Characterization of microRNAs differentially expressed during bovine follicle development

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Abstract

Several different miRNAs have been proposed to regulate ovarian follicle function; however, very limited information exists on the spatiotemporal patterns of miRNA expression during follicle development. The objective of this study was to identify, using microarray, miRNA profiles associated with growth and regression of dominant-size follicles in the bovine monovular ovary and to characterize their spatiotemporal distribution during development. The follicles were collected from abattoir ovaries and classified as small (4–8 mm) or large (12–17 mm); the latter were further classified as healthy or atretic based on estradiol and *CYP19A1* levels. Six pools of small follicles and individual large healthy ($n=6$) and large atretic ($n=5$) follicles were analyzed using Exiqon's miRCURY LNA microRNA Array 6th gen, followed by qPCR validation. A total of 17 and 57 sequences were differentially expressed (greater than or equal to twofold; $P<0.05$) between large healthy and each of small and large atretic follicles respectively. Bovine miRNAs confirmed to be upregulated in large healthy follicles relative to small follicles (bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873) were further characterized. Three of these miRNAs (bta-miR-144, bta-miR-202, and bta-miR-873) were also downregulated in large atretic follicles relative to large healthy follicles. Within the follicle, these miRNAs were predominantly expressed in mural granulosa cells. Further, body-wide screening revealed that bta-miR-202, but not other miRNAs, was expressed exclusively in the gonads. Finally, a total of 1359 predicted targets of the five miRNAs enriched in large healthy follicles were identified, which mapped to signaling pathways involved in follicular cell proliferation, steroidogenesis, prevention of premature luteinization, and oocyte maturation.

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Introduction

In monovular species, including humans, cattle and horses, waves of follicular growth occur periodically in the ovary in response to follicle-stimulating hormone (FSH) throughout a female's reproductive life (Donadeu & Pedersen 2008). An initial phase of common growth within a wave is followed by the selection of a single follicle, usually the largest (occasionally the two largest), which continues to grow as a dominant follicle while all other follicles (subordinate) cease development and undergo atresia. Subsequent maturation of the dominant follicle may be followed a few days later by ovulation and transformation of the follicle remnants into a corpus luteum. Each of these developmental steps involves sequential and profound changes in tissue structure and function, requiring finely tuned and coordinated changes in the expression of thousands of genes. Transcriptome analyses have identified many such genes during follicle selection, maturation, and the follicle–luteal transition (Gilbert *et al.* 2011, Rao *et al.* 2011, Christenson *et al.* 2013), although relatively

little is still known about the molecular mechanisms regulating their expression, particularly at the post-transcriptional level.

miRNAs are key post-transcriptional regulators during cell growth and differentiation (Huntzinger & Izaurralde 2011). Several studies have already reported effects of specific miRNAs on different aspects of granulosa cell function (Donadeu *et al.* 2012), including steroidogenesis (Yao *et al.* 2010, Xu *et al.* 2011, Yin *et al.* 2012, Dai *et al.* 2013), proliferation (Yao *et al.* 2010, Yan *et al.* 2012, Dai *et al.* 2013, Zhang *et al.* 2013), survival (Carletti *et al.* 2010, Lin *et al.* 2012, Yang *et al.* 2012), terminal differentiation (Kitahara *et al.* 2013), and cumulus expansion (Yao *et al.* 2014). With a few exceptions (Carletti *et al.* 2010, Kitahara *et al.* 2013), most evidence on follicular roles of miRNAs has been obtained using cultured cells, particularly rodent cells, and in some cases actual changes in the expression of these miRNAs during follicle development have not been demonstrated. Indeed, although genome-wide miRNA analyses of whole ovarian tissues (Landgraf *et al.* 2007, Ro *et al.* 2007, Mishima *et al.* 2008, Hossain *et al.* 2009,

Ahn *et al.* 2010, Juanchich *et al.* 2013) or follicular, or luteal tissues (Fiedler *et al.* 2008, Tesfaye *et al.* 2009, McBride *et al.* 2012, Miles *et al.* 2012, da Silveira *et al.* 2012, Donadeu & Schauer 2013, Schauer *et al.* 2013, Soheli *et al.* 2013) have been reported in several species, detailed spatiotemporal profiles encompassing several follicle developmental stages have not. Identifying such profiles will be an important step toward understanding the functional involvement of miRNAs during specific stages of follicle development.

With this in mind, this study aimed to use microarray analyses to identify miRNA profiles associated with the growth and atresia of dominant-size follicles in bovine and to further characterize the identified miRNAs by establishing their spatiotemporal patterns during development, as well as identify the follicular cell pathways putatively targeted by these miRNAs.

Materials and methods

Collection and processing of bovine tissues

All tissues were collected from beef cattle at an abattoir and transported on ice to the laboratory. After rinsing with 70% ethanol, ovarian pairs containing a visible corpus luteum (Ireland *et al.* 1980) were selected for dissection. Individual follicles measuring 3–17 mm in diameter were carefully dissected out and their diameter determined in two different planes using a Vernier caliper. Each follicle was then hemisected and the follicular fluid collected 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 snapped frozen in liquid nitrogen until RNA extraction. In some experiments, after hemi-dissection follicles were gently scraped with blunt-ended forceps to separately collect granulosa cell and theca wall compartments. Theca walls were washed repeatedly to remove any residual granulosa cells. Whenever needed, mural granulosa cells and cumulus–oocyte complexes (COCs) were collected separately from follicle pools using a 75 µm filter (Fisher Scientific, Loughborough, UK). Follicular components were then separately snapped frozen in liquid nitrogen.

Intrafollicular concentrations of estradiol (E_2) and progesterone were measured using the competitive double antibody RIA kits (Siemens Healthcare Diagnostics, Inc., Camberley, UK) following manufacturer's instructions. All assays were validated in our laboratory by showing parallelism between serial sample dilutions and the provided assay standard curve (range, 5–500 pg/ml E_2 and 100–4000 pg/ml progesterone). Sensitivity of the assays was 0.87 and 10 pg/ml, and the intra-assay coefficient of variations were 6 and 4.3% for E_2 and progesterone respectively.

RNA extraction

Total RNA was extracted from snap-frozen bovine tissues using the miRNeasy Mini Kit (Qiagen, Manchester, UK) following the

manufacturer's instructions and previous homogenization with ceramic beads (Lysing Matrix D, MP Biomedicals, Santa Ana, CA, USA) using FastPrep FP120 Cell disruptor (MP Biomedicals). Total RNA was quantified by the NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its quality was determined on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Wokingham, UK).

Follicular fluid was processed following Exiqon's guidelines for plasma RNA purification using the miRNeasy Mini Kit. In brief, 250 µl of follicular fluid were centrifuged at 9400 *g* for 5 min at 4 °C to pellet down cellular contents. Two hundred microliter of clear supernatant were taken and mixed with 750 µl of QIAzol lysis buffer containing MS2 carrier RNA (Roche Diagnostics Ltd, Burgess Hill, UK), and the mixture was vortexed vigorously for a minute. Following incubation for 5 min at room temperature, each sample was spiked-in with 5 fmol of synthetic cel-miR-39 (Qiagen) and RNA extracted as per manufacturer's instructions (Qiagen).

Microarray analyses

Microarray analyses were carried out on pools of follicles measuring 4–8 mm in diameter (small follicles; six pools, three to five follicles of different diameters per pool) and individual dominant-size follicles (12–17 mm) that had been previously classified as large healthy ($n=6$) or large atretic ($n=5$) based on E_2 and *CYP19A1* levels (Fig. 1). The microarray procedure was carried out at Exiqon Vedbaek, Denmark. In brief, 600 ng total RNA from each sample were labeled with Hy3 fluorescent label using the miRCURY LNA microRNA Hi-Power Labeling Kit, Hy3/Hy5 (Exiqon) as per the manufacturer's instructions. A pool of samples was labeled with Hy5 and used as reference sample. The Hy3-labeled samples and Hy5-labeled reference RNA sample were mixed pairwise and hybridized to the miRCURY LNA microRNA Array 6th gen (Exiqon), which contained 1488 capture probes targeting all microRNAs for human, mouse, or rat registered in miRBase 16.0. The hybridization was carried out according to the miRCURY LNA microRNA Array Instruction manual using a Tecan HS4800 hybridization station (Tecan, Grödig, Austria). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System and the image analysis was carried out using the ImaGene 9 (miRCURY LNA microRNA Array Analysis Software, Exiqon). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global Lowess regression algorithm.

The background threshold was calculated for each individual microarray slide as 1.2 times the 25th percentile of the overall signal intensity of the slide. MicroRNAs with intensities above threshold in <20% (or 2) of the samples were removed from the final dataset used for the expression analysis. The number of present calls obtained (between 460 and 540) was highly comparable among samples.

For each probe and sample, an *M*-value was calculated as the log₂ median of the ratio between Hy3 and Hy5 signals for

Figure 1 Mean ($\pm$ S.E.M.) follicular fluid levels of estradiol (E₂) and progesterone, and *CYP19A1* transcript levels in whole follicle tissues from cattle ($n=5-11$ samples/diameter category). There was a significant effect of follicle diameter for E₂ ($P<0.0001$), progesterone ($P=0.02$), and *CYP19A1* ($P<0.0001$). Means with different superscripts (a,b,c) are different ($P<0.05$).

each of the four spots present on a slide. These values were used to compare the expression of each miRNA probe between large healthy and small follicles and between large healthy and large atretic follicles using Student's *t*-test with Benjamini and Hochberg false discovery rate adjustment ($P<0.05$).

RT-qPCR

Total RNA obtained from tissues (500 ng) or cell-free follicular fluid (6 μ l) was analyzed with miScript II RT and miScript SYBR Green PCR Kits using miScript Primer assays, as per manufacturer's instructions (Qiagen). The MX3005P QPCR System (Stratagene, La Jolla, CA, USA) was used, and a dissociation curve was included at the end of each run to confirm the presence of a single product.

Relative miRNA abundance was obtained using MX3005P Software by extrapolation of Ct values from a standard curve prepared from a pool of tissue samples and run simultaneously. Endogenous *RnU6-2* or, in the case of follicular fluid, spiked-in cel-miR-39 was used for normalization of miRNA data.

Relative quantification of mRNA levels was carried out on the same cDNA used for miRNA analyses. Bovine-specific primers against *CYP19A1* (CGCAAAGCCTTAGAGGATGA and ACCATGGTGATGTACTTTCC), *FSHR* (TGTGCATTCA-ATGGAACCCAACTCG and TGACCGGTCCAGAGGCTCCC), *CYP17A1* (GGCGACCTTACCATTG and AGATGAGTT-GCGTCCC), *SPRED1* (ACGAAGCCAAGCCAATCAGA and CCTCCACTTTCCTTGGGCAT), *TGFBR2* (GAAGTCCTGCTG-GAGCAACT and AGGCAATCTTGGGGTCATGG), *ATG7* (GGCCTCTCCAGATTGCAGTT and GCTTCGTCTAGCC-GGTACTC), *CREB1* (AATCCTAGTGCCCAGCAACC and CGCTGTGCGAATCTGGTATG), and *LHCGR* (GGACTCTAG-CCCGTAGG and ACACATAACCACCATACCAAG) were used with the SensiFAST SYBR Lo-ROX Kit (Bioline Reagents Ltd, London, UK) on the MX3005P QPCR system, as per the manufacturer's instructions. Relative miRNA abundance was calculated as indicated earlier and data were normalized using 18S values within each sample.

Dixon's Outlier test was applied to both qPCR and immunoassay data sets, and outlier values ($P<0.01$) were excluded from subsequent analyses. Non-normal data were log-transformed before analyses. Data were analyzed using the general linear model procedure by one- or two-way ANOVA followed by Tukey's pairwise comparison tests. Data sets involving only two experimental groups were analyzed using Student's *t*-tests. Spearman's rank correlation test was used to determine the relationship between miRNA levels in whole follicle tissues and follicular fluid. In all cases, statistical significance was considered at $P<0.05$.

In situ hybridization

The tissues were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 4 °C. After deparaffinization and rehydration, ovarian sections (5 μ m) were denatured with 5 μ g/ml proteinase K in 75 ml PBS, fixed in 4% PFA for 10 min and rinsed with 0.2% glycine in PBS. The ovarian sections were incubated with freshly prepared imidazole buffer (VWR International Ltd, Lutterworth, UK) and then placed in a humidified chamber. Freshly prepared 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich) was added to each slide for 1 h at room temperature, followed by 2 h of prehybridization with 50% formamide and 5 $\times$ SSC buffer at 35 °C. The sections were then incubated overnight with double digoxigenin-labeled LNA-modified oligonucleotide probes against bta-miR-202 (80 nM), *RnU6-2* (3 nM), or scrambled RNA (40 nM) (Exiqon) in a hybridization buffer solution (50% formamide, 5 $\times$ SSC, 10% Dextran sulfate salt) in a humidified chamber. After application of the probe, slides were covered with gel bond film and heated at 60 °C for 5 min. After overnight incubation in a humidifying chamber at 35 °C, the slides were washed with 2% BSA and 0.2 $\times$ SSC post hybridization buffer for 10 min three times at 4 °C to avoid unspecific binding, followed by

Figure 2 Principal component analyses plot performed on all follicular samples used for microarray, and on the top 50 microRNAs with the highest s.d. The normalized log ratio values were used for the analysis. The features were shifted to be zero centered, (i.e. the mean value across samples was shifted to zero) and scaled to have unit variance (i.e. the variance across samples was scaled to one before the analysis).

rinsing with $1 \times$ TBS. The slides were then incubated with 2% sheep serum and 1% BSA for 1 h at room temperature followed by incubation with anti-digoxigenin antibody (1:200; Roche) for 2 h at room temperature, and color development with NBT/BCIP (Roche) at 4 °C for up to 16 h. The samples were observed under light microscope at $20\times$ (Leica 800). Three independent analyses were carried out using different ovarian sections.

Granulosa cell culture

Granulosa cells collected from follicles measuring 4–8 mm were passed through a filter mesh (70 μ m; Fisher Scientific) to separate out COCs. The cells were then treated with red blood cell lysis buffer (8.3 gm/l NH₄Cl in 0.01 M Tris–HCl buffer of pH 7.5), washed with fresh McCoy's 5a medium, and cultured in 96-well plates (Fisher Scientific) with modified McCoy's 5a medium containing 0.02 M HEPES, 3 mM L-glutamine, 0.1% BSA, and 1% Pen–Strep, and supplemented with 10 ng/ml bovine insulin, 2.5 mg/l transferrin, 4 μ g/l sodium selenite, and 100 nM androstenedione (Gutierrez *et al.* 1997) in a humidified atmosphere at 37 °C with 5% CO₂. Twenty-four hours after plating, the cells were left untreated or were treated with Forskolin (10 mM), bovine insulin (1 mg/ml), and FCS (1%, v/v) for up to 6 days, with media replacement every 2 days. On different days during culture, the cells were harvested and processed for quantification of miRNA levels as described earlier. Three different experiments with cultured granulosa cells were carried out.

Results

A total of 94 antral follicles were collected and analyzed. As expected, the levels of E₂ and *CYP19A1* transcript were higher in follicles ≥ 9 mm (dominant-size follicles classified as healthy) than in follicles 3–8 mm in diameter (Fig. 1). E₂ and *CYP19A1* levels remained low in a subset of ≥ 9 mm follicles, classified as atretic, which also had relatively high mean levels of progesterone.

Microarray analyses

A total of 545 probes yielded hybridization intensities above the background threshold across the follicular samples analyzed. These corresponded to 523 unique miRNAs including 191 sequences registered as bovine in miRBase release 18. The results of comparative analyses of follicular groups (large healthy vs small and large healthy vs large atretic) are shown in Supplementary Table 1, see section on supplementary data given at the end of this article (raw microarray data have been deposited in the NCBI's GEO repository, GSE54692). Principal Component Analysis revealed clustering of samples largely according to follicle class (Fig. 2). A total of 17 and 57 miRNA sequences were differentially expressed (greater than or equal to twofold; adjusted *P* value < 0.05) between large healthy and small follicles and between large healthy and large atretic follicles respectively (Tables 1 and 2). Out of these, eight and 20 miRNAs, respectively, corresponded to bovine sequences registered in miRBase. A total of seven miRNAs were upregulated in large healthy follicles relative to small follicles; these miRNAs were also expressed at higher levels in large healthy than in large atretic follicles. Moreover, out of a total of ten miRNAs

Table 1 miRNAs differentially expressed (greater than or equal to twofold) between small and large healthy follicles in bovine.

miRNA	Fold change	Adj. <i>P</i> value ^a
Downregulated in large healthy follicle		
hsa-miR-3178	3.60	0.002
hsa-miR-1246/bta-miR-1246	2.97	0.027
hsa-miR-1290	2.82	0.035
hsa-miR-1275	2.29	0.020
hsa-miR-625-3p	2.21	0.006
hsa-miR-3621	2.17	0.002
hsa-miR-483-3p/bta-miR-483	2.17	0.002
hsa-miR-1469	2.15	0.014
hsa-miR-498	2.14	0.003
hsa-miR-4279	2.05	0.012
Upregulated in large healthy follicle		
bta-miR-202	4.18	0.002
hsa-miR-876-5p/bta-miR-876	3.79	0.002
hsa-miR-876-3p	3.09	0.002
hsa-miR-873-5p/bta-miR-873	2.70	0.003
hsa-miR-451a/bta-miR-451	2.65	0.014
hsa-miR-144-3p/bta-miR-144	2.35	0.009
hsa-miR-652-3p/bta-miR-652	2.13	0.003

^aFDR; Benjamini and Hochberg adjustment.

downregulated in large healthy follicles relative to small follicles, eight were also downregulated relative to large atretic follicles. Of all sequences upregulated between

Table 2 miRNAs differentially expressed (greater than or equal to twofold) between large atretic follicles and large healthy follicles in bovine.

miRNA	Fold change	Adj. P value ^a
Downregulated in large healthy follicle		
hsa-miR-659-3p	6.05	0.000
hsa-miR-4285	5.69	0.001
hsa-miR-1469	5.11	0.001
hsa-miR-625-3p	4.94	0.000
hsa-miR-642b-3p	4.62	0.004
hsa-miR-765	4.11	0.001
hsa-miR-675-5p	4.08	0.000
hsa-miR-422a	3.89	0.000
hsa-miR-498	3.69	0.000
hsa-miR-665	3.66	0.003
hsa-miR-483-3p/bta-miR-483	3.64	0.001
hsa-miR-1909-3p	3.61	0.001
hsa-miR-3178	3.36	0.001
hsa-miR-149-3p	3.12	0.001
hsa-miR-3685	3.11	0.006
bta-miR-21*	3.09	0.002
hsa-miR-2116-5p	3.09	0.001
hsa-miR-4279	2.96	0.001
hsa-miR-551b-5p	2.85	0.000
hsa-miR-1236	2.83	0.000
hsa-miR-4258	2.78	0.000
hsa-miR-373-5p	2.74	0.000
hsa-miR-1915-3p	2.71	0.000
hsa-miR-4292	2.58	0.001
hsa-miR-150-5p/bta-miR-150	2.54	0.001
hsa-miR-371a-5p	2.50	0.001
bta-miR-21	2.39	0.001
hsa-miR-204-3p	2.36	0.000
bta-miR-409a	2.36	0.000
hsa-miR-744-5p/bta-miR-744	2.36	0.002
hsa-miR-4306	2.34	0.002
hsa-miR-1275	2.28	0.003
hsa-miR-361-3p	2.27	0.001
hsa-miR-3621	2.21	0.001
hsa-miR-638	2.18	0.000
hsa-miR-4268	2.17	0.001
hsa-miR-3676-3p	2.15	0.000
hsa-miR-2115-3p	2.09	0.002
hsa-miR-142-5p/bta-miR-142	2.03	0.001
Upregulated in large healthy follicle		
bta-miR-202	9.98	0.000
hsa-miR-876-5p/bta-miR-876	5.99	0.000
hsa-miR-31-3p	4.90	0.001
hsa-miR-31-5p/bta-miR-31	3.94	0.001
hsa-miR-876-3p	3.92	0.000
hsa-miR-873-5p/bta-miR-873	3.53	0.000
hsa-miR-652-3p/bta-miR-652	3.50	0.000
hsa-miR-450b-5p	3.17	0.012
hsa-miR-210/bta-miR-210	2.98	0.002
hsa-miR-451a/bta-miR-451	2.49	0.031
bta-miR-34b	2.22	0.028
hsa-miR-190a/bta-miR-190a	2.10	0.004
hsa-miR-15b-5p/bta-miR-15b	2.10	0.006
hsa-miR-424-5p	2.07	0.033
hsa-miR-4275	2.06	0.001
hsa-miR-33a-5p/bta-miR-33a	2.04	0.005
hsa-miR-144-3p/bta-miR-144	2.02	0.032
hsa-miR-487b/bta-miR-487b	2.01	0.003

^aFDR; Benjamini and Hochberg adjustment.

large healthy and small follicles all but one corresponded to bona-fide bovine miRNAs (bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, bta-miR-873, and bta-miR-876) and thus this set of miRNAs (Table 3) was chosen for further characterization.

Validation of microarray data by qPCR

Confirmatory qPCR analyses were carried out across a wide range of developmental stages to more precisely identify the follicle diameter categories involved in the differences in miRNA expression observed by microarray. These analyses revealed an increase in the expression of bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873 in large healthy relative to small follicles (Fig. 3A), in agreement with the results of microarray analyses. A significant increase in the levels of these miRNAs occurred at the 12–14 mm stage or later, except for miR-873 for which expression levels increased above fourfold between 6–8 and 9–11 mm follicles. A decrease in expression levels between large healthy and large atretic follicles involving one or more diameter categories for each of these follicle types was confirmed for three of the five miRNAs analyzed (miR-144, miR-202, and miR-873). The expression of bta-miR-876 was presumably too low in bovine follicles and could not be detected by qPCR.

Changes in the expression of selected miRNAs (bta-miR-483, bta-miR-150, bta-miR-21, bta-miR-409a, bta-miR-378, and bta-miR-142) that were present at lower levels in large healthy follicles than in large atretic follicles (Table 2 and Supplementary Table 1) were also validated by qPCR using samples from the follicles measuring 9–17 mm in diameter at different stages of growth or atresia (Fig. 3B). For five of the six miRNAs analyzed, the results of qPCR were in agreement with those of microarray, in that their levels were significantly higher in all or some of the large atretic follicle categories relative to large Healthy follicles. An exception was bta-miR-483, for which levels were not significantly different between large healthy and large atretic follicles.

Localization of miRNAs within follicular compartments

We next sought to determine the localization of miRNAs within follicular compartments. Granulosa (including COCs) and theca fractions were separately collected from individual healthy or atretic follicles measuring 9–17 mm in diameter (Fig. 4). Relative abundance of FSHR and CYP17A1 transcripts in each cell fraction was used to ensure the lack of cross-contamination between fractions collected from the same follicle (Fig. 4A). As shown in Fig. 4B, three of the five miRNAs upregulated in large healthy follicles relative to small follicles, such as miR-144, miR-202, and miR-873, were expressed at

Table 3 Bovine miRNAs sequences upregulated (greater than or equal to twofold) between large healthy follicle and small follicles.

miRNA	Mature sequence	Chromosome	Genomic location start-end (strand)	Locus property
bta-miR-202	UUCCUAUGCAUUAUACUUCUUU	26	25988240–25988338 (+)	Intergenic
bta-miR-876	UGGAUUUCUUUGUGAAUCACCA	8	15183224–15183304 (+)	Intergenic
bta-miR-873	GCAGGAACUUGUGAGUCUCCU	8	15159560–15159636 (+)	Intergenic
bta-miR-451	AAACCGUUACCAUACUGAGUUU	19	20796860–20796930 (–)	Intergenic
bta-miR-144	UACAGUAUAGAUGAUGUACUAG	19	20797022–20797105 (–)	Intergenic
bta-miR-652	AAUGGCCGCCACUAGGGUUGUG	X	62939283–62939380 (+)	TMEM164, intron 1

much higher levels in granulosa cells than in theca cells. In contrast, miR-652 was expressed at similar levels in the two cell types whereas miR-451 was present at slightly higher levels in theca cells. To determine whether, as presumed, the enrichment of miRNAs in the granulosa compartment reflected the expression by mural granulosa cells rather than by COCs, we analyzed these two compartments in small and large follicles. The results (Fig. 4C) showed that an increase in the levels of all five miRNAs in large follicles could indeed be attributed to a large extent to an increase in miRNA expression in mural granulosa cells rather than in the COC.

To confirm the results of qPCR, we then carried out *in situ* hybridization of bta-miR-202. We chose this miRNA as it has been reported to be expressed specifically in the ovary in other species and moreover its expression within bovine follicles appeared to be clearly compartmentalized (Fig. 4B). Consistent with qPCR data, the miR-202 probe hybridized most intensely to granulosa cells, with some signal also detected in the theca compartment but not in the ovarian stroma (Fig. 5A). miR-202 had a more diffuse cellular distribution than the nucleolar positive control, *RnU6-2*; however, in contrast to *RnU6-2*, the miR-202 signal was strongest around the nucleus (Fig. 5A), indicating a high miRNA abundance in the scant granulosa cell cytoplasm, as expected.

To determine whether, as shown in other studies (da Silveira *et al.* 2012), changes in follicular cell expression of miRNAs would be reflected by follicular fluid levels, the levels of bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873 were compared between matched samples of fluid and whole tissue from large healthy and large atretic follicles. Owing to limiting follicular fluid volumes, small follicles could not be included in these analyses. Moreover, follicular fluid levels of bta-miR-144 and bta-miR-451 were not detected. Of the other three miRNAs, only the levels of bta-miR-202 were significantly correlated ($P < 0.01$), albeit moderately ($r = 0.46$), between whole follicle tissues and follicular fluid (data not shown).

Body-wide expression of miRNAs

QPCR screening of an array of body tissues (Fig. 6) showed that bta-miR-144, bta-miR-451, bta-miR-652, and bta-miR-873 were present in tissues across the bovine body, with levels of all these miRNAs except

bta-miR-873 being relatively low in the ovary. In contrast, bta-miR-202 was not expressed in tissues other than testis and ovary, consistent with results in other species (Landgraf *et al.* 2007, Ro *et al.* 2007, Armisen *et al.* 2009, Juanchich *et al.* 2013).

Functional analyses

To identify cellular processes putatively targeted by miRNAs in large healthy follicles, lists of predicted bovine gene targets were obtained from TargetScan (release 6.2) and analyzed using Ingenuity Pathway Analysis (<http://www.ingenuity.com/>). A total of 1359 transcripts were identified, with bta-miR-144 having the highest number of predicted targets (836) followed by bta-miR-873 (302), bta-miR-202 (191), bta-miR-451 (22), and bta-miR-652 (8). Results of ontology analyses of the entire set of target genes are given in Supplementary Table 2, see section on supplementary data given at the end of this article.

We then sought to determine the expression profiles of selected miRNA target genes across follicle development stages. We chose four genes (*SPRED1*, *TGFRB2*, *ATG7*, and *CREB1*) that were predicted (Supplementary Table 3, see section on supplementary data given at the end of this article) to be simultaneously targeted by two or more of the miRNAs, bta-miR-144, bta-miR-202, and bta-miR-873, whose expression most dynamically changed across follicular stages (Fig. 3A). The expression of all of *SPRED1*, *TGFRB2*, *ATG7*, and *CREB1* was significantly lower in large healthy than in small or large atretic follicles, whereas the levels of the three miRNAs were highest in large healthy follicles (Fig. 7). These profiles were consistent with *SPRED1*, *TGFRB2*, *ATG7*, and *CREB1* being the targets of bta-miR-144, bta-miR-202, and bta-miR-873 in large healthy follicles. In contrast, levels of *LHCGR*, a predicted nontarget, were highest in large healthy follicles and lowest in large atretic follicles, as expected.

To more precisely investigate the actual functions of miRNAs in follicular cells, we first tested whether miRNA expression patterns during follicle development (Fig. 3) could be modeled *in vitro*. Granulosa cells from small follicles were cultured under conditions that prevent early luteinization (Gutierrez *et al.* 1997) and 1 day later cells were treated with Forskolin to induce differentiation or were left untreated, as in previous *in vitro* miRNA validation studies (McBride *et al.* 2012).

Figure 3 Relative abundance (mean $\pm$ S.E.M.) of miRNAs in bovine follicles ($n=4-11$ samples/diameter category). Bovine miRNAs whose expression was upregulated in large healthy follicle relative to small follicles in the microarray analyses are shown in (A) and selected miRNAs whose expression was downregulated in large healthy follicles relative to large atretic follicles are shown in (B). There was a main effect of follicle diameter ($P<0.01$) for all miRNAs except bta-miR-483 ($P>0.1$). Means with different superscripts (a,b,c) are different ($P<0.05$).

Among those miRNAs upregulated in large healthy follicles, the expression of bta-miR-144, bta-652, and bta-miR-873 quickly decreased upon culture and their levels were consistently nondetectable after 1–2 days. In contrast, the levels of bta-miR-202 and bta-miR-451 were readily detectable by qPCR for up to 6 days of culture; however, treatment with Forskolin did not result

in an increase in the levels of either miRNA in granulosa cells (Fig. 8). We also determined in granulosa cell cultures the expression of some of the miRNAs that were downregulated in large healthy follicles relative to large atretic follicles, namely, bta-miR-409a, bta-miR-150, and bta-miR-21. We found that although the levels

Figure 4 Relative abundance (mean $\pm$ S.E.M.) of (A) *FSHR* and *CYP17A1* and (B) miRNAs in granulosa and theca cell fractions of bovine healthy follicles (9–17 mm; $n=10$ follicles). Mean differences are indicated by * $P<0.05$ or ** $P<0.01$. (C) Relative abundance of the same miRNAs in mural granulosa and cumulus-oocyte complex fractions from small (4–8 mm) and large (9–17 mm) follicles ($n=4-6$ pools/follicle group, three to five samples per pool). There was an interaction of cell fraction $\times$ follicle category for all miRNAs ($P<0.001$). Means with different superscripts (a,b) are different ($P<0.05$).

Figure 5 Representative images showing results of *in situ* hybridization of bta-miR-202 in bovine follicular wall. Formalin-fixed paraffin embedded ovary sections were hybridized with DIG-labeled LNA probes for bta-miR-202 (A), the small nucleolar RNA, RnU6-2 (B), or a scrambled oligonucleotide sequence (C). Perinuclear areas with strong miR-202 hybridization in granulosa cells are indicated by arrows. GC and TC indicate the granulosa and theca compartments of the follicular wall.

of bta-miR-409a were quickly downregulated upon culture, bta-miR-150 and bta-miR-21 continued to be expressed and either did not change or, in the case of bta-miR-150, decreased overtime in response to Forskolin (Fig. 8). These results, while preventing further *in vitro* functional analyses of miRNAs enriched in large healthy follicles, also indicated that, overall, miRNA expression may be regulated differently *in vivo* and *in vitro*, as already reported for miR-21 and other miRNAs (Carletti *et al.* 2010, McBride *et al.* 2012).

Discussion

An involvement in the regulation of follicle function has been proposed for several miRNAs (reviewed in Donadeu *et al.* (2012)), although most evidence to date derives from results using granulosa cell cultures. Moreover, physiological changes in the expression of miRNAs supporting putative roles during follicle development have often not been demonstrated (Hu *et al.* 2012, Yang *et al.* 2012, Dai *et al.* 2013), and detailed spatiotemporal miRNA profiles during development are not available. In a previous study (McBride *et al.* 2012), we used sequencing to characterize the developmental changes in miRNA expression in ovine follicles, although our focus was on terminal differentiation (follicle–luteal transition) rather than on follicle growth and atresia. In this regard, the present findings are important as they provide novel information on the dynamic changes in miRNA levels across a wide range of follicle developmental stages in a monovular species, the cow, identifying a subset of miRNAs that are putatively involved in growth and regression of dominant follicles.

Our first objective was to use expression profiling to identify miRNA populations uniquely associated with steroidogenically active dominant-size follicles. We reasoned that by comparing miRNA profiles between large healthy follicles and each of small and large atretic follicles we would identify subsets of miRNAs putatively involved in follicle growth and atresia respectively. Using a heterologous microarray approach we were able to profile a total of 191 known bovine miRNAs in follicular tissues. In a previous study using sequencing, we identified a total of 212 unique miRNAs across different stages of follicle and luteal development in sheep (McBride *et al.* 2012), whereas Fiedler *et al.* (2008) earlier detected 206 different miRNA transcripts by microarray analyses of mouse granulosa cells.

Relative to large healthy follicles, a significantly larger number of sequences were differentially expressed in large atretic follicles than that in small follicles, although only a fraction of these corresponded to registered bovine miRNAs (Tables 1 and 2). Many of the bovine miRNAs differentially expressed in large atretic follicles are reportedly involved in regulating the proliferation and survival of different cell types (miR-483, miR-150, miR-21, miR-409a, miR-31, miR-34b, miR-33a; Yamakuchi *et al.* 2008, Bertero *et al.* 2011, Liu *et al.* 2011, Brabletz 2012, Cirera-Salinas *et al.* 2012, Weng *et al.* 2012) as well as immune cell activation (miR-150, miR-21, miR-31; Xiao *et al.* 2007, Xue *et al.* 2013, Das *et al.* 2014), which is consistent with an active role of these miRNAs in follicular fate decisions. The involvement of miR-21 in the promotion of cell survival is well established, including in mouse granulosa cells during ovulation (Carletti *et al.* 2010). In this study, miR-21 levels were on average sixfold higher in bovine large atretic follicle than that in large healthy follicles (Fig. 3B). Interestingly, earlier expression profiling of equine

Figure 6 Relative abundance of miRNAs in pools of different bovine tissues (minimum of three animals per pool). CL, corpus luteum; PBMC, peripheral blood mononuclear cells.

follicular fluid found increased mean miR-21 levels in subordinate follicles relative to dominant follicles (Schauer *et al.* 2013), and in seasonally anovulatory follicles relative to ovulatory follicles (Donadeu & Schauer 2013). Taken together, these data suggest a role for miR-21 in follicular atresia; in light of previous

information showing an involvement of this miRNA in the mediation of immune and fibrotic responses in other body systems (Kumarswamy *et al.* 2011), the role of miR-21 in follicular death warrants further investigation.

Many of the differences in miRNA expression between large healthy follicles and small follicles were also found

Figure 7 Relative abundance (mean $\pm$ s.e.m.) across different categories of bovine follicles ($n=9-19$ samples/diameter category) of (A) miR-144, miR-202, and miR-873 and (B) selected transcripts (*SPRED1*, *TGFBR2*, *ATG7*, and *CREB1*) predicted to be targeted by those miRNAs, as well as a predicted nontarget (*LHCGR*). There was an effect of follicle category ($P<0.01$) for all endpoints. Means with different superscripts (a,b,c) are different ($P<0.05$).

Figure 8 Changes in the levels (mean $\pm$ S.E.M.) of miR-202, miR-451, miR-150, and miR-21 in bovine granulosa cells cultured in the presence of Forskolol (10 μ M), insulin (1 μ g/ml), and FCS (1%) or left untreated for 4 days. Fold changes in miRNA levels relative to levels before the onset of treatment (day 0) are shown. There was an interaction of treatment $\times$ day for miR-451 ($P=0.04$) and miR-150 ($P=0.005$). Means with different superscripts (a,b) are different ($P<0.05$).

between large healthy follicles and large atretic follicles. Principal component analyses showed three clearly distinct follicle populations; however, because the health status of small follicles could not be determined, samples within this category might have presumably contained atretic as well as healthy follicles, which could account for some of the similarities in miRNA patterns between the small follicles and large atretic follicle groups. Most of the sequences upregulated in large healthy follicles relative to small follicles corresponded to bona-fide bovine miRNAs (bta-miR-144, bta-miR-451, bta-miR-652, and bta-miR-873). QPCR analyses confirmed that at least three of these miRNAs were also upregulated significantly in some or all of the large healthy follicle categories relative to large atretic follicles, thus identifying this set of miRNAs as very strong candidates with a role in the development of bovine dominant follicles. miR-144, miR-202, miR-451, and miR-652 were reported earlier to be expressed in the ovary (Hossain *et al.* 2009, McBride *et al.* 2012, Kitahara *et al.* 2013, Schauer *et al.* 2013), although their expression patterns within follicular tissues were not studied in detail. In this study, bta-miR-202 was restricted to the bovine gonad; moreover, its expression within the follicle was highest in granulosa cells while levels in ovarian stroma were negligible. These findings are consistent with data showing gonad-restricted expression of miR-202 in humans, rodents, frogs, and fish (Landgraf *et al.* 2007, Ro *et al.* 2007, Armisen *et al.* 2009, Juanchich *et al.* 2013) and the expression of miR-202 predominantly in Sertoli cells within the mouse testis (Wainwright *et al.* 2013). Studies on chicken and

mice showed sexually dimorphic expression of miR-202 in primordial gonads under regulation of estrogen or *Sox9*, suggesting a role in early testis development. The present result of a greater than fourfold increase in bta-miR-202 levels between 9–11 and 15–17 mm healthy follicles builds on those earlier findings by indicating a role for this miRNA during folliculogenesis in the adult bovine ovary. In addition to miR-202, biological roles for the clustered miR-144 and miR-451 have also been proposed, but only in relation to non-ovarian cells; those include regulatory roles in erythropoiesis (Pase *et al.* 2009), differentiation of vascular smooth muscle cells (Turczyńska *et al.* 2013) and, potentially relevant during follicular differentiation, cholesterol trafficking in macrophages (Ramirez *et al.* 2013). Interestingly, both miR-144 and miR-451, as well as miR-652, were upregulated in rat ovaries following an ovulatory hCG bolus, although no attempt was made to identify the specific cell type(s) involved (Kitahara *et al.* 2013).

To gain insights into their spatiotemporal involvement during follicle development, the expression of bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873 was determined over a wide range of developmental stages and cell compartments in bovine follicles, providing information comparatively more detailed than in any previous study. An increase in the levels of all of these miRNAs in large follicles was accounted for increases in mural granulosa cells rather than in COC; this was not surprising as the latter contains a very small fraction of the total cells in the follicle, and therefore microarray analyses of whole follicle tissues might have missed changes in miRNA levels in the COC. Indeed, miRNAs differentially expressed in bovine COCs, cumulus cells or oocytes, in addition to changes in miRNA expression during oocyte maturation, have been reported (Hossain *et al.* 2009, Tesfaye *et al.* 2009, Miles *et al.* 2012), and they did not include any of the miRNAs identified as enriched in large healthy follicles in this study. Nonetheless, our results suggest a potential involvement of bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873 in the regulation of mural granulosa cell function during maturation of the dominant follicle, as their expression in all cases increased in healthy follicles once they reached a diameter of 12–14 mm. In contrast to the other miRNAs, a sharp increase in the levels of bta-miR-873 occurred as early as the 9–11 mm stage in healthy follicles and was associated with a significant reduction in bta-miR-873 levels in all large atretic follicle categories; these temporal relationships suggest a potential role of this miRNA in selection of the dominant follicle, a possibility that warrants further investigation, particularly because of the lack of functional data on miR-873 in any cell type or species.

To identify cell functions and pathways potentially targeted by bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652 and bta-miR-873 in dominant follicles, we

carried out ontology analyses for the complete set of predicted mRNA targets obtained by TargetScan (Supplementary Table 2). Many of the signaling pathways overrepresented by these targets corresponded to growth factors putatively involved in follicular cell proliferation and steroidogenesis and prevention of premature luteinization including HGF, IGF1, TGF β , estrogen, neurotrophin/TRK, PEDF, and NGF (Parrott *et al.* 1993, Salas *et al.* 2006, Glister *et al.* 2010, Mani *et al.* 2010, Garcia-Rudaz *et al.* 2011, Chuderland *et al.* 2013) and others involved in oocyte maturation including neuregulin, GDNF, and EGF (Conti *et al.* 2006, Noma *et al.* 2011, Linher-Melville & Li 2013), the fine-tune regulation of which is of key importance during the final stages of follicle development. Also present were several core cell cycle regulatory pathways (cell cycle regulation by BTG family proteins, estrogen-mediated S phase entry, and G1/S checkpoint regulation), a finding which is consistent with the notion that the development of the dominant follicle involves a substantial decrease in granulosa cell proliferation (Lussier *et al.* 1987, Gaytan *et al.* 1996).

Bioinformatics analyses were complemented by demonstrating that the changes in the levels of selected transcripts during follicle development were consistent with these being the targets of miRNAs in large healthy follicles, specifically, a decrease in the expression of TGFBR2 and CREB1, two transcripts involved in TGF β and cAMP signaling and which are involved in follicular differentiation (Somers *et al.* 1995, Wehrenberg *et al.* 1998), and SPRED1 and ATG7, two transcripts involved in RAS/MAPK signaling and autophagy, respectively, and which expression during follicular development has not been previously characterized. Unfortunately, changes in miRNA expression during bovine follicle development could not be replicated in cultures of granulosa cells, which prevented further *in vitro* validation of genes and signaling pathways putatively targeted by miRNAs. Nonetheless, our results provide useful information by showing that bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and miR-873 may in combination play a key role in the final maturation of the bovine dominant follicle in preparation for the subsequent follicle–luteal transition.

In summary, this study identified for the first time genome-wide miRNA profiles associated with growth and atresia of dominant-size follicles in bovine. Five miRNAs were upregulated in large healthy follicle relative to small follicle categories (bta-miR-144, bta-miR-202, bta-miR-451, bta-miR-652, and bta-miR-873) and three of these (bta-miR-144, bta-miR-202, and bta-miR-873) were also expressed at higher levels in large healthy follicle than in large atretic follicle categories. These three miRNAs were predominantly expressed in the mural granulosa cell compartment of the follicle, and one of them, bta-miR-202, was not expressed in body tissues other than the gonad. Finally, gene ontology analyses of predicted miRNA targets

revealed that, within the growing dominant follicle, these miRNAs putatively target multiple signaling pathways involved in follicular cell proliferation, steroidogenesis, and prevention of premature luteinization, in addition to oocyte maturation. In conclusion, the present data identify a specific set of miRNAs that may play a key role during the maturation of dominant follicles in the bovine ovary.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0140>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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