Identification and expression of Dmrt1 and Sox9 during the gonadal differentiation of Rana chensinensis

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Abstract: Gonadal sex differentiation proceeds by the interplay of various genes including the transcription factors and secretory factors in a complex network. Dmrt1 and Sox9 were considered to be the essential genes involved in the testicular differentiation in mammals, birds, reptiles, and fishes. Here, we have successfully isolated and characterized cDNAs encoding the Rana chensinensis orthologs of Dmrt1 and Sox9, and we have determined patterns of expression during gonadal differentiation and adult tissues. At the molecular level, a clear sexual dimorphism of rcDmrt1 and rcSox9 was observed in the gonad-mesonephros complex or gonads of tadpoles during stages 26–46. The rcDmrt1 transcript was first detected in undifferentiated gonads at stage 30 and became stronger at stages 32–36. Thereafter, rcDmrt1 was observed in males and gradually increased from stage 42 to stage 46. By contrast, rcSox9 was observed in the whole gonadal differentiation period (stages 26–46) of males and females. In adult frogs, Dmrt1 was merely detected in testes, while rcSox9 was more highly expressed in the brain, liver, and testes. These results showed that both Dmrt1 and Sox9 participate in gonadal differentiation, and Dmrt1 is more closely implicated in testicular differentiation.

Key words: Dmrt1, Sox9, stage-specific gene expression, gonadal development, Rana chensinensis

1. Introduction

Sexual development is a fundamental process of life, which is a regulatory cascade of spatial-temporal expression of multiple genes. In mammals, the sex-determining region of the Y chromosome (SRY) initiates male sex determination, which is a testis-determining factor (Koopman et al., 1990; Polanco and Koopman, 2007). In contrast, Sry does not exist in nonmammalian vertebrates (Tiersch et al., 1991; Denny et al., 1992). In previous studies, genetic sex determination and environmental sex determination were performed in a range of vertebrate amphibian, reptile, and fish species, but the molecular mechanism triggering the sexual differentiation in these species remained unclear (Czerwinski et al., 2016; Geffroy et al., 2016). Thus, investigation of the molecular mechanism determining the sex of nonmammalian vertebrates remains a primary challenge and will be important for evolutionary biology. The double-sex and mab-3-related transcription factor (Dmrt) is a homologous gene family to Doublesex (Dsx) of Drosophila melanogaster and Maleabnormal-3(Mab-3) of Caenorhabditis elegans, which encodes a conserved double-sex and mab-3 (DM) domain, containing a novel zinc module and disordered tail (Shen and Hodgkin, 1988; Burtis et al., 1989; Zhu et al., 2000). The zinc finger structure of the Dmrt transcription regulation factor binds to a specific DNA sequence, which could function as a regulator in sex determination and differentiation (Zhu et al., 2000; Kim et al., 2007). Dmrt1 has been reported in mouse (Matson et al., 2010); chicks (Smith et al., 2003, 2009; Lambeth et al., 2014); turtles Chelydra serpentina (Rhen et al., 2007), Trachemys scripta (Barske and Capel, 2010), and Lepidochelys olivacea (Torres Maldonado et al., 2002); frogs Xenopus laevis and Xenopus tropicalis (Yoshimoto et al., 2006), Rana temporaria (Rodrigues et al., 2017), and Rana rugosa (Shibata et al., 2002); and fishes Oryzias latipes (Kobayashi et al., 2004), Danio rerioze (Guo et al., 2005), Xiphophorus maculates (Veith et al., 2006), Oreochromis niloticus (Kobayashi et al., 2008), and Acanthopagrus schlegelli (Wu et al., 2012). In most species, Dmrt1 is conserved in genetic sex-determining signal pathways of mammals, birds, reptiles, etc. (Raymond et al., 1999a; Shan et al., 2000; Murdock et al., 2003, 2006; Rhen et al., 2007). Dmrt1 plays a key role in initializing testis differentiation and later maintenance of male development; it is first expressed in the genital ridge of embryos, and then becomes male-specific and restricted to the seminiferous tubules of the testis (Pask et al., 2003; Abramyan et al., 2009). These results suggested
that the upregulation of Dmrt1 might play a role in male sexual development. Dmrt1, the male sex determination-related gene, appears to be more directly involved in sex differentiation than sex determination. In mice, Dmrt1 is expressed in all stages of spermatogonia, localized in Sertoli cells and some developing germ cells in testes (Brunner et al., 2001; Shibata et al., 2002; Kim et al., 2007; Abramyan et al., 2009). In addition, knockout of the Dmrt1 could lead to a reduced number of germ cells, which indicates that Dmrt1 is essential for spermatogenesis (Matson et al., 2010).

Sex-determining region Y box 9 (Sox9), a Sox superfamily member, has a conserved high mobility group (HMG) DNA-binding motif shared with SRY and several related proteins. Among the vertebrate Sox superfamily members, Sox9 is known to be critical for many aspects of development including sex determination and gonadal differentiation, nervous system development, heart valve development, and chondrogenesis (Hong and Saint-Jeannet, 2005; Lincoln et al., 2007; Akiyama et al., 2011; Jakob et al., 2011; Nakamura et al., 2012). Defects of Sox9 in XY individuals with campomelic dysplasia (CD) showed complete or partial sex reversal (Meyer et al., 1997; Michel-Calemard et al., 2004; Katoh-Fukui et al., 2015). In adult individuals, Sox9 was strongly expressed in the onset of Sertoli cell differentiation, whereas it was downregulated in the female gonads (Fröjdman et al., 2000). In addition, Sox9 could upregulate the transcription of the anti-Müllerian hormone gene and maintain spermatogenesis and fertility in the testes (Josso et al., 1993; Mackay et al., 2000; Josso et al., 2001; Ortega et al., 2015), which induced the transdifferentiation of granulosa cells into Sertoli-like cells in the ovary of adult mouse (Couse et al., 1999; Dupont et al., 2003; Barske and Capel, 2010). These results suggest that Sox9 may be critical for Sertoli cell and testicular differentiation.

In the present study, the full-length cDNAs of Dmrt1 and Sox9 from testes of the frog Rana chensinensis were cloned and their sequence features were analyzed. In addition, the tissue expression and distribution of rcDmrt1 and rcSox9 were examined during gonadal differentiation. Finally, all data were used to analyze the role of Dmrt1 and Sox9 in the regulation of gonadal differentiation and maintenance of testicular function.

## 2. Materials and methods

### 2.1. Animals and treatments

Two-year-old adult Chinese brown frogs (Rana chensinensis) were obtained from the Qinling Mountains of Shaanxi, China, in November and December of 2016. Males (n = 5; 7.7–8.1 g) and females (n = 5; 9.2–10.0 g) were fed Tenebrio molitor twice a day and cultured at 25 ± 2 °C in tanks containing 2 L of dechlorinated tap water. The light/dark cycle was 14 h/10 h. After 10 days of environmental adaption, brains, livers, kidneys, testes, and ovaries were dissected immediately following ether anesthesia (euthanasia). Samples were frozen in liquid nitrogen and stored at −80 °C until further analysis.

Tadpoles were collected in March and April 2017 from the same area as the adult frogs, and they were raised in similar conditions as adult frogs while being fed with boiled lettuce every other day ad libitum. According to Gosner stages (Gosner, 1960), the gonad-mesonephros complex (GMC) of tadpoles at stages 26–28, the undifferentiated gonads at stages 30–40, and the testes and ovaries at stages 42–46 were isolated under a dissecting microscope (Figure 1), and then specimens were stored at −80 °C. All procedures involving animals were in accordance with the ethical standards of the Animal Research Ethics Committee of Shaanxi Normal University, China.

### 2.2. Nucleic acid extractions and RT-PCR

Total RNA was isolated using RNAsio Plus (TaKaRa) as described by the manufacturer. All RNA samples were quantified using the absorption of light at 260 and 280 nm by Thermo Scientific NanoDrop 2000 (Thermo Fisher).

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**Figure 1.** Images of undifferentiated gonads and differentiated testes and ovaries of R. Chensinensis tadpoles. A, Stage 28; B, stage 46, testis; C, stage 46, ovary. Wd, Wolffian duct; Gmc, gonad-mesonephros complex; Pe, perisome; Ki, kidney; Te, testis; Ov, ovary.
Nucleic acids of ratio 1.7–1.9 were used for semiquantitative RT-PCR analysis. In order to clone Dmrt1 and Sox9 in testes of *R. chensinensis*, specific primers (Table 1) were designed referring to the relevant cDNA sequences of *Rana rugosa* (AB272609.1, AB035887.1), *Rana nigromaculata* (EF524050.1), and *Bufo marinus* (Fj697174.1). PCR was performed in a final volume of 50 µL containing 10 µL of cDNA, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 5 µL of 10X PCR buffer (Mg2+), 4 µL of dNTP mix, 0.5 µL of TaKaRa Taq, and 28.5 µL of ddH2O. The amplified products were analyzed on a 1.5% agarose gel and purified using the MiniBEST Agarose Gel DNA Extraction Kit, Ver. 4.0 (TaKaRa), followed by ligation with the pGEM-T easy vector (Promega) and transformation into DH5α *E. coli* cells. In order to obtain accurate sequences, at least 3 individuals of positive clones were selected for sequencing. Finally, SeqMan of DNAStar software was used for sequence assembly.

### 2.3. Sequence and phylogenetic analysis

Full-length amino acid sequences of *rcDmrt1* and *rcSox9* were deduced from the obtained nucleotide sequences. Potential functional motifs were analyzed by SMART (http://smart.embl.de/smart/set_mode.cgi?NORMAL=1) (Letunic et al., 2012). Signal peptide prediction was performed with SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). Secondary structure was predicted using PSIPRED v3.3 (http://bioinf.cs.ucl.ac.uk/psipred/). Molecular weight and pI were predicted using Compute pI/Mw (http://web.expasy.org/compute_pi/). In order to construct phylogenetic trees, multiple sequence alignments were performed by ClustalX (Thompson et al., 1997). Neighbor-joining phylogenetic trees of Dmrt1 and Sox9 from different amphibians and reptiles were created by the MEGA 5.1 program, in which we applied a bootstrap test of 2000 replicates to check the robustness of the phylogram.

#### 2.4. *rcDmrt1* and *rcSox9* expression analysis

Expressions of *rcDmrt1* and *rcSox9* were analyzed in adult tissues including testis, ovary, kidney, liver, and brain by RT-PCR using *Dmrt1* and *Sox9* primers. To standardize each experiment, the amount of a specific transcript was divided by the amount of *rp18* transcript. PCR reactions were performed and the amplified products were analyzed as previously described. In tadpoles, expression of *rcDmrt1* and *rcSox9* in the GMC during stages 26–46 was examined just like in adult frogs.

### 2.5. In situ hybridization

Digoxigenin (DIG)-labeled RNA probes of *rcDmrt1* used for in situ hybridization were synthesized by in vitro transcription with a DIG RNA labeling kit (Roche Diagnostics). The transcriptions were performed from linearized plasmids using either T7 (antisense) or SP6 (sense) RNA polymerases. The testes were sectioned at a thickness of 8 µm. In the in situ hybridization protocol was performed essentially as described previously (Wilhelm et al., 2007; Abramyan et al., 2009).

### 3. Results

#### 3.1. Identification and characterization of *rcDmrt1* and *rcSox9*

Through sequence assembly, we obtained a cDNA of 1118 bp from *R. chensinensis*, which was named *rcDmrt1* (GenBank Accession No. KC439687). The *rcDmrt1* sequence was composed of a 5' untranslated region of 4 bp, 3' untranslated region of 109 bp, and ORF of 1005 bp, which encoded 334 amino acids (Figure 2). The predicted amino acid sequence of *rcDmrt1* had a conserved DM domain, a male-specific motif, and a P/S-rich element (Figure 2), which showed high homology to *G. rugosa* (93%), *X. tropicalis* (66%), *X. laevis* (65%), *H. sapiens* (53%), and *M. musculus* (52%) (Table 2). The identities

### Table 1. Oligonucleotide primers used for PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dmrt1</em>-1</td>
<td>AAAATGCCTAACACGGGAGGA</td>
<td>TATTCTTCACCCAGGATTCAC</td>
<td>1079</td>
</tr>
<tr>
<td><em>Dmrt1</em>-2</td>
<td>ATGCCTAACAGCGAGGAGCCAT</td>
<td>CGCCACGCCAGTCTAAATAATGTC</td>
<td>628</td>
</tr>
<tr>
<td><em>Dmrt1</em>-3</td>
<td>GCATGCTACAGGGAGGGCT</td>
<td>CTCCACAGCTACTAGTCTCG</td>
<td>325</td>
</tr>
<tr>
<td><em>Dmrt1</em>-4</td>
<td>ATGCCAAATCTTCCTCGTG</td>
<td>ACCTGTAAGGGTCACGGAAGGCA</td>
<td>301</td>
</tr>
<tr>
<td><em>Sox9</em>-1</td>
<td>GCATGCTACAGGGAGGGCT</td>
<td>CTCCACAGCTACTAGTCTCG</td>
<td>325</td>
</tr>
<tr>
<td><em>Sox9</em>-2</td>
<td>CTCCACAGCTACTAGTCTCG</td>
<td>GGTGCTACTTTGTGTCG</td>
<td>511</td>
</tr>
<tr>
<td><em>Sox9</em>-3</td>
<td>AAGAACAAACACCGGACTGCA</td>
<td>TCCATAGAGGCTACGGAAGGCA</td>
<td>980</td>
</tr>
<tr>
<td><em>Sox9</em>-4</td>
<td>ATGTCATGACTTCGGATCCCT</td>
<td>GGTGCTACTTTGTGTCG</td>
<td>511</td>
</tr>
<tr>
<td><em>Sox9</em>-5</td>
<td>AAGAACAAACACCGGACTGCA</td>
<td>TCCATAGAGGCTACGGAAGGCA</td>
<td>980</td>
</tr>
<tr>
<td><em>Sox9</em>-6</td>
<td>CCAGGGCTACGACTTACTGACT</td>
<td>TTTACGGGAAGGCTACGGAAGGCA</td>
<td>115</td>
</tr>
<tr>
<td><em>rp18</em></td>
<td>GCTGTCGACCTTGCCGAAAGGCA</td>
<td>ACCTGTAAGGGTCACGGAAGGCA</td>
<td>115</td>
</tr>
</tbody>
</table>
of amino acid sequences of the DM domain and male-specific motif showed 82%–100% and 68%–91% identities, respectively, while the P/S-rich element showed 27%–90% identities among the tested species except *B. gargarizans*, *C. palustris*, *C. mydas*, and *T. scripta* (Table 2). The start and stop codon positions of *Dmrt1* of *R. chensinensis* and *G. rugosa* were similar to each other as well as no frame shifts. There was no N-terminal signal sequence of rcDmrt1, which suggested that rcDmrt1 was a nonsecretory protein. The molecular weight of rcDmrt1 was 36.8 kDa with an isoelectric point of 8.45. The secondary structure of rcDmrt1 contained 14.97% alpha helix, 22.75% extended strand, 0.84% beta turn, and 62.28% random coil.

In addition, we also obtained cDNA of 1580 bp for *R. chensinensis*, which was named *rcSox9* (GenBank Accession No. KC442293). The *rcSox9* sequence was composed of a 5′ untranslated region of 3 bp, 3′ untranslated region of 128 bp, and ORF of 1449 bp, which encoded 482 amino acids (Figure 3). The predicted amino acid sequence of *rcSox9* had a conserved HMG-box domain and PQA-rich element (i.e. rich in proline, glutamine, and alanine), which displayed high identities to *G. rugosa* (97%) and some reptiles, such as *T. scripta* (87%) and *C. palustris* (86%) (Figure 3; Table 2). Amino acid sequences of the HMG-box domain and PQA-rich element showed 97%–99% and 37%–85% identities among the tested species. The start and stop codon positions of the *Sox9* ORF of *R. chensinensis* and *G. rugosa* were similar to each other as well as no frame shifts. There was no N-terminal signal sequence of rcSox9, which suggested that rcSox9 was also a nonsecretory protein. The molecular weight of rcSox9 was 53.6 kDa with an isoelectric point of 6.32. The secondary structure of rcSox9 contained 17.12% alpha helix, 2.92% extended strand, 0.84% beta turn, and 79.12% random coil.

### 3.2. Multiple alignments and phylogenetic tree construction
Multisequence alignments of Dmrt1 and Sox9 of *R. chensinensis* and other tested species were performed by DNAman software. Results showed that the identity of rcDmrt1 with other amphibians, fishes, and reptiles was 53%–93%, 39%–41%, and 28%–40% (Table 2), respectively. In addition, the identity of rcSox9 with other amphibians, fishes, and reptiles was 82%–97%, 74%–77%, and 86%–88% (Table 2), respectively.
Phylogenetic analysis indicated that rcDmrt1 and rcSox9 were grouped in the clades of amphibian Dmrt1 and Sox9, respectively (Figure 4). Furthermore, rcDmrt1 and rcSox9 respectively constituted a sister group with the Dmrt1 and Sox9 of *G. rugosa* (Anura), showing an identity of approximately 93%–97% (Figure 4; Table 2), but a relatively distant relationship was detected for *P. waltl* (Caudata), *D. rerio* (fishes), and *Chelonia mydas* (reptiles) (Figure 4).

3.3. Expression of rcDmrt1 and rcSox9 in gonads of tadpoles

As Figure 5 shows, expression of rcDmrt1 and rcSox9 was examined in the GMC of tadpoles during stages 26–28 and in the gonads at stages 33–46 by RT-PCR. The rcDmrt1 transcript was first detected in undifferentiated gonads at stage 30, which became stronger at stage 32, and the strongest expression was identified at stages 34–36. At stage 38, rcDmrt1 had approximately the same expression level as stage 30, and there was no rcDmrt1 transcript observed at stage 40. From stage 42, rcDmrt1 expression was examined and it became stronger at stage 46 in testes of the males, but it was not observed in ovaries of the females. In contrast, the rcSox9 transcript was detected in all GMC samples and became stronger at stages 32–38 and 42–46. The rcSox9 transcript was detected in both males and females at stages 42–46, whereas rcDmrt1 was only detected in males (Figure 5).

3.4. Organ and tissue distributions of rcDmrt1 and rcSox9 in adults

Expression of rcDmrt1 and rcSox9 was detected in various tissues including testis, ovary, liver, brain, and kidney of adults by RT-PCR while rpl8 was an internal control. The results showed that Dmrt1 had a higher expression level in the testis, but it was not examined in the ovary or any other tissues (Figure 6). As Figure 6 also shows, rcSox9 was expressed in adult testes, ovaries, kidneys, livers, and brains, while stronger expressions of rcSox9 were detected in testes and brains, respectively. In addition, we found that rcSox9 had higher expression than rcDmrt1 in testes.

3.5. Location of rcDmrt1 expression in testes

Expression of rcDmrt1 was detected in adult testes by in situ hybridization. In the sections, strong positive signals of rcDmrt1 were mainly detected in interstitial cells, Sertoli cells, and developing germ cells (Figure 7A). In contrast,
no signals were detected in the negative control under the DIG RNA labeling sense probe (Figure 7B).

4. Discussion

4.1. Characteristic analysis of rcDmrt1 and rcSox9

The sex determination mechanism of amphibians mainly includes genetic sex determination and environmental sex determination. Dmrt1, Daxl, Sox3, Sox9, and FoxI2 are sex determination-related genes in amphibians (Clarkson and Harley, 2002; Nakamura, 2009). In the present study, we have obtained and characterized a Dmrt1 cDNA from R. chensinensis, named rcDmrt1. The deduced amino acid sequence of rcDmrt1 showed more than 92% identity to Dmrt1 homologs of G. rugosa and P. nigromaculatus in anurans, and 59% identities to Dmrt1 of P. waltl in Caudata. Dmrtl was reported in human, mouse, chick, turtle (Chelydra serpentina, Trachemys scripta), frog (Xenopus laevis, Xenopus tropicalis, Rana rugosa), and fish (Raymond et al., 1999; Guan et al., 2000), which contained a DM domain, a male-specific motif, and a P/S-rich element. The DM domain, a novel DNA binding motif, contained the conserved cysteines, histidines, and chelated zinc, which combined with the DNA minor groove (Zhu et al., 2000). Through the zinc finger structure, Dmrt1 as...
Figure 4. The neighbor-joining phylogenetic trees of Dmrt1 and Sox9. Phylogenetic tree was constructed on the basis of alignment of the amino acid sequences of Dmrt1 and Sox9 homologs, showing the evolutionary relationship of rcDmrt1 (A) and rcSox9 (B) with other species of the Dmrt1 and Sox9 family. Numbers at branch nodes are percentages of bootstrap confidence values derived from 2000 replications. GenBank Accession Nos. of various species are shown in Table 2.

a regulator bound to the specific DNA sequence and was involved in sex determination and differentiation (Raymond et al., 1999). The male-specific motif, a sex specific motif near the C-terminus, was well conserved among numerous Dmrt1 and mainly involved in male sex determination (Guan et al., 2000). The P/S-rich element as a C-terminal domain rich in proline and serine residues was detected in the Dmrt1 of many species. As Hoshi and Nakao (2008) showed, the homology within the P/S-rich region of rcDmrt1 was high throughout the other vertebrates, except fish.

Members of the Sox superfamily were characterized with the presence of a Sry-related HMG box, which was involved in various developmental processes of animals, particularly in organogenesis (Bowles et al., 2000; Jakob et al., 2011; Nakamura et al., 2012; Wei et al., 2016). Here, we also identified and characterized the complete coding sequence of rcSox9. The conserved HMG-box domain and PQA-rich element were found in rcSox9, respectively, and their relative positions were similar to Sox9 homologs throughout the investigated species (McDowall et al., 1999; Clarkson and Harley, 2002; Smith and Koopman,
The HMG-box as a DNA binding domain has a high affinity with the minor groove of non-B-type DNA structures, which was involved in the regulation of DNA-dependent processes such as transcription, replication, recombination, and DNA repair (Stros et al., 2007). It has been confirmed that Sox9 bound to a minimal DNA element in intron 1 of Col2a1 and acted as a potent activator in the differentiation program of chondrocytes (Lefebvre et al., 1997). The PQA-rich element as a C terminus domain consisted entirely of proline, glutamine, and alanine, which contributed to maximal transactivation (McDowall et al., 1999; Smith and Koopman, 2004). In humans, mutations of Sox9 caused CD, and many of these mutations could result in truncations and frameshifts in the C terminus of Sox9 (McDowall et al., 1999).

In phylogenetic trees, Anura formed a cluster by itself, whereas Caudata and reptiles formed another two separate clusters. The evolutionary relationships were consistent with the one in traditional taxonomy. All these results revealed that rcDmrt1 and rcSox9 were orthologous
to the Dmrt1 and Sox9 of G. rugosa in the evolutionary relationships, respectively. In addition, rcDmrt1 and rcSox9 were well conserved in the living organisms.

4.2. Expression analysis of rcSox9 and relationships, respectively. In addition, rcDmrt1 to the rcDmrt1 this study, we examined the expression of during stages 43–46 (Gosner, 1960; Hogan et al., 2008). In addition, this expression was examined and became increased gradually of the tail occurs and the formation of the juvenile frog is still not examined and confirmed. Basing on Gosner stages, the role of rcDmrt1 in sex determination of R. chensinensis is still not examined and confirmed. Basing on Gosner stages, the development of tadpoles can be divided into 4 periods. There is no sex differentiation during stages 27–30 and the gonads come into differentiation at stages 31–36, the end of sex determination systems are often highly dynamic, although genetic effects have been documented in common frogs (Rodrigues et al., 2014). However, the basic mechanism of sex determination and gonadal differentiation is still unknown. In X. laevis, Dmrt1 was transcribed in gonads at stages 53–62, which was expressed exclusively in primordial gonads and continued its expression after sex determination rather than in the sex determination period (Osawa et al., 2005; Yoshimoto et al., 2006; Uno et al., 2008). In G. rugosa, Dmrt1 was first expressed in differentiating testes at stage XXV (unlike Gosner stages), which only existed in spermatogonia cells (Shibata et al., 2002). The role of rcDmrt1 in sex determination of R. chensinensis is still not examined and confirmed. Basing on Gosner stages, the gonads come into differentiation at stages 31–36, the end of gonadal differentiation. At stages 37–42 the disappearance of the tail occurs and the formation of the juvenile frog is during stages 43–46 (Gosner, 1960; Hogan et al., 2008). In this study, we examined the expression of rcDmrt1 in R. chensinensis tadpoles. It was specifically expressed in the primordial gonads at stages 30–36. In addition, rcDmrt1 expression was examined and became increased gradually at stages 42–46 in males, but it was not observed in the females. These results showed that Dmrt1 transcription was restricted in the gonadal differentiation period of tadpole. Therefore, we supposed that rcDmrt1 played a key role in the early sex differentiation of R. chensinensis. Nevertheless, it will be meaningful to determine whether rcDmrt1 is involved in sex determination pathways or directly regulates the synthesis of sex steroids.

A previous study showed that Dmrt1 was expressed in the gonads of Bufo marinus, and Dmrt1 mRNA was restricted to Sertoli cells (Abramyan et al., 2009). In mammals, Dmrt1 was localized in the germ cells and Sertoli cells of the testes in Macropus eugenii, but in addition it was detected in Leydig cells, peritubular myoid cells, and the acrosome of sperm heads (Pask et al., 2003). In this study, rcDmrt1 was only expressed in testes of adult frog R. chensinensis, and rcDmrt1 mRNA was localized in interstitial cells, Sertoli cells, and some developing germ cells in testes. All these results suggest that Dmrt1 has a key role in spermatogenesis and maintaining the function of testes (Guan et al., 2000; Brunner et al., 2001; Shibata et al., 2002; Veith et al., 2006; Ge et al., 2017, 2018).

Individual development was strictly regulated by the gene regulatory networks, in which the specific genes and other related genes were involved, and any obstacle would lead to abnormal development. In vertebrates, Sox9 was expressed in the GMC of both sexes during gonadal differentiation (Barske and Capel, 2010; Dumond et al., 2011), which indicated that Sox9 might be involved in gonadal development. Moreover, Sox9 clearly showed sexually dimorphic patterns in adults; the defect of Sox9 of XY individuals in CD showed complete or partial sex reversal (Meyer et al., 1997; Michel-Calemard et al., 2004; Katoh-Fukui et al., 2015). Here, we found that the different expressions of rcSox9 were not only related to the sexes but also the development stages. rcSox9 was detected in all the tested tissues, with the highest expression found in the testes and ovaries. In addition, rcSox9 has also been expressed in the GMC of larvae at stages 32–45. These results suggest that Sox9 plays a role in gonadal differentiation and the maintenance of the functioning of adult sexual glands. Sox9 and forkhead box L2 (Foxl2) could have synergism and antagonism for each other in regulating the expression of Cyp19 and the conversion of testosterone to estradiol (McCleavey et al., 1993; Lavery et al., 2012; Navarro-Martín et al., 2012). However, the absence of foxl2 would lead to Sox9 expression, testis development, and XX sex reversal (Veitia, 2010). Therefore, we speculated that Sox9 may indirectly affect steroid hormone synthesis and regulate gonadal development.

In summary, this study described the cloning and characterization of the R. chensinensis Dmrt1 and Sox9 genes. Their mRNA transcripts presented a tissue-specific manner and natural linear flow in regulation of gonadal development of R. chensinensis, which indicated their critical roles in gonadal development and maintenance of gonadal function. In addition, RcDmrt1 was only detected in the testes of adult frogs, and RcDmrt1 mRNA was located in interstitial cells, Sertoli cells, and germ cells of adult testes. These results show that both Dmrt1 and Sox9 participate in gonadal differentiation and maintain testicular function, and Dmrt1 is more closely implicated in testicular differentiation. These explorations have provided novel molecular insights to understand the mechanism of sex determination of nonmammalian vertebrates.

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