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A network of miRNAs expressed in the ovary are regulated by FSH

Nan Yao[1,2,3], Cai-ling Lu[2,3], Jian-jun Zhao[1,2,3], Hong-fei Xia[1,3], Da-guang Sun[1,2,3], Xin-quan Shi[1,3], Chong Wang[1,2,3], Dan Li[1,2,3], Yi Cui[1,3], Xu Ma[1,2,3]

[1]Graduate School of Peking Union Medical College, Beijing, China, [2]Department of Genetics, National Research Institute for Family Planning, Beijing, China, [3]World Health Organization Collaborative Center for Research in Human Reproduction, Beijing, China

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1. ABSTRACT

The process of folliculogenesis requires a tightly regulated series of gene expression that are a pre-requisite to the development of ovarian follicle. Among these genes, follicle-stimulating hormone (FSH) is notable for its dual role in proliferation and development of follicles as well as differentiation of granulosa cells. The post-transcriptional expression of these genes is under the control of microRNAs (miRNAs), a class of small, endogenous RNAs that negatively impact gene expression. This study was carried out to determine the role of several miRNAs including mir-143, let-7a, mir-125b, let-7b, let-7c, mir-21 in follicular development in the mouse. The expression of these RNAs was very low in primordial follicles but these became readily detectable in the granulosa cells of primary, secondary and antral follicles. We show that this expression is under negative control of FSH. FSH inhibited the expression of some (mir-143, let-7a, mir-125b) but not all of these RNAs. Together, these findings suggest that FSH regulates folliculogenesis by a network of miRNAs.

2. INTRODUCTION

Ovary serves two main functions; generation of fertilizable, developmentally competent oocytes and the secretion of steroid hormones which support fertilization and pregnancy. Follicles within the ovary act as the primary functional unit that support ovulation. Close to birth in rodents, oocytes get surrounded by pre-granulosa cells and form primordial follicles (1). Within such follicles, oocytes get arrested at meiosis I and remain in this state until the time of ovulation (2). Mammalian folliculogenesis is a complex process through which primordial follicles develop first into preovulatory follicles followed by a maturation process that allows them to be released at ovulation. During this process, pre-granulosa cells undergo size changes, proliferate and are subjected to hormone-regulated gene expression (3). Progression through the successive stages of follicular development requires many extracellular and intracellular signaling molecules, most notably follicle-stimulating hormone (FSH), activin and growth and differentiation factor (GDF)-9. The

Table 1. Sequences of probes

miRNA	**Sequence 5'- 3'**
Northern Blotting	
mir-21	TCAACATCAGTCTGATAAGCTA
mir-143	TGAGCTACAGTGCTTCATCTCA
mir-125b	TCACAAGTTAGGGTCTCAGGGA
mir-320	TTCGCCCTCTCAACCCAGCTTTT
let-7a-1	ACTATACAACCTACTACCTCA
let-7b	AACCACACAACCTACTACCTCA
let-7c	AACCATACAACCTACTACCTCA
U6	CGTTCCAATTTTAGTATATGTGCTGCCGAAGCGA
***In situ* hybridization**	
mir-21	tcAacAtcAgTcTgAtaAgcTa
mir-143	tgAgcTacAGtGcTtCatCtca
mir-125b	tcAcAagTtAgGgTctCagGga
let-7a-1	aCtaTacAaCcTacTacCtca
let-7b	aAccAcAcAaCcTaCtaCctCa
let-7c	aaCcAtAcAaCcTaCtaCctca
scramble	cAttAatGtcGGAcaActCaat

development of early follicles is likely regulated but is not solely dependent on FSH. By contrast, FSH is crucial to the proper development of follicles past the antral stage, acting as the predominant survival factor (4-5).

miRNAs are a class of 17–24 base single-stranded RNA molecules that are highly conserved from plants to animals (6). miRNAs have an important role in the translational regulation and degradation of mRNAs by base pairing to the 3'- untranslated regions (UTRs) of the target mRNAs. Translation blockade versus mRNA degradation depends on the extent of base pairing with the target genes (7). miRNAs have been implicated in many processes in vertebrates, including cell growth, proliferation, apoptosis, fat metabolism, neuronal patterning, and tumorigenesis (8-11). In higher eukaryotes, miRNAs are equally important as transcriptional regulators of gene expression. Recently, it was shown that some miRNAs are highly enriched in the ovary suggesting that these RNAs may play an important role in follicular development and might underlie female infertility (12-14). Here, we characterized the expression of seven miRNAs (let-7a, mir-143, mir-21, mir-125b, let-7b, let-7c and mir-320) in the ovary that are shown to be highly enriched during follicular development. We also examined the effect that FSH on the expression of these regulatory RNAs.

3. MATERIALS AND METHODS

3.1. Tissue collection and processing

The use of the animals was in accordance with the institutional Guide for the Care and Use of Laboratory Animals. Organs were removed from adult female ICR mice after they were sacrificed by cervical dislocation. Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA). Ovaries were fixed in 10% buffered formalin, dehydrated through ascending series of alcohol, embedded in paraffin, and serially sectioned at 5 μm thickness.

3.2. Northern blotting

Cells were homogenized in Trizol reagent, and total RNA was isolated using chloroform extraction and isopropanol precipitation. After being washed with 70% (v/v) ethanol, RNA pellets were dissolved in DEPC-treated water. Total RNA (fForty micrograms) from each tissue sample was fractionated in 15% denaturing (8 M urea) polyacrylamide gels, transferred to Hybond N^+ membranes (Amersham, UK) and cross linked by UV exposure. Membranes were probed with ^{32}P-labeled oligonucleotides complementary to miRNAs. The miRNA sequences are provided at http://microrna.sanger.ac.uk and the sequences of probes used in Northern blotting are shown inTable 1. U6 served as a loading control. Pre-hybridization and hybridization were carried out at 29°C using ExpressHyb Hybridization Solution (Clontech, CA) according to the manufacturer's instruction. Membranes were washed at room temperature, twice in 2X SSC, 0.1% SDS, once in 0.5X SSC, 0.1% SDS, and once in 0.1X SSC, 0.1% SDS. The blots were exposed to Molecular Dynamics Phosphorimager screens. Blots were re-probed by stripping the probe by boiling in 0.5% SDS for 20 min.

3.3. Locked nucleic acid (LNA)-modified-*In situ* hybridization for miRNAs

Locked nucleic acid (LNA)-modified, digoxigenin (DIG)-labeled probes complementary to mouse mature miRNAs were generated by Takara (Shiga, Japan). The LNA technology chemically modifies the sugar-phosphate backbone of the oligonucleotide, increases thermal stability, and improves hybridization properties. The sequences of probes for *in situ* hybridization are shown in Table 1. Sections were de-paraffinized and were treated for 20 min with proteinase K (10 μg/ml). After a washing in PBS, sections were re-fixed in 4% paraformaldehyde for 10 min, washed twice in PBS, and prehybridized for 1 hr in hybridization buffer (Roche, Mannheim, Germany). Tissues were hybridized overnight in the presence of 0.4 pmol/μl probe at 50°C. Slides were washed twice in 2X SSC at 37 °C and finally washed in a high stringency 50% formamide-2X SSC at hybridization temperature.

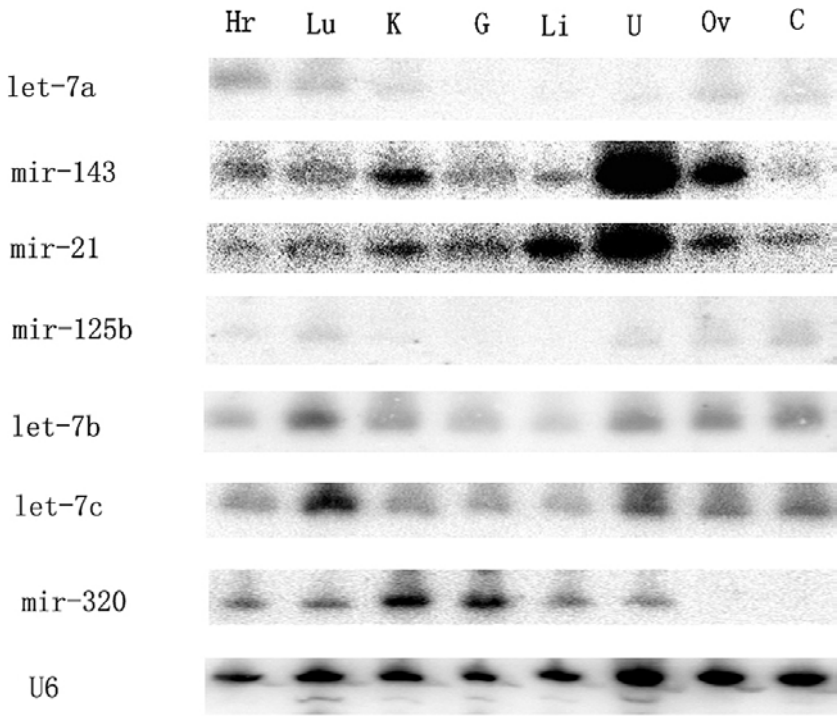

Figure 1. The expression levels of let-7a, mir-143, mir-21, mir-125b, let-7b, let-7c and mir-320 was analyzed by Northern blotting. Total RNA was obtained from heart (Hr), lung (Lu), kidney (K), gut (G), liver (Li), uterus (U), ovary (Ov) and cerebrum (C) of adult female mice. U6 snRNA was used as a detection control.

Immunological detection was carried out using the anti-DIG Fab conjugated to alkaline phosphatase (Roche, Mannheim, Germany) according to the manufacturer's protocol. Sections were developed in a mixture of alkaline phosphatase (AP), nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP; Invitrogen, Carlsbad, CA).

3.4. Cell culture

The regulation of expression of miRNAs was characterized in KK-1 cells a cell line originally derived from an ovarian tumor of inhibin-α-driven, T-antigen transgenic mice. KK-1 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. and cultured in F12/DMEM supplemented with 10% fetal bovine serum, containing 50 mIU/ml penicillin and 0.5 mg/ml streptomycin. KK-1 cells were treated with or without 10 ng/ml human FSH (Sigma, Saint Louis, USA) and harvested at 0, 6, 12, 24, 48 hr.

3.5. TaqMan miRNA expression assays

cDNA was synthesized from total RNA extracts using specific miRNA stem-loop primers and a TaqMan miRNA reverse transcription kit according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems, Foster City, Calif., USA). Real-time PCR was performed using the Applied Biosystems 7000 Sequence Detection System. Twenty microliter PCR reaction mixtures included 1.33 μl of RT product, 1X TaqMan Universal PCR Master Mix and 1 μl of primers. These were mixed according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems). The threshold cycle data were determined using default threshold settings. The expression level of each miRNAs was normalized against the expression level of the house-keeping gene, U6, and presented as the mean normalized expression level

4. RESULTS

4.1. miRNA expression in tissues of adult female mice

Based on the results of previous studies using microarrays and small RNA cloning, we chose a panel of seven miRNAs that are highly enriched in the normal tissues in the adult female mice. For each of these, eight different RNA samples were used. As shown in Figure 1, six of the seven miRNAs, let-7a, let-7b, let-7c, mir-143, mir-21 and mir-125b, were expressed in the ovary of the adult mice, while mir-320 was not been detected. Furthermore, none of the seven miRNAs was specifically expressed solely in the ovary.

4.2. Expression of miRNAs in the follicles of adult female mice

Analysis of expression of miRNA in the ovaries of adult females was carried out by *in situ* hybridization. Regardless of the size of follicles, there was positive signals in the granulosa cells of follicles, while no staining was observed in oocytes (Figure 2). Small oocytes of the primordial follicles were surrounded by a layer of flattened pre-granulosa cells (Figure 2A1). Larger oocytes of the primary follicles were surrounded by a single layer of

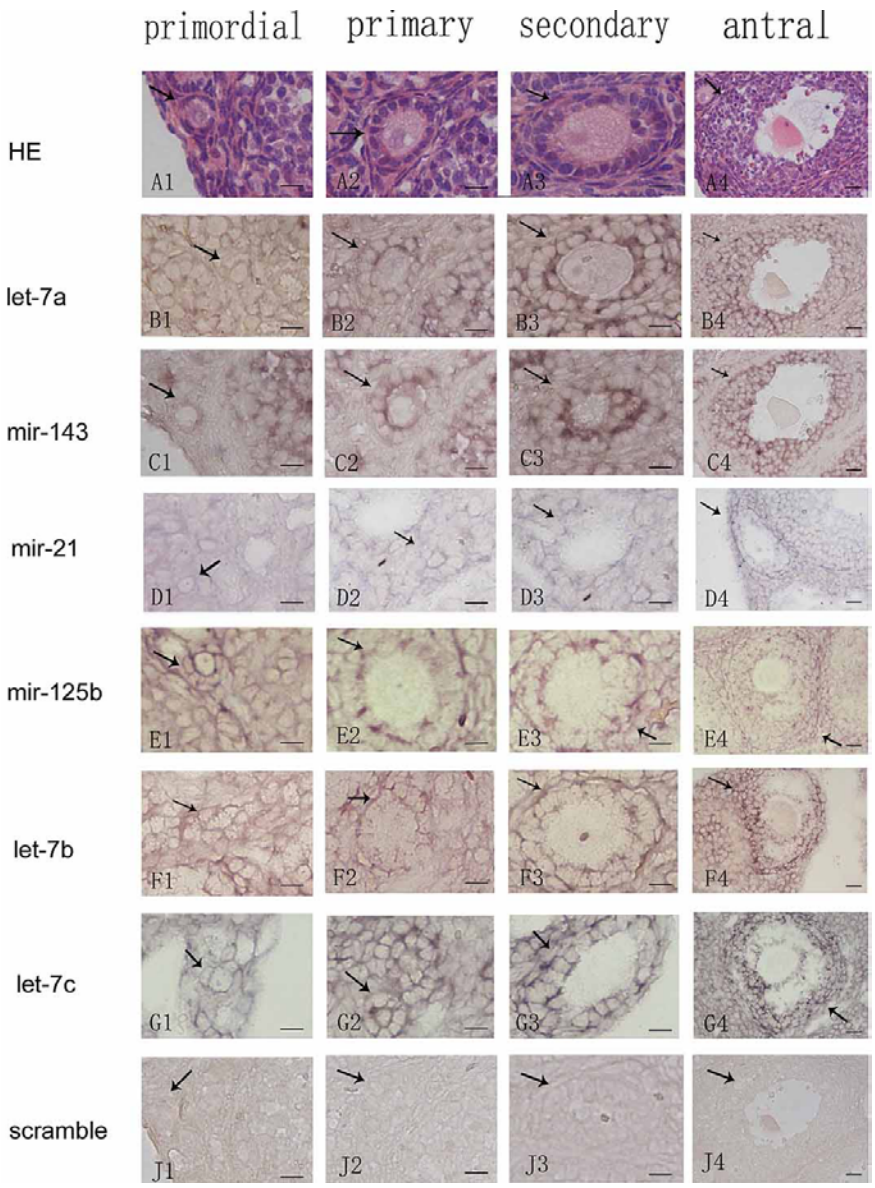

Figure 2. *In situ* hybridization analysis of expression of miRNAs in the mouse ovary during follicle development. B-G: *In situ* hybridization with DIG-labeled antisense probes for let-7a (B), mir-143 (C), mir-21 (D), mir-125b (E), let-7b (F) and let-7c (G) and a scrambled oligonucleotide sense (J) control. Arrows point to follicles. Bar=10 micrometer

cuboidal granulosa cells (Figure 2A2). Follicles surrounded by a multi-layer of cuboidal granulosa cells were classified as secondary follicles (Figure 2A3). These follicles were surrounded by theca cells. Antral follicles (Figure 2A4) were comprised of a large oocyte surrounded by cumulus cells, a fluid-filled antrum, granulosa cells, and theca cells. Among the detected miRNAs, while less staining was seen in primordial follicles, let-7a (Figure 2B), mir-143 (Figure 2C), mir-21 (Figure 2D), mir-125b (Figure 2E), let-7b (Figure 2F) and let-7c (Figure 2G) was readily detectable in granulosa cells of primary, secondary and antral follicles. In addition, in antral follicles, in which mir-21, mir-7b and mir-7c were detected, the intensity of hybridization signal appeared to be higher than in pre-antral follicles. By contrast, let-7a, mir-143 and mir-125b were expressed in primary and secondary follicles at higher levels.

4.3. Regulation of the expression of miRNAs by FSH

In order to elucidate whether FSH affects the expression level of miRNAs, the ovarian cell line, KK-1 was subjected to a time course treatment with FSH. After 6, 12, 24 and 48 hr treatment, the expression of let-7a, mir-143 and mir-125b was assessed by RT-PCR TaqMan assay. The expression level of mir-143 was significantly ($P<0.01$) decreased after FSH treatment (Figure 3B). Although, the expression level of let-7a and mir-125b was slightly decreased, these decreases were not statistically significant (Figure 3A, Figure 3C).

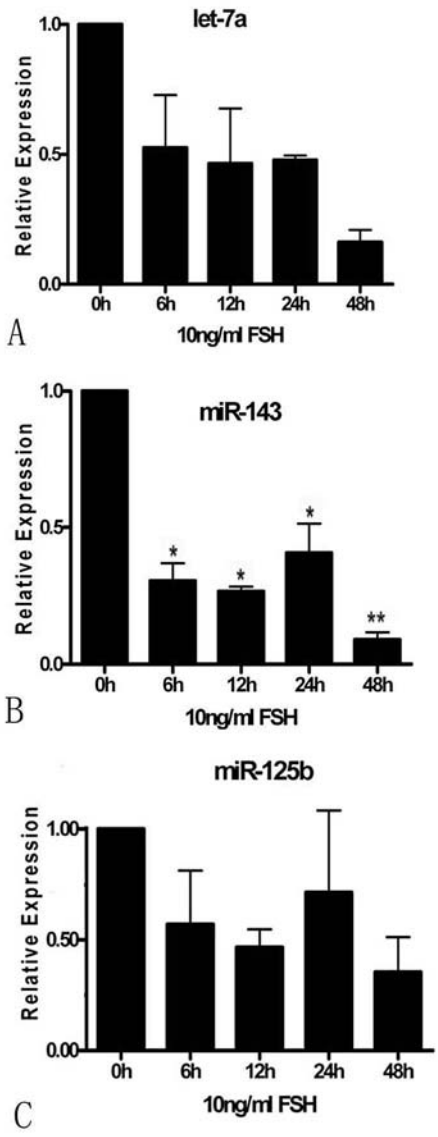

Figure 3. Expression of let-7a (A), mir-143 (B) and mir-125b (C) after exposure to FSH (10 ng/ml) for 3h, 6h, 12h, 24h and 48 h. Data are presented as mean normalized expression (MNE) levels ±S.E.M of triplicate determinations from three independent experiments. **: $p < 0.01$, and ***: $p < 0.001$ when compared with the control sample.

5. DISCUSSION

miRNAs are 22-nucleotide single-stranded non-coding RNA molecules that are abundant, highly conserved, and tissue specific (15). Mature miRNAs appear to function as post-transcriptional suppressors by binding to their target mRNAs via base pairing and subsequently induce either translational repression or mRNA destabilization (6). Thus far, 122 and 141 miRNAs have been cloned respectively from mouse ovary and testes suggesting that miRNAs might be involved in translational repression in the reproductive organs (16, 17). We recently generated an expression atlas of miRNAs which identified miRNAs that were significantly expressed in the ovary during progression from primordial follicles to the antral stage. The distinct expression pattern of miRNAs in the follicles at each stage of follicular development suggested that the detected miRNAs might affect highly specific mechanisms in folliculogenesis. To further analyze the role of these RNAs, we chose seven miRNAs that were highly enriched in the ovary (12- 14). Among these, six miRNA, let-7a, mir-143, mir-21, mir-125b let-7b, and let-7c were expressed at a high level in the adult female mouse ovary, while mir-320 was not expressed. Because the level of expressed mir-320 as ascertained by microarray was quite low as compared with the other six miRNA, we analyzed the expression of these RNAs by Northern blotting. However, similar results were obtained (16). This is not surprising, given the fact that granulosa cells account for a high proportion of ovarian cells.

The expression levels of let-7a, mir-143, mir-21, mir-125b, let-7b and let-7c in the granulosa cells of primary follicles were higher than that expressed in the granulosa cells of primordial follicles. Majority of primordial follicles were still in a quiescent state (18). These early steps of folliculogenesis are critical, as primordial follicles are considered to be the fundamental reproductive units of the ovary that give rise to all dominant follicles (2). Numerous genes namely, Foxl2, Kitl and NGF, are expressed in granulosa cells, and play critical functions during early folliculogenesis (2). The development of pre-antral follicles is slow, likely due to suppression of expression of genes that contribute to follicular growth, development and differentiation (19). One primary reason for this suppression might lie in the expression of miRNAs which control gene expression. These miRNAs may function as transcriptional regulators through their interactions with some endocrine, paracrine and/or autocrine factors, such as FSH, activin and GDF-9, which are important factors during folliculogenesis. As shown here, FSH is likely to influence the development of pre-antral follicles by virtue of its effect on expression of selective miRNAs. Growth beyond the small-antral stage, however, becomes critically dependent on FSH support (20). The expression of miRNAs during folliclular development suggests that miRNAs are likely subject to endocrine regulation, particularly by FSH.

The granulosa cells play a key role in follicular development and oocyte maturation, and the normal process of ovulation depends on the proliferation and growth of granulosa cells (21). It is well known that FSH induces granulosa cell proliferation and differentiation. The induction of a decreased expression level of several specific miRNAs (let-7a, mir-143 and mir-125b) by FSH suggests that some effects of FSH in granulosa cells is regulated by miRNAs. Further analysis of the miRNAs that are expressed in the ovay likely provides an insight into the regulation of folliculogenesis.

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Send correspondence to: Xu Ma, National Research Institute for Family Planning, 12 Da-hui-si, Hai Dian, Beijing, 100081, China, Tel: 86-010-62179059, Fax: 86-010-62179059, E-mail: genetic@263.net.cn

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