuals, respectively), whereas treatment of the tissue fragments with r-eSRS21 increased the germ cell number of both sexes in a dose-dependent manner (Fig. 5). On the other hand, culture of tissue fragments in *misr2-AS*-injected embryos with and without r-eSRS21 significantly inhibited proliferation of germ cells in both sexes compared with the uninjected embryos ($P < 0.05$ and $P < 0.01$ in XY and XX individuals, respectively). These results suggest that MIS functions to stimulate germ cell proliferation through MISRII immediately after they reach the gonadal primordium.



r-eSRS21(μl)	—	0	1.5	7.5	—	0	1.5	7.5	—	0	1.5	7.5
mis-AS	—	—	—	—	+	+	+	+	—	—	—	—
misr2-AS	—	—	—	—	—	—	—	—	+	+	+	+

FIG. 5. Effect of recombinant MIS on germ cell proliferation *in vitro*. The germ cells in the tissue fragments treated with r-eSRS21 protein or without (control) for 3 d were counted. Results are expressed as mean ± SEM of GFP-positive germ cell number ($n \geq 5$; *, $P < 0.05$; **, $P < 0.01$ when compared with the XY control, $^{++}$, $P < 0.01$ when compared with the XX control). −, Number of the germ cells before culture.

Discussion

In the present study, to elucidate the role of MIS signaling in early gonadal differentiation in medaka (teleost, *O. latipes*), lacking Müllerian ducts, we first isolated *mis* and *misr2* cDNAs from the testes and examined expression pattern of their mRNAs. *mis* mRNA was expressed specifically in the somatic cells surrounding PGCs in both sexes at stage 32 before PGCs reached the gonadal region, whereas *misr2* mRNA was expressed predominantly in the somatic cells of both sexes from stage 36 onward, when they arrived at the gonadal region, consistent with the recent report (17). These results suggest that MIS indirectly induces germ cell proliferation by stimulating the release of the proliferative factors in the somatic cells from around stage 36 in medaka. In mammals, *Mis* mRNA expression is detected in Sertoli cells of the testis after the onset of *Sry* expression and in granulosa cells of the ovary at d 6 after birth (4), whereas *Misr2* mRNA is detected in the mesenchymal cells adjacent to the Müllerian duct during embryogenesis, in Sertoli cells and granulosa cells (6–8). In birds, *MIS* mRNA is expressed at high levels in Sertoli cells of embryonic testes and in lower amounts in ovaries (34). In reptiles, *MIS* mRNA is expressed in the differentiating Sertoli cells of embryos incubated at the male temperature (33 C), but not in those incubated at the female temperature (30 C) (35). In teleost fish, Japanese eel MIS (eSRS21) is expressed in Sertoli cells of immature testes and acts in preventing spermatogenesis (14). These results indicate that MIS expression is predominant in the somatic cells surrounding the germ cells, and we suggest that MIS functions as a prerequisite regulator in sex differentiation and gametogenesis in vertebrates.

In the loss-of-function experiments using *olvas-GFP*$^{\text{STII-YI}}$

transgenic medaka strain, there were fewer germ cells in both sexes of MIS- and MISRII-defective embryos compared with controls after the proliferation of germ cells, but no significant differences were seen in PGC numbers of both sexes between controls and MIS- or MISRII-defective embryos before they reach the gonadal region. These findings were supported by histological observation and cell proliferation assay using BrdU labeling analysis. Thus, we provide the first demonstration that MIS signaling is required for germ cell proliferation in both sexes during early sex differentiation. Furthermore, we tested whether MIS protein induces germ cell proliferation in both sexes *in vitro*. Treatment of tissue fragments containing germ cells in the MIS-expressing embryos with r-eSRS21 resulted in a significant increase in the number of germ cells in both sexes compared with the control. On the other hand, culture of tissue fragments from the MIS- or MISRII-defective embryos inhibited proliferation of germ cells in both sexes. Moreover, treatment with r-eSRS21 in the MIS-defective embryos dose-dependently increased germ cell number of both sexes, whereas in the MISRII-defective embryos, it did not permit proliferation of germ cells. These results suggest that MIS functions to stimulate germ cell proliferation solely through MISRII in medaka. In mice, MIS-deficient males develop as internal pseudohermaphrodites, possessing a complete male reproductive tract, and also a uterus and oviducts (10), similar to the phenotype of MISRII mutant males (9). Moreover, the phenotype of MIS ligand/ MISRII double mutant males is indistinguishable from those of each single mutant, suggesting that MIS is the only ligand of the MISRII *in vivo*, and also that the MIS signaling pathway is simple in mammals. Therefore, the MIS signaling pathway may be conserved among vertebrates in the ligand/ receptor system.

PGC is a precursor stem cell that develops into the gametes. PGCs segregate from the somatic cells early in development and migrate through the somatic tissues to reach the developing gonads. After PGCs become associated with gonadal somatic cells, they divide and then differentiate into definitive gametes. Discovering molecular signals that controls germ cell division and differentiation should elucidate gonadal sex differentiation and gametogenesis. In medaka, PGCs are found in the somatic mesoderm at stage 32, and subsequently reach the gonadal region at stage 36 (26, 27). Although the number of PGCs scarcely increases during the migratory period, they acquire proliferative activity after the completion of migration (26). In XY gonads, germ cells double in number by the hatching stage (stage 39), whereas in XX gonads, they show about a 4-fold increase (Fig. 3). XY-specific signal for *DMY/dmrt1bY* mRNA is initially detected in the somatic cells surrounding germ cells at stage 36 and persists in Sertoli cell lineage cells (22, 27). Recently, it has been reported that *DMY/dmrt1bY* functions as an inhibitory regulator in germ cell proliferation in a sex-specific manner (36). However, the mechanism whereby their proliferation is stimulated remains unclear. In the present study, we found that both *mis* and *misr2* mRNAs were expressed in the somatic cells surrounding the germ cells of both sexes, and MIS indirectly stimulated germ cell proliferation through MISRII in the somatic cells from around stage 36. Moreover, loss-of-function of either MIS or MISRII resulted in suppression of germ cell proliferation during sex differentiation. These results indicate that MIS functions as an indirect initiator in germ cell proliferation in both sexes immediately after PGCs reach the gonadal primordium. Recently, MISRII mutant has been identified and characterized in medaka (37). The mutant fish display excessive proliferation of germ cells in both sexes soon after hatching, and one half of the homozygous XY mutant undergo sex reversal, which accompanies the expression of the female-characteristic *aromatase* gene in the gonadal somatic cells (37); germ cells normally enter mitotic arrest in XY fish after hatching, whereas they maintain proliferative activity and go into early meiosis in XX fish (25). In contrast, as we have shown previously, the MISRII- and MIS-defective embryos showed loss of germ cell proliferation in both sexes before hatching, but not after hatching. Collectively, these lines of evidence suggest that there could be differences in molecular mechanisms whereby germ cell proliferation in gonadal development is regulated before and after hatching. The mechanism underlying this difference may be elucidated by investigating correlations of MIS signaling with several factors, such as *aromatase*, which are expressed from around the hatching stage. Thus, MIS is likely to possess multiple regulatory functions in gonadal development in medaka, similar to mammals (38).

In mice, PGCs migrate along the hind gut toward the genital ridge, reaching it by embryonic d (E) 11.5. Thereafter, the number of the germ cells increases rapidly to 20,000–25,000 cells by E 13.5 (39), and enter mitotic arrest in the developing testis or meiosis in the ovary. Although several growth factors have increased proliferation and the survival of mouse PGCs in culture, including stem cell factor (40–42), leukemia inhibitory factor (42), and basic fibroblast growth factor (43, 44), it remains unclear whether they are responsible for the regulation of germ cell proliferation during early sex differentiation in the intact embryos (45). In mice, *Mis* transcripts first appear in Sertoli cells of fetal testes between E 11.5 and 12.5, but not in other male fetal tissues or in female embryos (4); however, the cellular localization of *Misr2* transcripts at the stage has not been described. Recently, in organ cultures of E 13 XY gonads from rats, treatment with all-*trans*-retinoic acid decreased MIS expression in Sertoli cells and dramatically reduced the number of gonocytes (46), suggesting that retinoic acid and MIS signalings are involved in regulating gonocyte number during the early gonadal differentiation of XY individuals. Therefore, MIS signaling also may be involved in germ cell proliferation during early gonadal differentiation in mammals, though further investigation is needed.

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