[Frontiers in Bioscience, Landmark, 18, 588-597, January 1, 2013]

miR-143 is critical for the formation of primordial follicles in mice

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1. ABSTRACT

Although microRNAs (miRNAs) have been implicated in fine-tuning gene networks, the roles of mmu-mir-143 (miR-143) in mammalian ovary development have not been studied *in vitro*. We investigated the expression and function of miR-143 in the mouse ovary during primordial follicle formation. Real-time polymerase chain reaction analysis showed that miR-143 expression increased during primordial follicle formation from 15.5 days post-coitus to 4 days post-partum. miR-143 was located in pregranulosa cells by *in situ* hybridization. To study the function of miR-143 in primordial follicle formation we established an electroporation transfection model *in vitro* that allowed miR-143 expression to be efficiently upregulated and inhibited in cultured ovaries. Further studies showed that miR-143 inhibited the formation of primordial follicles by suppressing pregranulosa cell proliferation and downregulating the expression of genes related to the cell cycle. These findings suggest that miR-143 is critical for the formation of primordial follicles and regulates ovarian development and function.

2. INTRODUCTION

The mammalian ovary is composed of germ cells and somatic cells. Folliculogenesis begins with the breakdown of germ cell clusters and the formation of primordial follicles between 17.5 days post-coitus (dpc) and 4 days post-partum (dpp); these follicles are thought to represent the only available source of oocytes during the entire reproductive lifespan of females (1-2). Primordial follicles are the smallest ovarian follicle units and are continuously recruited to grow into primary and more advanced ovarian follicles. The ovaries of newborn mice are densely packed with oocytes, most of which are present in clusters with no surrounding granulosa cells (3-4). One to two days after birth, a number of primordial follicles appear and the number of germ cell clusters declines (1). By postnatal day 3–4, a class of primary follicles is discernible by the presence of cuboidal granulosa cells. Currently, the mechanisms of primordial follicle arrest and activation are the subject of intense investigation. A number of key factors, including Gdf 9, Nobox, Figα, and FoxL2 (5-7), have been reported to regulate the breakdown of germ cell clusters and formation of primordial follicles. However, there is little information regarding the function of microRNAs (miRNAs) in early follicle development.

Table 1. Sequences of miRNAs and the primers applied for reverse transcription and quantitative real-time PCR

Name	**Sequence of primers 5′→3′**
Sequences of probes for *in situ* hybridization	
miR-143	tgAgcTacAGtGcTtCatCtca
Scramble	cAttAatGtcGGAcaActCaat
Primers for miRNAs	
miR-143 RT Primer	CTCAACTggTgTCgTggAgTCggCAATTCAgTTgAggAgCTACA
miR-143 Forward Primer	CACTggTCTgAgATgAAgCAC
miR-143 Reverse Primer	CTCAACTGGTGTCGTGGAGTC
U6 RT primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGACAAA
U6 PCR Forward primer	ACACTCCAGCTGGGGGAGCCCTTCGGGGAC
Primers for genes	
Cyclin B1 Forward primer	TGGCCTCACAAAGCACATGA
Cyclin B1 Reverse primer	GCTGTGCCAGCGTGCTAATC
Cyclin D2 Forward primer	AAGCCTGCCAGGAGCAAA
Cyclin D2 Reverse primer	ATCCGGCGTTATGCTGCTCT
Cyclin E2 Forward primer	TGCTGCCGCCTTATGTCATT
Cyclin E2 Reverse primer	TCCGAGATGTCATCCCATTCC
Cdk 2 Forward primer	CTGCCATTCTCACCGTGTCC
Cdk 2 Reverse primer	AGCTTGATGGACCCCTCTGC
Cdk 4 Forward primer	GGCCTTTGAACATCCCAAT
Cdk 4 Reverse primer	TCAGTTCGGGAAGTAGCACAG
Cdk 6 Forward primer	TGCGAGTGCAGACCAGTGG
Cdk 6 Reverse primer	AGGTCTCCAGGTGCCTCAGC
GAPDH Forward primer	TGTGTCCGTCGTGGATCTGA
GAReverse primer	TTGCTGTTGAAGTCGCAGGAG

Abundant evidence suggests that miRNAs play critical roles in controlling cell processes such as cell proliferation, apoptosis, differentiation, and tumorigenesis (8-12). In the ovary, a number of miRNAs, including miR-143, have been cloned and described, and the results have suggested that miRNAs play important roles in follicle development (13-15).

Studies of miR-143 have mainly focused on cancer tissues such as colorectal cancer (16), prostate cancer (17) and human ovarian cancer because miR-143 downregulates cancer cell proliferation (11) and its expression is reduced in cancer tissues (18). miR-143 expression has been detected in the granulosa cells of primary and secondary follicles in the mouse ovary (19). Hence, we hypothesized that miR-143 may participate in the regulation of follicle development. We examined miR-143 expression during primordial follicle formation, and investigated its functions through electroporation transfection. Our results demonstrate for the first time that miR-143 is critical for primordial follicle formation in mice.

3. MATERIALS AND METHODS

3.1. Animals (ethics statement)

All experiments were conducted using Kunming white mice purchased from the Laboratory Animal Center of the Institute of Genetics in Beijing. Mice were housed in a facility at China Agricultural University according to the guidelines for laboratory animals approved by the Chinese Association for Laboratory Animal Science. Adult female and male mice were mated during the late afternoon at a ratio of 1:1 to induce pregnancy and female mice were checked for vaginal plugs the next morning. Female mice with vaginal plugs the day after mating were considered to be at day 0.5 of pregnancy.

3.2. *In situ* hybridization

Ovaries were collected from mice at 15.5 dpc, 17.5 dpc, 1 dpp, and 4 dpp and fixed in 4% paraformaldehyde. Cryosections (10 μm thick) were prepared and an *in situ* hybridization (ISH) assay was carried out as described previously (20). The ISH probe sequences are shown in Table 1. The sections were examined under a DMRB light microscope (Leica, Vertrieb, Germany).

3.3. Real-time polymerase chain reaction assay

Total RNA was extracted from ovaries and cultured cells using TRIzol (Invitrogen, USA). cDNA was synthesized from 1 μg purified RNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with an Applied Biosystems 7500 Sequence Detection System using a SYBR Premix Ex Taq II Kit (Takara Bio, Inc., Shiga, Japan). Each sample was analyzed in triplicate. The primers for miR-143, U6, Cyclins B1, D2 and E2, Cdks 2, 4, and 6, and GAPDH are listed in Table 1. The PCR conditions were as follows: 95°C for 15 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s, and 95°C for 15 s. Expression levels were normalized to GAPDH expression. TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) were used to quantify miR-143 expression levels. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The miR-143 expression level was normalized to the level of expression of the housekeeping gene U6 and is presented as the mean normalized expression level. All experiments were performed in at least triplicate.

3.4. Isolation, electrotransfection and cultivation of mouse ovary

Ovaries were isolated from mouse embryos at 15.5 dpc. The ovaries, kidneys, and trunk were washed with phosphate-buffered saline (PBS). miR-143 mimics or inhibitors (10 μM) were injected into the ovary using a thin glass needle with a mouthpiece. For each injection, the

optimal volume was 0.3 µL per ovary. Corresponding negative control (NC) mimics and inhibitors were used as negative controls. miRNA mimics and inhibitors were chemically synthesized by GenePharma (Shanghai, China). After injection, ovaries were placed between the Tweezertrodes (diameter, 5 mm; BTX, USA), but not directly touching them. The anterior–posterior axis of the genital ridge was parallel to the Tweezertrodes. Then a 20-V, 50-ms rectangular pulse was charged five times in 150-ms intervals by an ECM 2001 Electro Cell Manipulator (BTX). After electroporation, the ovaries were placed on the membrane of a culture insert of a 24-well dish and were cultured in serum-free Dulbecco's modified Eagle's medium F12 (DMEM/F12; Gibco-BRL, Rockville, MD, USA) at 37°C in a humidified atmosphere containing 5% CO2. The culture medium was replaced every 2 days.

3.5. Immunohistochemistry

Isolation, electrotransfection, and cultivation of fetal ovaries were performed as above and the tissues were harvested after 8 days of *in vitro* culture (equivalent to postnatal day 4). Briefly, the ovaries were fixed and embedded, and 5-µm continuous sections were created using a microtome. After dewaxing, rehydration, and antigen retrieval with 0.01% sodium citrate buffer (pH 6.0), the sections were immunostained through incubation with rabbit anti-Vasa antibody (dilution 1:100; Santa Cruz) overnight at 4°C. Next, biotinylated goat anti-rabbit IgG (1:300; Zymed Laboratories, San Francisco, CA, USA) and peroxidase-conjugated streptavidin (1:300; Zymed Laboratories, San Francisco, CA, USA) were applied, and the sections were exposed to diaminobenzidine (Zhongshan Company) for 1 min. The sections were finally counterstained with hematoxylin and examined under a light microscope.

3.6. Ovarian follicle counts

Ovarian follicles were counted using the most widely used approach. In brief, every fifth section was analyzed for the presence of follicles, and only follicles in which the nucleus of the oocyte was clearly visible were scored. The starting section was usually selected randomly. The cumulative follicle counts were multiplied by 5 for the four-fifths of the ovary that were not analyzed. The follicles were manually counted by two individuals. Oocytes surrounded by pregranulosa cells or a mixture of squamous and cuboidal somatic cells were scored as primordial follicles.

3.7. BrdU analysis

To evaluate the relationship between miR-143 and pregranulosa cell proliferation, mouse ovaries were isolated at 15.5 dpc, electrotransfected with different miRNAs, cultured as above, and treated with 5'-bromo-2'-deoxy-uridine (BrdU, 100 µM) for 24 h. Then they were fixed, embedded in paraffin, and serially sectioned to a thickness of 5 µm. Every fifth section was analyzed for BrdU-positive cells; only cells with clearly visible nuclei were scored. The starting section was usually selected randomly. Cumulative BrdU-positive cell counts were obtained as above.

3.8. Cell culture and miRNA transfection

To determine the effect of miR-143 on the expression of cell cycle-related genes, we isolated somatic cells from 1 dpp mouse ovaries and cultured them for miRNA transfection. Briefly, isolated ovaries were treated with 1 mg/mL collagenase I and 1 mg/mL collagenase IV in DMEM/F12 supplemented with 4 mg/mL bovine serum albumin (BSA), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C for 40 min. Then they were treated at 37°C with 0.2% trypsin and 1.4 mg/mL DNase for 15 min followed by 0.25% trypsin and 0.02% EDTA for 20 min until clusters of granulosa cells were dispersed as individual cells. The granulosa cells were cultured with DMEM/F12 supplemented with 10% FBS for 6–8 h after plating to allow attachment of the cells to the plastic culture dish, and then were transfected using Lipofectamine 2000 (Invitrogen) for 12 h. Pregranulosa cells were transfected with miR-143 mimics and inhibitors and control siRNAs at a final concentration of 100 nM. All transfections were independently repeated at least three times.

3.9. Statistical analysis

Experiments were performed at least three times and the values are given as the mean±SEM. Data were analyzed by ANOVA using StatView software (SAS Institute, Inc., Cary, NC, USA). When a significant F ratio was identified by ANOVA, the differences among groups were compared using Fisher's protected least significant difference post hoc test. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. miR-143 expression during the formation of primordial follicles

As mouse primordial follicle formation occurs between 17.5 dpc and approximately 4–5 dpp, ovary miR-143 expression during this period was examined by real-time PCR and ISH. Real-time PCR analysis showed that miR-143 was expressed at low levels at 15.5 and 17.5 dpc, increased at 1 dpp, and remained elevated until 4 dpp (Figure 1B). Then we localized miR-143 using a digoxigenin-labeled LNA probe (a scrambled probe was used as a negative control). The miR-143 signal was weak at 15.5 and 17.5 dpc, and much stronger from 1 to 4 dpp (Figure 1A-L), in accordance with the real-time PCR results (Figure 1M). In addition, miR-143 was localized to pregranulosa cells, but not oocytes. These results suggest that miR-143 may regulate the formation of primordial follicles by acting on somatic cells.

4.2. Electroporation transfection of miR-143 mimics and inhibitors

To assess the effect of miR-143 on the formation of primordial follicles, we overexpressed and silenced miR-143 in 15.5 dpc ovaries through electroporation transfection of miR-143 mimics and inhibitors, respectively. After transfection, the ovaries were dissected and cultured *in vitro* for analysis of morphology. The overexpression and silencing efficiencies in the cultured ovaries were determined 1, 2, 5, and 8 days after transfection by real-time PCR. miR-143 expression was 6.8-, 3.31-, 2.48-, and 2.25-fold higher in the overexpressed group than in the controls (Figure 2A), while the silencing efficiency was

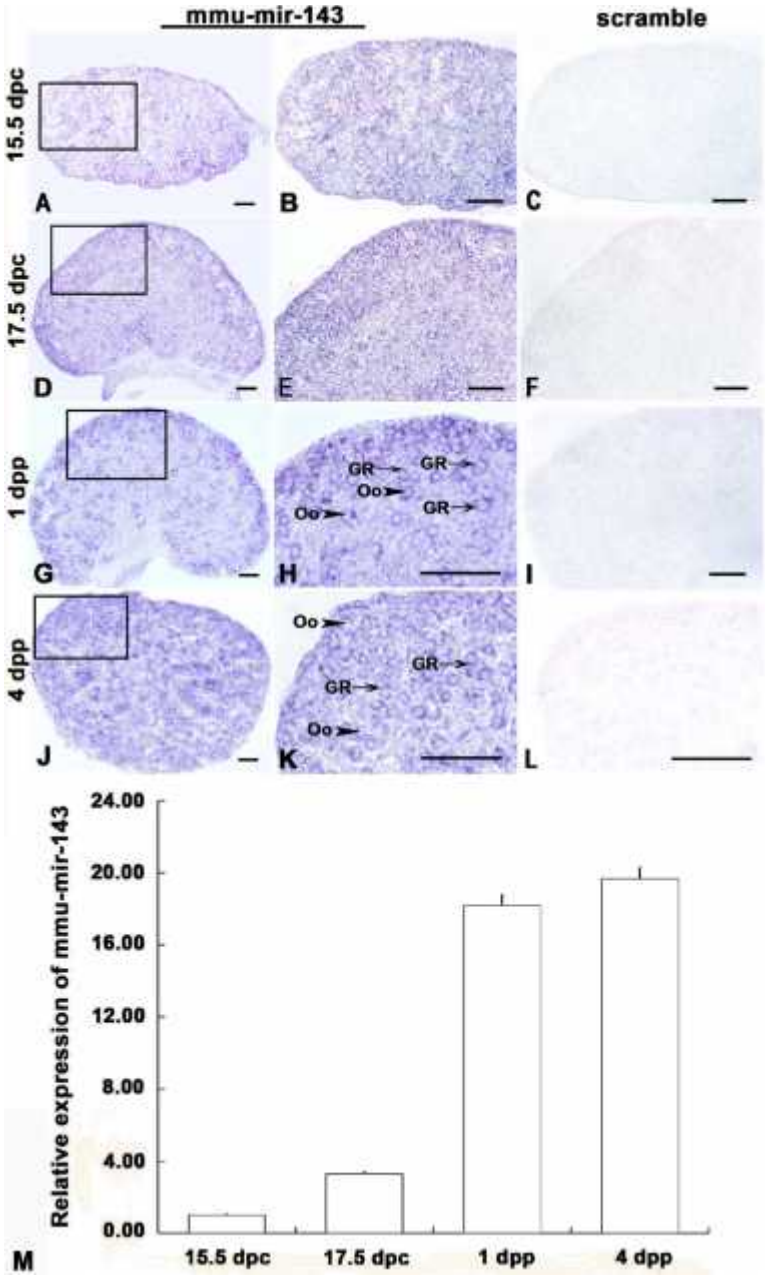

Figure 1. Detection of miR-143 in mouse ovaries by ISH (A-L) and analysis of miR-143 expression levels in ovaries from 15.5 dpc to 4 dpp by real-time PCR (M). ISH analysis of ovaries at 15.5 dpc (A-C), 17.5 dpc (D-F), 1 dpp (G-I), and 4 dpp (J-L) for miR-143 with DIG-labeled antisense probes. (C, F, I and L) ISH analysis performed with a scrambled oligonucleotide sense control. Arrowheads, oocytes; arrows, pregranulosa cells. Oo, oocyte; GR, pregranulosa cell. Bar=100 μm.

99.2%, 98.6%, 90.3%, and 82.8% compared to controls in the same stages (Figure 2B). These data indicate that electroporation transfection in cultured ovaries is effective and long-lasting.

4.3. miR-143 inhibits the formation of primordial follicles

To assess the effect of miR-143 on the formation of primordial follicles, miR-143 mimics and inhibitors were transfected into 15.5 dpc ovaries by electroporation. Then the tissues were cultured *in vitro*, harvested 8 days after transfection, and subjected to histological examination. In the miR-143 mimics group, the germ cells retained a cortical distribution but had a loose arrangement. In contrast, the germ cells in the miR-143 inhibitors group were more densely packed (Figure 3A-H). In addition, we counted and compared the numbers of primordial follicles and germ cells. The number of primordial follicles was significantly lower in miR-143 mimics than in controls (2636±129 vs. 4885±94) but higher in miR-143 inhibitors

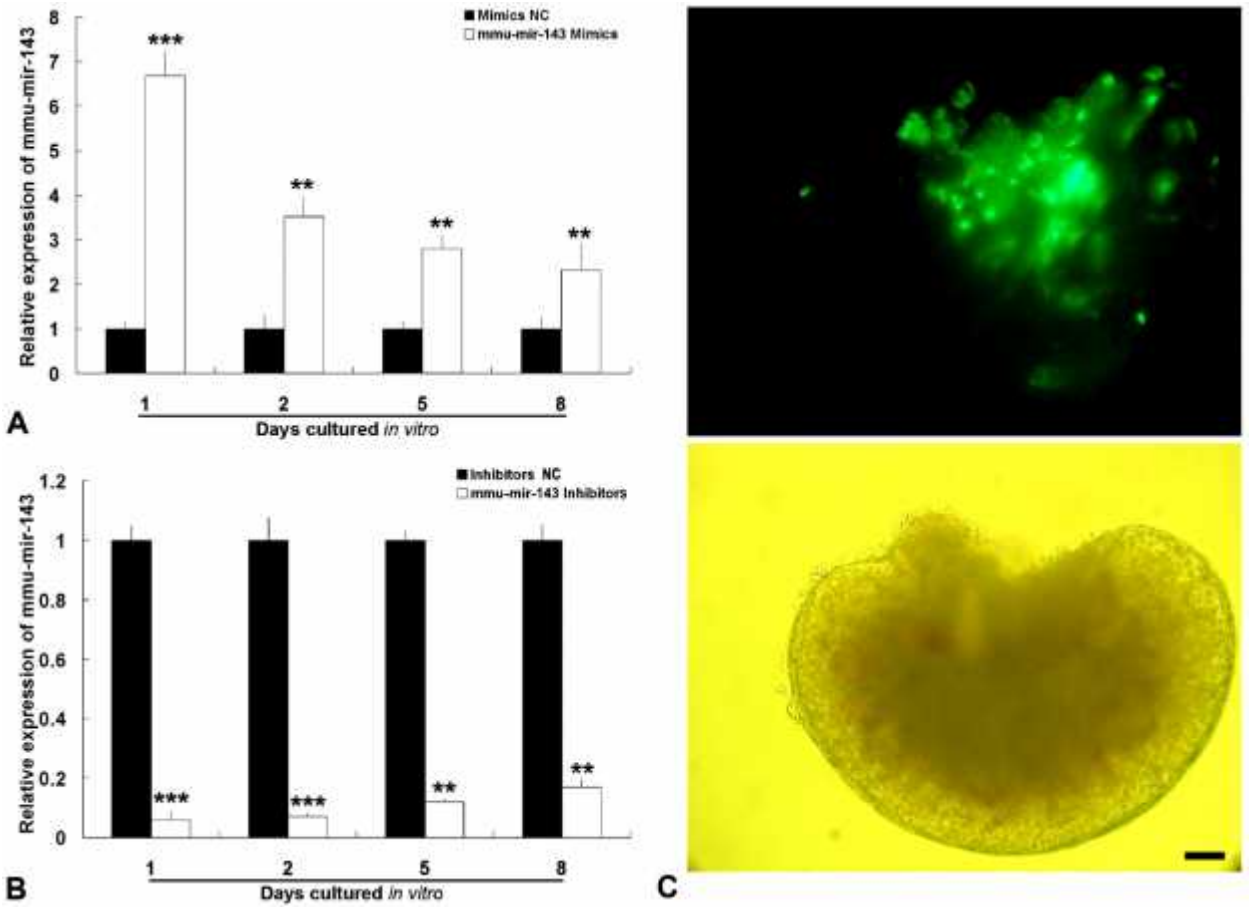

Figure 2. Real-time PCR analysis of miR-143 electroporation transfection efficiency in 15.5 dpc mouse ovaries cultured *in vitro* for 1, 2, 5, and 8 days and expression analysis using green fluorescent protein (GFP). Relative miR-143 mRNA expression levels after electroporation-mediated transfection of miR-143 mimics (A) and miR-143 inhibitors (B). (C) Expression of GFP in miR-143 mimics in 15.5 dpc mouse ovaries cultured for 5 days. Top, fluoroscope; bottom, light microscope. Stars indicate statistically significant differences between groups (***P<0.01*, ****P<0.001*). Values are the mean±SEM of three separate experiments. Bar=100 μm.

than in controls (6430±49 vs. 4542±101; ***P<0.01*). However, the total number of germ cells did not differ between treatment groups (miR-143 mimics, 5135±96; NC mimics, 5633±85; miR-143 inhibitors, 6513±165; NC inhibitors, 5572±83) (Figure 3I). These data suggest that miR-143 has the capacity to inhibit the formation of primordial follicles.

4.4. miR-143 inhibits the proliferation of pregranulosa cells

To elucidate the function of miR-143 in primordial follicle formation, we investigated its role in pregranulosa cell proliferation, which is crucial in primordial follicle formation. BrdU staining was carried out after transfection of miR-143 mimics or miR-143 inhibitors and culture for 8 days. The number of BrdU-positive cells per ovary were counted. There were fewer positive cells per ovary in miR-143 mimics than in controls (924±58 vs. 1180±55), whereas miR-143 silencing increased the number compared to controls (1428±60 vs. 1148±40) (Figure 4E). These data suggest that miR-143 inhibits the proliferation of pregranulosa cells.

4.5. Effects of miR-143 on the expression of genes related to the cell cycle

To further investigate the function of miR-143 in pregranulosa cell proliferation, we analyzed its effects on the expression of genes related to the cell cycle, including Cyclins B1, D2, and E2 and Cdks 2, 4, and 6 in isolated pregranulosa cells after transfection with miR-143 mimics and inhibitors. The mRNA expression of all compounds except Cdk 2 was significantly lower in miR-143 mimics than in NC mimics (Figure 4A), as well as in those transfected with miR-143 inhibitors (Figure 4B) (***P<0.01*, **P<0.05*).

5. DISCUSSION

Previous studies on the functions of miR-143 have mainly focused on its expression and relation to tumorigenesis in humans (21-25). The potential regulatory function of miR-143 in the ovaries of mice was illustrated in a recent study (26). The present study was the first to define the miR-143 expression pattern in the developing

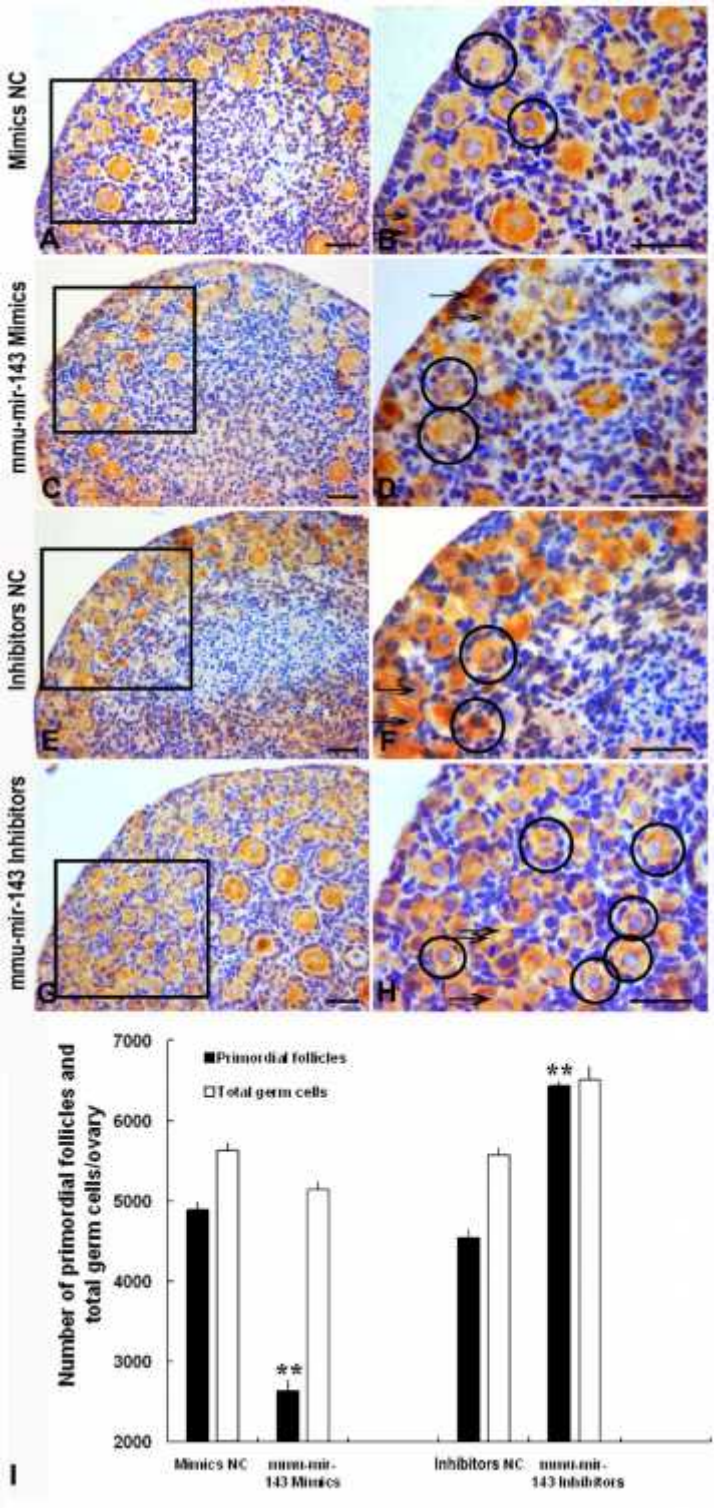

Figure 3. Effect of miR-143 on primordial follicle formation. Oocytes were marked by Vasa immunolocalization (dark brown). Ovaries in NC mimics, miR-143 mimics, NC inhibitors, and miR-143 inhibitors were cultured *in vitro* for 8 days. (A and B) Ovaries from NC mimics. (C and D) Ovaries from miR-143 mimics. (E and F) Ovaries from NC inhibitors. (G and H) Ovaries from miR-143 inhibitors. (I) Numbers of primordial follicles formed and total numbers of germ cells in ovaries. Arrow, germ cell; ring, primordial follicle. Stars indicate statistically significant differences between two groups (***P<0.01*). Values are the mean±SEM number of primordial follicles and total germ cells. Each set of data comes from six ovaries. Bar=100 μm.

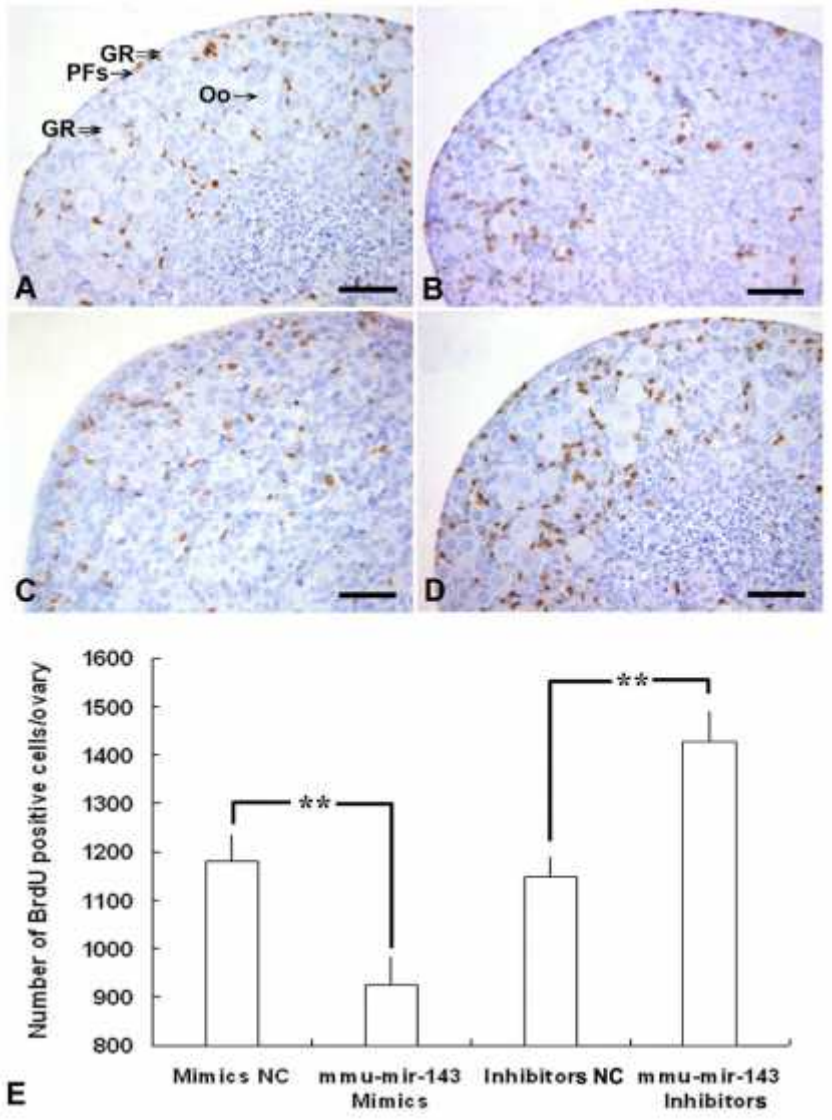

Figure 4. Effect of miR-143 on pregranulosa cell proliferation. Immunolocalization of BrdU-labeled pregranulosa cells in ovaries in NC mimics, miR-143 mimics, NC inhibitors, and miR-143 inhibitors cultured *in vitro* for 8 days. (A) NC mimics. (B) miR-143 mimics. (C) NC inhibitors. (D) miR-143 inhibitors. (E) Number of BrdU-positive cells per ovary. Values are the mean±SEM number of positive cells. Stars indicate statistically significant differences between groups (***P<0.01*, **P<0.05*). Each set of data comes from six ovaries. Bar=100 µm.

mouse ovary. The results show that miR-143 expression increases from 15.5 dpc to 4 dpp, the period during which primordial follicle formation occurs. The cells expressing miR-143 in the ovary were pregranulosa cells, in accordance with results from adult mice (19). Our findings suggest that miR-143 may regulate primordial follicle formation by acting on somatic cells.

Knockout animal models are effective experimental tools for studying the function of specific miRNAs. For example, Dicer-knockout mice have been used to study the function of miR-143/145 in smooth muscle *in vivo* (27). However, these mice are not suitable for analysis of early follicular development because knocking out Dicer causes delayed development of the embryo, and mutant embryos die before the body axis forms (28). To study the function of miR-143 in the developing ovary, we first established an ovarian miRNA electroporation transfection model according to previously described methods (39-30). The transfection efficiencies for miR-143 mimics and inhibitors in cultured ovaries were high, and the overexpression and silencing effects persisted for as long as 8 days. Furthermore, the development of cultured ovaries, especially *in vitro* primordial follicle formation in the control group, was similar to *in vivo* ovary development. This proves that the model established in this study is reliable and effective. Most importantly, this model will be of practical use, not only in functional studies of

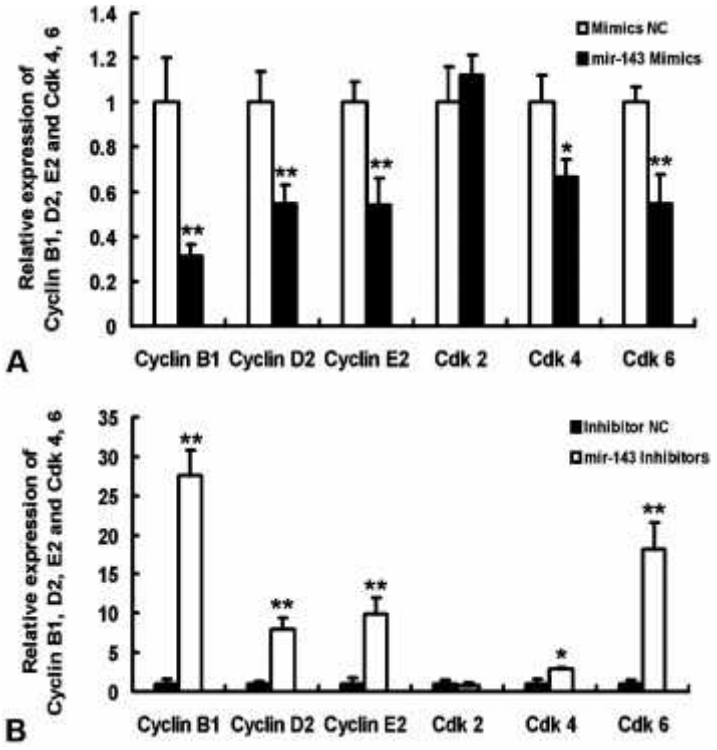

Figure 5. Effect of miR-143 on the relative expression of genes related to the cell cycle. The relative expression of Cyclin B1, Cyclin D2, Cyclin E2, Cdk 2, Cdk 4, and Cdk 6 in isolated somatic cells after transfection with miR-143 mimics (A) or miR-143 inhibitors (B) for 24 h using Lipofectamine 2000, as assessed by real-time PCR. GAPDH mRNA was used as an internal reference. Stars indicate statistically significant differences between groups (***P<0.01*, **P<0.05*).

miRNAs, but also in analyses of the roles of genes and other factors in early follicle development, and could be applied to functional studies of other tissues/organs *in vitro*.

The results presented here demonstrate that miR-143 influences the formation of primordial follicles by inhibiting pregranulosa cell proliferation. Primordial follicles are oocytes surrounded by flat squamous pregranulosa cells, the formation of which involves the invasion of pregranulosa cells into a single package of oocytes. Pregranulosa cell proliferation is indispensable for this process. We found that miR-143 is localized in the pregranulosa cells of primordial follicles and that miR-143 disrupts primordial follicle formation by inhibiting pregranulosa cell proliferation. The results of our analysis of the role of miR-143 in pregranulosa cell proliferation are consistent with previous reports of its inhibitory effects on ovarian, colorectal (16, 18), and prostate cancers (17). These data collectively suggest that miR-143 plays important roles in early follicle development.

The regulation of cell proliferation is a tightly controlled process that depends on numerous endocrine, paracrine, and autocrine factors, notably the expression of cycle proteins (Cyclins) and protein kinase (Cdk) activity (31). In the ovary, Cyclin D2 is the predominant cyclin in granulosa cells and its expression is increased by FSH, estradiol, and insulin (32-34). Cyclin D2-knockout mice are infertile due to the inability of granulosa cells to proliferate in response to FSH (35-36). Cyclin E/Cdk 2 is a critical factor in cell cycle progression (37), while overexpression of Cyclin E has been noted in ovarian cancers (38-39). Recent studies have shown that the proliferation of granulosa cells in rats is accompanied by increases in the expression and activities of Cyclin D2/Cdk 4 and Cyclin E/Cdk 2 in cumulus granulosa cells (40-41). In the present study, miR-143 reduced the expression of Cdks 4 and 6 and Cyclins B1, D2, and E2 in pregranulosa cells. These findings suggest that miR-143 may inhibit the proliferation of pregranulosa cells in the mouse ovary during primordial follicle formation by downregulating Cyclin D2 and Cdks 4 and 6. However, the relationships between miR-143 and Cyclins/Cdks need to be further elucidated in future studies.

In conclusion, this study provides a novel and effective miRNA overexpression and suppression model for *in vitro* analysis of fetal mouse ovaries. Our findings demonstrate that miR-143 is critical for the formation of primordial follicles in mice, and deepen our understanding of the role of miRNAs in early folliculogenesis in the fetal ovary.

6. ACKNOWLEDGEMENTS

Jianfang Zhang and Xiaowen Ji contributed equally to this article. This work was supported by the National Basic Research Program of China

(2012CB944700) and the Natural Science Foundation of China (31172288).

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Key Words: miR-143; Formation of primordial follicle; Mice; Pregranulosa cells; Proliferation; Electroporation transfection

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