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Short Communication

MIR-378 INDUCES APOPTSIS OF GRANULOSA CELLS DURING FOLLICLE DEVELOPMENT IN CATTLE

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ABSTRACT

MicroRNAs (miRNAs) are small endogenous molecules that are involved in diverse cellular processes. However, little is known about the function of miRNAs in bovine ovary development. Previous study in our lab indicated miR-378 is an apoptosis-associated miRNA that participates in the regulation of bovine corpus luteum development. In addition, whether miR-378 regulates the apoptosis of bovine granulosa cells (bGCs) remains undetermined. Accordingly, this study evaluated the expression of miR-378 in granulosa cells (GCs) of dominant and subordinate follicles and analyzed the apoptosis rates of miR-378 mimics and mimics NC-transfected GCs by flow cytometry method. Results revealed that miR-378 was significantly expressed in the subordinate follicles compared with the dominant follicles, and the apoptosis rate and cleaved caspase-3, a hallmark of apoptosis, increased in the miR-378 mimics-transfected GCs in vitro. This study is the first to report that apoptotic miR-378 plays a regulatory role in bGCs, which will help to find a new way in the research of follicular atresia.

Keywords: miR-378; cattle; granulosa cell; follicle; ovary.

INTRODUCTION

Follicle development in cattle undergoes two or three follicular waves during each estrous cycle, which includes growing follicle recruitment, selection and growth of leading follicles, ovulation of the preovulatory dominant follicle, and degeneration of anovulatory subordinate follicles (Fortune, Sirois, Turzillo, and Lavoir, 1991). During the common growth within a wave, the largest follicle grows as a dominant follicle and becomes ovulatory, whereas all other follicles (subordinate) cease development and undergo atresia. Apoptosis is a major mechanism in the physiological processes of follicular atresia(Hernandez-Coronado *et al*., 2015; Portela *et al*., 2015; Yang, Xu, Zhao, and Li, 2012), which is controlled spatio-temporally at the transcriptional level; however, the mechanism of apoptosis at the post-transcriptional level is poorly understood.

miRNAs are a class of small non-coding endogenous RNA molecules (−22 nt in length) that function at the post-transcriptional level and regulate gene expression in a sequence-dependent manner(Ambros, 2001; Bartel, 2004). miRNAs regulate organism development, cellular differentiation and proliferation, and cell apoptosis and death (Andreas *et al*., 2016; Pan, Toms, Shen, and Li, 2015; Xu, Linher-Melville, Yang, Wu, and Li, 2011).

In cattle, batches of miRNAs are present in the ovary (Hossain *et al*., 2009), and miRNAs demonstrate spatio-temporal expression in oocytes, granulosa cells (GCs), cumulus cells, and preimplantation embryos (Abd El Naby *et al*., 2013). Numerous miRNAs, such as human miR-93(Jiang *et al*., 2015), mouse miR-320(Yin *et al*., 2014) and miR-224(Yao *et al*., 2014), porcine miR-378(Toms, Xu, Pan, Wu, and Li, 2015), miR-92a(da Silveira, Carnevale, Winger, and Bouma, 2014) and miR-34a(Tu *et al*., 2014), have been associated with the apoptosis of GCs.

miRNA transcriptome analysis has been used to identify numerous miRNAs (e.g., GC miRNA) that are differentially expressed during the development of dominant or subordinate bovine follicles (Gebremedhn *et al*., 2015; Sontakke, Mohammed, McNeilly, and Donadeu, 2014). However, little is known about the specific functions of miRNAs in bovine granulosa cells (bGCs).

Our previous study proved that miR-378 serves an important function in the corpus luteum development of bovines(Ma *et al*., 2011), However, whether miR-378 also regulates follicle development remains unknown. Thus, the present study explored the expression of miR-378 in dominant and subordinate follicles, and identified whether miR-378 can induce the apoptosis of bGCs.

MATERIALS AND METHODS

GC and CL collection: Simmental heifers were around 18-month old and 450kg. $PGF_{2\alpha}$ was used to synchronize estrous cycle. Ovaries were obtained from estrous synchronized Simmental heifers and collected by cutting the vaginas. Follicles were isolated from the

ovary by blunt dissection using scissors and forceps. The size of each follicle was measured using a caliper, and each follicle was classified as dominant or subordinate depending on the diameter as previously recommended with minor modifications(Ireland, Murphee, and Coulson, 1980). Follicles ≤11 and ≥12 mm in diameter were classified as subordinate and dominant, respectively (Spicer *et al*., 2011). The oocytes were removed using a mouth-operated micropipette under a microscope, and the GCs were stored at −80 °C.

RNA extraction and RT-PCR: Total RNA was extracted from the GCs of bovine dominant and subordinate follicles using the mirVana™ miRNA Isolation Kit (Ambion). First-strand cDNA was synthesized by the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's protocols. Relative quantification of miR-378 was performed using SYBR® Green Real-time PCR Master Mix (Toyobo: QPK-201) as the standard protocol. U6 small nuclear RNA served as endogenous control. The primers of miRNA-specific RT and Real-time PCR were designated as primer 5 (Table 1).

Table 1. RT-PCR and Real-time PCR primers for miR-378 and U6 detection.

Primer	Sequence (10 pmol/μL)	Annealing Temp (°C)	Product Size (bp)
miR-378	RT: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGTGTG-3′ F: 5′-GGGGTTTGGCAATGGTAGAACT-3′ R: 5′-GGGGTATGGCACTGGTAGAAT-3′	59	66
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ R(RT): 5′-CGCTTCACGAATTTGCGTGTCAT-3′	59	89

Apoptosis analysis of GCs by flow cytometry

All the bGCs was cultrued in DMEM medium with 10% (V/V) Fetal Bovine Serum in 37 °C and 5% CO_2 incubator. The cell was passaged when the confluency reach 90%. All the cells in this reserch was within 5 passages. One day before transfection, 0.5-2×10^5GCs were plated in 12-well culture plate, When the cells reached around 50% confluency, 100μM miR-378 mimics or control from RiboBio (Guangzhou, China) was transfected to bGCs strictly following the Lipofectamine 2000 protocol.After 48 h, the cells were enzymatically digested and washed with PBS. Then, the cells were measured through AnnexinV-FITC/PI double staining using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Pharmingen) in accordance with the manufacturer's instructions. Results were analyzed using cell flow cytometry.

Activity assay of activated caspase-3 of GCs

The transfection of miR-378 mimics or control to the GCs is the same as above. After 48 h, the cells were analyzed using the Caspase 3 Activity Assay Kit (Beyotime, China).

Statistical analysis: All experiments for miR-378 or the control were conducted in triplicates. All data are presented as means ± SE, and statistical evaluation of the data was conducted with SPSS 14.0. Statistical analysis was performed with the independent-sample Student's t-test to compare the two groups. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Expression analysis of miR-378 in GCs: The expression of miR-378 in the bGCs of dominant and subordinate follicles was detected by real-time RT PCR (Figure 1). miR-378 was significantly upregulated in the subordinate follicles than in the dominant follicles ($P < 0.01$). Results demonstrated that miR-378 may be involved in the apoptosis of bovine ovarian follicles.

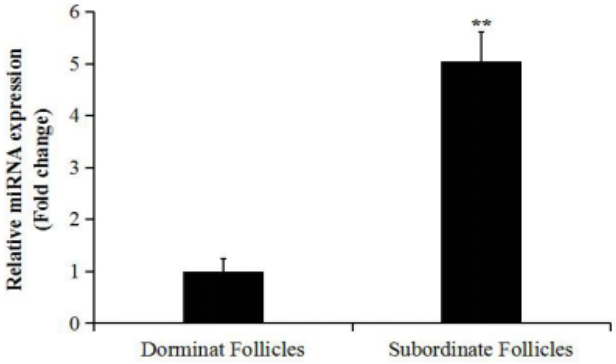

Figure 1.Expression of miR-378 in bovine ovarian granulosa cells. The relative expression levels of miR-378 were measured in the dominant and subordinate follicles using qRT-PCR. U6 was used as a loading control to normalize the expression levels. Data were expressed as mean ± s.d. ($n = 3$). $^{*}P<0.05$, $^{**}P<0.01$.

Effects of miR-378 on GC apoptosis: GCs can decide whether the follicles grow further or undergo apoptosis(Matsuda, Inoue, Manabe, and Ohkura, 2012). To test the potential role of miR-378 in GC apoptosis in the bovine follicles, the isolated bGCs were incubated in vitro and transiently transfected with miR-378 mimics. The apoptotic rate was estimated by Annexin V-FITC/PI double staining. Results indicated that miR-378 mimics remarkably increased the apoptosis rate of bGCs compared with the control ($P < 0.01$, Figure 2), suggesting that miR-378 induced apoptosisin bGCsin vitro. The activated caspase-3 was also estimated by ELISA, and the caspase-3 activity of the GCs was higher in the miR-378 mimics-transfected group than in the control group ($P < 0.05$, Figure 3).

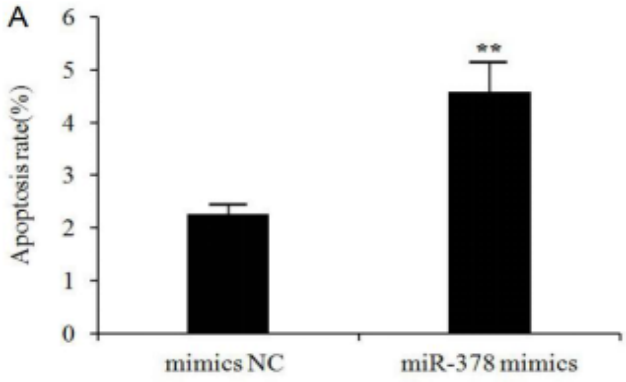

Figure 2.Apoptosis rate of bGCs transfected with miR-378 mimics or mimics NC.(A) bGC apoptosis level after miR-378 mimics or mimics NC treatment was determined by fluorescence activated cell sorting (FACS) 48 h after transfection. (B) bGC apoptosis level after transfection with miR-378 mimics or mimics NC. Data are presented as the mean ± s.d. (n = 3). $^{*}P<0.05$, $^{**}P<0.01$.

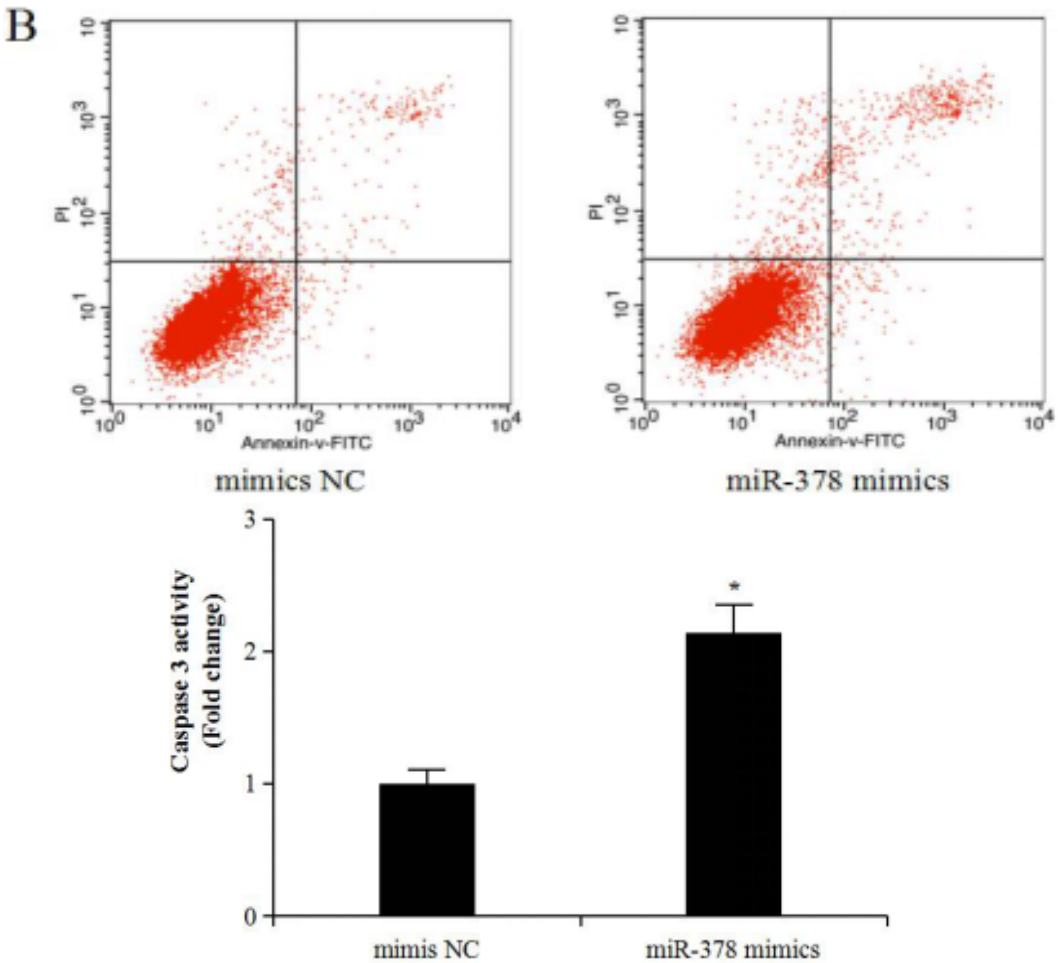

Figure 3.Caspase-3 activity of bGCs transfected with miR-378 mimics or mimics NC. After the bGCs were transfected with miR-378 mimics or mimics NC, the activity of caspase-3 was detected by ELISA. Data are presented as mean ± s.d. (n = 3). $^{*}P<0.05$, $^{**}P<0.01$.

DISCUSSION

In the present study, the effect of miR-378 on the regulation of bGC apoptosis was investigated.The expression level of miR-378 significantly increased in the subordinate follicle GCs than in the dominants. Flow cytometry was used to evaluate the in vitro effect of miR-378 on the apoptosis of bovine ovarian GCs. Results indicated that the upregulation of miR-378 in bGCs in vitro increased the rate of apoptosis in bGCs, suggesting that miR-378 may induce the apoptosis of bGCs.

miRNAs post-transcriptionally mediate ovary gene expression in farm animals (Gebremedhn *et al*., 2015). Using Illumina miRNA deep sequencing, Gebremedhn *et al*. identified 30 miRNAs (e.g., miR-378) that are abundantly expressed in the bGCs of dominant follicles(Gebremedhn *et al*., 2015), which agrees with the present results. These findings indicate that numerous miRNAs play crucial roles in the regulation of folliculogenesis.In cultured primary porcine GCs, miR-378 overexpression significantly decreased the protein and mRNA levels of PGR. This observation is the first evidence showing that miR-378 functions in porcine ovary and post-transcriptionally regulates the gene expression of progesterone receptor (Toms *et al*., 2015). In pigs, miR-378 knockdown increased cumulus expansion and oocyte progression to MII and suppressed aromatase (CYP19A1) expression in cumulus cells. Estradiol can reverse the function of miR-378, indicating that this miRNA may decrease estradiol production by suppressing the gene expression of aromatase to regulate the maturation of cumulus-oocyte complexes(Pan *et al*., 2015; Xu *et al*., 2011). Using the TargetScan online tool (www.targetscan.org), 185 transcripts with conserved sites, containing a total of 187 conserved sites and 85 poorly conserved sites were predicted the target gene of miR-378. Within these genes, miR-378 may interact with apoptosis-related gene, like bone morphogenetic protein 2 (BMP2)(Liu *et al*., 2013), to regulate the cell fate of bGCs. Thus, the target of miR-378 must be identified to explore in depth the functions of miR-378.

In conclusion, miR-378 can induce the apoptosis of bovine ovarian GCs. This study provided insights into the molecular mechanisms of apoptotic miR-378-mediated follicular development.

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