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Expression analysis of differentially expressed miRNAs in male and female chicken embryos

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ABSTRACT. MicroRNAs (miRNAs) are small non-coding RNA molecules that play key roles in the regulation of development processes of many tissues and organs at the post-transcriptional level. However, little is known about how they affect chicken gonadal development. We examined the expression of four miRNAs (miR-218, -200b, -196, and -206) in chicken embryonic gonads at embryonic days 3.5-6.5. Their target genes were predicted by miRDB, TargetScan and PicTar algorithms. The expression levels of these four miRNAs differed with sex to varying degrees; miR-200b was expressed at a significantly higher level in female gonads during the entire interval. The whole mount *in situ* hybridization result showed considerably higher expression of miR-200b in females than in males in E5.5 embryos. The miRNA target scanning results indicated several genes with functions in gonad development and gonad function. We conclude

that miR-200b is involved in the regulation of gonad development and sexual differentiation of chicken embryos.

Key words: Chicken; MiRNAs; Sexual differentiation; Gonad development

INTRODUCTION

MicroRNAs (miRNAs) have been more and more of a concern after the discovery of the first miRNA lin-4 which can regulate the timing of the development of *Caenorhabditis elegans* embryos in the late stage (Lee et al., 1993). The characteristics and functional mechanism of miRNAs have been well elucidated with further research. They are a group of 20-24-nt endogenous non-coding and single-stranded RNAs that are cleaved from 70-80 nt hairpin pre-miRNA by the Dicer enzyme in cytoplasm, and mainly function at the post-transcriptional level by binding to the 3' untranslated region of their target genes through perfect or imperfect base-pairing. The most important is that miRNAs play wide and critical roles in controlling many animal biological processes in the development of tissues and organs, including developmental timing (Reinhart et al., 2000), muscle development (Chen et al., 2006), the development of nervous system (Li et al., 2006), and so on.

Animal gonadal differentiation and development are important biological processes for reproductive function. In recent years, miRNAs have been identified to be active during the developmental processes of the embryonic gonads, reproductive tissues and germ cells (Fu et al., 2008; Hong et al., 2008; Hossain et al., 2009; Torley et al., 2011). However, there are few studies focusing on miRNA function in chicken embryonic gonads. Until now, we and Smith's research group have reported that some miRNAs (miR-202*, miR-363, miR-101, and miR-31) show significant differential expression during chicken gonad sex differentiation (Bannister et al., 2009; Huang et al., 2010; Cutting et al., 2012). In addition, miR-202* has been inferred to control testicular genesis (Bannister et al., 2009). Apparently, more information is needed to clarify miRNA function in chicken sexual differentiation.

In our previous study, some miRNAs with sexual dimorphic expression were screened by miRNA chips. Here, the expression profiles of four chosen miRNAs (miR-218, -200b, -196, and -206) were constructed by using RT-PCR and WISH (whole-mount *in situ* hybridization), and the characteristics of their target genes were then analyzed to further understand the possible functions of specific miRNAs during chicken early gonadal differentiation.

MATERIAL AND METHODS

Gonad tissue collection and treatment

Fresh fertilized white leghorn eggs were incubated at 37.8°C with relative humidity of 60-70%. The embryo gonads at E3.5, E4.5, E5.5, and E6.5 were collected respectively (samples containing genital ridge at 3.5 and 4.5 days, urogenital system (UGSs) at 5.5 and 6.5 days) on super clean bench, and then immediately stored in RNAwait overnight at 4°C, and preserved at -20°C. DNA of every embryo was extracted from small parts of other tissues and used to sex the corresponding embryos by the duplex-PCR method (Feng et al., 2003). In the

WISH analysis, whole embryos at 4.5 days and UGSs at 5.5 days were isolated and sexed as above and then fixed overnight with 4% paraformaldehyde in PBS at 4°C, and the following treatment was according to Huang et al. (2010).

RNA extraction and cDNA synthesis

About 5-10 male or female embryonic gonad samples at each stage were pooled respectively, and total RNAs were then extracted with TRIzol reagent according to the operation manual (Invitrogen, USA). After determining the quality and concentration of extracted total RNAs, cDNAs were synthesized using miRNA-specific stem-loop RT primers with TransScript First-Strand cDNA Synthesis Super Mix kit (TransGen, China). Specific stem-loop primers of four miRNAs were designed according to the method described by Chen et al. (2005) and shown in Table 1. The mixture was as follows: 2 μg total RNA, 0.2 μL of each stem-loop RT-miRNA and R-5S primer (10 μM), respectively, 10 μL 2X TS Reaction Mix, and 1 μL TransScript RT/RI Enzyme Mix, adding RNase-free water to the total volume of 20 μL. The mixtures were then incubated in an Applied Biosystems 2720 Thermal Cycler for 50 min at 42°C, 15 min at 70°C.

Table 1. Primers used for amplifying mature miRNAs by stem-loop method.

Primer name	Primer sequences (5'-3')	Tm (°C)	Cycles	Size (bp)
Universal primer	AACTGGTGTCGTGGAGTCGGC	-	-	-
L-5S	CCATACCACCCTGGAAACGC	60	26	69
R-5S	TACTAACCGAGCCCGACCCT	60	26	69
RT-miR-196	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAACAAC	-	-	-
RT-miR-200b	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATCATCAT	-	-	-
RT-miR-206	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCACACAC	-	-	-
RT-miR-218	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACATGGTT	-	-	-
L-miR-196	TAGGTAGTTTCATGTTGTTGGCTC	60	33	54
L-miR-200b	TACCCTGTAGAACCGAATTTGT	59.8	33	62
L-miR-206	GGTGGAATGTAAGGAAGTGTGTGG	59	32	57
L-miR-218	GTTGTGCTTGATCTAACCATGTCTC	58.7	33	55

Semi-quantitative analysis of expression profiles

Chicken 5S rRNA was used as the internal reference for analyzing the expression profile of each miRNA. A universal sequence of stem-loop RT primers was used as reverse primer for amplifying specific miRNA. PCR was carried out in a 15-μL mixture including 1 μL cDNA, 0.05 μL dNTP (10 mM), 1.5 μL Mg^{2+} buffer (25 mM), 0.2 μL of each specific miRNA primer (L-miR) and reverse primers (10 μM), and 0.1 μL *Taq*DNA polymerase (5 U/μL). The PCR amplification procedure was as follows: 95°C for 5 min (95°C for 15 s, optimum annealing temperature for 15 s and 72°C for 20 s) x optimum cycling number, and final extension at 72°C for 5 min.

The amplified products were run on 2.5% agarose gels with 110 V for 25 min, and gels were photographed by the Bio-Rad gel imaging system (Bio-Rad, USA). The optical density values of miRNA and 5S rRNA bands in gels were calculated by using the Quantity-One software, and the relative expression of detected miRNAs were then standardized by using the optical density values of miRNAs to those of corresponding 5S rRNA. The variabilities of dif-

ferent expression between the two sexes at the same stage and between different stages in the same sex were analyzed by a *t*-test. Finally, the temporal expression patterns of each miRNA were plotted based against the above analysis results.

Spatial expression analysis by WISH

The 20-nt antisense RNA oligonucleotide probe that is the complement to miR-200b and the sense probe that is the homologue to miR-200b were synthesized respectively according to the instructions of mirVana™ miRNA probe construction kit (Ambion) and labeled with UTP-digoxin (Roche, Switzerland). Three chicken samples of each group were pooled in a 1.5-mL tube for hybridization. The WISH procedure followed the method described by two research groups (Thisse et al., 2004; Huang et al., 2010). Images of whole-mount embryos/UGSs were captured using a stereomicroscope (LEICA MZ 75).

Target analysis of miRNAs

The analysis of predicted targets of chicken miRNAs was done by using three algorithms: TargetScan 5.1 (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/) and PicTar (http://pictar.mdc-berlin.de/). The common targets predicted by the three different programs were then identified.

RESULTS

Temporal expression profiles in chicken embryos

The amplification electropherogram and corresponding temporal expression profiles of four miRNAs during chicken early gonadal development are shown in Figure 1. Figure 1A displays the results of gel electrophoresis of the miRNAs tested and 5S rRNA. Every sample at each stage had triplicate biological repeats. The amplification results of 5S rRNA and miRNA tested during the period of E3.5-E6.5 in female embryonic gonads were shown respectively at the left of marker, the right ones were the results of males.

The temporal expression profiles of miR-218, -206, -196, and -200b are shown in Figure 1B-E, respectively. During the developmental process of E3.5-E6.5, the expression levels of miR-218 in female embryonic gonads were always higher than those in males, and maintained the same expression trend in both sexes, which showed slight downregulation at first, then slight upregulation and downregulation again (Figure 1B). However, miR-206 expressed at a higher level in males than in females without significant difference (Figure 1C). The expression trends of miR-196 were similar in the two sexes going up dramatically from E3.5 to E4.5, then dropping down sharply and rising slowly later. The expression levels were lower in females than those in males at E3.5 and E4.5, with a highly significant difference ($P < 0.01$) at E3.5, but higher in females at E5.5 and E6.5 with a significant difference ($P < 0.05$) at E6.5 (Figure 1D). Similar to miR-218, the expression levels of miR-200b in female embryonic gonads were always higher than those in males, and the expression difference between the two sexes was significant ($P < 0.05$) at both E3.5 and E6.5, and an even highly significant difference ($P < 0.01$) was observed at E5.5 (Figure 1E).

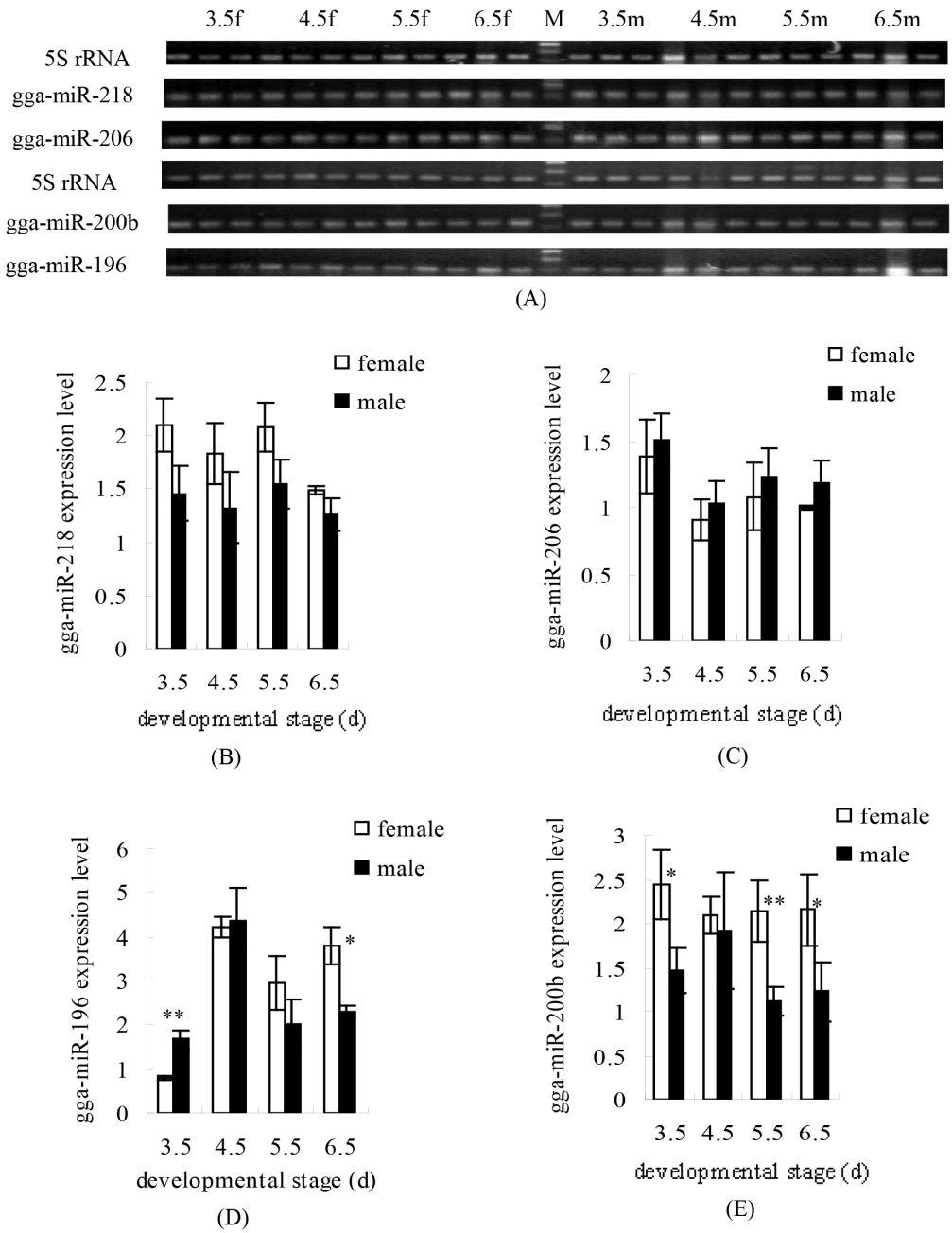

Figure 1. Expressions of miR-218, -206, -196, and -200b during sex differentiation in chicken embryo (**A**). Electrophoretogram of miR-218, -206, -196, -200b, and 5S rRNA, triple per sample. f = female, m = male; M = marker, 50 bp. **B. C. D. E.** Developmental expression profiles of miR-218, -206, -196, and -200b, respectively; *t*-test. *Significant difference ($P < 0.05$), **extremely significant difference ($P < 0.01$). (d) = days.

Spatial expression patterns by WISH

Semi-quantitative results of miR-200b showed a significant difference in E5.5 embryos between the two sexes, so its spatial expression in E4.5 embryos and E5.5 UGSs were further studied by WISH. The results are shown in Figure 2, where no positive expression was detected in tissues hybridized with control sense probe at either E4.5 or E5.5. In E4.5 chicken embryos hybridized with anti-sense probe, miR-200b was expressed in limb bud, notochord, ventral aspect, and brain in both sexes. In E5.5 embryos, visible hybridization signals appeared in gonad and the surrounding kidney, and obviously higher expression was in females than in males.

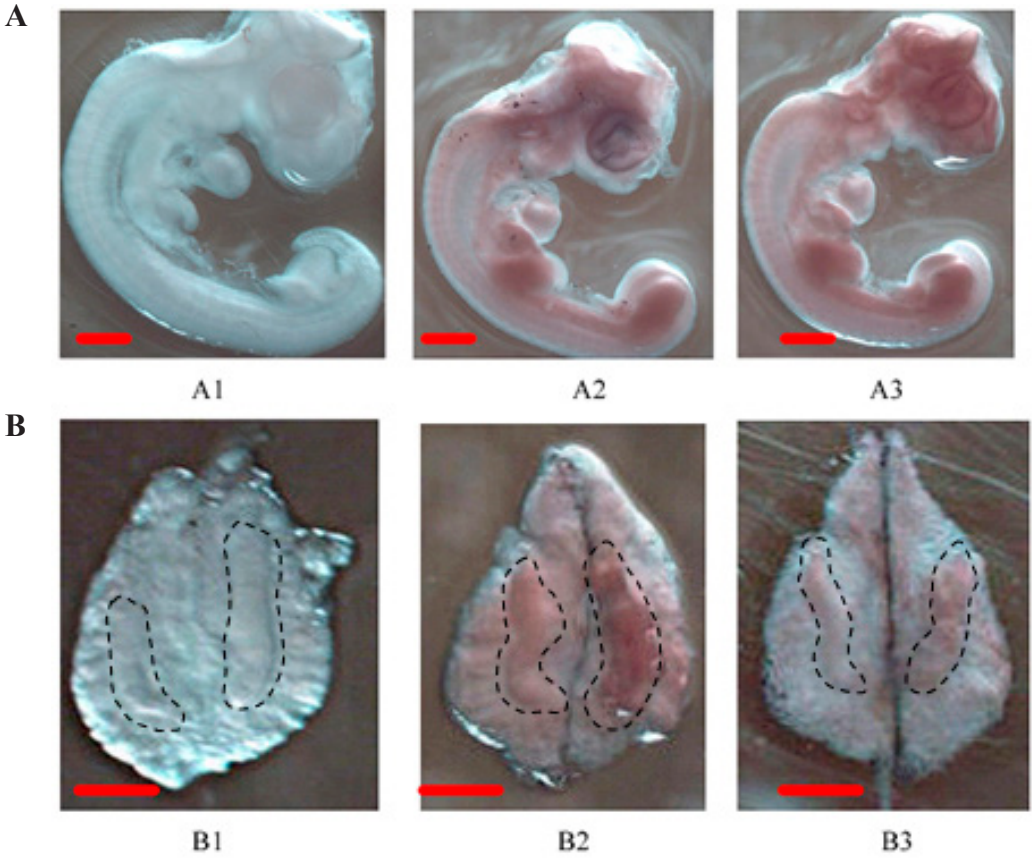

Figure 2. Analysis of miR-200b expression by WISH **A** and **B** showed whole chicken embryos from E4.5 and UGSs from E5.5 probed with RNA sense probe (**A1** and **B1** = negative control) and anti-sense probe (**A2** and **B2** were females, **A3** and **B3** were males). Positive detection of miRNA expression was indicated by brown staining. Dashed = gonad. Bar = 500 μm.

Putative miRNA target gene prediction

TargetScan 5.1, miRDB and PicTar programs were used to predict the target genes of

the four miRNAs tested, and genes associated with animal gonad development and reproductive function among the common targets of each miRNA are displayed in Table 2.

Table 2. Putative target genes and function for miRNAs.

miRNA name	Putative targets	Function
miR-218	*NMT2* (N-myristoyltransferase 2)	Be involved in testis development (Quintero-Rivera et al., 2007).
	HAPLN1 (Hyaluronan and proteoglycan link protein 1)	May promote periovulatory granulosa cell survival (Liu et al., 2010).
	RNF139 (Ring finger protein 139)	Plays a critical function during male germ cells meiosis (Cho et al., 2005).
	CLK3 (CDC-like kinase 3)	Be related to the acrosome reaction of mature spermatozoa (Menegay et al., 1999).
miR-196	*CDKN1B* [Cyclin-dependent kinase inhibitor 1B (p27, Kip1)]	Determining mammalian ovarian development (Wang et al., 2010).
miR-206	*CLTC* [Clathrin, heavy chain (Hc)]	Initiating the formation of tubulobulbar complexes which function during sperm release and the translocation of spermatocytes in rat (Young et al., 2009).
	VAMP4 (Vesicle-associated membrane protein 4)	Be important for acrosome biogenesis during spermiogenesis (Guo et al., 2010).
	CLCN3 (Chloride channel 3)	Be involved in the chloride channels in volume regulation of spermatozoa after ejaculation (Cooper and Yeung, 2007).
miR-200b[1]	*FMR1* (Fragile X mental retardation 1)	Having functions in regulating ovarian aging and determining ovarian reserve (Gleicher and Barad, 2010).

[1]Common targets of miR-200b predicted only by miRDB and TargetScan.

DISCUSSION

The expression information of any molecules including miRNAs at specific developmental stages as well as in specific tissues is of great significance for understanding their functions. In the present study, results of temporal expression showed that miR-196 and miR-200b were differentially expressed according to sex in chicken embryos at some critical stages of sex differentiation, especially the higher continued expression of miR-200b in females at all stages tested. Furthermore, the higher expression of miR-200b in female gonads shown by WISH was consistent with that of temporal expression. The expression profiles of miRNA-196 and -200b indicated that they may have some regulatory functions in sexual differentiation or gonadal development in the embryonic chicken.

The four miRNAs tested in this study have been reported to be involved in the process of organ (tissue) development. For instance, miR-196 has been found to play a role in patterning the axial skeleton in chicken (McGlinn et al., 2009); miR-206 has been demonstrated to be involved in regulating proliferation and differentiation of skeletal muscle satellite cells in mammals (Chen et al., 2010). Although some predicted targets of all these four miRNAs (listed in Table 2) were found to be involved in the developmental process of the animal gonad and maintenance of gonadal functions, they were not reported to have any function in animal gonadal development in previous studies except for miR-200b.

In present study, we found that miR-200b was universally expressed in some tissues in E4.5 chicken embryo, which was similar to the results of other studies in zebrafish (Wienholds et al., 2005) and chicken (Darnell et al., 2006). Accordingly, miR-200b has been shown to have important functions in many organs, including the development of olfactory bulb and palate (Bak et al., 2008; Shin et al., 2012), renal tubule maturation (Patel et al., 2012), some cancer development (Kan et al., 2012; Kurashige et al., 2012; Dai et al., 2013), etc. Recently, miR-200b and its two other family members were observed to be highly overexpressed in the SEOC

(serous epithelial ovarian cancer) cell lines, and suggested to correlate with serous epithelial ovarian cancer (Kan et al., 2012). This conclusion indicated that the normal expression of miR-200b is important to ovary development. Additionally, FMR1, the predicted target gene of miR-200b, was considered to be a regulator of ovarian function. Certainly, the interaction between miR-200b and FMR1 should be further identified. We can suppose that miR-200b could be involved in gonad (ovary) development in chicken embryo.

In this study, the expression pattern at chicken embryonic gonads and the targets of four miRNAs were analyzed. According to the combined results of gonadal expression of miRNAs in chicken embryos and the functions of their putative targets analyzed on the basis of reported research, we can conclude that miR-200b may be involved in the regulation of chicken gonadal development. This finding provides information that miR-200b could serve as a candidate for further studies to understand the regulatory function of miRNAs during the process of chicken sex differentiation and gonadal development.

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