Müllerian Inhibiting Substance in the Caudate Amphibian *Pleurodeles waltl*

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Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone), is a key factor of male sex differentiation in vertebrates. In amniotes, it is responsible for Müllerian duct regression in male embryos. In fish, despite the absence of Müllerian ducts, MIS is produced and controls germ cell proliferation during gonad differentiation. Here we show for the first time the presence of MIS in an amphibian species, *Pleurodeles waltl*. This is very astonishing because in caudate amphibians, Müllerian ducts do not regress in males. Phylogenetic analysis of MIS *P. waltl* ortholog revealed that the deduced protein segregates with MIS from other vertebrates and is clearly separated from other TGF-β family members. In larvae, MIS mRNA was expressed at higher levels in the developing testes than in the ovaries. In the testis, MIS mRNA expression was located within the lobules that contain Sertoli cells. Besides, expression of MIS was modified in the case of sex reversal: it increased after masculinizing heat treatment and decreased after estradiol feminizing exposure. In addition to the data obtained recently in the fish medaka, our results suggest that the role of MIS on Müllerian ducts occurred secondarily during the course of evolution. **(*Endocrinology* 154: 3931–3936, 2013)**

In most vertebrate species, male and female reproductive tracts, the Wolffian and Müllerian ducts, respectively, are both present in the embryo before gonad differentiation. In males, in addition to testosterone that allows the Wolffian ducts to persist and to differentiate as spermiducts and accessory organs, the differentiating testes also produce Müllerian inhibiting substance (MIS) that induces Müllerian duct regression (1). In female embryos, in the absence of MIS, Müllerian ducts are maintained and will differentiate into oviducts under the influence of ovarian estrogens.

MIS is a member of the TGF-β family (2). This homodimeric disulfide-linked glycoprotein must be cleaved at a proteolytic site to release the C-terminal domain, in which resides the biological activity. Like TGF-β, MIS signals through 2 distinct serine/threonine receptors: MIS binding induces the assembly of a receptor complex in which the type II receptor phosphorylates and activates the type I receptor. Once activated, the type I receptor activates cytoplasmic proteins, the Smads, which bind to a common partner, Smad 4, allowing nuclear translocation, and together with coactivators, stimulate the transcription of target genes (3). In humans, in cases of mutations affecting MIS or its receptor, Müllerian ducts are retained in males, a condition known as the persistent Müllerian duct syndrome (4–7).

MIS has been found in reptiles, birds, and mammals (8). In teleost fishes, MIS and its receptor are expressed in males despite the absence of Müllerian ducts, and the hormone controls germ cell proliferation (9–11). To date, MIS has never been described in Amphibia, and its existence in the Caudata is questionable because in this order, Müllerian ducts are maintained in adult males.

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Abbreviations: DMC1, disrupted meiotic cDNA 1; E_2, estradiol; MIS, Müllerian inhibiting substance.

Pleurodeles waltl, the Iberian ribbed newt (Figure 1), is a member of the Salamandridae family. It is a gonochoristic species with genetic sex determination; males are determined by the monogametic ZZ genotype, whereas females display a heterogametic ZW genotype (12). At stage 53, the early differentiation of the gonadal sex can be detected histologically. In ZW larvae, oogonia stay in the cortex where they proliferate. At stage 54, these cells associate with somatic cells and constitute follicles. Most of the oogonia enter meiosis, and this process is arrested at prophase I. The ovary differentiates as an ovisac because medulla regression leaves a cavity (Figure 1). In ZZ larvae, at stage 53, germ cells migrate from the cortex toward the medulla. Together with differentiating Sertoli cells, they will constitute units named cysts that are themselves included into lobules (Figure 1). The cortex devoid of germ cells becomes albuginea, the testis envelope.

Genetic sex determination in *P. waltl* can be counteracted by temperature (12). For maximal efficiency, temperature should be maintained at 32°C from stage 42 to stage 54, a developmental window defined as the thermosensitive period (Figure 1) (13). Both Wolffian and Müllerian ducts are present in ZZ and ZW larvae (Figure 1). After metamorphosis (stage 56), in the case of ovary differentiation, estrogens will induce the differentiation of the Müllerian ducts into oviducts. In contrast to amniotes, Müllerian ducts will not disappear in ZZ larvae, and they can even differentiate into oviducts in the case of experimentally administrated estrogens.

Our aim was to determine whether MIS was expressed in *P. waltl* and to study its pattern of expression during normal development and sex reversal situations. Our results show that MIS is expressed in that species in which Müllerian ducts are maintained in males and suggest that the role of MIS on Müllerian ducts was probably acquired secondarily during the course of evolution.

Materials and Methods

P. waltl larvae and adults were reared in fresh water at 20°C ± 2°C. The Animal Care and Use Committee of Lorraine approved the experimental protocols, and guidelines for laboratory procedures were followed at all times. Developmental stages were

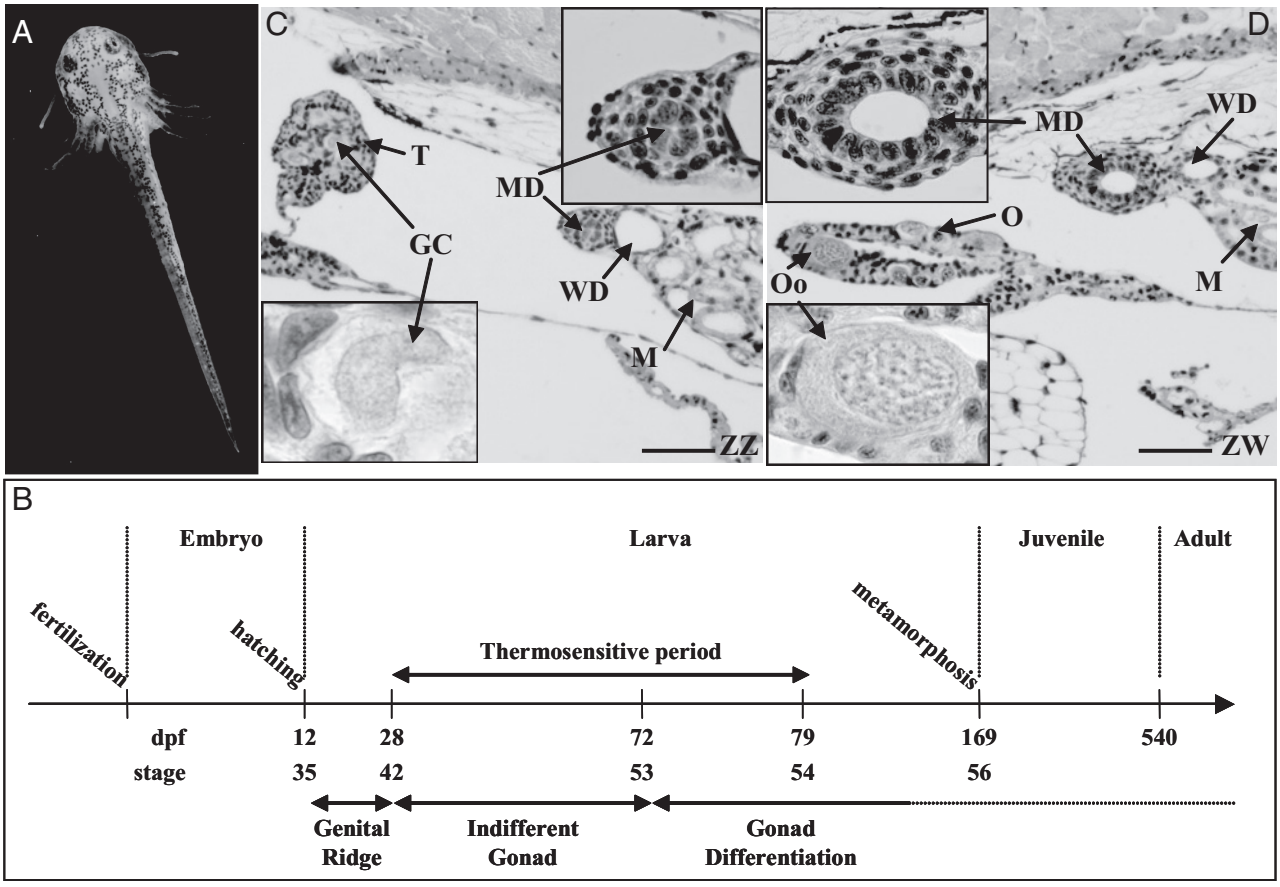

Figure 1. A, *P. waltl* larva. B, Timetable of *P. waltl* development. C and D, Histological sections of a male (ZZ) and a female (ZW) at the time of metamorphosis. On each picture, 2 inserts show at higher magnification, respectively, the Müllerian duct and 1 germ cell (nonmeiotic with a polylobular nucleus in the male and meiotic in the female). Abbreviations: dpf, days post fertilization; GC, germ cell; M, mesonephros; MD, Müllerian duct; O, ovary; Oo, oocyte; T, testis; WD, Wolffian duct. Scale bars, 50 μm.

determined by macroscopic observation (14). The sexual genotype was identified by electrophoretic patterning of the sex-linked peptidase-1 (15).

Juvenile testis MIS cDNA was PCR-amplified with degenerate primers 5′-TGCTTYACGMGNATGTTYCC-3′ and 5′-CCRCAYTCYTCNGCNACCATRTG-3′ with slight modifications of a previously described protocol (16). Briefly, the hybridization temperature of the touchdown PCR program was lowered from 60°C to 50°C, and amplification was pursued with 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1.5 minutes.

The detailed protocols for RNA extraction, reverse transcription, and PCR have been described previously (17, 18). The primers used are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online website at http://endo.endojournals.org).

For in situ hybridization, testes were fixed during 16 hours in paraformaldehyde (4%) (Sigma-Aldrich) prepared in 75mM phosphate buffer, embedded in Tissue-Tek O.C.T. (QIAGEN). A 238-bp MIS sequence (nucleotides 366–603) was inserted into a pGEM-T Easy (Promega) vector, which was used to generate digoxigenin-labeled RNA probes (Supplemental Table 1). Antisense and/or sense probes were diluted with hybridization buffer to 3.6 ng/μL, denatured at 95°C for 5 minutes, and applied on the cryosections (10 μm thickness) overnight at 55°C. After ribonuclease treatment and washes in formamide-containing (Sigma-Aldrich) buffer (30%), sections were saturated with blocking buffer for 1 hour before an overnight application of the alkaline phosphatase-conjugated antidigoxigenin antibody (Fab fragment; Roche) (1/500, 4°C). Phosphatase activity was revealed with Nitro blue tetrazolium-5-Bromo-4-chloro-3-indolyl-phosphate (NBT-BCIP) (Roche).

Organotypic cultures were performed in Leibovitz L15 medium supplemented with L-glutamine (2mM), penicillin (10 IU/mL), and streptomycin (10 μg/mL), at room temperature (20°C ± 2°C). Testes and liver were harvested from adult males. After 8 or 24 hours, conditioned medium was obtained after centrifugation (3000 rpm). After addition of 2× Laemmli sample buffer (vol/vol), samples were subjected to electrophoresis and Western blotting as reported previously (19).

Gene expression is expressed as mean ± SEM from several samples as indicated in the text. Differences among groups were

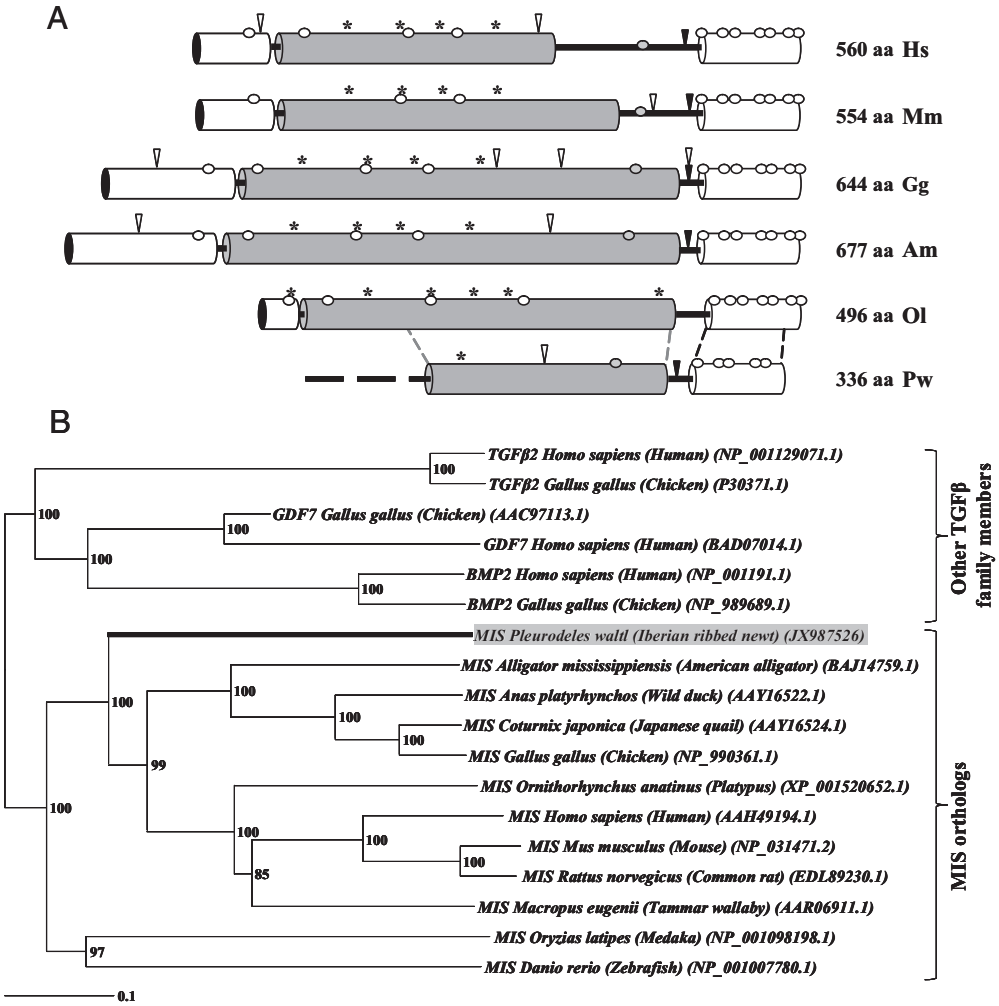

Figure 2. *P. waltl* MIS protein structure and phylogenetic analysis. A, Schematic representation of MIS from several vertebrates depicting signal peptide (dotted cylinder), MIS domain (gray cyclinder), TGF-β domain (white cylinder), conserved cysteines (white circles), position of introns (asterisks), cleavage site (black triangles), and N-glycosylation sites (white triangles). B, Phylogenetic tree showing the relations between *P. waltl* MIS and MIS of other species. We used the Phylowin software and applied a bootstrap test of 500 replicates to check the robustness of the phylogram. Bootstrapping values, distance scale, and Genbank accession numbers are given. Scale bar, 0.1 substitution per site.

tested using ANOVA and the Bonferroni least-significant difference test (SPSS version 12.0 software).

Results and Discussion

Using degenerate primers that were designed from conserved regions of vertebrate MIS cDNA sequences, we amplified a 1008-bp MIS cDNA fragment of *P. waltl*. This fragment was then cloned into a pGEM-T Easy Vector System (Promega) and sequenced (GenBank accession number JX987526). The deduced amino acid sequence contained 336 residues, with the typical TGF-β domain at the C terminus and the main part of the MIS domain usually located in the middle of the protein (Figure 2A and Supplemental Figure 1). The amphibian MIS sequence had an overall amino acid similarity of 34% to chicken, 33% to alligator, 32% to platypus, 24% to human or mouse, 23% to zebrafish, and only 16% to medaka. Amino acid similarity was higher in the proteolytically cleaved mature C terminus: 44% to chicken and platypus, 38% to mouse, 37% to alligator, 33% to human, 26% to zebrafish, and 20% to medaka. The predicted plasmin cleavage site (R/K-XX-R/K) between the N terminus and the mature C terminus was located between amino acids 235 and 238 (RQNR/STG). Finally, analysis of cysteine distribution throughout the *P. waltl* MIS protein showed that 5 of the 7 cysteines usually found in the C-terminal MIS fragment of most vertebrates were in conserved positions (Figure 2A and Supplemental Figure 1).

The partial MIS amino acid sequence of *P. waltl* was used to construct a phylogenetic tree using the Phylowin software (20). The amphibian MIS clusters within the clade of MIS proteins, well separated from other members of the TGF-β family (Figure 2B). Thus, the gene we have identified in *P. waltl* has the phylogenetic properties and structural features expected for an MIS ortholog.

To determine the expression pattern of MIS during development, we conducted real-time PCR analyses We found a higher level of MIS mRNA expression in male than in female gonad-mesonephros samples before (1.7-fold at stage 50, $P < .01$) and during gonad differentiation (3.6-fold at stage 54, $P < .01$) (Figure 3A). At stage (54+60d) when differentiated gonads were big enough to be analyzed separately from the mesonephros, this differential expression was more important (12.5-fold, $P < .01$) (Figure 3A). After metamorphosis, the level of MIS mRNA decreased in the testis in which the expression level appeared similar to that observed in the ovary (data not shown). In adult tissues, MIS mRNA was detected only in the gonads and was found not only in the testis but also in the ovary (Figure 3B).

To determine which testis compartment expressed MIS in the testis, we performed in situ hybridization. Testes of young juvenile animals were fixed in 4% paraformaldehyde

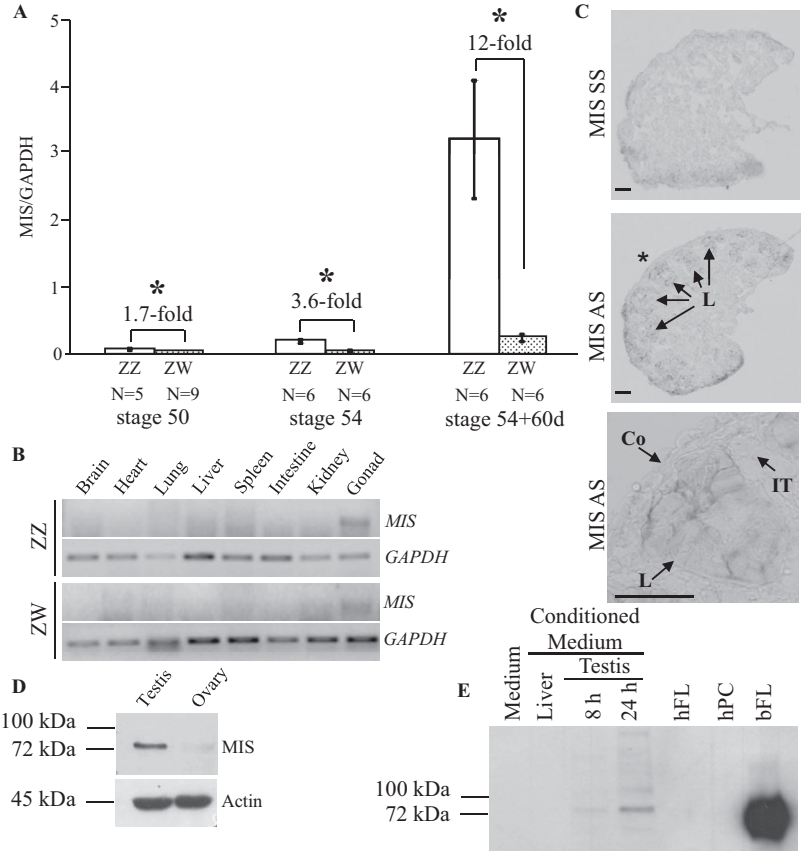

Figure 3. MIS mRNA expression. A, Real-time PCR analysis. Samples were pools of 4 gonad-mesonephros complexes for larvae before (stage 50) and at the beginning (stage 54) of gonad differentiation; they consisted of gonads only during the differentiation process (stage 54+60d). *, Significantly different from ZW samples ($P < .05$). B, RT-PCR analysis of different organs from an adult male and an adult female. C, In situ hybridization performed on testis sections of a young juvenile male. The lobule indicated by the asterisks is shown enlarged in the picture at the bottom. Bars, 50 μm. D, Western blot analysis of MIS expression in adult gonads. Actin is used as a loading control. E, Western blot analysis of MIS expression in conditioned medium. The analysis was performed on media conditioned by liver and testes from *P. waltl* adult males for 8 or 24 hours. Medium alone and recombinant human MIS were used as negative controls. Recombinant bovine MIS (full length = bFL) was used as positive control. Abbreviations: AS, antisense probe; Co, cortex; hFL, human full-length; hPC, human plasmin-cleaved; IT, interstitial tissue; L, lobule; SS, sense probe.

and cryosectioned. At this stage, the gonads contain lobules surrounded by interstitial tissue producing steroids. Within the lobules, germ cells are associated with somatic cells corresponding to Sertoli cells (21). Expression of MIS was detected in the lobules, in the position expected for Sertoli cells (Figure 3C).

To determine whether the MIS protein could be translated and secreted in *P. waltl* testis, we performed organotypic cultures and Western blot analyses. In adult testis, a monoclonal antibovine MIS antibody (Mab 168) that cross-reacts with ovine and caprine MIS detected a protein with a molecular mass similar to bovine MIS (72 kDa) (19). The intensity of this band was very low in the ovary (Figure 3D). In *P. waltl* adult testis-conditioned medium, the antibody detected a protein with a molecular mass similar to recombinant bovine MIS (72 kDa). No signal was observed in male liver-conditioned medium or in non-conditioned medium (Figure 3E). This suggested that *P. waltl* MIS protein could be translated and secreted.

We also studied MIS mRNA expression during treatments known to induce sex reversal. First, ZW larvae were grown at 32°C from stage 42 to 54 to induce a female to male sex reversal (13). At stage 54+60d, our real-time PCR analyses revealed that such a treatment induced a 3.6-fold increase in MIS mRNA gonadal expression in comparison with control ZW larvae (Figure 4A). At the same stage, to ascertain sex reversal, we studied the expression of aromatase, the estrogen-synthesizing enzyme, which is known to decrease in case of a masculinizing heat treatment (17, 22). As expected, a 7.7-fold decrease in gonadal aromatase expression was observed (Figure 4A). Sex reversal was confirmed by the absence of the recombinase disrupted meiotic cDNA 1 (DMC1) that is a meiosis entry marker specific of ovary differentiation at this stage (Figure 4A). We then studied ZZ larvae exposed to estradiol (E_2) from stage 51 to 54, a treatment known to have a feminizing action (12). At stage 56, we observed that MIS mRNA gonadal expression was inhibited in individuals exposed to E_2 at 100 μg/L, whereas it was not modified in the case of exposure to 1 μg/mL E_2 (Figure 4B). The inhibition of MIS mRNA expression was in agreement with the feminizing effect observed only in response to a high dose of E_2. Indeed, sex-reversed individuals expressed a high level of aromatase and the meiosis entry marker DMC1 (Figure 4B).

This work is the first to describe MIS in an amphibian species. Furthermore, our findings show that *P. waltl* is an original vertebrate in which males maintain Müllerian ducts despite the expression of MIS. These are new data following those showing the existence of MIS in medaka, a species without Müllerian ducts. Thus, it appears that the primitive function of MIS was to play a role in testis

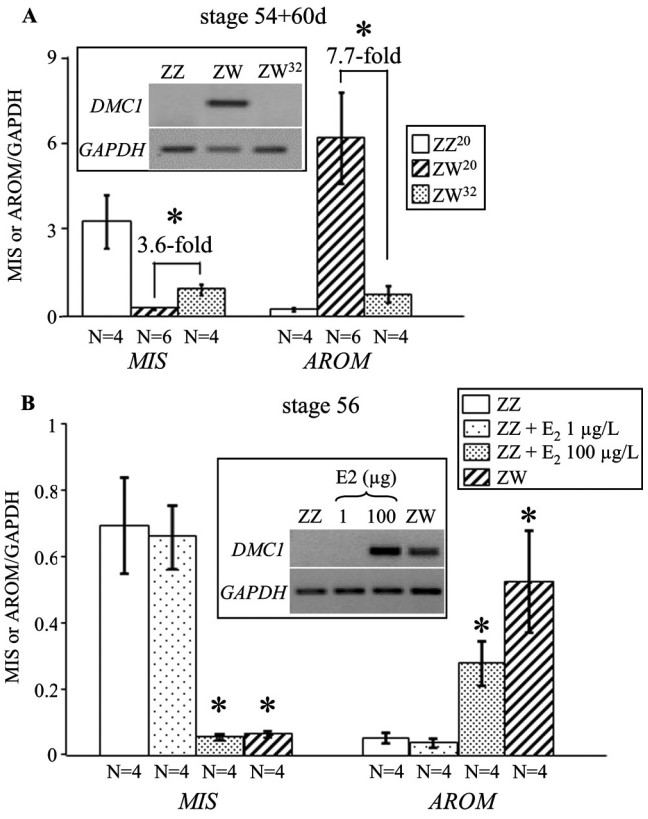

Figure 4. MIS mRNA expression in the case of sex reversal. A, Real-time PCR analysis of MIS and aromatase (AROM) expression at stage 54+60d in gonads of control larvae reared at 20°C (genetic males ZZ^{20}, genetic females ZW^{20}) and genetic female larvae reared at 32°C (ZW^{32}).*, Significantly different from control females (ZW^{20}) ($P < .05$). B, Real-time PCR analysis of MIS and aromatase expression at stage 56 in gonads of control larvae (genetic males ZZ, genetic females ZW) and E_2 benzoate-treated genetic male larvae (ZZ + E_2). *, Significantly different from control males (ZZ). The insets show the expression of the meiosis entry marker DMC1 that is specific of ovarian differentiation at this stage.

differentiation and that its property to induce Müllerian duct regression was probably acquired later during the course of evolution. The timing of MIS-increased expression in the differentiating testis of *P. waltl* could be related to a role in the control of germ cell proliferation as recently highlighted in the medaka (9–11). The production of MIS in *P. waltl* testis could also explain why experimental parabiosis associating genetically male and female embryos can mimic the well-known freemartinism observed in cattle (23–26). In the latter, the masculinized XX (genetically female) embryo is thought to arise due to the passage of MIS from the male (XY) twin via placental anastomoses. In a *P. waltl* parabiosis model, in the case of ZZ/ZW associations, at stage 54+60d, germ cell numbers in the ZW gonad were similar to those observed in the ZZ gonad and 2-fold lower than in a control ZW gonad (26). Recombinant MIS from *P. waltl* could be used in organotypic cultures of gonads to test the role of the hormone on

germ cell proliferation. It might be interesting now to study MIS receptors in *P. waltl* because an expression in the gonad would be in agreement with a role in gonad differentiation. It might be also interesting to determine whether other members of the Caudata are also MISsed amphibians.

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A.C. advised on molecular biology. D.C. contributed to the cloning, sex-reversing treatments, and the RT-PCR studies. H.D. contributed to the cloning. I.A-A. performed all the studies except the Western blotting and performed the statistical analysis. J.-Y.P. provided antibodies, and N.d.C. performed the Western blot studies. S.F. supervised the studies, performed organotypic cultures, harvested tissues, and wrote the manuscript. All authors performed critical reading of the manuscript.

Disclosure Summary: The authors have nothing to disclose.

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