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Original Article

Cloning, identification, and expression analysis at the stage of gonadal sex differentiation of chicken miR-363 and 363*

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miRNAs (microRNAs) are small, functional, non-coding RNAs and have been proved to implicate in regulation of diverse biological processes ranging from cell differentiation to organism development. With the purpose of exploring the roles of miRNAs on chicken embryo sexual determination and gonadal differentiation, we cloned and identified the stem-loop precursor structure (GenBank accession no. GU597370) of chicken miR-363 and 363* followed by studying their temporal and spatial expression patterns in chicken embryo at the stage of E3.5–6.5 d (embryonic days 3.5–6.5) by semi-quantitative RT-PCR and WISH (whole-mount *in situ* hybridization) in this study. The results showed that miR-363* located in cloned sequence of unknown segment in chicken genome, and flanking sequence of miR-363 and 363* according to the structural features of miRNAs precursor. Significantly differential expression ($P < 0.05$) of gga-miR-363 between female and male chicken embryonic gonads was found at E4.5 and 6.5 d, but the differential expression of gga-miR-363* from E3.5 to 6.5 d between both sexes fell short of significant level. The results of WISH indicated that expression signals of gga-miR-363 mainly appeared at limb bud, notochord, ectoderm, brain in E4.5 d chicken embryo, and urogenital systems (UGSs) at E6.5 d, and the expression level of E6.5 d was higher in the female than that in the male. It can be speculated that gga-miR-363 would involve in the gonadal development and gga-miR-363* might have transient regulatory functions during the early stages of chicken embryo development.

Keywords chicken; miRNAs; gonadal development; expression pattern; differential expression

Introduction

MicroRNAs (miRNAs) are a group of recently discovered, 19–25 nt endogenous non-coding RNAs that are cleaved from 70 to 100 nt hairpin precursors. These tiny RNAs regulate the expressions of target genes at post-transcriptional level by two kinds of different mechanism: triggering mRNA cleavage or inhibiting translation through perfect (in plants) or imperfect (in animals) complementarity to 3′ UTR (untranslated region) of target mRNA [1]. miRNAs have been found in *Caenorhabditis elegans*, *Drosophila*, chicken, mice, and human, suggesting an ancient and widespread role. They are believed to be critical in a wide range of biological processes, and they would target at many genes. In animals, the most comprehensive functions of miRNAs are to control development processes, such as regulation of development timing, differentiation, and proliferation of specified cells, formation of organ morphology [2,3].

Gonadogenesis, especially sex determination and differentiation, is the important developmental events in animals. Because the chicken embryo is visible and easily accessible for manipulation during embryogenesis, it has been a cornerstone of studying gonadal development, and this model system has contributed greatly to our understanding of vertebrate development process [4]. Studies on sexual determination and gonadal development in chicken always become hot spots in recently decade years. In chicken, male is homogametic sex (ZZ), whereas the female is the heterogametic (ZW), and the genetic pathways are differentially activated to initiate testis or ovary development [5]. However, the mechanism underlying sex determination remains unknown, and two different sex determination hypotheses, Z-dosage theory antagonized by W-dominance theory, is existing. Previously, several transcription factors that participated in sexual determination and gonadal development have been isolated, such as *AMH*, *SOX9*, *DMRT1*, and so on [6], but many pieces are missing and little information is available on how this process is regulated.

Up to date, studies on regulatory functions of miRNAs during gonad development are rarely reported. In maize, *Zea mays*, miR-172 encoded by *ts4* gene have been determined to control sex determination through interaction with

its target gene *IDS-1* [7]. In animals, some studies have indicated that miRNAs would participate in gonadal development, such as maintaining functions of germ stem cells and somatic cells in *Drosophila* [8] and controlling spermatogenesis in the simplified chordate, *Oikopleura dioica* [9] and mammals [10]. Moreover, in chicken embryo gonad, miR-202* was observed to be sexually dimorphic, with up-regulation in the developing testis from the onset of sexual differentiation, and suggested it would function in regulating testicular development [11]. However, few studies have examined the regulatory roles of miRNAs existed in sexual determination and gonadal differentiation in chicken, and further investigations will be needed.

In our previous work, differentially expressed miRNAs between male and female chicken embryo at E3.5 d were analyzed by microarray chip (CapitalBio Corp., Beijing, China). The microarray chip included 743 probes in triplicate, corresponding to 576 human (including 122 predicted miRNAs), 358 mouse, and 238 rat miRNA sequences. Thus, expressed miRNAs included some potentially novel chicken miRNA molecules, which were homologous with the corresponding sequences of human, mouse, or rat. In the present study, miR-363 and 363*, which is located in the same stem-loop precursor, but no relative information in chicken, existed in the miRBase database (http://microrna.sanger.ac.uk/sequences/), were used as experiment subjects. According to the homology analysis, we predicted the sequences of gga-miR-363 and 363*, and genome blast at UCSC (http://genome.ucsc.edu) indicated that predictive miR-363 sequence located in the antisense strand of chicken fourth chromosome, nevertheless, its flanking sequence containing miR-363* was unknown. Then we cloned and identified the sequence of precursor, carried out homology analysis, and studied temporal and spatial expression patterns of miR-363 and 363* in female and male chicken embryonic gonads at E3.5−6.5 d, in order to better understand their biological function *in vivo*.

Materials and Methods
Gonad tissue collection and embryo sexing
Freshly fertilized white leghorn chicken (*Gallus gallus*) eggs were incubated under 60−70% relative humidity at 37.8°C.

To analyze the temporal expression profiles of the chicken embryos, gonads were harvested at 3.5, 4.5, 5.5, and 6.5 d after hatching, respectively, on super clean bench (3.5 and 4.5 d samples obtained trunks containing genital ridge, 5.5 and 6.5 d samples obtained urogenital systems, UGSs). Samples were preserved in the RNA wait (Fastgen, Foster City, USA) overnight at 4°C, and then stored at −20°C. Meanwhile, a fraction of other tissue of each embryo was taken for the extraction of DNA for sexing by the duplex-PCR method established in our laboratory [12].

For the chicken embryo samples used for the analysis of spatial expression patterns, embryos were harvested (4.5 d samples obtained the whole embryos, 6.5 d samples obtained UGSs) and sexed as above. Then, the samples were fixed overnight with 4% PFA (paraformaldehyde) in PBS (phosphate-buffered saline) at 4°C, followed by rinsing twice in PBT (PBS with 0.1% Tween-20) for 10 min, dehydrating for 10 min in a series of methanol (25, 50, 75, 100%, dilution by PBT), and then stored at −20°C until whole mount *in situ* hybridization (WISH).

Cloning, identification, and homology analysis of miR-363 and 363* precursor
Primers [5′-CTGTGTCAGCGGATGAAGGT-3′ (L) and 5′-CTGTTAGATAAAGTGCGTCCCG-3′ (R)] were designed according to the anterior−posterior sequences of genome where chicken miR-363 existed. The unknown sequence of the precursor was amplified with chicken genome DNA as a template, followed by cloning and sequencing. Cycling parameters were 95°C for 5 min, 35 cycles of 95°C, 40 s; 60°C, 40 s; 72°C, 40 s, and final extension at 72°C for 5 min.

The 110 nt flanking sequence containing miR-363 and 363* sequences cloned from chicken genome was folded by RNAfold (version 3: http://frotend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi). Homology analysis of miR-363 and 363* precursor sequence between chicken and other several species (human, mouse, and rat) was carried out by ClustalW software (http://www.ch.embnet.org/software/ClustalW.html).

Analysis of temporal expression profiles by RT-PCR
Ten pairs of chicken embryonic gonads from E3.5 to E6.5 d were pooled, respectively, by sex, each with triplicate biological repeats. Total RNAs were isolated with Trizol reagent (Invitrogen, Carlsbad, USA), and treated with DNase for 20−30 min at 37°C according to the manual of RNA purification kit (Ambion, Austin, USA) to remove DNA in order to avoid contamination of trace amount of DNA. RNA concentrations were measured by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA). The total RNAs were polyadenylated with poly(A) tailing kit (Ambion), then purified with phenol and chloroform, and reverse-transcribed by SuperScript^{TM} III reverse transcriptase (Invitrogen) with 0.5 μg poly (T) adapter (5′-GCGAGCACAGAATTAATACGACTCACTATAGG(T)$_{12}$VN-3′ (V = A,G,C; N = A,G,C,T).

Chicken 5S rRNA was selected as the internal reference gene for the analysis of expression profiles. All reverse primers of miRNAs and internal reference gene were adapter primer of reverse transcription (5′-GCGAGCACAGAATTAATACGA-3′), and the forward primers were designed by primer 5.0 software based on the entire tested miRNAs sequences. The forward primers of miR-363,

363* and 5S rRNA were 5′-AATTGCACGGTATCCATC TGT-3′, 5′-CGGGTGGATCACGATGC-3′ and 5′-AATA CCGAGTGTCGTAGGCG-3′, respectively, and PCR products were expected to be about 65 bp. PCR reactions were carried out in a 15 μl mixture containing 1 μl cDNA, 0.1 μl dNTP (10 mM), 1.5 μl Mg^{2+} buffer (25 mM), 0.2 μl forward and reverse primers, respectively, 0.1 μl *Taq* DNA polymerase (5U/μl). PCR amplification was carried out by optimizing the annealing temperature and cycles. Cycling parameters were: 95°C for 5 min, (95°C for 15 s; annealing temperature for 15 s; 72°C for 40 s) ×cycles, 72°C for 5 min.

The PCR products were run on 2.5% agarose gels with 110 V voltage for 25 min. Gels were photographed using BIO-RAD gel imaging system (Hercules, USA), and optical density values of miRNA bands were calculated by Quantity-one software and standardized by the densities of corresponding 5S rRNA bands. After calculating the standard errors of expression levels of three independent experiments, variabilities between different sexes at the same stage and among different stages in the same sex were analyzed by *t*-test, and then relative expression profiles of each development stage were plotted.

Analysis of spatial expression patterns by WISH

WISH of miR-363 was performed as follows. The synthetic antisense RNA oligonucleotide probe with about 20 nt of complementarity to mature miR-363 was used to detect the expression signals in chicken embryo, and synthetic sense RNA oligonucleotide probe with about 20 nt of homogeny to miR-363 as a control. According to the instruction of mirVana™ miRNA probe construction kit (Ambion), probes were synthesized by *in vitro* transcription with UTP-digoxin (DIG) (Roche, Basel, Switzerland). The labeling efficiency and the amount of probes were detected by dot blot hybridization with an anti-DIG Fab fragment conjugated to AP (Roche).

For WISH, three to four embryonic chicken samples of each sex at each stage were pooled in a tube for hybridization. All the procedure followed the method described by Thisse *et al*. [13], and the protocol found on the website (http://geisha.arizona.edu/geisha/documents) except that the samples were treated with 6% H_2O_2 for 1 h (E4.5 d) and 10 min (E6.5 d), respectively, before proteinase K digestion. Images of whole-mount embryos/UGSs were captured using stereoscopic microscope (LEICA MZ 75), and the background was replaced to light blue with PhotoShop software.

Results
Cloning, identification, and homology analysis of miR-363 and 363* precursor

The length of the unknown sequence containing the precursor that we amplified by PCR was about 450 bp (**Fig. 1**, lane 2), and the precise length was 452 bp by sequencing. Chicken miR-363* sequence obtained through homology hybridization in chip was in the amplified unknown sequence after blasting with the result of sequencing, and in the upstream of genome sequence of miR-363 (**Fig. 2**).

Based on the result of cloning and the positions where miR-363 and 363* existed, their flanking sequence was folded by RNA-folding software. The results showed that the flanking sequence could form a stem-loop structure, miR-363 and 363* located at both arms of the hairpin, and the lowest free energy (ΔG) was −26.6kcal/mol (**Fig. 3**).

The homology of the precursor sequences of miR-363 or miR363* among chicken (gga), human (hsa), mouse (mmu), and rat (rno) was analyzed by ClustalW software. The results showed that the similarities between chicken and human, mouse or rat were 94, 89 and 92% respectively, and the mature miRNAs showed high homology with their counterparts in other species, with only one base difference (**Fig. 4**).

Analysis of temporal expression profiles

The expressions of miR-363 and 363* in female and male chicken gonads during the early stages of sex differentiation (E3.5−6.5 d) were detected by semi-quantitative RT-PCR. The amplification results of miR-363, 363* and
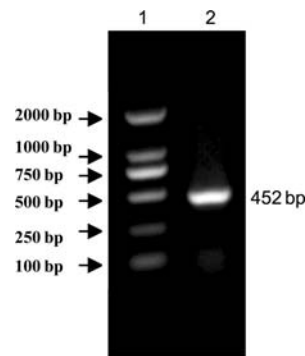

Figure 1 PCR amplification of unknown sequence including pre-miRNA   Lane 1, marker DL 2000; lane 2, PCR product.

Figure 2 Unknown sequence including pre-miRNA   miR-363* is boxed, miR-363 is indicated with shading and primers are underlined.

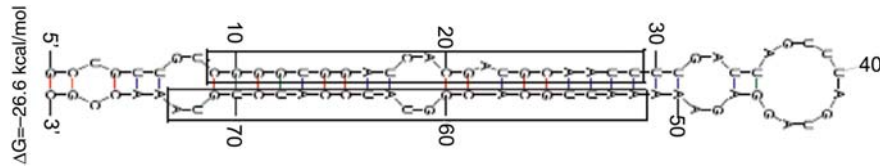**Figure 3 Precursor structure of miR-363 and miR-363*** miR-363 and miR-363* are boxed.


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gga-pre-miRNA    ----GCUGUUGUCGGGUGGAUCACGAUGCAAUUUUGAUUAGUUUAGUAGGAG
hsa-pre-miRNA    ------UGUUGUCGGGUGGAUCACGAUGCAAUUUUGAUGAGUAUCAUAGGAG
mmu-pre-miRNA    ------UGUUAUCAGGUGGAACACGAUGCAAUUUUGGUUGGGUGUAAUAGGAG
rno-pre-miRNA    UUUUGCUGUUAUCGGGUGGAUCACGAUGCAAUUUUGAUUAGAGUAAUGGGAG
                 * * * *  * * * * * * *   * * * * * * * * * * * * * * *   *    *   *  * * * *

gga-pre-miRNA    AAAAAUUGCACGGUAUCCAUCUGUAAACCGC----
hsa-pre-miRNA    AAAAAUUGCACGGUAUCCAUCUGUAAACC------
mmu-pre-miRNA    GAAAAUUGCACGGUAUCCAUCUGUAAACC------
rno-pre-miRNA    AGAAAUUGCACGGUAUCCAUCUGUAAACCGCAGGA
                 * * * * * * * * * * * * ***  * * ** * * * * * * **
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Figure 4 The homology analysis of precursor in chicken with human, mouse, and rat miR-363* is underlined and miR-363 is indicated with shading.

5S rRNA for each sample with triplicate repeats were shown in **Fig. 5(A)** and the corresponding expression profiles were given in **Fig. 5(B–D)**.

Figure 5(B) was the expression profile of gga-miR-363. In female chicken embryo, the expression level was down-regulated between E3.5 and 4.5 d, then upregulated remarkably, and maintained this high expression level during E5.5 and 6.5 d. The expression level between E4.5 d and other three stages was significantly different ($P < 0.05$). In male, the expression was inhibited step by step from E3.5 to 6.5 d, and the most obvious reduction appeared at E4.5 and 5.5 d, and the expression difference was significant between E4.5 and 6.5 d ($P < 0.05$). The higher expression in male than in female appeared at E3.5–4.5 d, but this difference was opposite in other two stages. In addition, the differential expression between sexes reached significant level at E4.5 and 6.5 d ($P < 0.05$).

The expression profile of gga-miR-363* was shown in **Fig. 5(C)**. In females, its expression was dramatically decreased from E3.5 to 4.5 d, increased from E4.5 to 5.5 d, and then stepped down a little from E5.5 to 6.5 d. Between E3.5 and 4.5, E3.5 and 6.5, E4.5 and 6.5 d, significant differential expressions ($P < 0.05$) were observed. In males, the expression was persistently downregulated until E5.5 d. At E3.5 and 5.5 d, the expression levels were higher in females than in males, whereas an opposite trend was observed at E4.5 d, and a considerable level existed at E6.5 d. But there were no significant differential expression between sexes at each stage.

The comparison of expression levels between miR-363 and 363* were shown in **Fig. 5(D)**. The results indicated that the expression level of miR-363 was higher than 363* in the same sex at the same stage, and the difference was most obvious with about 2 folds in each sex at E4.5 d. There were significant difference for the expression of miR-363 and 363* in E4.5 male and 6.5 d female ($P < 0.05$).

Analysis of spatial expression patterns by WISH

The expression of miR-363 was detected in whole chicken embryo in both female and male at E4.5 d. Compared with the control, which showed little background hybridizing with the sense probe [**Fig. 6(A)**], miR-363 was expressed obviously at limb bud (a), notochord (b), ectoderm (c), and brain (d) in both sexes [**Fig. 6(B,C)**]. There was no difference between the expression of miR-363 in female and male.

It was difficult to determine whether the hybridization signal appeared or not in UGS at the early stage (E4.5 d), so the gonad and kidney system of E6.5 d chicken embryo was used as materials to observe the expression signal. The visible hybridization signals of miR-363 appeared at gonad (dashed) and the surrounding kidney, and the signals were obviously higher in female than in male [**Fig. 6(E,F)**]. Little staining was detected in tissues hybridized with control sense probe [**Fig. 6(D)**].

Discussion

In chicken, the sequence of miR-363 had been obtained through homology searches using human miRNA gene sequences [14] and isolated from small RNA library of embryo at different stages [15,16]. However, the identification of structural features had not been done, and it was not approved by miRBase database. During the process of biosynthesis of miRNAs, miRNA and miRNA* were generated by cleaving single-strand RNA precursor with 70–80 nt hairpin, so the presence of hairpin structures
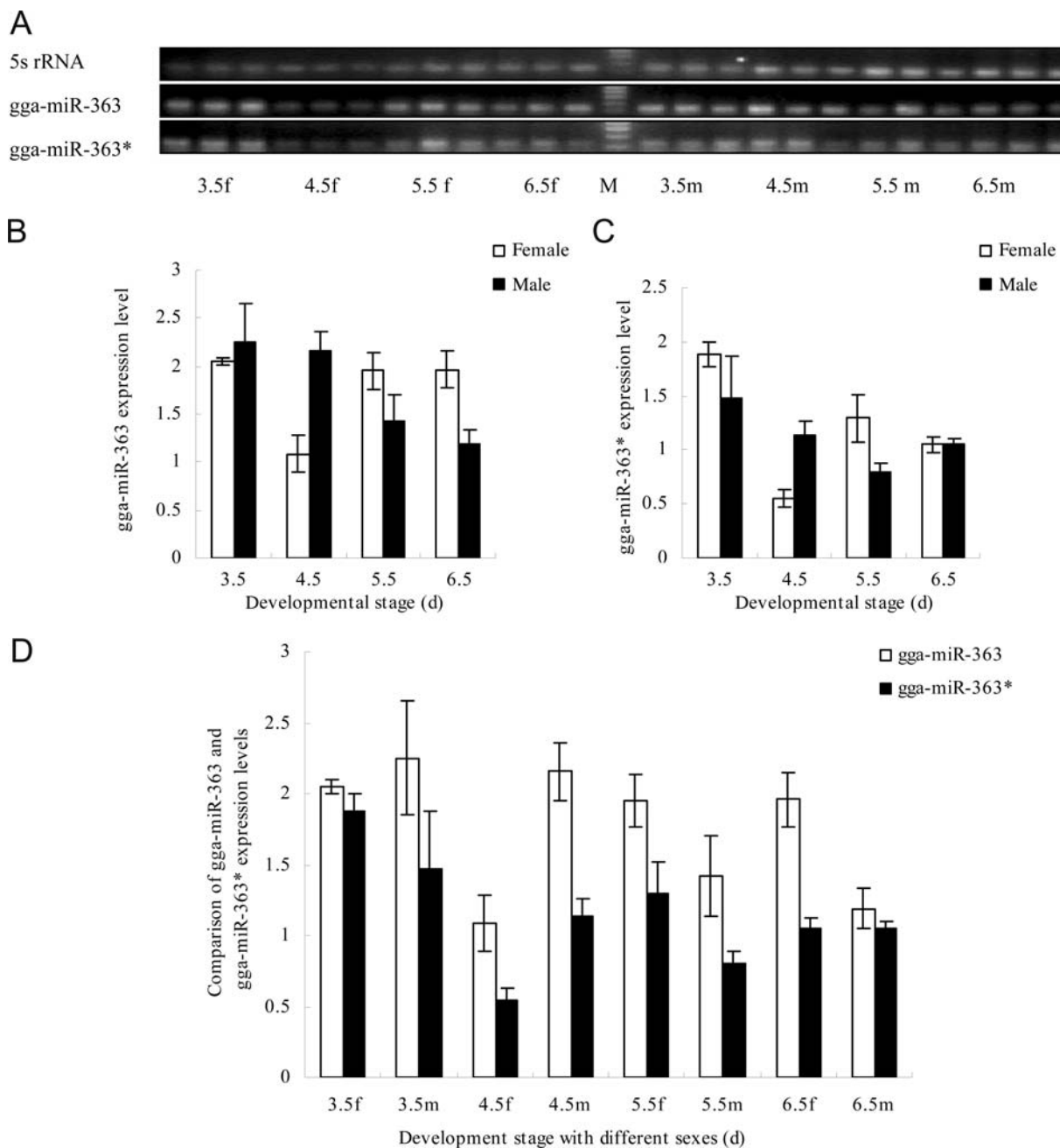
Figure 5 Comparison of expressions of gga-miR-363 and gga-miR-363* during sexual differentiation in chicken embryo (A) Electrophoretogram of gga-miR-363 and gga-miR-363*. All the samples were done in three independent experiments. f stands for female; m stands for male; maker, 50 bp. (B) and (C) The developmental expression profiles of gga-miR-363 and gga-miR-363*, respectively. (D) Comparison of gga-miR-363 and gga-miR-363* expression.

containing miRNAs was established as an important criteria to determine whether small RNAs were miRNAs. Meanwhile, the candidate miRNAs would be entirely within the arm of the hairpin [17], and research personnel of Massachusetts Institute of Technology pointed out that the lowest free energy was at least 25 kcal/mol (ΔG folding ≤ -25 kcal/mol) in miRscan method [18]. In this study, the flanking sequence containing miR-363 and 363* in chicken were cloned, and we confirmed that the flanking sequence could form the typical hairpin precursor structure

with mature miRNA sequences in each arm, which was predicted by RNA-folding software (**Fig. 3**). Simultaneously, the lowest free energy of this pre-miRNA was -26.6 kcal/mol and according to the feature of energy. In addition, multiple sequence blast of precursor and mature sequences showed that the similarities of precursor sequences between different species with chicken were 94, 92 and 89% respectively, and the mature sequences among chicken, mouse, rat, and human were evolutionarily conserved, which appeared consistent with
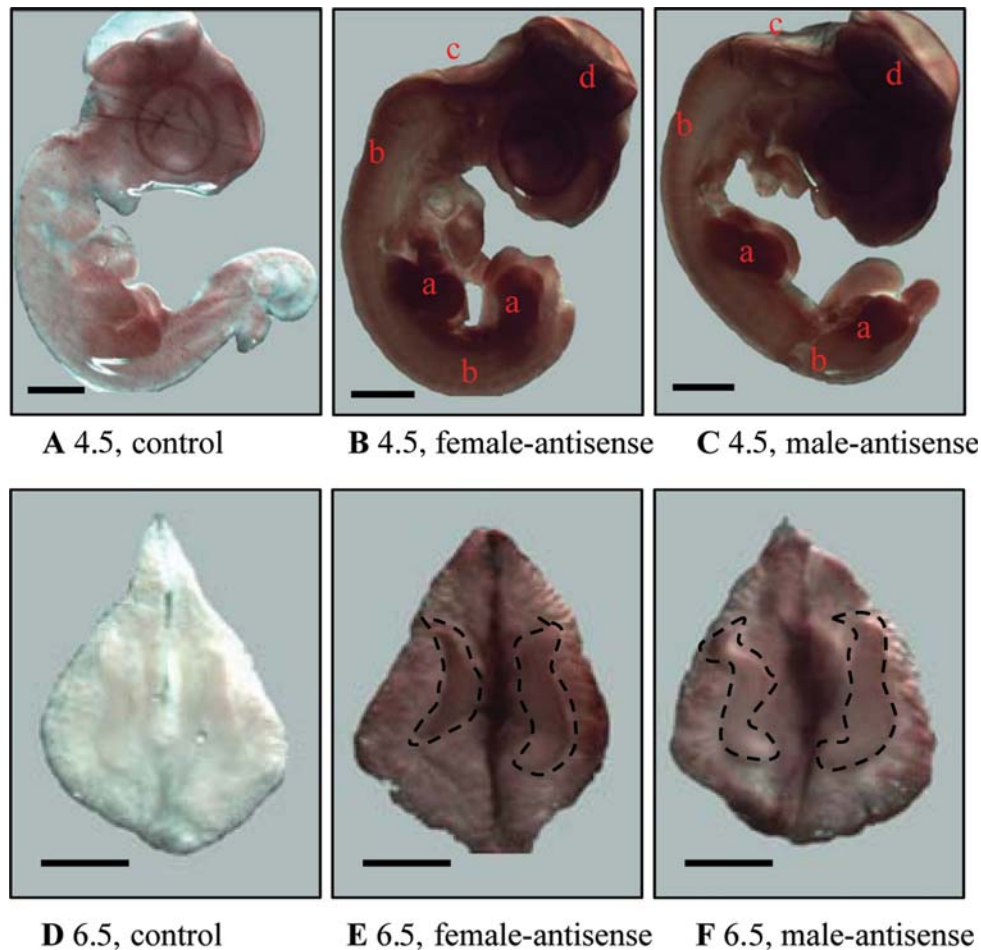
Figure 6 Analysis of miR-363 expression by WISH in whole-mount chicken embryos and UGSs from E4.5 d and E6.5 d respectively probed with RNA antisense and sense probe (negative control) Positive detection of miRNA expression was indicated by brown or purple staining, and signals appear at limb bud (a), notochord (b), ectoderm (c), and brain (d) of E4.5 d chicken embryo and at gonad (dashed) and kidney of E6.5 d UGSs. Images were taken approximately 20 min after addition of color solution. Bars = 500 μm.

the results observed by Hicks *et al*. [19]. and the opinions raised by Ason *et al*. [20] that high conservatism of evolution of miRNA was present between species, especially the part of stem, and more mutable point existed in the loop region. All the above was in compliance with criteria for miRNA annotation as bona fide miRNAs, so we took them as gga-miR-363 and gga-miR-363*.

It was important to study temporal and spatial expression patterns of miRNAs in specified tissue and development stage for understanding the roles of miRNAs during animal development process. In order to determine whether regulatory functions of miR-363 and 363* existed during gonad sexual differentiation of chicken embryo, their expression profiles during the important stages of sex differentiation (E3.5–6.5 d) were analyzed by RT-PCR. Meanwhile, we compared the expression levels of gga-miR-363 and 363*, which generated from the same precursor, and the different expressions of miR-363 and 363* observed in the gonads might be mediated by a selective processing mechanism. Reviewing the biological generation process of miRNAs,

pre-miRNAs were cut by Dicer into miRNA: miRNA* duplexes in the cytoplasm, and in most cases, one strand of the hairpin with lower thermodynamic stability in 5′ terminal was present as a mature miRNA, which involved in RNA-induced silencing complex (RISC) to educe functions, whereas another mature miRNA of the same hairpin derived from the complementary '*' stand was degraded [21]. However, recent studies suggested that some miRNA duplexes simultaneously encoded mature miRNAs on both strands that was resulted from the same thermodynamic stability of 5′ and 3′ terminals, and miRNA* constantly existed at an appropriate physiological level. So miRNA* could also integrate with Ago protein, and the expressions of miRNA and miRNA* emerged, although the types of miRNA* was much less abundant than corresponding miRNA [22,23]. As observed for both gga-miR-363 and 363* from our study, the miRNA: miRNA* segments were typically presented within the predicted precursor, paired to each other with 2 nt 3′ overhangs (**Fig. 3**). The expressions of miR-363 and 363* were simultaneously detected, and the

level of miR-363 expression was much higher than miR-363* in the same sex at each stages, which were concurrent with the latter opinion described above.

Some reports of temporal and spatial expressions of miR-363 were present in chicken and other animals. In the chicken embryo of E2.5−3 d, its expression signals were detected in the ectoderm, pharyngeal arches, notochord, limb bud and high expression existed in embryonic stage by WISH probed with LNA (locked nucleic acids) probes [14], suggesting a wide range of functions, such as regulating limb growth and patterning of embryo, controlling central nervous system development of embryo. Hicks et al. [20] also attained its high expression through small RNA library construction of 11 d chicken embryo, suggesting its function in embryonic development. Another research including small RNA library study and northern blot experiment described its expression signals were observed at lung, kidney, eyes, pancreas, liver, and spleen of adult chicken and in the chicken embryo at eight stages (E1−8 d) [15], suggesting a broad function in adult and embryonic chicken. In addition, the high expression of miR-363 during embryonic stages was observed in chicken and zebrafish [14,24]. This also was observed in our previous miRNAs chip experiment, with the results of high expression of miR-363 in E3.5 d chicken embryos. Moreover, our results of WISH indicated that positive signals mainly appeared in the limb bud, notochord, ectoderm, brain of E4.5 d chicken embryo, which was coincident with the research described by Darnell et al. [14]. In the UGSs of E6.5 d chicken embryo, its high expression appeared in gonad and surrounding kidney, and higher in female than male, which agreed with our semi-quantitative results. All these showed miR-363 had extensive functions in the early development of embryo, and its high expression in gonad obtained from our study further inferred it would participate in the sexual differentiation of gonad during the early stages of chicken embryo. Though our WISH could not exclude the possibility that we were detecting the presence of hairpin precursor as well, many researches had pointed out that in situ detection of miRNA mainly detected mature miRNA because of higher detection threshold of precursor and relatively lower sensitivity than that of mature miRNA of this assay [25,26]. So combining expressions of miR-363 studied in chicken and other species with our research, we inferred gga-miR-363 had extensive regulatory roles in chicken, and would be involved in gonad sexual differentiation during the early stages of chicken embryo. Up to now, few studies about miR-363* had been reported, and the prediction of its target genes in chicken was difficult, because of lack of appropriate software. So it was very difficult to predict its functions in the gonadal development of chicken embryo, and maybe it failed to play a key role in the process of gonadal development according to our study that the sexual differentially expression of gga-miR-363* during E3.5−E6.5 d in chicken embryo gonad fell short of significance level, and its expression tendency in female or male was scrambled.

It was an important tool to study spatial expression of miRNA or mRNA in development biology by ISH (in situ hybridization), and we developed a homely, cheap and new method. Although mature miRNAs were only approximately 22 nt in length, they were much more abundant than mRNA that informed to us that an oligonucleotide probe with a single hapten could bind to a sufficient number of copies of a specific miRNA [18]. Up to now, there were three different ways reported to detect mature miRNAs by ISH. Robert and David described a modified ISH method for miRNAs detection with RNA probes, in combination with specific wash conditions based on tetramethylammonium chloride and RNase A treatment to generate highly sequence specific conditions [27]. While this work was in progress, Obernosterer et al. [28] reported another ISH method, which focused on the use of LNA that allowed a significant increase in the hybridization temperature and thereby an enhanced stringency for short probes as required for miRNA detection. Recently, another new method that involved the labeled extension of miRNA hybridized to an approximately 100 nt long ultramer template containing the complementary sequence of the miRNA at its 3′ terminus has been described [29]. In our experiment, RNA probes were constructed by mirVana™ miRNA probe construction kit and labeled by DIG to detect miR-363. The process of probe preparation contained the rapid preparation of dsDNA templates and in vitro transcription of these templates to synthesize short (<100 nt) RNA transcripts including eight-base sequence identical to T7 promoter primer. Moreover, the results of WISH in this study suggested that the constructed probes could successfully detect miRNA, and signals were similar with the LNA probes.

In conclusion, gga-miR-363 and 363* had been identified and the analysis of sexually dimorphic miRNAs expressions in the gonads was constituted. Although there was significant difference of miR-363 expression between both sexes at E4.5 and 6.5 d in chicken embryos, and showed obvious hybridization signals in UGSs of E6.5 d chicken embryos, it is not yet clear whether miR-363 expression was involved in driving the progression of sexual differentiation. Identification of potential target genes and functional studies are now required to examine the role of miR-363 in gonadal development.

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