Original Paper

Ablation of the MiR-17-92 MicroRNA Cluster in Germ Cells Causes Subfertility in Female Mice

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Key Words

MicroRNA • MiR-17-92 cluster • Subfertility • Apoptosis

Abstract

Background/Aims: Oogenesis is a highly complex process that is intricately regulated by interactions of multiple genes and signaling molecules. However, the underlying molecular mechanisms are poorly understood. There is emerging evidence that microRNAs contribute to oogenesis. Here, we aimed to investigate the role of miR-17-92 cluster in regulating oogenesis. **Methods:** The miR-17-92 cluster was genetically ablated in germ cells of female mice by applying the *Cre-loxp* system for conditional gene knockout. Mating experiment, superovulation and histological analysis were used to assess the fertility of the model female mice. TUNEL assay was used to identify apoptotic cells in ovaries. The expression level of apoptosis- and follicular atresia- related genes was evaluated by qRT-PCR. Western blotting was performed to detect protein expression. Bioinformatics software and dual luciferase reporter assay were applied to predict and verify the target of miR-17-92 cluster. **Results:** Deletion of miR-17-92 cluster in germ cells of female mice caused increased oocyte degradation and follicular atresia, perturbed oogenesis, and ultimately led to subfertility. Genes involved in follicular atresia and the mitochondrial apoptotic pathway were obviously up-regulated. Furthermore, we verified that miR-19a regulated oogenesis at the post-transcriptional level by targeting *Bmf* in the ovaries of miR-17-92 cluster conditional knockout female mice. **Conclusion:** The miR-17-92 cluster is an important regulator of oogenesis. These findings will assist in better understanding the etiology of disorders in oogenesis and in developing new therapeutic targets for female infertility.

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Introduction

As a public health problem, infertility affects as many as 48.5 million couples worldwide [1]. Disorders in oogenesis are the main cause of female infertility. Such disorders are caused by many risk factors, including genetic, epigenetic and environmental ones, making investigation of the pathophysiology of these disorders extremely challenging. Oogenesis is a highly complex, yet robust process that is intricately regulated by interactions of multiple genes and various signaling molecules. However, the underlying molecular mechanisms remain largely obscure.

MicroRNAs (miRNAs)—small, endogenous, noncoding RNA molecules—are known to negatively regulate gene expression either by translational suppression or by destabilizing messenger RNA (mRNA) molecules [2-4]. Currently, many miRNAs have been identified and characterized in the ovaries of various mammalian species, including mice [5, 6], humans [7], cows [8], goats [9], sheep [10] and pigs [11]. Dicer is the ribonuclease III used in the biogenesis of mature functional miRNAs [12]. Global depletion of miRNA functions using Dicer ablation results in increased follicular atresia [13] and lower ovulation rate [12] in Dicer conditional knockout (cKO) mice. miRNAs are suggested to play fundamental roles in the formation of primordial follicles, follicular recruitment and selection, follicular atresia, oocyte–cumulus cell interaction, granulosa cell function, and luteinization [14].

A typical multifunctional gene cluster, miR-17-92, encodes six miRNAs (miR-17, 18a, 19a, 20a, 19b, and 92a). Emerging evidence has shown that this cluster is essential for many developmental and pathogenic processes [15], such as type B lymphopoiesis [16], lung development [16], heart development [17], neurogenesis [18, 19] and tumorigenesis [20]. It is involved in the processes of cell proliferation, apoptosis, differentiation and metastasis. Several research groups have reported that the miR-17-92 cluster contributes to spermatogenesis [21-27]. He *et al.* found that miRNA-20 and miRNA-106a are preferentially expressed in mouse spermatogonial stem cells (SSCs). miRNA-20 and miRNA-106a regulate SSCs renewal at the post-transcriptional level by targeting STAT3 and Ccnd1 [26]. In 2010, Björk *et al.* verified that miR-18 targets heat shock transcription factor 2 in spermatogenesis [24]. In 2011, Tong *et al.* reported that, compared with wild-type (WT) controls, male germ cell-specific miR-17-92 knockout (KO) mice exhibited small testes, lower numbers of epididymal sperm, and mild defects in spermatogenesis [25]. Similarly, in 2016, Xie *et al.* showed that targeted disruption of miR-17-92 in the testes of adult mice resulted in severe testicular atrophy, many empty seminiferous tubules, and depressed sperm production [27]. They further demonstrated that dysregulated mTOR signaling contributed to the pathogenesis of testicular anomalies [27]. However, little is known about the functions of miR-17-92 in the regulation of oogenesis.

Here, we genetically ablated the miR-17-92 cluster in the germ cells of female mice by applying the *Cre-loxp* system for conditional gene KO (cKO). These cKO mice displayed much reduced fecundity with increased follicular atresia and lower ovulation numbers compared with controls. Moreover, genes involved in follicular atresia and the mitochondrial apoptotic pathway were obviously upregulated. We further verified that miR-19a regulated oogenesis at the post-transcriptional level via targeting *Bmf* (BCL2-modifying factor) in the ovaries of miR-17-92 cKO female mice. Our results demonstrate that the miR-17-92 cluster is an important regulator controlling oogenesis.

Materials and Methods

Mice

Floxed miR-17-92 transgenic mice (referred to as miR-17-92^{flax/flax} mice) [16] were supplied by the laboratory of Dr. Tyler Jacks, MIT, USA. The *Ddx4-Cre* mice carrying the Asp-Glu-Ala-Asp (DEAD) box polypeptide 4 (*Ddx4*) promoter-mediated Cre recombinase, which is specifically expressed in germ cells [28], were purchased from the Model Animal Research Center of Nanjing University, P. R. China. To delete

the miR-17-92 cluster in mouse germ cells, first, male *Ddx4-Cre* mice were bred with female miR-17-92^{fllox/fllox} mice. Then, male offspring with the genotype miR-17-92^{fllox/+}; *Ddx4-Cre* were bred with female miR-17-92^{fllox/fllox} mice to generate miR-17-92^{fllox/Δ}; *Ddx4-Cre* mice, called here miR-17-92 cKO mice. C57BL/6 mice were purchased from SLAC Laboratory Animal Co., Shanghai, P. R. China. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, and were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Mouse Genotyping

For mouse genotyping, tail-tip biopsies were subjected to polymerase chain reaction (PCR) amplification using a primer pair specific for *Cre* to amplify a 240-bp *Cre* fragment, primer pair F4/R5 to amplify 255-bp (miR-17-92 wide type allele, miR-17-92⁺) and/or the 289-bp (miR-17-92 floxed allele, miR-17-92^{fllox}) fragment, and primer pair F4/AV145 to amplify the 441-bp miR-17-92 deleted allele fragment (miR-17-92^Δ) (Table 1).

In Situ Hybridization

miRNA expression patterns were detected by *in situ* hybridization (ISH) on paraffin wax sections according to previously published methods with modifications [29], using digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes. Briefly, after being treated with proteinase K, sections were hybridized with LNA probes diluted with Enzo ISH buffer (Enzo Life Sciences, USA) at 37 °C overnight. Then, the slides were washed in 0.2 x saline-sodium citrate with 2% bovine serum albumin at 4 °C for 5 min. Subsequently, a horseradish peroxidase (HRP)-based signal amplification system was hybridized to the probes before color development with 3, 3'-diaminobenzidine tetrahydrochloride. Sections were then counterstained with hematoxylin. Images were obtained with a Leica DM2500 microscope (Leica, Germany). Sections hybridized without probes were used as negative control.

Superovulation

For superovulation, adult miR-17-92 cKO female mice were injected intraperitoneally with pregnant mare serum gonadotropin (Sansheng, China; 10 IU) and human chorionic gonadotropin (Sansheng, China; 10 IU) 48 h apart. Mice were euthanized by cervical dislocation and metaphase II oocytes were collected from oviducts 14 h post human chorionic gonadotropin injection. miR-17-92^{fllox/fllox} females at the same age as the miR-17-92 cKO females were used as controls.

Fertility Assessment

Eight- to 12-week-old miR-17-92 cKO female mice were subjected to a continuous mating study. Two female mice were housed with one 8- to 10-week-old known fertile WT male mouse. The numbers of offspring per litter were recorded. Female miR-17-92^{fllox/fllox} mice were used as controls.

Histology

Mouse ovaries were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4 °C overnight, embedded in paraffin wax, and sectioned at 6 μm. Slides used for histological analysis were stained with hematoxylin. Images were obtained with a Leica DM2500 microscope and a Leica DFC 550 digital camera. Follicle numbers (including atretic follicles) were quantified as described [30, 31].

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

To identify apoptotic cells in ovaries, assays were performed using the TUNEL BrightRed Apoptosis Detection Kit (Vazyme, China) on 6 μm paraffin wax sections, according to the manufacturer's instructions. Slides incubated in the absence of terminal deoxynucleotidyl transferase were used as negative controls.

Table 1. Primers for PCR-based genotyping

Primer symbol	Primer sequence
cre-forward	5'-CACGTGCAGCCGTTTAAGCCGCGT-3'
cre-reverse	5'-TTCCCATTTCTAAACAACACCCCTGAA-3'
F4	5'-TCGAGTATCTGACAATGTGG-3'
R5	5'-TAGCCAGAAGTTCCAAATTGG-3'
AV145	5'-ATAGCCTGAAACCAACTGTGC-3'

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated from the ovaries of WT, controls and miR-17-92 cKO mice using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. Reverse transcription was performed using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China). qRT-PCR was performed using FastStart Universal SYBR Green Master Mix kits (Roche, Germany) on an ABI PRISM 7500 system (Applied Biosystems, USA) according to the procedure used by Sun *et al.* [32]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes within the ABI 7500 System Software (V2.0.4) and the gene expression levels were normalized to GAPDH or U6. The primers used for qRT-PCR are shown in Table 2.

Luciferase Assay

The 3'-untranslated region (UTR) of *Bmf* containing the binding sites for miR-19a was amplified by PCR using the following primers: forward (F), 5'-ACG TCT CGA GCT CCC TTT AGC TTT CAG CTA GG-3'; and reverse (R), 5'-ATT TGC GGC CGC CAC CTA GCA AGG TTG CTG AAG-3'. The PCR fragment was inserted into the psi-CHECKTM-2 vector (Promega, USA) by digestion with XhoI and NotI. For mutagenesis of miRNA, three nucleic acids from the seed sequence of the miR-19a mature sequence were mutated using QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, USA). The primer used for mutagenesis was: miR-19a-mut, 5'-GCA GCC CTC TGT TAG TTT TGC ATA GTA GAG TAC AAG AAG AAT GTA GTT CTA CTA ATC TAT CAA AAC TGA TGG TGG CCT GC-3'. To generate miRNA expression constructs, miR-19a fragment was inserted into the pcDNA3.1 as described [18, 19]. Plasmids were quantified by Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA), and then transfected into 293T cells using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. Cells were harvested 48 h after transfection and detected for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay Kit (Promega, USA) according to the manufacturer's instructions.

Western Blotting Analysis

Protein extracts were harvested by lysing the ovaries isolated from miR-17-92 cKO and control mice with RIPA lysis buffer (Beyotime, China) containing an EDTA-free protease inhibitor cocktail (BioTools, USA). The protein concentration was determined by using Bioepitope Bicinchoninic Acid protein assay kits (Bioworld, China). Equal amounts of protein (50 µg/ lane) were loaded onto 12% SDS-PAGE gels. Western blotting was performed as described [33].

The following primary antibodies were used: anti-Bmf (rabbit, 1:1,000; Ab181148, Abcam, USA), and anti-β-tubulin (mouse, 1:100; sc-55529, Santa Cruze, USA). HRP-conjugated secondary antibodies (SA-00001-1 and SA-00001-2, Proteintech Biotechnology, USA) were used at 1:2000. The intensities of the bands were quantified using Image J software.

Statistics

All experiments were repeated at least three times. The results are presented as means ± standard error of the mean (SEM). Means were compared using two-tailed, unpaired Student's *t* tests using IBM SPSS statistics (v. 20.0; IBM Corp., USA); *P* < 0.05 was considered statistically significant.

Table 2. List of the qRT-PCR primers used in this study

Gene Symbol	Primer Sequence
Bmf	F: 5'-GTGAGCGGCTGCTTGTCTGG-3' R: 5'-CAAAACAGGTACAGCAGAGAGC-3'
Bak	F: 5'-CAACCCCGAGATGGACAACCTT-3' R: 5'-CGTAGCGCGGTTAATATCAT-3'
Bax	F: 5'-GCGATGAGATGGACCACAAC-3' R: 5'-CTTCCAGATGGTGACCGAGG-3'
Bad	F: 5'-AAGTCCGATCCCGGAATCC-3' R: 5'-GCTCACTCGGCTCAAAGTCT-3'
Bik	F: 5'-GCGATGAGATGGACCACAAC-3' R: 5'-CTCTGACACCTGTCCGGCTG-3'
Bid	F: 5'-GCCGAGCACATCACAGAC-3' R: 5'-TGGCAATGTTGTGGATGATTTCT-3'
Bim	F: 5'-CTCCCTACAGACAGAACC-3' R: 5'-CGTTGAACCTCGTCTCCGATC-3'
Bcl-2	F: 5'-GAGGATTGTGGCCTTCTTT-3' R: 5'-CGTTATCCTGGATCCAGGTG-3'
Caspase3	F: 5'-GCAGGAGACCATCGAGGATG-3' R: 5'-GACTGGATGAACACACGACCC-3'
Caspase8	F: 5'-TGCTTGGACTACATCCCACAC-3' R: 5'-TGCAGTCTAGGAAGTTGACCA-3'
Caspase9	F: 5'-GCTGAGACGCTGCTCGGTG-3' R: 5'-GAGGAAGTGCAGGCCACCTC-3'
Cyp11a1	F: 5'-GACCCTTACAAGTATTTGGTCTG-3' R: 5'-GGTATCCAGAGCCAGTAACCT-3'
Egr-1	F: 5'-TCGGCTCCTTCCCTCACTCA-3' R: 5'-CTCATAGGGTTGTTCGCTCGG-3'
Fas	F: 5'-GCGGGTTCGTGAAATGATAA-3' R: 5'-GCAAAATGGGCTCCTTGATA-3'
FasI	F: 5'-TCCGTGAGTTTACCACCAACA-3' R: 5'-GGGGGTTCCCTGTTAAATGGG-3'
Noxa	F: 5'-ACGCGAAGACTGGGACTCT-3' R: 5'-AGCCCTGTAAAGTACATCCTA-3'
Gapdh	F: 5'-CATGGCCTTCCGTGTTCTTA-3' R: 5'-GCCTGCTTACCACCTTCTT-3'
U6	F: 5'-CTCGCTTCGCGAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
mmu-miR-17(20a)	F: 5'-GAACCTCACCTTGGGACTGA-3' R: 5'-TGCTACAAAGTGCCTCACTG-3'
mmu-miR-18a	F: 5'-CTAAGGTGCATCTAGTGCA-3' R: 5'-CATGCTTCTGCTGACTTGG-3'
mmu-miR-19a	F: 5'-AGTTGTGTTTGCAGCCCTCT-3' R: 5'-GAAGTAAATAGCAGGCCACCA-3'
mmu-miR-19b	F: 5'-GCAGATTTGCAGTTTACGCTA-3' R: 5'-GGTGTCATTACGATGCAGA-3'
mmu-miR-92a	F: 5'-GGGAAATCAAACCCCTTTC-3' R: 5'-ATCCCCCACCACCACTCAACAG-3'

Results

Expression Levels of miR-17-92 Cluster Members in Mouse Ovaries

The miR-17-92 cluster transcribes into a single polycistronic transcript, which yields six individual mature miRNAs, including miR-17, 18a, 19a, 20a, 19b, and 92a (Fig. 1A) [34]. According to the sequence homology and seed conservation, the six miR-17-92 components can be grouped into four distinct miRNA families, miR-17 (including miR-17 and 20a), miR-18, miR-19 (including miR-19a and 19b), and miR-92 family [34] (Fig. 1A). Given the known function of the miR-17-92 cluster in regulating spermatogenesis [21-27], we predicted that it might also be essential for oogenesis. To investigate the role of miR-17-92 cluster in mouse female germ cell development, we first examined expression profiles of members of miR-17-92 cluster in ovaries from 5-, 10-, 15-, 20-, 40- and 160-d-old WT mice using qRT-PCR. All members of the miR-17-92 cluster were expressed in mouse ovaries, with relatively high levels in 5- and 40-d-old mice (Fig. 1B). To further characterize the expression patterns in ovaries, we performed ISH on 8-week-old WT mouse ovaries using DIG-labeled LNA probes. The miR-17-92 cluster was expressed mainly in oocytes and ovarian stromal cells (Fig. 1C). These results suggested that the miR-17-92 cluster is involved in mouse oogenesis.

The miR-17-92 cluster is Efficiently Deleted in Female Mouse Germ Cells Using *Ddx4-Cre* Mice

To study the functional roles of the miR-17-92 cluster in female germ cell development *in vivo*, we generated mouse models in which the miR-17-92 cluster was ablated genetically by applying the *Cre-loxp* system using *Ddx4-Cre* mice with specific Cre recombinase activity in the germ cells [28]. *Ddx4-Cre* mice were crossed with miR-17-92^{lox/lox} transgenic mice. After two rounds of crossing as shown in Fig. 2A, miR-17-92 cKO mice, with a genotype of miR-17-92^{lox/Δ}; *Ddx4-Cre*, were obtained. Because the *Ddx4* promoter drives the expression of Cre recombinase in germ cells, the genotype of germ cells of miR-17-92^{lox/+}; *Ddx4-Cre* mice should be miR-17-92^{Δ/+}; *Ddx4-Cre*, indicating deletion of miR-17-92 in one allele of their germ cell genomic DNA. Consequently, the miR-17-92 cluster was deleted from the germ cells of cKO mice (miR-17-92^{lox/Δ}; *Ddx4-Cre*) with genomic deletion of one allele of miR-17-92 in other parts of the body. DNA from mouse tail tips was extracted to perform genotyping using appropriate primers (Fig. 2B and Table 1). The results of genotyping are shown in Fig. 3A.

Fig. 1. The miR-17-92 cluster is expressed in the mouse ovary. (A) The genomic structure of miR-17-92 cluster on mouse chromosome 14. miRNAs with the conserved seed sequence are indicated by the same color codes. The sequence of each mature miRNA is shown, and their seed sequences are highlighted in red. (B) The relative expression levels of the miR-17-92 cluster in mouse ovaries at different postnatal stages were determined by qRT-PCR. Expression of the small RNA U6 was used as an internal standard for normalization. Primers for miR-17 also recognized miR-20a: n = 3 per group. (C) Expression pattern of the miR-17-92 cluster was detected by ISH in 8-week-old WT mouse ovaries. The probe for miR-17 also recognized miR-20a. N.C., negative control. Scale bar, 40 μ m.

Fig. 2. The strategy of generating miR-17-92 conditional knockout mice using the Ddx4-Cre line. (A) Outline of two rounds of crossing to generate miR-17-92 conditional knockout (cKO) mice. (B) A schematic representation of deletion of miR-17-92 cluster in germ cells by using the *Ddx4* promoter-mediated Cre transgenic mice. Primers F4, R5, and AV145 were used for genotyping.

Fig. 3. Germ cell-specific deletion of miR-17-92 cluster in mice. (A) Genotyping was performed on genomic DNA extracted from tail-tip biopsies. (B) Expression levels of the individual components of miR-17-92 cluster in ovaries of miR-17-92 cKO adult mice were analyzed by qRT-PCR. $n = 3$ per group. (C) Expression of individual components of the miR-17-92 cluster in oocytes of miR-17-92 cKO adult mice were analyzed by RT-PCR. KO: miR-17-92 cKO adult mice. Control: miR-17-92 *flox/flox* female mice with the same age of miR-17-92 cKO females. * $P < 0.05$, ** $P < 0.01$, compared with those of controls, using two-tailed unpaired Student's *t* tests.

To determine the efficiency of deletion of the miR-17-92 cluster in female germ cells, ovarian tissues collected from adult cKO and control miR-17-92 *flox/flox* mice were subjected to qRT-PCR. As shown in Fig. 3B, the expression levels of the individual components of miR-17-92 cluster were significantly downregulated in adult ovaries of cKO mice, compared with controls. Single oocytes were collected by puncturing the ovaries of cKO and control mice with a sharp needle in M2 medium (Merck, USA), selected using a mouth pipette, and then lysed further to perform RT-PCR to detect the expression of the miR-17-92 cluster as described [35]. However, no amplification band could be obtained, indicating no expression of individual components of the miR-17-92 cluster in the oocytes of cKO mice. For the oocytes of control miR-17-92 *flox/flox* mice, RT-PCR revealed that the miR-17-92 cluster was expressed (Fig. 3C). Our results confirmed successful germ cell depletion of the miR-17-92 cluster from miR-17-92 *flox/Δ*; *Ddx4-Cre* female mice.

Fig. 4. Subfertility of miR-17-92 cKO adult female mice. (A) The mean body weight of miR-17-92 cKO female mice was indistinguishable from that of controls (female littermates of miR-17-92 cKO female mice). (B) Whole ovarian images of 8-week-old miR-17-92 cKO mice and control. Both left and right ovaries from one representative female mouse were shown. (C) The relative ovarian weight of miR-17-92 cKO mice was indistinguishable from controls. (D) Litter sizes from miR-17-92 cKO female mice mated with WT male mice were smaller than those of control female mice mated with WT male mice. (E) The mean number of ovulated oocytes per female mouse after hormonal stimulation. KO: miR-17-92 cKO adult mice. Control: miR-17-92^{flax/flax} female mice at the same age as miR-17-92 cKO females. Data are means $\pm$ SEM, n = 3–5 mice per group. *P<0.05, **P<0.01, *** P<0.001, and n.s.: not significant.

Germ Cell Ablation of the miR-17-92 Cluster Causes Subfertility in Female Mice

The miR-17-92^{Δ/Δ} newborn mice invariably died within minutes after birth [16]. The miR-17-92 null fetuses were small with severely hypoplastic lungs and clear ventricular septal defects [16]. The miR-17-92 cKO mice we generated had lost the miR-17-92 cluster from their germ cells with genomic deletion of one allele of miR-17-92 in other parts of the body. These miR-17-92 cKO female mice displayed no significant differences in body weight during the 0–8 week's postnatal development compared with control littermates (Fig. 4A). The miR-17-92 cKO mice showed normal ovarian morphology (Fig. 4B). The size of their ovaries was indistinguishable from that of controls (Fig. 4B). In addition, there was no significant difference in the ovary-to-body weight ratio between miR-17-92 cKO adult mice and controls (Fig. 4C). The fertility of 8- to 12-week-old miR-17-92 cKO female mice and controls was tested by mating experiments and the numbers of offspring per litter were recorded. Although they gave birth to heterozygous offspring when crossed with WT male mice, they displayed much reduced fecundity (Fig. 4D). Moreover, ovulated oocytes after hormonal stimulation were significantly reduced in the miR-17-92 cKO female mice compared with controls (Fig. 4E).

Because the miR-17-92 cKO female mice were subfertile, histological sections of their ovaries were examined. Many follicles contained degraded oocytes and more empty cavities were observed (Fig. 5A), suggesting that more follicles underwent atresia in the ovaries of cKO mice compared with the controls (Fig. 5B). The TUNEL assays revealed severe apoptosis in the ovaries of miR-17-92 cKO female mice compared with controls (Fig. 6A).

Follicular atresia is an apoptotic process [36, 37]. It is initiated by oocyte or granulosa cell apoptosis [38]. Thus, the expression level of apoptosis- and follicular atresia- related genes in the ovaries was evaluated. The qRT-PCR analysis revealed the expression of most

Fig. 5. Morphological comparison of ovaries from miR-17-92 cKO female mice and controls. (A) Representative images of hematoxylin staining of adult ovary sections. a and c: ovarian sections of control miR-17-92^{flox/flox} female mice; b and d: ovarian sections of miR-17-92 cKO mice. Scale bar, 40

µm. Representative images of atretic follicles and empty cavity-like structures are indicated by arrows. (B) Statistical analysis of the percentages of atretic follicles including empty cavities in total follicles per ovarian section of miR-17-92 cKO female mice. KO: miR-17-92 cKO adult mice. Control: miR-17-92^{flox/flox} female mice at the same age of miR-17-92 cKO females. Data are means ± SEM, n = 5 mice per genotype. *P<0.05.

Fig. 6. Germ cell-specific deletion of the miR-17-92 cluster in mice resulted in severe cell apoptosis. (A) Detection of apoptotic cells in the ovaries of miR-17-92 cKO and control (miR-17-92^{flox/flox}) females using TUNEL assays. N.C. negative control. Scale bars, 40 µm. Representative images of TUNEL-positive follicular cells are indicated by arrows and insets. (B) The expression levels of apoptosis- and follicle atresia-related genes in the ovaries of miR-17-92 cKO and control (miR-17-92^{flox/flox}) female mice were detected by using qRT-PCR. n = 3. *P<0.05, **P<0.01.

of the proapoptotic and follicular atresia-related genes were upregulated (Fold change>2; $P < 0.05$) in the ovaries of miR-17-92 cKO female mice, including *Bax*, *Bak*, *Bik*, *Bmf*, *Bim*, *Bid*, *Bad*, *Caspase 3*, *Caspase 8*, *Caspase 9*, *Noxa*, *Cyp1a1*, *Egr-1*. However, *Fas*, *FasL*, *Bcl-2* displayed no differential expression (Fig. 6B). Previous studies have demonstrated that miR-17-92 plays an anti-apoptotic role in many physiological processes [39-41]. These results suggest that increased apoptosis induced by miR-17-92 deletion is likely one contributing factor for the more severe follicular atresia in miR-17-92 cKO female mice.

Identification of miR-17-92 Targets Involved in the Aberrant Ovarian Phenotypes Observed in miR-17-92 cKO Mice

miRNAs negatively regulate gene expression at the post-transcriptional level either by promoting degradation of the target mRNAs or by suppression of translation [2]. To further explore the direct molecular mechanisms underlying such reduced fecundity of miR-17-92

Fig. 7. The miRNA-mRNA network focused on oogenesis and cell apoptosis-related signaling pathway. Red diamond nodes represent miRNAs; green cycle nodes represent mRNAs, and yellow cycle nodes represent mRNAs which are connected by at least three edges.

Fig. 8. Identification of *Bmf* as target gene of miR-19a. (A) A TargetScan snapshot showing the predicted targeting site of miR-19a on the 3'-UTR of *Bmf*. (B) Luciferase assays of miR-19a targeting effects on the *Bmf* 3'-UTR. miR-19a but not its mutant form (miR-19a-mut) recognized the 3'-UTR of *Bmf* and reduced luciferase activity. $n = 3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (C) The protein level of BMF was increased in the ovaries of miR-17-92 cKO adult mice, detected by western blotting assays. miR-17-92^{flox/flox} females of the same age were used as controls. Data are presented as the mean $\pm$ SEM; $n \geq 3$ in all genotypes; $*P < 0.05$, $**P < 0.01$ relative to controls.

cKO female mice, putative target genes of the members of the miR-17-92 cluster were predicted. Three web-accessible databases, TargetScan (<http://www.targetscan.org/>), miRDB (<http://www.mirdb.org>) and miRanda (<http://www.microrna.org>) were searched. These predicted target genes were submitted to the KEGG pathway database (<http://www.genome.jp/kegg/>) for pathway enrichment analysis. Genes enriched in the canonical pathways related to follicular development and cell apoptosis were selected to construct an mRNA-miRNA interaction network (Fig. 7).

Among the target genes, *Bmf* awakened our interest. BMF is a proapoptotic protein belonging to the BH3-only subgroup of the BCL-2 family [31]. Liew *et al.* demonstrated that *Bmf*^{-/-} mice contained more primordial and growing follicles than those from WT female mice [31]. Loss of *Bmf* also prolonged the fertile life span in female mice [31]. Vaithyanathan *et al.* reported that *Bmf* promoted germ cell loss during murine oogenesis [30]. Therefore, the detailed interaction between miR-19a and *Bmf* were analyzed. We found that the 3'-UTR of *Bmf* contains a targeting site for miR-19a (Fig. 8A). The sequence alignment between miR-19a and the *Bmf* 3'-UTR is well conserved among different species (Fig. 9). To validate the results of the algorithm prediction, a dual luciferase reporter assay was performed. The *Bmf* 3'-UTR was inserted into the psiCHECKTM-2 dual-luciferase reporter vector and co-transfected with the miR-19a overexpression vector into 293T cells. Luciferase activities were detected. While luciferase activities in constructs containing the 3'-UTR of *Bmf* were not affected by the mutated miR-19a or control empty vectors, they were significantly reduced by the expression of miR-19a. The dual luciferase experiments confirmed that miR-19a could bind to the 3'-UTR sequence of *Bmf* (Fig. 8B). Furthermore, qRT-PCR and western blot analysis revealed increased expression of the *Bmf* in the ovaries of miR-17-92 cKO female mice, indicating that this gene is a miR-19a target and is negatively regulated by miR-19a (Fig. 6B and 8C). Our results suggest a specific targeting regulation by miR-19a on *Bmf* activity in the ovaries of miR-17-92 cKO female mice, contributing to the phenotype of follicular atresia.

Discussion

Oogenesis is a precise biological process and any disorder in it will result in female infertility. Identification of specific molecules that regulate oogenesis would contribute to our better understanding of the pathophysiology of infertility caused by oogenesis disorders. Emerging studies have shown that, like coding genes, miRNAs play critical roles in germ cell development [42, 14]. Gene KO mouse models are powerful tools to gain insight into the involvement of miRNAs in the function of germ cells and their contribution to gametogenesis. We generated mouse models in which the miR-17-92 cluster was genetically ablated in germ cells by applying the *Cre-loxp* system. Even though the miR-17-92 cKO female mice gave birth to heterozygous offspring when crossed with WT male mice, they displayed much reduced

Fig. 9. Sequence alignment of the miR-19a base-pairing site on the 3'-UTR of *Bmf*. The region complementary to the miR-19a is highly conserved among 23 species. The predicted binding sites of miR-19a with *Bmf* target sequences are highlighted in red.

Fig. 10. A TargetScan snapshot showing the predicted targeting sites of miR-17, miR-19a, miR-19b, miR-20a and miR-92a on the 3'-UTR of *Bim*.

fecundity. These mice show subfertility rather than infertility, which is likely because miRNAs negatively regulate target gene expressions post-transcriptionally by fine tuning [14].

Lower ovulation numbers and increased follicular atresia were observed in the cKO female mice than in controls. It is believed that apoptosis is the major mechanism involved in follicular atresia. There are two apoptotic pathways in mammalian cells: the intrinsic pathway (also known as the mitochondrial control of apoptosis signaling pathway) and the extrinsic apoptotic pathway [43]. Several studies have suggested that germ cell apoptosis is mediated by the intrinsic apoptosis pathway [44], which is regulated by the relative levels and activities of pro- and anti-apoptotic members of the BCL2 protein family [43]. The expression of pro-apoptotic BH3-only genes (*Noxa*, *Bmf*, *Bid*, *Bik*, *Bad* and *Bim*), the pro-apoptotic effector protein gene (*Bax* and *Bak*), initiator caspases (*Caspase 8* and *Caspase 9*) and executioner caspase (*Caspase 3*) were upregulated in the ovaries of miR-17-92 cKO female mice. Moreover, some follicular atresia-related genes (*Cyp11a1* and *Egr-1*) were also upregulated. However, *Fas*, *FasL* and *Bcl-2* displayed no differential expression. These data support the hypothesis that deletion of miR-17-92 cluster in the germ cells results in severe follicle atresia via the intrinsic apoptotic pathway.

When considering the physiology of follicular atresia, one might expect genes for apoptosis to be inversely regulated by miRNAs. Here we identified *Bmf* as a target gene of the miR-17-92 cluster in the cKO mouse ovaries. Likewise, *Bim* (also known as *Bcl2l11*) also belongs to the pro-apoptotic BH3-only BCL-2 family member, and it acts as an apoptotic activator [40]. The 3'-UTR of *Bim* contains targeting sites for miR-17, miR-19a, miR-19b, miR-20a and miR-92a (Fig. 10). Previous studies identified *Bim* as direct targets of miR-17 [40, 45], miR-20a [27, 40], and miR-92a [40]. Xie *et al.* identified *Bim* as the direct target of miR-20a in the testes of miR-17-92 cKO male mice, and demonstrated that it is implicated in the process of spermatogenesis [27]. Moreover, Wang *et al.* reported *Bim* induces porcine follicular atresia [46]. qRT-PCR analysis revealed the increased expression of *Bim* in the ovaries of miR-17-92 cKO female mice, suggesting that *Bim* acts as the target gene of miR-17-92 in cKO mice ovaries, contributing to follicular atresia. The elevated oocyte degradation and follicular atresia in the ovaries of miR-17-92 cKO female mice appears to be at least in part due to upregulated expression of *Bmf* (a putative target of miR-19a) and *Bim* (a putative target of miR-17, miR-19a, miR-19b, miR-20a and miR-92a). Thus, the normal regulatory function of the miR-17-92 cluster is to promote oocyte survival. These results are consistent with previous reports that miR-17-92 plays an anti-apoptotic role in many physiological processes [39-41].

miRNAs are frequently transcribed together as a polycistron unit that is processed into multiple individual mature miRNAs [34]. The miR-17-92 cluster is a typical example yielding

six individual mature miRNAs belonging to four distinct miRNA families [34]. Moreover, a single miRNA can simultaneously target multiple genes. Therefore, it is possible that there might be more genes directly affected by the miR-17-92 cluster involved in oogenesis other than *Bim* and *Bmf*. We intend to identify more target genes and construct an interaction network for miR-17-92 and its targets, which collaborate to regulate the process of oogenesis.

In this study we have demonstrated an essential role of the miR-17-92 cluster in regulating oogenesis. Deletion of this cluster in germ cells of female mice caused increased oocyte degradation and follicular atresia, perturbed proper oogenesis, and ultimately resulted in subfertility. Our results reveal an important molecular mechanism of miR-17-92 in modulating murine oogenesis by targeting *Bmf*, which promotes germ cell loss during murine oogenesis [30]. These findings will assist in better understanding the etiology of disorders in oogenesis and in developing new therapeutic targets for female infertility.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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