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The adequate corpus luteum: miR-96 promotes luteal cell survival and progesterone production

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Abstract

Context: Inadequate progesterone production from the corpus luteum is associated with pregnancy loss. Data available in model species suggest important roles of miRNAs in luteal development and maintenance.

Objective: To comprehensively investigate the involvement of miRNAs during the ovarian follicle-luteal transition

Design: The effects of specific miRNAs on survival and steroid production by human luteinized granulosa cells (hLGCs) were tested using specific miRNA inhibitors. Candidate miRNAs were first identified through microarray analyses of follicular and luteal tissues in a bovine model.

Setting: UK academic institution associated with teaching hospital

Patients or other participants: hLGCs were obtained by standard transvaginal follicular fluid aspiration from 35 women undergoing assisted conception

Intervention(s): Inhibition of candidate miRNAs in vitro

Main outcome measure(s): Levels of miRNAs, mRNAs, FOXO1 protein, apoptosis and steroids were measured in tissues and/or cultured cells.

Results: Two specific miRNA clusters, miR-183-96-182 and miR-212-132, were dramatically increased in luteal relative to follicular tissues. miR-96 and miR-132 were the most upregulated miRNAs within each cluster. Database analyses identified FOXO1 as a putative target of both these miRNAs. In cultured hLGCs, inhibition of miR-96 increased apoptosis and FOXO1 protein levels, and decreased progesterone production. These effects were prevented by siRNA-mediated downregulation of FOXO1. In bovine luteal cells, miR-96 inhibition also led to increases in apoptosis and FOXO1 protein levels.
Conclusions: miR-96 targets FOXO1 to regulate luteal development through effects on cell survival and steroid production. The miR-183-96-182 cluster could provide a novel target for the manipulation of luteal function.

Introduction

In monovular species, such as humans, ovulation involves rupture of the wall of a mature follicle and release of the contained oocyte for fertilization. Following ovulation, the follicular remnants undergo profound remodeling resulting in formation of a highly vascular, highly steroidogenic corpus luteum (CL), with a critical role in establishment/maintenance of pregnancy. Luteal development involves fine-tuned changes in proliferation, survival, migration and differentiation simultaneously affecting a multitude of cell types (1). Particularly critical is the differentiation of estrogen-producing follicular cells into luteal cells with the ability to produce high levels of progesterone. The importance of this is reflected in the association of luteal insufficiency and/or suboptimal progesterone levels with pregnancy failure in species including cattle, sheep and horses (2-4). Although a similar association has been proposed in humans (5), controversy exists on whether the CL is actually a primary cause of infertility in women (6). A much-needed understanding of molecular regulation of luteal development in humans would provide clarification and assist in identifying novel therapeutic targets for fertility manipulation.

miRNAs are ubiquitously involved in post-transcriptional gene regulation during tissue development/differentiation. Global miRNA profiles during follicular development have been reported in cattle, sheep and mice (7-9), and numerous miRNAs were shown to regulate follicular proliferation, survival and steroidogenesis (10-12). In addition, a significant number of miRNAs change in expression during the follicle-luteal transition and luteal maturation (7,9,13,14), however, although specific roles have been demonstrated for some of these miRNAs in rodents, including in luteal angiogenesis (miR-17-5p and let-7b (15)), survival (miR-21 (16)), and LHCGR downregulation (miR-
136-3p and miR-122 (17,18)), very limited information exists on their involvement in other species, particularly humans (14).

A greater understanding of the roles of miRNAs in normal luteal development, and in particular the follicle-luteal transition, could provide important insight into human reproductive health. Considering this, we performed analysis both in cells from human patients and in tissues collected from cattle. Being a monovular species, cattle provide a convenient model to study human ovarian physiology, importantly allowing study of clinically-relevant follicular/luteal tissues difficult to access in women. Our studies identified a miRNA cluster which is highly expressed in the CL and plays a role in promoting luteal cell survival and steroidogenesis, providing a potential target for future interventions in human reproductive health.

**Methods**

**Tissue collection**

Human luteinized granulosa cells (hLGCs) were obtained (19) from 35 donors undergoing assisted conception at the Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, UK. Ethical approval was given by the regional medical research ethics committee (2005/R/RM/11 and SR431), all women gave informed consent and cells were analyzed anonymously. Cells were centrifuged through Ficoll Paque Plus solution (1.077 g/cm³, GE Healthcare, UK), then the middle layer was collected and washed before culture. Cell viability was ~70%.

Bovine tissues were collected at an abattoir. Ovarian pairs containing a visible CL were used to collect individual follicles >10 mm in diameter (8). Follicular walls and follicular fluid were snap-frozen in LN2 and frozen at -80°C, respectively. Corpora lutea corresponding to days 1-4 of an estrous cycle (20) were collected and snap-frozen. Bovine granulosa cells were obtained from follicles 4-8 mm in diameter (8,21). Luteal cells (steroidogenic and other cells including fibroblasts, endothelial and immune) were isolated using a modification of published protocols (22). In brief, the CL was minced and digested twice in collagenase-II and BSA (both from Sigma-Aldrich, UK) at 37°C for 45 min.
Supernatants were filtered (100-µm) then incubated with DNase-I (Sigma-Aldrich) for 10 min at 37°C. After digestion, cells were filtered again (70-µm), washed and incubated with red cell blood lysis buffer (8.3 gm/l NH₄Cl in 0.01 M Tris-HCl, pH= 7.5; Sigma-Aldrich) for 1 min. Cell viability was >80%.

**Cell culture**

Both hLGCs and bovine luteal cells were cultured in 24- or 12-well plates (Thermo-Fisher, UK; 100,000 and 500,000 cells/well) in DMEM/F-12 (Life Technologies, UK) containing 2.5mM L-Glutamine, 15mM HEPES, 1% Pen-Strep, Fetal bovine serum (FBS; 10% v/v for the first 24 h, 2% thereafter), ITS (Insulin (5 µg/ml), Transferrin (5 µg/ml) and sodium selenite (5 ng/ml)) and Fungizone (2.5 µg/ml; all from Sigma-Aldrich) in a humidified atmosphere at 37°C with 5% CO₂. Twenty-four hours later, cells were transfected with locked-nucleic acid (LNA) anti-miRNAs (Exiqon, Denmark; 50 nM), two human FOXO1 siRNAs (ID#106654 and 106653; Life Technologies; 100 nM each) and/or a scrambled oligonucleotide (AllStars-Negative Control; Qiagen, UK; 50 or 100 nM) using Hiperfect reagent (Qiagen), and 1 or 2 days later they were collected for RNA, protein or Caspase 3/7 activity analyses while culture media were frozen. In some instances, after 6 days in culture hLGCs were treated with hCG (100 ng/ml; Serono Laboratories, UK) for 4 days after which RNA was collected. Progesterone and estradiol levels in culture media were quantified using Coat-A-Count radioimmunoassay (Siemens Healthcare Diagnostics Inc., USA) and DIAsource E2-RIA-CT (DIAsource ImmunoAssays S.A., Belgium) kits, respectively. Sensitivities and coefficients of variance were 0.01 ng/ml and 1 pg/ml, and 4.3% and 2.6%, respectively, for each assay.

Bovine granulosa cells were cultured as described (21) and 24 h later were either left untreated or treated with forskolin (10 µM), bovine insulin (1 mg/ml) and FBS (1% v/v; all from Sigma-Aldrich) for up to 4 days to induce luteinization.

**RNA Extraction**
Total RNA was extracted from snap-frozen tissues using the miRNeasy Mini kit (Qiagen) following homogenization with ceramic beads using FastPrep FP120 Cell disruptor (MP Biomedicals, UK). RNA was analyzed using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Willmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies Inc., UK). RNA from cultured cells was isolated using TRIzol® Reagent (Life Technologies) and concentrations were determined using Quant-iT™ RiboGreen® RNA kit (Life Technologies).

Microarray Analyses

Bovine samples from 6 large (diameter, 12-17 mm), steroidogenically-active follicles (classified based on CYP19A1 and estradiol levels (8)) and 6 early corpora lutea were analyzed with the miRCURY LNA™ microRNA Array 6th generation (contained 1488 capture probes targeting all miRNAs for human, mouse or rat in miRBase 16.0) by Exiqon Services (Denmark), as described in detail (8). Differences in miRNA expression were determined using Student’s t-test with Benjamini and Hochberg False Discovery Rate (FDR) adjustment. Raw microarray data were deposited in NCBI’s GEO repository, GSE54692.

RT-qPCR

Individual miRNAs were analyzed using miScript II RT and miScript SYBR Green PCR kits, and miScript Primer Assays (Qiagen). mRNA levels were quantified on the same cDNA using species-specific primers (Table S1) and the SensiFAST™ SYBR Lo-ROX Kit (Bioline Ltd., UK). The MX3005P QPCR system (Stratagene, CA, USA) was used. Relative transcript abundance was obtained using MX3005P software by extrapolating Ct values from a standard curve prepared from a sample pool. Endogenous RnU6-2 was used for normalization of miRNA, and 18S or GAPDH were used to normalize mRNA data.
In Situ Hybridization

In situ hybridization of frozen ovarian tissues was performed using a modified protocol (8) with double digoxigenin-labeled LNA probes (Exiqon) against bta-miR-132 (80nM), RnU6-2 (3nM) or a scrambled RNA sequence (40nM). Independent analyses were performed on 3 different sections.

Western blotting

Total protein was obtained by adding buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH=6.8; Sigma-Aldrich) to cultured cells at 60°C and then scraping off. Samples were boiled for 5 min and electrophoresed in a 12% SDS-PAGE gel with Color-plus pre-stained marker (BioRAD, UK) in Mini Trans-Blot® Cell (BioRAD) at 150 volt for 90 min. Gels were transferred to a 0.2 μM Nitrocellulose membrane (GE healthcare, UK) using Trans-Blot® SD Semi-Dry Transfer Cell (BioRAD) at 15 volt for 60 min. After blocking, the blot was incubated with anti-FOXO1 (#2880; Cell Signaling, MA, USA; 1:500) or anti-β-Tubulin (#2146; Cell Signaling; 1:1000) overnight at 4°C, followed by washing and incubation with IRDye®680RD Donkey anti-rabbit IgG (926-68073; LI-COR Biosciences, UK; 1:10000) for 1h and visualization with LI-COR Odyssey infrared imaging scanner. Signal intensities were quantified using Image Studio Lite 5.0 (LI-COR).

Apoptosis assays

Apoptosis was measured in triplicate in 96-well plates (2x10^4 cells/well) using Caspase-Glo 3/7 assays (Promega Ltd, UK) and a Synergy BioTek micro-plate reader. In addition, cells grown on coverslips were stained with Annexin-V-Fluos staining kit (Roche, UK) and visualized using a Leica DMLB fluorescence microscope.
Statistical analyses

Data was analyzed using the GLM procedure by one-way or two-way ANOVA followed by Tukey’s pairwise comparison tests or, whenever only two experimental groups were compared, Student’s t-tests. In all cases, statistical significance was considered at P<0.05.

Results

The miR-183-96-182 and miR-212-132 clusters are highly upregulated during the follicular-luteal transition. To identify miRNAs potentially involved in the follicular-luteal transition in the monovular ovary we collected bovine large antral follicles and early cycle corpora lutea. Expression profiles of selected genes were consistent with those naturally encompassing the follicle-luteal transition (Figure 1A). Upon microarray analyses, a total of 545 probes yielded hybridization intensities above background across all samples, corresponding to 523 unique miRNAs including 191 sequences registered as bovine in miRBase 18. Results of comparative analyses are shown in Figure 1B,C and Table S2.

A total of 11 and 22 unique miRNAs were up- and down-regulated, respectively (≥2.5 fold; FDR<0.01), in corpora lutea relative to large antral follicles (Table 1). The top 4 differentially expressed sequences corresponded to the homologues of human miR-183-5p, miR-96-5p, miR-182-5p and miR-132-3p, and were all upregulated in CL (Figure 1D,E). These sequences derive from two different miRNA clusters, miR-183-96-182 and miR-212-132. Only one of the -3p homologues in the miR-183-96-182 cluster, miR-183, was also detected by microarray, and was slightly upregulated in the CL (1.15 fold; Table S2). In addition, the homologues of human miR-132-5p and miR-212-3p (none registered as a bovine sequence in miRBase 18), but not miR-212-5p, were also detected and were upregulated in CL (Table 1; Figure 1D,E). Since bta-miR-96 and bta-miR-132 were the top upregulated miRNAs within each cluster (Figure 1D) and, based on Ct values, were also the most abundant in ovarian tissues, our subsequent analyses focused on these two miRNAs, for simplicity hereafter referred to as miR-96 and miR-132.
QPCR screening across bovine tissues revealed neither miR-96 nor miR-132 was restricted to the ovary (Figure 2A). Nevertheless, miR-132 was expressed at highest levels in CL, although in situ hybridization showed that within the CL this miRNA was broadly distributed, not restricted to any particular cell type (Figure 2B).

Next, to ascertain whether the increase in miR-96 and miR-132 during luteinization involves granulosa-derived cells, the major source of luteal progesterone, we induced bovine granulosa cells to luteinize in culture (7) and showed that indeed this was associated with a distinct increase in the expression of miR-96, miR-132 and other miRNAs from the same genomic clusters (Figure 2C).

**FOXO1 is a putative target of miR-96 and miR-132 during the follicle-luteal transition.** To investigate the roles of miR-96 and miR-132 we first used TargetScan 7.1 and miRTarBase 6.0 to obtain lists of computationally predicted targets in human and bovine, and experimentally validated targets (available from humans and rodents), respectively. To identify high-confidence targets we selected a subset of genes that 1) were predicted targets of both miRNAs, 2) contained conserved target sites and 3) were known to be involved in luteal development (http://okdb.appliedbioinfo.net/), and we then determined their relative expression in ovarian tissues (Figure 2D). In this way, the transcription factor, FOXO1, a critical regulator of cell survival and metabolism (23), was identified as high-confidence target based on its clearly decreased expression in CL relative to follicles. The levels of other genes analyzed were only slightly lower (ACVR1A), not different (RASA1 and FOXO3) or higher (CDKN1A, MMP9 and HB-EGF) in CL than in follicles, indicating they may not naturally mediate the effects of those miRNAs during the follicle-luteal transition.

**miR-96 has an anti-apoptotic effect in hLGCs mediated by FOXO1.** To investigate the involvement of these miRNAs in the human ovary, we first determined changes in miRNA expression in hLGCs (corresponding to an early stage of luteinization) that had been treated with hCG to induce further differentiation in culture (24). Results showed that, as in bovine (Figure 2C), the two miRNAs are up-regulated in response to a luteinization stimulus in human cells (Figure 3A).
Next, we investigated whether, as suggested by the results of our miRNA target analyses, these two miRNAs may regulate luteal cell survival. We transfected hLGCs with anti-miRNAs (Figure 3B) and determined the effects on apoptotic responses to serum removal. We found that anti-miR-96 but not anti-miR-132 led to a significant mean increase (1.6-fold) in the Caspase 3/7 activation response to serum starvation (Figure 3C). Interestingly, similar Caspase 3/7 responses were obtained even in non-stressed cells maintained in serum; in addition, under those conditions, simultaneous inhibition of both miRNAs produced a Caspase 3/7 response similar to that induced by inhibition of miR-96 only (Figure 3D). The pro-apoptotic effect of miR-96 inhibition was confirmed by V-Annexin staining (Figure 3E).

We then determined whether the effects of miR-96 could be mediated by its putative target, FOXO1. Indeed, inhibition of miR-96 induced a robust mean increase (1.8-fold) in FOXO1 protein one day after transfection (Figure 3F), with a slightly smaller (1.7-fold) although significant increase induced also by miR-132 inhibition; again, simultaneous inhibition of the two miRNAs did not have a synergistic effect on FOXO1 levels. To confirm a causal involvement of FOXO1 in the observed apoptotic response to anti-miR-96, we transfected cells simultaneously with anti-miR-96 and FOXO1 siRNA. We showed that this effectively prevented both an increase in FOXO1 protein (Figure 3G) and the activation of Caspase 3/7 (Figure 3H) in response to anti-miR-96 thus indicating that miR-96 promotes hLGC survival by targeting FOXO1.

miR-96 promotes progesterone production by hLGCs through targeting FOXO1. Given the reported involvement of miRNAs in steroidogenesis (10,11), we investigated the short-term effects of miR-96 and miR-132 on progesterone and estradiol production by analyzing spent culture media of hLGCs transfected with anti-miR-96 and/or anti-miR-132 (Figure 4A). All treatments resulted in a decrease in mean progesterone levels at 24h, however, this was significant only in response to anti-miR-96, alone or in combination with anti-miR-132 (>1.6-fold, P<0.01). Moreover, the effects of anti-miRs on progesterone were transient as differences were no longer detected 48h after transfection (P>0.1; not shown). In contrast, significant changes in estradiol levels were not detected (P>0.1) in response to transfection with anti-miRNAs.
We then determined whether the observed stimulatory effects of miR-96 on progesterone may involve repression of FOXO1. This was indeed the case, as transfection with FOXO1 siRNA prevented the temporary reduction in progesterone levels by anti-miR-96 (Figure 4B), indicating that, in hLGCs, an inhibitory effect of FOXO1 on progesterone synthesis is relieved by an increase in miR-96 upon luteinization.

To investigate the mechanisms behind the observed effects of miR-96 and FOXO1 on steroid levels, we quantified the expression of several genes involved along the cholesterol and steroid synthesis pathways (Figure 4C), the transcript levels of which were previously shown to be regulated by FOXO1 in rodent granulosa cells (25). We did not detect significant differences in the levels of any of the transcripts analyzed in response to inhibition of miR-96 in the absence or presence of FOXO siRNA, indicating that distinct molecular mechanisms, which could possibly include changes in protein levels and/or activity of steroidogenic gene products, may account for the effects of FOXO1 in steroid production in hLGCs.

The anti-apoptotic effects of miR-96 are conserved in bovine luteal cells. Finally, to investigate the functional conservation of miR-96 during the follicle-luteal transition in other species we established whether the observed effects of this miRNA in human ovarian cells also occurred in bovine. We collected cells from bovine corpora lutea and cultured them in the presence or absence of anti-miR-96. Consistent with data in human (Fig 3C), inhibition of miR-96 (Fig 5A) led to an increase in Caspase 3/9 activity in response to serum deprivation (Fig 5B), together with an increase in FOXO1 protein levels (Fig 5C). In contrast, progesterone production by bovine cells was not affected by miR-96 inhibition (not shown).

Discussion

Little is known about the molecular regulation of the follicle-luteal transition in humans, to a large extent due to the limited availability of healthy ovarian tissues for study. In this regard, monovular species such as cattle can provide extremely valuable insight, particularly when compared to common
rodent models with much more distinct ovarian physiology (26,27). In this study, we demonstrate the
value of this comparative approach by identifying, using the bovine model, a novel miRNA-mediated
mechanism involved in functional regulation of the human corpus luteum.

The finding of an increase in the expression of the miR-183-96-182 and miR-212-132 clusters, as
well as miR-21, during luteinization is consistent with results of previous studies in other species,
including bovine (7,13,28-31), overall suggesting conserved roles of these miRNAs during the follicle-
luteal transition. However, although an effect of miR-21 in promoting cell survival during ovulation
has already been demonstrated, at least in mice (16), the precise roles of the miR-183-96-182 and miR-
212-132 clusters, the most upregulated miRNAs during the follicle-luteal transition, had so far not been
defined during that transition.

Studies in rodents showed FOXO1 to regulate different granulosa cell pathways involved in
proliferation, survival and differentiation (25,32-34), and ovarian FOXO1 expression to rapidly
terminate in response to the ovulatory gonadotropin surge (35). Moreover, FOXO1 was experimentally
validated as miR-96 target in human (36,37) and cattle (29). Indeed, the latter was the only study so far
to show targeting of FOXO1 by the miR-183-96-182 cluster specifically in granulosa cells, which
reportedly enhanced cell proliferation. In contrast, we found no evidence of an effect of miR-96 or miR-
132 on proliferation of luteal cells (not shown). Cell cycle arrest (rather than proliferation) associated
with activation of survival pathways is a hallmark of luteinization(1), consistent with the anti-apoptotic
effects of miR-96 in both human and bovine cells in our study. Taken together, our results and those
of (29) indicate that the effects of the miR-183-96-182 cluster may depend on the stage of follicle/luteal
development. Most importantly, we identify miR-96 as a novel mediator of a key effect of the ovulatory
LH surge, promotion of luteal cell survival, through targeting of FOXO1.

Granulosa-derived cells are the main source of luteal progesterone. The observed decrease, albeit
short-lasting, in progesterone production by hLGCs in response to anti-miR-96 indicates a stimulatory
effect of miR-96 on this crucial function. In contrast, a previous study reported an inhibitory effect of
miR-96 on progesterone production by human non-differentiated granulosa cells, suggesting the effects
of this miRNA may be developmental stage-specific(12) Moreover, we show the inhibitory effects of
miR-96 to be mediated through downregulation of FOXO1. Studies in rodent granulosa cells (25) provided evidence that FOXO1 may target several genes along the cholesterol/steroid synthesis pathway (including hmgcs1, nr5a1, star, cyp11a1, cyp19a1 and cyp27a1) acting to prevent a premature increase in follicular steroid production before luteinization. Our results implicate miR-96 in modulating the effect of FOXO1 on steroidogenesis in humans, however, since we did not detect significant changes in the transcripts for the above cholesterol/steroid-producing genes, further investigation of the downstream mechanisms involved is warranted. In contrast to hLGCs, an effect of miR-96 inhibition on progesterone production by bovine luteal cells was not observed. Since, compared to human cells, bovine cells were collected from relatively mature corpora lutea, our finding suggests the stimulatory effects of miR-96 may occur only during the initial stages of luteinization; alternatively, these results may be explained by the heterogeneous nature of the bovine luteal cell preparations used in our study (containing not only granulosa-derived but also other luteal cell types) or may reflect intrinsic differences in regulation of luteal progesterone production between humans and cattle, e.g., the distinct dependence of human granulosa lutein cells on LH (1).

Because of its relative high abundance in luteal cells allowing robust downregulation using LNAs, we focused our analyses on miR-96. However, it should be noted that all miR-183-96-182 cluster miRNAs have similar seed sequences, and there is evidence they coordinately regulate some target genes (29,36,38); thus, all 3 miRNAs are expected to contribute to luteal regulation in vivo. Further complexity in predicting physiological effects of these miRNAs is provided by the fact that they presumably act in coordination with other miRNAs, for example, the anti-apoptotic miR-21 (16), thus ensuring robust follicular differentiation.

Finally, in contrast to anti-miR-96, miR-132 inhibition had in our study only small, non-significant effects on hLGCs, particularly in relation to cell survival, and in addition did not enhance the effects of miR-96 alone. Yet, albeit to a slightly lower extent than anti-miR-96, miR-132 inhibition did induce an increase in FOXO1 protein, in agreement with this being an experimentally validated target of miR-132 (39,40). Although we do not have an explanation for the relatively minor cell responses to miR-132 compared to miR-96 in the face of similar inhibitory effects on FOXO1, this may reflect differences in
specific effector mechanisms elicited by the two miRNAs, a possibility that should be investigated in the future. Moreover, in line with our results, inhibition of miR-132 and miR-212 in a previous study had no obvious effects on steroid production by mouse granulosa cells, overall suggesting the miR-212-132 cluster may instead be involved in other aspects of the follicle-luteal transition.

In summary, using a cross-species approach we identified miR-96 as a novel regulator of the follicle-luteal transition, through FOXO1-mediated promotion of luteal cell survival and progesterone production. Reported wider roles for FOXO1 in the ovary suggest that miR-96, and indeed the miR-183-96-182 cluster, likely has broader effects during the follicle-luteal transition. Such effects may be important not only in ensuring a normal luteal phase but also in regulating luteal rescue and the establishment of pregnancy, a potential key link for investigation in future studies.

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**Figure legends**

Figure 1. A) Relative mean (± SE) transcript levels of *LHCGR*, *CYP19A1* and *HSD3B1* in bovine ovulatory size follicle (12-17 mm; n = 6) and early corpora lutea (n = 6) samples used for microarray analyses. B, C) Heat map representation (B) and Principal Component Analyses Plot (C) of top 50 miRNA probes with highest standard deviation in bovine ovulatory size follicles and early corpora lutea. Each row in the heat map represents a miRNA and each column represents a sample. The color scale illustrates the relative expression level of miRNAs. Red color represents an expression level below the reference channel and green color represents expression higher than the reference. For PCA plot analysis, the normalized log ratio values were used. The features were shifted to be zero centered, (i.e.
the mean value across samples was shifted to 0) and scaled to have unit variance (i.e. the variance across samples was scaled to 1 before the analysis). Raw microarray data were deposited in the NCBI’s GEO repository, GSE54692. D) Levels (mean ± SE), obtained by qPCR, of top transcripts identified by microarray as up- or down-regulated in early CL relative to ovulatory-size follicles. E) Comparative mean fold changes in miRNA expression between bovine early CL and ovulatory-size follicles obtained by microarray and qPCR. In all panels, differences between two means, determined by t-test, are shown by * (P<0.05) and ** (P<0.01).

Figure 2. A) Relative abundance of miR-96 and miR-132 across several bovine tissues. For each tissue, RNA samples from 3 to 5 animals were pooled and analyzed by qPCR. CL– corpus luteum, PBMC – polymorphonuclear cells. B) Localization of miR-132 within the bovine CL by in situ hybridization. Frozen ovarian sections were hybridized with DIG-labelled LNA probe against scrambled RNA (negative control, left panel), bta-miR-132 (middle panel) and RnU6 snRNA (positive control, right panel), scale = 100 um, 20x. C) Changes in relative miRNA levels (mean ± SE) during in vitro luteinization of bovine granulosa cells. Granulosa cells were cultured for 4 days with or without media containing forskolin, insulin and FBS to induce luteinization (n = 5 experiments). Data are shown relative to expression values on Day 0. Group means with different letters (a, b, c) are different (P<0.05). D) Relative mean (± SE) transcript levels, quantified by qPCR, of predicted common targets of miR-96 and miR-132 in bovine ovulatory size follicles (9-17 mm; n = 6) and early corpora lutea (n = 6). For each gene, mean differences (P<0.05) are shown by *.

Figure 3. A) Relative levels of miR-96 and miR-132 in human luteinized granulosa cells treated with hCG (100 ng/ml) for 4 days or left untreated. B) Relative levels of miRNAs following transfection of human luteinized granulosa cells with the indicated oligonucleotides. C, D) Caspase 3/7 activity in transfected human luteinized granulosa cells with (C) or without (D) previous serum removal for 12h. E) Representative pictures (scale = 100 um, 20x) showing Annexin V staining (green; PI staining is shown in red) of human luteinized granulosa cells 24h after transfection with the indicated oligonucleotides. F) Relative levels of FOXO1 protein after transfection of human luteinized granulosa
cells with the indicated oligonucleotides. A representative western blot is shown.  G) Representative FOXO1 and B-tubulin western blots of human luteinized granulosa cells transfected with the indicated oligonucleotides for one day. H) Caspase 3/7 activity following transfection of human luteinized granulosa cells with the indicated oligonucleotides. In all experiments, oligonucleotides were transfected at the following concentrations: scramble oligonucleotide (50 or 100 nM, negative control), anti-miR-96 (50 nM), anti-miRNA-132 (50 nM), anti-miR-96 + anti-miR-132 (25 nM and 25 nM) and FOXO siRNA (100 nM). Values shown for each time-point were in all cases normalized to the corresponding value at the time of transfection (Day 0 or Hour 0). Data are shown as mean ± SE (n=3 - 6 experiments). Significant differences (P<0.05) between two means are shown by a star (A). For comparisons involving more than two means, different letters (a,b,c) are used to indicate significance (P<0.05). In F, mean FOXO1 values were compared across groups within each Day.

Figure 4. A, B) Levels of progesterone (A, B) and estradiol (A) in spent culture media one day after transfection of human luteinized granulosa cells with the indicated oligonucleotides (n = 6 experiments). C) Transcript levels of different genes involved in cholesterol and steroid production, quantified by qPCR in human luteinized granulosa cell samples collected one day after transfection with the indicated oligonucleotides (n = 3 experiments). Mean (± SE) values are shown and were normalized to values on the day of transfection (Day 0). Group means with different letters (a,b,c) are different (P<0.05).

Figure 5. A,B,C) Relative levels (mean ± SE) of miR-96 (A), Caspase 3/7 activity (B) and FOXO1 protein (C) following transfection of bovine luteal cells with scramble oligonucleotide (50 nM, negative control) or anti-miR-96 (50 nM) at the indicated times (n=3 experiments). A representative western blot is shown in C. Values shown for each time-point were in all cases normalized to the corresponding value at the time of transfection (Day 0 or Hour 0). Significant differences (P<0.05) between means are shown by different letters (a,b,c). In C, mean FOXO1 values were compared between groups within each Day, and differences are indicated by * (P<0.05).
Table 1. miRNAs differentially expressed (≥ 2.5 fold) between early corpora lutea and pre-ovulatory size follicles in cow.

<table>
<thead>
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<th>miRNA</th>
<th>Fold-change</th>
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miRNA nomenclature according to miRBase 21
n = 6 animals per tissue type
P-value (Benjamini and Hochberg adjusted) <0.01 in all cases
Figure 1

A

Figure 1A shows a bar graph comparing transcript levels normalized to RPS5 between Follicle and Corpus luteum. The x-axis represents different genes (LHCGR, CYP19A1, HSD3B1), and the y-axis represents transcript levels. The bars indicate higher transcript levels in Follicle compared to Corpus luteum.

B

Figure 1B is a heatmap indicating the expression levels of various miRNAs (hsa-mir-376b, hsa-mir-378a-3p, hsa-mir-106b-5p, etc.) in Corpora lutea and Follicles. The colors range from green to red, with green indicating lower expression and red indicating higher expression.

C

Figure 1C is a PCA plot with PC1 and PC2 axes. The plot distinguishes between Follicles and Corpora lutea, with clustered data points indicating differentiation in gene expression patterns.

D

Figure 1D presents a bar graph comparing transcript levels (normalized to RPS5) between Follicle and Corpus luteum. The y-axis represents transcript levels, and the bars indicate significant differences with ** markers.

E

Figure 1E is a histogram showing fold-change in transcript levels between Corpus luteum and Follicle, with Microarray and RT-qPCR data. The fold-change values are indicated on the y-axis, with the histogram bars showing the distribution of fold-changes.
Figure 2

A

miR-96

miR-132

Transcript levels (normalized to RNU44)

Liver Heart Kidney Spleen Skin Testis Early CL Blood PMSG

B

Non-luteinized Luteinized

miR-96

miR-132

Transcript levels (normalized to RNU44)

Day of luteinization

C

miR-212

miR-183

Transcript levels (normalized to RNU44)

Day of luteinization

D

Follicle Corpus luteum

Transcript levels (normalized to 18S)

FOXO1A ACOVR1A RASA1 FOXO3 CDKN1A MMP9 HB-EGF
Figure 5

A

miR96 levels (normalized to RNU6-2)

Days post-transfection

Scramble
Anti-miR-96

B

Caspase 3/7 activity

36h post-transfection
(12h after serum removal)

Scramble+serum
Scramble
Anti-miR-96

C

FOXO1 protein levels (normalized to β-tubulin)

Days post-transfection

Scramble
Anti-miR-96

*