Micro-RNA378 (miR-378) Regulates Ovarian Estradiol Production by Targeting Aromatase

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Estradiol is a steroid hormone that not only plays an important role in ovarian follicular development but also is associated with many reproductive disorders. Owing to the importance of aromatase in the production of estradiol, the regulation of aromatase gene expression at the transcriptional level has been an extensive area of study for over two decades. However, its regulation at the posttranscriptional level has remained unclear. Here, we show that micro-RNA378 (miR-378) is spatiotemporally expressed in porcine granulosa cells, the cells that generate estradiol in the ovary during follicular development, in an inverse manner compared with the expression of aromatase. *In vitro* overexpression and inhibition experiments revealed that aromatase expression, and therefore estradiol production, by granulosa cells, is posttranscriptionally down-regulated by miR-378. Furthermore, site-directed mutation studies identified two binding sites in the 3′-untranslated region (3′-UTR) of the aromatase coding sequence that are critical for the action of miR-378. Interestingly, overexpression of the aromatase 3′-UTR enhanced aromatase expression at the protein level in granulosa cells, possibly mediated by the binding of miR-378 within this region, thereby reducing the binding of this micro-RNA to the endogenous aromatase 3′-UTR. **(*Endocrinology* 152: 3941–3951, 2011)**

Micro-RNA (miRNA) are small (19–25 bp) RNA that diversely regulate gene expression through their control of mRNA stability or translation (1). The functions of these noncoding RNA, until recently, have remained relatively unknown, and they are now emerging as important cellular regulators that influence growth, development, differentiation, and apoptosis. The role of miRNA in the ovary was indicated by the fact that knockout of Dicer, the ribonuclease III that is responsible for the synthesis of mature functional miRNA in the ovary, resulted in the dysfunction of folliculogenesis, oocyte maturation, ovulation, and infertility (2–6). miRNA precursors (pre-miRNA) generated in the nucleus are exported to the cytoplasm, where they are converted to a single-stranded mature miRNA. A mature miRNA, as part of an RNA-induced silencing complex, can bind to 3′-untranslated regions (3′-UTR) of target mRNA and induce their degradation, translational repression, or both (7–11). It has been estimated that up to 30% of mRNA may be subjected to miRNA regulation, and individual miRNA are predicted to target up to several hundred genes (12, 13). Many highly regulated mRNA contain multiple miRNA binding sites, often targeted by different miRNA, which may enhance the effectiveness of regulation (14).

Ovarian follicular development is dependent on the proliferation and differentiation of granulosa cells (reviewed in Ref. 15). Estradiol is synthesized from androgen in granulosa cells via aromatization by cytochrome P450 aromatase. Estradiol represents one of the key ovarian hormones produced by the developing ovulatory follicle and reflects the differentiation of granulosa cells. Estradiol is required for female reproduction, because severe fertil-

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Abbreviations: CMV, Cytomegalovirus; F, forward; FBS, fetal bovine serum; miR-378, miRNA378; miRNA, micro-RNA; R, reverse; 3′-UTR, 3′-untranslated region.

ity defects arise when its synthesis or action are suppressed (16, 17). In the ovary, estradiol acts in concert with FSH to facilitate folliculogenesis and steroid production. As a secreted hormone, estradiol modulates the structure and function of female reproductive tissues, such as the uterus and oviduct. It is also one of the principal determinants of the function of pituitary neurons and is critical in enabling these cells to exhibit fluctuating patterns of biosynthetic and secretory activity, allowing them to generate the preovulatory surge of LH (reviewed in Ref. 18). Thus, the regulated expression of aromatase, the key enzyme for estradiol synthesis, plays an important role in fertility. Although the regulation of aromatase expression at the transcriptional level has been studied in detail (18), it was unknown whether its expression is also regulated at the posttranscriptional level. The current study used a porcine model to investigate whether aromatase expression, and therefore estradiol production, is regulated by small, noncoding RNA in granulosa cells. A second objective was to further determine the underlying mechanisms of miRNA378 (miR-378) action in ovarian function.

Materials and Methods

Granulosa cell culture

Porcine ovaries were collected from prepubertal gilts in PBS from a local slaughterhouse, transported to the laboratory within 2 h while maintained at room temperature, and rinsed three times with sterile 1× PBS. Granulosa cells were aspirated from small (1–3 mm in diameter) and large (4–6 mm in diameter) follicles using a 20-ml syringe fitted to an 18-gauge needle. The follicular fluid was centrifuged at 500 × *g* for 5 min to obtain a cell pellet. This pellet was then washed with a large volume of DMEM/F12 (Life Technologies, Inc.-Invitrogen, Carlsbad, CA) supplemented with extra 1× antibiotic/antimycotic (Life Technologies Invitrogen) by pipetting up and down several times to disperse the cells well, followed by centrifugation. This process was repeated two more times. Although this is an established procedure for pig granulosa cell isolation (19–25), the possibility that the resultant granulosa cell samples may contain a small number of theca cells is not excluded. After trypan blue staining (HiMedia, Mumbai, India) to obtain the viable cell number, cells were seeded at 1.6×10^6 per well in six-well plates or 0.3×10^6 cells per well in 24-well tissue culture-treated plates in 2 or 0.5 ml DMEM/F12 supplemented with extra 1× antibiotic/antimycotic and 10% fetal bovine serum (FBS) (Life Technologies Invitrogen) and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 38.0 C. After 24 h culture, the medium was aspirated to eliminate any clusters of nonadherent dead cells on top of the viable monolayer, the cells were rinsed with 1× PBS, and fresh DMEM/F12 with 10% FBS was added to continue the primary culture.

miRNA target prediction

The binding regions for miR-378 were identified in the 3′-UTR sequences for human, pig, and mouse using the RNA22 program (http://cbcsrv.watson.ibm.com/rna22.html) and the species-specific miR-378 sequence. All three mature miRNA are identical except for an additional nucleotide at the 3′ end of the pig miRNA. These three binding regions were then aligned together to determine the conservation between species. The accession numbers of the 3′-UTR of the aromatase gene are as follow: Ensembl ENSSSCT00000011478 (pig), NM_007810 (mouse), and NM_000103 (human).

Cloning of the lentiviral gene transfer plasmid pL-SIN-Lenti-H1-miR-378-EF1α-EGFP

The self-inactivating lentiviral gene transfer plasmid (pL-SIN-Lenti-EF1α-EGFP) was a kind gift from Dr. James Ellis (26). To clone the H1 promoter-miR-378 fragment into the pL-SIN-Lenti-EF1α-EGFP construct, two primers, *Bam*HI-miR-378 forward (F) 5′-**GGATCC**CCCGGGCTGAGGAATT-3′ and *Bam*HI-miR-378 reverse (R) 5′-**GGATCC**AAGCTTAAAA-AAAGGCCTTC-3′, with *Bam*HI *underlined in bold*, were used to amplify a 415-bp product by PCR using pre-miR-378 (27) as template. This product was subcloned into the pGEM-T-Easy vector (Promega, Madison, WI) and sequenced. The *Bam*HI insert was excised and ligated into a *Bam*HI-cut pL-SIN-Lenti-EF1α-EGFP vector, resulting in the final lentiviral gene transfer plasmid pL-SIN-Lenti-H1-miR-378-EF1α-EGFP. The orientation of the insert was verified by restriction digestion with *Sma*I.

Production of recombinant lentiviral particles

Production of recombinant lentiviral particles was carried out as described previously (28).

Lentiviral transductions

Cells were transduced with lentivirus when they reached 40–50% confluence, and 2 or 0.5 ml medium with lentivirus and 2 or 0.5 μl polybrene (4 μg/μl; Sigma Chemical Co., St. Louis, MO) were added to each well of a six- or 24-well plate, respectively. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 38.0 C for 24 h. The medium was then replaced with fresh DMEM/F12 supplemented with 10% FBS to continue the culture for 48 h, followed by collection of the cells and spent medium, which were stored at −80 C.

miR-378 inhibitor treatment

Anti-miR miRNA inhibitors (Ambion, Austin, TX) bind to, and inhibit the activity of, endogenous miRNA when introduced into cells. These RNA-based inhibitors are chemically modified both to increase their stability and to improve their activity. At 40–50% confluence, granulosa cells were transfected with either anti-miR miR-378 inhibitor (Ambion) or the negative control AM17010 (Ambion) using the Lipofectamine 2000 reagent following the manufacturer's protocol. At 48 h after transfection, cells and spent medium were collected and stored at −80 C.

Reverse transcription and real-time RT-PCR

Reverse transcription and real-time RT-PCR was performed as described previously (29). Primers used were as follows: aromatase-F, 5′-AGTGCATCGGCATGTATGAG-3′, and aromatase-R, 5′-GTCCACGTAGCCCAAGTCAT-3′, product size 191 bp, GenBank NM_214430.1; RNA polymerase II (RPII)-F, 5′-CAGGAGTGGATCCTGGAGAC-3′, and RPII-R, 5′-GGA-GCCATCAAAGGAGATGA-3′, product size 181 bp (30). A reaction in which cDNA was replaced by water was applied to

confirm no potential contamination of amplicons. Product sizes were verified by agarose gel electrophoresis, and all products were sequenced to confirm identity. The RPII housekeeping gene was amplified for each sample to verify the presence of cDNA and as an internal control to calculate the relative level of target gene expression using the $2^{-\Delta\Delta Ct}$ method (31).

miR-378 detection

Total RNA of granulosa cells was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Approximately 1 μg total RNA was used in each reverse transcription reaction using the miScript reverse transcription kit (QIAGEN). Real-time PCR was performed using the miScript SYBR Green PCR kit (QIAGEN), with each reaction consisting of 2 μl cDNA, 10 μl 2× QuantiTect SYBR Green PCR master mix, 2 μl 10× miScript universal primer (QIAGEN), 2 μl 10× miScript primer assay [Hs_miR-378_1 catalog no. MS00006909, Hs_RNU6B_2 (*U6*), catalog no. MS00014000; QIAGEN], and 4 μl ribonuclease-free water. Reactions were run on an Mx3005P System (Stratagene, La Jolla, CA) for 40 total cycles. As a housekeeping gene, *U6* was amplified for each sample to verify the presence of cDNA and as an internal control to calculate the relative level of miR-378 expression by the $2^{-\Delta\Delta Ct}$ method (31).

Western blotting

Western blotting was performed as described (32). Granulosa samples were electrophoresed on a 10% polyacrylamide gel. Proteins were transferred onto polyvinylidine difluoride membranes. The blots were then incubated in antibody against aromatase (1:100; Acris GmbH, Herford, Germany) or glyceraldehyde-3-phosphate dehydrogenase (1:10,000; AbCam, Cambridge, MA).

Analysis of estradiol levels by ELISA

Granulosa cells were transduced with lentivirus or transfected with plasmid DNA. After 24 h, the medium was changed and replaced with DMEM/F12 supplemented with 10% FBS and testosterone (Sigma) at 10^{-6} M, followed by continued culture for another 48 h. Spent medium was collected and assayed for the presence of estradiol by ELISA (EA 70; Oxford Biomedical Research, Oxford, MI) according to the manufacturer's protocol. Each experiment was carried out in triplicate.

Construction of luciferase reporter constructs

To clone the 3′-UTR of aromatase (+1525 to +1796; Ensembl ENSSSCT00000011478, where +1 represents the transcriptional start site) containing the putative miR-378 binding sites (Ensembl ENSSSCT00000011478, from +1589 to +1609, from +1607 to +1627, and from + 1627 to +1647) into the cytomegalovirus (CMV)-driven luciferase reporter p-miR-Report (Addgene, Cambridge, MA) to generate CMV-luciferase-aro3′-UTR (p-miR-Report + WT miR-378 BS in the aromatase 3′-UTR), the 3′-UTR was amplified by PCR using the following primers: aro-F 5′-**ACTAGT**TGGGACATTGCTCACCTC-3′ and aro-R 5′-**AAGCTT**ATTGTGTTAATGTTTAATA-3′, product size 284 bp, with *Spe*I and *Hin*dIII sites *underlined in bold*, which are also present in the multiple cloning site of the p-miR-Report vector. PCR was performed using porcine granulosa cell cDNA as template. The PCR product was subcloned into the pGEM-T-easy vector (Promega) and sequenced to confirm identity. A *Spe*I and *Hin*dIII insert was excised and ligated into a *Spe*I- and *Hin*dIII-cut p-miR-Report vector. As a negative control sequence (conURT), an unrelated sequence was amplified from the mouse glial cell line-derived neurotrophic factor coding sequence (GenBank BC119031.1, from +115 to +476, not in-frame and therefore does not code for the glial cell line-derived neurotrophic factor protein). The RNA22 program was employed to computationally confirm the absence of a binding site for miR-378 in this control fragment. The conUTR sequence was cloned using primers conUTR-F 5′-**ACTAGT**GTTATGGGATGTCGTGGCT-3′ and conUTR-R: 5′-**AAGCTT**TGGCAGTTAAAACGCACCC-3′, product size 374 bp. The control vector (CMV-luciferase-conUTR or p-miR-Report + conUTR) was constructed using the same strategy as described above for CMV-luciferase-aro3′-UTR.

Mutagenesis

Mutagenesis reactions on the p-miR-Report + WT miR-378 BS in the aromatase 3′-UTR construct were performed using the overlap PCR method. Mutations were introduced into putative miR-378 binding sites located in the 3′-UTR of the aromatase coding sequence [mutation A (MUA) from +1604 to +1608, mutation B (MUB) from + 1622 to +1625, and mutation C (MUC) from +1641 to +1645]. The primer sequences used to construct the p-miR-Report + MUA miR-378 BS in aromatase 3′-UTR, p-miR-Report + MUB miR-378 BS in aromatase 3′-UTR, and p-miR-Report + MUC miR-378 BS in aromatase 3′-UTR mutants were as follows: aro-mutA-F 5′-GCCT**GCTATTCCCCCAATAAGGTCT** GAACACCCAACC-3′(TCCAG to AGGTC) and aro-mutA-R 5′-CTAGTGGAGGTTGGGTGTTC**AGACCTTATTGGGGGAAT**-3′ (CTGGA to GACCT); aro-mutB-F 5′-CCCAATATCC**AGTGAACACCCAACCAGGTCT**AGAGTTTCCTG-3′ (TCCA to AGGT) and aro-mutB-R 5′-GTGAACCTCAGGAAACTCT**AGACCTGGTTGGGTGTTC**-3′ (TGGA to ACCT); aro-mutC-F 5′-CCAC**TAGAGTTTCCTGAGCAGGTCT**GCACACGTTTATC-3′ (GTTCA to CAGGT) and aro-mutC-R 5′-GAATATTAGATAAACGTGTGC**AGACCTGCTCAGGAAACTC**-3′ (TGAAC to ACCTG), each with the putative miR-378 biding sites *underlined in bold*. The mutagenesis PCR comprised three steps to generate each fragment with the desired mutation. First, primers mut-F and aro-R (for construction of the luciferase reporter) were applied to amplify PCR product A, and primers aro-F (for construction of the luciferase reporter) and mut-R were used to amplify PCR product B using the p-miR-Report + WT miR-378 BS in aromatase 3′-UTR vector as template. Next, product A and B were used as template to amplify for 11 cycles in the absence of primer, followed by the addition of primers aro-F and aro-R (for construction of the luciferase reporter), generating the product with the mutation. The PCR product with the mutation was then subcloned into the pGEM-T-easy vector (Promega), and the presence of each mutation was confirmed by DNA sequencing. A *Spe*I and *Hin*dIII insert was excised from the pGEM-T-easy vector and ligated into the *Spe*I- and *Hin*dIII-digested p-miR-Report vector. The sequence identity was confirmed by DNA sequencing.

Transient transfections and dual luciferase reporter gene assays

Freshly isolated granulosa cells from large follicles were seeded into wells of a 24-well plate. When the cells reached 40–50% confluence, they were transduced with lentivirus, and 24 h

after transduction, the medium was changed. Cells were then cotransfected with the firefly luciferase reporter constructs [p-miR-Report + WT miR-378 BS in aromatase 3′-UTR, p-miR-Report + conUTR and pGL3-Basic (promoterless; Invitrogen, Carlsbad, CA); p-miR-Report + MUA miR-378 BS in aromatase 3′ UTR, p-miR-Report + MUB miR-378 BS in aromatase 3′ UTR, p-miR-Report + MUC miR-378 BS in aromatase 3′ UTR, p-miR-Report + conUTR control and pGL3-Basic] and pRL-TK, a vector encoding the renilla luciferase reporter gene. Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol and included a total of 0.55 μg DNA (0.5 μg of each firefly luciferase construct and 0.05 μg renilla luciferase reporter plasmid). After 30 h, granulosa cell lysates were harvested by direct lysis. In the inhibitor study, cells that had been subjected to lentiviral transduction were cotransfected with the appropriate firefly luciferase reporter constructs (p-miR-Report + WT miR-378 BS in aromatase 3′-UTR, p-miR-Report + conUTR as a control and pGL3-Basic) and pRL-TK and treated with either anti-miR miR-378 inhibitor or negative control AM17010 using the same method described above. Luciferase activity was measured in triplicate using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized by renilla luciferase activity, and each result was presented as the fold relative to pGL3-Basic as described previously (30). Each experiment was performed in triplicate.

Generation of the aromatase 3′-UTR expression construct and expression analysis

The luciferase coding sequence in the CMV-luciferase-aro 3′-UTR vector described above was removed using a *Spe*I and *Bam*HI digestion, resulting in the generation of the CMV-aro 3′-UTR expression construct. A similar strategy was used to generate the CMV-3′-UTR control from CMV-luciferase-conUTR described above. Granulosa cells were transduced with miR-378 lentivirus. After 24 h, the medium was changed and cells were transfected with the aromatase 3′-UTR overexpression vector using the Lipofectamine 2000 reagent following the manufacturer's protocol. At 48 h after transfection, a cell sample was taken for Western blotting. In addition, the spent culture medium was collected for the estradiol ELISA.

Statistical analyses

Mean fold changes in mRNA levels were calculated relative to granulosa cells derived from small follicles transduced with the EGFP lentivirus (control). Luciferase activity for the various 3′-UTR and mutants were calculated relative to pGL3-Basic empty vector. All experiments were repeated at least three times, with data representing the mean ± SEM of all repeats. Results from each experiment were based on at least three independent collections of ovaries, and each experiment was carried out using cells isolated from separate collections. At least 80 ovaries were used to isolate separate batches of granulosa cells. Data were analyzed by *t* test or one-way ANOVA followed by either the Bonferroni test for selected pairs comparisons (see Figs. 2C and 3B) for *t* tests or the Tukey test for multiple comparisons (all other figures) for ANOVA to determine statistical differences between groups using GraphPad Prism analysis software. Results were considered significant at $P < 0.05$.

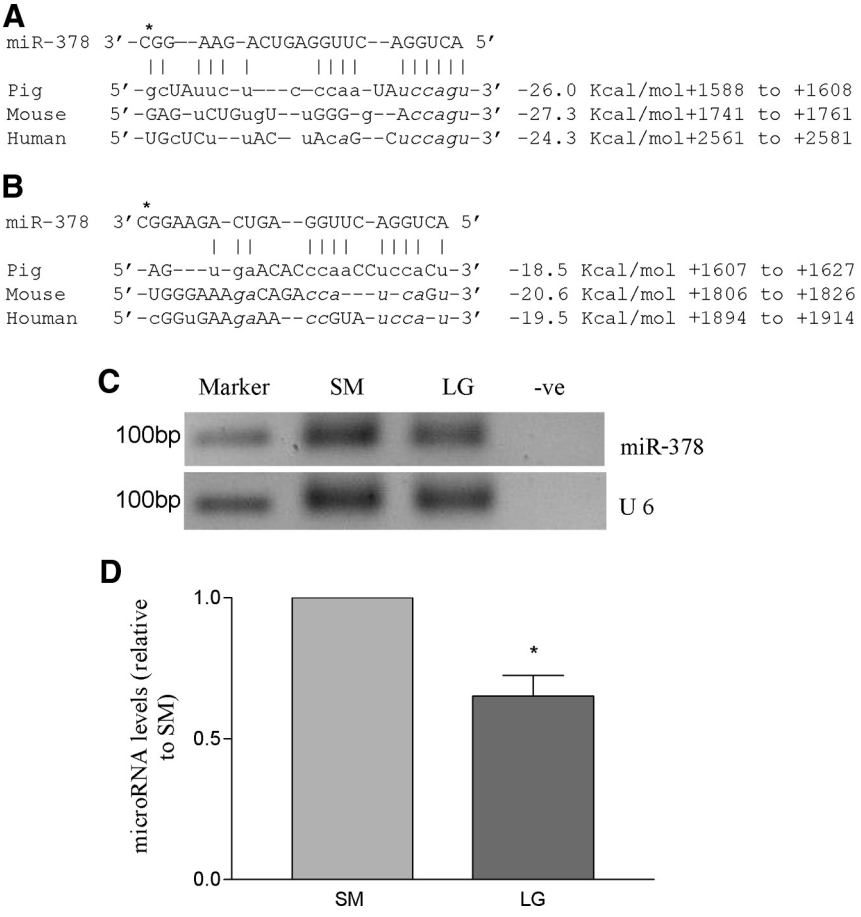


FIG. 1. Putative miR-378 binding sites in the 3′-UTR and expression of miR-378 in freshly isolated granulosa cells. A and B, Cross-species homology of miR-378 targeting the aromatase 3′-UTR. Putative miR-378 binding sites (potential complementary residues shown in *lowercase letters*); +1 is the transcriptional start. A and B represent two different putative binding sites. *, Additional nucleotide in the pig miR-378. The rest of the miR378 mature sequences are identical across the three species. C, Representative results of RT-PCR for mature miR-378 using RNA prepared from granulosa cells derived from small follicles (SM, 1–3 mm) and large follicles (large, 3–6 mm). U6 expression was used as a housekeeper. D, Relative quantitation of real-time PCR results. Negative control in which reverse transcriptase was omitted from the reaction is indicated by -ve. Data represent the mean ± SE of three independent experiments. *, Statistical differences between groups ($P < 0.05$).

Results

miR-378 decreases aromatase protein expression and estradiol production in granulosa cells

Using the RNA22 miRNA target detection program, the 3′-UTR of aromatase mRNA of pig, human, and mouse

were aligned with the sequence of mature miR-378. Putative target sites for miR-378 were identified in this region across the three species with similar folding energy (Fig. 1, A and B). We first sought to investigate whether miR-378 is physiologically expressed in granulosa cells and whether its expression is dependent on follicular maturation. RNA was extracted from freshly isolated porcine granulosa cells derived from small (1–3 mm) and large (3–6 mm) follicles, and RT-PCR was performed. As shown in Fig. 1, C and D, miR-378 is expressed in granulosa cells during follicular development, suggesting that it is locally available and therefore may play a role in the differentiation of ovarian follicles. We next overexpressed miR-378 to study its role in granulosa cells. To allow high-efficiency delivery to primary cells to achieve overexpression, we constructed a lentiviral expression vector in which two copies of pre-miR-378 were cloned in tandem to the 3′ end of the H1 promoter and transduced the recombinant lentivirus (Lenti-miR-378) into granulosa cells. As shown in Fig. 2, A and B, transduction of Lenti-miR-378 resulted in significant increases in miR-378 expression in granulosa cells isolated from small and large follicles with the lentiviral control (Lenti-GFP). To investigate whether miR-378 regulates aromatase expression, we evaluated changes at the mRNA and protein levels by real-time RT-PCR and Western blotting, respectively. Although no significant change was detected at the mRNA level between the Lenti-control and Lenti-miR-378 groups (Fig. 3A), aromatase protein levels were significantly decreased in the Lenti-miR-378 group in granulosa cells derived from both small and large follicles (Fig. 3, B and C). To investigate the effect of the miRNA on estradiol production, one of the key functions of granulosa cells, we collected spent media from the cultured miR-378-transduced and empty vector-transduced cells, and performed an ELISA to determine changes in the concentration of estradiol. Overexpression of pre-miR-378 via lentivirus transduction decreased the concentra-

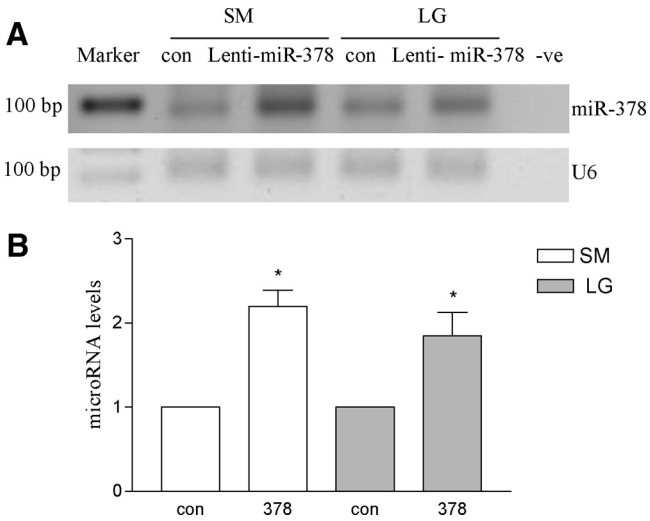


FIG. 2. Overexpression of miR-378 in ovarian granulosa cells. A, RT-PCR was performed on RNA isolated from small (SM) and large (LG) granulosa cells transduced with miR-378 lentivirus (Lenti-miR-378) or control EGFP virus (con). B, Relative quatitation of real-time PCR results depicting the increased expression of miR-378 imparted by miR-378-lentiviral transductions. –ve is a negative control in which reverse transcriptase was omitted from the reaction. Data represent the mean ± SE of three independent experiments, with a * indicating statistical differences from respective controls ($P < 0.05$).

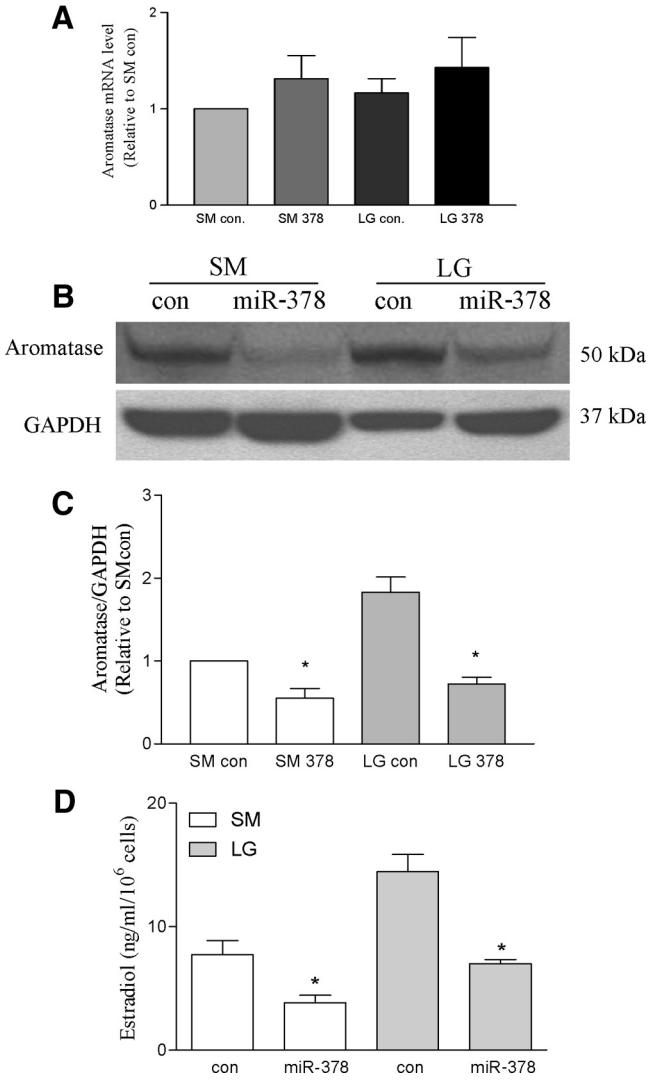
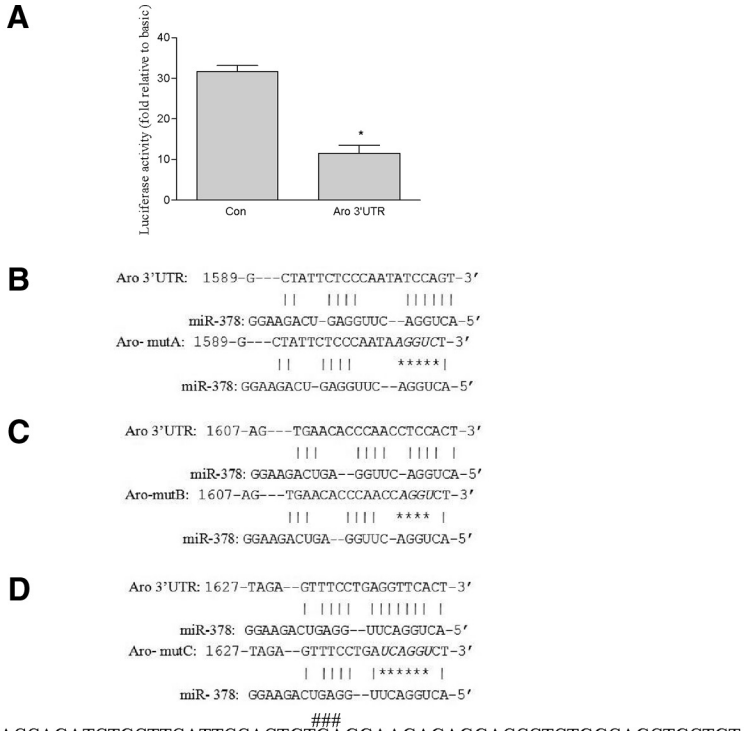


FIG. 3. MiR-378 suppresses aromatase protein expression and estradiol production in ovarian granulosa cells. A) RT-PCR analysis of aromatase transcript expression in small (SM) and large (LG) granulosa cells transduced with either miR-378-lentivirus (miR-378) or control EGFP virus (con). B, Representative Western blot of aromatase expression in SM and LG granulosa cells transduced with either miR-378-lentivirus (miR-378) or control EGFP virus (con). The expression of glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. C, Densitometric quantitation depicting decreased expression of aromatase after miR-378-lentiviral transduction. D, Levels of estradiol in the spent culture medium collected from miR-378-transduced (miR-378) and EGFP-transduced control (con) groups of granulosa cells. Data represent the mean ± SE of five (C) and three (D) independent experiments. *, Significant differences from each respective control ($P < 0.05$).



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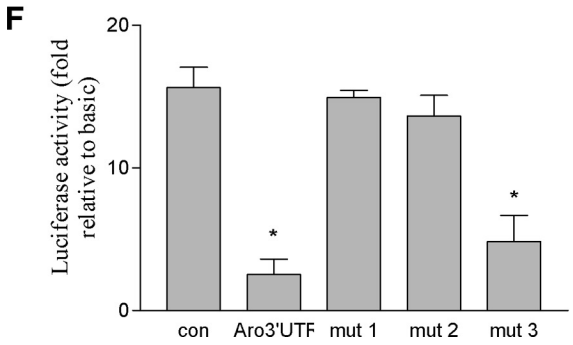

FIG. 4. Identification of putative miR-378 binding sites within the aromatase 3′-UTR. A, The aromatase 3′-UTR decreases luciferase activity. Granulosa cells isolated from large follicles were first transduced with lenti-miR-378, transfected with either the aromatase 3′-UTR-linked luciferase vector or a control (a luciferase construct in which the 3′-UTR of aromatase was replaced with a random sequence without any miR-378 target sites), and a luciferase assay was then performed. B–D, Computationally identified putative miR-378 binding sites A (+1589 to +1609), B (+1607 to +1627), and C (+ 1627 to +1647); sites were predicted by the RNA22 miRNA target detection program. *Top lines* indicate wild-type 3′-UTR sequences, whereas *bottom lines* represent mutated sequences. The mutated nucleotides are indicated by *italics* and the mismatches by *asterisks*. The position of each binding site is indicated on the *right*. E, 3′-UTR of aromatase showing the location of the three sites: site A, *italic*; site B, *lowercase letters*; site C, *underlined*. The three nucleotides shared by both site 1 (B) and 2 (C) are both in *italic* and *lowercase letters*. #, Translational stop. F, Granulosa cells were first transduced with lenti-miR-378 and then transfected with the luciferase-aromatase 3′-UTR vector or a control vector (a construct in which the 3′-UTR of aromatase was replaced with a random sequence without any miR-378 target sites), and luciferase activity was measured. Mut 1, mut 2, and mut 3 represent luciferase expression constructs in which the miR-378 binding sites in the luciferase-aromatase 3′-UTR vector were mutated as indicated in B, C, and D, respectively. Data represent the mean ± SE of three independent experiments. *, Statistical differences between groups ($P < 0.05$).

tions of estradiol in both small and large follicle-derived primary granulosa cells (Fig. 3D).

MiR-378 suppresses aromatase by targeting to specific sites within the aromatase 3′-UTR

Because miR-378-mediated regulation of aromatase expression appeared to take place at the posttranscriptional level, we further sought to examine whether this miRNA targeted the 3′-UTR of aromatase mRNA. The 3′-UTR was isolated via RT-PCR using granulosa cell-derived RNA and cloned into the multiple cloning site of the p-miR-Report vector (Ambion). This manipulation resulted in the linking of the aromatase 3′-UTR to a CMV-driven luciferase reporter construct, thereby acting as a substitution for the 3′-UTR of the reporter cDNA. As a control vector, a random sequence of similar length containing no binding sites for miR-378 was cloned into the same vector using a similar strategy. Both vectors were transfected into large follicle-derived, miR-378-overexpressing granulosa cells, and luciferase activity was determined 30 h after transfection. As shown in Fig. 4A, luciferase activity decreased by over 60% when the aromatase 3′-UTR was linked to the 3′ end of the luciferase cDNA, suggesting that this region is the target of miR-378, mediating the inhibition of aromatase expression. To further identify the specific sites of miR-378 action within the aromatase 3′-UTR, the three computationally identified putative miR-378 target sites in this region were mutated individually as outlined in Fig. 4, B–D. miR-378-transduced granulosa cells were transfected with either wild-type or each of the three mutated aromatase 3′-UTR luciferase expression constructs, and luciferase activities were subsequently analyzed. As shown in Fig. 4F, consistent with our earlier finding, miR-378 decreased luciferase activities via specific sites within the 3′-UTR of the aromatase transcript. This

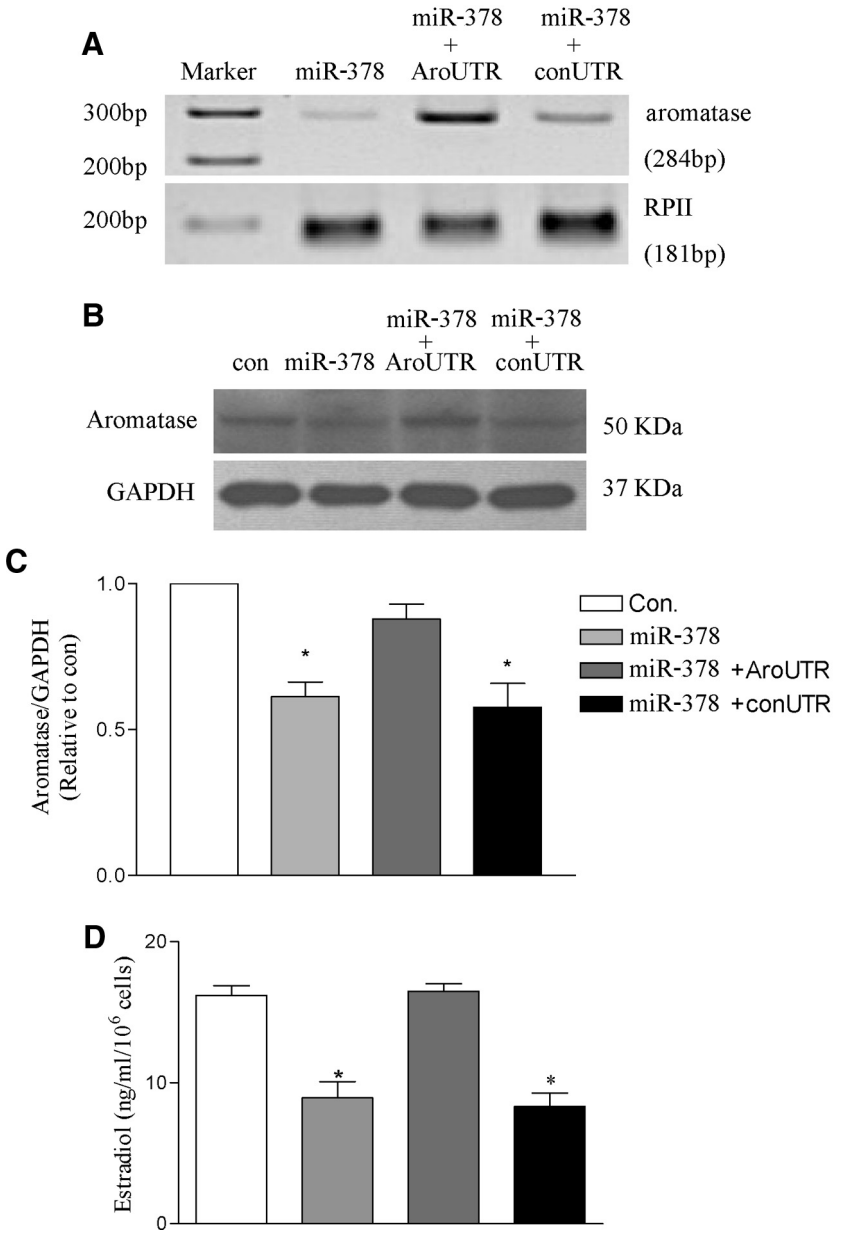

FIG. 5. Overexpression of the aromatase 3′-UTR reverses the repression on aromatase protein expression and estradiol production by miR-378 in ovarian granulosa cells. A, RT-PCR analysis of aromatase 3′-UTR transcript miR-378-lentivirus (miR-378)-transduced granulosa cells after transfection with the indicated constructs; B, representative Western blot of aromatase protein levels in the miR-378-transduced large follicle-derived granulosa cells after transfection with the indicated constructs; C, densitometric analysis revealed that the repression of aromatase expression at the protein level mediated by miR-378-lentiviral transduction was reversed by the overexpression of the aromatase 3′-UTR; D, estradiol levels in control and treatment groups indicated in C. Data represent the mean ± SE of six independent experiments. *, Significant differences compared with controls ($P < 0.05$).

inhibitory effect was abolished when binding sites 1 and 2 were mutated, whereas mutation of site 3 had no effect on the miR-378-mediated suppression of luciferase activities. This suggests that sites 1 and 2 are the targets of miR-378 action on the aromatase 3′-UTR. In addition, we reasoned that if the aromatase 3′-UTR is targeted by miR-378, overexpression of this region alone should result in the binding of miR-378 to the recombinant 3′-UTR. This binding would in turn lead to a decrease in the opportunity for miR-378 to bind to the 3′-UTR of the endogenous aromatase transcript, reversing the repression of miR-378 on endogenous aromatase protein expression. To test this notion, the region of the porcine aromatase 3′-UTR (Ensembl ENSSSCT00000011478, +1525 to +1796) was cloned and inserted 3′ to the CMV promoter, generating a construct that allows overexpression of the aromatase 3′-UTR. The overexpression of the 3′-UTR was confirmed by RT-PCR (Fig. 5A). Overexpression of the aromatase 3′-UTR in miR-378-transduced granulosa cells resulted in a reversal of the repressing effect of miR-378 on aromatase protein levels (Fig. 5, B and C). In contrast, transfection with a control construct in which the aromatase 3′-UTR was replaced with a random sequence without a miR-378 targeting site failed to reverse the miR-378 inhibitory effect on aromatase protein expression, indicating specificity of the aromatase 3′-UTR effect (Fig. 5, B and C). Moreover, the miR-378-mediated suppression of estradiol production in granulosa cells was also reversed by overexpression of the aromatase 3′-UTR, as determined by ELISA (Fig. 5D). These data further verified that the 3′-UTR of aromatase is indeed the target region in which miR-378 represses aromatase expression.

The suppression of miR-378 is reversed by it inhibitor

Anti-miR miRNA inhibitors (Ambion) are designed to bind to and inhibit the activity of endogenous miRNA when introduced into cells. If miR-378 indeed down-regulates aromatase expression, and thus estradiol production in granulosa cells, one would expect the miR-378 inhibitor, anti-miR miR-378, to reverse this effect. As shown in Figure 6, A and B, addition of anti-miR miR-378 resulted in an increase in aromatase protein levels compared with a control oligo AM17010, which contains a random sequence with the same length as the specific inhibitor but does not target any

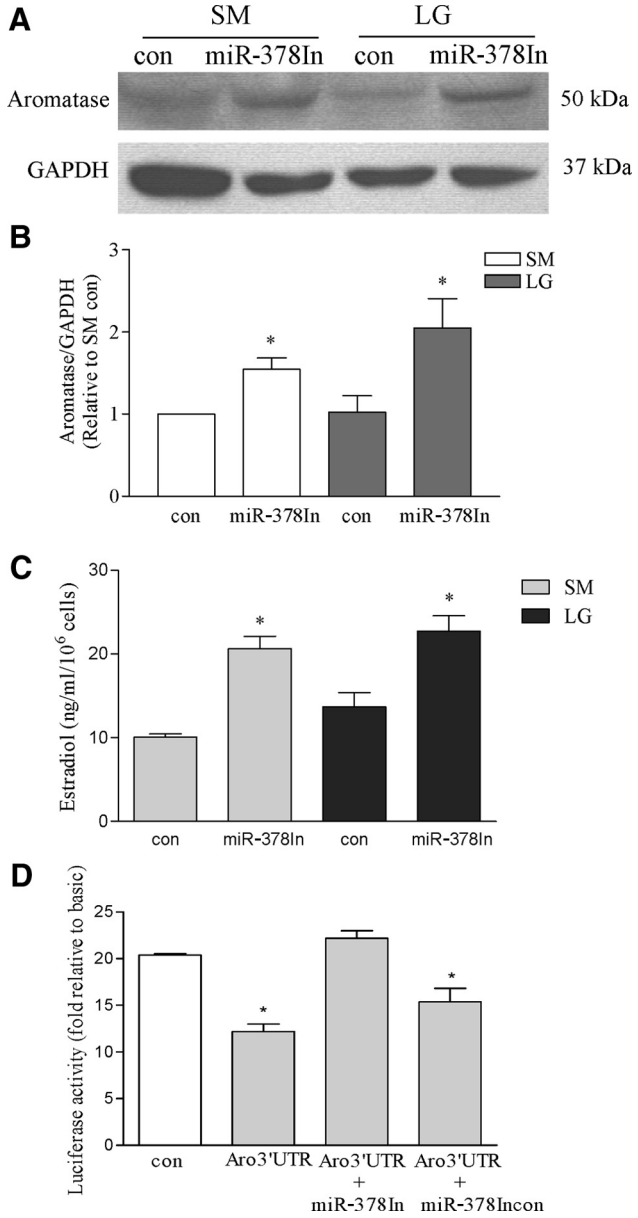

FIG. 6. An miR-378 inhibitor increases aromatase expression and estradiol levels and reverses the repressive effect of miR-378 on aromatase 3′-UTR-linked luciferase activity. A, A representative Western blot of aromatase expression in the presence (miR-378In) and absence (con) of the miR-378 inhibitor in small (SM) and large (LG) follicle-derived granulosa cells; B, densitometry showing the increase in aromatase protein expression mediated by the miR-378 inhibitor; C, changes in estradiol levels in the spent medium collected from SM and LG follicle-derived granulosa cells in the presence (miR-378In) and absence (con) of the miR-378 inhibitor; D, granulosa cells isolated from large follicles were first transduced with lenti-miR-378 and then transfected with either the luciferase-aromatase 3′-UTR-linked vector (aro 3′-UTR) or a control vector (con; luciferase construct in which the 3′-UTR of aromatase was replaced with a random sequence without any miR-378 target sites), followed by luciferase activity assays. Cells transfected with Aro 3′-UTR cultured in the presence (aro3′-UTR+miR-378In) and absence (aro3′-UTR+miR-378Incon) of the miR-378 inhibitor are indicated. Data represent the mean ± SE of four (B), three (C), and four (D) independent experiments. *, Statistical differences from respective controls ($P < 0.05$).

known miRNA. A similar result was also observed for estradiol production by granulosa cells from both small and large follicles (Fig. 6C). This inhibition approach was also used to confirm the specificity of the action of miR-378 on the 3′-UTR of aromatase. As shown in Fig. 6D, consistent with our earlier finding, luciferase activity was decreased when the luciferase reporter 3′-UTR was replaced with the aromatase 3′-UTR. This inhibitory effect was reversed in the presence of the miR-378 inhibitor, anti-miR miR-378.

Discussion

Previous studies have reported on the presence of numerous miRNA in ovarian cells, (33, 34). The potential involvement of miRNA in the control of ovarian function via a gene profiling approach has also been investigated by comparing different miRNA profiles in normal and malignant ovarian cells (35) as well as in mouse granulosa cells before and after human chorionic gonadotropin treatment (33). More recently, the role of miRNA in the regulation of steroidogenesis, apoptosis, and ovarian cancer has also been described (36–39). However, the molecular mechanisms underlying how miRNA are involved in regulating ovarian function remain largely unknown. Our study identified that miR-378 down-regulates ovarian granulosa cell aromatase protein levels and estradiol production via binding within the 3′-UTR of the aromatase transcript. Our finding is supported by the following: 1) overexpression of miR-378 decreased aromatase expression in granulosa cells derived from both small and large follicles; 2) the levels of estradiol were also lower in granulosa cells in which miR-378 was overexpressed; 3) the repressive effects of miR-378 were reversed in the presence of an miR-378 inhibitor; 4) linkage of the aromatase 3′-UTR to the 3′ end of the luciferase reporter cDNA resulted in decreased luciferase activity, as well as decreased aromatase protein levels, in granulosa cells derived from both small and large follicles; 5) mutation of each of two of three computationally predicted miR-378 binding sites in the 3′-UTR of aromatase abolished the inhibitory effect on luciferase activities; and 6) overexpression of the aromatase 3′-UTR alone enhanced aromatase expression and estradiol production, likely via competitive binding to/exhaustion of the endogenous miR-378 pool in granulosa cells.

Ovarian steroid hormones such as estradiol play key roles in ovarian follicular development, oocyte maturation, ovulation, luteogenesis, and embryo development (17, 40–42). In addition, the dysfunction of estradiol is known to be implicated in the development of

polycystic ovary syndrome (43) and estradiol-related cancers (44). Steroidogenesis in most mammals involves the two-cell model, in which androgens are synthesized from cholesterol in theca interna cells, followed by conversion to estradiol in granulosa cells. Estradiol production is the hallmark of preovulatory follicular development, reflecting FSH-induced expression of cytochrome P450 aromatase (CYP19), which converts androgens to estradiol in granulosa cells (45). *In vivo* and *in vitro* evidence derived from both animal models and human studies suggest that estradiol biosynthesis is closely linked to preovulatory follicle development (reviewed in Ref. 40). On the other hand, it has also been reported that oocytes of follicles that have been exposed to excessive levels of estradiol are associated with lower fertilization rates and altered levels of global oocyte methylation (46), indicating the importance of the precise regulation of estradiol on the acquisition of an oocyte's developmental competence. Owing to the importance of aromatase in the production of estradiol, the regulation of aromatase gene expression has been an intense area of research (reviewed in Ref. 18), resulting in a detailed understanding of its transcriptional regulation. To our knowledge, our study is the first to report on the regulation of aromatase expression at the posttranscriptional level. In addition to miR378, computational analysis of the 3′-UTR sequence of aromatase also revealed potential binding target sites for multiple miRNA, including miR-539, let-7/miR-98, miR-140, miR-423, and miR-17. Their possible involvement and how they cooperate with each other to regulate aromatase expression certainly warrant further study.

In addition to the extensive list of stimulators of estradiol production that have already been identified (38, 47–49), the inhibitory role of miR-378 on ovarian aromatase expression may also represent an important physiological mechanism within the ovary that allows precise control over the levels of estradiol. In addition, it is known that over 99% of ovarian follicles undergo atresia, which is associated with decreased levels of estradiol in follicular fluid (50, 51). The suppressive role of miR-378 on estradiol production may also be one of the mechanisms involved in follicular atresia. Interestingly, using a large-scale platform approach, it was recently shown that 51 miRNA have suppressive effects on estradiol production, although their mechanisms of action are unclear (37). Our finding that the levels of miR-378 are higher in granulosa cells derived from small compared with large follicles is consistent with its role in repressing aromatase expression and thus estradiol production during the early stage of follicular development. It is possible that higher levels of miR-378 may be one of the mechanisms that control optimal estradiol levels in the follicular fluid as a means to ensure that oocyte growth/competence is not compromised.

Three putative miR-378 binding sites were identified in the porcine aromatase 3′-UTR using a computer-based miRNA target detection program. These sites are located in proximity to each other, with the first site (site 1) sharing a three-nucleotide overlap with a second site (site 2; Fig. 4E). The finding that mutations in sites 1 and 2 in the aromatase 3′-UTR abolished the inhibitory effect of miR-378 suggest that these sites are the targets of miR-378 that result in posttranscriptional repression. It appears that both of these sites are required for this miRNA to exert its inhibitory effects, because mutation of each site individually resulted in a full reversal of inhibition. It is possible that binding to both sites is required, or alternatively, binding to one site is sufficient, but binding is dependent on the intact configuration of the adjacent sequence at the other site.

miR-378 has previously been reported to be expressed in gastric (52) and adipose (53) tissues. Its demonstrated functions include enhancing cell survival and promoting tumor growth and angiogenesis via targeting the expression of SuFu and Fus-1 (27), regulating nephronectin expression to modulate osteoblast differentiation (54), and repressing the expression of cytochrome P453 (CYP) 2E1 (55). Our results revealed that miR-378 also plays a functional role in the ovary, demonstrating that this miRNA regulates aromatase expression in granulosa cells. Understanding how miRNA control steroidogenesis in the ovary may provide a means to develop potential novel treatments for steroid-related reproductive disorders. For example, targeted delivery of miR-378 (*i.e.* expression driven by a granulosa cell-specific promoter) may be an alternative to the aromatase inhibitors that are currently used for the treatment of breast cancer, which have considerable side effects (56). In addition, the 3′-UTR of aromatase or miR-378 inhibitors may be used as adjuvants for or to replace exogenous estradiol, which currently is used to treat menopausal women as a means to compensate for the loss of sufficient estradiol levels in aging ovaries (57). Our finding that miR-378 targets aromatase in granulosa cells adds important insights into the molecular mechanisms underlying the regulation of ovarian estradiol production.

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