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Differential expression of the MHM region and of sex-determining-related genes during gonadal development in chicken embryos

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ABSTRACT. The chicken (*Gallus gallus*) embryo has been used as a classic model system for developmental studies because of its easy accessibility for surgical manipulation during embryonic development. Sex determination in birds is chromosomally based (ZZ for males and ZW for females); however, the basic mechanism of sex determination is still unknown. Here, the dynamics of expression of candidate genes implicated in vertebrate sex determination and differentiation were studied during embryonic chicken gonadal development. Gene expression profiles were obtained before, during, and after gonadal sex differentiation in females and males for *DMRT1*, *SOX3*, *SOX9*, *DAX1*,

SCII, *HINTZ*, *HINTW*, and the male hypermethylated (MHM) region. Transcripts for the *HINTZ*, *DMRT1*, *DAX1*, *SCII*, and *SOX9* genes were observed in both sexes, but expression was higher in male gonads and may be correlated with testicular differentiation. The expression patterns of *HINTW*, *SOX3*, and MHM suggest that they may act in ovary development and may be involved in meiosis entry. MHM was upregulated and *DMRT1* was downregulated in females at the same developmental stage. This may indicate a regulation of *DMRT1* by MHM ncRNA. Similar dynamics were observed between *HINTW* and *HINTZ*. This study reports on the MHM expression profile during gonadal development and its correlation with the expression of genes involved in vertebrate sex determination.

Key words: Gonad; Development; Chicken; Epigenetics; Dosage compensation

INTRODUCTION

The sex of mammals and birds is determined upon fertilization, through the inheritance of sex-specific genes on the sex chromosomes, representing a gene-based sex determination model. Mammalian males carry XY sex chromosomes and females have XX sex chromosomes, whereas in birds, the homogametic sex is the male (ZZ) and the heterogametic one is the female (ZW) (Ohno et al., 1964). In chicken (*Gallus gallus*), gonad morphological differentiation begins between day 5.5 and 6.5 [corresponding to Hamburger and Hamilton developmental stages HH28-HH30 (Hamburger and Hamilton, 1992; Sanes, 1992)]. In female embryos, only the left gonad develops into an ovary, while the right one regresses. In male embryos, bilateral testis differentiation occurs (Carlon and Stahl, 1985).

Gonadogenesis is a relatively conserved process, and much of the sex-determining pathway is thought to be shared between mammals, birds, and other groups. *SRY* (Sex-determining Region on Y-chromosome) is a testis-determining gene conserved in mammals, but has not been identified in birds (Graves, 1998). However, other genes considered to be important for gonadogenesis in mammals like Doublesex and Mab-3 Related Factor 1 (*DMRT1*), *SRY*-related HMG-box 3 (*SOX3*), *SRY*-related HMG-box 9 (*SOX9*), and dosage-sensitive sex reversal-adrenal hypoplasia critical region on chromosome X gene 1 (*DAX1*) have been observed in birds (Smith et al., 1999, 2000).

In addition, bird-specific genes associated with sex determination and differentiation include the histidine triad nucleotide binding proteins, W- and Z-linked (*HINTW* and *HINTZ*, respectively). HINTW and HINTZ belong to the HIT superfamily of nucleotide transferases and hydrolases that are homodimers acting on the α-phosphate of ribonucleotides (Brenner, 2002).

Some dosage-compensated genes in birds are concentrated in a region of the short arm of the Z chromosome, near the male hypermethylated (MHM) locus (Arnold et al., 2008; Mank and Ellegren, 2009; Melamed and Arnold, 2007, 2009). The MHM region is hypermethylated in both ZZ chromosomes in males but it is unmethylated in females (Teranishi et al., 2001). This locus encodes an MHM non-coding RNA (ncRNA) that is expressed only in females and specifically accumulates near its transcriptional site (Teranishi et al., 2001).

Here, we investigated the expression patterns of the *DMRT1*, *SOX9*, *SOX3*, *DAX1*, *HINTW*, *HINTZ*, and *SCII* genes and the MHM region in gonads of chicken embryos before, during, and after sex determination.

MATERIAL AND METHODS

Animals

All procedures for animal management (of adult animals and embryos of *G. gallus*) were approved by the Ethics and Animal Research Committee (process No. 075/2007) and by the Internal Biosafety Committee (process No. CBQ-FMRP: 0030/97-08.49-I) of Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo.

Sample collection

Freshly laid chicken (*G. gallus*) eggs were incubated under humid conditions at 37.5°C. The embryos were removed from the eggs, staged according to the criteria of Hamburger and Hamilton (1992), decapitated, and their gonadal, mesonephric, and cardiac tissues excised. Samples were harvested before (HH20, 72 h and HH25, day 4), just before (HH28, day 5.5), during (HH35, day 8), and after (HH40, day 14) the period of sexual differentiation (Figure 1). The gonads, kidneys, and hearts of adult animals were also harvested. After sexing, the samples were pooled (five samples per pool) by stage, sex, and tissue. The gonadal and mesonephric region were excised together at HH20, HH25, and HH28.

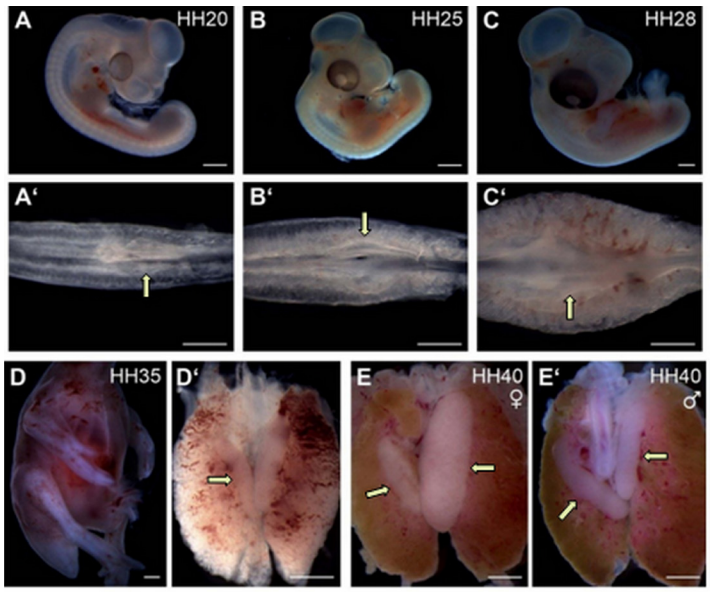

Figure 1. A.-D. Embryos staged according to the criteria of Hamburger and Hamilton (HH; 1992). **A'.-D'.** Gonadal (arrows) and mesonephric regions of the corresponding stages. **E.** Female and male gonads (**E'**) (arrows). Observe the asymmetry of the HH40 female gonads. Scale bars = 100 µm.

Molecular analysis

Genomic DNA and total RNA were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions, and stored at -80°C. The sex of embryos was determined by polymerase chain reaction (PCR) amplification of sex chromosome-specific sequences of *CHD* genes (Griffiths et al., 1998). The PCR mix (25 µL) consisted of 100 ng DNA template, 10 pmol of each primer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10X PCR buffer, and 1 U Taq DNA polymerase (Invitrogen, Brazil). The amplification conditions were 1 cycle of 94°C for 1 min, 45°C for 1 min, and 1 min at 72°C, followed by 35 cycles of 94°C for 40 s, 45°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were separated by 10% polyacrylamide gel electrophoresis. Fluorescence-based quantitative PCR (qPCR) was conducted to measure the expression of the *DMRT1*, *HINTZ*, *HINTW*, *SOX9*, *SOX3*, *SCII*, and *DAX1* genes and of the MHM region. Total RNA was subjected to DNase (Invitrogen) treatment to remove genomic DNA, and 400-1000 ng of each sample were reverse transcribed using Superscript III First-Strand Synthesis (Invitrogen) and random hexamers (Invitrogen) as primers, in accordance with manufacturer instructions. The first strand of complementary DNA (cDNA) synthesized was quantified, diluted to 50 ng/µL, and tested for genomic DNA contamination by PCR with β-actin genomic primers (Table 1). Samples were then subjected to qPCR. PCR primers were designed on known sequences of *G. gallus* using Gene Runner (3.5 version, Hasting), and the Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) software (Table 1). All reactions were performed in, at least, duplicate, and cDNA was amplified using primer concentration of 10 pmol for target genes and 5 pmol for the endogenous control gene (β-actin gene), using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a StepOne PCR Detection System (Applied Biosystems). The qPCR condition was initiated by heat denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 59.5°C in a volume of 10 µL. The gene-specific products were identified by melting curve analysis. Each amplification set was first performed with standard curves to confirm primer efficiency (90-110%). The threshold cycle (C_T) of each gene analyzed was normalized to the C_T of β-actin, and 18S RNA was used as a second reference control (data not shown). Relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Data were analyzed using the Statistical Analysis System (SAS), version 8.02 (SAS Institute, Cary, NC, USA). The Student *t*-tests were used to determine statistical significance of differences between means, and differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The *SOX3* gene showed periods of expression in female gonadal tissue (HH20, HH35) and basal levels in males during the initial stages of development (Figure 2A and Table 2). Its expression was also detected in mesonephric tissue (Table 3), but not in cardiac tissue (Table 4).

SOX9 transcripts were detected in gonadal tissue in males and females (Figure 2B and Table 2). *SOX9* was not expressed in male gonads during the HH40 stage. However, *SOX9* expression during other stages was higher in males (Figure 2B). We also detected *SOX9* expression in mesonephric and cardiac tissue (Tables 3 and 4).

Table 1. Primer information.

Gene	Number	Primer sequence (5'-3')	Length (bp)
β-actin	X00182	F: 5'-CgT gCT gTg TTC CCA TCT AT-3'	103 cDNA
		R: 5'-TTg CTC Tgg gCT TCA TCA C-3'	421 gDNA
DAX1	AF202991	F: 5'-ggA CAT CAg CAC CAA ggA gT-3'	117
		R: 5'-gCT TCC TgT gCT TCC TTC Tg-3'	
DMRT1	AF123456	F: 5'-gAA gCC TCC CAg CAA CAT AC-3'	113
		R: 5'-Cgg gTA gTA ggA gCA CAT C-3'	
HINTW	NM204688	F: 5'-AgA TTg Tgg CgC ACC TCT TC-3'	51
		R: 5'-CAC TTC TCg CCA ACA ATC ATC A-3'	
HINTZ	AB026676	F: 5'-ATA TTC Tgg gAg gTC gTC AgT Tg-3'	77
		R: 5'TTG TAC ACA TGC AGC ATC TCT TGT-3'	
MHM	AB046699	F: 5'-ACA gAC AAC CAA ggg CAg TC-3'	59
		R: 5'-TCA AAC gCT ACg ATg gAA AT-3'	
SCII	X80792	F: 5'-CAg TTC CTT gTg gTg TCC CT-3'	144
		R: 5'-gTT gTT gCT ggC TTC ACT CA-3'	
SOX9	NM204281	F: 5'-gAg gAA gTC ggT gAA gAA Cg-3'	124
		R: 5'-gCT gAT gCT ggA ggA TgA CT-3'	
SOX3	NM204195	F: 5'-AAg gAC AAA TAC TCg CTg CC-3'	100
		R: 5'-ggg CgT AAg TgT CAA TCC TC-3'	

Number = GenBank acession number; length = amplicon length; cDNA = complementary DNA; gDNA = genomic DNA.

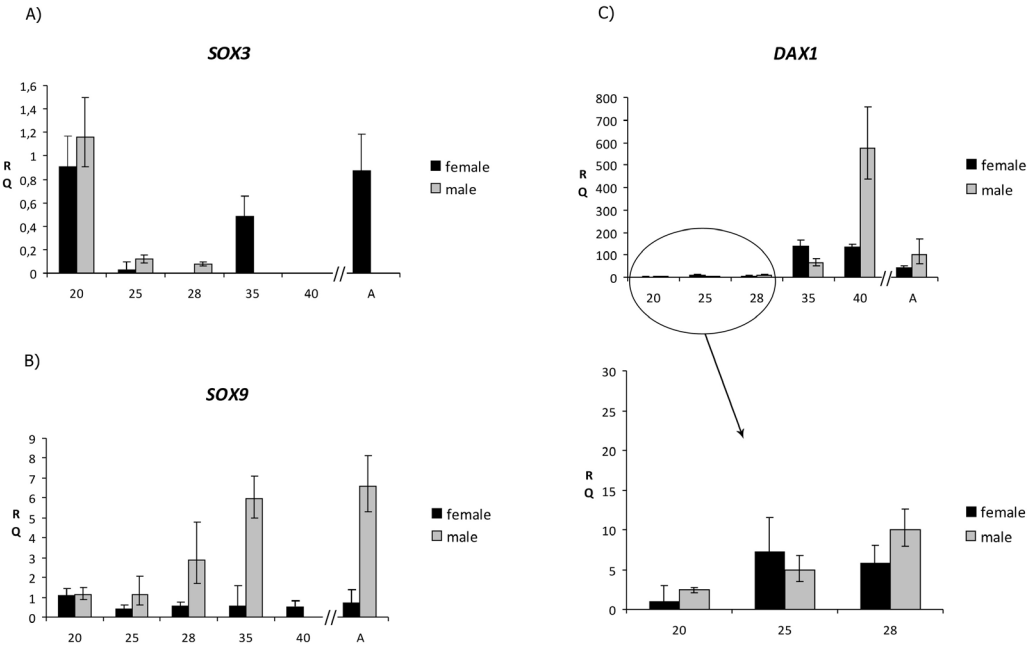

Figure 2. Quantitative real-time PCR analysis of chicken *SOX3* (**A**), *SOX9* (**B**) and *DAX1* (**C**) genes during testicular and ovarian development. cDNA from male and female gonads on embryonic stages HH20, HH25, HH28, HH35, and HH40, tests and ovary from adult animals (A) was amplified with gene-specific primers. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. RQ = relative quantification.

Table 2. Gene expression in gonadal tissue during the embryonic development (HH20-40 stages) and in adult individuals (A).

Sex	Stage	*SOX3*	*SOX9*	*DAX1*	*SCII*	*HINTZ*	*HINTW*	*DMRT1*	MHM
	20*	0.89	1.11	1.08	1.83	2.29	2.61	4.49	3.18
	25*	0.03	0.41	7.32	0.99	0.39	1.56	12.60	1.55
F	28*	-	0.54	5.89	0.83	0.66	1.23	15.00	2.15
	35	0.49	0.58	136.61	39.70	122.10	24.31	624.14	0.56
	40	-	0.52	133.46	17.95	97.54	18.79	577.61	13.55
	A	0.89	0.74	41.98	0.78	0.98	0.85	-	1.88
	20	1.16	1.17	2.43	0.49	2.49	-	3.80	-
	25	0.12	1.14	4.92	0.69	1.14	-	18.00	-
M	28	0.08	2.96	10.02	2.50	2.28	-	30.00	-
	35	-	5.96	65.13	2.40	5.51	-	701.33	-
	40	-	-	575.17	111.60	1271.41	-	4871.89	-
	A	-	5.99	101.08	18.31	7.38	-	2841.16	-

The relative quantification was obtained by real-time PCR, through $2^{-\Delta\Delta Ct}$ method. F = female; M = male; (-) = no expression detected. The gonadal and mesonephric region were analyzed together at HH20, HH25, and HH28.

Table 3. Gene expression in mesonephric tissue during the embryonic development (HH20-40 stages) and in adult individuals (A).

Sex	Stage	*SOX3*	*SOX9*	*DAX1*	*SCII*	*HINTZ*	*HINTW*	*DMRT1*	MHM
	20*	0.89	1.11	1.08	1.83	2.29	2.61	4.49	3.18
	25*	0.03	0.41	7.32	0.99	0.39	1.56	12.60	1.55
F	28*	-	0.54	5.89	0.83	0.66	1.23	15.00	2.15
	35	-	-	10.06	1.27	1.14	1.39	29.67	4.76
	40	-	24.97	22.55	1.28	4.65	8.31	22.11	12.75
	A	-	0.99	2.41	-	2.93	4.37	4.77	22.48
	20	1.16	1.17	2.43	0.49	2.49	-	3.80	-
	25	0.12	1.14	4.92	0.69	1.14	-	18.00	-
M	28	0.08	2.96	10.02	2.50	2.28	-	30.00	-
	35	-	0.23	13.23	1.67	3.62	-	23.22	0.01
	40	0.41	0.72	26.53	1.74	5.48	-	8.11	0.95
	A	-	-	47.90	1.96	14.79	-	-	-

The relative quantification was obtained by real-time PCR, through $2^{-\Delta\Delta Ct}$ method. F = female; M = male; (-) = no expression detected. The gonadal and mesonephric region were analyzed together at HH20, HH25, and HH28.

Table 4. Gene expression in cardiac tissue during the embryonic development (HH20-40 stages) and in adult individuals (A).

Sex	Stage	*SOX3*	*SOX9*	*DAX1*	*SCII*	*HINTZ*	*HINTW*	*DMRT1*	MHM
	20*	-	-	-	-	1.94	5.04	-	8.09
	25*	-	-	-	-	-	3.89	-	-
F	28*	-	0.24	3.34	1.30	3.56	2.93	12.43	4.86
	35	-	0.39	9.29	-	4.83	2.81	6.19	8.00
	40	-	-	6.08	1.43	2.85	2.70	5.21	4.04
	A	-	-	-	-	-	40.63	-	179.71
	20	-	-	-	2.00	4.73	-	-	-
	25	-	1.15	-	2.12	7.24	-	-	-
M	28	-	0.60	-	2.61	4.71	-	8.83	-
	35	-	0.49	3.17	4.80	6.52	-	3.09	0.01
	40	-	0.36	6.01	3.00	11.92	-	-	1.29
	A	-	-	-	-	35.46	-	-	-

The relative quantification was obtained by real-time PCR, through $2^{-\Delta\Delta Ct}$ method. F = female; M = male; (-) = no expression detected. The gonadal and mesonephric region were analyzed together at HH20, HH25, and HH28.

We observed transcripts of *DAX1* in gonadal tissue from the earliest stage examined (HH20) in both males and females (Figure 2C and Table 2). Significant *DAX1* expression in males and females was first seen at HH28, which corresponds to the onset of morphological

sex differentiation. *DAX1* expression was upregulated in male and female gonads at HH35, but it was more pronounced in females (Figure 2C). Male gonads showed the highest value of expression for *DAX1* at stage HH40, and its expression in male gonads was 3-fold higher than in female gonads.

Expression of *SCII* was high in cardiac and mesonephric male tissues (Tables 3 and 4). In gonadal tissue, *SCII* expression was upregulated in females (HH20-25); it was higher in males at HH28, and increased at HH40 (Figure 3A and Table 2). *HINTZ* was expressed in embryos at almost all stages and tissues studied (Tables 2-4 and Figure 3B). *HINTW* was expressed in all female tissues analyzed (Tables 2-4 and Figure 3C).

Our results further showed that *DMRT1* was expressed in male and female gonadal tissues (HH20–28) (Figure 4A). Prior to gonad morphological differentiation (HH20-25), gene expression was similar in males and females. At HH28, *DMRT1* expression was upregulated in males and expression levels were maximal at HH40. Mesonephric tissue showed *DMRT1* expression during all stages of development analyzed (Table 3) however at lower levels than in gonadal tissue (Table 2). Cardiac tissue showed *DMRT1* expression at HH28 and HH35 in both sexes and at HH40HH only in females (Table 4). MHM transcripts were detected almost exclusively in the female tissues (Figure 4B and Tables 2-4).

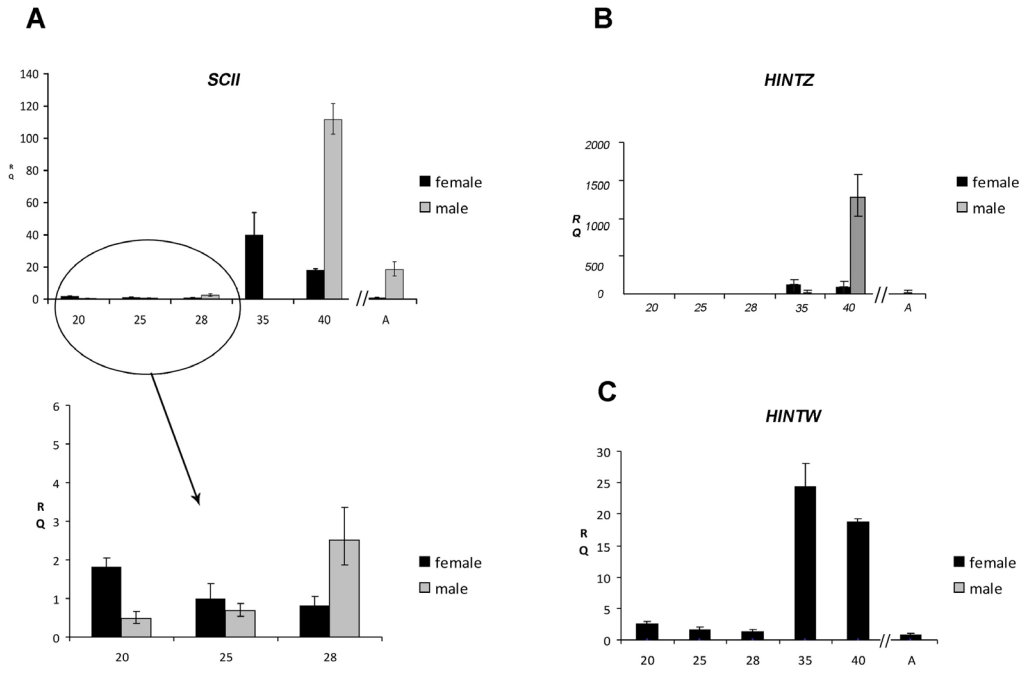

Figure 3. Quantitative real-time PCR analysis of chicken *SCII* (**A**), *HINTZ* (**B**) and *HINTW* (**C**) genes during testicular and ovarian development. cDNA from male and female gonads on embryonic stages HH20, HH25, HH28, HH35, and HH40, tests and ovary from adult animals (A) was amplified with gene-specific primers. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. RQ = relative quantification.

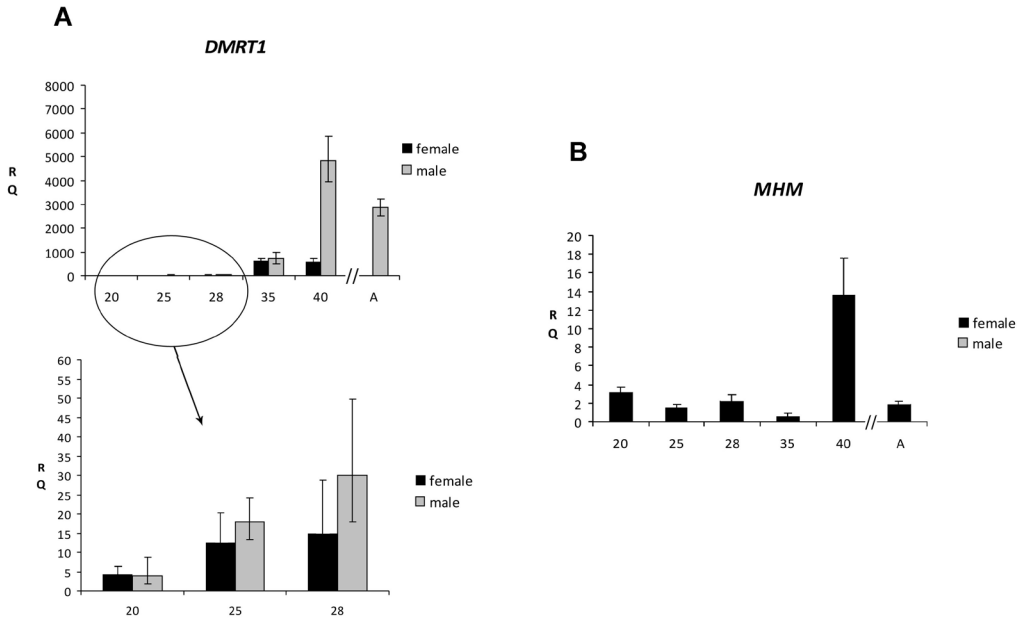

Figure 4. Quantitative real-time PCR analysis of chicken *DMRT1* gene (**A**) and MHM region (**B**) during testicular and ovarian development. cDNA from male and female gonads on embryonic stages HH20, HH25, HH28, HH35, and HH40, tests and ovary from adult animals (A) was amplified with gene-specific primers. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. RQ = relative quantification.

DISCUSSION

The *SOX3* gene maps to chromosome 4 of *G. gallus* and is expressed in both sexes during HH28 and HH40 stages with elevated levels at the time of sexual differentiation (i.e., between HH30 and HH35), indicative of a role of *SOX3* in both sexes (Smith et al., 1999). Our results suggest that *SOX3* expression in the female gonads may have an important role in sex differentiation. Further studies in chicken (between 30 and 35HH stages) may elucidate the function of *SOX3* in birds. Its expression detected in mesonephric tissue (somatic cell lineage) was in agreement with previous expression data for mammals (Weiss et al., 2003).

The *SOX9* gene is expressed during testicular development in birds, reptiles, amphibians, and fish. Unlike *SRY*, the *SOX9* gene has been hailed as the first truly conserved element of the vertebrate sex-determining cascade, controlling Sertoli cell differentiation and responding to distinct upstream triggers in the different groups (Smith and Sinclair, 2001). Our data were in agreement with semi-quantitative PCR studies previously performed by other authors (Smith et al., 1999). *SOX9* displayed differential expression patterns in gonadal tissue (Figure 2B), and it was also expressed in mesonephric and cardiac tissues (Tables 3 and 4), indicating other functions of this gene besides its role in gonadal development. According to our results and reports from the literature (Smith and Sinclair, 2001; Agrawal et al., 2009; Sekido and Lovell-Badge, 2009; Bagheri-Fam et al., 2010; Carré et al., 2011), *SOX9* expression in birds may be important for testicular development.

Other studies have shown that *DAX1* expression was higher in females than in males at HH30–HH32 (Smith et al., 1999, 2000; Yamamoto et al., 2003). Accordingly, our data showed elevated female expression of *DAX1* at HH35, but also higher expression in male gonads at HH40 (Figure 2C). Our data may indicate a role of *DAX1* in ovarian development and some function related to male development, indicated by *DAX1* upregulation in male gonads at HH40.

The higher *SCII* expression in cardiac and mesonephric male tissues indicated that *SCII* eludes a probable dosage compensation mechanism for gene expression from the Z chromosome. In gonadal tissue, *SCII* expression was upregulated in females before the onset of morphological differentiation (HH20-25), and at HH28 (corresponding to the start of morphological differentiation), the levels of expression were higher in males and further increased at HH40 (Figure 3A and Table 2). The expression dynamics in gonadal tissues observed here suggest a role for *SCII* in gonadal development or in sex-determination itself. The expression pattern of *SCII* did not indicate that this gene is related only to testicular differentiation, as suggested previously (McQueen et al., 2001), and *SCII* may participate in ovarian development.

HINTZ expression was slightly upregulated at the HH35 stage in gonadal tissue in females (Figure 3B). The highest level of *HINTZ* expression was noted in males at the HH40 stage (post-differentiation) and decreased in gonadal adult tissues of both sexes (Figure 3B). Previous studies (Yamada et al., 2004) and our results showed low *HINTW* expression in gonadal regions, during the HH20, HH25, and HH28 stages (Figure 3C). We describe here for the first time that *HINTW* expression in female gonads peaked at HH35. Interestingly, at HH40, the male gonad showed strong upregulation of *HINTZ*. These results could indicate an inhibitory effect of *HINTZ* that could regulate the *HINTW* expression on females at HH40. Until now, endogenously produced HINTW protein has not been detected, although recombinant HINTW could be over-expressed in bacteria (Moriyama et al., 2006). One possibility is that *HINTW* transcripts are not translated and act as an ncRNA. In addition, *HINTW* is expressed throughout the embryo at extragonadal sites, such as the central nervous system and myotomes (Scholz et al., 2006, Smith, 2007), and our data indicated expression in mesonephric and cardiac tissue (Tables 3 and 4).

In chicken, the *DMRT1* gene maps to the Z chromosome and has no homologue on the W chromosome (Nanda et al., 1999; Raymond et al., 1999). In the present study, we observed that *DMRT1* expression was upregulated in males and its levels were maximal at HH40 (4-fold higher than in females). The adult testis showed similarly high levels of *DMRT1* expression, which could indicate that *DMRT1* functions in spermatogenesis. Our data were in agreement with results of a recent study where expression of *DMRT1* was knocked down with the use of RNA interference in early embryos, resulting in feminization of the embryonic gonads in genetically male (ZZ) embryos (Smith et al., 2009). In conclusion, *DMRT1* is probably involved in the regulation of testicular differentiation.

Similar to some studies that have shown female-specific expression of MHM, we detected its expression only in the female gonadal tissue (Teranishi et al., 2001; Bisoni et al., 2005; Roeszler et al., 2012) (Figure 4B). We detected MHM expression almost exclusively in the female tissues (Figure 4B and Tables 2-4), as has also been observed in previous studies (Teranishi et al., 2001; Bisoni et al., 2005; Roeszler et al., 2012). We did not detect MHM expression in male gonadal tissues, which contradicts observations by Carré et al. (2011). However, mesonephric and cardiac tissues at HH35 and HH40 showed basal levels of MHM transcripts (Tables 3 and 4), which may be explained by our method having higher sensitivity than those

used in previous analyses (Teranishi et al., 2001), and this may be related to differential, tissue-dependent methylation of MHM. The expression profile of the MHM region in the gonadal region showed that its expression was unchanged during the initial stages and that MHM expression was upregulated at HH40 when gonadal differentiation takes place. MHM expression decreased in the adult ovary (Figure 4B).

The expression of MHM in females, and its accumulation near its locus, may be important in female development, and provide a regulatory mechanism similar to *Xist* RNA in mammals and to *roX1* and *roX2* RNAs in *Drosophila*, which represent two well-studied examples of ncRNA transcript components as part of mechanisms for compensating gene dosage.

In our study, the expressions from the MHM region (Figure 4B) and of the *DMRT1* gene (Figure 4A) were compared in gonadal tissue, and we verified that in females, RNA levels of MHM were increased at the same time as *DMRT1* expression was downregulated. Similarly, recombinant expression from an exogenous plasmid containing 2 kb of the chicken MHM region in the left testes of 13-week-old roosters resulted in decreased *DMRT1* expression (Yang et al., 2010). Nevertheless, expression of others candidate genes, such as *SOX9*, did not change in these tissues (Yang et al., 2010). Recently, Roeszler et al. (2012) observed that retroviral-mediated mis-expression of MHM in males induces disruption of testis development, but does not induce feminization. MHM mis-expression also affects the gonadal expression of *DMRT1* (Roeszler et al., 2012). This suggests that MHM ncRNAs could specifically regulate the expression of its neighboring genes, including *DMRT1* (Teranishi et al., 2001; Yang et al., 2010). Recently, *DMRT1* was shown to be a sex-determining gene (Zhao et al., 2010), and from our data and those of the literature we suggest that direct epigenetic regulation of *DMRT1* by the MHM region may be involved in determining sex in *G. gallus*.

CONCLUSIONS

In this study, we have described the expression profiles of several genes during chicken gonadal sex differentiation; the genes investigated were the following: *DMRT1*, *SOX3*, *SOX9*, *DAX1*, *SCII*, *HINTZ*, *HINTW*, and the MHM region. *HINTZ*, *DMRT1*, *DAX1*, *SOX9*, and *SCII* showed similar expression patterns with transcripts levels being higher in males than in females, which indicated putative roles of these genes in testis development or spermatogenesis. On the other hand, *SOX3*, *HINTW*, and the MHM region may act in ovary development or in suppressing male development. The increase in expression of the MHM region in females coincided with a decrease in *DMRT1* expression, which may reflect silencing by MHM-derived ncRNA. A similar mechanism could be involved in the *HINTZ* repression by the *HINTW* gene. Using qPCR, this study has investigated expression of the MHM region in different tissues and at different stages of gonadal development, and we have identified correlations in expression among genes involved in vertebrate sex determination. This report suggests the existence of epigenetic mechanisms, maybe involving ncRNAs in the regulation of gonadal development and sex determination in chicken.

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