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A miRNA target network putatively involved in follicular atresia

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ABSTRACT

In a previous microarray study, we identified a subset of micro RNAS (miRNAs), which expression was distinctly higher in atretic than healthy follicles of cattle. In the present study, we investigated the involvement of those miRNAs in granulosa and theca cells during atresia. Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) confirmed that miR-21-5p/-3p, miR-150, miR-409a, miR-142-5p, miR-378, miR-222, miR-155, and miR-199a-5p were expressed at higher levels in atretic than healthy follicles (9–17 mm, classified based on steroidogenic capacity). All miRNAs except miR-21-3p and miR-378 were expressed at higher levels in theca than granulosa cells. The expression of 13 predicted miRNA targets was determined in follicular cells by RT-qPCR, revealing downregulation of *HIF1A*, *ETS1*, *JAG1*, *VEGFA*, and *MSH2* in either or both cell types during atresia. Based on increases in miRNA levels simultaneous with decreases in target levels in follicular cells, several predicted miRNA target interactions were confirmed that are putatively involved in follicular atresia, namely miR-199a-5p/miR-155-*HIF1A* in granulosa cells, miR-155/miR-222-*ETS1* in theca cells, miR-199a-5p-*JAG1* in theca cells, miR-199a-5p/miR-150/miR-378-*VEGFA* in granulosa and theca cells, and miR-155-*MSH2* in theca cells. These results offer novel insight on the involvement of miRNAs in follicle development by identifying a miRNA target network that is putatively involved in follicle atresia.

1. Introduction

The overwhelming majority of follicles recruited from the primordial pool during a female's reproductive life will undergo atresia before they can reach the ovulatory stage. Atresia is an active process involving not only cell death but also resorption of follicular tissue and its replacement by stromal and new follicular tissue; these processes involve infiltration by immune and other cells, very much resembling wound healing [1]. Among several key regulators of wound healing and tissue remodeling processes across body tissues are microRNAs (miRNAs) [2].

The involvement of miRNAs in different aspects of follicle development has been demonstrated in numerous studies [3,4]. Much of the existing evidence has been obtained using follicular cell cultures, mostly granulosa cells. Often reported effects of miRNAs include either the promotion or suppression of granulosa cell apoptosis [5–10]. Yet, in many cases, the site of expression, if any, of these miRNAs within follicles (ie, granulosa and theca compartments) or whether their expression actually changes during follicle atresia, supporting their physiological role, has not been clarified. Moreover, although the posttranscriptional effects of miRNAs in tissues often involve targeting of a common gene simultaneously by several miRNAs and, at the same time, a single miRNA can simultaneously target multiple genes, previous functional studies in follicles have often used a one miRNA–one target approach thus providing limited information on the wider biological effects of miRNAs expressed simultaneously acting in coordination.

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In a previous study, we used microarray to profile miRNA expression across a wide range of antral follicle development stages in cattle [11], a species which follicular physiology closely resembles the human, particularly when compared with rodents; by comparing miRNA profiles between steroidogenic-active and steroidogenic-inactive follicles, we identified a subset of miRNAs that are putatively involved in the growth of healthy dominant follicles. In the present study, we focused our attention on those miRNAs identified as upregulated during follicle atresia in our previous study. Specifically, we established and compared the expression of miRNAs and their putative targets within the follicular granulosa and theca compartments to gain insight into their involvement in follicle atresia.

2. Materials and methods

2.1. Collection and processing of bovine tissues

Follicles from ovaries of cycling beef cattle obtained at an abattoir were collected as part of a separate study [11]. Individual follicles 9–17 mm in diameter were dissected out, and the follicular fluid aspirated and centrifuged at 800 *g* for 10 min. The resulting supernatant was stored at −80°C until further analyses, and the cell pellet was combined with the follicular wall free of surrounding stroma and snap-frozen in liquid nitrogen until RNA extraction. Alternatively, after hemidissection, follicles were gently scraped with blunt-ended forceps to collect granulosa and theca wall compartments. Theca walls were washed repeatedly to remove any residual granulosa cells. Theca and granulosa cells from each individual follicle were then separately snapped frozen in liquid nitrogen.

Intrafollicular concentrations of estradiol and progesterone were measured using competitive double antibody radioimmunoassay kits (Siemens Healthcare Diagnostics Inc) following the manufacturer's instructions. All assays were validated in our laboratory by showing parallelism between serial sample dilutions and the provided assay standard curve. Sensitivity of the assays was 0.56 ng/mL and 0.01 ng/mL, and the intra-assay coefficient of variations were 6% and 4.3% for estradiol and progesterone, respectively.

2.2. RT-qPCR

Total RNA was extracted using the miRNeasy Mini kit (Qiagen, UK) and reverse-transcribed using the miScript II RT kit (Qiagen), as described [11]. Messenger RNA levels were quantified using the SensiFAST SYBR Lo-ROX Kit (Bioline Reagents Ltd, UK) and bovine-specific primers (Table 1). For miRNA quantification, the miScript SYBR Green PCR kit and miScript Primer Assays (Qiagen) were used. All PCRs were run on a MX3005P QPCR system (Stratagene, CA) using a standard curve to calculate copy numbers from Cq values [11]. Messenger RNA data were normalized using *18S* values within each sample, and miRNA data were normalized using endogenous *RnU6-2*. Mean intra-assay coefficient of variations for miRNA and mRNA qPCRs were 9.5% and 11.3%, respectively.

Table 1
Primer sequences used in messenger RNA analyses.

Gene	Sequence (5′-3′) sense/antisense
18S	GCTGGCACCAGACTTG/GGGGAATCAGGGTTCG
CYP19A1	CGCAAAGCCTTAGAGGATGA/ACCATGGTGATGTACTTTCC
E2F2	TCGCTATGACACATCGCTGG/CGTCACGTAGGCCAGTCTCT
ETS1	CACAGTCTCTCCGGCAAAGT/GTGGATGATAGGCCGACTGG
HIF1A	CAGAAGAACTTTTGGGCCGC/TCCACCTCTTTTGGCAAGCA
IGF1	AGTGCTGCTTTTGTGATTTCTTGA/ GCACACGAACTGGAGAGCAT
IGF1R	AAGCTGAGAAGCAGGCAGAG/CGGAGGTTGGAGATGACAGT
JAG1	GAGTGTGAGTGTTCTCCGGG/TTGGCCTCGCATTCATTTGC
LHCGR	GGACTCTAGCCCGTAGG/ACACATAACCACCATACCAAG
MSH2	TGGGCAGAAGTGTCCATTGT/CCCACGCTAATCCAAACCCA
MYD88	AAGTTGTGCGTGTCTG/GGAAATCACATTCCTTGCT
PAPPA	TTGCTGCGCTTCTACAGTGA/GCACAGTCACCCTGTAGGTC
RECK	GTGCTTCCTTCTCTTGTCTGGA/GGCTTGACAGTATTCTCGGC
SIRT1	GCTTACAGGGCCTATCCAGG/TATGGACCTATCCGAGGTCTTG
TIMP3	GGATTCACCAAGATGCCCCA/GAGCTGGTCCCACCTCTCTA
VEGFA	TGTAATGACGAAAGTCTGGAG/TCACCGCCTCGGCTTGTCACA

2.3. Micro RNA target identification

Identification of putative miRNA targets was done using miRTarBase release 6.0 and TargetScan release 7.0 to select targets experimentally validated in human and/or rodents (by reporter assay, Western blot and/or RT-qPCR, as detailed in http://mirtarbase.mbc.nctu.edu.tw/) and computationally predicted targets within the bovine genome (http://www.targetscan.org), respectively. For convenience, each identified miRNA target interaction was classified as high-, medium-, or low-confidence based on whether it was present in both miRTarBase and TargetScan, miRTarBase only or TargetScan only.

2.4. Statistical analyses

Robust regression and outlier removal (ROUT) test was applied to datasets, and outlier values ($P < 0.01$) were excluded from subsequent analyses. Gene expression data were assessed for normality using the D'Agostino and Pearson normality test and were log-transformed before statistical analysis where necessary. Two-way analysis of variance followed by unpaired *t*-tests to identify differences in gene expression between healthy and atretic follicles within each cell type were used. Significance was considered at $P < 0.05$, whereas differences with P values <0.1 were considered to approach significance. Nomenclature according to miRBase release 21 is used throughout the manuscript. All miRNAs referred to are bovine (bta-) except otherwise specified.

3. Results

3.1. Micro RNA expression analyses in follicular tissues

In a previous study in cattle [11], microarray analyses yielded a total of 11 unique bovine sequences, which were expressed in greater abundance (>1.5-fold) in atretic than in healthy preovulatory size follicles (Table 2). The status of the follicles analyzed in that study had been predetermined on the basis of steroidogenic capacity and