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Opposite roles of *DMRT1* and its W-linked paralogue, *DM-W*, in sexual dimorphism of *Xenopus laevis*: implications of a ZZ/ZW-type sex-determining system

Shin Yoshimoto[1], Nozomi Ikeda[1], Yumi Izutsu[2], Tadayoshi Shiba[1], Nobuhiko Takamatsu[1] and Michihiko Ito[1,*]

SUMMARY

A Y-linked gene, *DMY/dmrt1bY*, in teleost fish medka and a Z-linked gene, *DMRT1*, in chicken are both required for male sex determination. We recently isolated a W-linked gene, *DM-W*, as a paralogue of *DMRT1* in *Xenopus laevis*, which has a ZZ/ZW-type sex-determining system. The DNA-binding domain of DM-W shows high sequence identity with that of DMRT1, but DM-W has no significant sequence similarity with the transactivation domain of DMRT1. Here, we first show colocalization of DM-W and DMRT1 in the somatic cells surrounding primordial germ cells in ZW gonad during sex determination. We next examined characteristics of DM-W and DMRT1 as a transcription factor in vitro. DM-W and DMRT1 shared a DNA-binding sequence. Importantly, DM-W dose-dependently antagonized the transcriptional activity of DMRT1 on a DMRT1-driven luciferase reporter system in 293 cells. We also examined roles of DM-W or DMRT1 in gonadal formation. Some transgenic ZW tadpoles bearing a DM-W knockdown vector had gonads with a testicular structure, and two developed into frogs with testicular gonads. Ectopic DMRT1 induced primary testicular development in some ZW individuals. These observations indicated that DM-W and DMRT1 could have opposite functions in the sex determination. Our findings support a novel model for a ZZ/ZW-type system in which DM-W directs female sex as a sex-determining gene, by antagonizing DMRT1. Additionally, they suggest that *DM-W* diverged from *DMRT1* as a dominant-negative type gene, i.e. as a 'neofunctionalization' gene for the ZZ/ZW-type system. Finally, we discuss a conserved role of DMRT1 in testis formation during vertebrate evolution.

KEY WORDS: Sex determination, DMRT1, Ovary, Amphibian, Transactivation, Neofunctionalization

INTRODUCTION

In the genetic sex-determining system of vertebrates, heterogametic sex chromosomes determine the male (XY) or female (ZW) fate. In the XX/XY sex-determining system, the Y-linked *SRY* gene of most mammals and the *DMY/dmrt1bY* genes of the teleost fish medaka act as sex-determining genes that trigger formation of the testis, leading to male sexual development (Sinclair et al., 1990; Koopman et al., 1991; Matsuda et al., 2002; Nanda et al., 2002; Matsuda et al., 2007). By contrast, the molecular mechanism for the ZZ/ZW sex-determining system remains largely unclear, although recent work suggested the chicken Z-linked *DMRT1* gene as a sex (male) sex-determining gene (Smith et al., 2009).

We recently isolated a female genome-specific (W-linked) gene, *DM-W*, in the South African clawed frog *Xenopus laevis* (Yoshimoto et al., 2008). Although *X. laevis* uses the ZZ/ZW system (Chang and Witschi, 1956), little has been published on the molecular mechanisms of sex determination and primary gonadal differentiation in this animal. *DM-W* is a paralogue of *DMRT1* (Yoshimoto et al., 2008), which encodes a transcription factor involved in testis formation in non-mammalian vertebrates (Ferguson-Smith, 2007; Smith et al., 2009). The testis-forming *DMY/dmrt1bY* gene of the medaka fish is a co-orthologue (or paralogue) of *DMRT1* (Matsuda et al., 2002; Nanda et al., 2002; Matsuda et al., 2007). In the chicken, which has the ZZ/ZW system, the Z-linked *DMRT1* is required for male sex determination, as described above, indicating that its gene dose may induce male development. In *X. laevis*, we showed that *DM-W* is expressed transiently during sex determination, and that some transgenic ZZ tadpoles carrying DM-W expression vectors have primary ovarian structures (Yoshimoto et al., 2008). Moreover, exogenous DM-W in ZZ gonads upregulates the expression of at least two ZW gonad-specific genes, *Foxl2* and the aromatase gene *Cyp19*, during and after sex determination (Okada et al., 2009). These results suggest that the W-linked *DM-W* is a candidate for a sex (ovary)-determining gene for the ZZ/ZW-type system in *X. laevis*.

Although the DNA-binding domains of DM-W and DMRT1 share high sequence identity, their C-terminal regions have no significant sequence similarity (Yoshimoto et al., 2008). The C-terminal region of DMRT1 contains a transactivation domain (Yoshimoto et al., 2006), suggesting that DM-W functions as a competitor of DMRT1 for its target genes in the primordial ZW gonads. Interestingly, *DM-W* mRNA is more abundant than *DMRT1* mRNA in the ZW gonads early in sex determination (Yoshimoto et al., 2008). Here, to clarify the functional relationship between DM-W and DMRT1 in sex determination and gonadal development, we analyzed transgenic *X. laevis* carrying a DM-W knockdown or DMRT1 expression plasmid, and examined the effect of DM-W on the transcriptional activity of DMRT1 in vitro. Our findings lead us to propose a novel molecular mechanism of sex determination, in which DM-W and DMRT1 mediate a ZZ/ZW sex-determining system.

[1]Department of Bioscience, School of Science, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan. [2]Department of Biology, Faculty of Science, Niigata University, Nishiku Igarashi 8050, Niigata 950-2181, Japan.

*Author for correspondence (ito@sci.kitasato-u.ac.jp)

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MATERIALS AND METHODS

Plasmid constructs

For the bacterial expression vectors, pET34b-DM-W(122-194) and pMAL1-DMRT1(130-336) were constructed by inserting the region encoding residues from 122 to 194 of DM-W into pET34b (Novagen) and residues from 130 to 336 of DMRT1 into pMAL1 (New England Biolabs), respectively. The pcDNA3-myc or pEF1/GST2 plasmid was constructed by inserting the sequence encoding the myc epitope or glutathione-S-transferase into pcDNA3 or pEF1 (Invitrogen), respectively. For the DMRT1 and DM-W vertebrate expression vectors, pcDNA3-myc-DMRT1 or -DM-W, pcDNA3-FLAG-DM-W, and pEF/GST2-DMRT1 or -DM-W were constructed by inserting the ORF of DMRT1 or DM-W in-frame downstream of the tag sequence of the pcDNA3-myc, pcDNA3-FLAG (Ito et al., 1999) and pEF1/GST2 vectors, respectively. The luciferase reporter plasmid p4xDMRT-luc was constructed by inserting double-stranded oligonucleotides (5′-AAGCTT**TTGATACATTGTTGC**GAGAT**TTGATACATTGTTGC**GAGAT**TTGATACATTGTTGC**CTACC**TTGATACATTGTTGC**-3′), including four copies of 5′-TTGATACATTGTTGC-3′ (bold), into pLuc-MCS (Stratagene). For the *DM-W* knockdown vectors, we first amplified the *X. tropicalis* U6 promoter using 5′-CCCGTGTGGCAGATGATTGTTACCT-3′ and 5′-GAAACTCCAGCGAGCAGCACC-3′ as primers, and replaced the H1.2 promoter with the U6 promoter in pRNATin-H1.2/Hygro (Invitrogen). Next, the double-stranded oligonucleotides corresponding to 5′-CCGTAAGTTGGAGTACAGATATAGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCAAA-3′, 5′-CCGTAAATCCATCCTTGTTGGAGTTTGATATCCGACTTCAACGAGGATGGGTTTATTTTTTCCAAA-3′ and 5′-CCGTCATCGCAGACATCATATTGATTGATATCCGTCAGTATGGTGTCTGTGATGATTTTTTCCAAA-3′ were inserted into the *Bam*HI and *Hin*dIII sites of the resultant vector to generate psh-control(EGFP), psh-DM-W1 and psh-DM-W2, respectively.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using antisense RNA probes for *DMRT1* (nucleotides 598-1287) or *DM-W* (nucleotides 15-679), as described previously (Yoshimoto et al., 2008).

Production of antibodies and immunohistochemistry

The recombinant proteins for DM-W and DMRT1 were produced in *E. coli* BL21(DE3) harbouring pPET34b-DM-W(122-194) or pMAL1-DMRT1(130-336), and purified following the manufacturer's instructions. Each purified protein was injected into kbl:kw rabbits to raise an anti-DM-W or -DMRT1 antiserum. Each antibody was purified by affinity chromatography using the corresponding recombinant protein as the antigen. Gonads were fixed in Bouin's solution, dehydrated with methanol, embedded in paraffin wax and cut into 7 μm sections. Sections of primordial gonads at stage 50 were incubated with the anti-DM-W antibody, followed by treatment with an Alexa 488-conjugated secondary anti-rabbit antibody (Invitrogen). They were then incubated with a rhodamine-conjugated anti-DMRT1 antibody, which was produced using the EZ-label Rhodamine Protein Labelling kit (Pierce), and examined by fluorescence microscopy BZ-8000 (Keyence). Alternatively, the sections were incubated with the anti-DM-W or -DMRT1 antibody, followed by treatment with Alexa488 and/or Alexa546-conjugated anti-rabbit and -mouse antibodies.

Electrophoretic mobility shift assay (EMSA)

The two oligonucleotides, 5′-TCGAGATTTGATACATTGTTG-3′ and 5′-GGGCAACAATGTATCAAATCTCGA-3′ were annealed and labelled with ^{32}P, using the Klenow fragment. The DM-W or DMRT1 protein was produced by TNT Quick Coupled Transcription/Translation System (Promega), using pcDNA3-FLAG-DM-W or pcDNA3-Myc-DMRT1. The resultant DNA and protein were mixed in a reaction buffer [10 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM spermidine, 0.075% TritonX-100, 5 mM spermidine, 5 mM dithiothreitol, 1 μg/ml poly (dI/dC), 0.01% bovine serum albumin, 10% glycerol], and incubated at room temperature for 20 minutes. The samples were resolved by electrophoresis and subjected to autoradiography.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy mini kit (Qiagen) from the primordial gonads and other tissues, and was reverse transcribed with PowerScript (Clontech). PCR was carried out using the resultant first-strand cDNA as a template and specific primer pairs for the target genes, as follows: *DM-W*, 5′-CATTGCAAAGACAGCAAGCT-3′ and 5′-TCTGTGTTGCAGCATCAGCA-3′ following 5′-GAAGCTGGACTGCAGTAACT-3′ and 5′-AGACTACTAGACGAGGAGTG-3′; *DMRT1*, 5′-ATCACAGAAACCATCCAGCTG-3′ and 5′-TGGGTGGAGAAAGCACACTT-3′ following 5′-TACACAGACAACCAGCACAC-3′ and 5′-TGGGTGGAGAAAGCACACTT-3′; *Sox9*, 5′-AAGTCCACTCTCCTGGAGAA-3′ and 5′-CAGGTTGAAGGAGCTGTAGT-3′ following 5′-AAGTCCACTCTCCTGGAGAA-3′ and 5′-GAGTAGAGACCAGAATTCTG-3′; *P450cArom*, 5′-CCAGCTTATTGCATGGGACT-3′ and 5′-GTCTGGTTTCAGAAGCAGAG-3′; *xSox3*, 5′-GCTGGACTAATGGCGCTTAT-3′ and 5′-TTGGTGTACACTGTGCAGTC-3′; *xVLG-1* (*vasa*-like gene-1), 5′-GCTGCCAGAGGCTTGGATAT-3′ and 5′-ACTTGCTCTGCTTGCGATTG-3′; *xmegs* (meiotic meta-phase expressing gene in spermatogenesis), 5′-TCCGTGGTGTCCCAAATTCA-3′ and 5′-TATTGCTGATCCCATGGTCCT-3′ following 5′-TCCGTGGTGTCCCAAATTCA-3′ and 5′-ATTGCCTCAGGAAGGTTTGC-3′; *SP4* (sperm-specific protein 4), 5′-AATGAGCAAAGTGAGTGGCG-3′ and 5′-TGTGGACTTTGGATGTTGCG-3′; *EF-1O* (oocyte-specific form of elongation factor-1), 5′-TGCATCATGAGGCCTTGCAA-3′ and 5′-GCTTCTTGCCACTTCTTCTG-3′. As controls, *EF-1S* (somatic form of elongation factor-1) or *ODC-1* (ornithine decarboxylase 1) gene expression was examined by PCR using specific primers as follows: 5′-CCAGATTGGTGCTGGATATG-3′ and 5′-TTCTGAGCAGACTTTGTGAC-3′ for the amplification of *EF-1S*, or 5′-GGTTCAGAGGATGTGAAACT-3′ and 5′-CATTGGCAGCATCTTCTTCA-3′ for the amplification of *ODC* mRNA.

Determination of the ZW or ZZ status of individual animals

Genomic DNA was isolated from the tail of a tadpole or liver of an adult frog, and PCR was carried out to amplify the *DM-W* gene, to determine the ZW type, as described previously (Yoshimoto et al., 2008).

Luciferase reporter assays

Human 293 cells (3×10^3 cells) were co-transfected with the luciferase reporter plasmid p4×DMRT-luc (500 ng) and the expression vector for pcDNA3-FLAG-DMRT1 and/or pcDNA3-FLAG-DM-W, as indicated, together with the *Renilla* luciferase vector, pRL-SV40 (10 ng), using TransIT-LT1 (Mirus), according to the manufacturer's procedure. The total DNA was kept at 700 ng per transfection with pcDNA3-FLAG empty vector. After 24 hours, the luciferase activities were measured. Each firefly luciferase activity was normalized to the *Renilla* luciferase activity using the dual luciferase assay system (Promega).

Production of transgenic *X. laevis*

Transgenic tadpoles or frogs were produced using restriction-enzyme-mediated integration (REMI), as described previously (Yoshimoto et al., 2006). To detect the transgenes, PCR was performed using the genomic DNA from the liver of an individual frog or tadpole as the template, and specific primer pairs as follows: pcDNA3-FLAG-DMRT1, 5′-CAGCCAGGATTCTGGAGTTA-3′ and 5′-CAACTAGAAGGCACAGTCG-3′; psh-DM-W1/2, 5′-CCCGTGTGGCAGATGATTGTTACCT-3′ and 5′-TTTAGAAGGCACAGTCGAGG-3′. We confirmed that DNA fragments of the predicted length were not produced in the PCR reaction using the primer pairs described above and genomic DNA from the wild-type ZZ or ZW liver.

Animal care and use

The Institutional Animal Care and Use Committee of Kitasato University approved all experimental procedures involving *X. laevis*.

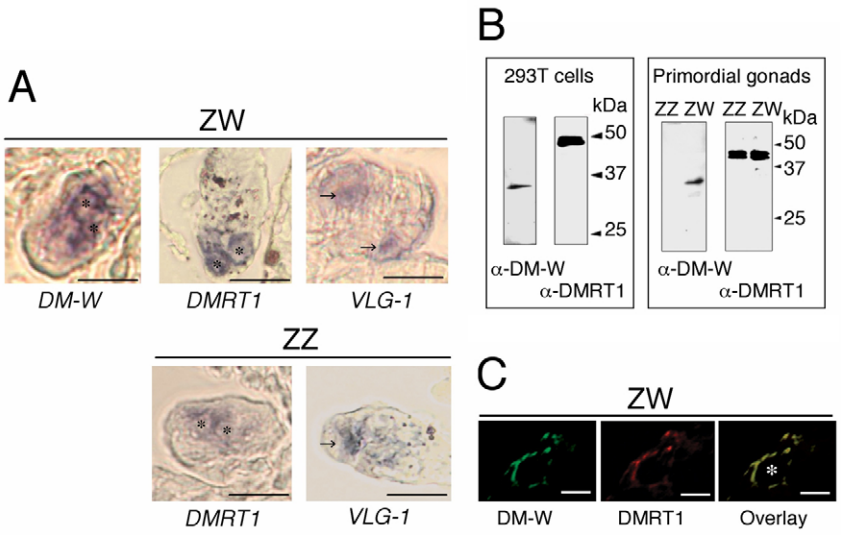

Fig. 1. Colocalization of the DM-W and DMRT1 proteins in somatic cells surrounding the PGCs of the ZW gonad at stage 50. (**A**) In situ hybridization analyses of *DM-W*, *DMRT1* and *XVLG-1* in cross-sections of primordial ZW (upper) or ZZ (lower) gonads at stage 50. Asterisks indicate primordial germ cells (PGCs). Scale bars: 5 μm. (**B**) Western blot analysis using the anti-DM-W and anti-DMRT1 antibodies. 293T cells were transiently transfected with pcDNA3-FLAG-DM-W or pcDNA3-FLAG-DMRT1. The extracts of the 293 cells (left) and primordial ZZ and ZW gonads (stages 50-52) (right) were examined by immunoblotting with each antibody followed by an HRP-conjugated anti-rabbit antibody. DM-W was only detected in the ZW samples. (**C**) Immunofluorescence analysis using anti-DM-W and anti-DMRT1 antibodies on cross-sections of primordial ZW gonads at stage 50. Scale bars: 2 μm. Asterisk indicates a PGC.

RESULTS

DM-W and DMRT1 were colocalized in the somatic cells surrounding primordial germ cells (PGCs) in ZW gonads during sex determination

DM-W and DMRT1 were specifically expressed in primordial gonads (Yoshimoto et al., 2008). Therefore, we first examined the DM-W- or DMRT1-expressing cells in tissue sections following whole-mount in situ hybridization performed at the sex-determining stage (stage 50). DM-W and DMRT1 were expressed in the somatic cells surrounding the primordial germ cells (PGCs) of the gonads (Fig. 1A). We next examined localization of DM-W and DMRT1 by immunohistochemistry using anti-DM-W and anti-DMRT1 antibodies (see Materials and methods). The specificity of the antibodies to DM-W and DMRT1 was confirmed by Western blot analysis using the extracts of primordial ZZ and ZW gonads (Fig. 1B). Fig. 1C indicated that both DM-W and DMRT1 were specifically expressed and colocalized in the somatic cells surrounding PGCs in a ZW gonad at stage 50 during sex determination, whereas in a ZZ gonad at the same stage, only DMRT1 expression showed the same pattern.

DMRT1 interacts with the same DNA sequence as DM-W in vitro

The DNA-binding domain of DMRT1 has high sequence identity (89%) with that of DM-W (Yoshimoto et al., 2008). We then examined whether DMRT1 and DM-W could bind the same DNA sequence by an in vitro binding analysis using the mammalian DMRT1-binding DNA cis element (Murphy et al., 2007). An electrophoretic mobility-shift (EMSA) assay using an in vitro transcription/translation product for FLAG-tagged DM-W indicated that two shifted bands were detected, which might be derived from a monomer and a dimer of DM-W interacting with the ^{32}P-labeled probe. The bands were supershifted by an anti-FLAG antibody (Fig. 2A). We also detected two shifted bands corresponding to complexes between Myc-tagged DMRT1 and the probe. Similarly, an anti-Myc antibody supershifted them (Fig. 2A). When excess unlabeled probe was added to the DNA-protein binding reaction as a competitor, the complex formation between DM-W or DMRT1 and the labeled probe decreased in a dose-dependent manner (see Fig. S1 in the supplementary material). These results indicted that DM-W and DMRT1 bound to the same DNA element in vitro.

DM-W can antagonize the transcriptional activity of DMRT1

DM-W has no significant sequence similarity with the transactivation domain of DMRT1 (Yoshimoto et al., 2008). Then we next examined the effects of DM-W on the transcriptional activity of DMRT1 using a luciferase reporter plasmid carrying four copies of the DMRT1-binding DNA cis element (Murphy et al., 2007). The 293 cells were transiently transfected with the reporter or empty reporter plasmid and the expression plasmid for DM-W and/or DMRT1 (Fig. 2B). The luciferase activity was enhanced by the expression of DMRT1 in a dose-dependent manner. By contrast, DM-W expression did not increase the promoter activity. Rather, the DMRT1-driven transcriptional activity decreased with DM-W expression, in a dose-dependent manner.

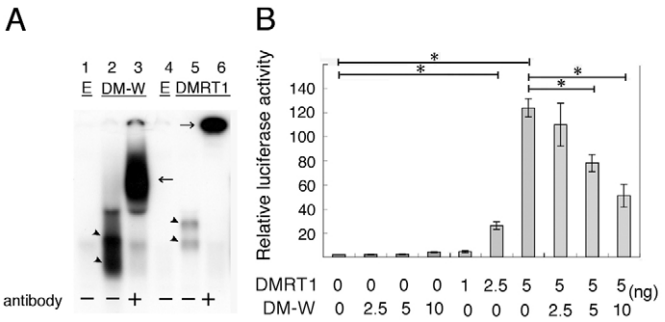

Fig. 2. DM-W can antagonize the transcriptional activity of DMRT1. (**A**) Electrophoretic mobility shift assay (EMSA) of in vitro translated DM-W and DMRT1. The in vitro translated product of FLAG-tagged DM-W (lane 2) or Myc-tagged DMRT1 (lane 5) and the ^{32}P-labeled DMRT1-binding sequence were analyzed on a native gel. Supershift analyses were performed using antibodies against FLAG (lane 3) and Myc (lane 6). The arrowheads show the predicted positions of DM-W or DMRT1 and the DNA complexes. The arrows indicate the predicted positions of the supershifted bands. (**B**) Effect of DM-W on the transcriptional activity of DMRT1 in transfected 293 cells. The luciferase reporter plasmid used included four copies of the DMRT1-binding sequence as described in the Materials and methods. Relative luciferase activity is shown as the fold increase compared with the value obtained with 200 ng of pcDNA3-FLAG empty vector. The data represent the mean ± s.e.m. from three separate experiments. *P<0.01.

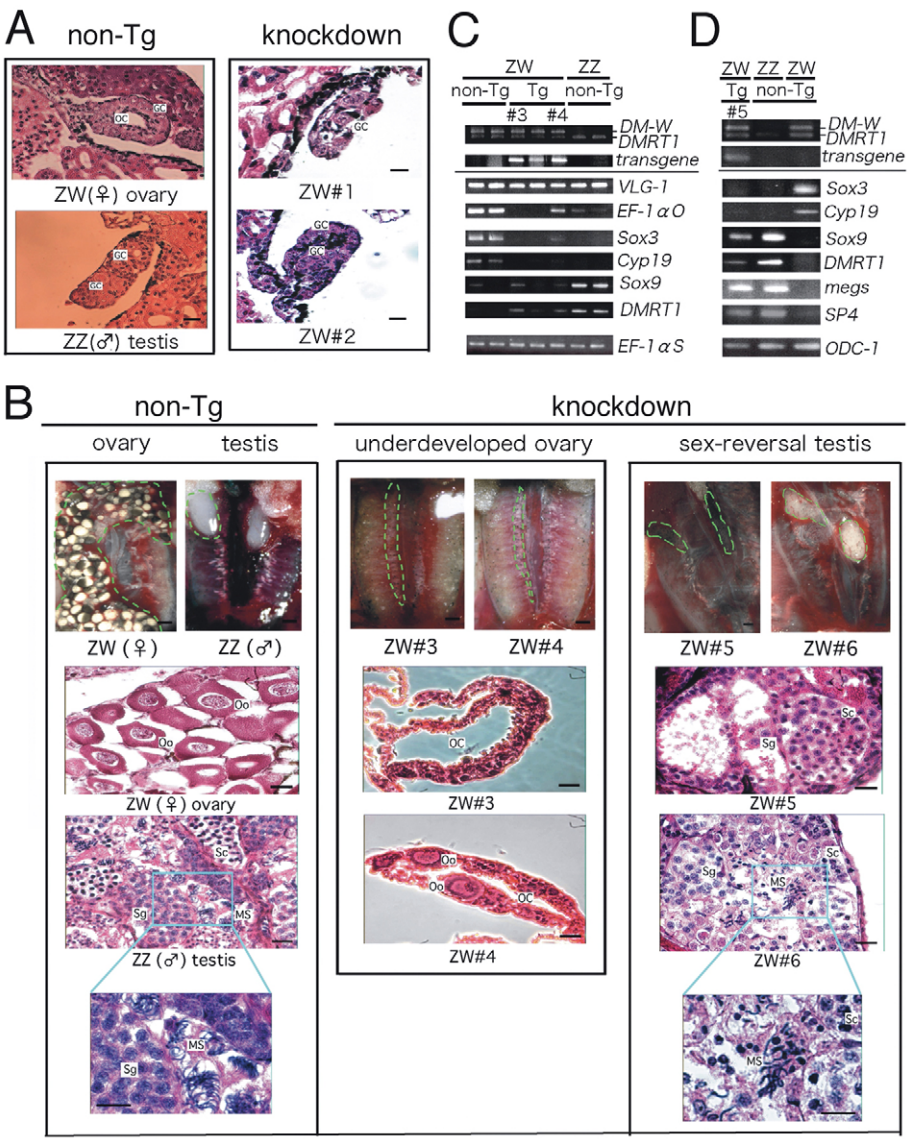

Fig. 3. Analyses of transgenic *X. laevis* individuals carrying the knockdown vector for *DM-W*. (**A**) Sections of ovarian gonads from two non-transgenic and two transgenic (knockdown) ZW tadpoles carrying psh-DM-W1 and -W2, at stage 56. GC, germ cell; OC, ovarian cavity. Scale bars: 20 μm. (**B**) Non-transgenic normal gonads (left), and underdeveloped ovaries (middle) and testicular gonads (right) of transgenic ZW frogs six months after metamorphosis. The gonad is within the dotted line. MS, mature sperm; OC, ovarian cavity; Oo, oocyte; Sc, spermatocyte; Sg, spermatogonium. Scale bars: 0.5 mm (upper); 20 μm (middle and lower). (**C**,**D**) RT-PCR of gonadal marker genes in normal ZW ovary, ZZ testis and transgenic ZW (#3-#5) gonads. ZW#3-#5 correspond to the individual animals in B. Genomic PCRs for the transgene insertion and genetic sex are shown in the upper two panels. The data in C and D are from two or one non-transgenic ZZ and ZW individuals, and three or one transgenic ZW individuals. The marker genes were as follows. *VLG-1*, germ cells; *EF-1O*, oocytes; *Sox3* and *P450cArom*, ovary-specific cells; *DMRT1* and *Sox9*, testis-specific cells; *megs* and *SP4*, spermatogenic cells; *ODC-1* and *EF-1S*, control.

DM-W knockdown in ZW individuals can induce male development

To examine the role of DM-W in ovary formation in *X. laevis*, we tried to perform a loss-of-function analysis of DM-W in ZW individuals using RNA interference (RNAi). Li and Rohrer (Li and Rohrer, 2006) have reported transgenic-based RNAi using U6 promoter-driven short hairpin (sh) RNA vector. Similarly, we constructed the shRNA-type knockdown vectors for the three sequences, and confirmed high knockdown efficiency in the mixture of psh-DM-W1 and -W2 in *Xenopus* A6 cells (see Fig. S2 in the supplementary material). Using the mixture, we examined the effect of *DM-W* downregulation on the primary sex differentiation in ZW individuals. At the time of sex determination (stage 50), some of the transgenic ZW tadpoles retained the endogeneous level for *DM-W*, and others showed a little or great decrease of DM-W expression (see Fig. S3 in the supplementary material). During primary gonadal differentiation, the germ cells propagated and differentiated in the medulla of the ZZ gonads, whereas in the non-transgenic ZW gonads, the germ cells, including the oogonia and oocytes, remained in the cortex around the ovarian cavities. Intriguingly, three out of the 11 transgenic ZW tadpoles did not form an ovarian cavity, and their germ cells were located in the medullar region of the gonads (Fig. 3A); eight of the transgenic ZW tadpoles showed normal ovarian development. The control ZZ and ZW tadpoles bearing the knockdown vector for an enhanced green fluorescence protein gene had developing gonads with normal testicular and ovarian structures, respectively (see Fig. S4 and Table S1 in the supplementary material).

We next evaluated the effects of the *DM-W* knockdown in adults at 6 months post-metamorphosis. We produced a total of 135 adults (83 ZW and 52 ZZ) of which 66 (38 ZW and 28 ZZ) were transgenic. Of the 45 non-transgenic ZW individuals, 42 had normal ovaries with mature oocytes (Table 1). By contrast, 10 out of 38 transgenic ZW individuals had morphologically abnormal gonads. The gonads of five of these frogs had almost completely regressed and contained no germ cells (data not shown); the other five frogs had underdeveloped ovaries with few or no oocytes (#3 and #4 in Fig. 3B). Most of the transgenic and non-transgenic ZZ individuals had normal testes, and the negative-control ZW frogs carrying the empty knockdown vector had normal ovaries.

Table 1. Transgenic *X. laevis* carrying the DM-W knockdown vector

Genetic sex	ZZ	ZZ	ZW	ZW
Transgenesis	+	–	+	–
Number of adults	28	24	38	45
Number of individuals with underdeveloped gonads (%)	3 (10.7%)	4 (16.7%)	10 (26%)	3 (7%)
Number of individuals with sex-reversed gonads (%)	0 (0%)	0 (0%)	2 (5%)	0 (0%)

The genetic sex and transgenesis were determined by the PCR detection of *DM-W* and transgenes, using genomic DNA from the liver, as described in the Materials and methods. psh-DM-W1/2 vectors were used for the knockdown of *DM-W*.

RT-PCR revealed that the underdeveloped ovaries of the ZW transgenics (#3 and #4) expressed transcripts of the germ-cell marker *VLG-1*, and a higher level of *DMRT1* than normal ZW ovaries. We previously showed that *Sox3* is expressed at high levels expressed in the immature ovary (Koyano et al., 1997). Here, *Sox3* mRNA was downregulated in the ZW transgenic gonads, compared with normal ZW ovaries (Fig. 3C). More importantly, the gonads of two transgenic ZW frogs had a morphologically testicular structure, indicating that the *DM-W* knockdown in ZW frogs could cause male development. One developed both incomplete and complete seminiferous tubules containing spermatogonia and spermatocytes (ZW#5 in Fig. 3B). This gonad expressed fewer transcripts of *Sox3* and *Cyp19*, the estrogen synthase (aromatase) gene, than did normal ZW ovaries, but more mRNAs of *DMRT*, *Sox9* and the spermatogenic-cell markers, *megs* (Ikema et al., 2002) and *SP4* (Hiyoshi et al., 1991) (Fig. 3D). The other ZW frog developed complete seminiferous tubules with some sperm cells (ZW#6 in Fig. 3B). We could not perform RT-PCR using the RNA of this testis because it had been sectioned for histological analysis. We also could not determine to what extent *DM-W* was downregulated by the knockdown vector in the primordial ZW#5 and #6 gonads during the sex-determining period. However, as shown in Fig. S3B in the supplementary material, the *DM-W* expression was almost completely repressed in the adult testis of ZW#5. By contrast, we detected some *DM-W* transcripts in the underdeveloped ovary of ZW#4, although the expression level was lower than in the normal ZW ovary. Because there were different levels of efficiency in knocking down endogenous *DM-W* among the gonads from the transgenic ZW tadpoles at the sex-determining stage (stage 50), as shown in Fig. S3A in the supplementary material, the phenotypic difference between the testicular gonads and underdeveloped ovaries might reflect the difference.

Exogenous *DMRT1* can induce primary development into testes in ZW individuals

We next examined whether DMRT1 is involved in testis formation and differentiation in *X. laevis* by producing transgenic individuals carrying a *DMRT1* expression vector behind a cytomegalovirus (CMV) promoter (pCMV-DMRT1). At about 1 month post-metamorphosis, we examined the gonads of the young adults. Of the 84 frogs used (68 ZW and 16 ZZ), 19 (15 ZW and 4 ZZ) carried the transgene. All four of the transgenic ZZ individuals had normal developing testes just like those of the non-transgenic ZZ individuals. As shown in Fig. 4, the wild-type testis contained some developing seminiferous tubules in the medula region, whereas the wild-type developing ovary had an ovarian cavity and some cysts involving a large number of oocytes in the cortex region. Interestingly, eight of the 15 ZW transgenic individuals developed gonads with both testicular and ovarian structures. About half of the transverse sections of the 15 ZW transgenic gonads showed no ovarian cavities or oocytes, and the germ cells were localized to both the medulla and the cortex (Fig. 4). These results suggest that exogenous *DMRT1* induced testicular structures in some ZW frogs, indicating that DMRT1 could act as a testis-forming gene. The result coincided with the finding that exogenous *DMY/dmrt1bY* induces testis formation in genetically female (XX) medaka fish (Matsusda et al., 2007), and that reduction of DMRT1 expression in vivo leads to feminization of the embryonic gonads in genetically male (ZZ) chickens (Smith et al., 2009).

DISCUSSION

DM-W, which is a paralogue of *DMRT1*, is involved in primary ovary development in *X. laevis* (Yoshimoto et al., 2008). The N-terminal region (amino acids 1-123), including the DNA-binding domain of DM-W, which is encoded by exons 2 and 3, has relatively high sequence identity (82%) with the corresponding region of DMRT1. By contrast, *DM-W* has no homologous region to exons 4-6 of *DMRT1*, which includes the sequence encoding the transactivation domain (Yoshimoto et al., 2006; Yoshimoto et al., 2008). The sequence of the *DM-W* exon 4, including an in-frame stop codon, contained no significant homology among genomic and cDNA sequences derived from vertebrate species, including *X.*

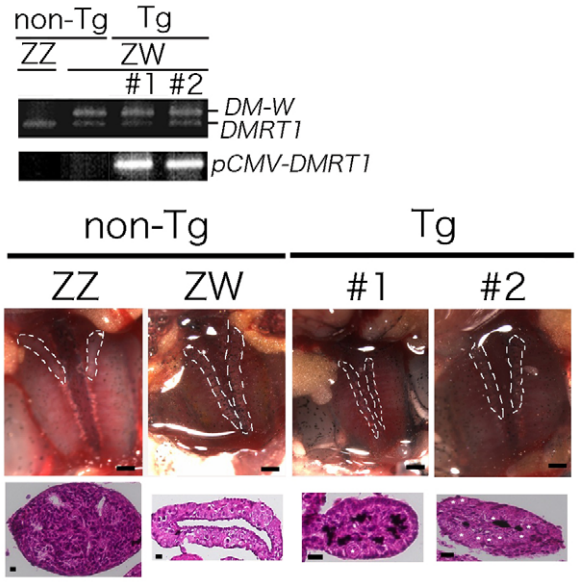

Fig. 4. Analysis of transgenic ZW frogs carrying an expression vector for *DMRT1*. Non-transgenic normal ZZ and ZW gonads (left), and transgenic ovotestis (middle and right) carrying pcDNA3-FLAG-DMRT1, 1 month after metamorphosis, are shown. Genomic PCRs for transgene insertion and genetic sex are shown in the upper two panels. The gonad is inside the dotted line. The asterisks indicate germ cells. Scale bars: 0.5 mm (upper); 20 μm (lower).

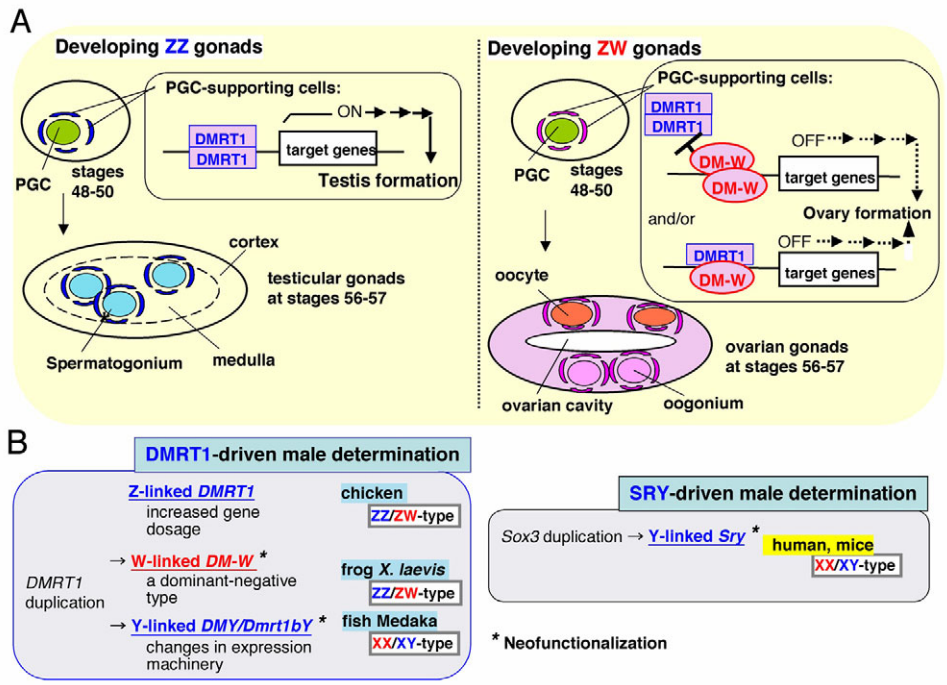

Fig. 5. Proposed model for sex determination mediated by sex chromosome-linked *DMRT1* subfamily members in vertebrates. (**A**) A model for the ZZ/ZW sex-determining system in *Xenopus laevis*. In the PGC-supporting cells of the ZZ primordial gonads during the sex-determination period (stages 48-50), DMRT1 transactivates one or more target genes that participate in testis formation. At stages 56-57, male germ cells migrate into medulla and differentiate into spermatogonia. In the ZW primordial gonads during sex determination, DM-W and DMRT1 are colocalized in the PGC-supporting cells. The DM-W homodimer and/or DM-W/DMRT1 heterodimer bind the target gene(s) for DMRT1, preventing the gene from interacting with the DMRT1 homodimer, leading to transcriptional repression. As a result, DM-W inhibits the gene cascades for testis formation, resulting in ovary formation. At stages 56-57, female germ cells, including oogonia and oocytes, remain in the cortex around the ovarian cavities, which is a hallmark of developing ovarian ZW gonads. (**B**) Sex determination mediated by sex chromosome-linked *DMRT1* subfamily members in non-mammalian vertebrates. The Y-linked *DMY/dmrt1bY*, W-linked *DM-W* and Z-linked *DMRT1* genes seem to play a crucial role in sex determination in fish medaka, frog *X. laevis* and chicken, respectively. Because *DM-W* is a dominant-negative type of a testis-forming gene *DMRT1* in *X. laevis*, *DMRT1* might be involved in male sex determination in various species of non-mammalian vertebrates. *Sry* or *DMY* and *DM-W* may have evolved from *Sox3* or *DMRT1*, respectively, as a case of 'neofunctionalization' for sex determination.

laevis and *X. tropicalis*. Therefore, we proposed that the unique exon 4 of *DM-W* might have emerged through or after a duplication of *DMRT1* in an ancestral species of *X. laevis*, resulting in the functional difference between the two protein on sex determination and/or differentiation in *X. laevis* (Yoshimoto et al., 2008). To clarify the difference, we performed expression and in vitro analyses of DM-W and DMRT1, and analyzed transgenic individuals bearing the DM-W knockdown or DMRT1 expression vector.

Immunohistochemical analysis using anti-DM-W and anti-DMRT1 antibodies revealed that DM-W and DMRT1 were colocalized in somatic cells surrounding PGCs in ZW primordial gonads at the sex-determining stage (Fig. 1C). The observation coincided with the report in medaka fish by Kobayashi et al. (Kobayashi et al., 2004) that sex-determining gene *DMY* showed specific expression in the PGC-supporting cells before morphological sex differentiation. Moreover, we found that DM-W or DMRT1 interacted with the same DNA sequence in vitro (Fig. 2A). Taken together, these findings suggest that DM-W could bind the same target gene (or an overlapping set of genes) as DMRT1 in PGC-supporting cells of primordial ZW gonads. Interestingly, two shifted bands were observed in the binding experiment between the probe DNA and DM-W or DMRT1 (Fig. 2A). Because mammalian or *Drosophila* DM domain proteins could dimerize (An et al., 1996; Erdman et al., 1996; Murphy et al., 2007), the two bands might be derived from the complexes of the probe DNA with a monomer and a dimer of DM-W or DMRT1.

DM-W antagonized the transcriptional activity of DMRT1 in the DMRT1-driven luciferase reporter assay in 293 cells (Fig. 2B), suggesting that *DM-W* could be a dominant-negative gene of *DMRT1*. In the future, it will be necessary to isolate target gene(s) for DMRT1 and examine an antagonistic role of DM-W in the transcriptional regulation of the gene(s) by DMRT1 on sex determination. Importantly, *DM-W* knockdown or ectopic expression of *DMRT1* induced testicular development in some transgenic ZW individuals (Fig. 3; Fig. 4), indicating opposite roles of DM-W and DMRT1 in sexual dimorphism. However, we also observed normal development of gonads, which coincided with the genotypes, in some transgenic individuals carrying the DM-W knockdown vector or the DMRT1 expression vector. In addition, some ZW transgenic frogs carrying the knockdown vector exhibited underdeveloped gonads (Table 1). We could not determine to what extent *DM-W* was downregulated by the knockdown vector in the primordial ZW#5 and #6 gonads during the sex-determining period, but we found that the *DM-W* expression was almost completely repressed in the adult testis of ZW#5. By contrast, we detected some *DM-W* transcripts in the underdeveloped ovary of ZW#4, although the expression level was lower than in the normal ZW ovary (see Fig. S3 in the

supplementary material). In fact, we observed that there was different efficiency of the endogenous knockdown of *DM-W* in the transgenic ZW gonads at the sex-determining stage (see Fig. S2 in the supplementary material). These findings suggest that the phenotypic difference would reflect different efficiencies of endogenous *DM-W* knockdown or different expression level of ectopic *DMRT1* in the transgenic primordial gonads at the sex-determining stage.

Overall, this study suggest that DM-W antagonizes the transcriptional regulation of some target gene(s) by DMRT1 in the primordial ZW gonads, and lead us to propose the following model, in which ZZ/ZW sex determination in *X. laevis* is mediated by the W-linked *DM-W* and the testis-forming gene *DMRT1* (Fig. 5A). In ZW primordial gonads during sex determination, a DM-W homodimer can overlie the regulatory element of target gene(s) for DMRT1, and prevent DMRT1 from interacting with its binding site. This model is based on the assumption that DM-W can compete with DMRT1 for binding to the regulatory element. This situation may result from the much higher expression of DM-W than that of DMRT1 at the early stage (stage 48) of sex determination (Yoshimoto et al., 2008); and/or, a DM-W/DMRT1 heterodimer could bind the DMRT1 target sequence, leading to transcriptional repression, which may occur when DM-W and DMRT1 are expressed as almost equal levels at the late stage (stage 50) of sex determination (Yoshimoto et al., 2008). In fact, we detected the homo- and heteromer formation of DM-W and DMRT1 in 293T cells (see Fig. S5 in the supplementary material). Anyway, DM-W inhibits the gene cascades for testis formation or anti-ovary formation, resulting in ovary formation. Thus, in our novel model of sex determination, sex is determined in some species by a gene that inhibits testis formation, i.e. an anti-testis gene.

Treating *X. laevis* larvae with estrogen causes male-to-female sex reversal (Villalpando and Merchant-Larios, 1990). Assuming that DM-W is indeed a DMRT1 antagonist (Fig. 5A), estrogen probably acts downstream of DM-W in the normal ovary-formation cascade. In fact, we recently showed that ectopic DM-W upregulates the expression of *Cyp19*, which encodes the estrogen-synthesizing enzyme P450 aromatase, and of *Foxl2* in ZZ gonads (Okada et al., 2009). DMRT1 may directly decrease *Foxl2* and/or *Cyp19* expression as a repressor, or indirectly as an activator of another factor, in ZW gonads; in either case, DM-W may function to antagonize this activity.

Some DM domain genes, including vertebrate *DMRT1* and *Drosophila double sex* are involved in sex determination and/or sexual development (Voff et al., 2003), suggesting evolutional implications mediated by DM domain proteins in sexual dimorphism in triploblastic animals. *DMRT1* might be directly involved in testicular formation or development during vertebrate evolution. Each species of non-mammalian vertebrates may have developed a different regulatory system for DMRT1 expression or function, contributing to species diversity (Fig. 5B). *DMY/Dmrt1bY*, a Y-linked co-orthologue of *DMRT1*, may have emerged as a sex (testis)-determining gene, probably by changing expression machinery for its transcript in teleost fish medaka (Otake et al., 2008). Avian *DMRT1* may have become Z-linked to exert its gene dose effect, probably leading to male development (Smith et al., 2009). *X. laevis DM-W* may have emerged on the W-chromosome following the duplication of *DMRT1*, as a sex (ovary)-determining gene for a dominant-negative regulator of DMRT1 (Fig. 5). Medaka *DMY/Dmrt1bY* and *X. laevis DM-W* may have evolved from *DMRT1* for an XX/XY-type or ZZ/ZW-type sex-determining system as a case of 'neofunctionalization'. In the neofunctionalization model of gene duplication, one copy retains the original function, and the other evolves a new function (Lynch et al., 2001). Because the sex (testis)-determining gene *Sry* evolved from *Sox3* as a case of 'neofunctionalization' during mammalian evolution (Fig. 5B), it will be intriguing to understand evolutionary relationships in vertebrates between sex-determining genes as a case of 'neofunctionalization' and sex-determining systems.

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Supplementary material
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Competing interests statement
The authors declare no competing financial interests.

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Table S1. Transgenic tadpoles carrying the knockdown control vector

Genetic sex	ZZ	ZZ	ZW	ZW
Transgenesis	+	–	+	–
Number of tadpoles	14	21	15	14
Number of individuals with abnormal or sex-reversed gonads (%)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)

A psh-control(EGFP) vector was used as a negative control of the *DM-W* knockdown experiment. The genetic sex and transgenesis of the tadpoles at stage 59 were determined by the PCR detection of *DM-W* and transgenes, using genomic DNA from the tail, as described in the Materials and methods.