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TEMPOROSPATIAL EXPRESSION OF *Dmrt1* IN CHICKEN UROGENITAL SYSTEM *(GALLUS GALLUS)* USING WHOLE MOUNT *IN SITU* HYBRIDIZATION

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Doublesex and *mab-3*-related transcription factor 1 *(Dmrt1)* is a Z-linked gene that putatively determines the phenotype of gonads in birds. The sex differential expression of *Dmrt1* was examined using whole-mount *in situ* hybridization *(WISH)* in the urogenital systems during embryogenesis. The results revealed that *Dmrt1* showed dimorphic expression in chicken gonads, which increased from day 6.5 to day 10.5. The expression of *Dmrt1* in male (ZZ) gonads was not twice as much as in female (ZW) gonads, suggesting the existence of other regulatory mechanisms in addition to Z chromosome dosage effect.

Keywords: Dmrt1 gene – whole-mount *in situ* hybridization – chicken embryos – testes – ovaries

INTRODUCTION

Sex in vertebrates is determined by various factors, such as environment and gene. The influence of gene on sex determination is accomplished at fertilization by the constitution of the sex chromosome. In mammals, the male is heterogametic (XY chromosomes), whereas the female is homogametic (XX chromosomes). The sex-determining region Y (SRY) is the testis-determining gene in mammals [8, 19]. By contrast in birds, the male is homogametic (ZZ chromosomes) while the female is heterogametic (ZW chromosomes). Sex chromosomes in birds are not homologous to those of mammals, and SRY gene has not been identified. The mechanism underlying chicken sex determination is still uncertain and two possible mechanisms have been proposed–the W chromosome may carry a dominant-acting ovary determinant [23] or it may depend upon the Z chromosome dosage (two for male, one for female). One Z-linked gene, *Dmrt1,* has received considerable attention, as it is involved in Z chromosome dosage hypothesis in avian sex determination [22].

Dmrt1 is homologous to the *Drosophila* sex regulator gene *doublesex* and the nematode worm sex regulator *mab-3* [16]. It has been identified in diverse species including mammals, chickens, monotremes, turtles, frogs and fishes [7]. Multiple alternative splicing of chicken *Dmrt1* exists in gonads [28] and each isoform has the

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same DM domain. It has been increasingly demonstrated to play an important role in sexual development in many different species [5, 20]. In *Drosophila* and worm, the homologues of DM domain, *doublesex* and *mab-3,* have male-specific functions [16]. In reptiles in which sex is determined by temperature, the expression of *Dmrt1* is upregulated at male producing temperatures when sex is being determined [6, 18]. In the medaka fish *(Oryzias latipes),* a duplicated copy of *Dmrt1, dmy/dmrt1b,* is the main testis determinant [11], and a W-linked copy, *dmw,* is involved in ovarian development in an amphibian *(Xenopus laevis)* [26]. *Dmrt1* null mutants lead to differentiation defect of germ and Sertoli cells starting at birth and finally result in a highly dysgenic testis in mouse [9]. In humans, the deletion of *Dmrt1* causes testicular dysgenesis [7, 14]. These function-oriented researches have proved a close relation between testes development and *Dmrt1* which is found to be a conserved sex related gene across different phyla.

WISH is a useful tool in development biology, which can detect specific messenger ribonucleic acids (mRNAs) within an embryo or intact tissue fragment. Currently, expression of *Dmrt1* has mainly focused on day 3 to day 7.5 by RT-PCR and *in situ* hybridization [17, 20, 21, 25]. However, the dynamic changes of *Dmrt1* in chicken urogenital systems at day 6.5 to day 10.5 have not been elucidated systematically. In our research, partial chicken *Dmrt1* sequence was cloned, then the exact expression pattern of *Dmrt1* was investigated in chicken urogenital systems at day 6.5, day 8.5 and day 10.5 using *WISH*. Meanwhile the expression of *Dmrt1* was confirmed on sections. The analysis of the data through *WISH* and the sections is expected to yield useful information on the temporospatial expression of *Dmrt1* in male and female chicken embryos.

MATERIAL AND METHODS

Animals

Fertilized white leghorn chicken eggs were obtained from the department of animal science of Huazhong agricultural university and incubated at 37.8 °C in a humid incubator. Urogenital systems of chicken embryos were isolated at days 6.5, 8.5 and 10.5 of incubation. The sex of each embryo was identified as described previously [4]. All experiments were performed in accordance with institutional guidelines for animal welfare.

Dmrt1 *antisense and sense probes synthesis*

The template RNA was extracted from chicken urogenital systems and converted into cDNA by reverse transcription (RT). Fragments *a* and *b* of *Dmrt1* (*Dmrt1* isoforms, accession number: AF123456) [28] were identified and amplified from this cDNA using anti-sense primer: 5-TATGAGGTGGGCGGGTAG-3 and sense primer:

5-CAGCAGAGGGCACTTGGA-3. The PCR cycling conditions were 35 cycles, each for 40 s at 94 °C, 40 s at 60 °C and 40 s at 72 °C in a 15 μl reaction mixture containing 0.25 mM dNTP, 0.2 μM primers, 1 μl genomic DNA and 1 U Taq polymerase (Fermentas Life Sciences, USA). Amplified DNA fragments were analyzed using 1.2% agarose gel electrophoresis and then reclaimed by a gel recovery kit (Transgen Biotech, China). Then they were cloned by a TA Cloning® dual promoter vector (pCR®II) (Invitrogen catalogue No CK2070-20, USA) and sequenced (Sangon, China). The sense and antisense probes were synthesized by SP6/T7 RNA polymerase (Fermentas, USA) and then detected using 1.2% agarose gel electrophoresis. After that, the labeling efficiency of the sense and antisense probes was detected guided by the Roche instruction manual (Roche Diagnostics).

The amplified *Dmrt1* fragments were cloned into recombinant plasmids *Dmrt1*/pCR®II. The sequence of *Dmrt1* in recombinant plasmids was 100% homologous with the chicken *Dmrt1* in GenBank database. The labeling efficiency of antisense and sense probes was detected by dot assay (data not shown).

Whole-mount in situ *hybridization*

Twelve embryonic urogenital systems (six male and six female) were collected at every stage. The samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline plus 0.1% Tween 20 (PBST) at 4 °C overnight, and then dehydrated through a series of methanol/PBST solutions (25%, 50%, 75% and 100% methanol), each for 10 min and stored at –20 °C until hybridization. The *WISH* protocol [1, 24] was used with the following modifications: (1) the samples were treated with 20 μg/ml proteinase K in PBST for 10 min at room temperature. (2) the samples were examined every 15 min during coloration and then rinsed twice in NTMT (100 mmol/L Tris-HCl, pH 9.5, 50 mmol/L $MgCl_2$, 100 mmol/L NaCl, 0.1% Triton X-100) for 10 min. The time of coloration was the same for all the samples. Then they were rinsed with PBST for at least six times to stop the staining reaction and photographed in a stereomicroscope (LEICA MZ 75). All the samples were photographed under the same microscope parameters.

Microscopic analysis of hybridization signal on sections

To identify further the hybridization signal, the urogenital systems after hybridization were cut into sections and observed under the inverted fluorescence microscope (Nikon, Japan). The urogenital systems were selected and fixed in 4% PFA overnight, then routinely dehydrated in an ethanol series, subsequently embedded in paraffin. After that, the samples were cut to 4 μm serial sections and photographed with a camera (Canon G11).

RESULTS

The expression of Dmrt1 *in chicken urogenital system*

The expression of *Dmrt1* at different stages was displayed in Figure 1. At day 6.5, *Dmrt1* was expressed higher in male gonads than female (Fig. 1 A1–B1). However, at day 8.5 and day 10.5, the expression of *Dmrt1* was stronger in male gonads than female but not notably (Fig. 1 A2–B2, A3–B3). There was nearly no signal in the urogenital systems hybridized with sense probe (Fig. 1 C1–D1, C2–D2, C3–D3). *Dmrt1* was not only expressed in gonads but also on the surface of mesonephros.

The microscopic observation in sections of urogenital systems after hybridization confirmed the results of *WISH* further. We could observe apparent hybridization signal in gonads and on the surface of mesonephros. The intensity of signal was higher

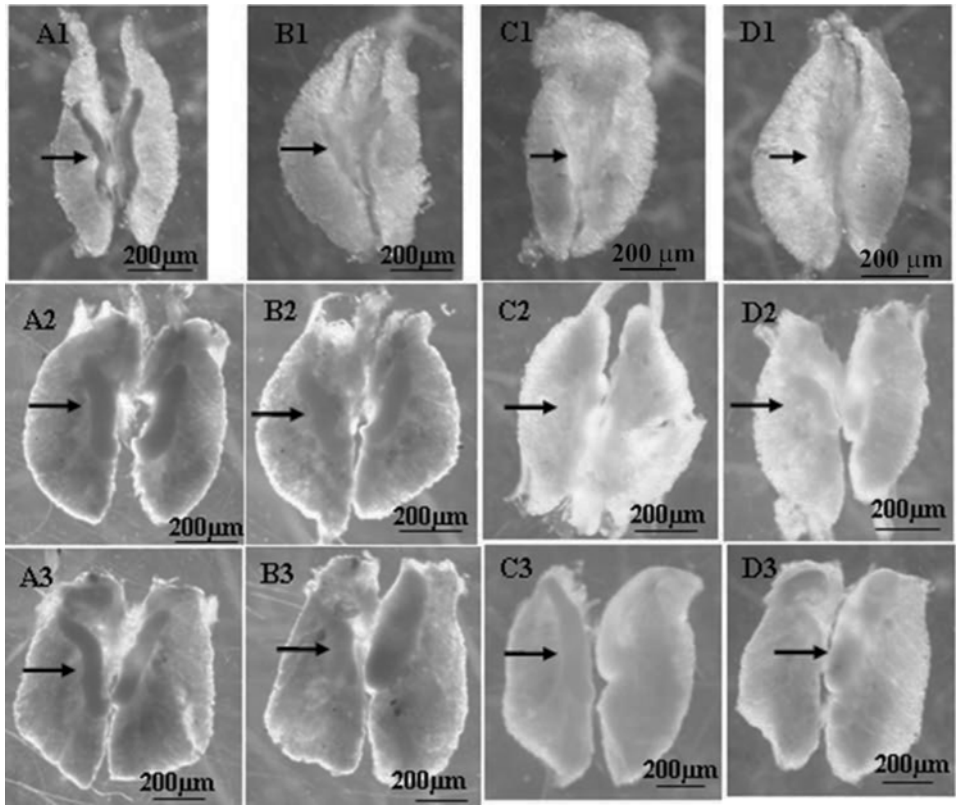

Fig. 1. Dmrt1 mRNA expression in chicken urogenital systems at day 6.5, day 8.5 and day 10.5. A1–A3: male embryos, detected by RNA antisense probe; B1–B3: female embryos, detected by RNA antisense probe; C1–C3: male embryos, detected by RNA sense probe; D1–D3: female embryos, detected by RNA sense probe. *Dmrt1* was expressed in both gonads and mesonephros hybridized with antisense probe. There was nearly no signal in the urogenital systems hybridized with sense probe. The dashed areas and arrows represented gonads and beside them were mesonephros

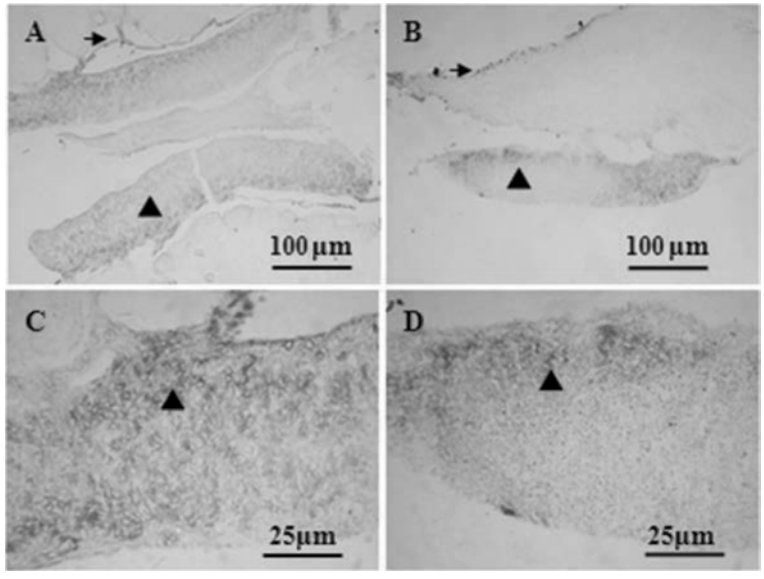

Fig. 2. The sections of male (A) and female (B) urogenital systems hybridized with antisense probe at day 6.5, showing hybridization signal in gonads (triangle) and on the surface of mesonephros (arrow). C was the amplification of A, D was the amplification of B

in testes than ovaries at different stages. It was nearly blank in the sections of urogenital systems hybridized with sense probe. Figure 2 showed the hybridization signal in the sections of male and female urogenital systems hybridized with antisense probe at day 6.5.

DISCUSSION

In this study, both the results of *WISH* and microscopic analysis showed that *Dmrt1* had sexually dimorphic expression in chicken embryos and the expression of *Dmrt1* was higher in male than in female urogenital systems at day 6.5 to day 10.5. Smith et al. [21] found similar results in mouse. Although McQueen and Clinton [12] who defended the existence of incomplete dosage compensation, our results seemed to agree with most of studies [2, 3, 28] who suggested that there might be no Z chromosome dosage compensation mechanism in chicken. Similarly, Oréal et al. [13] reported that *Dmrt1* began to show dimorphic expression on day 6.0 and on day 7.0 it showed more than twice expression in male than in female gonads. We suggested that an additional mechanism rather than Z chromosome dosage effect may be at work in chicken sex determination, in accordance with Smith et al. [20] who presumed that there may be a dominant gene on the W chromosome. Zhao et al. [28] believed that *Dmrt1 b* rather than *a* was expressed in gonads from day 6.0 to day 8.0, while other

study [20] showed that *Dmrt1 a* and/or *b* was expressed since day 7.5. Our result is consistent with the former study which showed no *Dmrt1 a* expression from day 6.5 to day 8.5.

In chicken embryos, the gonads form about day 3.5, maintain bipotential from day 3.5 to day 6.5, and after day 6.5 the gonads begin to differentiate into testes or ovaries. We found that *Dmrt1* was up-regulated from day 6.5 to day 10.5. It is presumed that *Dmrt1* maintained high level before day 10.5 to induce testes and ovaries formation. However, in contrast to our findings, Shan et al. [17] showed that *Dmrt1* expression in ovaries became weaker from day 6.5 onwards and was undetectable on day 10.0 and day 14.0. We believed that their results may result from the low concentration of probes which reduced the detectability of the signals. In Smith's study [20], they detected *Dmrt1* in both male and female gonads using *WISH* at mRNA level and immunofluorescent at protein level, which was consistent with our study and supported our deduction.

In addition to the expression of the *Dmrt1* in gonads, its expression was also found in mesonephros from day 6.5 to day 10.5 during embryonic development. The result is opposite to Raymond's study [15] but coherent with Smith's investigative images though they did not mention the expression in mesonephros in their articles [20, 21]. A recent article claimed that sex is determined in gonads and the sexual differentiation in non-gonadal tissue and organs is initiated by hormones in mammals, while the sex determining pathways is directly act on both cells and tissues throughout the body in lower invertebrates [10]. Chicken is a species between mammals and invertebrate, it is presumed that chicken sexual development is different from both mammals and invertebrate. A recent study proposed that somatic cells in birds possessed an inherent sex identity and its sexual differentiation was cell autonomous [27], which seems to support our reasoning. It is presumed that as a part of urogenital system the mesonephros may express some important sex determination and differentiation genes.

In conclusion, we have confirmed dimorphic expression of the *Dmrt1* gene in chicken embryos. *Dmrt1* is expressed more abundantly but not twice as much in ZZ as in ZW gonads. It suggests that an additional regulatory mechanism may be at work besides Z chromosome dosage effect. These findings will undoubtedly help to better understand molecular mechanisms acting in the sex determination and sex differentiation of birds.

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