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The regulatory role of Dicer in folliculogenesis in mice☆

Lei Lei[a], Shiying Jin[a], Gabriel Gonzalez[b], Richard R. Behringer[b], Teresa K. Woodruff[a,*]

[a] Department of Obstetrics and Gynecology, Northwestern University, Chicago, IL, USA
[b] Department of Genetics, University of Texas, MD Anderson Cancer Center, Houston, TX, USA

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ABSTRACT

Dicer is the ribonuclease III for synthesis of mature functional microRNAs (miRNAs), which play an important role in regulating cell development. In the mouse ovary, the Dicer1 protein was expressed in both oocyte and granulosa cells of the follicle. In the present study, the role of miRNAs in mouse ovarian development was explored by using *Dicer1* conditional knockout (cKO) mouse ovarian tissue (*Amhr2 Cre/-; Dicer flox/flox*), in which *Dicer1* is deleted specifically in follicular granulosa cells. The morphology and gene expression profile of cKO and wild type (WT) mouse ovaries at various stages of development (day 4, day 8, 8 weeks and 8 months) were examined. Comparative analysis of the follicle number indicated that conditional inactivation of *Dicer1* in the follicular granulosa cells led to an increased primordial follicle pool endowment, accelerated early follicle recruitment and more degenerate follicles in the cKO ovaries. In addition, significant differences were noted in the expression of some follicle development-related genes between cKO and WT mouse ovaries, such as *Amh*, *Inhba*, *Cyp17a1*, *Cyp19a1*, *Zps*, *Gdf9* and *Bmp15*, suggesting the function of miRNAs in regulating gene expression is time- and gene-dependent. With the *Dicer1* inactivation, mmu-mir-503, a miRNA that is more abundant in mouse ovary than in other tissues, was down-regulated significantly. Meanwhile, the expression of mmu-mir-503 decreased notably with follicle development in the gonadotropin-primed mouse ovary. Up-regulation of mmu-mir-503 in primary cultured granulosa cells resulted in the decreased expression of both the target gene and non-target gene at the transcriptional level, which involve genes related to granulosa cell proliferation and luteinization. In conclusion, Dicer1 plays important roles in follicular cell development through the differential regulation of gene expression.

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1. Introduction

MicroRNAs (miRNAs) are endogenous, short non-coding RNAs (~22 nt) that have been identified in a variety of organisms from plants to mammals, and play important roles in development, cancer, stress responses, and viral infections through regulating the function of eukaryotic messenger RNAs (mRNAs) (Yajaira et al., 2007; Buchan and Parker, 2007). Central to the RNA interference (RNAi) machinery is the RNase III-containing enzyme, Dicer (Bernstein et al., 2001). Dicers (Dicer1 and Dicer2) are required to process microRNA precursors or long double-stranded RNA (dsRNA) into mature effector interference RNA molecules for, miRNA and small interference RNA (siRNA) pathways, respectively (Carmell and Hannon, 2004; Jin and Xie, 2007). Dicer1 is required for miRNA production in mammals (Kanellopoulou et al., 2005).

The miRNA regulatory pathway was previously found to be essential for mammalian development. Disruption of the *Dicer1* gene in mice results in lethality at embryonic day 7.5 due to a loss of pluripotent stem cells (Bernstein et al., 2003). A different *Dicer1* knockout caused lethality much later in development, with impaired angiogenesis (Yang et al., 2005). Similarly, *Dicer1* mutant germline stem cells (GSCs) cannot be maintained and are lost rapidly from the niche without discernable features of cell death (Jin and Xie, 2007). In follow-up studies, which used the conditional knockout mouse model that silences *Dicer1* expression in a tissue- or cell-specific manner, Dicer1 was found to play an important role in T-cell differentiation (Muljo et al., 2005), limb morphogenesis (Harfe et al., 2005), lung epithelium morphogenesis (Harris et al., 2006), and hair follicle development (Andl et al., 2006).

The role of Dicer1 in mammalian reproduction has been explored in several studies. In the germ cell-specific *Dicer1* knockout mouse model, in which Cre recombinase is driven by the zona pellucida 3 (Zp3) promoter, *Dicer1*-deficient oocytes are unable to complete meiosis, and arrest with defects in meiotic spindle orga-

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* Corresponding author at: Northwestern University Feinberg School of Medicine, Robert H. Lurie Comprehensive Cancer Center, 303 East Superior Street, Suite 4-115, Chicago, IL 60611, USA. Tel.: +1 312 503 1928; fax: +1 312 472 4798.
E-mail address: tkw@northwestern.edu (T.K. Woodruff).

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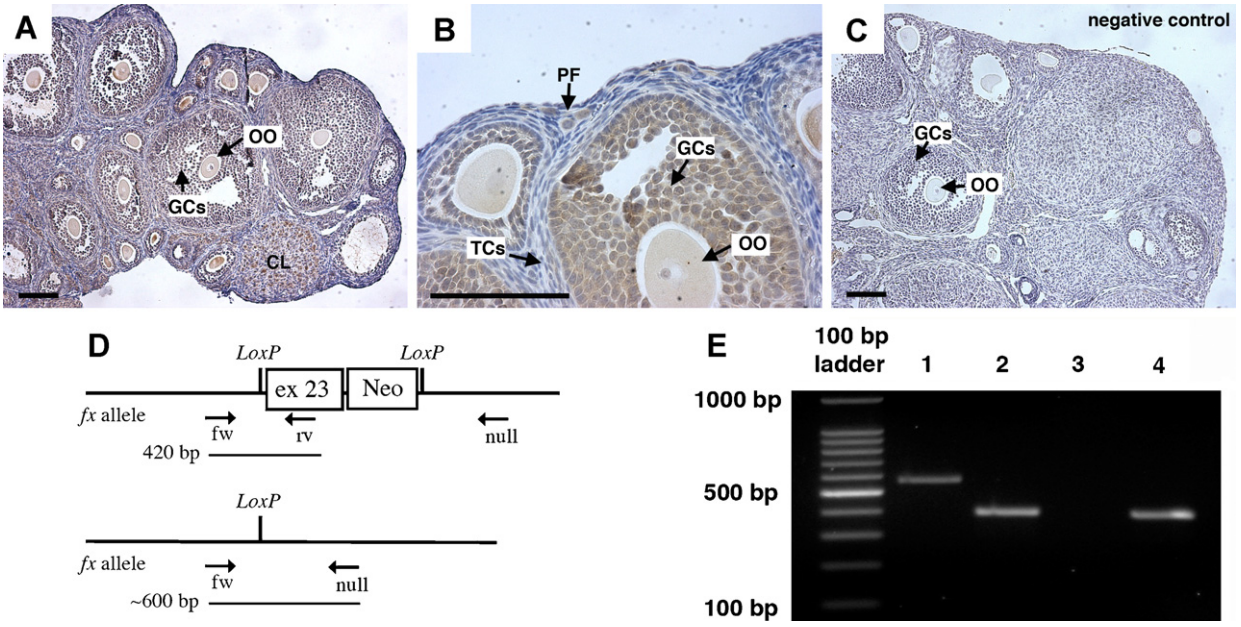

Fig. 1. The expression of Dicer1 protein in mouse ovaries measured by immunohistochemistry and verification of *Dicer* knock out in mouse ovaries with genomic DNA PCR. (A) The expression of Dicer1 could be detected in follicle oocytes, granulosa cells, luteal cells of the CL and stromal cells in 8-week WT mouse ovaries. (B) Higher magnification (40×) showed that Dicer1 is expressed in the oocytes of all stage follicles, but absent in the granulosa cells of primordial follicles. (C) Negative control. Bar = 100 μm. OO: oocyte; GCs: granulosa cells; TCs: theca cells; PF: primordial follicle; CL: corpus luteum. (D) PCR strategy for verification of *Amhr2-cre*-mediated deletion of *Dicer1* exon 23 in the cKO mouse ovary (Gonzalez and Behringer, 2009). (E) PCR amplification of genomic DNA extracted from the ovaries of cKO mice with *fw/null* primers generated a ~600-bp band (Lane 1) and amplification with *fw/rv* primers generated a ~420-bp band (Lane 2). No band was observed when genomic DNA from WT mouse ovaries was amplified with *fw/null* primers (Lane 3) and a ~420-bp band was generated when genomic DNA from WT ovaries was amplified using *fw/rv* primers (Lane 4).

nization and chromosome congression (Murchison et al., 2007). The mouse mutant with a hypomorphic *Dicer* allele (*Dicer d/d*) resulted in *Dicer1* deficiency with subsequent female infertility due in part to the insufficiency of corpus luteum (CL) function (Otsuka et al., 2008). In four recent parallel studies, anti-Müllerian hormone receptor type 2 (Amhr2) promoter is used to generate conditional *Dicer1* knockout (cKO) mice. Based on the expression of *Amhr2*, *Dicer1* is conditionally inactivated postnatally in ovarian granulosa cells, and in the mesenchyme-derived cells of oviducts and uterus. Deletion of *Dicer1* in these cell types results in multiple reproductive defects including decreased ovulation rates, compromised oocyte and embryo integrity, delayed early embryonic development, prominent bilateral paratubal (oviductal) cysts, and impaired development of the female reproductive tract (Nagaraja et al., 2008; Hong et al., 2008; Gonzalez and Behringer, 2009; Pastorelli et al., 2009). Although multiple defects are found in the reproductive tract in the *Dicer1* cKO mouse ovary, impaired early embryonic development suggests that folliculogenesis/oogenesis is dysfunctional when *Dicer1* is conditionally deleted in follicle granulosa cells. Notably, all the *Dicer1*-deficient female mice are infertile.

The regulatory effect of miRNAs on gene expression occurs at both at the transcriptional and translational levels (Buchan and Parker, 2007). The possibility that many mammalian miRNAs play important roles during development and other processes is supported by their tissue-specific or developmental stage-specific expression patterns as well as their evolutionary conservation (Lewis et al., 2003). By using miRNA amplification profiling (mRAP), Takada et al. (2006) identified 73 miRNAs in adult mouse ovary, and mmu-mir-503 is abundantly expressed in adult mouse ovary and placenta (same sequence as mmj-157 in original paper). Searching the target genes of mmu-mir-503 in miRBase (http://microrna.sanger.ac.uk/sequences/) and Targetscan (http://www.targetscan.org/), 882 target genes were found that are involved in cell communication, receptor activity, ion binding, nucleic acid binding, protein binding, cellular physiological processes, system development, metabolism, etc. Among the identified target genes, *Gdf9*, *Fshr*, *Esr2*, *ActRIIa*, *Ccnd2* and *ActRIIb* are involved in ovarian follicle development.

In the present study, the role of *Dicer1* in mouse ovarian follicle development was explored. Follicular development and its related gene expression were analyzed comparatively between wild type (WT) and *Dicer1* cKO mouse ovaries. Dicer was found to play an important role in primordial follicle endowment, follicle initiation, follicle atresia, and CL formation.

2. Materials and methods

2.1. Genomic DNA PCR

The *Dicer1* conditional null mice were generated and maintained as described (Gonzalez and Behringer, 2009). To verify the deletion of *Dicer1* driven by the *Amhr2* promoter in the mouse ovary, wild type (WT: *Amhr2 +/+; Dicer flox/flox*) and *Dicer1* conditional knockout (cKO: *Amhr2 Cre/+; Dicer flox/flox*) mouse ovaries were collected for genomic DNA isolation using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). The PCR strategy is described in Fig. 1D and in a previous study (Gonzalez and Behringer, 2009). The primers used for PCR are as follows: *fw* (CCTGACAGTGACGGTCCAAAG), *rv* (CATGACTCTTCAACTCAAACT), and *null* (CCTGAGCAAGGCAAGTCATTC).

2.2. Histology and follicle counts

Ovaries of WT and cKO mice were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections (5 μm) of each ovary were stained with hematoxylin and eosin (H & E) for follicle counting and morphological analysis. For each ovary, the middle sections of all ovarian sections were chosen for imaging. The follicles of different developing stages: primordial follicle, primary follicle, two-layer secondary follicle (2-secondary), tertiary follicle, and antral follicle were counted as described previously (Bristol-Gould et al., 2006). Follicles between two-layered secondary to antral stage follicles and with degenerated oocyte structures were tallied as degenerate follicles (Myers et al., 2004). Based on the average follicle diameter (150 μm), the degenerate follicles were counted every 30 sections and summed to obtain the total number or degenerate follicles for each ovary. Using the average CL size of 400 μm, every 80 sections were counted and the numbers were summed to obtain the total CL number in one ovary (Numazawa and Kawashima, 1982).

Table 1
Fold change in gene expression changes in *Dicer1* cKO mouse ovaries relative to WT mouse ovaries.

Gene	Description	Fold changeday 4	Fold changeday 8	Fold change 8 weeks
Primordial follicle development				
Foxl2	Forkhead box L2	1.29	−1.44*	−1.80*
Nobox	NOBOX oogenesis homeobox	−1.49*	−1.39*	1.21*
Kit	Kit oncogene	−1.04	−1.42*	−1.09
Kitl	Kit ligand	−1.06	1.13	−1.05
Amh	Anti-mullerian hormone	1.14	1.09	−1.44*
Bcl2	B-cell lymphoma/leukemia-2	1.47*	−1.28*	−1.60*
Bax	Bcl-2 associated X protein	1.21*	−1.27*	−1.48*
Oocyte specific gene				
Gdf9	Growth differentiation factor 9	1.10	−1.11	−1.41*
Bmp15	Bone morphogenetic protein 15	1.23	−1.20*	−1.50*
Zp1	Zona pellucida glycoprotein 1	–	−1.06	−1.13
Zp2	Zona pellucida glycoprotein 2	–	−1.24*	−1.41*
Zp3	Zona pellucida glycoprotein 3	–	−1.07	−1.59*
Activin/inhibin related				
Inha	Inhibin alpha subunit	–	−1.46*	1.20*
Inhba	Inhibin beta A subunit	1.86*	−6.37*	4.81*
Inhbb	Inhibin beta B subunit	–	−1.41*	−1.38*
ActRIIa	Activin receptor IIA	–	−1.25*	−1.48*
ActRIIb	Activin receptor IIB	–	−1.08	−1.14
Hormone-related				
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	–	1.30*	−2.48*
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	–	1.09	3.97*
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1	–	−1.55*	5.23*
Esr2	Estrogen receptor 2 (beta)	–	−1.27*	−1.40*
Ar	Androgen receptor	–	−1.16	−1.66*
Fshr	Follicle stimulating hormone receptor	–	−1.25	1.20
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	–		−1.55*
Granulosa cell development				
Ccnd2	CyclinD2	–	1.13	−1.38*
Cdkn1b	Cyclin-dependent kinase inhibitor 1B	–		−1.62*
Casp3	Caspase3	–	−1.20	−1.41*

* Indicates significant differences with $P < 0.05$.

2.3. Immunohistochemisty and TUNEL

The expression of Dicer in WT mouse ovaries was examined by immunohistochemistry using the M.O.M. kit (Vector, Laboratories, Inc., Burlingame, CA). The primary antibody used for detection of Dicer1 is mouse monoclonal (13D6) to Dicer-chip grade at 1:200 dilution in PBS (ab14601, Abcam Inc., Cambridge, MA). Mouse IgG1 monoclonal antibody [NCM1] – isotype control (ab27479, Abcam Inc.) was used as negative control at the same dilution. For the detection of inhibin alpha-subunit, the primary antibody was rabbit polyclonal anti-alpha subunit antibody at the dilution of 1:100 in PBS (provided by Dr. W. Vale, The Salk Institute, La Jolla, CA). Rabbit sera at the same dilution were used as the negative control. The secondary antibody was biotinylated goat anti-rabbit at 1:500 dilution in PBS (Vector Laboratories, Inc.). DAB substrate was applied followed by hematoxylin counterstaining. TUNEL staining was performed using the TACS-XL In Situ Apoptosis Detection Kit (R&D, Minneapolis, MN) following the manufacturer's protocol. Sections were then counterstained with hematoxylin.

2.4. GRMO2 granulosa cell line culture and siRNA transfection

GRMO2 cells are derived from a mouse granulosa cell line (provided by N.V. Innogenetics, Ghent, Belgium) and were cultured as described (Burkart et al., 2006) in HDTIS (1:1 mixture of DMEM and Ham's F12 medium, 10 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin) supplemented with 2% fetal bovine serum and sodium pyruvate (100 mg/l) in a humidified incubator at 37 °C and 5% CO_2. Cells were transfected with siPORT™ *NeoFX*™ according to the manufacturer's protocol (Ambion, Inc., Austin, TX). To knockdown *Dicer1* levels in the GRMO2 cell line, On-TARGET plus SMART pool L-040892-00-0005, Mouse DICER1, NM_148948 (DHARMACON, Inc., US) was used at 10 nM concentration for transfection. siGENOME Non-Targeting siRNA Pool #2 (D-001206-14-05, DHARMACON, Inc., US) was used as the negative control at 10 nM. After cells were incubated in transfection solution for 24 h, media was changed to fresh HDTIS. At 96 h after transfection, when *Dicer1* was proven to be knocked down by immunoblotting, both siDicer transfected cells and control transfected cells were collected for gene expression assay by real-time PCR.

2.5. RNA isolation and real-time PCR

For gene expression analysis, total RNA was isolated from the ovaries (WT and cKO) and from the GRMO2 cell line using a Qiagen mini prep RNA isolation kit (Qiagen, Valencia, CA). On column DNase digestion was performed using RNase-free DNase (Qiagen) to eliminate DNA contamination. 1 μg of total RNA of each sample was reverse transcribed with MMV reverse transcriptase (Promega, Madison, WI). The expression of follicular development-related genes (Table 1) was examined by real-time PCR using the Taqman gene expression assay according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA). The expression of *Gapdh* was used as an internal control. For the examination of mmu-mir-503, total RNA (including small RNAs) was isolated from ovaries and primary cultured granulosa cells with the miRNeasy Mini RNA isolation kit (Qiagen). 10 ng of total RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems Inc.). The level of mmu-mir-503 was examined by real-time PCR with the Taqman microRNA assay (mmu-mir-503) using the manufacturer's protocol (Applied Biosystems Inc.).

2.6. mmu-mir-503 in situ hybridization

All the solutions and manipulations used for mmu-mir-503 *in situ* detection were performed under RNase-free conditions. Ovaries from 21-day-old (C57 × BCA) mice were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (8 μm) were placed on Tissue Path Superfrost* Plus Gold Slides (Fisher Scientific, Pittsburgh, PA) and dried in a 37 °C incubator overnight. The protocol for non-radioactive *in situ* hybridization on paraffin sections using DIG-labeled miRCURY™ detection probes used for microRNA detection was obtained from Exiqon (Denmark). After sections were deparaffinized, deproteinated, and pre-hybridized for 2 h, the sections were incubated with the miRCURY™ LNA Detection probe for mmu-miR-503 (Exiqon) at a concentration of 20 nM in a humidified 56 °C chamber overnight. The miRCURY™ LNA Detection probe for U6 (Exiqon) was used as a negative control, and sections were incubated at a concentration of 20 nM in a humidified 54 °C chamber overnight. The next day, after rinsing with a 2×saline-sodium citrate buffer (SSC) at hybridization temperature, the slides were washed in wash buffer (50% formamide, 2×SSC) at hybridization temperature with 3 times for 30 min, followed by 5 times for 5 min in PBST at room temperature. All sections were then incubated with blocking buffer for 1 h, followed by overnight incubation with anti-DIG-AP fragments in blocking buffer at a 1:2000 dilution. On the third day, sections were incubated with NBT/BCIP for color development for about 5–8 h. To delineate ovarian structures, sections with a positive signal were counterstained with nuclear fast red. After dehydration briefly in ethanol, sections were covered with coverslips for microscopic examination.

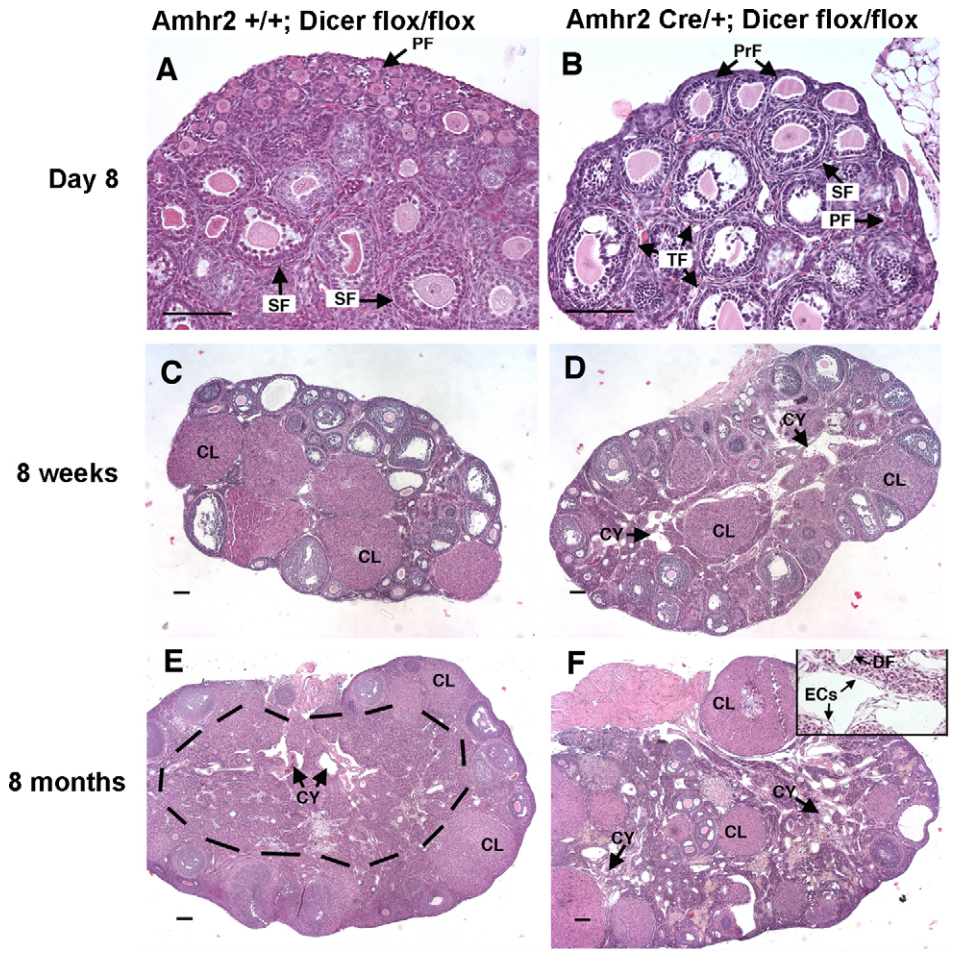

Fig. 2. The morphology of the WT and *Dicer1* cKO mouse ovaries at different developmental stages. Representative sections of ovaries of WT mice at day 8 (A), 8 weeks (C), and 8 months (E), and ovaries of *Dicer1* cKO mice at day 8 (B), 8 weeks (D) and 8 months (F) are shown. The magnified cyst structure seen in *Dicer1* cKO ovaries, characterized by a single layer of epithelial cells is indicated by the arrow in (F). PF: primordial follicle; PrF: primary follicle; SF: secondary follicle; TF: tertiary follicle; CL: corpus luteum; CY: cyst; DF: degenerated follicle; ECs: epithelial cells. The area enclosed by the dashed line indicates a large area of stromal cells in the 8-month-old WT mouse ovary. Bar = 100 μm.

2.7. Primary granulosa cell culture and mmu-mir-503 over-expression

Granulosa cells were collected using the method modified from Briers et al. (1993). Day 21 mouse ovaries (C57 × BCA) were collected 46 h after priming with 5 IU equine chorionic gonadotropin (eCG, Sigma–Aldrich, St. Louis, MO). Granulosa cells were released by puncturing large antral follicles with insulin-gauge needles in DMEM/F12 media supplemented with 3 mg/ml BSA. A single-cell suspension was acquired by gently pipetting up and down several times. Granulosa cells were cultured for 8 h in growth media: DMEM/F12 with 10 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin and 5% FBS prior to transfection.

Transfection was performed with siPORT™ *NeoFX*™ according the manufacturer's protocol (Ambion, Inc.). First, the Silencer® Cy™3-labled Negative Control #1 was used to test the efficiency of the small RNA transfection in primary cultured granulosa cells. Primary cultured granulosa cells were transfected with Pre-miR™ miRNA Precursor for mmu-miR-503 (Ambion, Inc.) at 30 nM in the growth media but without FBS. After a 24 h incubation, the transfected primary granulosa cells were cultured in growth media. As a transfection control, Pre-miR™ Precursor Molecules-Negative Control #1 was transfected at the same concentration and incubation time. Both mmu-mir-503 overexpressing cells and control transfected cells were collected 48 h after transfection for gene expression analysis by real-time PCR.

3. Results

3.1. The expression of Dicer1 in mouse ovary

The expression of Dicer1 protein in mouse ovary was examined by immunohistochemistry in 8-week mouse ovaries. As shown in Fig. 1, Dicer was detected in the oocytes of primordial follicles, and in both granulosa cells and oocytes of the follicles from primary to antral stage in WT ovaries. Dicer1 expression was also noted in the CL and some stromal cells. To verify the *Dicer1* deletion driven by *Amhr2* promoter in the mouse ovary, we employed the same PCR strategy (Fig. 1D) as previously described, which identifies the loss of *Dicer1* exon 23 mediated by *Amhr2-cre* in the reproductive tract of *Dicer1* cKO mice (Gonzalez and Behringer, 2009). As shown in Fig. 1E, PCR amplification of cKO (*Amhr2 Cre/+; Dicer flox/flox*) ovarian DNA with *Dicer1* deletion-specific primers (*fw* and *null*) generated a ~600-bp band (Lane 1), confirming that exon 23 of *Dicer1* had been deleted in the cells within the cKO ovary (i.e., granulosa cells). cKO ovarian DNA was then PCR amplified using the *fw* and *rv Dicer1*-specific primers, which resulted in a 420-bp band from ovarian cells with intact *Dicer1* (i.e., oocytes and stromal cells; Lane 2). As controls, the same PCR amplifications were carried out using DNA from the WT mouse ovary (*Dicer flox/flox* females that did not inherit the Cre allele: *Amhr2 +/+; Dicer flox/flox*); amplification with the *Dicer1*-specific primers (*fw* and *rv*; Lane 4) generated a 420-bp band and amplification with the *Dicer1* deletion-specific primers (*fw* and *null*, Lane 3) generated no detectable bands.

3.2. Histological analysis of the Dicer1 cKO ovary

Changes in ovarian histology due to *Dicer1* deletion were monitored at three developmental stages by H & E staining (Fig. 2). At day 8, WT ovaries contained mainly primordial follicles, pri-

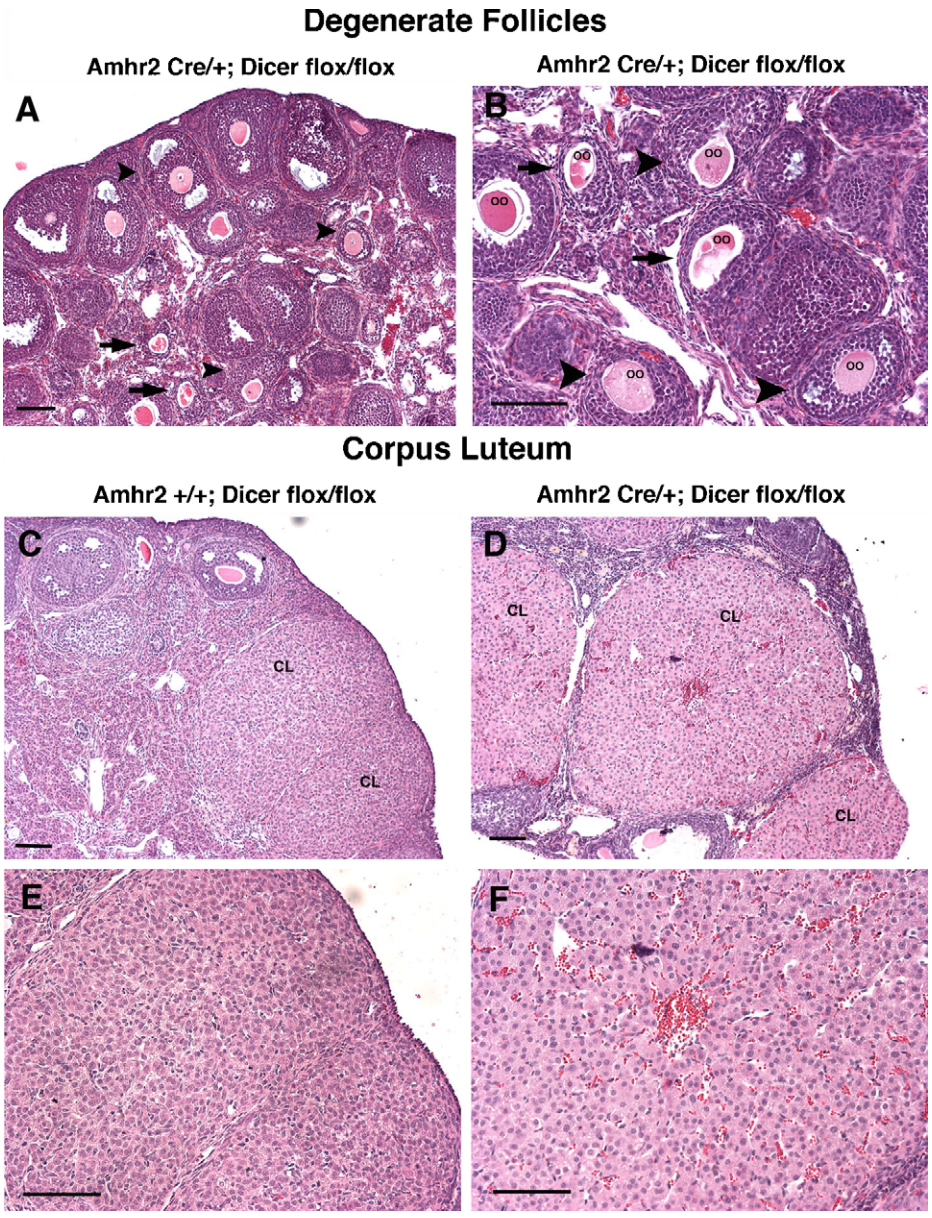

Fig. 3. The morphology of degenerate follicles and CL in *Dicer1* cKO mouse ovaries. (A and B) The histological structure of 8-week-old mouse ovaries shows healthy developing follicles with intact oocytes (arrowhead), and degenerate follicles with fragmented (degenerated) oocyte structures (arrow). The histological morphology of the CL in 8-month-old WT mouse ovaries (C and E) and 8-month-old *Dicer1* cKO mouse ovaries (D and F). Compared with WT ovaries, the CL in *Dicer1* cKO ovaries have more red blood cells, and the luteinized granulosa cells have larger cytoplasms. Bar = 100 μm. OO: oocyte; CL: corpus luteum.

mary follicles, and a few secondary follicles (Fig. 2A), whereas the *Dicer1* cKO ovaries had more tertiary follicles, which developed beyond the two-layer secondary stage with obvious antrum-like spaces (Fig. 2B). The ovaries from 8-week-old *Dicer1* cKO animals had follicles representing all developmental stages and CL resulting from ovulation (Fig. 2D). More degenerate follicles were found in cKO ovaries as compared to WT ovaries (Fig. 2C). Degenerate follicles were characterized by their developmental stage between two-layer secondary to tertiary follicles, with one layer of flattened granulosa cells close to degenerated oocytes (Fig. 3A and B). These early degenerate follicles were TUNEL negative but had inhibin alpha-subunit expression in granulosa cells (Supplementary Fig. 1). In addition, there were obvious cysts in the center of the 8-week *Dicer1* cKO ovaries (Fig. 2D). By 8 months in the WT mouse ovaries, there were only a few developing follicles and the ovaries showed a large area of stromal cells in the center (Fig. 2E). In ovaries from 8-month-old cKO mice, more developing follicles could still be observed, and similar to cKO 8-week-old ovaries, more obvious cysts were found in cKO ovaries than those of WT mice (Fig. 2F). The structure of cyst was characterized by a single layer of epithelial cells (Fig. 2F). Differences in the histological structures of CL were observed in WT and cKO 8-month-old ovaries (Fig. 3C–F). In cKO mouse ovaries, there was obvious abnormal vascularization confirmed by the presence of more red blood cells in the CL, and luteal cells in the cKO ovaries had larger cytoplasmic areas. Compared with cKO, the WT ovaries had less vascularization in the CL, with little or no large cytoplasmic areas in the luteal cells.

3.3. Folliculogenesis in the Dicer1 cKO mouse ovary

To further evaluate the differences in follicular development between WT and cKO mouse ovaries, the follicle number at different developmental stages was counted in the ovaries of day 4,

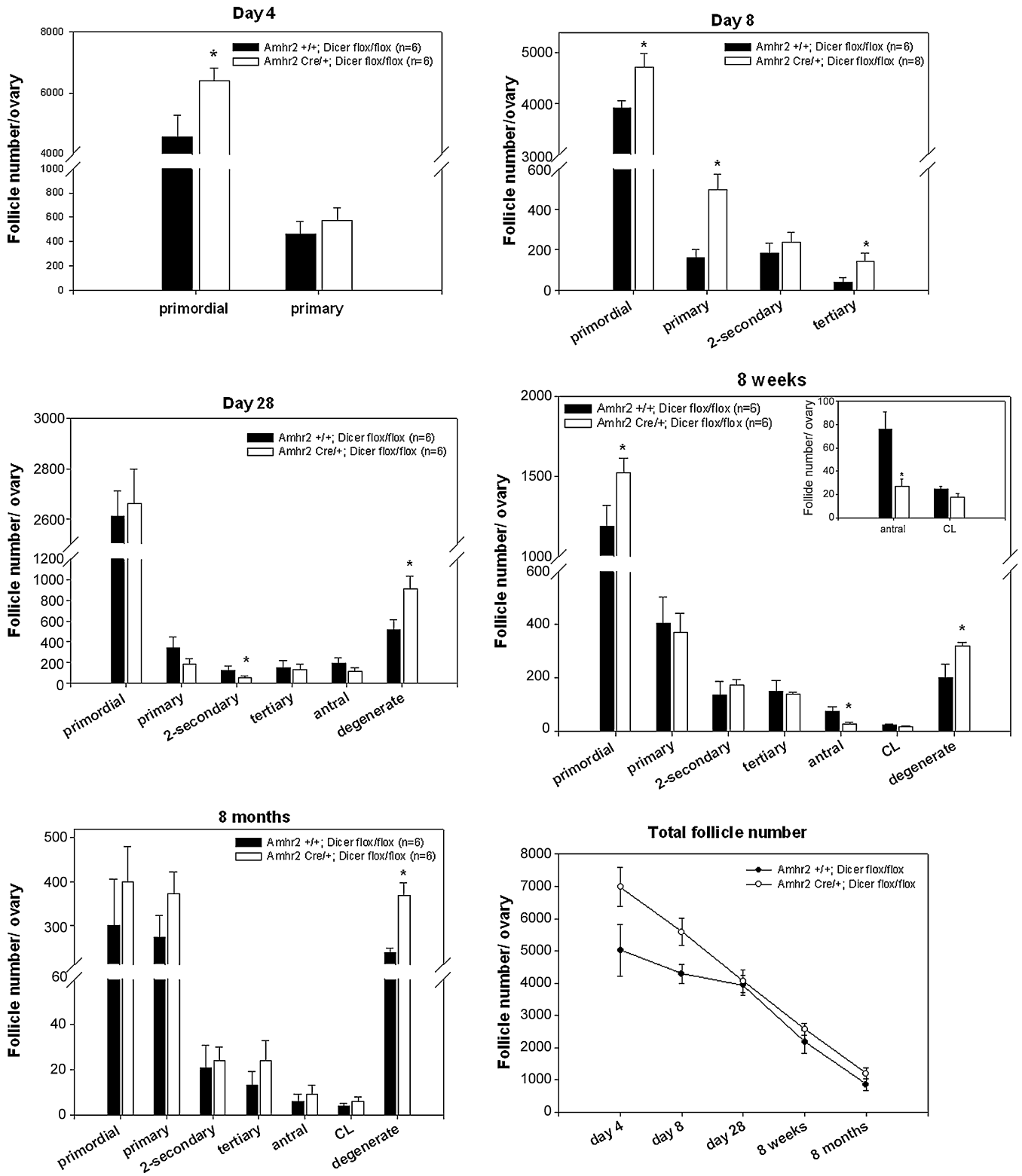

Fig. 4. Comparative analysis of follicle numbers at the different stages of development (day 4, day 8, day 28, 8 weeks, and 8 months) in WT and *Dicer1* cKO mouse ovaries.

day 8, day 28, 8-week and 8-month-old animals, with the time points chosen based on previous mouse ovarian development studies (Halpin et al., 1986; Eppig and Wigglesworth, 2000; Reddy et al., 2008). As shown in Fig. 4, at day 4, when the primordial follicle pool is established in the neonatal ovary, a significant difference was found in the number of primordial follicles between WT and cKO mouse ovaries, suggesting that cKO ovaries were endowed with a bigger primordial follicle pool. Although more primary follicles were found in the cKO mouse ovaries, the difference was not statistically significant. By day 8, with the initiation of follicle development, significantly more primary follicles were found in *Dicer1* cKO mouse ovaries as compared to WT. Most importantly, although there was a comparable level of secondary follicles in WT and cKO mouse ovaries, more tertiary follicles with three layers of gran-

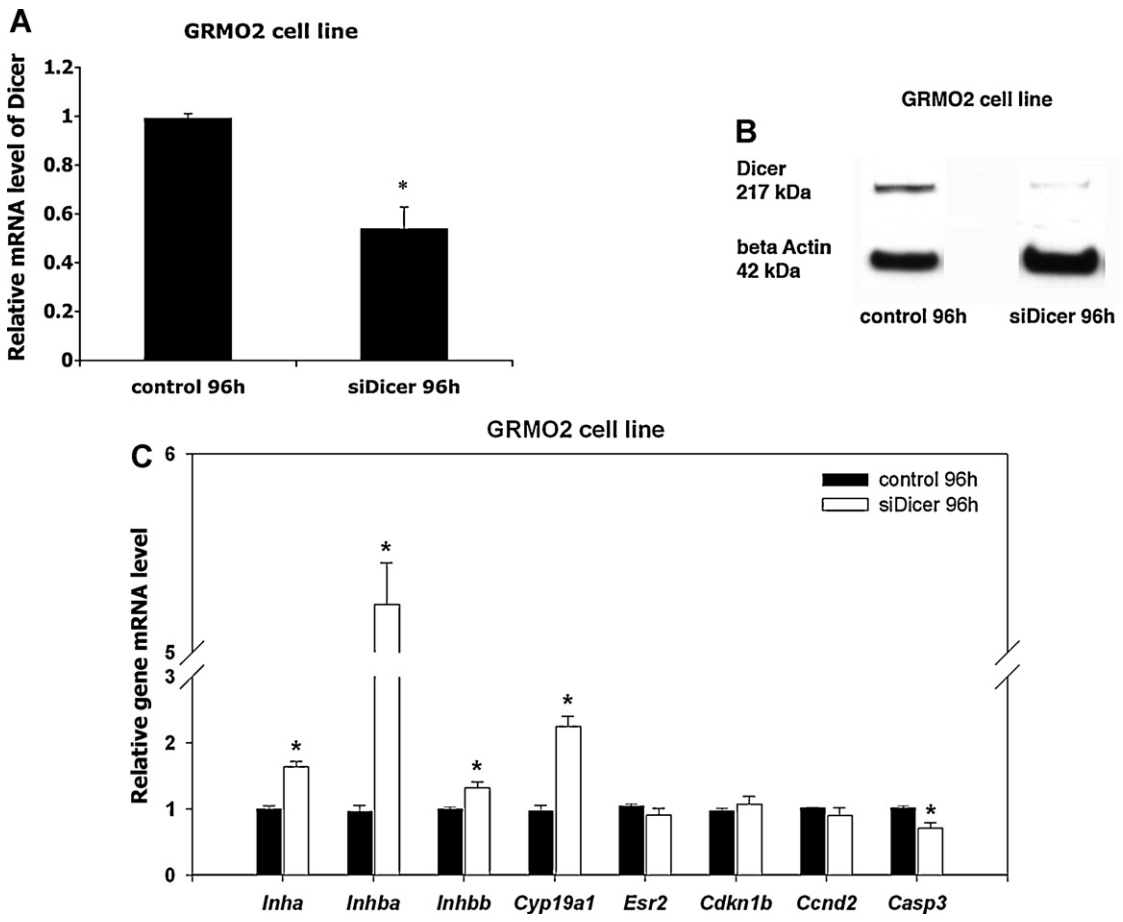

Fig. 5. The role of Dicer in granulosa cell development demonstrated by *Dicer1* knockdown in GRMO2 mouse granulosa cell line. The down-regulation of Dicer1 in the GRMO2 cell line at the mRNA level (A) and protein level (B) after 96 h siDicer transfection. (C) The expression of follicle development-related gene expression in GRMO2 cells after *Dicer1* was knocked down through siDicer transfection. GRMO2 cells transfected with control non-targeting siRNA were used as controls.

ulosa cells and obvious small antrums were detected in the cKO mouse ovaries. There were 142 ± 40 tertiary follicles in cKO ovaries *vs.* 40 ± 25 in WT ovaries ($P < 0.05$). By puberty (day 28), the follicle numbers in different stages were comparable between WT and cKO mouse ovaries. Significantly more degenerate follicles were observed in the *Dicer1* cKO ovaries. By 8 weeks, the time of reproductive maturity with regular estrous cycles in mice, as well as the number of primary, two-layer secondary and tertiary follicles, were comparable between WT and cKO mice. Significantly lower numbers ($P < 0.05$) of antral follicles suggest that more antral follicles underwent atresia in cKO mouse ovaries before they were able to ovulate. By 8 months, the equivalent of advanced reproductive age for mice, the total follicle number was relatively higher in cKO ovaries; however, this difference did not reach statistical significance (Danilovich and Sairam, 2005). Similar to the ovaries from younger animals, more degenerate follicles were found in 8-month-old cKO mouse ovaries compared to WT.

Although the *Dicer1* cKO mice were endowed with a larger number of primordial follicles at day 4, the rapid initiation of follicle development and accompanying follicle degeneration during prepuberty resulted in a comparable total follicle number between WT and cKO by day 28. From day 4 to day 28, the average total follicle number decreased from 6984 to 4066 (41.78% loss) in cKO mice. In WT mouse ovaries, the total follicle number only decreased from 5020 to 3937 (21.57% loss). After puberty, from day 28 to 8 months, with comparable follicle numbers, a similar trend in follicle loss was observed in cKO and WT mouse ovaries, although in the cKO ovaries there were more follicles present than in WT ovaries.

3.4. Gene expression in Dicer1 cKO mouse ovary

As Dicer1 is involved in the synthesis of miRNAs, which in turn regulate ovarian gene expression, genes involved in folliculogenesis in the WT and *Dicer1* cKO ovaries were examined by real-time PCR (Table 1). Three stages of ovaries were examined: day 4, day 8, and 8 weeks, based on the observed differences in folliculogenesis between the WT and cKO mouse ovary (Fig. 4). In the day 4 ovaries, expression levels of four genes were significantly different between WT and cKO mouse ovaries: *Bcl2*, *Bax*, *Nobox*, and *Inhba*. In the day 8 ovaries, genes involved in primordial follicle recruitment, inhibin/activin-related genes and hormone-related genes showed obvious differences between the cKO and WT mouse ovaries. Two genes involved in oocyte development, *Zp2* and *Bmp15*, had significantly lower expression levels in cKO ovaries than in WT ovaries. By 8 weeks, most genes examined had significantly lower expression in cKO *vs.* WT ovaries, particularly the genes involved in activin/inhibin (*Inhba*) assembly, hormone synthesis (*Cyp11a1*, *Cyp17a1* and *Cyp19a1*), granulosa cell development (*Casp3*, *Ccnd2* and *Cdkn1b*), and oocyte-specific genes (*Zp2*, *Zp3*, *Gdf9* and *Bmp15*).

3.5. The effect of Dicer1 knockdown on granulosa cell development

In the *Dicer1* cKO mouse ovary, *Dicer1* is deleted starting from the fetal stage of development, the time when *Amhr2* is active in the mouse ovary (Jorgez et al., 2004). Although *Dicer1* was knocked out specifically in follicular granulosa cells, the interaction between

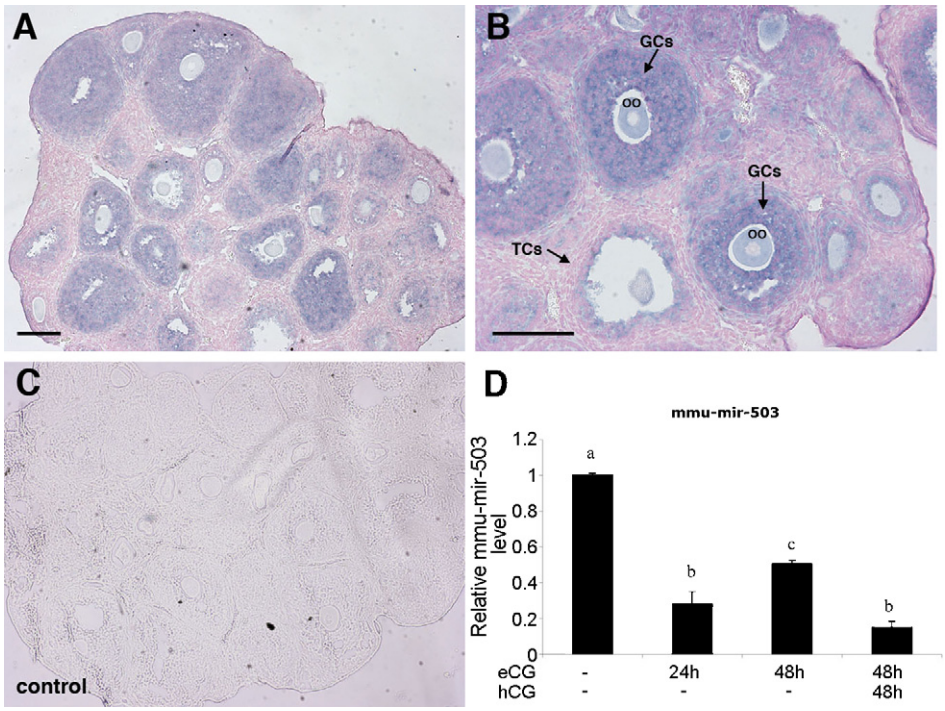

Fig. 6. The expression of mmu-mir-503 during follicle development in the mouse ovary. (A and B) *In situ* hybridization of mmu-mir-503 in day 28 mouse ovaries. mmu-mir-503 could be detected in the cytoplasm of both oocytes and granulosa cells. (C) Negative control for *in situ* hybridization incubated with U6 probe that has no known miRNA targets. (D) mmu-mir-503 levels in mouse ovaries after priming with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to stimulate follicle development and ovulation. mmu-mir-503 was down-regulated with follicle development to the antral stage and decreased further when the CL formed in the ovary. OO: oocyte; GCs: granulosa cells; TCs: theca cells.

the oocyte and granulosa cells plays an important role in regulating granulosa cell development. To demonstrate the function of miRNAs in granulosa cell development, *Dicer1* was knocked down in growing granulosa cells by transfecting siDicer into the GRMO2 mouse granulosa cell line. The knockdown efficiency was first examined by real-time PCR and immunoblot. After 96 h of transfection, the level of *Dicer1* was down-regulated significantly in the GRMO2 cells at both the mRNA level and the protein level (Fig. 5A and B). GRMO2 cells with down-regulated *Dicer1* were collected to examine gene expression by real-time PCR. As shown in Fig. 5C, similar to the gene expression seen in the 8-week ovaries, *Inha*, *Inhba*, *Cyp19a1*, and *Casp3* showed significant differences between control and *Dicer1* knockdown groups. Notably, changes in expression of these four genes in the *Dicer1* knockdown GRMO2 cells showed similar trends to those seen in the 8-week cKO mouse ovaries. Unlike the cKO ovaries, *Inhbb* was up-regulated in the Dicer1 knockdown GRMO2 cells, and there was no difference in the expression of *Cdkn1b* and *Ccnd2* in *Dicer1* knockdown GRMO2 cells.

3.6. The expression of mmu-mir-503 in WT mouse ovaries

The expression of the miRNA mmu-mir-503 in the mouse ovary was examined by *in situ* hybridization. Expression of mmu-mir-503 was found in the cytoplasm of both granulosa cells and oocytes (Fig. 6A and B), but was absent in the CL of 8-week-old mouse ovaries (data not shown). To examine the changes of mmu-mir-503 levels during follicle development, 21-day-old mice were injected with eCG to stimulate follicle development to the large antral stage, followed by hCG-induced ovulation and the formation of CL in the mouse ovary. As shown in Fig. 6D, the mmu-mir-503 levels in 21-day-old mouse ovaries were used as controls. After ovaries were primed with eCG for 24 h to stimulate growth of FSH-responsive follicles, the mmu-mir-503 level was down-regulated significantly. After 48 h of eCG injection, at which point many pre-antral follicles in the ovary reach the large antral stage, the expression of mmu-mir-503 increased, but only to about half of the level of mmu-mir-503 in 21-day-old mouse ovaries. The granulosa cells in ovulated follicles luteinized 48 h following hCG injection. The level of mmu-mir-503 was down-regulated significantly further to about 1/10 of the level in 21-day-old mouse ovaries (control ovaries). The significant down-regulation of mmu-mir-503 expression during follicle granulosa cell luteinization was consistent with the absence of mmu-mir-503 expression in CL in 8-week-old ovaries.

3.7. The effect of mmu-mir-503 over-expression on granulosa cell gene expression

The level of mmu-mir-503 in *Dicer1* cKO mouse ovary was examined by real-time PCR. Because of the *Dicer1* deletion, the levels of mmu-mir-503 in cKO ovaries were significantly lower than that in WT mouse ovaries (Fig. 7A). Since the mmu-mir-503 levels decreased with follicle development in the mouse ovaries, mRNA duplex (pre-mir 503) was transfected in primary cultured mouse granulosa cells isolated from antral follicles to up-regulate mmu-mir-503 levels. Transfection efficiency of small RNA in primary cultured granulosa cells was verified by the expression of cy3-labled small RNA in the cells (Supplementary Fig. 2). After 48 h of mRNA duplex transfection, the level of mmu-mir-503 increased significantly (Fig. 7B). Granulosa cells with mmu-mir-503 over-expression were collected to examine follicular development-related gene expression. As shown in Fig. 7C, high levels of mmu-mir-503 in transfected granulosa cells resulted in significant down-regulation of both target and non-target genes that are involved in granulosa cell proliferation and luteinization.

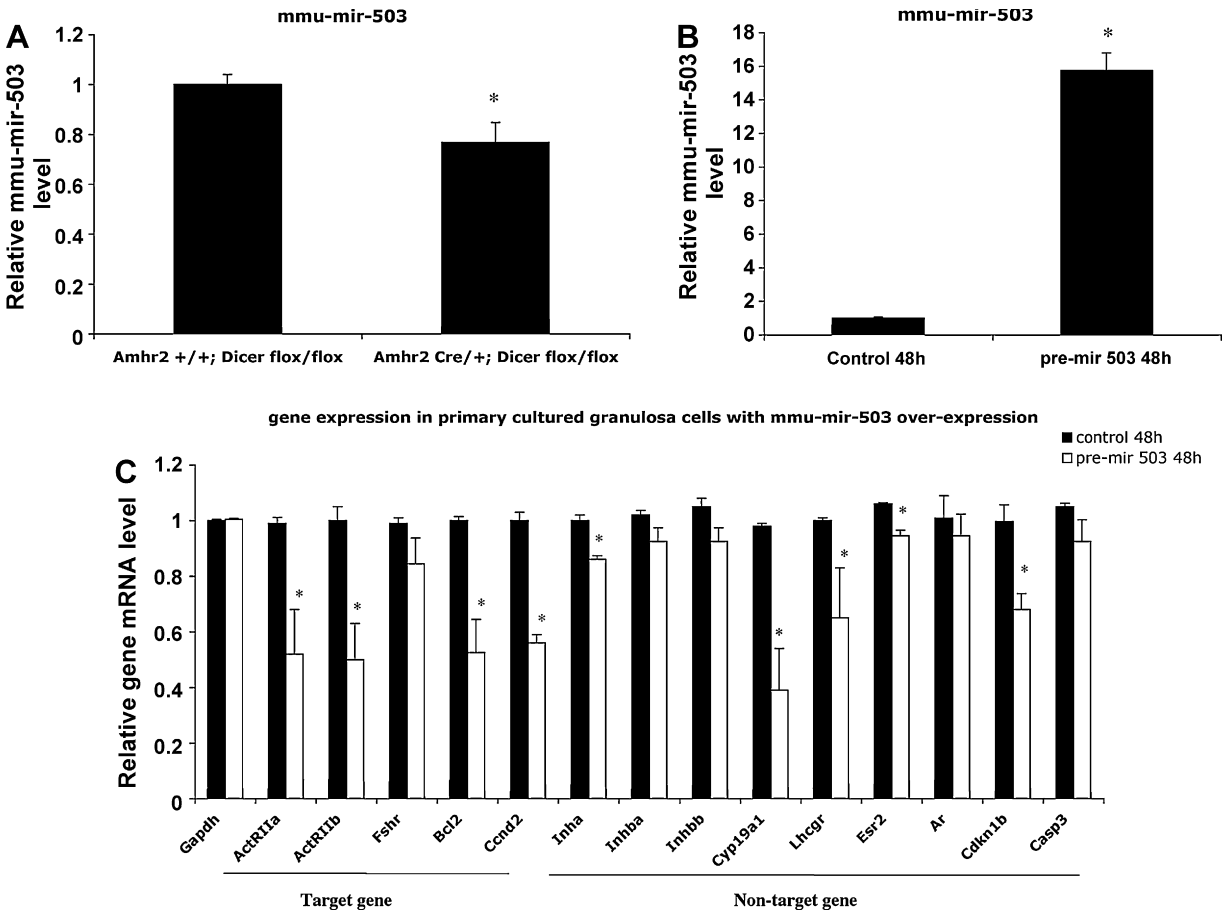

Fig. 7. The regulatory role of mmu-mir-503 in granulosa cell development. (A) Down-regulation of mmu-mir-503 in *Dicer1* cKO mouse ovaries. (B) The transfection of duplex mRNA led to the up-regulation of mmu-mir-503 levels in primary cultured mouse granulosa cells acquired from day 28 mouse ovaries after priming with gonadotropins. (C) The examination of follicular development-related gene expression at the mRNA level after up-regulation of the mmu-mir-503 level in primary cultured granulosa cells. Primary cultured granulosa cells transfected with Pre-miR™ Precursor Molecules-Negative Control were used as controls.

4. Discussion

Dicer is the critical enzyme in small RNA-regulated cell development. Recent research revealed that small RNAs play important regulatory roles in cell proliferation, differentiation, and apoptosis. During ovarian development, both oocytes and granulosa cells undergo differentiation, and at the same time acquire responsiveness to hormones and growth factors. The proliferation of follicular granulosa cells is critical to provide support to the oocytes (Erickson and Sorensen, 1974). Through different pathways, the apoptosis of both oocytes and granulosa cells leads to follicle loss at different developmental stages (Kaipia and Hsueh, 1997; Tingen et al., 2009). In the present study, the role of miRNAs in ovarian follicle development was examined in *Dicer1* cKO mouse ovaries to extend the work of previous studies and specifically examine follicle development and gene expression.

In mice, *Amhr2* is expressed in the fetal and postnatal gonads, the coelomic epithelium of the mesonephros, and the mesenchyme surrounding the Müllerian ducts (Arango et al., 2008). In the adult ovary, *Amhr2* is expressed specifically in the granulosa cells of preantral and small antral follicles (Baarends et al., 1995). By using a knock-in line in which Cre recombinase is driven by the *Amhr2* promoter, this gene could be conditionally inactivated in follicular granulosa cells of the mouse ovary, providing a means to study this gene's function specifically during ovarian development (Jorgez et al., 2004; Jeyasuria et al., 2004; Pangas et al., 2008). Although in the present and previous studies *Dicer1* deletion was verified by genomic DNA PCR without quantification, the infertility associated with phenotypes present in the *Dicer1* cKO mouse ovary and oocyte suggested that granulosa cell expressed Dicer plays important roles in mouse ovarian follicle development (Gonzalez and Behringer, 2009).

Based on the expression of *Amhr2* in mice, *Dicer1* is not only conditionally knocked out in the granulosa cells of *Amhr2 Cre/+; Dicer flox/flox* mouse ovaries, but also in the somatic cells of the oviduct and uterus. Previous parallel studies determined that the female *Dicer1* cKO mouse is infertile due to defects in ovarian function and the presence of paratubal cysts. Superovulation of immature females yields only half the number of oocytes compared with WT mice, suggesting an abnormal response to gonadotropin stimulation or defects in ovulation (Nagaraja et al., 2008). This hypothesis is consistent with our examination of the expression of hormone receptor genes: both androgen receptor (*Ar*) and luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) were down-regulated significantly in 8-week-old *Dicer1* cKO mouse ovaries (Table 1) Although follicles at all developmental stages were found in 8-week-old cKO ovaries, there were significant differences in gene expression of the hormone-related genes, which may be the reason for abnormal estrous cycles in cKO mice (Nagaraja et al., 2008). At the mRNA level, three key enzymes involved in hormone synthesis in the ovary showed obvious changes with *Dicer1* inactivation in granulosa cells. Expression of *Cyp19a1*, the enzyme for estrogen synthesis, was about five-fold higher in cKO mouse ovaries compared with WT ovaries, and the estrogen levels were higher in cKO mice, but these differences were not significant (Gonzalez and Behringer, 2009). In contrast, expression of *Cyp11a1*, the enzyme

for the synthesis of progesterone, was about two-fold lower in cKO mouse ovaries than in WT ovaries (Table 1). Progesterone levels in cKO mice were slightly higher but not significantly different in our cooperative study (Gonzalez and Behringer, 2009). Impaired progesterone synthesis, as a result of *Dicer1* deficiency, was also found in the previous study. Disrupted development of the CL and low progesterone are thought to be the major reason for infertility in *Dicer1*-deficient mice (Otsuka et al., 2008). Notably, in addition to the abnormal luteal cell morphology in the cKO mouse ovary (Fig. 3F), significant changes in luteal cell-related gene expression (i.e., *Cyp11a1*, *Cdkn1b*) also reflect the loss of normal luteal cell function due to *Dicer* deletion in mouse ovarian granulosa cells.

The communication between oocyte and granulosa cells plays a very important role in follicle development (Matzuk et al., 2002). As demonstrated in the present study, although *Dicer1* was knocked out in follicular granulosa cells, the development of the oocyte was also seriously disrupted. Although ovulation could occur despite the deletion of Dicer1 in follicle granulosa cells, impaired egg development is one of the major reasons for the infertility of cKO mice (Nagaraja et al., 2008; Gonzalez and Behringer, 2009). In the collaborative study, from which the ovaries examined in the present study were derived, oocytes collected from the isthmus showed abnormal egg structures with dark condensed oocytes inside the zona pellucida, or empty zona pellucida (Gonzalez and Behringer, 2009). Interestingly, similar defects in early embryos were found in a parallel study using a similar mouse model (Nagaraja et al., 2008). These investigators observed that there was no embryo in the uteri of adult *Dicer1* cKO female mice on 3.5 day after copulation. However, between 15 and 100 zona pellucida remnants and several embryos were found when the paratubal cysts were flushed (Nagaraja et al., 2008). Similar fragmentation and degeneration associated with *Dicer1* deletion in granulosa cells were also observed in early embryonic development, even when the embryos from *Dicer1* cKO mice were cultured *in vitro* (Hong et al., 2008). All of these studies confirm our hypothesis that in the *Dicer1* cKO ovary, impaired oocyte development is the major reason for the lack of healthy eggs and infertility. In addition to impaired functional gene expression in granulosa cells of *Dicer1* cKO mice, the down-regulation of oocyte-specific gene expression as the result of impaired oocyte-granulosa cell communication leads to the failure of oocyte development and maturation in the *Dicer1* cKO mouse ovaries.

Based on our assessment of the follicle numbers in WT and *Dicer1* cKO ovaries, *Dicer1* was found to play an important role in folliculogenesis, including follicle endowment, initiation, and depletion. It was suggested by the gene expression data in various developmental stage ovaries that miRNAs has a differential regulatory effect among target genes. For instance, *Kitl*, which is involved in primordial follicle formation and development, did not show obvious changes during ovarian development in the absence of *Dicer1* (Driancourt et al., 2000). In contrast, *Inba*, which encodes a subunit of Activin A/Inhibin A and hence plays a critical role in folliculogenesis, varied significantly at the three stages of *Dicer1* cKO ovarian development examined (Knight and Glister, 2006).

Although the *Dicer1* cKO mouse ovaries were endowed with larger primordial follicle pools at day 4, the follicles showed rapid initiation in 8-day-old cKO mouse ovaries. However, a greater number of follicles were found degenerated because of failed oocyte development. Based upon histological analysis, the degenerate follicles were characterized by obviously degenerated oocytes but functional granulosa cells showing positive staining for inhibin alpha-subunit (Supplementary Fig. 1). This suggests that although it is the granulosa cells that have the defective miRNA regulatory pathway, the subsequent effect on oocyte development may be the determinative reason for early follicle degeneration. At the mRNA level, all the oocyte-specific genes were down-regulated in *Dicer1* cKO mouse ovaries. Most interestingly, in both hypomorphic *Dicer1* mutant mice and Zp3 conditional *Dicer1* deletion in oocytes, there are no significant defects in the morphology of oocytes and early embryos. The function of *Dicer1* in oocytes was determined to be the organization of spindles and congression of chromosomes (Murchison et al., 2007). The deletion of *Dicer1* in granulosa cells led to degenerate oocytes with obvious morphological defects and down-regulation of oocyte-specific genes, suggesting that Dicer plays different roles in regulating oocyte and granulosa cell development and hence follicle development.

In mice, the miRNA mmu-mir-503 has been found to be more abundant in the ovary and placenta than in other tissues (Takada et al., 2006). Although the expression of mmu-mir-503 was localized to both granulosa cells and oocytes, the level of mmu-mir-503 was significantly lower when follicular development was primed by gonadotropins, suggesting a role of mmu-mir-503 in follicle granulosa cell proliferation and differentiation, and especially luteinization. Gene expression profiles in granulosa cells with overexpressed mmu-mir-503 showed that mmu-mir-503 mediated repression of target gene expression at the level of RNA accumulation, and *Ccnd2*, which is essential for the proliferation of follicle granulosa cells, is down-regulated significantly (Gilchrist et al., 2006). High levels of mmu-mir-503 also led to the decrease of non-target gene expression, perhaps because of the interaction of signal pathways. *Cdkn1b* is an important gene involved in granulosa cell luteinization (Hampl et al., 2000), and showed decreased expression with high levels of mmu-mir-503 expression in granulosa cells. The inhibitory effect of mmu-mir-503 on granulosa cell proliferation and luteinization is consistent with the down-regulation of mmu-mir-503 in gonadotropin-primed ovaries.

In conclusion, through the regulation of follicle cell proliferation, differentiation and apoptosis, the Dicer1-regulated miRNA signal pathway plays an important role in mouse follicular development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2009.09.021.

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