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Molecular characterization, tissue distribution, and expression of two ovarian Dicer isoforms during follicle development in goose (*Anser cygnoides*)

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ABSTRACT

Dicer plays a key role in the biogenesis of microRNAs and small interfering RNAs, which control the coordinated expression of multiple of genes during follicle development. In this study, the cDNAs encoding two Dicer isoforms (gDicer-a and gDicer-b, respectively) were isolated and cloned from goose ovary using RT-PCR. This is the first time a new *Dicer* splice variant has been characterized at the molecular level in vertebrates. Sequence analysis indicated that both of the two isoforms consist of seven conserved functional domains, where gDicer-b lacks a linker sequence between DEAD box and helicase C domain composed of 158 amino acids. Each domain of gDicer-a/gDicer-b showed higher than 89.5% identity to corresponding domain of Dicers from chicken, human, and mouse. The ubiquity of transcripts of *gDicer-a*/*gDicer-b* was found in all tested tissues by real time PCR with the pituitary, oviduct, and hypothalamus being the predominant site of expression of *gDicer-a*. A similar expression profile of the *gDicer-a*/*gDicer-b* mRNAs was found during follicle development. The abrupt changes in transcripts of *gDicer* in 2–4 mm, 9–10 mm, F5, and F1 follicles support its participation in the process of follicle recruitment, selection, dominance, and ovulation. However, high mRNA levels of *gDicer-b* and *caspase-3* were detectable in atretic and post-ovulatory follicles, where expression of *gDicer-a* was considerably low. These findings suggest that gDicer is required for follicle development, and structural differences in the helicase domain of two gDicer isoforms might contribute to their different roles in controlling granulosa cell apoptosis.

1. Introduction

The most representative characteristic of ovarian follicle development in oviparous species like birds is the existence of a well-distinguished follicular hierarchy as a prerequisite for follicle maturation, ovulation, and oviposition, which is tightly coupled with the morphological and functional alternation in oocyte and somatic cells (Johnson and Woods, 2009; Johnson, 2012). The fate of each follicle is highly dependent upon the regulatory activities of numerous extra- and intra-ovarian factors that mainly function in an endocrine, paracrine, and autocrine manner (Onagbesan et al., 2009). The dynamic and highly regulated process of follicle development requires the coordinated actions of a great number of genes, which is orderly orchestrated at the transcriptional and post-transcriptional levels (Baley and Li, 2012). Recent years have seen some major advances in our understanding of post-transcriptional gene regulation in modulating ovarian functions with the discovery of small non-coding RNAs including microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Carletti and Christenson, 2009; Baley and Li, 2012). MiRNAs and siRNAs exert their effects through specific incorporation into RNA-induced silencing complexes (RISCs) to bind target mRNA and facilitate post-transcriptional regulation (Carthew and Sontheimer, 2009). Increasing evidence supports the vital role of small RNAs in the animal gonad by guarding genomes and guiding development (Lau, 2010), and miRNAs/siRNAs are crucial for controlling cell proliferation, differentiation, steroidogenesis, and apoptosis in the ovary (Luense et al., 2009; Baley and Li, 2012).

As a member of the RNase III family, Dicer plays a central role in the biogenesis of miRNAs and siRNAs by cleaving the double-strand RNA (dsRNA) into 21–25 bp RNA duplexes, and it is thus inferred that Dicer is required for follicle development (Luense et al., 2009). With the construction of knockout mouse models, growing evidence has been gathered to support the role of Dicer in female reproductive tract development and fertility. The description of abnormal phenotypes in female reproductive tissues caused by *Dicer* deletion has been generalized by Luense et al., which could be mainly attributed to the effect of loss of *Dicer* on the generation of small RNAs (Luense et al., 2009). In addition, Dicer is also essential for oocyte maturation (Liu et al., 2010),

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spermatogenesis (Yadav and Kotaja, 2013), embryonic development (Yang et al., 2005), chromatin remodeling (Fukagawa et al., 2004), stem cell maintenance (Forstemann et al., 2005), and immune regulation (Cobb et al., 2006). Recently, a new role of Dicer in *Caenorhabditis elegans* (*C. elegans*) has been revealed to be involved in fragmenting chromosomal DNA by a caspase-mediated conversion from an RNase to a DNase during apoptosis (Nakagawa et al., 2010). Although the similar results have not been reported in higher vertebrate animals, it is still of most interest to investigate the interactions between Dicer and caspase throughout follicle development regarding the fact that greater than 99% of ovarian follicles undergo atresia during life reproduction (Hsueh et al., 1994).

Despite the important role of Dicer in controlling ovarian functions, to our knowledge, up to date expression of Dicer during follicle development in the avian species has not been reported and is poorly understood. Therefore, this study aimed to firstly characterize the cDNAs encoding *Dicer* in the geese (*Anser cygnoides*), an excellent biological model for studying follicle development on the basis of the large size of ovarian follicles and consecutive ovulation, and subsequently to determine the developmental expression of *Dicer* mRNAs and its possible relationship with expression of *caspase-3* throughout follicle maturation. These data could be crucial for getting a better understanding of the role of small RNAs in the avian ovary.

2. Materials and methods

2.1. Experimental animals and sample collection

The healthy maternal line of Tianfu meat geese (*A. cygnoides*), 35–45 weeks of age and laying in regular sequences of at least 2–3 eggs, was used in the present study. The geese were kept under natural conditions of light and temperature at the Experimental Farm of Waterfowl Breeding of Sichuan Agricultural University (Sichuan, China) and were provided with free access to feed and water. Individual laying cycles were monitored for each goose throughout the laying sequence. Geese were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. The liver, hypothalamus, pituitary, adrenal glands, ovary, oviduct, and follicles at different stages of development including prehierarchical follicles graded in single millimeter follicle diameter increments: 1) <2 mm, 2) 2–3 mm, 3) 3–4 mm, 4) 4–5 mm, 5) 5–6 mm, 6) 6–7 mm, 7) 7–8 mm, 8) 8–9 mm, and 9) 9–10 mm; hierarchical follicles designated as F5–F1 (measuring F1 > F2 > F3 > F4 > F5 in diameter); post-ovulatory follicles (POF); and atretic follicles were collected, rapidly frozen in liquid nitrogen, and finally stored at −80 °C until RNA extraction. All procedures in this study were approved by the Beijing Animal Welfare Committee.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from all the samples using Trizol (Invitrogen, USA) according to the manufacturer's instructions, and the quality of the resulting RNA was assessed by visualizing the ribosomal RNA bands via agarose gel electrophoresis. cDNA was obtained using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instruction with 1 μg of total RNA as a template.

2.3. Primer design

According to the reported cDNA sequences of the chicken, zebra finch, human, and mouse *Dicer* gene (GenBank accession numbers: NM001040465, NM001163403, NM001195573, and NM148948, respectively), nine pairs of primers (P1–P9) were designed to amplify nine overlapping fragments of the full-length goose *Dicer* coding sequence (illustrated in Suppl. Fig. 1A). Based on the sequences obtained using primers (P1–P9), P10 and P11 were used to quantify the relative mRNA expression of *gDicer-a* and *gDicer-b* (illustrated in Suppl. Fig. 2). Based on goose *caspase-3* (GenBank accession number: KF787121), P12 was used to assess the relative mRNA expression of *caspase-3*. In addition, P13, based on goose *β-actin* (M26111), and P14, based on goose *18S* rRNA (L21170), were used as internal controls. All of the primers above (shown in Table 1) were designed using Primer Premier 5 software (Primer Biosoft International, USA) and synthesized by Invitrogen Corporation (Applied Invitrogen, Shanghai, China).

Table 1
Primer sequences used in this study.

Primer name		Sequence (5′ to 3′)	Tm (°C)	Size (bp)
RT-PCR				
P1	*Dicer*-1F	CCAGTAAACTCCAAATTGATGTCTCAGCAG	68.9	1065
	Dicer-1R	CAGCCTTGCTAGGATCCAGATAGCACAT		
P2	*Dicer*-2F	AACCAGCCACTGCTGGATGTGGAC	68.4	949
	Dicer-2R	GAAGAATGAGACCAGGATTAGGACCAAGAG		
P3	*Dicer*-3F	TGCGCTTGATTACCAAGTGATCCATTTAC	69.5	707
	Dicer-3R	TCAGCTTCTGTGGGTTTGAACTCTAGTGC		
P4	*Dicer*-4F	ATGCTGAAACTGCAACTGATTTGGTGGTCT	69.0	833
	Dicer-4R	TGCTCGAAATTTTCTAAGAACCTCTTCCTG		
P5	*Dicer*-5F	CTGGACATGGCATTGGCAAGAATCAG	70.6	1588
	Dicer-5R	CCCTTGTGATTCAAATGGCGAGGAGTC		
P6	*Dicer*-6F	ATGTGCTATCTGGATCCTAGCAAGGCTGT	69.7	1247
	Dicer-6R	TCTCAGCTGTTTGGGACCTGAGGTTG		
P7	*Dicer*-7F	CATGAAAAGCCCTGCTTTG	56.2	951
	Dicer-7R	CACCAGGGTCCCAGAACA		
P8	*Dicer*-8F	ACGCAATGACAGAAACACTG	53.9	807
	Dicer-8R	ATGGTCCACAGTCTACAACA		
P9	*Dicer*-9F	TACGATGGAAAGGTCAGAGT	52.1	490
	Dicer-9R	CCTGGCCTTCTAACACTAAG		
Real time PCR				
P10	*Dicer-a*-F	GCTGGGATGATGGTGAGAGAG	61.2	142
	Dicer-a-R	CAAGTGAGGCAGGTGAGAAGTG		
P11	*Dicer-b*-F	TGACTGCAACGTATCTGTGCAT	60.5	161
	Dicer-b-R	CTGCTTGTTACGAGGCTGATTCT		
P12	*Caspase-3*-F	CTGGTATTGAGGCAGACAGTGG	62	158
	Caspase-3-R	CAGCACCCTACACAGAGACTGAA		
P13	*18S*-F	TTGGTGGAGCGATTTGTC	53.9	129
	18S-R	ATCTCGGGTGGCTGAACG		
P14	*β-actin*-F	CAACGAGCGGTTCAGGTGT	59.6	92
	β-actin-R	TGGAGTTGAAGGTGGTCTCGT		

F, sense primers; R, antisense primers.

2.4. RT-PCR

Using total cDNA transcribed from the ovary as template, nine fragments of the goose *Dicer* were amplified with corresponding primer pairs by reverse transcription (RT)-polymerase chain reaction (PCR). The PCR was performed in a Bio-Rad thermal cycler (Bio-Rad Laboratories, USA) with the following program: denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at the primer-specific annealing temperature (listed in Table 1), and 72 °C for 1 min, followed by a final extension for 10 min at 72 °C. PCR products were evaluated by DNA electrophoresis (illustrated in Suppl. Fig. 1B) in a 1.5% agarose gel and purified using a gel extraction kit (Watson Biomedical Inc., Shanghai, China). Target cDNAs were then cloned into the pMD19-T vector (Invitrogen, USA) and sent to Invitrogen Corporation (Applied Invitrogen, Shanghai, China) for sequencing.

2.5. Real-time PCR

The mRNA expression levels of *gDicer-a*, *gDicer-b*, and *caspase-3* in goose various tissues and follicles at different stages of development were measured by quantitative real-time PCR (qRT-PCR). The qRT-PCR was performed in a 96-well iCycler IQ5 (Bio-Rad Laboratories, USA) using a Takara ExTaq RT-PCR kit and SYBR Green as the detection dye (Takara, Dalian, China). qRT-PCR was carried out on the cycler system in 1 cycle of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and